# QUALICHEK™ Melamine ELISA

**REF** : KBFP1026

*Designed and Developed as per AOAC Official Methods of Analysis Guidelines (Method 971.22)*

ISO9001:2016 ISO13485:2016

Ver 1.1

**Validated and Complies for Testing as Per FSSAI requirements*.**

Quantitative testing of Melamine in samples, such as milk powder, tissue, feed, eggs, etc.

| | | | |
|---|---|---|---|
| **NOT FOR DIAGNOSTIC OR HUMAN USE** | | **REF** | **Catalog Number** |
| | **Store At** | **LOT** | **Batch Code** |
| | **Manufactured By** | | **Biological Risk** |
| | **Expiry Date** | | **Consult Operating Instructions** |

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**REF** **KBFP1026** **Σ 96 tests**

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**Introduction:**

Melamine is an organic base chemical most commonly found in the form of white crystals rich in nitrogen. It is widely used in plastics, adhesives, countertops, dishware, and whiteboards. Melamine is added into the milk to increase the protein count falsely in milk and dairy products. The addition of melamine increases the nitrogen content of the food products and therefore its apparent protein content. Irritability, blood in urine, little or no urine, signs of kidney infection, high blood pressure are the common symptoms of melamine poisoning.

*As per FSSAI standards the contaminant-Melamine shall not exceed the Maximum Level prescribed for the article of food as mentioned in the table below:

| Food | Maximum Level (mg/Kg) |
|---|---|
| Powdered infant formula | 1.0 |
| Liquid infant formula | 0.15 |
| Other foods | 2.5 |

**Intended Use:**

This QUALICHEK™ Melamine ELISA kit is for quantitative testing for Melamine in sample such as milk powder, tissue, feed, eggs, etc.

**Principle:**

The QUALICHEK™ Melamine ELISA kit uses Competitive-ELISA as the method. This kit is composed of ELISA Microtiter plate, HRP conjugate, antibody, standard and other supplementary reagents. The microplate provided in this kit has been pre-coated with Melamine antigen. During the reaction, Melamine in the samples or standard competes with Melamine antigen on the solid phase plate for Anti-Melamine antibody. Then Horseradish Peroxidase (HRP) conjugate is added to each micro plate well, and TMB substrate is for color development. There is a negative correlation between the OD value of samples and the concentration of Melamine. The concentration of Melamine in the samples can be calculated by comparing the OD of the samples to the standard curve.

**Materials Provided:**

1. Melamine Coated Microtiter plate - 1 x 96 wells
2. Melamine Standards, 1 ml/vial - 0, 2, 6, 18, 54, and 162 ppb
3. HRP Conjugate - 5.5 ml
4. Antibody Working Solution - 5.5 ml
5. (20X) Wash Buffer - 40 ml
6. 2X Reconstitution Buffer - 50 ml
7. TMB Substrate A - 6 ml
8. TMB Substrate B - 6 ml
9. Stop Solution - 6 ml
10. Instruction Manual – 1 no

**Material required but not provided:**

1. Microtiter plate reader
2. Homogenizer
3. Nitrogen Evaporators/Water bath
4. Oscillators
5. Centrifuge
6. Graduated pipette
7. Balance (sensibility 0.01 g)

8. High-precision pipettes: Single channel (20-200 ul, 100-1000 ul), Multichannel (300 ul) micropipettes
9. N-hexane
10. Acetonitrile
11. NaOH
12. Concentrated HCl
13. Methanol.

**Storage Information:**

1. Store main kit components at 2-8°C Avoid freeze / thaw cycles.
2. Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components to appropriate storage conditions.

