Malachite Green Assay

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1. Background:

The malachite green essay is a colormetric assay used to detect inorganic phosphate. Phosphate is bound to molybdenum with then complexes with the malachite dye. Advantages of this method are that it only requires two reactants and has a low detection limit. Disadvantages of this method are that malachite is toxic to humans; it can cause lung and liver related illnesses. Therefore, be sure to wear a mask when you are weighing out the salt.

2. Consumables:

* Concentrated sulfuric acid
* Ammonium heptamolybdate (NH46Mo7O24·4H2O)
* Polyvinyl alcohol (PVA 72000)
* Malachite green oxalate (C25H25N2·C2HO4·0.5C2H2O4, MW = 463.50)
* Potassium phosphate monobasic (KH2PO4, MW = 136.07) or bought phosphate standard
* Clear plate reader plates (Greiner 96 flat transparent – GRE96ft or similar such as Corning 384 flat transparent – COS384ft or Costar 96 flat transparent – COS96ft)
* Pipette tips and racks (for 2-10 ul and 20-200 ul pipette)
* Millipore/MilliQ water or distilled water (DI) if MilliQ is not available
* Aluminum foil
* Ice and container to hold ice

3. Equipment:

* Tecan plate reader or similar
* Volumetric flasks (3, 100 ml and 1, 50 ml)
* 250 ml beaker
* Graduated cylinder (1-100 ml)
* 1, 100 ml glass Pyrex bottle
* 1, 50 ml glass Pyrex bottle
* Combo-stir/hot plate and magnetic bar
* Thermometer
* 2-10 ul and 20-200 ul pipette
* Multi-channel pipette (if available, makes your life easier)
* Gloves, goggles, and mask
* Fume hood

4. Preparing Malachite Reagent Solutions:

4.1 Malachite 1 (M1) – makes 100 ml

1. Prepare M1 by first using a graduated cylinder to measure out 50 ml of MilliQ/DI water and pour it into a 100 ml volumetric flask.
2. Place the flask in the ice bath **in the fume hood**. Measure out 16.5 ml of concentrated sulfuric acid with a graduated cylinder and slowly add it to the beaker in the ice bath.
3. Place acid solution in the freezer for approximately 20 min.
4. In a second container (you can use the 100 ml Pyrex bottle to make cleaning up easier), add 25 ml of MilliQ/DI water and 1.755 g of ammonium molybdate. Mix well.
5. Place the 100 ml Pyrex container with the ammonium molybdate in the freezer for approximately 20 min. Do not let it freeze.
6. When both solutions are cold, pour the ammonium molybdate solution from the Pyrex bottle into the acid solution in the 100 ml flask and top it off to 100 ml with MilliQ/DI water. Return to Pyrex bottle.
7. **Store the M1 solution in the fridge (4C)** in the Pyrex bottle. Lasts several months.

4.2 Malachite 2 (M2) – makes 50 ml

1. Prepare M2 by using a graduated cylinder to measure out 50 ml of MilliQ/DI water and pouring into a 250 ml beaker.
2. Place beaker on a combo-hot/stir plate and heat until 80C using a thermometer.
3. Turn heat down and add 175 mg of PVA.
4. Mix with the stir plate until the PVA dissolves (approximately 30 min) to ensure that PVA dissolves.
5. Wearing a mask and gloves, weigh out 17.5 mg of malachite green in the fume hood. **It is important to wear a mask because inhaling malachite dusk can be harmful to your health.**
6. Turn off the head and add the malachite green to the PVA solution.
7. Stir for 5 minutes and transfer to a 50 ml Pyrex bottle.
8. **Store solution at room temperature wrapped in aluminum foil** to keep it from photodegrading. Lasts several weeks but if white precipitate forms, dischard.

