Melamine (MEL) ELISA Kit

Technical Manual

(ELISA)

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1 Principle and Application

This kit adopts the method of indirect competitive enzyme-linked immunoassay (ELISA) to detect Melamine (MEL) in the sample such as milk powder, milk, tissue, feed, eggs and serum. The kit is composed of Microtiter Plate coated with coupled antigens, HRP conjugate, antibodies, standards and other supporting reagents. During the detection, with adding standards or samples, the MEL in the samples will compete with the coupled antigens to combine with anti-MEL antibodies. After adding HRP conjugate, take coloration with TMB substrates. Absorbance value of the samples is a negative correlation with MEL content. Lastly, by comparing the obtained absorbance values with the standard curve, we can calculate the content of MEL in the sample.

2 Technique Data

2.1 Kit Sensitivity: 2ppb (ng/mL)

2.2 Reactive Mode: 25°C, 30min ~ 15min

2.3 Detection Limits:

Sample	Detection Limits
Milk (treatment 1)	54ppb
Milk powder (treatment 1)	40ppb
Milk/milk powder (treatment 2)	2ppb
Tissue(Chicken/pig/duck/fish/shrimp/liver etc.)	4ppb
Animal feed	200ppb
Eggs	40ppb
Serum	8ppb

2.4 Cross-reaction Rate:

Melamine 100%

Cyanuric acid 60%

Triazine, Diamine triazine ..... <1%

2.5 Sample Recovery Rate:

Sample	Recovery rate
Milk/milk powder	90±20%
Tissue	85±20%
Animal feed	85±20%
Eggs, serum	80±20%

3 Composition of the Kit

Reagent	Specification
Microtiter Plate	8wells× 12strips
Standard: 0ppb, 2ppb, 6ppb, 18ppb, 54ppb, 162ppb (black cap)	1.0mL each
High standard (red cap)	1ppm
Antibody solution (blue cap)	1×5.5mL
HRP conjugate (red cap)	1×5.5mL
Substrate Reagent A (white cap)	1×6mL
Substrate Reagent B (black cap)	1×6mL
Stop Solution (yellow cap)	1×6mL
Concentrated Wash Buffer (20×)(white cap)	1×40mL
Concentrated Reconstitution Buffer (2×) (yellow cap)	1×50mL
Instruction	1
Adhesive Membrane	1
Sealed bag	1

4 Materials Required but Not Supplied

4.1 Equipment: microplate reader, printer, grinder (for homogenizing solid samples), nitrogen evaporator, vortex mixer (for shake and mix), centrifuge, graduated transfer pipette, and balance with a division value of 0.01 g, constant temperature device;

4.2 Micropipette: single-channel (20-200μL, 100-1000μL), and multi-channel 300μL;

4.3 Reagents: acetonitrile, sodium hydroxide, concentrated hydrochloric acid, n-Hexane, methanol.

5 Experimental preparation

Restore all reagents and samples to room temperature (adjust to around 25°C) for more than 30 min before use. This is a crucial step to ensure there is no precipitation in the reagents.

5.1 Notice Before Sample Processing:

Please note that the labware must be clean. Use disposable pipette tips to avoid contamination of interference results.

5.2 Solution preparation:

Solution 1: 1M HCl Solution

Dilute 8.6mL of concentrated hydrochloric acid to 100mL with deionized water.

Solution 2: 0.1M NaOH Solution

Dilute 0.4g of sodium hydroxide to 100mL with

deionized water.

Solution 3: Acetonitrile-0.1M NaOH Solution

Mix 84mL of acetonitrile with 16mL of 0.1M NaOH Solution.

Solution 4: 1M NaOH Solution

Dilute 4g of sodium hydroxide to 100mL with deionized water.

Solution 5: Reconstitution Buffer

Dilute the Concentrated Reconstitution Buffer (2×) 2 times (Concentrated Reconstitution Buffer (2×) /Deionized water= 1:1). It can be stored for one month at 4°C.

Solution 6: Working Wash Buffer

Dilute the concentrated wash buffer (20×) by a factor of 20 (Concentrated wash buffer/Deionized water= 1: 19).

5.3 Sample pretreatment steps:

5.3.1 Milk treatment 1.

1) Transfer 600µL of sample into a 2mL centrifuge tube, add

1mL of acetonitrile, shake thoroughly; and centrifuge at 4000 rpm for 5 minutes at room temperature.

2) Take 100µL of the supernatant, add 900µL of Reconstitution Buffer (Solution 5), and mix well.