**Reagent Preparation:**

1. **1 M HCl:**
   Dilute 8.6 ml of Concentrated HCl with deionized water to 100 ml.
2. **Acetonitrile-0.1 M NaOH Solution:**
   Mix 84 ml of Acetonitrile and 16 ml of 0.1 M NaOH solution fully.
3. **0.1 M NaOH Solution:**
   Dissolve 0.4 g of NaOH in 100 ml deionized water.
4. **1 M NaOH Solution:**
   Dissolve 4 g of NaOH in 100 ml of deionized water.
5. **Reconstitution Buffer:**
   Dilute the 2X Reconstitution Buffer with deionized water. (2X Reconstitution Buffer (v): Deionized water (v) = 1:1). The Reconstitution Buffer can be stored at 4°C for 1 month.
6. **Wash Buffer:**
   Dilute 20X Concentrated Wash Buffer with deionized water. (20X Concentrated Wash Buffer (v): Deionized water (v) = 1:19)

**Sample Pretreatment:**

Bring all reagents and samples to room temperature before use.
Open the microplate reader in advance, preheat the instrument, and set the testing parameters.
Experimental apparatus should be clean, and the pipette should be disposable to avoid cross-contamination during the experiment.

**Pretreatment of milk sample:**

1. Take 600 ul of milk sample into 2 ml tube and add 1 ml of acetonitrile, oscillate until mixed fully. Centrifuge at 4000 rpm for 5 min.
2. Take 100 ul of supernatant and add 900 ul of Reconstitution buffer. Mix fully.
3. Take 50 ul for analysis.

*Note: Sample dilution factor: 27, minimum detection dose: 27 ppb.*

**Pretreatment of Milk Powder:**

1. Weigh 2±0.05 g of milk powder sample into a 50 ml centrifuge tube, add 4 ml of methanol, and oscillate until mixed fully.
2. Centrifuge at 4000 rpm for 5 min. Take 100 ul of supernatant and add 900 ul of Reconstitution buffer. Mix fully.
3. Take 50 ul for analysis.

*Note: Sample dilution factor: 20, minimum detection dose: 20 ppb.*

**Pretreatment for Milk / Milk Powder** (alternate method 2)**:**

1. Take 2 ml of milk sample or 2 g of milk powder sample into a centrifuge tube.
2. Add 8 ml of Acetonitrile-0.1 M NaOH solution and oscillate fully for 2 min. Centrifuge at 4000 rpm for 10 min. Take 4 ml of the upper layer liquid and dry at 50-60°C under a gentle stream of nitrogen or water bath.
3. Add 1 ml of n-hexane to dissolve the remaining dry material, then add 1 ml of Reconstitution buffer. Oscillate strongly for 30 sec and centrifuge to remove the upper layer n-hexane phase.
4. Take 50 ul of the lower layer liquid for analysis.

*Note: Sample dilution factor: 1, minimum detection dose: 1 ppb.*

**Pretreatment for Tissue (chicken, porcine, duck, fish, shrimp, liver):**

1. Weigh 2±0.05 g of homogenate tissue sample into a 50 ml centrifuge tube.
2. Add 8 ml of Acetonitrile-0.1 M NaOH solution and oscillate fully for 2 min. Centrifuge at 4000 rpm for 10 min. Take 2 ml of the upper liquid layer and dry at 50-60℃ under a gentle stream of nitrogen or water bath.
3. Add 1 ml of n-hexane to dissolve the remaining dry material, and then add 1 ml of Reconstitution buffer. Oscillate strongly for 30 seconds and centrifuge to remove the upper layer n-hexane phase.
4. Take 50 ul of the lower layer liquid for analysis.

*Note: Sample dilution factor: 2, minimum detection dose: 2 ppb*

**Pretreatment for Feed:**

1. Weigh 2±0.05 g of crushed feed sample into a centrifuge tube. Add 2 ml of 1 M HCl solution and 16 ml of deionized water, then homogenate the sample.
2. Swirl for 1 min and oscillate for 2 min with vortex.
3. Centrifuge at 4000 rpm for 15 min. Take 10 ml of the supernatant and adjust the pH to 6~8 with 1 M NaOH. (The added amount of 1 M NaOH is different according to the feed sample. The needed amount is generally between 0.5 ml~1 ml.)
4. Centrifuge at 4000 rpm for 15 min. Take the supernatant (It is recommended to increase the centrifuge speed or filter the supernatant with filter paper if the supernatant is muddy).
5. Dilute the supernatant 10 times with the Reconstitution buffer (Take 100 ul of supernatant and add 900 ul of Reconstitution buffer. Mix fully.)
6. Take 50 ul for analysis.

