Melanin content assay

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Done according to the protocol in Wasmeier et al Rab38 and Rab32 control post-Golgi trafficking of melanogenic enzymes. J Cell Biol. 2006 Oct 23;175(2):271-81. The relevant portion of the Materials and Methods from that paper is reproduced here:

Melanin assay
Cells were disrupted by sonication in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, and protease inhibitors. Pigment was pelleted at 20,000 *g* for 15 min at 4°C, rinsed once in ethanol/ether (1:1), and dissolved in 2 M NaOH/20% dimethylsulfoxide at 60°C. Melanin content was measured as optical density at 492 nm.

We will independently determine the melanin content of the cells and their protein amount (via the Bio-Rad DC protein assay) and graph the results as OD492/mg protein.

1. Count suspended cells and make pellets. Label the tubes with the cell count, for calculating the resuspension volume. Can freeze the pellets at -20 for later use.

2. Can make a large stock solution of the melanin content resuspension buffer **without the DTT and protease inhibitors** and store at room temperature (50 mM Tris-HCl, pH 7.4, 2 mM EDTA, 150 mM NaCl). When you're ready to do the assay, take an aliquot and add DTT to 1 mM and protease inhibitors to 1X.

3. Resuspend pellets with 50 ul buffer/1E6 cells. Use the probe sonicator to break up the DNA.

4. Centrifuge the lysates at 20,000 g (the highest speed on the small refrigerated centrifuge) for 15 min at 4°C.

5. While the tubes are spinning, label a second set of tubes for collecting the supernatants for protein determination.

6. While the tubes are spinning, prepare the 1:1 EtOH:ether mixture to wash the pellets. Make enough for 500 ul per tube. Ether is stored in the flammable cabinet. Use a syringe and a needle narrower than 16-gauge (higher numbers are narrower) to withdraw the desired amount of ether. Ether drips very easily out of the syringe and it is a strong-smelling anesthetic, so do this in the fume hood!

6. When the tubes have finished spinning, carefully remove the supernatant to your labeled tubes and store at 4°C or -20°C while you finish the melanin determination.

7. Wash the melanin pellets (some white membrane may also be present) with the EtOH/ether mixture and centrifuge again.

8. After the tubes finish spinning, remove the EtOH/ether from all the tubes to a conical and place it in the fume hood to safely evaporate.

9. Dissolve the pellets in 1 ml 2 M NaOH/20% DMSO (this can also be made as a stock and stored at room temperature). Place the tubes in the 60°C heat block.

10. Once melanin has fully dissolved, vortex to mix and read the absorbance at 492 nm. If necessary, dilute the melanin with the 2 M NaOH/20% DMSO buffer so that the reading is < 0.35 (I don't trust the linearity of the spec above that range.) As an example, when I had a big pellet of melan-Ink cells, I had to dilute the melanin 20-fold to get into this range. Record the OD492 and the dilution factor you used for each tube.

11. For the protein determination, you need a standard curve of known protein concentrations to compare your sample tubes against. Thaw a tube of 10% BSA (not 10X or 100X BSA) from the common freezer. 10% = 10 g/100 ml = 100 mg/ml.

Preparing the BSA standards
a. Use the 100 mg/ml BSA stock to make 600 ul of 1.5 mg/ml BSA, **diluted in the melanin content buffer** (the Tris/EDTA/NaCl one).

600 ul * 1.5 mg/ml = X ul * 100 mg/ml
X = 900/100 = 9 ul of 10% BSA diluted with 591 ul buffer

b. Use the 1.5 mg/ml buffer to make 150-ul tubes at 0.2, 0.5, 0.75, 1.0, and 1.25 mg/ml.

0.2 mg/ml: 20 ul of the 1.5 mg/ml solution + 130 ul buffer
0.5 mg/ml: 50 ul of the 1.5 mg/ml solution + 100 ul buffer
0.75 mg/ml: 75 ul of the 1.5 mg/ml solution + 75 ul buffer
1.0 mg/ml: 100 ul of the 1.5 mg/ml solution + 50 ul buffer
1.25 mg/ml: 125 ul of the 1.5 mg/ml solution + 25 ul buffer
Keep the rest of the 1.5 mg/ml solution for the final data point.

12. Pipet 100 ul of each sample and each standard into a fresh, labeled tube that can hold at least a 5ml volume. Also make a tube that has 0 mg/ml BSA (that is, just 100 ul of the Tris/EDTA/NaCl buffer).

13. Add 500 ul of the DC Protein Assay Reagent A to each tube and vortex.

14. Add 4 ml reagent B to each test tube and vortex immediately.

15. Wait 15 minutes for color to develop. Read absorbances at 750 nm (use the 0 mg/ml tube to set the reference first). If any samples give readings that are higher than the one given by the 1.5 mg/ml standard, dilute those samples in additional Tris/EDTA/NaCl buffer and re-read them. The absorbances will be stable for at least 1 hour.

16. Open the Melanin content assay Excel document and replace the values that you have recorded for

BSA standards OD750 reading
Cell counts
Resuspension volume
Melanin dilution factor
OD492 melanin reading
OD750 protein reading

17. Add a line of best fit to the standard curve graph and check its equation and R^2 value. The closer to 1 the better. Rearrange the line's equation to be able to calculate the protein concentration for a given OD750 reading. Plug these parameters into the row of the table that calculates the protein concentration.

18. The graphs should automatically update with the new numbers.