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1 TITLE:

Measuring interactions of globular and filamentous proteins by nuclear magnetic resonance spectroscopy (NMR) and microscale thermophoresis (MST)

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SUMMARY:
Here, we present a protocol for the production and purification of proteins that are labeled with stable isotopes, and subsequent characterization of protein-protein interactions using Nuclear Magnetic Resonance (NMR) spectroscopy and MicroScale Thermophoresis (MST) experiments.

ABSTRACT:
Filamentous proteins such as vimentin provide organization within cells by providing a structural scaffold with sites that bind proteins containing plakin repeats. Here, a protocol for detecting and measuring such interactions is described using the globular plakin repeat domain of envoplakin and the helical coil of vimentin. This provides a basis for determining whether a protein binds vimentin (or similar filamentous proteins) and for measurement of the affinity of the interaction. The globular protein of interest is labeled with ^{15}N and titrated with vimentin protein in solution. A two-dimensional NMR spectrum is acquired to detect interactions by observing changes in peak shape or chemical shifts, and to elucidate effects of solution

conditions including salt levels, which influence vimentin quaternary structure. If the protein of interest binds the filamentous ligand, the binding interaction is quantified by MST using the purified proteins. The approach is a straightforward way for determining whether a protein of interest binds a filament, and for assessing how alterations, such as mutations or solution conditions, affect the interaction.

INTRODUCTION:

Interactions between proteins allow the formation of molecular machines that create order within cells. The individual interactions are often weak but usually contribute to multivalent complexes that can be cooperative and dynamically regulated. Sensitive assays that provide atomic resolution and quantitative information about such complex interactions are needed to deduce mechanisms and design interventions such as drug-like molecules. NMR spectroscopy is an efficient method for obtaining such information about protein interactions, and also is used for fast screening for ligands including those that bind weakly[1]. The NMR methods used can be categorized into those that are protein observe or ligand observe. This manuscript uses the former approach in which a spectrum of a stable-isotope labeled protein that is comparatively small (usually under 20 kDa) is acquired and the unlabeled ligand is titrated. This allow the labeled residues involved in the interaction to be mapped in favorable cases. Once the complex forms, there are changes in the chemical environments of interacting residues that manifest themselves as changes in the chemical shift and shape of their NMR signals. The extent of such changes correlates with the degree of involvement of these groups in the interaction. Chemical shift perturbations (CSPs) can be measured by comparing a series of NMR spectra of the protein collected in the absence and presence of varying amounts of the ligand. For larger ligands or complex interactions, the change in peak shape or intensity can be measured to deduce interactions.

The most common 2D experiment used for detecting ligand interactions is the ^{15}N-heteronuclear single quantum correlation (HSQC) experiment[2]. This requires that one protein be uniformly labeled with ^{15}N, which is typically achieved by expressing them as affinity-tagged versions in *E. coli* bacterial cultures grown in ^{15}N-enriched media. Binding is apparent when the HSQC spectra collected during the titration are superimposed, revealing peak changes for a subset of residues involved in the complex formation. The interaction can occur in the fast exchange regime where the free and ligand-saturated state signals collapse into one population averaged peak. Alternatively, in the case of slow exchange between the states, both signals are observed with integrals that represent their relative amounts. While NMR lineshape analysis can be used to estimate the binding affinities in some cases, methods such as MST have also proven convenient and provide cross-validation of genuine interactions.

The example provided is of two proteins found within desmosomes. They mediate junctions between cell surfaces and the cytoskeleton and mediate multivalent interactions between cell adhesion machines and intermediate filaments to maintain the integrity of skin and heart tissues and withstanding of shear forces. Diseases can result when desmosomal proteins such as desmoplakin or vimentin are compromised by mutations or autoantibodies, leading to destabilization of cell-cell junctions, and hence their interactions are of critical importance[3]. The

structural basis of ligand binding by desmosomal proteins can be characterized by NMR spectroscopy, while the interactions can be quantified by MST. Methods herein were used to characterize the interactions between plakin repeat domains (PRDs) which often are present as tandem sets that offer basic grooves, and vimentin, an intermediate filament that interacts through an acidic surface offered by its helical bundle[4]. These complexes are formed at the cell membrane where they anchor for intermediate filaments of the cell cytoskeleton to desmosomes that connect to adjacent cells, thus forming a network of adhesive bonds that radiates throughout a tissue.

PROTOCOL:

1. Recombinant Protein Expression

1.1 Expression of Envoplakin PRD (E-PRD) and Vimentin 99-249 (VimRod)

1.1.1 Transform *E. coli* BL21(DE3) cells with the plasmid containing the desired gene. Spread the cells on agar plates containing 100 µg/mL ampicillin. Incubate the plates at 37 °C overnight.

1.1.2 Pick a single colony and inoculate 20 mL of Terrific broth (TB) containing 100 µg/mL ampicillin to select for the plasmid. Grow the culture at 37 °C with shaking (180 rpm) overnight.

