Malachite Green (MG) ELISA Kit

Introduction

Malachite green (MG), originally used as a dyeing agent of textiles, was introduced as an ectoparasiticide, fungicide and antiseptic in aquaculture. The broad fungicidal and anti-parasitical spectrum has made MG very popular among fish culturists. The potential carcinogenic, mutagenic and tetragenic properties were demonstrated in several animal species and cell lines. The U.S. FDA nominated MG as a priority chemical for carcinogenicity testing under the National Toxicology Program. Though the use of MG has been banned in several countries and U.S. FDA, it is still being used in many countries due to its low cost, ready availability and efficacy.

In fish, MG is easily absorbed into tissues during waterborne exposure and extensively metabolized to the reduced, colorless compound, Leucomalachite green (LMG). According to the European Commission, methods that can be used for the determination of MG residues in fish muscles should meet a minimum required performance limit of 2ug/Kg for sum of MG and LMG. Several analytical methods have been described for MG and its major metabolite LMG detection, such as HPLC and LC-MS. These techniques need expensive apparatus and reagents and are also time-consuming. A sensitive, rapid and inexpensive method for detection of MG is needed.

An indirect competitive ELISA kit was designed to detect MG residues in fish muscle and water by GlycoNex. The detection limit for MG is 0.05 ppb and there is no cross reaction to other antibiotics and sulfa drugs. The MG ELISA kit is an enzyme immunoassay intended for use in the qualitative and semiquantitative analysis of MG residues in fish muscle. This kit uses a cutoff level of 1 ppb to distinguish positive from negative samples. The assay provides a rapid preliminary analytical test result. We suggest that all positive samples must be confirmed by LC-MS analysis.

The MG ELISA kit is less laborious and more convenient for the screening of MG residues in fish muscle. The extraction and analysis can be carried out in 2 hrs and more then 80 samples can be tested in one kit.

Principle

The MG ELISA kit is based on the specific immuno-chemical reactions between antigens and antibodies. In the test procedure, the sample and the anti-MG antibodies are added into the MG-antigen immobilized microtiter plates. If the MG is present in the sample, it will inhibit the binding of anti-MG antibodies to the limited amount of MG-antigen, which is immobilized on the microtiter well. Consequently the secondary antibody reaction will be limited. Thus, the enzyme-substrate reaction will not generate and only light color shown, indicating a positive result. If there is a negative sample, it will generate deep color after the enzyme-substrate reaction.

Product Content

1. MG Microtiter well plate: 8 microwells/strip, 12 strips/plate.
2. MG Standard: 0 ppb, 0.05 ppb, 0.1 ppb, 0.2 ppb, 0.5 ppb, 1 ppb, 2 ppb, 1 ml/bt
3. Sample Diluent : 1 bt, 50ml/bt.
4. Washing buffer(10X): 1 bt, 50 ml/bt.
5. MG Antibody (10X): 1 vial, 1.2 ml/vial.
6. MG Antibody Diluent : 12 ml/bt
7. Tracer (10X): 1 vial/kit, 1.2 ml/vial.
8. Tracer Diluent : 1 bt, 12 ml/bt
9. Substrate Solution(TMB): 1 bt, 12ml/bt
10. Stop Solution: 1 bt, 12 ml/bt

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Operation Procedure

A. Reagent preparation:

1. Prior to use, warm all reagents to ambient temperature by allowing them to stand on the benchtop for one hour. Gently mix all reagents.
2. Extraction buffer : to 1 ml glacial acetic acid add 99 ml methanol.
3. 1X washing solution: dilute the Washing Solution (10X) with 9 volumes of distilled water and mix.
4. 1X MG Antibody: dilute the MG Antibody (10X) with 9 volumes of MG Antibody Diluent and mix before use.
5. 1X tracer: dilute the tracer (10X) with 9 volumes of Tracer Diluent and mix before use.

B. Sample preparation:

1. To 1 g homogenized fish muscle add 10 ml of extraction buffer.
2. Vortex for 3 minutes.
3. Centrifuge at 3500 rpm (~2000g) for 10 minutes.
4. Transfer 1 ml of supernatant to a polypropylene tube and evaporate to dryness under a stream of nitrogen at 70°C
5. Reconstitute the residue with 500 ul of sample diluent and vortex for 10 seconds.
6. Centrifuge at 3500 rpm (~2000g) for 10 minutes.
7. The clear solution is ready to test.

