Published in final edited form as:

Methods Mol Biol. 2014 ; 1140: 169–178. doi:10.1007/978-1-4939-0354-2_13.

Screening proteins for NMR suitability

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Summary

NMR spectroscopy is an invaluable tool in structural genomics. Identification of protein samples that are amenable to structure determination by NMR spectroscopy requires efficient screening. Here, we describe how we prepare multiple samples in parallel and screen by NMR. The method described here is applicable to large structural genomics projects but can easily be scaled down for application to small structural biology projects since all the equipments used are those commonly found in any NMR structural biology laboratory.

Keywords

NMR screening; autoinduction; structural genomics; NMR spectroscopy; NMR sample

Introduction

NMR spectroscopy and x-ray crystallography are the two major methods commonly used to solve atomic resolution protein structures in structural genomics projects. These two methods are complementary and together are able to yield structures of more proteins than either method alone(1-6). While rapid, parallel structure determination in solution by NMR is limited to proteins of ~20 kDa or less, the NMR samples allow immediate and insightful follow-up experiments to identify functional molecular interactions and map binding surfaces (7). The major bottleneck or restriction point in the NMR structural genomics pipeline is the identification of a protein sample that is amenable to multidimensional 3D-NMR spectroscopy using ^{15}N and ^{13}C -stable isotope labels (2,3). Once such a sample has been identified, the probability of solving its 3D structure is very high, and all subsequent investment of resources into such a sample will be well spent. This key bottleneck/decision point is analogous to that of obtaining a well-diffracting crystal in the crystallography pipeline. Importantly, identification of good quality NMR samples can be performed in a rapid, parallel screening procedure for many samples and/or constructs (and conditions, if desired), early in the NMR pipeline.

Here we describe our protocol for identifying proteins amenable to NMR structure determination. This protocol has been refined in our laboratory over approximately 15 years,

¹¹A “poor” ^{15}N -HSQC is characterized by broad overlapping peaks in the middle of the ^{15}N -HSQC whereas a soluble, but “unfolded” protein will have sharp intense peaks in the middle. From our experience so far, targets falling under these categories almost always require construct re-design.

on over 3000 samples of archaeal, bacterial and eukaryotic proteins leading to over 500 “structure quality” samples. Importantly, this procedure does not require sophisticated automation, and can be readily implemented in most laboratories with standard commercially available equipment. The protocol makes use of the powerful pET expression system for recombinant proteins in *E.coli* (8), combined with hexa-histidine tagged target protein for rapid affinity purification. The hexa-his-tag usually does not interfere with the NMR spectrum of the target protein (2), and can be readily cleaved off with TEV protease if desired. Our optimized NMR screening buffer has a high salt content because we have observed that we can avoid aggregation and/or increase solubility for many more proteins compared to low salt buffers. While higher salt concentration may compromise the performance of some cryogenic NMR probes, we are able to obtain many more structure-quality samples (leading to high quality solution structures) using high salt as opposed to low-salt buffers.

We use 2D ^{15}N -HSQC spectra to assess the NMR suitability of the target (2) because it offers (1) greater information content than 1D ^1H NMR, (2) is relatively fast and inexpensive, and (3) accurately reflects the amenability of the protein for subsequent ^{15}N , ^{13}C -based spectroscopic structure determination. In a well-folded protein that adopts a single conformation in solution, backbone amide ^1H - ^{15}N pairs (and Tyrosine indole NHs) appear as single, well dispersed peaks, while side chain NH_2 groups appear as two peaks sharing a common ^{15}N chemical shift. Thus, the number of peaks, their degree of chemical shift dispersion and their uniformity of intensity provide key information on the biophysical properties of the protein, including whether it is fully or partially folded, aggregated, or in multiple conformations (1,2).

