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Abraxis LLC

ABRAXIS

Malachite Green/Leucomalachite Green Plate Kit

PN 52251B

Instructional Booklet

Read Completely Before Use.

INTENDED USE

The Abraxis Malachite Green/Leucomalachite Green plate kit is a competitive ELISA for the quantitative analysis of Malachite green and Leucomalachite Green in aquatic products.

Malachite Green (MG) is a non-permitted (North America, Europe, etc.) anti-fungal agent which is sometimes used in hatcheries for the treatment of external fungal and parasitic infections of fish eggs, fish, shellfish and as a general hatchery disinfectant. Leucomalachite Green (LMG) is a metabolite of MG and is persistently found in fish tissue long after MG may no longer be detected. Where MG and/or LMG is detected, appropriate product action will be taken by regulatory agencies, which could include recalls.

As with any analytical technique (GC, HPLC, etc…..) positive results requiring some action should be confirmed by an alternative method.

ASSAY PRINCIPLES

The Abraxis Malachite Green/Leucomalachite Green ELISA plate kit is a competitive enzyme-labeled immunoassay. Because the residue is found primarily as the reduced Leuco compound in tissue and this ELISA detect primarily MG, an oxidation step is needed (all reagents are supplied in the kit) to convert this residue (LMG) to MG.. MG and LMG are extracted from a sample by blending or shaking with extraction solvent, samples are oxidized using oxidation reagents provided in the kit. The diluted/oxidized Malachite Green sample extract and calibrators are pipetted into test wells followed by malachite green antibody to initiate the reaction. After 30 minute incubation, the wells are washed with laboratory grade water. Malachite Green HRP conjugate is then added and incubated for 30 minutes. Following a second wash after the incubation, a clear substrate is added to the wells and any bound enzyme conjugate causes the conversion of the colorless substrate/chromogen to a blue color. After 20-30 minute incubation, the reaction is stopped and the amount of color in each well is read. The color of the unknown samples is compared to the color of the calibrators and the Malachite green/Leucomalachite Green concentration of the samples is determined by interpolation.

SPECIFICITY

The Abraxis Malachite green Plate Kit can not differentiate between the various Malachite Greens metabolites, but detects their presence to differing degrees. The following table shows the % cross reactivity of Leucomalachite versus Malachite green.

Compound	%CR
Malachite green	100%
Leucomalachite green	<1%, 100% after oxidation
Crystal Violet	120%
LeucoCrystal Violet	<1%

DETECTION LIMIT

Aquatic products: 0.25ppb

REAGENTS AND MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2-8℃.

•Plate containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating dessicant.

•1 vials containing 0.4 ml of Malachite Green calibrator stock solution 100µg/L(ppb) of Malachite Green.

•1 vial containing 0.8 ml Malachite Green HRP Enzyme Conjugate concentrate X 25.

•1 vial containing 9 ml of anti-Malachite Green antibody.

•1 vial containing 16 ml of Substrate.

•1 vial containing 12 ml of Stop Solution (Caution 1N HCI . Handle with care.)

•1 vial containing 15ml HRP conjugate diluent.

•2 X 25 mL bottles of Sample Dilution Buffer

•1 bottle containing 100 mL of Wash Solution concentrate 5X. Needs dilution prior to use.

•Reagent A (4X) (for Sample preparation)15 ml. **Remove from kit, aliquot and freeze upon receipt of kit.**

•Reagent B (for Sample preparation)12 g.

•Alumina , Neutral 100-200 mesh, 130 g.

•Instructions.

PRECAUTIONS

1. Each reagent is optimized for use in the Abraxis Malachite Green/Leucomalachite Green Plate Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Abraxis Malachite Green/Leucomalachite Green Plate Kits with different lot numbers.
2. Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
3. Do not use reagents after expiration date.

4. Reagents should be brought to room temperature, 20-28℃ (62-82℉) prior to use. Avoid prolonged (>24hours) storage at room temperature.
5. Malachite green is an antibiotic and should be treated with care.
6. For longer time storage, Reagent A should be removed from kit upon arrival, aliquoted (2 mL/vial) and frozen at – 20 °C. One of the frozen vials can be used for dilution (**Preparation of Reagents section**).
7. The Stop Solution is 1N hydrochloric acid, avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.

MATERIALS REQUIRED BUT NOT PROVIDED

1. Laboratory quality distilled or deionized water.
2. Graduated cylinder, 100 ml or larger .
3. Glassware for sample extraction and extract collection.
4. Acetonitrile (**Important:** HPLC grade, 99.9%**)**.
5. Pipet with disposable tips capable of dispensing 50µl
6. Multi-channel pipet; 8 channel capable of dispensing 50 and 100 µL
7. paper towels or equivalent absorbent material
8. Microwell plate or strip reader with 450nm filter
9. Timer.
10. Vortex mixer.

Preparation Reagents

Reagent A: Dilute Reagent a 1:4 with Acetonitrile (100%). Prepare fresh before each assay.

