



Malachite green ELISA Kit (OKAO00134) Instruction for Use

For the quantitative detection of Malachite green (SDM) concentration in tissue
(Fish, shrimp).

This product is intended for research use only.

Table of Contents

1. Background	2
2. Assay Summary	错误! 未定义书签。
3. Storage and Stability.....	2
4. Kit Components	3
5. Required Materials Not Supplied	3
6. Precautions	3
7. Technical Application Tips	4
8. Reagent Preparation.....	5
9. Sample Preparation	6
10. Assay Procedure	7
11. Calculation of Results.....	8
12. Typical Expected Data	8
13. Technical Resources	10

1. Background

Principle

Aviva Systems Biology Malachite green ELISA Kit (OKAO00134) is based on standard competitive inhibition enzyme-linked immuno-sorbent assay technology. Malachite green has been pre-coated onto a 96-wellplate (12 x 8 Well Strips). Standards or test samples are added to the wells along with a rabbit anti-Malachite green antibody and an anti-rabbit / HRP Conjugated antibody, then incubated. Wells are washed and detection substrates are added and incubated. An enzymatic reaction is produced through the addition of TMB which is catalyzed by HRP generating a blue color product that changes yellow after adding acidic stop solution. The density of yellow coloration read by absorbance at 450 nm which is quantitatively proportional to the amount of detector antibody bound in the well and inversely proportional to the amount of Malachite green in the sample.

Background

Malachite green is an organic compound that is used as a dyestuff and has emerged as a controversial antimicrobial used in aquaculture. Malachite green is traditionally used as a dye for materials such as silk, leather, and paper. Although called malachite green, This dye is not prepared from the mineral malachite – the name just comes from the similarity of color. Malachite green is classified in the dyestuff industry as a triarylmethane dye and also using in pigment industry. Formally, malachite green refers to the chloride salt $[C_6H_5C(C_6H_4N(CH_3)_2)_2]Cl$, although the term malachite green is used loosely and often just refers to the colored cation. The oxalate salt is also marketed. The anions have no effect on the color. The intense green color of the cation results from a strong absorption band at 621 nm (extinction coefficient of 105 M⁻¹cm⁻¹).

General Specifications

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Specificity	Malachite green <u>CAS#:</u> 569-64-2 <u>PubChem#:</u> 11294 <u>Alias:</u> MG; MALACHITE GREEN; Basic Green 4; Victoria Green B; Malachite green chloride; Aniline Green; Grenoble Green
Detection Range	0.5-8 ppb
Limit of Detection	0.03 ppb

2. Storage and Stability

- Upon receipt store kit at 4°C. Do not use past expiration date.
- The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% within the expiration date under appropriate storage condition.

3. Kit Components

- The following reagents are the provided contents of the kit.

Description	Quantity	Storage Conditions
Malachite green Microplate	96 Wells (12 x 8 Well)	4°C Do not use past expiration date
Malachite green Standards	6 x 1 mL	
Malachite green Rabbit Detector Antibody	1 x 7 mL	
Anti-Rabbit / HRP Conjugate	1 x 700 uL	
Detection Reagent A	1 x 7 mL	
Detection Reagent B	1 x 7 mL	
2X Sample Prep Buffer	1 x 6 mL	
20X Wash Buffer	2 x 15 mL	
Sample extraction agent1	1 x 10 mL	
Sample extraction agent2	1 x 10 mL	
Stop Solution	1 x 7 mL	

4. Required Materials Not Supplied

- Microplate reader capable of reading absorbance at 450 nm.
- Automated plate washer (optional).
- Pipettes capable of precisely dispensing 0.5 µL through 1 mL volumes of aqueous solutions.
- Pipettes or volumetric glassware capable of precisely measuring 1 mL through 100 mL of aqueous solutions.
- New, clean tubes and/or micro-centrifuge tubes for the preparation of standards or samples.
- Absorbent paper or paper toweling.
- Distilled or deionized ultrapure water.
- Heater water bath
- Centrifuge
- Reagents: NaOH, ethyl acetate, n-Hexane, Acetonitrile (CH₃CN), deionized water.

5. Precautions

- Read instructions fully prior to beginning use of the assay kit.
- Any deviations or modifications from the described method or use of other reagents could result in a reduction of performance.
- Reduce exposure to potentially harmful substances by wearing personal protective lab equipment including lab coats, gloves and glasses.
- For information on hazardous substances included in the kit please refer to the Material Safety Data Sheet (MSDS).