The cost of ^{13}C -labeled carbon sources combined with the low frequency (25-10%) of each sample having sufficient NMR quality, necessitates an initial ‘screening sample’ using only ^{15}N -labeled protein. However, an important consideration in the initial ^{15}N -NMR screening protocol is the degree to which the results are reproducible for a subsequent ^{15}N - ^{13}C -labeled sample for full 3D-NMR data collection and structure determination. Thus, the media (especially carbon and nitrogen sources) should be as similar as possible for both the ^{15}N screening sample and the ^{15}N - ^{13}C structure determination sample. Our cost estimates showed that ^{13}C -glucose is by far the least expensive source of ^{13}C for uniform protein labeling in *E.coli*. We also wanted to take advantage of autoinduction during the screening phase when we are dealing with a large number of samples, but be able to switch to IPTG-induction with ^{13}C -glucose as the sole carbon source for the smaller number of targets prepared for full NMR data collection, with high reproducibility. We therefore optimized our screening protocol for a glucose-based media, but taking advantage of an autoinduction strategy to achieve optimal induction of protein expression for multiple cultures that may grow at different rates, without the need to monitor optical density.

Autoinduction takes advantage of the fact that the presence of glucose in the media prevents the transport of lactose into the cell (9). The original autoinduction media of Studier and colleagues (10-12) uses more glycerol and lactose than glucose as carbon source, which is expensive for ^{13}C -labeling (13). To ensure reproducibility of the yield and behavior of the

protein between ^{15}N -screening cultures and ^{15}N - ^{13}C -“structure production” cultures we developed an autoinduction protocol in which glucose is used as the main carbon source for *E. coli* growth, when the glucose in the media runs out, the remaining lactose will induce transcription of our protein target at high cell densities. Using a test set of 6 different proteins, we optimized the glucose/lactose ratio in our autoinduction media so that we obtained very similar growth curves and induction levels at the point of harvest compared with IPTG-induction. Since implementing this protocol in 2007, we have screened hundreds of targets using our glucose-autoinduction media and deposited well over 30 NMR structures in the PDB that resulted from this screening method (14).

Developments in NMR technology over the past decade have also increased the feasibility and productivity of NMR sample screening. Automatic sample changers (such as the Bruker B-ACS) coupled with software for automated acquisition of multiple spectra (we use Bruker ICONNMR) have increased productivity per spectroscopist and per spectrometer. The availability of 1.7 mm cryogenic microprobe allows the use of less protein per screening sample, and/or the screening of more conditions per protein without the need to increase protein production. In this chapter, we describe our current NMR screening procedure using a Bruker 500 MHz spectrometer equipped with 1.7 mm cryogenic microprobe and B-ACS sample changer. The procedure is also applicable to screening with conventional 5 mm probe spectrometers but would require increased protein per sample, or higher field strength.

Materials

^{15}N -labelled autoinduction minimal media

Stock solutions: All solutions are prepared using deionized water. Store the stock solutions in a screw cap container at room temperature.

1M ZnSO_4 : dissolve 1.44g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 5 mL of water.

1M MgSO_4 (Warning: add the water slowly, the solution gets hot and could boil over): dissolve 12.32g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 50 mL of water.

1M CaCl_2 : dissolve 7.35 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 50 mL of water.

Media per target

In an autoclavable 2L flask, if using shaker, or 1L centrifuge bottle, if using the LEX system, dissolve 6.8 g of Na_2HPO_4 , 3g of KH_2PO_4 , 0.5g of $^{15}\text{NaCl}$, and 0.6g of N-labelled NH_4Cl , in 500 mL water and autoclave the media.

In a separate small beaker or falcon tube, mix the following: 1 mL of 1M MgSO_4 stock solution; 5 mg of biotin; 5 mg of thiamine-HCl; 10 μL of 1M ZnSO_4 stock solution; 100 μL of 1M CaCl_2 stock solution; 2.5 g of glucose; 1 g of lactose and enough water to make 10 mL. Stir or shake until all components dissolve. Filter sterilize through a 0.2 micron syringe filter into the autoclaved ^{15}N -labelled solution.

