Tilmicosin (TIM) 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 Tilmicosin (TIM) in the sample such as tissue, liver, honey, eggs and milk. The kit is composed of Microtiter Plate coated with coupled antigens, HRP enzyme conjugates, antibodies, standards and other supporting reagents. During the detection, with adding standards or samples, the TIM in the samples will compete with the coupled antigens to combine with anti-TIM antibodies. After adding enzyme conjugates, take coloration with TMB substrates. Absorbance value of the samples is a negative correlation with TIM content. Lastly, by comparing the obtained absorbance values with the standard curve, we can calculate the content of TIM in the sample.

2 Technique Data

2.1 Kit Sensitivity: 0.5ppb (ng/mL)

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

2.3 Detection Limits:

Sample	Detection Limits
Animal tissue (higher detection limit)	0.5ppb
Animal tissue (lower detection limit)	10ppb
Liver (chicken liver, pork liver, etc.)	10ppb
Honey	1ppb
Eggs, milk	5ppb

2.4 Cross-reaction Rate:

Tilmicosin 100%

Tylosin 1%

2.5 Sample Recovery Rate:

Sample	Recovery rate
Animal tissue (higher detection limit)	90±20%
Animal tissue (lower detection limit)	90±20%
Liver (chicken liver, pork liver, etc.)	75±15%
Honey	95±25%
Eggs, milk	95±25%

3 Composition of the Kit

Reagent	Specification
Microtiter Plate	8wells× 12strips
Standard: 0ppb, 0.5ppb, 1.5ppb, 4.5ppb, 13.5ppb, 40.5ppb	1.0mL each
High Standard：1ppm(red cap)	1×1.0mL
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 to 200μL and 100 to 1000μL, and multi-channel 300μL;

4.3 Reagents: anhydrous sodium carbonate, $NaHCO_3$, Methanol, Ethyl Acetate, n-Hexane.

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: 0.1M CB Buffer (pH = 10.6)

Dissolve 0.932g anhydrous sodium carbonate and 0.1g sodium bicarbonate in 100mL of deionized water and mix well.

Solution 2: 0.5M CB Buffer (pH = 10.6)

Dissolve 4.66g anhydrous sodium carbonate and 0.5g sodium bicarbonate in 100mL of deionized water and mix well.

Solution 3: Reconstitution Buffer

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

Solution 4: Milk and Egg Diluent

Mix reconstitution buffer (Solution 3) with methanol in a 19:1 volume ratio (i.e., 19 parts reconstitution buffer + 1 part methanol).

Solution 5: 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 Animal Tissue like Pork, Chicken, Beef, Lamb, Fish, Shrimp (Higher Detection Limit) treatment.

1) Weigh 2g ± 0.05g of homogenized tissue sample into a 50mL polystyrene centrifuge tube. Add 2mL of 0.1M CB buffer (Solution 1) and 8mL of ethyl acetate. Vortex for 3 minutes, then centrifuge at room temperature at 4000r/min for 5 minutes.

2) Transfer 4mL of the upper organic phase to a clean, dry 10mL glass tube. Evaporate to dryness under nitrogen or air at 50-60°C.

3) Add 1mL of n-hexane to the dried residue, vortex for 30 seconds. Then add 1mL of the reconstitution buffer (Solution 3), vortex for 30 seconds, and centrifuge at room temperature at 4000r/min for 5 minutes.

4) Discard the upper layer and take 50μL of the lower aqueous phase for analysis.

Dilution times of the sample:1 Detection limits:0.5ppb

5.3.2 Animal Tissue like Pork, Chicken, Beef, Lamb, Fish, Shrimp (Lower Detection Limit) treatment.

1) Weigh 1g ± 0.05g of homogenized sample into a 50mL polystyrene centrifuge tube. Add 4mL of the working wash solution (Solution 5), vortex for 3 minutes, then centrifuge at room temperature at 4000r/min for 5 minutes.

2) Transfer 200μL of the upper clear liquid to a new container, then add 600μL of the reconstitution buffer (Solution 3), and vortex for 30 seconds.

3) Take 50μL of the mixture for analysis.

Dilution times of the sample:20 Detection limits:10ppb

5.3.3 Liver (Chicken, Duck, Pig Liver) treatment.

1)Weigh 1g ± 0.05g of homogenized liver sample into a 50mL polystyrene centrifuge tube. Add 2mL of 0.5M CB buffer (Solution 2) and 8mL of ethyl acetate, then vortex for 3 minutes. Then centrifuge at room temperature at 4000r/min for 5 minutes.

2)Transfer 2mL of the upper organic phase to a clean, dry 10mL glass tube, then evaporate to dryness under nitrogen or air at 50-60°C.

3)Transfer 2mL of the upper organic phase to a clean, dry 10mL glass tube, then evaporate to dryness under nitrogen or air at 50-60°C.

4)Add 1 mL of n-hexane, shake for 30 seconds. Then add 1 mL of reconstitution buffer (Solution 3), shake for 30 seconds. Centrifuge at 4000 r/min at room temperature for 5 minutes.

5)Remove the upper phase, take 100μL of the lower aqueous phase, and add 400μL of reconstitution buffer (Solution 3), then vortex for 30 seconds to mix well.

6)Take 50μL for analysis.

Dilution times of the sample:20 Detection limits: 10ppb

5.3.4 Honey treatment.

1) Weigh 2g ± 0.05g of honey sample into a 50mL polystyrene centrifuge tube. Add 2mL of 0.1M CB buffer (Solution 1) and 8mL of ethyl acetate, then vortex for 3 minutes. Then centrifuge at room temperature at 4000r/min for 5 minutes.

2) Transfer 2mL of the upper organic phase to a clean, dry 10mL glass tube, then evaporate to dryness under nitrogen or air at 50-60°C.

3) Add 1mL of reconstitution buffer (Solution 3), then vortex for 30 seconds to mix well.

4) Take 50μL for analysis.

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

5.3.5 Egg treatment.

1) Homogenize the egg sample at low speed using a homogenizer.

2) Take 100μL of the homogenized egg sample and add 900μL of egg diluent (Solution 4), then vortex for 30 seconds to mix well.

3) Take 50μL for analysis.

Dilution times of the sample:10 Detection limits: 5ppb

5.3.6 Milk treatment

1) Take 100μL of fresh milk sample and add 900μL of milk diluent (Solution 4), then vortex for 30 seconds to mix well.

2) Take 50μL for analysis.

Dilution times of the sample:10 Detection limits: 5ppb

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 into each 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 in the dark.

Step 3: Washing: Uncover the adhesive membrane carefully, discard liquid in the wells, pipette 350μL of Working Wash Buffer (Solution 5) 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 tapping 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 color is too pale.)

Step 5: Stop the reaction: Pipette 50μL of Stop Solution to each well, and shake gently by hand (or use a microplate shaker). 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 TIM 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.