Alignment Media Preparation

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Brief Description

^{1}H-^{15}N residual dipolar couplings (RDCs) are easily acquired for the purpose of protein structure validation and refinement. The data can be collected on samples labeled only with ^{15}N. Obtaining RDCs in two different alignment media greatly improve the quality of refinement and can aid in dimer structure determination; 500µl of a 0.5-1mM sample is usually sufficient for this purpose.

Data Acquisition and RDC Calculation

Refer to the linked pages for detailed descriptions of the Jmodulation Experiment RDC and HSQCTROSY RDC Measurement and their respective RDC calculation methods.

Alignment Media Preparation

Isotropic Sample

The first sample to be observed is an isotropic sample. You may want to dilute the isotropic sample by one third (to 75% of its original concentration) to match the concentration of most aligned samples. In the case of a weak dimer, this may be important.

PEG Bicelle

Alignment of the protein sample in PEG($C_{12}E_5$/hexanol) is used as a first alignment media because it produces primarily steric alignment (useful in dimer geometry predictions), the success rate is high, and it can be doped with other charged detergents to give a second alignment media .

Chemicals used for this preparation:

Sigma Aldrich 76437, Pentaethylene glycol monododecyl ether ($C_{12}E_5$ PEG)
Sigma Aldrich H13303, Hexanol
Sigma Aldrich 855820, Cetyltrimethylammonium bromide (CTAB)
Sigma Aldrich O4003, Sodium octyl sulfate (SOS)

The preparation procedure is as follows:

- Prepare the 16% PEG stock solution by first mixing 50µl of $C_{12}E_5$ (pentaethylene glycol monododecyl ether) with 200µl of buffer and 50µl of D_2O by vortexing.[1]

- Add approximately 16µl of hexanol to the stock solution, in aliquots of 2µl with vortexing after each addition. The solution will go from clear to milky, then to translucent and viscous with lots of bubbles. Continue to add hexanol until the solution goes clear again. If it becomes milky/turbid again, you have gone past the nematic phase.
- Sample Content (for 220 µL sample):

    145 µL of Protein stock solution
    55 µL of 16% PEG stock solution
    20 µL of D_2O

        Final PEG concentration is 4.2%.

- Record the ^2H splitting by running the s2pul expt with tn='lk' (for Varian instruments). The range of the splitting should be around +/-20Hz.
- PEG can be doped with either cetyltrimethyl ammonium bromide (CTAB) for positively charged proteins or sodium octyl sulphate (SOS) for negatively charged proteins. Charging the medium to be like the protein prevents association and gives higher resolution spectra. A suitable ratio of PEG:CTAB/SOS is ~30:1.

Pf1 Phage

Preparation of a Pf1 phage alignment sample is fairly straightforward.[2] The protein sample is diluted by the alignment medium.

Chemicals used for this preparation:

ASLA Biotech P-50-P, Pf1 phage 50 mg/mL

The preparation procedure is as follows:

- Start with a protein stock 0.5-1mM and a pf1 phage stock of 50 mg/mL. Prepare a sample of 12.5 mg/mL of phage.
- Sample Content (for 220 µL sample):

    145 µL of Protein stock solution
    55 µL of Pf1 phage 50 mg/mL stock solution
    20 µL of D_2O

        Final phage concentration is 12.5 mg/mL

- Record the ^2H splitting by running the s2pul expt with tn='lk' (for Varian instruments). The range of the splitting should be around +/-8-10 Hz.

Polyacrylamide Gel (Compressed and Stretched)

The preparation of polyacrylamide gel samples is a two step process. First the gels must be polymerized, equilibrated to the correct pH and cut to the appropriate size before being dried. Second the gels are re-hydrated using the protein stock in the appropriate NMR tube.

Chemicals used for this preparation:

Bio-Rad 161-0144, 40% Acrylamide/Bis solution 19:1
Bio-Rad 161-0733, 10X TBE
Bio-Rad 161-0700, APS
Bio-Rad 161-0800, TEMED
Sigma Aldrich M7279, N,N'-methylenebisacrylamide (BIS)
Sigma Aldrich 448281, (3-Acrylamidopropyl)trimethylammonium chloride solution
Sigma Aldrich 282731, 2-Acrylamido-2-methyl-1-propanesulfonic acid

Other materials used for this preparation:

Wilmad P-4.4965M-6.5135-0-0, 4.4965mm +/-0.0065mm ID x 6.5135mm +/-0.0065mm. Ground polished and buffed OD. 40mm +/- 0.5mm long. Both ends wet saw cut only.

Preparation of Dried Acrylamide Gels

The preparation procedure is as follows:

- Prepare the positively and negatively charged 40% 19:1 bis:acrylamide solutions.
- Positively charged stock solution (2mL):

  31 mg of N,N'-methylenebisacrylamide (BIS)
  961 µL of (3-acrylamidopropyl)trimethylammonium chloride solution
  1039 µL of H_2O

- Negatively charged stock solution (2mL):

  31 mg of N,N'-methylenebisacrylamide (BIS)
  802 mg of 2-acrylamido-2-methyl-1-propanesulfonic acid
  2000 µL of H_2O

Standard formulas for preparing compressed and stretched polyacrylamide gels

Gel type	% acrylamide	% charged	Vol. 40% charged stock solution (µL)	Vol. 40% neutral stock solution (µL)	Vol. 10x TBE (µL)	Vol. 10% APS (µL)	Vol. TEMED (µL)	Vol. per casting tube (µL)	Type of casting tube
charged compressed	7	50	43.75	43.75	400	7.5	5	140	2.8 mm ID plastic
charged stretched	5	50	62.5	62.5	860	10	5	300	3.2 mm ID glass
neutral stretched	5	0	0	125	860	10	5	490	4.5 mm ID glass

Note: The APS solution should be prepared fresh and polymerization will begin as soon as TEMED is added.