- Kit cannot be used beyond the expiration date on the label.

6. Technical Application Tips

- Do not mix or substitute components from other kits.
- To ensure the validity of experimental operation, it is recommended that pilot experiments using standards and a small selection of sample dilutions to ensure optimal dilution range for quantitation.
- Samples exhibiting OD measurements higher than the highest standard should be diluted further in the appropriate sample dilution buffers.
- Prior to using the kit, briefly spin component tubes to collect all reagents at the bottom.
- Replicate wells are recommended for standards and samples.
- Cover microplate while incubating to prevent evaporation.
- Do not allow the microplate wells dry at any point during the assay procedure.
- Do not reuse tips or tube to prevent cross contamination.
- Avoid causing bubbles or foaming when pipetting, mixing or reconstituting.
- Completely remove of all liquids when washing to prevent cross contamination.
- Prepare reagents immediately prior to use and do not store, with the exception of the top standard.
- Equilibrate all materials to ambient room temperature prior to use (standards exception).
- For optimal results for inter- and intra-assay consistency, equilibrate all materials to 37°C prior to performing assay (standards exception) and perform all incubations at 37°C.
- Pipetting less than 1 μL is not recommended for optimal assay accuracy.
- Once the procedure has been started, all steps should be completed without interruption. Ensure that all reagents, materials and devices are ready at the appropriate time.
- Incubation times will affect results. All wells should be handled in the same sequential order and time intervals for optimal results.
- Samples containing precipitates, fibrin strands or bilirubin, or are hemolytic or lipemic might cause inaccurate results due to interfering factors.
- TMB Substrate is easily contaminated and should be colorless or light blue until added to plate. Handle carefully and protect from light.
- To minimize influences on the assay performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly controlled. It is also strongly suggested that the whole assay is performed by the same operator from the beginning to the end.

7. Reagent Preparation

- Equilibrate all materials to room temperature prior to use and use prepare immediately prior to use.

7.1 1X Anti-Rabbit / HRP Conjugate

- 7.1.1 Prepare the **1X Anti-Rabbit / HRP Conjugate** immediately prior to use by diluting the **10X Avidin-HRP Conjugate** 1:10 with **Conjugate Diluent**.
- 7.1.2 For each well strip to be used in the experiment (8-wells) prepare 500 μ L by adding 50 μ L of **10X Avidin-HRP Conjugate** to 450 μ L **Conjugate Diluent**.
- 7.1.3 Mix thoroughly and gently. Hold no longer than 2 hours prior to using in procedure. Do not store at 1X concentration for future use.

7.2 1X Wash Buffer

- 7.2.1 If crystals have formed in the **20X Wash Buffer** concentrate, equilibrate to room temperature and mix gently until crystals have completely dissolved.
- 7.2.2 Add the entire 20 mL contents of the **20X Wash Buffer** bottle to 380 mL of ultra-pure water to a clean > 1,000 mL bottle or other vessel.
- 7.2.3 Seal and mix gently by inversion. Avoid foaming or bubbles.
- 7.2.4 Store the **1X Wash Buffer** at room temperature until ready to use in the procedure. Store the prepared **1X Wash Buffer** at 4°C for no longer than 1 week. Do not freeze.

7.3 Microplate Preparation

- Micro-plates are provided ready to use and do not require rinsing or blocking.
- Unused well strips should be returned to the original packaging, sealed and stored at °4C.
- Equilibrate microplates to ambient temperatures prior to opening to reduce potential condensation.

8. Sample Preparation

8.1 Sample Preparation and Storage

- Mix diluted samples gently and thoroughly.
- Pipetting less than 2 μL is not recommended for optimal assay accuracy.
- Untreated samples should be stored at -20°C .
- Prepared samples can be stable for 1 week at $+4^{\circ}\text{C}$.

8.1.1 **Tissue** (meat, chicken, duck, liver, shrimp, fish, egg, etc.)

8.1.1.1 Homogenize the sample at 10,000 rpm for 1 min.

8.1.1.2 Weigh 3 ± 0.05 g of the homogenized sample, place into centrifugal tube, add 3 mL **0.02 M PB Buffer**, mix gently and thoroughly.

8.1.1.3 Add 4 mL ethyl acetate and 2 mL Acetonitrile (CH_3CN), mix gently and thoroughly for 5 minutes.

8.1.1.4 Centrifuge at above 4,000 rpm at room temperature ($20\text{-}25^{\circ}\text{C}$) for 10 min.

8.1.1.5 Transfer 2 mL of supernatant (approx 1 g sample) into a new centrifugal tube, dry completely with a gentle stream of nitrogen or rotary evaporation at 56°C .