1.1.3 Transfer the entire 20 mL culture to 1 L of TB containing 50 µg/mL ampicillin. Incubate the culture at 37 °C with shaking at 180 rpm until the OD_{600} = 0.6-0.8.

1.1.4 Reduce the temperature to 18 °C and induce protein expression by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Continue incubation at 18 °C with shaking at 160 rpm overnight to allow protein expression.

1.1.5 Harvest the cells by centrifuging the culture at 8000 x g for 15 min. Decant and discard the supernatant.

1.1.6 Wash the harvested cells by resuspending the cell pellet in approximately 40 mL of phosphate buffered saline solution (PBS: 20 mM phosphate buffer, pH 7.4, 120 mM NaCl). Transfer the resuspension to a 50 mL tube. Centrifuge again at 8000 x g for 15 min.

1.1.7 Decant and discard the supernatant. Either immediately begin the purification protocol or freeze the cell pellets at -20 °C for future use.

1.2. Expression of Isotopically Labeled Protein

1.2.1 Transform *E. coli* BL21(DE3) cells and prepare a 20 mL starter culture as in 1.1.1-1.1.2.

1.2.2 Transfer the entire 20 mL culture to 1 L of enriched TB containing an additional 4.0 g tryptone, 5.0 g NaCl, and 100 µg/mL ampicillin. Incubate the culture at 37 °C with shaking at 160 rpm until the OD_{600} = 1.6-1.9.

1.2.3 Harvest the 1 L culture by centrifugation at 8000 x g for 15 min. Decant and discard the supernatant.

1.2.4 Wash the cell pellet by gently resuspending in approximately 40 mL of PBS and transfer the resuspension to a 50 mL tube.

1.2.5 Centrifuge again at 8000 x g for 15 min. Decant and discard the supernatant.

1.2.6 Resuspend the cell pellet in 20 mL of M9 minimal media (**Table 1**) and transfer to the remainder of the 950 mL of M9 minimal media containing 100 µg/mL ampicillin.

1.2.7 Add 50 mL of filter sterilized nutrient mix (**Tables 2 and 3**).

1.2.8 Acclimatize the culture to 18 °C for 30 min before adding IPTG to a final concentration of 1 mM.

1.2.9 Incubate overnight at 18 °C with shaking at 160 rpm.

1.2.10 Harvest the cells as in section 1.1.4-1.1.6.

2. Immobilized Metal Affinity Chromatography (IMAC) Purification of VimRod and E-PRD

2.1 Purification of His6-tagged VimRod

2.1.1 Resuspend the cell pellet in 5 mL/g of PBS containing a protease inhibitor cocktail lacking EDTA. Homogenize with 12 strokes in a Dounce tissue homogenizer to improve the cell lysis in the following step.

2.1.2 On ice, sonicate the cell suspension at a pulse of 1 s on/1 s off, 80% amplitude for a total of 1.5 min. Repeat the sonication two additional times, swirling gently on ice between runs to prevent overheating.

2.1.3 Centrifuge the sample at 75 000 x g for 45 min. Decant and filter the supernatant using a syringe filter (0.45 µm).

2.1.4 Equilibrate a 5 mL IMAC column with 5 column volumes (CV) of binding buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 10 mM imidazole) at a flow rate of 1 mL/min using a fast protein liquid chromatography (FPLC) system.

2.1.5 Load the filtered supernatant onto the column at a flow rate of 0.5 mL/min.

2.1.6 Wash the column with 5 CV of Wash buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 50 mM imidazole) at a flow rate of 1 mL/min.

2.1.7 Elute the protein with 3 CV of elution buffer (20 mM HEPES, pH 7.5, 500 mM NaCl, 350 mM imidazole) at a flow rate of 0.5 mL/min. Collect 1.5 mL fractions. If available, select the up-flow elution mode to increase the concentration of eluted protein.

2.1.8 Identify the fractions from the FPLC chromatogram that contain the protein of interest by SDS-PAGE and use standard methods to measure the protein concentration[5].

2.1.9 Pool and concentrate the elution fractions containing the highest amounts of protein using a centrifugal ultrafiltration device (MWCO 3 kDa, 5 mL) to 2 mL. Centrifuge at 21 000 x g to remove any precipitate and pass through a 0.22 µm filter.

2.1.10 Equilibrate a 120 mL size exclusion chromatography (S) column with 2 CV of S buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 0.5 mM TCEP) at a flow rate of 1 mL/min using an FPLC.

2.1.11 Inject the concentrated protein from 2.1.9 onto the column and elute with 1 CV of S buffer at a flow rate of 0.5 mL/min, collecting 1 mL fractions.

