MALACHITE GREEN TOTAL/CRYSTAL VIOLET ELISA

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A competitive enzyme immunoassay
for screening and quantitative analysis of
malachite green, leucomalachite green, crystal
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BRIEF INFORMATION

The EuroProxima Malachite Green Total/Crystal Violet ELISA is a competitive enzyme immunoassay for the screening and quantitative detection of malachite green, leucomalachite green, crystal violet and leucocrystal violet in fish and shrimp. Samples and standards are measured in duplicate. A total of 40 samples can be analysed with one kit.
Malachite Green Total/Crystal Violet ELISA contains all required reagents, including standards, to perform the test. Some reagents required for sample preparation are also included.

1. INTRODUCTION

Triphenylemethane dyes such as malachite green (MG) and crystal violet (CV) are a group of organic dyes widely used in industry for dying purposes. They have been also used in aquacultures to treat and prevent fungal and parasitic infections in fish, however due to their toxic effects they have never been authorised to be used in fish for human consumption. These dyes are extensively absorbed and metabolised to the reduced leuco forms: leucomalachite green (LMG) and leucocrystal violet (LCV) that can persist in tissues for a long time. The detection of the residue of these dyes in aquaculture products indicate illegal use as only zero levels are accepted. In the European Union the minimum required performance limit for a method for the detection of a sum of malachite green and leucomalachite green is 2 µg/kg (Commission Decision 2002/657/EC). In accordance with Commission Decision 2002/994/EC any imports of aquaculture fishery products from China should be tested for the presence of malachite green, crystal violet and their metabolites.
To provide a new method for simultaneous detection of residues of both malachite green and crystal violet, R-Biopharm Nederland developed the Malachite Green Total/Crystal Violet (MGT) ELISA. The test is applicable for analysis of fish and shrimp samples. During the sample preparation procedure, malachite green, crystal violet, leucomalachite green and leucocrystal violet are first extracted from the homogenised samples. Then leucomalachite green and leucocrystal violet are converted to their parent compounds in a fast oxidation step. Samples are finally analysed by Malachite Green Total/Crystal Violet ELISA and results are expressed as total MG concentration: a sum of malachite green, crystal violet, leucomalachite green and leucocrystal violet.

2. PRINCIPLE OF MALACHITE GREEN TOTAL/CRYSTAL VIOLET ELISA

The microtiter plate based Malachite Green Total/Crystal Violet ELISA consists of one precoated plate with antibody against MG (12 strips, 8 wells each). Horseradish peroxidase (-HRP) labeled MG and standard solution or sample are added to the wells. Free MG/CV from the samples or standards and MG-HRP conjugate compete for the specific antibody binding sites (competitive enzyme immunoassay). After an incubation time of 30 min at room temperature, the non-bound reagents are removed in a washing step. The amount of bound MG-HRP conjugate is visualized by the addition of a substrate/chromogen solution (tetramethylbenzidine, TMB). During the incubation the colourless chromogen is converted by the peroxidase enzyme into a blue reaction product. This blue colour is inversely proportional to the amount of bound MG/CV. The more MG/CV is present in the standard solution or sample, the less colour is developed. The enzymatic reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at a wavelength of 450 nm.

3. SPECIFICITY AND SENSITIVITY

The cross-reactivity pattern of the antibody is as follows:

Malachite green (MG)	100%
Crystal violet (CV)	65%
Leucomalachite green (LMG)	100% (after oxidation)
Leucocrystal violet (LCV)	65% (after oxidation)

The cross-reactivities are determined in a buffer system. The reported values may be different in samples due to matrix effects.
The test cannot discriminate between analytes and cross-reactive substances.

The Limit of detection (LOD) and the detection capability (CCβ) are determined under optimal conditions. Cut-off criteria need critical consideration.