Purification buffers

Lysis Buffer: 10 mM imidazole, 20 mM tris, 500 mM NaCl, 10 μM ZnSO_4 , pH 8.5

Wash Buffer : 30 mM imidazole, 20 mM tris, 500 mM NaCl, 10 uM ZnSO₄, pH 8.5

Eluitor Buffer : 500 imidazole, 20 mM tris, 500 mM NaCl, 10 uM ZnSO₄, pH 8.5

NMR buffers

Buffer stock solutions are prepared using deionized water. Store the solutions at room temperature. See Note 1:

1M sodium acetate, adjust pH to 5.0 with acetic acid.

1M tris, adjust pH to 7.7 with HCl or NaOH.

Base NMR screening Buffer: 450 mM NaCl, 10 mM DTT, 10 uM ZnSO₄, 1 mM benzamidine, 0.1% Sodium azide, 10% D₂O.

Methods

Fermentation for screening

We use an Innova 4000 incubator shaker for the Starting Cultures and a larger Innova 4430 incubator shaker for the Large Scale Culture (Figure 1). This part is implemented in parallel, we grow up to 24 different targets per incubator using the modified LEX system (Harbinger Inc., see Figure 1). The same incubator shaker can hold up to 12 fermentation flasks if using shaking system for aeration. Fermentation in minimal media takes 3 days per batch of targets. In our laboratory one technician can ferment up to 3 batches of 24 targets per week using a pair of Innova 4000 and 4430 shakers. The steps below are for a single batch of 24 cultures carried out by one technician in parallel.

1. Add appropriate amount of sterile antibiotic solution(s), depending on the vector and competent cells used, to 24 flasks/bottles of prepared ¹⁵N-labelled minimal media. These are the **Large Scale Cultures**.
2. Transfer 20 mL aliquots of the Large Scale Culture into 24 100 mL flasks.
3. Inoculate each 100 mL flask with a single colony from a plate of freshly transformed plasmid, or anywhere from 20 to 50 uL of glycerol stock of *E. coli* harboring the desired plasmid. This is the **Starting Culture**.
4. Incubate the Starting Cultures with shaking at 37C, 200 rpm overnight. See Note 2.
5. Inoculate each Large Scale Cultures with an entire Starting Culture and aerate, or shake at 200 rpm, at 37C for 24 to 30 hours.
6. Harvest cells by centrifugation at 7000 rpm for 20 minutes at 4C.

¹ 3-(N-morpholino)propanesulfonic acid (MOPS) buffer at pH 6.5 is one of our standard screening buffers but a 1M stock solution turns yellow at room temperature in less than a week, so only make enough for immediate use. Any left over MOPS stock may be stored at 4°C for up to 2 weeks, but after prolonged storage even at 4°C this buffer will eventually turn yellow. We do not use yellow MOPS solution.

² Make sure the starting culture is very cloudy before proceeding to the next step. From our observations, overnight incubation suffices for starting culture inoculated with a freshly transformed colony from a plate. For those inoculated with glycerol stocks, it depends on the cell density of the glycerol stock used as well as the condition of the glycerol stock (i.e., how long has it been in the -80C freezer and how many times has it been freeze-thawed). If the starting culture does not grow well, it can be re-inoculated with more glycerol stock and incubated again overnight.

7. Freeze cell pellets in a falcon tube at –80C until ready for purification.

NMR samples for screening

This part is implemented in parallel. Ideally, there is no limit as to how many targets can be purified per batch. Our group typically purifies 12 targets at a time (see Note 3). We use a Branson digital sonifier with a 4-prong horn to lyse the cells, Sorvall Evolution RC centrifuge equipped with SLA600TC rotor for spinning the falcon tubes. The method described here is for his-tagged proteins. If a different tag is used, purify the proteins accordingly and proceed to step 12.