*Note: Sample dilution factor: 100, minimum detection dose: 100 ppb.*

**Pretreatment for Eggs:**

1. Homogenate the egg sample with homogenizer to mix the egg whites and yolks fully.
2. Weigh 2±0.05 g of homogenate egg sample into a centrifuge tube. Add 8 ml of Acetonitrile-0.1 M NaOH solution and oscillate fully for 2 min.
3. Centrifuge at 4000 rpm for 10 min at room temperature. Take 1 ml of the upper layer liquid and dry at 50-60℃ under a gentle stream of nitrogen or water bath.
4. Add 1 ml of n-hexane to dissolve the remaining dry material, and then add 1 ml of Reconstitution buffer. Oscillate strongly for 30 seconds and centrifuge to remove the upper layer n-hexane phase.
5. Take the lower phase solution and dilute it with Reconstitution buffer for 4 times (50 ul of sample + 50 ul of 1X Reconstitution Buffer), mix fully.
6. Take 50 ul for analysis.

*Note: Sample dilution factor: 20, minimum detection dose: 20 ppb.*

**Assay Procedure:**

Bring all reagents to room temperature for 30 min before use. **All the reagents should be mixed thoroughly by gently swirling before pipetting. Avoid foaming.**

1. Number the sample and standard in order (multiple well), and keep a record of standard wells and sample wells. Standards and Samples should be tested in duplicates.

2. Add **50 ul of Standard or Sample** into respective wells
3. Add **50 ul of HRP Conjugate** to each well.
4. Add **50 ul of Antibody working Solution** to each well. Mix well.
5. Incubate at Room Temperature 25°C for 30 min.
6. Aspirate and wash plate 5 times with Wash Buffer (1X) and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.
7. Add **50 ul of TMB Substrate A** to each well, followed by **50 ul of TMB Substrate B**. Mix well.
8. Incubate in dark for 15 min at Room Temperature 25°C. (The reaction time may be shortened or prolonged according to the depth of the color).
9. Add **50 ul of stop solution** to each well, oscillate gently to mix well.
10. Read the plate at 450 nm with a microplate reader within 10 min after adding stop solution.

**Calculation of Results:**

**Absorbance (%) =A/$A_0$ × 100%**

A: Average absorbance of standard or sample
$A_0$: Average absorbance of 0 ppb Standard

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Graph Paper, plot the average value (absorbance 450 nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Concentration.

If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit is best recommended for automated results.

**Notes:**

1. The overall OD value will be lower when reagents have not been brought to room temperature before use or room temperature is below 25°C.
2. If the wells turn dry during the washing procedure, it will lead to bad linear standard curve and poor repeatability. Operate the next step immediately after wash.
3. Mix thoroughly and wash the plate completely. The consistency of wash procedure can strongly affect the reproducibility of this ELISA kit.
4. ELISA Microtiter plate should be covered by plate sealer. Avoid the reagents to strong light.
5. Do not use expired kit or reagents of different batches.
6. TMB should be abandoned if it turns color. When OD value of standard (concentration: 0) ＜ 0.5 unit ($A_{450nm}$＜0.5), it indicates the reagent may be deteriorated.
7. Stop solution is caustic; avoid contact with skin and eyes.
8. As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test.
9. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.

10. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
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, etc. can be used for quantitative confirmation

**Performance Characteristics:**

**Sensitivity:** 1 ppb (ng/ml)

**Reaction Mode:** 25°C, 30min~15 min

**Detection Limit:**
Milk powder - 20 ppb
Milk - 27 ppb
Milk / Milk powder (using alternate method 2) - 1 ppb
Tissue (chicken, porcine, duck, fish, shrimp, liver) - 2 ppb
Feed - 100 ppb
Eggs - 20 ppb

**Reactivity:**
Melamine (MEL) - 100%
Cyanuric Acid - 60%
Trizine <1%

**Sample Recovery Rate:**
Milk powder, Milk - 90±20%,
Tissue - 85±20%
Feed - 85±20%
Eggs - 80±20%

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