

**United States Department of Agriculture  
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Screening for Malachite Green and Crystal Violet by ELISA		
Revision: 02	Replaces: CLG-MGCV1.01	Effective: 06/20/2016

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**A. INTRODUCTION**

1. Background

Malachite green (MG) and crystal violet (CV) are triphenylmethane dyes. MG has been used as a topical fungicide and antiprotozoal agent in aquaculture. CV is also known to be effective in the treatment of fungal infections. Both compounds possess mutagenic properties and are banned for use in aquaculture. They are readily absorbed into fish tissue and metabolically reduced to leucomalachite green (LMG) and leucocrystal violet (LCV), respectively. These metabolites persist in the tissue of exposed fish.

2. Summary Of Procedure

Malachite green, leucomalachite green, crystal violet, and leucocrystal violet are extracted from fish tissue. Leucomalachite green and leucocrystal violet are then oxidized back to malachite green and crystal violet, respectively, and detected as malachite green and crystal violet using a competitive enzyme immunoassay. The assay is carried out on microtiter plates and the resulting color intensity is measured at 450 nm in a plate reader. The assay will not differentiate between malachite green, leucomalachite green, crystal violet, and leucocrystal violet. The result will be the sum of all four compounds.

3. Applicability

This method is suitable for the analysis of malachite green, leucomalachite green, crystal violet, and leucocrystal violet in fish of the order Siluriformes (catfish) at levels  $\geq 1$  ppb.

*Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA*

**B. EQUIPMENT**

Note: Equivalent apparatus or instrumentation may be substituted unless specified for any of the following.

1. Apparatus

- a. Malachite Green (MG)/LMG ELISA Test Kit-Cat. No.1019-06, Bioo Scientific Corp., Austin, TX.

Note: No substitution for this kit is recommended. Kits are stored in the refrigerator at 2 - 8 °C. If you are not planning to use the kit for over 1 month, store 100X MG- Biotin Conjugate and Malachite Green Standards in a freezer as noted in kit instructions.

This kit contains:

- i. Malachite green antibody-coated microtiter plates (96 wells).

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- ii. Malachite green standards (1.8 mL each, ready to use): 0 ppb, 0.05 ppb, 0.15 ppb, 0.5 ppb, 1.5 ppb, 4.5 ppb; 100 ppb fortification solution of malachite green (1.8 mL); 50 ppb fortification solution of leucomalachite green (1.8 mL) – upon special request see D.1.c.
  - iii. 100X MG Biotin Conjugate (80 µL).
  - iv. MG Biotin Conjugate Diluent (6 mL).
  - v. 100X Streptavidin-HRP (200 µL).
  - vi. Streptavidin-HRP Diluent (15 mL).
  - vii. 20X Wash Solution (28 mL).
  - viii. Stop Buffer (20 mL).
  - ix. TMB Substrate (12 mL).
  - x. Concentrate of Sample Extraction Buffer A (20 g containing Reagent I and II).
  - xi. 10X Sample Extraction Buffer B (10 mL).
  - xii. 10X Sample Extraction Buffer D (15 mL).
  - xiii. 10X Oxidant Solution (1.8 mL).
  - b. Centrifuge tubes - 50 mL and 15 mL with graduation, polypropylene, Falcon.
  - c. Timer - Control Company.
  - d. Vortex mixer - Vortex Genie, Fisher.
  - e. N-EVAP - Organomation Associates, Inc.
  - f. Centrifuges - Eppendorf (50 mL tubes) and VWR (15 mL tubes).
  - g. Pipettors - capable of dispensing volumes from 1 µL to 5 mL, Rainin and Gilson.
  - h. Multi-channel pipettor (50-300 µL) - Rainin.
  - i. Graduated cylinder - 100 mL.
  - j. Low actinic volumetric flasks - 100 mL, 50 mL, and 10 mL.
  - k. Transfer pipettes (Pasteur pipettes).
  - l. Glass bottles - 100 mL.
  - m. Dispensette - 2.5 to 25 mL (Easy calibration brand).
2. Instrumentation
- a. Microplate reader - EL 808 Ultra, Bio-Tek Instruments.