3) Take out 50µL for test.

Dilution times of the sample:27 Detection limits: 54ppb

5.3.2 Milk powder treatment 1.

1) Weigh 2g±0.05g of homogenized samples into a 50mL centrifuge tube, add 4mL of methanol, shake thoroughly; and centrifuge at 4000 rpm for 10 minutes at room temperature.

2) Take 100µL of the supernatant, add 900µL of Reconstitution Buffer (Solution 5), and mix well.

3) Take out 50µL for test.

Dilution times of the sample:20 Detection limits: 40ppb

5.3.1 Milk/milk powder treatment 2.

1) Take 2mL of milk or 2g ± 0.05g of milk powder into a centrifuge tube.

2) Add 8mL of Acetonitrile-0.1M NaOH Solution (Solution 3), shake thoroughly for 2 minutes, centrifuge at 4000 rpm for 10 minutes at room temperature, take 4mL of the supernatant, and evaporate it to dryness under nitrogen or air at 50-60°C.

3) After dissolving the dried residue (obtained from previous step) in 1mL n-hexane, add 1mL of Reconstitution Buffer (Solution 5), mix for 30 seconds, centrifuge at 4000 rpm for 5 minutes at room temperature, and remove the upper layer liquid.

4) Take 50µL of lower layer liquid for analysis.

Dilution times of the sample:1 Detection limits: 2ppb

5.3.4 Tissue (Chicken/pig/duck/fish/shrimp etc.) treatment.

1) Take 2g ± 0.05g of sample into a centrifuge tube.

2) Add 8mL of Acetonitrile-0.1M NaOH Solution (Solution 3), shake thoroughly for 2 minutes, centrifuge at 4000 rpm for 10 minutes at room temperature, take 2mL of the supernatant, and evaporate it to dryness under nitrogen or air at 50-60°C.

3) After dissolving the dried residue (obtained from previous step) in 1mL n-hexane, add 1mL of Reconstitution Buffer (Solution 5), mix for 30 seconds, centrifuge at 4000 rpm for 5 minutes at room temperature, and remove the upper layer liquid.

4) Take 50µL of lower layer liquid for analysis.

Dilution times of the sample:2 Detection limits: 4ppb

5.3.5 Animal feed treatment.

1) Add 2g ± 0.05g of crushed sample to 2mL of 1M HCl Solution (Solution 1), and then add 16mL of deionized water, shake for 2min; and centrifuge at 4000 rpm for 15 minutes at room temperature.

2) Take 10mL of the supernatant, add 1M NaOH Solution (Solution 4) to adjust the pH to 6-8. (Note: Due to variations in feed samples, the amount of 1M NaOH Solution added may differ and should be adjusted accordingly, typically ranging between 0.5mL to 1mL.)

3) Centrifuge at 4000 rpm for 15 minutes at room temperature.

4) Mix 100µL of the supernatant with 900µL of Reconstitution Buffer (Solution 5).

5) Take out 50µL for test.

Dilution times of the sample:100 Detection limits: 200ppb

5.3.6 Egg treatment.

1) Homogenize the sample (Egg white, egg yolk, or whole egg.)

2) Take 2g ± 0.05g of homogenized sample into a centrifuge tube, add 8mL of Acetonitrile-0.1M NaOH Solution (Solution 3), shake thoroughly for 2 minutes. (As egg white contains a high protein content, it may form a gelatinous mass after the addition of the Solution 3. This is a normal occurrence and does not affect the experimental results.)

3) Centrifuge at 4000 rpm for 10 minutes at room temperature, take 1mL of the supernatant, and evaporate it to dryness under nitrogen or air at 50-60°C.

4) After dissolving the dried residue (obtained from previous step) in 1mL n-hexane, add 1mL of Reconstitution Buffer (Solution 5), mix for 30 seconds, centrifuge at 4000 rpm for 5 minutes at room temperature, and remove the upper layer liquid.

5) Mix 50µL of lower layer liquid with 150µL of

Reconstitution Buffer (Solution 5), then shake for 30 seconds.

6）Take out 50μL for test.

Dilution times of the sample:20 Detection limits: 40ppb

5.3.7 Serum treatment.

1) Transfer 0.5mL of sample into a 50mL centrifuge tube, add 2mL of Acetonitrile-0.1M NaOH Solution (Solution 3), thoroughly shake for 2 minutes; and centrifuge at 4000 rpm for 10 minutes at room temperature. Then take 1mL of the supernatant, and evaporate it to dryness under nitrogen or air at 50-60°C.

