

## Making microcrystalline DNP samples

### Stock Biradical solutions

Biradical	MW	50 mM	100 mM
AMUPol	727.4	36.4 mg/mL	72.7 mg/mL
AsymPolPOK	630.2 (Na form)	31.5 mg/mL	63 mg/mL

Biradicals can be stored as stock 100 mM solutions in H<sub>2</sub>O at -20°C or -80°C for several months.

### Protocol

Protein should be prepared as microcrystals in buffer with desired D<sub>2</sub>O/H<sub>2</sub>O ratio, precipitating agents, and 10% (by volume) d<sub>6</sub>-DMSO.

#### Day 1

- (1) Spin down crystals in tabletop centrifuge. Realiquot sample so each eppendorf tube contains ~40 µL of microcrystals and ~40 µL of excess buffer. Excess buffer beyond what is needed in individual samples should be saved for making biradical solution and for recovery washes.
- (2) Biradicals are stored as 10x solutions in H<sub>2</sub>O. Determine the amount of biradical needed to make DNP samples for a particular protein. 10 mM AMUPol and 5mM AsymPolPOK biradical gives excellent enhancements and faster DNP buildup times but may shorten nuclear-spin T<sub>2</sub>'s. 5 mM biradical is a good compromise of DNP buildup time and nuclear T<sub>2</sub>. To each DNP sample, 9 µL of 10x biradical will be added.
- (3) Measure out the needed amount of biradical in H<sub>2</sub>O, freeze and lyophilize.

#### Day 2

- (4) Add appropriate amount of protein crystallization buffer to lyophilized biradical to make a 10x biradical solution (i.e. 50 mM or 100 mM).

*STEPS 5-10* are for each DNP sample:

- (5) To each 80 µL aliquot of protein that will be used as a DNP sample add 9 µL of 10x biradical solution.
- (6) Resuspend the crystals in buffer with vortexing.
- (7) Place a clean DNP rotor with rotor packing adaptor in an eppendorf tube. Add 45 µL of resuspended protein microcrystals containing biradical and centrifuge the sample to pack the crystals in the rotor.
- (8) Remove excess buffer from the rotor/rotor adaptor and add remaining 45 µL of resuspended protein microcrystals. Centrifuge to pack the remaining crystals. Remove excess buffer.

- (9) At this point the rotor should contain crystals beyond the fill line. Carefully remove excess crystals and store with excess buffer containing biradical that was recovered from the sample. You should leave a very slight excess to be displaced by the silicone plug to ensure the sample has no air bubbles.
- (10) Using the rotor plug tool, add a silicone plug to the top of the rotor. The rotor plug tool ensures the plug is inserted to the proper depth. It is best to have a very slight excess of buffer/crystals in the rotor which will be displaced by the plug to ensure there are no air bubbles. Carefully remove excess displaced sample using a Kimwipe and store the clean rotor in an eppendorf tube with an appropriate, detailed label. The rotor cap can be added just prior to the DNP experiment.
- (11) To insert the cap without damaging the fins, set the cap into the rotor body so it is well aligned. Set the rotor, drive cap end down, into the cap placement tool. Press firmly and evenly on a flat surface. Take out the rotor and check that there is no visible gap between drive cap and rotor body.

#### **DNP day(s)**

- (12) Check the biradical content of rotors using the benchtop X-band instrument. If there is an EPR spectrum of the same radical and sample concentration, compare the intensity and lineshape of the EPR spectrum to ensure DNP viability.
- (13) Make sure the rotor is well marked, and confirm the sample spins smoothly using the benchtop MAS spin-test station. The sample is now ready to go in the DNP probe. The DNP system at the MagLab uses a MAS-2 unit in the cooling cabinet, which requires that the sample must be inserted and manually spun using the cooling cabinet controls. If you have not been trained in cryogenic MAS control, please ask for help from the DNP staff.

#### Data collection - DNP setup

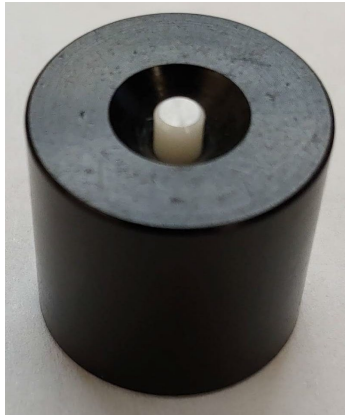
DNP enhancement and buildup time must be measured before MAS-NMR experiments may be carried out.

- (14) **Microwaves on/off** - Take enough scans with microwaves off for important peaks to be visible. Take the same number of scans with microwaves on in a new file. Overlay spectra (in TopSpin use 'md' command) and scale the MW-off spectrum to obtain an estimate of DNP enhancement. If the MW-off spectrum uses an excessively large number of scans, the number of MW-on scans can be reduced by a factor of 4 or 16 - remember that signal-to-noise scales as (number of scans)<sup>0.5</sup>.
- (15) **Buildup time** - to find the optimal DNP-buildup time, with microwaves on, acquire a pseudo-2D spectrum in which the DNP polarization time of the pulse sequence is varied. Plot the peak intensity as a function of DNP polarization time to determine optimal DNP-buildup time. If the DNP buildup-time is unusually long for the radical, the sample may need to be degassed by freeze-thaw cycling.

## Cap removal

- (16) To remove the cap without damaging the drive cap fins, use the cigar-cutter type cap remover, but be careful with the sharp internal edge. It can cut into the side of the plastic drive fins and affect spinning. Hold the rotor so that the drive cap is facing up and hold the tool so the blades are near the bottom of the tool. Slide the body of the rotor onto the cap tool, with light pressure on the sides of the cap tool, until the blade clicks into the notch between cap and rotor. Insert the rotor body into the rotor vice and tighten while keeping the cap remover blade in the notch. Use small, even turns on both sides of the vice until cap is free.

Cap placement tool



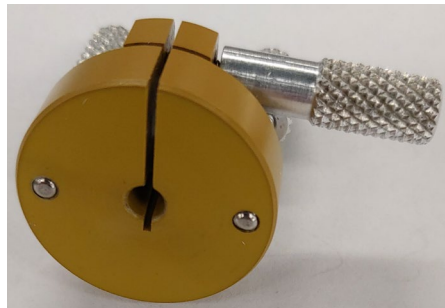
“Cigar cutter-type” cap remover, top and bottom. The blades are visible in the bottom view



Top

Bottom

Rotor vice



A video showing the how to use the “Cigar cutter-type” cap removal tool – starting at 1 min 29s. <https://www.youtube.com/watch?v=bNFJj2g0UjI>

## **Notes**

Stable cofactors, inhibitors, or substrates can be added after *step 9* before the plug is added in *step 10*. These should be at 10-20x concentration so that only 1-2  $\mu\text{L}$  need to be added to the rotor. Add the reagent to the top of the rotor and allow it to diffuse for 5-10mins (on ice if protein has limited stability). Continue at *step 10* above.

Unstable reagents can be added just prior to an experiment. That is do not add the plug until the day of the experiment.

Protein can be recovered using the rotor adaptor— place the sample upside down in a clean eppendorf tube and centrifuge.

## **Biradical references**

AmuPol: <https://onlinelibrary.wiley.com/doi/full/10.1002/anie.201304657>

AsymPolPOK: <https://pubmed.ncbi.nlm.nih.gov/30095255/>