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**C. REAGENTS AND SOLUTIONS**

*Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependant on the expiration dates of the compounds used. The maximum length of time that a working reagent shall be used is 1 year unless the laboratory has produced extension data.*

1. Reagents

- a. Dichloromethane - HPLC grade, Fisher.
- b. Acetonitrile - HPLC grade, Acros.
- c. n-Hexane - HPLC grade, Burdick and Jackson.
- d. Water - HPLC grade, Fisher.

2. Solutions

Note: All solutions should be stored refrigerated at 2 - 8 °C.

- a. Preparation of 1X oxidant solution:

Mix 1 volume of 10X oxidant solution with 9 volumes of acetonitrile. For example, this can be achieved by pipetting 500 µL of 10X oxidant solution into 4.5 mL acetonitrile (4.5 mL acetonitrile can be pipetted with a 5.0 mL pipettor).

- b. Preparation of 1X sample extraction buffer A:

Carefully open sample extraction buffer A concentrate bag, remove reagent I and II bags and mix them in a glass bottle. Add 100 mL of distilled or HPLC grade water to dissolve the reagents.

Note: Follow example from C.2.a. to prepare solutions C.2.c. through C.2.h.

- c. Preparation of 1X sample extraction buffer B:

Mix 1 volume of 10X sample extraction buffer B with 9 volumes of distilled or HPLC grade water.

- d. Preparation of 1X sample extraction buffer D:

Mix 1 volume of 10X sample extraction buffer D with 9 volumes of distilled or HPLC grade water.

- e. Extraction buffer D/acetonitrile solution:

Mix 4 volumes of 1X sample extraction buffer D with 1 volume of acetonitrile (4:1 v/v).

- f. Preparation of 1X wash solution:

Mix 1 volume of 20X wash solution with 19 volumes of distilled or HPLC grade water.

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- g. Preparation of 1X MG-biotin conjugate:  
Mix 1 volume of 100X biotin conjugate with 99 volumes of biotin conjugate diluent. **IMPORTANT:** prepare fresh within 5 minutes of use and prepare only the amount needed.
- h. Preparation of 1X streptavidin-HRP:  
Mix 1 volume of 100X streptavidin-HRP with 99 volumes of streptavidin-HRP diluent. **IMPORTANT:** prepare fresh within 10 minutes of use and prepare only the amount needed.
- i. 50:50 acetonitrile/water:  
Mix 1 part acetonitrile with 1 part water. Use a graduated cylinder.

**D. STANDARDS**

*Note: Equivalent standards / solutions may be substituted. Purity and counterions are to be taken into account when calculating standard concentrations. The stability time frame of the solution is dependant on the expiration date of the components used. In-house prepared standards shall be assigned an expiration date that is no later than the expiration date of the earlilest expiring component or no later than the stability stated in the method, whichever ends soonest. The maximum length of time that an in-house prepared standard shall be used is 1 year unless the laboratory has produced extension data.*

- 1. Source
  - a. Leucomalachite green (Cat. No. 125660) - Sigma-Aldrich
  - b. Leucocrystal violet (Cat. No. 21915) - Sigma-Aldrich.
  - c. Leucomalachite green at 50 ng/mL- (special order Cat. No. 1019), Bioo Scientific.

2. Preparation

Note: Equivalent standard solutions may be prepared.

- a. Stock standard solutions of leucomalachite green at 100 µg/mL:  
Weigh approximately 10 mg of leucomalachite green (D.1.a) into a 100 mL low actinic volumetric flask. Record the exact weight. Bring to volume with acetonitrile. Store at 2 - 8 °C for 3 months.
- b. Stock standard solutions of leucocrystal violet at 100 µg/mL:  
Weigh approximately 10 mg of leucocrystal violet (D.1.b) into a 100 mL low actinic volumetric flask. Record the exact weight. Bring to volume with acetonitrile. Store at 2 - 8 °C for 3 months.

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- c. Intermediate standard solution of leucomalachite green at 1 µg/mL (prepared from stock standard solution):

Note: The amount of the stock standard solution pipetted depends on the actual weighed amount.

Use a low actinic flask. Based on the desired volume of intermediate standard, calculate the volume of stock standard solution needed to produce a 1 µg/mL intermediate standard solution. Add this volume to the flask and dilute to volume with the acetonitrile/water (50:50) mixture. For example: the standard weight is 15 mg/100 mL; pipet 6.7 µL of stock standard into a low actinic flask and add 993 µL acetonitrile/water (50:50 v/v). Store at 2 - 8 °C for 1 month.