2) After dissolving the dried residue (obtained from previous step) in 1mL n-hexane, add 1mL of Reconstitution Buffer (Solution 5), mix for 30 seconds, centrifuge at 4000 rpm for 5 minutes at room temperature, and remove the upper layer liquid.

3) Take 50μL of lower layer liquid for analysis.

Dilution times of the sample:4 Detection limits: 8ppb

6 ELISA procedure

Place all reagents and samples to room temperature (adjust to around 25°C) for 30min. Gently shake the reagent bottles before use.

Take out the frame of the microplate along with the required number of wells. Then place the unused microplate wells into the sealed bag with the desiccant provided. Store the remaining kit in the refrigerator at 2-8°C.

Step 1: Number: Number the wells in sequence corresponding to the samples and standard, make 2-well parallel trials for each sample and standard, and record their locations.

Step 2: Incubation: Add 50μL of standard or sample into each numbered well, then add 50μL of HRP conjugate per well. Next, add 50μL of antibody solution per well. Finally, cover the Microtiter Plate with the adhesive membrane, shake gently by hand (or use a microplate shaker) for 5s and incubate for 30 min at 25°C.

Step 3: Washing: Uncover the adhesive membrane carefully, remove the liquid, pipette 350μL of Working Wash Buffer (Solution 6) to every well, let stand for 30 seconds then drain, repeat 5 times. Invert the plate and tap it against a thick absorbent paper (or lint-free cloth), with a soft towel placed underneath. (Bubbles that are not removed after patting dry can be punctured with a clean pipette tip).

Step 4: Color: Add 50μL of Substrate Reagent A to each well, then add 50μL of Substrate Reagent B per well. Shake gently by hand (or use a microplate shaker) for 5s, and allow to react for 15min at 25°C in the dark. (The reaction can be extended appropriately if the blue colour is too pale.)

Step 5: Stop the reaction: Pipette 50μL of Stop Solution to each well, shake gently by hand (or use a microplate shaker) for 5s. The reaction would be stopped.

Step 6: Calculate: Determine the Optical Density (OD value; absorbance value) at 450nm (Reference wavelength 630nm) with a microplate reader. Finish this step within 10min after stop the reaction.

7 Interpretation of result

7.1 Calculate the percentage of absorbance value

$$\text{Percentage of absorbance value}(\%) = \frac{A}{A0} \times 100\%$$

A—the average OD value of the sample or standard;

A0—the average OD value of the 0ppb standard.

It is used to calculate the percentage absorbance of a standard or sample.

7.2 Draw the standard curve and calculate

Take absorbance percentage (A/A0) of standards as Y-axis and the corresponding log of standards concentration (ppb) as X-axis.

Draw the standard semi-log curves with X-axis and Y-axis.

Take absorbance percentage of samples substitute into standard curve, then can get the corresponding concentration from standard curve. **Last, the resulting concentration values multiplied by the corresponding dilution times is the actual concentration of MEL of samples.**

If professional analysis software of the kit is used for calculation, it is more convenient for accurate and rapid analysis of a large number of samples.

8 Attention

8.1 Before test, the reagents and samples should be balanced to room temperature (25°C). If below 25°C, it will lead to all the standard OD value on the low side.

8.2 In the washing process, dry wells may result in non-linear standard curves and undesirable reproducibility. Therefore, proceed to the next step immediately after washing.

8.3 Please mix the contents within the wells uniformly and wash the plate thoroughly. The reproducibility is largely determined by consistency of washing step.

8.4 During the incubation, cover microplates with adhesive membrane to avoid light.

8.5 Do not use kits that are overdue. Do not mix reagents with those from other lots.

8.6 Substrate Reagent A/B is colorless. If not, please discard.

8.7 If absorbance value of 0ppb is below 0.5（A450nm< 0.5 ）, it means that the reagent may be metamorphic.

8.8 Stop solution is corrosives, please avoid contact with skin.

8.9 As the OD values of the standard curve may vary according to the conditions of actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test.

8.10 For the mentioned sample, fast and efficient extraction methods are included in the kit description. Please consult technical support for the applicability if other sample need to be tested.

8.11 The kit is used for rapid screening of actual samples. If the test result is positive, the instrument method such as HPLC, LC/MS can be used for quantitative confirmation.

9 Storage conditions

The kit shall be stored at 2-8 °C. Avoid freezing.

Shelf Life: 12 months. The date of manufacture is presented in the label of the box.