2.1.12 Identify the fractions containing the protein of interest as before.

2.1.13 Pool the fractions containing the highest amounts protein.

2.1.14 Store at 4 °C for short term use or add glycerol to 20% and store at -80 °C in small aliquots.

2.2. Purification of E-PRD Protein with the His6 Tag Removed

2.2.1 Follow the steps in 2.1.1-2.1.8 to purify His6-tagged E-PRD protein. Pool the peak fractions and determine the protein concentration.

2.2.2 Add tobacco etch virus (TEV) protease (1 mg/mL) at 2 µL/mg of pooled protein. Transfer to dialysis tubing (6 kDa) and dialyze in S buffer overnight at 4 °C. This step allows cleavage of the His6 tag and removal of the imidazole that will interfere with binding to the Ni-NTA resin in the next step.

2.2.3 Equilibrate 5 mL of Ni-NTA resin in a gravity column with 3 CV of S buffer. Drain excess buffer from the resin.

2.2.4 Pour the cleaved E-PRD protein onto the resin and incubate for 1 hour on a rocking platform to allow the uncleaved His6-tagged E-PRD and the cleaved His6 tag to bind. The TEV

protease is also His6-tagged and will to bind the resin. Collect the flow through, which contains the tag-free E-PRD. Wash the resin with 2 CV of S buffer to ensure all of the E-PRD is recovered.

2.2.5 Concentrate E-PRD in the flow through to 2 mL using a centrifugal ultrafiltration device (MWCO 3 kDa, 5 mL). Centrifuge at 21 000 x g to remove any precipitate and pass through a 0.22 µm filter.

2.2.6 Equilibrate a 120 mL S column with 2 CV of S buffer (20 mM HEPES, 150 mM NaCl, pH 7.5, 0.5 mM TCEP for MST or 20 mM Tris-HCl, 1 mM DTT, pH 7 for NMR) at a flow rate of 1 mL/min using an FPLC.

2.2.7 Inject the concentrated E-PRD protein from 2.2.5 onto the column and elute with 1 CV of S buffer at a flow rate of 0.5 mL/min, collecting 1 mL fractions.

2.2.8 Identify the fractions containing the protein of interest as before.

2.2.9 Pool the fractions containing the highest amounts protein.

2.2.10 Store at 4 °C for short term use or add glycerol to 20% and store at -80 °C in small aliquots.

3 NMR Methods

3.1 NMR Sample Preparation

3.1.1 Purify ^{15}N-labeled wild-type or R1914E E-PRD protein as previously described using 20 mM Tris-HCl, 1 mM DTT, pH 7 as the S buffer for step 2.2.6. Protein stock solutions usually range from 0.3 to 1 mM with volumes of about 1 mL.

Note: Protein can be concentrated to >100 µM using a MWCO 3 kDa, 5 mL centrifugal ultrafiltration device to bring the concentration into a suitable range for sample preparation.

3.1.2 Purify a sample of unlabeled VimRod protein using 20 mM Tris-HCl, 1 mM DTT, pH 7 as the S buffer for step 2.1.10.

3.1.3 In a final volume of 500 µL, add wild-type or mutant E-PRD protein to a final concentration of 100µM, deuterium oxide (D_2O) to a final concentration of 10% (v/v), and DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) to a final concentration of 20 µM. Bring the sample volume up to 500 µL using 20 mM Tris-HCl, 1 mM DTT, pH 7. A representative sample preparation is described in **Table 4**.

Note: The 0 ppm resonance of DSS is used to calibrate the ^{1}H chemical shifts as well as for indirect referencing of the ^{15}N chemical shifts of the protein[6]. D_2O is used for the deuterium lock signal to keep the spectrometer operating at a constant net magnetic field.

3.1.4 Make up a second sample of E-PRD, D_2O and DSS as in the previous step and add VimRod to a final concentration of 50 µM before bringing the volume up to 500 µL.

3.1.5 Transfer the 500 µL samples to a 5 mm wide NMR tubes for the experiment.

3.2 NMR Experimental Setup

3.2.1 Turn on the air flow with the eject command "ej"; this will bring the sample up from the magnet. Now, place the sample within a spinner on top of the magnet by the opening and insert with the command "ij". Wait until sample settles inside the magnet before proceeding.

3.2.2 Create a new dataset using the "edc" command and load standard ^1H NMR parameters by selecting experiment "ZGPR" (**Figure 1**). Fill in the NAME, EXPNO (experiment number) and PROCNO (processed data folder number) fields. Select the solvent in the "Set solvent" field and click on "Execute 'getprosol'" to read standard probehead and solvent dependent (prosol) parameters.

3.2.3 Lock the sample to the deuterated solvent, *i.e.,* D_2O, using command "lock" and wait until it is finished sweeping and achieves lock.

3.2.4 Correct the resonance frequency of the magnet by tuning the sample using the automatic tuning command "atma". Monitor the wobble curve until the automatic tuning is complete.