5. Making 1 ppm Phosphate Standard:

1. If you have a bought phosphate standard skip to step #2. If you do not have a bought standard you can make a 1000 ppm phosphate (as P) stock by adding 0.439 g of potassium phosphate monobasic to a 100 ml volumetric flask and topping it off to 100 ml with MilliQ/DI water.
2. Make a 1 ppm P solution from the 1000 ppm P stock solution by adding 0.1 ml of the 1000 ppm P solution to a 100 ml volumetric flask and topping it off to 100 ml with MilliQ/DI water. **It’s recommended that you calibrate your pipette before adding the 0.1 ml (0.1 ml = 0.1 g).**
3. Keep the 1 and 1000 ppm P stock solutions in the fridge (4C) with M1. The 1 ppm P standard should be remade every two weeks or so as biological activity could influence the concentration.

6. Sample Concentration Pre-Check

Before running all your samples, it is helpful to analyze some on a “test plate” to determine whether they are in the range of the standards (i.e., if you need to dilute them). Test dilutions for a Hedley fractionation might be something like adding 50, 20, and 10 ul of sample and topping each off to 200 ul. If you expect your sample concentrations to be high, you might want to add 10, 5, and 2 ul of each sample. When it comes to creating the final plate for analysis it may help to make dilutions in a second plate. If possible, try to work with larger volumes (e.g. 20 ul or more for a 100 ul pipette) or at least volumes that are in the mid-range of the pipette you are using so you can avoid pipetting errors.

7. Preparing and Analyzing a Plate

7.1 Standard Curve

Each time you analyze a plate you will need to create a standard curve using the 1 ppm P standard (see previous section). It is recommended that you use the first row of the plate (A1-A8) for your standard curve. All standards should add up to 200 ul. A typical standard curve may look something like this:

Table 1. Example standard curve preparation amounts and concentrations for a malachite green assay plate.

|  |  |  |  |
| --- | --- | --- | --- |
| Standard (Well ID) | Amount of MilliQ/DI Water Added (ul) | Amount of 1ppm P Standard Added (ul) | Actual Concentration (ppm phosphate-P)a |
| S1 (A1) | 200 | 0 | 0 |
| S2 (A2) | 180 | 20 | 0.1 |
| S3 (A3) | 160 | 40 | 0.2 |
| S4 (A4) | 140 | 60 | 0.3 |
| S5 (A5) | 120 | 80 | 0.4 |
| S6 (A6) | 100 | 100 | 0.5 |
| S7 (A7) | 80 | 120 | 0.6 |
| S8 (A8) | 0 | 200 | 1 |

a Can use the v1c1=v2c2 equation to calculate other concentrations.

Note: A good standard curve should have an R-squared value of at least three 9’s or more (e.g. 0.999).

7.2 Sample Layout Rules-of-thumb

1. As mentioned before, you should always run a standard curve on each plate. When planning to use the malachite\_calibration\_Rscript.R script, we suggest you plan to place standards across the first row from left to right in slots A1-A12 (or A1-A8 if you use the standards suggested in Table 1).
2. If you are planning to use the malachite\_calibration\_Rscript.R script, we suggest organizing samples across rows from left to right rather than down columns from top to bottom.
3. If possible, you’ll want to try to fit all samples from a particular analysis on one plate so they share the same standard curve. If you absolutely cannot, it might be a good idea to repeat a few samples between the two or more plates or compare standard curves so you can account for the variation between plates.
4. If possible, run your samples in triplicate (i.e. have three technical replicates) to assess the variation of the plate reader. You can typically fit four samples across a row (i.e. 12 columns divided by 3 replicates equals 4 samples). Standards do not have to be run in triplicate.
5. See section 6 for help on diluting samples so they are within the sample range.
6. The final volume of each sample (and standard) well should be 200 ul.
7. It does not matter whether you add the water for dilution or the sample first, just be consistent so it is easy for you to remember where you are in the plate.

7.3. Adding Reagents and Analyzing Samples

1. Once you have added the standards and samples to the plate (200 ul in each well), add 40 ul of M1.
2. Wait 15 minutes.
3. Add 40 ul of M2. Pipette up and down several times to mix well. Be careful to avoid creating bubbles because you will have to remove all the bubbles you create before analyzing on the plate reader.
4. Wait 1 hour.
5. Read on the plate reader. Set absorbance to 630 nm and number of readings per well to 10.

8. References

Penny, C. L. 1976. A simple micro-assay for inorganic phosphate. *Analytical Biochemistry*. 75:201-210.