Matrix	**LOD (ng/g)**	**CCβ (ng/g)**					
		MG	LMG	CV	LCV	MG+LMG	CV+LCV
Fish and shrimp	0.12	0.3	0.3	0.3	0.3	0.3[1]	0.3[1]

[1]0.15 ng/g of MG (or CV) and 0.15 ng/g of LMG (or LCV) = 0.30 ng/g total MG+LMG (or CV+LCV)

If the sample is found to be non-compliant, the results shall be verified by re-analysis of the sample using a confirmatory method.

4. HANDLING AND STORAGE

- Kit and kit components should be stored at 2°C to 8°C in a dark place. For repeated use store kit components as specified under chapter 9.
- After the expiry date of the kit and/or components has passed, no further quality guarantee is valid.
- Bring all kit components including the microtiter plate to ambient (room) temperature before use.
- Dilute the kit components immediately before use, but after the components are brought to ambient temperature.
- Avoid condensation in the wells of the plate. Bring the sealed plate to ambient temperature before opening the plate sealing.
- The substrate chromogen solution can be stored in a refrigerator (2ºC to 8ºC) until the expiry date stated on the label.
- Exposure of the chromogen solution to light should be avoided.

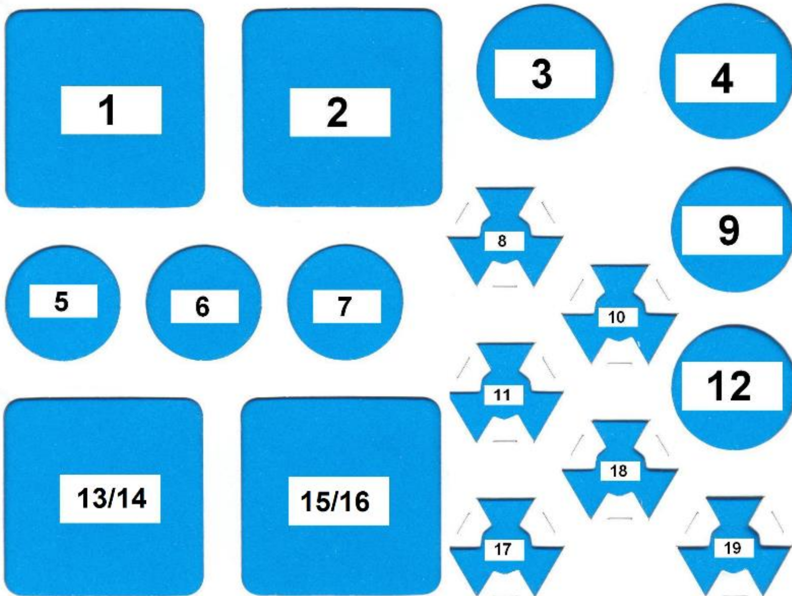
Degeneration of the reagents may have occurred when the following phenomena are observed:

- A blue colouring of the chromogen solution before transferring it into the wells.
- A weak or no colour reaction in the zero standard wells (E450nm < 0.8).

5. KIT CONTENTS

- Manual
- One sealed (96-wells) microtiter plate (12 strips, 8 wells each), coated with anti-MG antibody. Ready-to-use.
- One scoop spatula

Position of the reagents in the kit. For preparation of the reagents see Chapter 9.



1. **Extraction buffer** (50 ml, ready-to-use)
2. **Rinsing buffer** (30 ml, 20x concentrated)
3. **Substrate solution** (12 ml, ready-to-use)
4. **Stop solution** (12 ml, ready-to-use)
5. Not in use
6. Not in use
7. Not in use
8. **MG-HRP Conjugate** (100 µl, 100x concentrated)
9. **PBS buffer for conjugate** (13 ml, ready-to-use)
10. **MG standard** (500 µl, 10 µg/ml)
11. Not in use
12. Not in use

13/14. **Neutral aluminium oxide** (approx. 45 g)
15/16 **Sample dilution buffer** (40 ml, ready-to-use)
17. Not in use
18 **Oxidising solution** (1 ml, 10x concentrated)
19 **LMG spiking solution** (150 µl, 10 µg/ml)

6. EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- Scales and weighing vessels
- Gloves
- Fume hood
- Homogeniser
- Vortex mixer
- Centrifuge (2500 × g)
- Automated microplate washer or 8-channel micropipette 100 – 300 µl
- Microtiter plate shaker
- Microtiter plate reader with 450 nm filter
- Micropipettes 20 – 200 µl, 100 – 1000 µl
- Multipipette with 2.5 ml combitips
- Aluminium foil or parafilm
- Deionized or distilled water
- Acetonitrile
- Perchloric acid (70-72%)
- n-Hexane
- 15 ml polypropylene centrifuge tubes
- 4 ml glass test tubes with stoppers

7. PRECAUTIONS

- This kit may contain hazardous substances. For hazard notes please refer to the appropriate safety data sheets (SDS).
- Avoid contact of all biological materials with skin and mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area.
- Do not use components past expiration date and do not use components from different lots.
- Each well is ultimately used as an optical cuvette. Therefore, do not touch the under surface of the wells, prevent damage and dirt.
- All components should be completely dissolved before use. Take special attention to the substrate and rinsing buffer, which crystallize at +4ºC.
- Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain good precision and accuracy.

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8. SAMPLE PREPARATION

8.1 Fish and shrimp

- Weigh 0.5 g of homogenised sample into a 15 ml polypropylene centrifuge tube.
- Add 5 ml of acidified acetonitrile (see Chapter 9) and shake intensively or vortex for 2 min.
- Add 0.75 g – 1 g* of neutral aluminium oxide and 1 ml of the extraction buffer. Shake or vortex for further 1 min.
- Centrifuge for 5 min at 2500 × g.
- Transfer 1 ml of the upper solvent layer into a glass test tube and evaporate to dryness at 50°C under a stream of nitrogen.
- Reconstitute the residue in 100 µl of the diluted oxidising solution (see Chapter 9), vortex for 10 s, then add 300 µl of the sample dilution buffer immediately and vortex for 5 s.
- Add 400 µl of n-hexane to each sample and vortex for 5 s.
- Centrifuge for 10 min at 2500 × g.
- Remove the upper hexane layer and dilute the lower layer 5 times in the sample dilution buffer, i.e. 50 µl of the extracted sample + 200 µl of the sample dilution buffer.
- The sample is now ready to apply to the microtiter plate.

*Use the spatula provided with the kit. One flat scoop of the larger end (1 ml) is the amount of aluminium oxide that should be added to each sample.

9. PREPARATION OF REAGENTS

Before starting the assay, reagents should be brought to ambient temperature (20ºC - 25ºC). Any unused reagents should be stored immediately at +2ºC to +8ºC.
Prepare reagents freshly before use.

Microtiter plate
Bring the plate to ambient temperature before opening, to avoid condensation in the wells. Return unused strips into the zip resealable bag with desiccant and store at +2°C to +8°C for use in subsequent assays. Retain also the strip holder.

Rinsing buffer
The rinsing buffer is delivered 20 times concentrated. Prepare the dilution freshly before use. For each strip 20 ml of diluted rinsing buffer is used (1 ml concentrated rinsing buffer + 19 ml distilled water).

Sample dilution buffer
The sample dilution buffer for dilution of samples is supplied ready-to-use.

PBS buffer for Conjugate
The conjugate dilution buffer (PBS buffer) for diluting concentrated MG-HRP conjugate is supplied ready-to-use.

MG-HRP conjugate solution
The MG-HRP conjugate is delivered 100× concentrated. Spin down the conjugate in the vial by a short centrifugation step (1 min, 1000 × g). At least 0.8 ml of the diluted conjugate is required for two strips – 2 x 8 wells. Add 10 µl of the concentrated conjugate solution to 990 µl of PBS conjugate dilution buffer before use. Prepare the volume of diluted conjugate required for the number of strips to be used. Store unused concentrated conjugate at +2ºC to +8ºC.

Standards dilution buffer
Prepare 5% acetonitrile in the sample dilution buffer. Add 0.25 ml of acetonitrile to 4.75 ml of the sample dilution buffer and use it to prepare MG standards.