3.2.5 Shim the magnetic field using TOPSHIM (command "topshim"). Shimming is process of adjustments to magnetic field to achieve uniformity around the sample. It is good practice to store the shim values with the command "wsh" and read them using "rsh" before topshim, if using the same or similar samples.

3.2.6 Adjust the receiver gain with "rga" command to achieve maximum signal to noise ratio.

3.2.7 Place the center of the spectrum on the water resonance offset (o1) and set the 90 degree proton pulse (p1) at high power using "calibo1p1".

3.2.9 Collect the proton spectrum using the zero go "zg" command and process with "efp" which includes exponential multiplication ("em"), the free induction decay (FID) incorporating line broadening, "ft" fourier transformation of FID and "pk" to apply phase correction.

3.2.10 Apply the automatic phase correction "apk" and the automatic baseline correction "absn" using the polynomial without integration option.

3.2.11 Create a new dataset (as in 3.2.2) for the SOFAST HMBC experiment by selecting "SFHMQC3GPPH" in experiment.

3.2.12 Copy optimized P1 and O1 from proton spectrum and populate P1 dependent pulses by using command “getprosol 1H p1 plw1”, where p1 is the optimized P1 value and plw1 is the power level for P1.

3.2.13 Optimize the CNST54 constant to set the offset for amide chemical shift and CNST55 to define the bandwidth in order to encompass the spectral regions of interest which allows the receiver gain to be optimized (**Figure 2**). To select these parameters, extract the first FID (free induction decay) from the two-dimensional spectrum and look for the observed signal to define them. In addition, vary the relaxation delay (D1), number of scans (NS), and dummy scans (DS) to obtain acceptable signal sensitivity with command “gs”, which enables go and scan to monitor data quality in real time.

3.2.14 Record the spectra using Zero Go “zg”.

3.3 NMR Data Processing

3.3.1 Set the processing parameters to the size of the direct F2 (^{1}H) and indirect F1 (^{15}N) dimensions of the spectrum using “SI F2=2048, F1=512” with optional linear prediction in indirect dimension (**Figure 3**).

3.3.2 Select “QSINE” as the Window function and enter a Sine bell shift (SSB) of 2 to process the two-dimensional spectrum.

3.3.3 Enter the command “xfb” to process the data in both directions with window function and fourier transformation.

3.3.4 Use the command “apk2d” to carry out automatic phase correction in both directions. If the automatic process does not achieve a satisfactory level of phase correction, extract FIDs with the “rser” command, calculate phase values from 1D processing, and apply them to the 2D data.

3.3.5 Correct the baseline with the automatic baseline correction function “abs2” for 2D data. This applies a polynomial function between the ppm values defined in the processing parameters and will produce a 2D spectrum for further analysis.

3.3.6 If planning to perform serial processing for comparison of interaction data with another molecule, store the processing parameters with the command “wpar” and recall them with “rpar”. In this way all the datasets will be processed with same parameters and variations will not be introduced due to processing differences.

3.4 NMR Data Analysis

3.4.1 Enter the command “pp” to begin the process of peak picking.

3.4.2 Define the ppm range and minimum intensity/maximum number of peaks based on expected peaks (**Figure 4**). Click on OK and verify the results by visual inspection. If needed, re-run the process until results are satisfactory based on spectra quality.

3.4.3 Generate a peaklist with the "pp" command.

Note: This peaklist contains data height/peak intensity information by default and can be exported to subsequent spectrums and can be read by other programs.

3.4.4 Observe changes in the peak intensities or movement in chemical shifts in the protein HSQC spectra that indicate interaction with another molecule. If the interacting molecule is large, expect reductions in peak intensities along with disappearance of some peaks.

3.4.5 Import the peak list to the next data set by clicking on the "peaks" tab and selecting "import" with a right click in the peaks window.

3.4.6 Visualize the peaks over the spectrum and if needed shift them to new positions. Click on "reset intensities" for "complete table" to generate a peaklist for the spectrum with intensities (**Figure 5**). This peak list will carry over the position information from stored peak list.

3.4.7 Export the peak lists from different datasets to a spreadsheet or other mathematical program for analysis by selecting the "Export" function.

3.4.8 Calculate the change in peak intensities with the function "peak intensity in complex spectrum/peak intensity in protein spectrum" for each peak. Values can be converted to percentage change by multiplication of 100. Note that peak volumes are also useful although peak intensities are easier to measure for peaks that are positioned close to each other, as is usually the case for proteins with a high density of relatively broad peaks.

4. MicroScale Thermophoresis (MST)

4.1 Preparation of the Ligand Protein E-PRD

4.1.1 Exchange the ligand into an MST compatible buffer by dialyzing up to 800 µL of protein in a 3.5 kDa mini-dialysis unit suspended in 1 L of 20 mM HEPES, pH 7.5, 10 mM NaCl, stirring slowly at 4 °C overnight.