8.1.1.6 Add 1 mL N-hexane, then add 1 mL of the diluted 1X Sample Prep, shake strongly for 30 seconds.

8.1.1.7 Centrifuge at 4,000 rpm at room temperature for 5 min, remove the upper layer.

8.1.1.8 Assay samples neat (do not dilute).

9. Assay Procedure

- Equilibrate all reagents and materials to ambient room temperature prior to use in the procedure.
- Temperature regulation for all procedures and incubations to 25°C is recommended for optimal reproducibility.

- 9.1 Determine the required number of wells and return any remaining unused wells and desiccant to the pouch.
- 9.2 Add 50 µL of standards or samples into wells of the **Malachite green Microplate**. At least two replicates of each standard, sample or blank is recommended.
- 9.3 Immediately add 50 µL of the **Sulfamethoxine Rabbit Detector Antibody** and 50 µL of the **Anti-Rabbit / HRP Conjugate** to each well.
- 9.4 Cover the plate with the well plate lid, gently mix and incubate for 40 minutes.
- 9.5 Remove the plate lid and discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle or aspiration.
- 9.6 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
- 9.7 Wash plate 5 times with **1X Wash Buffer** as follows:
 - 9.7.1 Add 250 µL of **1X Wash Buffer** to each assay well.
 - 9.7.2 Incubate for 30 seconds.
 - 9.7.3 Discard the liquid in the wells by rigorously flicking into an acceptable waste receptacle.
 - 9.7.4 Gently blot any remaining liquid from the wells by tapping inverted on the benchtop onto paper toweling. Do not allow the wells to completely dry at any time.
 - 9.7.5 Repeat steps 10.7.1 through 10.7.4 **four** more times.
- 9.8 Add 50 µL of prepared **Detection Reagent A** and 50 µL of **Detection Reagent B** to each well.
- 9.9 Gently mix the plate and incubate for 15 minutes **in the dark**.
- 9.10 Add 50 µL of **Stop Solution** to each well and mix gently. Well color should change to yellow immediately. Add the **Stop Solution** in the same well order as done for the **Detection Reagents**.
- 9.11 Read the O.D. absorbance at 450 nm with a standard microplate reader within 5 minutes of stopping the reaction in step 10.10. If wavelength correction is available, set to 540 nm or 570 nm.

10. Calculation of Results

For analysis of the assay results, first derive the **Percent Relative Absorbance** for each sample or standard by calculating the mean absorbance between replicate wells. This value is then divided by the mean replicate Blank (0 ppb) absorbance, then multiplying by 100%:

$$\text{Percent Relative Absorbance (Sample or Standard)} = \frac{\text{Mean Absorbance (Sample or Standard)}}{\text{Mean Blank Absorbance}} \times 100\%$$

A standard curve is generated by plotting the **Percent Relative Absorbance** of each standard serial dilution point vs. the respective standard concentration. The Malachite green concentration contained in the samples can be interpolated by using linear regression of each sample **Percent Relative Absorbance** against the standard curve. This is best achieved using curve fitting software.

Note: If wavelength correction readings were available, subtract the readings at 540 nm or 570 nm from the readings at 450 nm. This may provide greater reading accuracy.

Note: If the samples measured were diluted, multiply the derived mean sample concentration by the dilution factor for a final sample concentration.

11. Typical Expected Data

11.1 Limit of Detection

Derived by linear regression of OD450 of the Mean Blank + 2xSD

Sample	Mean Recovery
Tissue	0.2 ppb

11.2 Reproducibility

Three samples of known concentrations were measured in 20 replicates within an assay plate to assess intra-assay reproducibility:

Mean Intra-Assay Precision - ≤5% (n = 3 x 20)

Three samples of known concentrations were measured across 40 replicate assays inter-assay reproducibility:

Mean Inter-Assay Precision - ≤12% (n= 3 x 40)

11.3 Cross-Reactivity Rate

Substance	Cross Reactivity Rate
Malachite green	100%
Leucomalachite green	100%

11.4 Recovery

The recovery of Malachite green spiked at levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay as directed in the Sample Preparation section.

Sample	Mean Recovery
Tissue	85 ±20%

12. Technical Resources

Technical Support:

For optimal service please be prepared to supply the lot number of the kit used.

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