Laboratory of Dr. Wayne L. Hubbell
Protocol adapted by Carlos J. López, PhD
Protocol can be downloaded from: http://www.biochemistry.ucla.edu/biochem/Faculty/Hubbell/

Apomyoglobin expression, purification, and spin labeling protocol

***Published in López et al.*[1]**

1. **Protein expression**: Start a 20ml overnight LB Amp culture from a BL21 (DE3) colony. Inoculate 1L of LB Amp with the overnight culture and incubate at 37˚C. Add IPTG to a final concentration of 1mM once the OD_{600} reached ~ 1AU to induce expression, increase the temperature to 42˚C, and incubate for ~ 3 hours. Spin down the cells at 6,000 rpm and resuspend the pellet in lysis buffer (50mM Tris-Cl, 1mM EDTA pH 7.5). Freeze the cells at -20˚C.
2. **Isolation of inclusion bodies:** Thaw the cells and add beta-mercaptoethanol to a final concentration of 20mM (28ul/20mL cell extract) and then add PMSF to a final concentration of 2mM (150mM stock solution in isopropanol).
3. **Note:** Use teflon tubes during for the centrifugation steps below as the other centrifuge tubes are damaged by the solvents used in step 8 (alternatively switch to the teflon tubes prior to step 7). Sonicate the crude extract for 5 minutes (sample should be on ice during the sonication steps!) and centrifuge for 15 minutes at 15,800 rpm. **Keep the pellet**! Note: Apomyoglobin should stay in the pellet (inclusion bodies), while the supernatant contains soluble proteins (*i.e.*, holomyoglobin and other contaminants).
4. Wash the inclusion bodies by resuspending the pellet in 20ml of lysis buffer (For the first wash, Triton X should be added to a final concentration of 1% v/v in order to remove weakly bound contaminants). Break the clumps with a glass rod and sonicate the sample for 5 minutes. Centrifuge for 15 minutes at 15,800 rpm. Discard the supernatant. Keep the pellet.
5. Repeat step 4 for an additional wash (this time without Triton X).
6. Resuspend the pellet in 20ml of lysis buffer and add urea to a final concentration of 1M (1.2g/20ml), break the clumps with a glass rod, incubate the resuspended pellet for 30 minutes at 4˚C. Following the 30 minutes incubation, sonicate the solution for 5 minutes.
7. Centrifuge for 15 minutes at 15,800 rpm and keep the pellet. The inclusion body pellet has been washed 3 times at this point to remove any soluble contaminants and it is ready for solubilization. This final inclusion body pellet can be frozen and stored until needed.
8. **Apomyoglobin purification:** To solubilize the inclusion body pellet, resuspend it in 10-20ml of solubilization solution (60% ddH_2O, 40% acetonitrile, 0.1% TFA) containing 5mM DTT. Use a glass rod to break the clumps and sonicate for 5 minutes. Centrifuge. The supernatant should be yellowish. In some cases, a higher amount of acetonitrile will be necessary to resolubilize the protein (the pellet should be very small); for those cases, repeat the resolubilization with a 50% solution of acetonitrile. Pool the apomyoglobin fractions and, if necessary, dilute the protein solution in ddH_2O to a final acetonitrile concentration below 40%.
9. Filter the resuspended protein fraction through a 0.2 µm membrane prior to loading into the reverse phase column.
10. Reverse Phase Chromatography (Vydac C4 column): Buffer A: (99.9% H2O, 0.1% TFA); Buffer B: (99.9% acetonitrile, 0.1% TFA). The following steps describe the general method I use during the reverse phase separation (for additional details on the use of the HPLC or the LabSolutions software see Evan Brooks). All solutions used in the HPLC stage should be HPLC grade (solvents can be filtered using a compatible membrane such as **nylon**).
    a. Equilibrate the column with 100mL (~ 4 CV) of (80% buffer A + 20% buffer B) solution.
    b. Inject the protein in 5mL installments (use the "Loading Method" programmed on the HPLC), collect the flow-through fractions.

c. Repeat step b until you have 5ml left.
d. Prior to loading the last 5ml of the resolubilized protein solution, load the purification method. Inject the last 5ml. Collect the flow-through. The program will automatically start the gradient (20-60%B over 10 minutes @ 4ml/min). The apomyoglobin peak should appear at around 50-60% Buffer B (*see Fig 1 below*).
e. For purification of additional mutants allow the column to equilibrate in 20% buffer B for 20 minutes, otherwise wash the column with 100% buffer B, and then equilibrate with, at least, 80mL of storage solution consisting of 50% acetonitrile + 50% water (no TFA!).