4.1.2 Concentrate the ligand using a centrifugal ultrafiltration unit (3 kDa MWCO) by centrifuging at 14 000 x g for 10 min. Transfer the concentrated protein to a clean tube.

4.1.3 Centrifuge the ligand at 21 000 x g for 10 min and carefully transfer the supernatant to a new tube to remove any precipitated protein. Determine the concentration of the ligand using the absorbance at 280 nm and the ligand extinction coefficient. Add 10% Tween-20 to give a final concentration of 0.015%. Tween-20 is added to the assay buffer to prevent adsorption to

the capillaries. The final assay buffer that is used for the MST experiments is 20 mM HEPES, pH 7.5, 10 mM NaCl, 0.015% Tween-20.

4.2 Preparation of Dye-labeled Target Protein VimRod

4.2.1 Reconstitute the RED-tris-NTA dye by adding 50 µL of 1x PBS-T supplied with the RED-tris-NTA to give a concentration of 5 µM. Dispense 2 µL aliquots into 200 µL tubes and store at -20 °C.

4.2.2 Dilute the target protein to 0.34 µM with assay buffer. Add 58 µL of target to a 2 µL aliquot of RED-tris-NTA dye and incubate for 30 min at room temperature. The final concentration of dye-labeled target is 0.33 µM. Centrifuge the labeled target at 21 000 x g for 10 min and carefully transfer the supernatant to a new tube to remove any precipitate. The RED-tris-NTA dye binds to proteins through the His6 tag and has a binding dissociation constant (K_D) in the sub-nanomolar range. It is effectively 100% bound to the target protein so no further purification is required.

4.2.3 Turn on the MST instrument and open the Control Software. Select the Red setting for the RED-tris-NTA dye. Turn on the temperature control to 25 °C.

4.2.4 Select the Pretest to validate the labeling of the target and check for aggregation or adsorption to the cuvettes. An assessment of these parameters is automatically provided.

4.2.5 Mix 17 µL of assay buffer and 3 µL of target protein and mix by pipetting. Fill two standard capillaries by dipping them in the diluted target and drawing up liquid into the center of the capillary. Place capillaries in the tray and into the instruments. Start the measurement.

4.2.6 Review the results looking for a sufficient level of fluorescence and no signs of adsorption (distortion in the capillary line shape) or aggregation (distortions in the MST trace) which will be flagged in the software analysis. If results are positive move on to the ligand steps, if not a different buffer must be tried or the amount of Tween-20 may be increased to 0.05% or higher.

4.3. Preparation of the E-PRD Ligand Two-fold Dilution Series

4.3.1 Prepare a dilution series by labeling 16, 200 µL tubes from 1-16.

4.3.2 Add 17 µL of the ligand at 1.17x greater concentration than the maximum ligand concentration desired to Tube1. This volume is twice the amount needed (8.5 µL) as an aliquot will then be transferred to the next tube in series. The final volume of the assay is 10 µL so 8.5 µL of 1.17X concentration of ligand will be diluted to 1x by addition of 1.5 µL of the target protein, VimRod. This maximum concentration chosen should be at least 20 times the estimated K_D value.

4.3.3 Add the 8.5 µL of assay buffer to Tubes2-16 using a new pipette tip for each aliquot. Reusing pipette tips can affect the accuracy (for advice on accurate pipetting see manufacturer's instructions). Transfer 8.5 µL of ligand from Tube1 to Tube2 slowly releasing the solution into the assay buffer without generating bubbles. Mix the ligand and buffer by pipetting up and down at least 6 times, again without generating bubbles. The E-PRD in the most concentrated tube was 1.5 mM and the labeled VimRod was present at a final concentration of 50 nM.

4.3.4 Transfer 8.5 µL of ligand from Tube2 to Tube3 and mix with the assay buffer. Repeat the serial dilution until all tubes have had ligand added. Discard 8.5 µL from Tube16 so that all tubes contain 8.5 µL of ligand in a series of two-fold dilutions.

4.4. Preparation of the Binding Reaction and MST experiment

4.4.1 Add 1.5 µL of the labeled target protein to each of the tubes and mix gently by pipetting up and down being careful to avoid bubbles. Incubate for 15 min.

4.4.2 Select the Expert Mode for the binding assay and enter in the parameters for a serial dilution series. Make sure temperature control is set to 25 °C, the excitation power is set to 40%, and the MST power to medium. These parameters must be optimized for other binding partners being studied.

4.4.3 Fill the capillaries with the binding reactions and place in the capillary tray. Load the tray into the instrument, wait for the temperature to regain 25 °C and start the measurement.

4.5. Data Analysis

4.5.1 Open the binding assay file in the Affinity analysis software. Review the capillary scans; Fluorescence levels should not vary more than 10% from the average, no aggregation or adsorption should be detected as described in section 3.6.