Standard dilution buffer: Mix 1 mL of acetonitrile with 9 mL of sample diluent provided. Cover and swirl to mix completely

HRP-conjugate solution: The conjugate provided in the kit is 25X concentrate. Before each assay, the conjugate should be diluted into conjugate diluent. For example mix 100ul of 25X concentrate conjugate with 2.4 ml of conjugate diluent to obtain the working conjugate.

CALIBRATOR PREPARATION

The Malachite green calibrator stock solution is diluted in **Standard dilution buffer** prior to use in the assay. Bring the stock Malachite Green calibrator stock solution to room temperature prior to diluting.

The stock standard will need to be diluted daily in 10% acetonitrile/sample dilution buffer (v/v) to prepare calibrators corresponding to 0, 0.005, 0.01, 0.02, 0.05, 0.2, and 0.5µg/L (ppb) of Malachite Green.

Label 6 clear 4 mL glass tubes or vials: “0.5”; “0.2”; “0.05”; “0.02”; “0.01”; “0.005”

- Standard at 0.5 ppb: 20 µL of 100 ppb stock into 3.98 mL of standard diluent

- Standard at 0.2 ppb: 400 µL of the 0.5 ppb standard into 600 µL of standard diluent
- Standard at 0.05 ppb: 200 µL of the 0.5 ppb standard into 1.8 mL of standard diluent
- Standard at 0.02 ppb: 400 µL of the 0.05 ppb standard into 600 µL of standard diluent
- Standard at 0.01 ppb: 400 µL of the 0.05 ppb standard into 1.6 mL of standard diluent.
- Standard at 0.005 ppb: 500 µL of the 0.01 ppb standard into 500 µL of standard diluent

Be sure to tightly cap the vials containing the stocks to minimize evaporative losses.

SAMPLE PREPARATION (Meat 1:50 Dilution)

1. Weigh 5g of tissue into a 20 or 40 mL glass vial
2. Add 10mL of Acetonitrile
3. Vortex (at max speed) for 1 minute
4. Place vial(s) in shaker/rotator for 10 minutes
5. Centrifuge vial(s) for 10 minutes at 4,000rpm
6. Transfer all of the supernatant into new vial(s)
7. Add 3g of Alumina (neutral) to vial(s)
8. Vortex for 1 minute
9. Centrifuge vial(s) for 10 minutes at 4,000rpm
10. Transfer 2mL of supernatant into new vial(s)
11. Evaporate supernatant at 60°C
12. Add 0.5mL of Reagent A (diluted 1:4) to dried vial(s)
13. Vortex for 30 seconds
14. Incubate vial(s) for 30 minutes
15. Add 0.1g of Reagent B to vial(s)
16. Vortex for 10 seconds and let stand for 15 minutes
17. Dilute 100 µl of supernatant with 1.9 mL of standard diluent (sample diluent buffer with 10% Acetonitrile)
18. Dilute 1:5 in sample diluent
19. Use 75 µl of sample in assay
20. Multiply the result by 50 to account for dilution.

TEST PROCEDURE (Note: Running calibrators and samples in duplicate will improve assay precision and accuracy.)

1. Allow reagents and sample extracts to reach room temperature prior to running the test. Fill a wash bottle with wash solution.
2. Place the appropriate number of test wells and into a microwell holder. Be sure to re-seal unused wells in the zip-lock bag with dessicant.
3. Using a pipet with disposable tips, add **75 µl of Calibrators or Sample extract** to the

appropriate test wells. Be sure to use a clean pipet tip for each. Add **75 µl of Antibody solution** to each well.

4. Rotate the plate gently for 30 seconds and incubate the test wells for **30 minutes**.
5. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and decant. Repeat 3X for a total of four washes. Tap the plate against absorbent paper.
6. Dispense **150 µL of Enzyme Conjugate** into each test well.
7. Rotate the plate gently for 30 seconds and incubate the test wells for **30 minutes**.
8. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with wash solution and decant. Repeat 3X for a total of four washes. Tap the plate against absorbent paper.
9. Dispense **150 µL of Substrate** into each well.
10. Incubate the wells for **20-30 minutes**.
11. Dispense **100 µL of Stop Solution** into each test well.
12. Read and record the absorbance of the wells at 450nm using a strip or plate reader.
13. Multiply ELISA results by appropriate sample dilution. If using the dilution given in the sample preparation procedure then multiply results by 50, resulting in an assay sensitivity of 0.25 ppb.

RESULTS INTERPRETATION

1. Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbance of the calibrator wells: Samples containing less color than a calibrator well have a concentration of Malachite Green/Leucomalachite Green greater than the concentration of the calibrator. Samples containing more color than a calibrator well have a concentration less than the concentration of the calibrator.
2. Quantitative interpretation requires graphing the absorbances of the calibrators (Y axis) versus the log of the calibrator concentration (X axis) on semi-log graph paper. A straight line is drawn through the calibrator points and the sample absorbances are located on the line .The corresponding point on the Y axis is the concentration of the sample. Samples with absorbances green than the lowest calibrator or less than the highest calibrator must be reported as <0.25 ppb or >25 ppb, respectively.

Alternatively, Abraxis can supply a spreadsheet template which can be used for data reduction. Please contact. Abraxis for further details.

GENERAL LIMITED WARRANTY

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