Standards
Prepare MG dilution series in the standards dilution buffer (5% acetonitrile in sample dilution buffer) starting from MG standard 10 µg/ml provided with the kit.
Intermediate dilutions MG:

100 ng/ml	10 µl of 10 µg/ml MG standard + 990 µl of standards dilution buffer
10 ng/ml	50 µl of 100 ng/ml + 450 µl of standards dilution buffer

Standard curve MG:

0.6 ng/ml	30 µl of 10 ng/ml + 470 µl of standards dilution buffer
0.24 ng/ml	200 µl of 0.6 ng/ml + 300 µl of standards dilution buffer
0.096 ng/ml	200 µl of 0.24 ng/ml + 300 µl of standards dilution buffer
0.0384 ng/ml	200 µl of 0.096 ng/ml + 300 µl of standards dilution buffer
0.01536 ng/ml	200 µl of 0.0384 ng/ml + 300 µl of standards dilution buffer
0.006144 ng/ml	200 µl of 0.01536 ng/ml + 300 µl of standards dilution buffer

Prepare the standards freshly before use.

Substrate/chromogen solution

The substrate/chromogen solution (ready-to-use) tends to precipitate at +4ºC. Take care that this vial is at room temperature when used (keep in the dark) and mix the content before pipetting into the wells.

Extraction buffer

The extraction buffer is supplied ready-to-use.

Oxidising solution

The oxidising solution is supplied 10× concentrated. Dilute the concentrated solution in acetonitrile freshly before use. At least 1 ml is required for 8 samples. Add 100 µl of the concentrated oxidising solution to 900 µl of acetonitrile and mix well. Prepare the appropriate volume of the oxidising solution required for the number of samples to be analysed. Prepare the diluted oxidising solution in glass vial, protect from light and use within one day. Store unused concentrated oxidising solution at +2ºC to +8ºC.

Acidified acetonitrile

Dilute concentrated (70-72%) perchloric acid 1250× in acetonitrile. In order to prepare enough for the extraction of 8 samples add 40 µl of perchloric acid to 50 ml of acetonitrile. Mix well and use it at the same day. Prepare the appropriate volume of the acidified acetonitrile needed for the extraction of the number of samples to be analysed.

10. ASSAY PROCEDURE

Rinsing protocol

Unbound components have to be removed efficiently between each incubation step in ELISAs. This is achieved by appropriate rinsing. Each rinsing procedure must be carried out with care to guarantee good inter- and intra-assay results.
Manual rinsing or rinsing with automatic plate wash equipment can be performed as follows:

Manual rinsing

1. Empty the contents of each well by turning the microtiter plate upside down followed by a firm short vertical movement.
2. Fill all the wells to the rims (300 µl) with rinsing solution.
3. This rinsing cycle (1 and 2) should be carried out 3 times.
4. Turn the plate upside down and empty the wells by a firm short vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove residual washing solution in the wells.
6. Take care that none of the wells dry out before the next reagent is dispensed.

Rinsing with automatic microtiter plate wash equipment
When using automatic plate wash equipment, check that all wells can be aspirated completely, that the rinsing solution is nicely dispensed and is reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute three rinsing cycles.

Assay Protocol

1. Prepare samples according to Chapter 8 and prepare reagents according to Chapter 9.

2. Pipette 100 µl of the standards dilution buffer in duplicate (wells H1, H2, blank). Pipette 50 µl of the zero standard (standards dilution buffer) in duplicate (wells A1, A2, maximal signal). Pipette 50 µl of each of the standard solutions in duplicate (wells B1,2 to G1,2 i.e. 0.006144, 0.01536, 0.0384, 0.096, 0.24, 0.6 ng/ml).