4.5.2 Select the MST analysis on the right panel and drag the assay data into the analysis set. If there are multiple runs of the same target-ligand binding assay under identical conditions they can be merged by dropping in the same set. Alternatively, each run can be dropped into analysis independently.

4.5.3 Move to the Dose Response Fit tab to graph the data. Choose the K_D model if a single binding site is expected. The Hill model is also an option for multiple binding sites with cooperative behavior. Outlier points that did not pass quality control can be removed from the fit at this stage. Merge sets with multiple assays will be averaged and errors calculated as the standard deviation.

4.5.4 Open the final tab and compare results between all plots on a single graph. Retrieve fitting results for each curve from the table that is generated. Export the data or fitted curves to other presentation or analysis software if desired.

REPRESENTATIVE RESULTS:

The E-PRD domain (residues 1822-2014 cloned into pProEX-HTC) of the human envoplakin gene and the VimRod domain (residues 99-249 cloned into pET21a) of human vimentin[4] were expressed with His6 tags and purified. **Figures 6 and 7** demonstrate the levels of purity of VimRod (18.8 kDa) and E-PRD (21.8 kDa) obtained from this method of protein purification. The removal of the His6 tag from the E-PRD construct is essential for the MST experiments as the VimRod protein is labeled using a His6 tag binding dye and any E-PRD retaining its His6 tag may compete for binding of the dye. The second IMAC column after cleavage of the tag with TEV protease removes the TEV protease, the cleaved tag and any uncleaved His6-E-PRD that remained. The final polishing step of the purification is size exclusion chromatography. Despite both proteins being of a similar size, the VimRod elutes from the column at a 51 mL while the E-PRD elution peak is centered at 72 mL where a protein monomer of this size would be expected. The apparent increase in size of VimRod is likely due to the its characteristics as a filamentous long rod shaped protein as analytically ultracentrifuge experiments demonstrated that VimRod was monomeric[4]. Lower yields of protein are obtained from the cultures grown in M9 than those from rich broth due to a lower amount of cells being produced in the minimal media. The initial growth of larger starter cultures for M9 preparations in TB allows improvement of cell yields while maintaining the extent of ^{15}N labeling necessary for the NMR experiments.

The ^{15}N-^{1}H HSQCs were acquired for the wild type and R1914E mutant of E-PRD in presence or absence of VimRod (**Figure 8A**-**8D**). The spectrum of E-PRD in **Figure 8A** shows the expected number of well resolved peaks, indicative of a properly folded protein. In the presence of VimRod (**Figure 8B**) the spectrum shows extensive line broadening and peak disappearance, corresponding to binding between the E-PRD and VimRod. This binding is lost by mutation of R1914E as evidenced by comparison of **Figure 8C and 8D**. Little change is observed in the spectrum upon addition of VimRod to the R1914E mutant indicating a lack of binding between this mutant E-PRD and VimRod. The E-PRD peak intensities in the presence/absence of VimRod were compared and plotted as the relative peak intensities in **Figure 8E**, which indicates the range of peak broadening in the E-PRD complex. The R1914E mutant of E-PRD (not shown) retained about 97% of peaks at 20% or higher peak intensities in presence of VimRod compared to about 20% for the wild type (**Figure 8E**). This represents a loss of function point mutant, with additional mutants having intermediate effects also having been studied[4].

To validate and quantitate the binding of VimRod and E-PRD MST analysis using His6-VimRod labeled with fluorescent RED-tris-NTA dye as the target mixed with decreasing concentrations of the ligand E-PRD from 1.28 mM to 39.1 nM were performed. Three binding titrations were carried out and the results are averaged and shown in **Figure 9**. The data were fit with a standard model of one-site ligand binding and gave a K_D of 25.7 ± 2.1 µM. Evaluation of the

binding between VimRod and E-PRD by surface plasmon resonance gave a similar K_D value of 19.1 ± 1.3 μM[4].

FIGURE AND TABLE LEGENDS:

Figure 1. **Screen Capture of the Setup of the NMR Experiment.** The window shown is used to set up a standard experiment to collect a HSQC dataset. Experiment parameters are read in adjacent to Experiment. The ZGPR experiment shown is chosen as an initial experiment to load the standard and solvent dependent proton parameters. The Title window is used to input experimental details for record keeping purposes. To collect the HSQC spectrum the ZGPR experiment is replaced with SFHMQC3GPPH.

Figure 2. **Adjusting of NMR Experimental Parameters.** The window shown is used for entering the basic parameters for the NMR pulse sequence in order to optimize the signal.

Figure 3. **NMR Data Processing.** Parameters used for processing each of the two dimensions of the NMR spectrum are shown, with arrows indicating those that are typically adjusted.

Figure 4. **Parameters for NMR Peak Picking.** The parameters used for picking NMR peaks in the processed NMR spectrum are shown with typical values. Adjust the ppm range, intensity and number of peaks to optimize the spectra.