- d. Intermediate standard solution of leucocrystal violet at 1 µg/mL (prepared from stock standard solution):

Note: The amount of the stock standard solution pipetted depends on the actual weighed amount.

Use a low actinic flask. Based on the desired volume of intermediate standard, calculate the volume of stock standard solution needed to produce a 1 µg/mL intermediate standard solution. Add this volume to the flask and dilute to volume with the acetonitrile/water (50:50) mixture. For example: the standard weight is 15 mg/100 mL; pipet 6.7 µL of stock standard into a low actinic flask and add 993 µL acetonitrile/water (50:50 v/v). Store at 2 - 8 °C for 1 month.

- e. Fortification standard solution of leucomalachite green at 100 ng/mL:

Mix 1 volume of intermediate standard solution (D.2.c) with 9 volumes of acetonitrile/water (50:50 v/v). For example: pipet 100 µL of intermediate standard solution into a flask and add 900 µL of acetonitrile/water (50:50 v/v); .Use low actinic flasks. Store at 2 - 8 °C for 1 month.

- f. Fortification standard solution of leucocrystal violet at 100 ng/mL:

Mix 1 volume of intermediate standard solution (D.2.d.) with 9 volumes of acetonitrile/water (50:50 v/v). For example: pipet 100 µL of intermediate standard solution into a flask and add 900 µL of acetonitrile/water (50:50 v/v); .Use low actinic flasks. Store at 2 - 8 °C for 1 month.

**E. SAMPLE PREPARATION**

Fish of the order Siluriformes (catfish) samples must be processed long enough to produce a homogenous blend of tissues, but not long enough to become warm.

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**F. ANALYTICAL PROCEDURE**

Note: The following steps are based on kit manufacturer instructions and may be subject to change. If any discrepancies exist, follow the current manufacturer instructions. Two recoveries are used. One is fortified with leucomalachite green and one with leucocrystal violet. Internal checks are fortified with either leucomalachite green or leucocrystal violet. Malachite green and crystal violet spikes are not necessary, because the “leuco” compounds are converted to the respective “parent” compound. Only the “parent” compound is detected by the kit.

**1. Preparation of Controls and Samples**

- a. Weigh  $2.0 \pm 0.1$  g of sample into a 50 mL polypropylene centrifuge tube..
- b. Weigh out  $2.0 \pm 0.1$  g portions of a known blank tissue into a polypropylene centrifuge tube for each of the following Quality Control samples as needed:
  - i. A tissue blank (negative control) - Three needed for each analytical batch.
  - ii. Leucomalachite Green Positive Control - Fortify the control sample with 20  $\mu$ L of the 100 ng/mL Fortification Standard Solution for a concentration of 1.0 ppb.
  - iii. Leucocrystal Violet Positive Control - Fortify the control sample with 20  $\mu$ L of the of the 100 ng/mL Fortification Standard Solution for a concentration of 1.0 ppb.
  - iv. An internal check sample (as needed).

**2. Extraction Procedure**

- a. Add 0.6 mL of 1X sample extraction buffer A, 0.4 mL of 1X sample extraction buffer B and 6.5 mL of acetonitrile.
- b. Vortex for 3 minutes at maximum speed.
- c. Add 3.0 mL of water and 2.0 mL of dichloromethane.
- d. Mix gently for 1 minute.
- e. Centrifuge the samples for 5 minutes at 4000 g..
- f. Transfer approximately 3 mL of the upper organic layer into a graduated 15 mL polypropylene centrifuge tube containing 100  $\mu$ L of 1X oxidant solution.
- g. Keep the samples at room temperature ( $\sim 20 - 25$  °C) for  $25 \pm 5$  minutes.
- h. Bring the samples to dryness on an N-EVAP by blowing nitrogen gas in a 50 - 60 °C water bath.
- i. Add 2 mL of n-hexane to dissolve the sample and then add 1.6 mL of extraction buffer D/acetonitrile solution.
- j. Mix the samples gently for 1 minute.

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- k. Centrifuge the samples for 10 minutes at 4000 g..
- l. Aspirate the upper organic layer completely.
- m. Use 90 µL of the lower aqueous layer for the ELISA.

Note: The prepared sample can be tested within 24 hours. The dilution factor is 2.0.