C. Operation procedure

1. Add 100 ul of the MG standard or fish extract to the appropriate microtiter wells.
2. Add 100 ul of 1X MG Antibody to each well, mix by knocking the edge of the plate gently and incubate for 30 minutes at 37℃.
3. Dump the reaction solution out of the microtiter wells.
4. Add washing buffer to fill the microtiter wells and dump it out. Repeat this procedure 3 times.
5. Pat it dry after the final washing step.
6. Add 100 ul of 1X tracer to each well, mix by knocking the edge of the plate gently and incubate for 30 minutes at 37℃.
7. Repeat step 3 -5.
8. Add 100 ul of the substrate solution (TMB) to each microtiter well and incubate for 10 minutes at 37℃ in the dark.
9. Add 100 ul of Stop Solution to each microtiter well.
10. Read the result at dual wavelength of 450 and 650 nm

D. Test Result Interpretation

1. Calculate the Percent Binding (B/B0%) for each calibrator or sample by following:

$$\frac{\text{Absorbance (standard or sample)}}{\text{Absorbance (0 ppb standard)}} \times 100 = \frac{B}{B0}\ \%$$

2. Prepare a calibration curve by plotting the B/B0 values versus their corresponding concentration on semi-log paper and drawing the best fit straight line. The graph should be similar to Fig. 1. Calculated the B/B0 values for each sample and interpolate the corresponding concentration from the calibration curve. The sample concentration should be corrected for dilution (5.5 folds).

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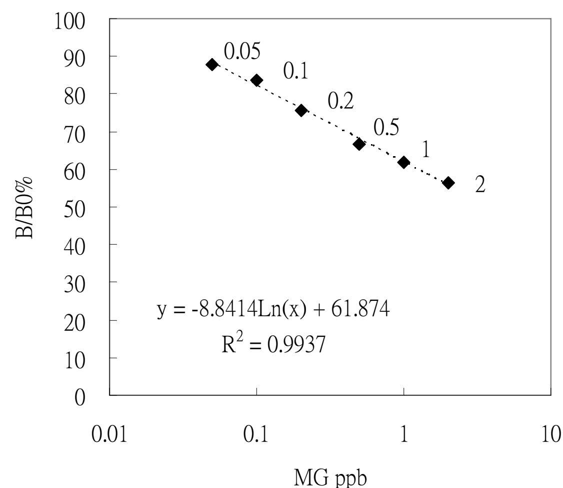

Figure 1. Standard curve (B/B0 % vs. concentration)

For example：

Divide the absorbance value of each sample by the absorbance of 0 ppb standard and multiply by 100 = y% ,the concentration = $e^{[(61.874-y)\ /\ 8.8414]}$ x 5.5 ppb （according to the Fig.1）.

* We suggest that positive samples must be confirmed by LC-MS analysis.

Limit of Detection

The limit of detection for the MG ELISA kit is 0.05ppb. It means the OD value of this concentration is significantly different from the negative Standard. (B/B0<90%)

Recovery : 70% ~ 110 %

Cross-reactivity

The following compounds were tested at 1 ppm and found not to cross-react significantly.

Clenbuterol	Sulfadiazine	Sulfaquinoxaline
Salbutamol	Sulfamonomethoxine	Sulfamethoxazole
Terbutaline	Sulfamethazine	Sulfanitran
Chloramphenicol	Sulfamethoxypyridazine	Penicillin G
Gentamicin	Sulfadimethoxine	Tetracycline
Oxytetracycline	Sulfathiazole	

Aromatic compounds (aniline, dimethylaniline, benzaldehyde)

Materials Required But Not Provided

1. glacial acetic acid
2. methanol
3. Polypropylene tubes

Warnings and Precautions

1. Assay reagents should be stored at 2-8℃.
2. Do not use reagents beyond expiration date.
3. A calibration curve must be performed with each assay.
4. Avoid contact **Stop Solution** and **Sample Diluent** with skin, eyes or clothing. If contact is made, wash with a large volume of water. If ingested, call a physician.

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