Figure 5. **Representative Peaklist with Intensities.** Each peak that is picked in the NMR spectrum is given a number, and its ^{1}H and ^{15}N chemical shifts and signal intensity are displayed. This peaklist can then be used to compare spectra obtained in the presence/absence of an interacting partner.

Figure 6. **Purification of His6-tagged VimRod by IMAC and S. A.** The chromatogram for the elution from the IMAC column shows one major peak of VimRod. B. The chromatogram for the elution from the S column shows one major peak. C. SDS-PAGE of fractions collected over the course of purification: MW standards with the MW indicated in kDa to the left of the gel (M), cell lysate (1), IMAC flow-through (2), wash (3), pooled elution (E1), pooled S elution (E2). Bands visible at higher molecular weights in lanes E1 and E2 are oligomers of pure VimRod as confirmed by western blot (data not shown).

Figure 7. **Purification of E-PRD by IMAC and S. A.** SDS-PAGE of the IMAC purification showing the molecular weight standards with the MW indicated in kDa to the left of the gel (M) and the eluate from the first IMAC column (E1), the TEV cleavage products (+TEV), and the flow through from the second IMAC column (FT). B. The chromatograph from the S column shows one major peak. C. SDS-PAGE of the molecular weight standards (M) and the fractions from S peak.

Figure 8. **HSQC Spectra of Wild-type and R1914E Mutant of E-PRD in the Presence and Absence of VimRod.** The HSQC spectra show wild-type E-PRD (100 μM) in 20 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, pH 7 in the absence (A) or presence of 50 μM VimRod (B). Panels C and D

are the HSQC spectra of the R1914E mutant (100µM) in the absence or presence of 50 µM VimRod, respectively. In panel E the relative ^{1}H-^{15}N peak intensities of the E-PRD with or without VimRod binding are shown as a function of the peak number, which is arbitrarily assigned and not based on sequence position. These values can be used to define a significance cutoff for peak intensity reduction upon addition of a ligand. If assignments are available, the significant values can often be seen to map to a binding area.

Figure 9. **Binding of E-PRD to VimRod.** E-PRD was diluted in a series of two-fold dilutions from 1.28 mM to 39.1 nM and incubated with labeled VimRod before performing MST analysis. Data from three independent assays were combined. The data were fit to a K_D model giving a K_D of 25.7 µM with a K_D confidence of ± 2.1 µM.

Table 1. M9 media for Isotopic Labeling.

Table 2. Nutrient Mix for Supplementation of M9 media.

Table 3. Metal Mix Supplement for Enriching the MT Nutrient Mix.

Table 4. NMR Sample Preparation.

DISCUSSION:

The 2D ^{15}N-resolved NMR experiment is one of the most widely used methods to show how two molecules interact. It is the most information-rich method that allows both partners' signals to be continuously monitored throughout a titration experiment in solution state. Although typically qualitative in the case of large complexes, the method can also be used in favorable cases to measure binding affinities where NMR signals can be tracked in high resolution spectra. Where assignments can be conveniently made, such as in the case of many proteins under 20 kDa in size, the binding sites can also be mapped. Complementary assays such as MST provide quantitative information about interactions in solution, and require less protein in unlabeled states. Comparison of mutant binding data is useful for providing controls to ensure that interactions evidenced by NMR line broadening are genuine and not artifacts of, for example, aggregation or viscosity changes.

Protein Expression

Streamlining the expression process reduces the amount of labor intensive protein production. Part of this optimization process involves identification of an appropriate strain of *E. coli* for the recombinant expression of protein. Strain preference depends on elements including the nature of the vector in use and, more specifically, the ultimate stability of the recombinant protein being expressed[7]. The risk of degradation of the heterologous protein by endogenous *E. coli* proteases can be reduced by use of protease deficient *E. coli* such as the BL21 strain. For genes containing rare codons, a strain such as BL21-CodonPlus (DE3)RIPL may be preferred. This strain combines the protease deficient nature of the BL21 strain with additional endogenous copies of rare codon tRNAs for arginine, isoleucine, proline, and leucine. Alternatively, rare codons that can compromise overexpression may be avoided by ordering a

codon-optimized construct from a commercial source. Many strains of *E. coli* are available for recombinant gene expression, each optimized for circumvention of a particular problem during expression[7]. In the case of this study, the standard protease deficient strain BL21(DE3) produced adequate quantities of soluble protein for subsequent purification and analysis.