3. ELISA

Note: Bring all reagents to room temperature (~ 20 - 25 °C) before use (about 2 hours). Return all reagents to 2 - 8 °C after use. Mix all reagents by gently inverting or swirling prior to use. Prepare volumes needed for the number of microwells used. Do not allow microwells to completely dry between working steps. Do not apply more than three test strips when a single step pipette is used. Run one 0 ppb, one 0.05 ppb, one 0.15 ppb and one 0.50 ppb standard with each set to establish plate acceptability. Use the kit supplied standards.

Insert sufficient number of wells into a microwell holder. Record standard and sample positions.

- a. Pipet 90 µL of standard or extracted sample into separate wells; use a new pipette tip for each standard or sample. Pipet standards in increasing concentration. Plate the sample extracts of the 3 tissue blanks in duplicate. Plate one set of duplicates at the beginning, one set in the middle and one set towards the end of the sample set. Plate the sample extracts of the recoveries in duplicate; plate them once at the beginning and once at the end of the set. Plate the sample extract of the internal check in duplicate.
- b. Pipet 30 µL of 1X MG-Biotin conjugate into each well.
- c. Mix well by gently rocking the plate manually for 1 minute.
- d. Incubate the plate for 30 minutes at room temperature (~ 20 - 25 °C) in the dark (i.e. lab counter drawer).
- e. Wash the plate 3 times with approximately 250 µL of 1X wash solution using a multi-channel pipettor or wash bottle. After the last wash, tap dry the plate on a paper towel. Do not allow the plate to air dry between working steps.
- f. Pipet 100 µL of 1X Streptavidin-HRP into each well and mix manually by gently rocking the plate.
- g. Incubate the plate for 15 minutes at room temperature (~ 20 - 25 °C) in the dark.
- h. Wash the plate 3 times with approximately 250 µL of 1X wash solution using a multi-channel pipettor or wash bottle. After the last wash tap dry the plate on a paper towel. Do not allow the plate to air dry between working steps.
- i. Pipet 100 µL of TMB substrate into each well. Any substrate solution showing discoloration is indicative of deterioration and should be discarded.

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- j. Time the reaction immediately after adding the substrate. Mix the solution by gently rocking the plate manually for 1 minute while incubating. Incubate for  $15 \pm 1$  minutes at room temperature ( $\sim 20 - 25$  °C) in the dark.
  - k. Pipet 100  $\mu$ L of stop buffer into each well.
  - l. Measure the absorbance at 450 nm against an air blank. Read within 10 minutes.
4. Instrumental settings  
Use a plate reader set at 450 nm for evaluation.
5. Sample Set  
For each batch of up to 20 samples on ELISA plate, include:
- a. Standards at 0, 0.05, 0.15, and 0.50 ppb
  - b. Positive control for leucomalachite green at 1 ppb
  - c. Positive control for leucocrystal violet at 1 ppb
  - d. 3 blank tissue controls
  - e. Check sample (if needed)
  - f. Samples

**G. CALCULATIONS**

Average the UV absorbance readings of the recoveries and internal check wells since they are run in duplicate.

Calculate the average (AVG), standard deviation (SD) and coefficient of variation (CV) for the six tissue blank UV absorbance readings. Calculate the decision level (DL) as follows:

Decision level (DL): Average tissue blank UV absorbance reading – 3 x standard deviation.

$$DL = \text{AVG}_{\text{Blank}} - 3 \times \text{SD}$$

Any sample having an UV absorbance reading of  $\leq$  DL is considered positive.

**H. SAFETY INFORMATION AND PRECAUTIONS**

- 1. Required Protective Equipment — Wear gloves, laboratory coat and safety glasses.
- 2. Hazards

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<i>Procedure Step</i>	<i>Hazard</i>	<i>Recommended Safe Procedures</i>
Dichloromethane	Vapors are harmful to skin, eyes, and respiratory system. Potential carcinogen and teratogen.	Work in fume hood.
n-Hexane	Flammable. Harmful if swallowed or inhaled. Causes respiratory, eye, and skin irritation.	Work in fume hood. Keep away from flame or heat.
Acetonitrile	Flammable. Harmful if swallowed or inhaled. Causes respiratory, eye, and skin irritation.	Work in fume hood. Keep away from flame or heat.
Leucomalachite green	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood.
Leucocrystal violet	Harmful if inhaled or ingested. Causes eye, skin, and respiratory irritation.	Work in fume hood.