11. Run SDS-PAGE of all the fractions and lyophilize the purified ApoMyb. If you have questions about the lyophilization procedure, see Evan Brooks.
12. **Resolubilization and refolding of purified apoMyb**: Weight the desired amount of lyophilized apoMb (I usually do about 6-10mg of lyophilized powder) and resolubilize it by adding 1ml of **freshly made** solubilization buffer consisting of 6M urea in 10mM sodium acetate and 5mM DTT at pH 6.1. Incubate for 30 min at 4 ˚C. To refold, dilute the solution seven-fold in refolding buffer (10mM sodium acetate, 5mM DTT pH 6.1). **Note: In some cases a higher yield of refolded protein can be achieved by dialysis refolding. For overnight dialysis refolding, use 4L of solubilization buffer without urea.**
13. **Spin labeling of apoMyb**: Filter the solution through a 0.2μm membrane and remove traces of urea and DTT by using HiTrap desalting column (GE Healthcare) equilibrated with 50mM sodium acetate + 100mM NaCl pH 6.1. Incubate the eluted protein overnight with 10-fold molar excess of MTSL (see steps a-b below for preparation of **holomyoglobin** samples, otherwise go to step 14).
    a. *If generating holomyoglobin*: make a fresh solution of 10mM bovine hemin (obtained from Sigma and stored at 4 ºC) in 0.1M NaOH and add a 2-fold molar excess to the protein. Incubate overnight at 4 ºC in the nutator.
    b. Filter out any precipitate (the unbound heme and precipitated protein will stick to the filter), wash out excess of hemin with buffer consisting of 10mM sodium acetate at pH 6.1, and do an UV-Vis wavelength scan to make sure $A_{409}/A_{280} \approx 5.0$ (Ideally it should be 5.06). A ratio above 4.8 is tolerated. This is the final step for the holo protein preparation. Skip to step 16.
14. Some ApoMyb mutants will form high molecular weight oligomers (Mr > 75kDa) during the refolding step, thus an additional gel filtration step (Superdex 75) is required to separate the 2 species (see figure 2). Note: for the holo protein samples this step is not necessary because the misfolded species precipitates out during the heme reconstitution step. For the gel filtration step, use the following buffer: 50mM sodium acetate and 100mM at NaCl pH 6.1 (**the salt is absolutely required to avoid nonspecific interaction of the protein with the column**). There will be, at least, 2 peaks in most cases (*see Fig 2 below*: Vr = 7.7mL and Vr = 12.6mL). Collect the 12.6mL fraction and do a buffer exchange (10mM sodium acetate, pH 6.1) before EPR studies. This is the final step in the preparation of apomyoglobin in the native state.
15. To generate the molten globule species, exchange buffer to 10mM sodium acetate + 10% EtOH at pH 4.1.
16. Concentrate the spin-labeled protein using the Amicon 10,000 MWCO to about 400μM for EPR studies.

ApoMb: ε_{280} = 15,400 M^{-1} cm^{-1}; MW = 17,200 Da (1 AU = 1.116 mg/ml)
HoloMb: ε_{280} = 31,000; ε_{409} = 157,000; MW ≈ 18,000Da (1 AU = 0.58 mg/ml)

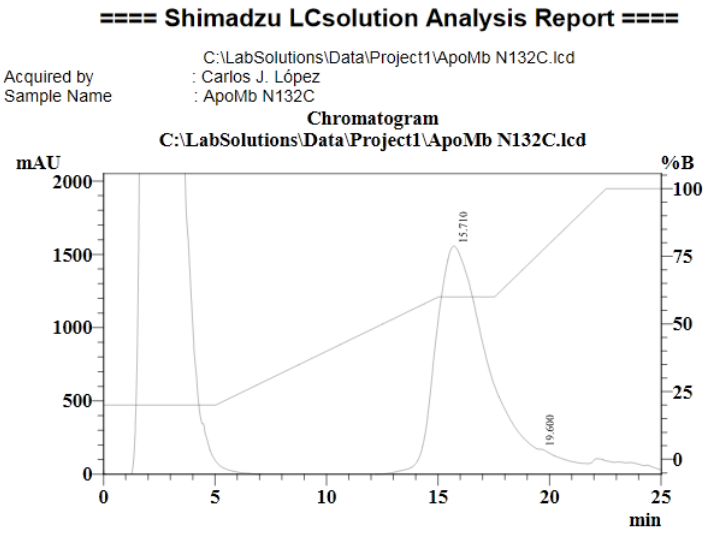

Figure 2: HPLC trace

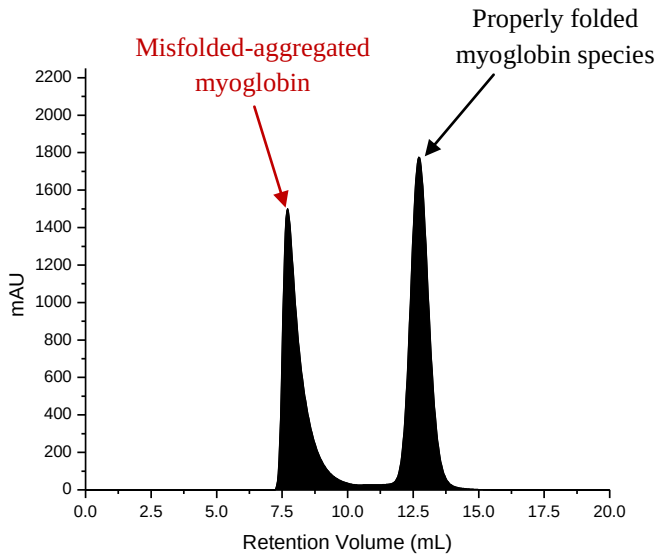

Figure 3: Gel filtration (Superdex 75)

Reference:

(1) López, C. J.; Oga, S.; Hubbell, W. L. *Biochemistry* **2012**, *51*, 6568.