1. Add 25 mL of lysis buffer into the frozen cell pellet. Shake the falcon tube vigorously, this will dislodge and thaw the frozen cell pellet from the bottom of the falcon tube and form a thick sludge.
2. Lyse the cells by sonication on ice.
3. Spin the cell debris at 12000 rpm for 20 min at 4C.
4. Decant the supernatant into another falcon tube and add 3 mL of Ni-NTA suspension to the latter. See Note 4. Discard the cell debris.
5. Rock the falcon tubes for an hour to allow the proteins to bind to the Ni-NTA beads at 4C.
6. Spin the beads down at 3000 rpm for 5 min. at 4C.
7. Carefully decant and discard the supernatant.
8. Wash the beads by adding 10 mL of Lysis Buffer, rock the falcon tubes for 10 to 15 min at 4C, spin the beads down and discard the supernatant.
9. Repeat the washing step 2 more times but use 10 mL of Washing Buffer each time.
10. In the last washing step, pour the beads into a gravity flow column (Biorad Econo-Pac).
11. Elute the protein from the beads with 5 mL of Elution Buffer.
12. Measure the protein yield. See Note 5.
13. Concentrate each protein to around 0.5 mL using centricon / microsep (Millipore / Pall). See Note 6.
14. Transfer 100 uL of each concentrated protein into a 100 uL dialysis button (Hampton Research). See Note 7.

³One sonifier can lyse 4 targets per batch (step 2) and the SLA600TC rotor can spin 14 falcon tubes per batch (steps 3, 6, and 8), so optimal number of targets per purification batch should be multiples of 12.

⁴Most Ni-NTA beads come in 30% ethanol solution. We remove the ethanol by exchanging the storage solution of Ni-NTA beads into our Lysis Buffer to make a 50% suspension.

⁵The number of NMR buffer conditions that can be tested will depend on the yield. We use the semi-quantitative Bradford assay because most of our targets for screening have His₆-tags and are eluted with high imidazole concentrations that interfere with using absorbance at 280 nm to fully quantify the protein.

⁶The final volume to which the sample should be concentrated to will depend on the yield as well as the NMR spectrometer's sensitivity. We try to achieve a final concentration of around 5 mg/mL for a 1.7mm microprobe at 500 MHz.

15. In a 50 mL falcon tube, prepare each NMR buffer by mixing 0.5 mL of the 1M buffer stock with 49.5 mL of NMR screening buffer. See Notes 1 and ⁸.
16. Put each dialysis buttons into its respective falcon tube and dialyze by rocking overnight at 4C.
17. Transfer each sample into an NMR tube (Bruker Z106463) using a hamilton syringe.

NMR screening

1. Measure the ¹⁵N-HSQC. We use the standard HSQC sequence in the Bruker pulse sequence library: hsqcetf3gpsi2 (15,16) with the following acquisition parameters: ¹H and ¹⁵N sweepwidth of 7000 and 2000 at 500 MHz, respectively; number of t1 increments = 64; number of scans per increment = 32. Total acquisition time per sample is approximately 3 hours.
2. Process the spectra using NMRpipe and NMRDraw software (17).
3. Classify the HSQC as “good”, “promising”, “poor”, or mostly “unfolded”. See Figure 2.

Acknowledgments

We acknowledge the support of NIGMS Protein Structure Initiative grant U54-GM094597 to NESG consortium and the Canada Foundation for Innovation for infrastructure funding.

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⁷We number each of our dialysis buttons so we can identify which contain what protein when all the buttons are dialyzed in the next step.

⁸Before our group acquired the 1.7 mm microprobe, the yield from one fermentation flask would only allow us to screen with one buffer condition because of the sample volume requirement of 0.5 mL. We use the protein's theoretical pI (<http://web.expasy.org/protparam>) to decide which of our 3 standard buffers (acetate 5.0; MOPS 6.5; tris 7.7) to exchange against. With the lower volume requirement of the microprobe, we can usually screen against all of our 3 standard buffers and sometimes other buffers as well, such as Sodium Phosphate (Na₂HPO₄), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2,2-Bis(hydroxymethyl)-2,2',2''-nitrilotriethanol (Bis-tris), etc.