- Mix stock solutions of neutral and charged 40% acrylamide/bis 19:1 to achieve the desired overall charge ratio.
- Dilute the mixtures 10x with TBE buffer (0.9 M TRIS, 0.9 M borate, 0.02 M EDTA, pH 8.2) to a final 7% or 5% concentration for compressed or stretched gels, respectively.
- Polymerization is initiated by the addition of ammoniumperoxide sulfate (APS) (0.15% or 0.1% for compressed or stretched, respectively) and tetramethylethylenediamine (TEMED) (0.1% or 0.05% for compressed or stretched, respectively).
- Pipet the mixture into the casting tubes carefully, to avoid bubbles, and keep them 1-2 hrs, allowing polymerization to occur.
- Use a 200 µL pipet with a trimmed pipet tip (to avoid hitting the gel) and water to carefully push the compressed gels out of the casting tubes into a 1L Erlenmeyer flask filled with deionized water. For the stretched gels, use a 1000 µL pipet to carefully push the stretched gels out of the casting tubes into prepared racks (no more than 4-5 gels per rack) braced in 2L nalgene beakers filled with deionized water.
- Wash the polymerized gels extensively in deionized water (two cycles over a period of 1 day). The gels will increase in size due to electro-osmotic swelling. Use cheesecloth to drain the water from the flask for the compressed gels. Be careful when draining and adding new deionized water not to damage the gels. The racks holding the stretched gels may be moved to a new 2L nalgene beaker with fresh deionized water.
- Equilibrate the polymerized gels to the desired pH (to match the pH of the protein stock) by washing extensively in buffered solution (two cycles over a period of 1 day). The buffer should not contain only the major buffering species and no salts.
- Wash the polymerized gels in deionized water overnight to allow them to swell to full size.
- Select the gels which have no cracks or imperfections and measure the diameter of the fully swollen gels and trim each gel to a length 5.7 times its diameter.

- Dry the gels over a 2 day period at room temperature on a teflon pan. If the gels do not dry well (straight, uniform width), but do not break, you can add a few drops of deionized water to slightly rehydrate them and allow them to dry again.

Preparation of Aligned Compressed Gel Samples

- Sample content (compressed gel):

    1 dried compressed gel equilibrated to the same pH as the protein stock
    200 µL of Protein stock
    20 µL of D_2O

- Measure a height of 12-13 mm for the sample volume in a 5 mm shigemi tube and mark with a sharpie.
- Add one dried compressed gel to the tube, followed by the protein stock and D_2O.
- Insert the plunger to the marked height and hold in place with parafilm.
- Allow the gel to swell in the fridge for 1 day. Inspect the final sample for cracks before collecting data.

Preparation of Aligned Stretched Gel Samples

Chemicals used for this preparation:

Sigma Aldrich 440272, Dichlorodimethylsilane

Other materials required for this preparation:

Wilmad 528-PP-2.8mm/Stem, 528-PP NMR tube sealed to a 50mm long stem section (stem dimensions 2.8mm +/-0.025mm ID x wall 0.38mm +/-0.015mm). Overall length, 8 inches. Both ends open.

Wilmad 528-PP-3.6mm/Stem, 528-PP NMR tube sealed to a 50mm long stem section (stem dimensions 3.6mm +/-0.025mm ID x wall 0.38mm +/-0.015mm). Overall length, 8 inches. Both ends open.

Wilmad 528-PP-3.9mm/Stem, 528-PP NMR tube sealed to a 50mm long stem section (stem dimensions 3.9mm +/-0.025mm ID x wall 0.38mm +/-0.015mm). Overall length, 8 inches. Both ends open.

Wilmad 5mm microprobe NMR tube style, Upper tube 528-PP section 7 inches long sealed to a 50mm long stem section (427-PP). Both ends open.

- Sample content (stretched gel):

    1 dried stretched gel equilibrated to the same pH as the protein stock
    300 µL of Protein stock
    20 µL of D_2O

- Prepare an NMR tube by washing the inside 3 times with dichlorodimethylsilane, then rinse with deionized water and dry.
- Place a dried stretched gel near the top of the 5mm end of the NMR tube and attach a syringe with tubing to the bottom of the tube.
- Carefully pipet in the protein stock and D_2O allowing the solution's surface tension to hold it in place in the tube.
- Use the syringe to adjust the position of the solution, such that the gel is centered in the column of solution. Cap the NMR tube, and let swell at room temperature 1-2 days. Capping the tube may cause the solution to shift, it's position should be readjusted with the syringe, to keep the gel centered.
- Once the gel is swollen, use the syringe to gently pull the gel down to the lower stem of the tube. Insert a small rubber stopper in the bottom of the tube to prevent the gel from drying. Carefully insert a shigemi plunger in the top of the tube, to the top of the gel or the bottom of the upper stem, whichever is higher.

References

1. Ruckert M and Otting G (2000), JACS, 122, 7793-7797
2. Hansen MR, Mueller L, Pardi A (1998), Nat Struct Biol, 5, 1065-1074

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