3. Pipette 50 µl of each sample solution in duplicate into the remaining wells of the microtiter plate.

4. Pipette 50 µl of the diluted MG-HRP conjugate into all wells, except the blank H1 and H2.

5. Seal the microtiter plate and shake the plate for a few seconds on a microtiter plate shaker.

6. Incubate for 30 minutes in the dark at room temperature (20°C -25°C).

7. Discard the solution from the microtiter plate and wash 3 times with rinsing buffer.
8. Pipette 100 µl of substrate solution into each well.
9. Incubate 30 minutes at room temperature (20ºC-25ºC).
10. Add 100 µl of stop solution into each well.
11. Read the absorbance values immediately at 450 nm.

11. INTERPRETATION OF RESULTS

Subtract the mean optical density (O.D.) of the wells H1 and H2 from the individual O.D. of the wells containing the standards and the samples.

The O.D. values of the six standards and the samples (mean values of the duplicates) are divided by the mean O.D. value of the zero standard (wells B1 and B2) and multiplied by 100. The zero standard is thus made equal to 100% (maximal absorbance) and the other O.D. values are quoted in percentages of the maximal absorbance.

O.D. standard (or sample)
-----------------------------------x 100 = percentage maximal absorbance
O.D. zero standard

<u>Calibration curve:</u>
The values in percentage of the maximal absorbance calculated for the standards are plotted (on the Y-axis) versus the MG equivalent concentration (ng/ml) on a logarithmic X-axis.

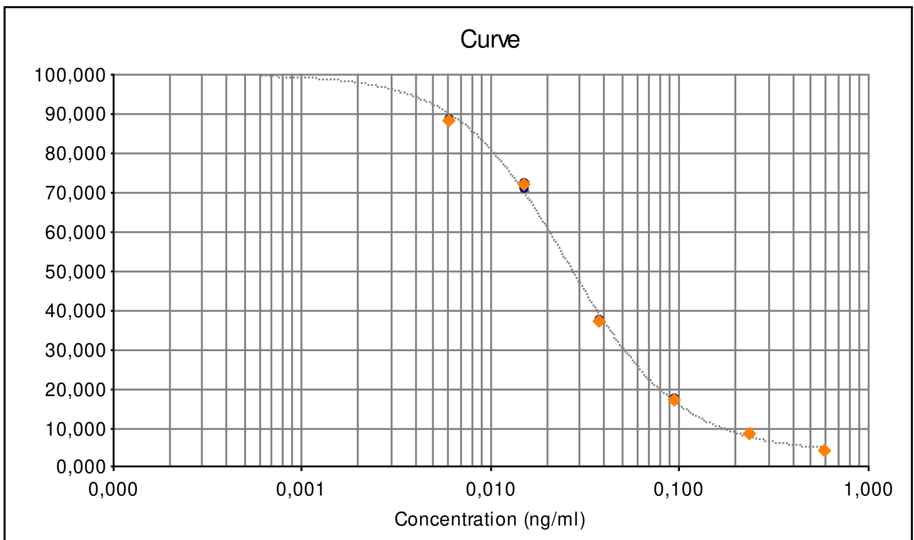

Figure 1: Example of a calibration curve.

<u>Calculation factor</u>

To obtain the total MG concentration in a sample (ng/g), the MG equivalent read from the calibration curve has to be multiplied by a factor 20.

12. LITERATURE

Commission Decision of 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). OJ L 221, 17.8.2002, p. 8. (with 8 amendments as on 1 July 2015).

Commission Decision of 20 December 2002 concerning certain protective measures with regard to the products of animal origin imported from China (2002/994/EC) OJ L 348, 21.12.2002, p. 154. (with 2 amendments as on 22 December 2003).

Wendy C. Andersen, José E. Roybal, Sherri B. Turnipseed. (2004) Determination of Malachite Green and Leucomalachite Green in Salmon with *In-Situ* Oxidation and Liquid Chromatography with Visible Detection. FDA/ORA/DFS Laboratory Information Bulletin No. 4334, 20(11), 1-13.

13. ORDERING INFORMATION

For ordering the EuroProxima Malachite Green Total/Crystal Violet ELISA, please use catalogue code 5161MGT.

14. REVISION HISTORY

Product name has been changed from EuroProxima Malachite Green Total ELISA to EuroProxima Malachite Green Total/Crystal Violet ELISA.

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