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Figure 1.

Innova 4430 incubator shaker modified into a LEX fermentation system (Harbinger Inc.) by introducing air lines that aerate each bottle via a glass frit sparger (available from Harbinger Inc.). This modification does not alter the shaking mechanism of the Innova 4430. Another advantage of this LEX system, aside from increasing the fermentation capacity of each incubator, is that the centrifuge bottle serves as the fermentation vessel and hence, facilitates the harvesting of the cells. The LEX system is well suited to minimal media, compared with rich media like TB or LB media because foaming is limited in minimal media and hence, eliminates the need to add anti-foam during fermentation. Good aeration also facilitates growth in minimal media.

Figure 2.

Sample ^{15}N -HSQC spectra that we would classify as (a) GOOD, see Note 8. Protein construct of the PHD domain of human methyltransferase MLL5 (GI:33636768) from residues 109 to 188. The structure was subsequently solved (PDB: 2LV9) (b) PROMISING, see Note 9. This is an alternate construct of the PHD domain of methyltransferase MLL5 (GI:33636768) from residues 82 to 194. (c) POOR. Full length protein construct of *L. monocytogenes* lmo0240 (GI:16802286). (d) MOSTLY UNFOLDED, see Notes 10. Protein construct of *C. rodentium* EspB (GI:5802672) from residue 124 to 231. Spectra are plotted with ^1H chemical shift on the horizontal axis from 6 to 10.5 ppm and ^{15}N chemical shift on the vertical axis from 105 to 136 ppm.

⁹A “good” ^{15}N -HSQC spectra should display good peak dispersion, roughly uniform peak intensities and the number of peaks should correspond roughly to the expected number of NH groups based on the protein sequence. We rarely observe the NHs for the His₆-tag and so do not count those residues in our analysis. For most of the targets that fall into this category, we would proceed with uniform ^{13}C and ^{15}N labeling of the protein and determine its structure with ease. However, because we screen the targets at a much lower concentrations than that required for full NMR data acquisition, some samples “fail” at the structure determination stage due to limited solubility at higher concentrations.

¹⁰A “promising” ^{15}N -HSQC spectra shows a hint of peak dispersion and most of the time the peak intensities are non-uniform, and the number of peaks do not match the expected peak count based on the protein sequence, indicative of multiple conformations. For targets under the promising classifications, as well as the few failures in “good” classification (see Note 8), it is worthwhile to screen for more buffer or pH conditions. If the protein function is known, screening with suspected ligands is an obvious route for recovery of the sample. If the number of peaks observed is more than expected, check the number of cysteines in the protein sequence and make sure enough DTT is added or perhaps use a stronger reductant such as TCEP. For example see PDB_id 2KKX and 2KKY (18). If reductant is not the issue, and the protein function is of unknown function, a bioinformatic search such as remote sequence homology to a PFAM family may give a clue as to what ligand to try (example: PDB_id 2K5T). For cases in which too few NH resonances are observed, sequence the plasmid DNA and acquire the mass spectra for the protein sample to ensure that the protein in the NMR sample is not truncated, if the protein sample is the correct, expected size, we assign the resonances that are observable and solve the parts of the protein that we can “see” by NMR. An example is PDB_id 1JJG (19), in which less than 80% of the expected peaks in the ^{15}N -HSQC spectra were observed and even those peaks are non-uniform in intensity. Thus, a structure or part of a structure can still be determined for proteins that give only a “promising” ^{15}N -HSQC. If different buffer conditions and a ligand search fails to stabilize the sample, then an alternative constructs should be cloned and then re-screened. In cases of domains within larger eukaryotic proteins we routinely screen multiple constructs of the target domain in parallel, in order to find the best construct for NMR. Figure 2A and 2B show the remarkable difference in stability and ^{15}N -HSQC with small changes in protein domain boundary.