



## 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 (meat) 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 detects 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 a 30 minute incubation, the wells are washed with 1X wash solution. 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 cannot differentiate between the various Malachite Greens metabolites, but detects their presence to differing degrees. The following table shows the % cross reactivity of the assay.

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



## DETECTION LIMIT

Aquatic (meat) 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°C.

- Microtiter Plate containing 12 test strips of 8 wells each in a re-sealable pouch with desiccant.
- 1 vial containing 0.4 mL of Malachite Green calibrator stock solution (100 µg/L(ppb) of Malachite Green).
- 1 vial containing 0.8 mL Malachite Green HRP Enzyme Conjugate 25X concentrate.
- 1 bottle containing 9 mL of anti-Malachite Green antibody.
- 1 bottle containing 16 mL of Substrate.
- 1 bottle containing 12 mL of Stop Solution (Caution 1N HCl. Handle with care.)
- 1 bottle containing 15 mL of HRP conjugate diluent.
- 1 bottle containing 75 mL of Sample Dilution Buffer.
- 1 bottle containing 100 mL of Wash Solution 5X concentrate. Needs to be diluted with deionized water prior to use (see Preparation of Reagents).
- 1 bottle containing 15 mL of Reagent A (4X). **Remove from kit, aliquot (2 mL/vial) and freeze upon receipt of kit.** Needs to be prepared (diluted) fresh prior to sample preparation (see Preparation of Reagents).
- 1 bottle containing 12 g of Reagent B (for sample preparation).
- 1 bottle containing 130 g of Alumina, Neutral 100-200 mesh.
- 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°C (62-82°F) 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 thawed and diluted for sample preparation immediately before use (see **Preparation of Reagents**).



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 with skin 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. 4 mL clear glass vials with Teflon-lined caps for calibration standard dilutions
5. Acetonitrile (**Important:** HPLC grade, 99.9%)
6. Pipet with disposable tips capable of dispensing 20 - 200  $\mu\text{L}$
7. Multi-channel pipet; 8 channel capable of dispensing 50 - 150  $\mu\text{L}$
8. Paper towels or equivalent absorbent material
9. Microtiter plate or strip reader with 450nm filter
10. Timer
11. Vortex mixer

### **PREPARATION OF REAGENTS**

- **Wash Solution:** The wash solution is provided as a 5X concentrate solution. Dilute at a ratio of 1:5 with deionized water prior to use. If using the entire bottle (100 mL), add to 400 mL of deionized water.
- **Reagent A:** Dilute Reagent A 1:4 with Acetonitrile (HPLC grade, 99.9%) prior to use. For example, dilute 1 mL of Reagent A with 3 mL of Acetonitrile. Dilute fresh for each batch of samples to be prepared. Do not use previously diluted Reagent A as this may produce inaccurate results.
- **Standard Diluent:** Sample Dilution Buffer diluted 10% with Acetonitrile (HPLC grade, 99.9%). For example, mix 1 mL of Acetonitrile with 9 mL of the provided Sample Dilution Buffer. Cover and swirl to mix completely.
- **HRP-conjugate solution:** The conjugate provided in the kit is a 25X concentrate. Before each assay, the conjugate needs to be diluted into conjugate diluent. For example, mix 100  $\mu\text{L}$  of 25X concentrate conjugate with 2.4 mL of conjugate diluent to obtain the working conjugate.

### **CALIBRATION STANDARDS PREPARATION**

The Malachite Green calibrator stock solution is diluted in **Standard Diluent** (see Preparation of Reagents) prior to use in the assay. Bring the Malachite Green calibrator stock solution to room temperature prior to diluting.

The stock standard will need to be diluted daily in **Standard Diluent** (see Preparation of Reagents) to prepare calibrators corresponding to 0, 0.005, 0.01, 0.02, 0.05, 0.2, and 0.5  $\mu\text{g/L}$  (ppb) of Malachite Green.



Label 6 clear 4 mL glass vials (with Teflon-lined caps): “0.5”; “0.2”; “0.05”; “0.02”; “0.01”; “0.005”. Dilute the calibration standards as follows:

- Standard at 0.5 ppb: 20  $\mu$ L of 100 ppb stock into 3.98 mL of Standard Diluent
- Standard at 0.2 ppb: 400  $\mu$ L of the 0.5 ppb standard into 600  $\mu$ L of Standard Diluent
- Standard at 0.05 ppb: 200  $\mu$ L of the 0.5 ppb standard into 1.8 mL of Standard Diluent
- Standard at 0.02 ppb: 400  $\mu$ L of the 0.05 ppb standard into 600  $\mu$ L of Standard Diluent
- Standard at 0.01 ppb: 400  $\mu$ L of the 0.05 ppb standard into 1.6 mL of Standard Diluent
- Standard at 0.005 ppb: 500  $\mu$ L of the 0.01 ppb standard into 500  $\mu$ L of Standard Diluent

Be sure to tightly cap the vials containing the solutions to minimize evaporative losses. Remaining Standard Diluent will be used for the Standard at 0 ppb.

### **SAMPLE PREPARATION (Aquatic Meat 1:50 Dilution)**

1. Weigh 5 g of tissue into a 20 or 40 mL glass vial.
2. Add 10 mL of Acetonitrile (HPLC grade, 99.9%).
3. Vortex (at max speed) for 1 minute.
4. Place vial in shaker/rotator for 10 minutes.
5. Centrifuge vial for 10 minutes at 4,000rpm.
6. Transfer all of the supernatant into new vial.
7. Add 3g of Alumina (neutral) to vial.
8. Vortex for 1 minute.
9. Centrifuge vial for 10 minutes at 4,000 rpm.
10. Transfer 2 mL of supernatant into new vial.
11. Evaporate supernatant at 60°C.
12. Add 0.5 mL of diluted Reagent A (see Preparation of Reagents) to dried vial.
13. Vortex for 30 seconds.
14. Incubate vial for 30 minutes.
15. Add 0.1 g of Reagent B to vial.
16. Vortex for 10 seconds and let stand for 15 minutes.
17. Dilute 50  $\mu$ L of supernatant with 0.95 mL of Standard Diluent (see Preparation of Reagents).
18. Dilute 1:5 by adding 100  $\mu$ L to 400  $\mu$ L Sample Dilution Buffer in a new vial.
19. Use 75  $\mu$ L of sample (from step 18) in assay.
20. Multiply the result by 50 to account for dilution.



**TEST PROCEDURE** (Note: Analysis of calibrators and samples in duplicate is recommended to 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  $\mu$ L of Calibrators or Sample extract** to the appropriate test wells. Be sure to use a clean pipet tip for each. Add **75  $\mu$ L 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  $\mu$ L 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  $\mu$ L of Substrate** into each well.
10. Incubate the wells for **20-30 minutes**.
11. Dispense **100  $\mu$ L 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 factor. 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 greater 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.



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R040517

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