3. Disposal Procedure

Follow federal, state and local regulations

**I. QUALITY ASSURANCE PLAN**

1. Performance Standard

- a. A plate must meet all of the following criteria:
  - i. No false negatives for the 1 ppb recoveries or false positives for the negative controls
  - ii. The absorbance must decrease from the 0 ppb to the 0.50 ppb standard wells.
  - iii. A variability in absorbance of less than  $\pm 25\%$  between duplicate measurements of the recoveries and internal check is acceptable. This is determined as follows:  $|x-y| / [(x+y)/2] * 100$
  - iv. The coefficient of variation (CV) of the six tissue blank measurements has to be  $\leq 20\%$ .

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2. Critical Control Points and Specifications

<i>Record</i>	<i>Acceptable Control</i>
a. Incubation time (F.3.j.)	15 minutes ± 1 minute
b. TMB substrate (F.3.i.)	Discard if discolored
c. Incubation time (F.2.i.)	25 minutes ± 5 minutes

Note: See “Warnings and Precautions” in the kit manual for additional instructions.

3. Intralaboratory Check Samples

- a. System, minimum contents.
  - i. Frequency: One per week per analyst when samples analyzed.
  - ii. Records are to be maintained.
- b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

  - i. Investigate following established procedures.
  - ii. Take corrective action as warranted

4. Sample Condition upon receipt: cold, unspoiled and sealed from air.

**J. APPENDIX**

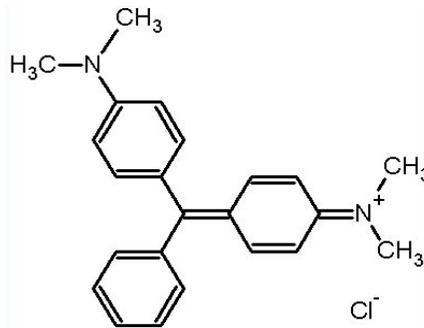
1. References

- a. Test kit instructions Malachite Green/LMG, Bioo Scientific
- b. Confirmatory analysis of malachite green, leucomalachite green, crystal violet and leucocrystal violet in salmon by liquid chromatography-tandem mass spectrometry. Geraldine Dowling, Patrick P.J. Mulder, Conor Duffy, Liam Regan, Malcolm R. Smyth, *Analytica Chimica Acta* **586**, (2007), 411-419 --for standard stability information.

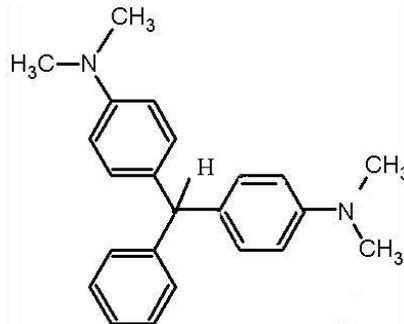
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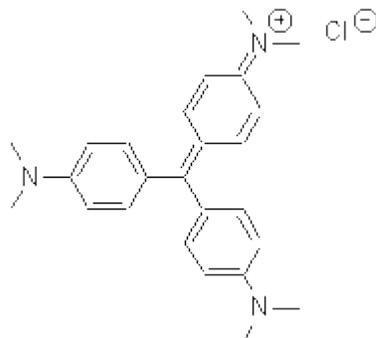
2. Structure



Malachite green



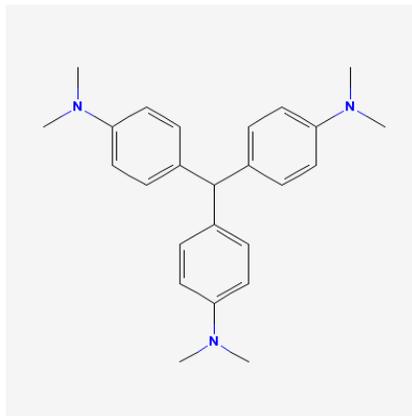
Leucomalachite green



Crystal violet

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Leucocrystal violet

3. Minimum Level of Applicability (MLA)
  - a. Leucomalachite green, malachite green, crystal violet, and leucocrystal violet:  
1 ppb

**K. APPROVALS AND AUTHORITIES**

1. Approvals on file.
2. Issuing Authority: Director, Laboratory Quality Assurance Staff.