Protein Purification

The purification protocol for a given protein is often unique in the sense that each protein remains stable and soluble under different conditions such as temperature, salt concentration, or pH. The overall effectiveness of purification through affinity chromatography is also sensitive to the concentration of eluting species such as imidazole at various steps during the purification process. In this work, critical buffer conditions for IMAC were pH for the E-PRD, and imidazole concentration for the VimRod. A pH of 7.5 was required to avoid precipitation of the E-PRD following initial elution from the IMAC column. For the IMAC purification of VimRod, increasing the concentration of imidazole from 30 to 50 mM during the column wash step was found to have a substantial improvement in the purity of the final elution fractions. For the elution step, increasing the concentration of imidazole from 250 to 350 mM also was found to improve the yield of the final elution. Initial attempts to elute protein using 250 mM imidazole led to incomplete elution of VimRod as revealed by a final 1 M imidazole strip of the column (data not shown). Increasing the imidazole concentration to 350 mM for the elution was sufficient to recover all of the protein bound to the column. S can serve a dual purpose because it acts as a polishing step for protein purification while simultaneously performing buffer exchange. Buffer exchange is a critical step for subsequent binding analysis since it removes the imidazole used to elute His6-tagged protein. It also serves as an opportunity to change conditions such as salt concentration or pH, which may impact the efficacy of certain downstream techniques or assays. Protein thermal shift (PTS) can be used to identify optimal buffers for downstream assays, especially for those requiring stable protein for prolonged periods of time at room temperature[8,9].

Binding Analysis

Protein that is freshly prepared is critical for accurate binding assays, although frozen protein can also be used as long as the results are compared. Filamentous proteins such as vimentin multimerize in a salt and pH dependent fashion, and hence the solution condition need to be optimized and the oligomeric state estimated by a method such as SEC[10,11], dynamic light scattering[12] or analytical ultracentrifugation[13-15]. NMR spectroscopy is well suited for measuring ligand interactions of small proteins at atomic resolution. However, when a protein interacts with larger molecule, slower tumbling ensues, and this results in loss of signals, which can confirm binding although it does not necessarily allow mapping of binding sites, which would also require assignment of at least backbone resonances. In this scenario, NMR experiments do not allow identification of the interaction site. Hence site directed mutagenesis is applied to identify the critical residues needed for the binding. Such mutants therefore do not exhibit signal loss. In this protocol, a mutant form with a substitution at position 1914 retains the peak intensities in presence of VimRod and therefore confirms disruption of the interaction of E-PRD and VimRod. Assignment of the backbone and sidechain resonances would add value to this approach, particularly as the structure for the free E-PRD has been solved by X-ray

crystallography[4]. Future applications of NMR include characterization of complex interactions between larger molecules and will benefit from ultra high field magnets and the use of other observable groups such as ^{13}C-labeled and trifluoro methyl groups as reporters.

MST has a number of advantages for studying binding interactions[16]. The binding partners are free in solution and not immobilized. Analysis of the quality of the samples is built into the software with quality control reporting of aggregation, adsorption to the capillaries or insufficient fluorescent labeling of the target molecule. Small amounts of the target are typically used, the concentration of the labeled target is usually between 20-50 nM in a 10-20 µL volume/reaction. This protocol uses very small reaction volumes (10 µL) to maximize the concentration of ligand that can be achieved in the titrations allowing weak binding interactions to be characterized. This necessitates accurate pipetting and care being taken to avoid introducing bubbles while still thoroughly mixing. Adequate mixing is critical for accurate, consistent fluorescence measurements along the set of serial dilutions. The amount of Tween-20 in the MST experiments was reduced from a standard 0.05% to 0.015% to lower the tendency to create bubbles and improve mixing.

The RED-Tris-NTA dye provides a quick, easy and convenient way to fluorescently label any protein that has a His tag. The labeling is effectively complete in only 30 minutes and is very tight so that no dye removal procedure is necessary. No modifications are made to amino acid residues in the protein that might alter the ligand binding properties. A caveat is that only the protein to be labeled should have a His6 tag. This required the cleavage of the tag from the ligand protein, E-PRD, and the removal of the tag and uncleaved E-PRD with a second IMAC column step. If possible, the ligand protein should be prepared without the use of a His tag. Alternatively, proteins may be covalently labeled with a fluorophore through amine coupling to lysine residues or thiol coupling to cysteine residues. However, care must be taken when using such systems since the covalent attachment of a fluorophore may affect electrostatic or polar binding interactions relying on lysine or cysteine residues. The quantification of binding affinity between the VimRod and E-PRD by MST was unusually sensitive to salt concentration. This problem was mitigated by initially dialyzing both the target and ligand into the same batch of assay buffer. Nonetheless, saturation of the MST binding curve could not be achieved when performing the MST assay in the presence of 150 mM NaCl due to the complex behavior of VimRod. Reliable, complete data was obtained once the concentration of NaCl was lowered to 10 mM allowing accurate calculation of the K_D. Hence, careful optimization of solution conditions and comparison with complementary assays are recommended to achieve robust results. Furthermore, MST may be used to quantify the salt dependence for a given interaction, quantify stoichiometric properties of protein interactions, monitor protein folding, and probe into enzyme kinetics[17].

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DISCLOSURES:

The authors disclose no conflicts of interest.

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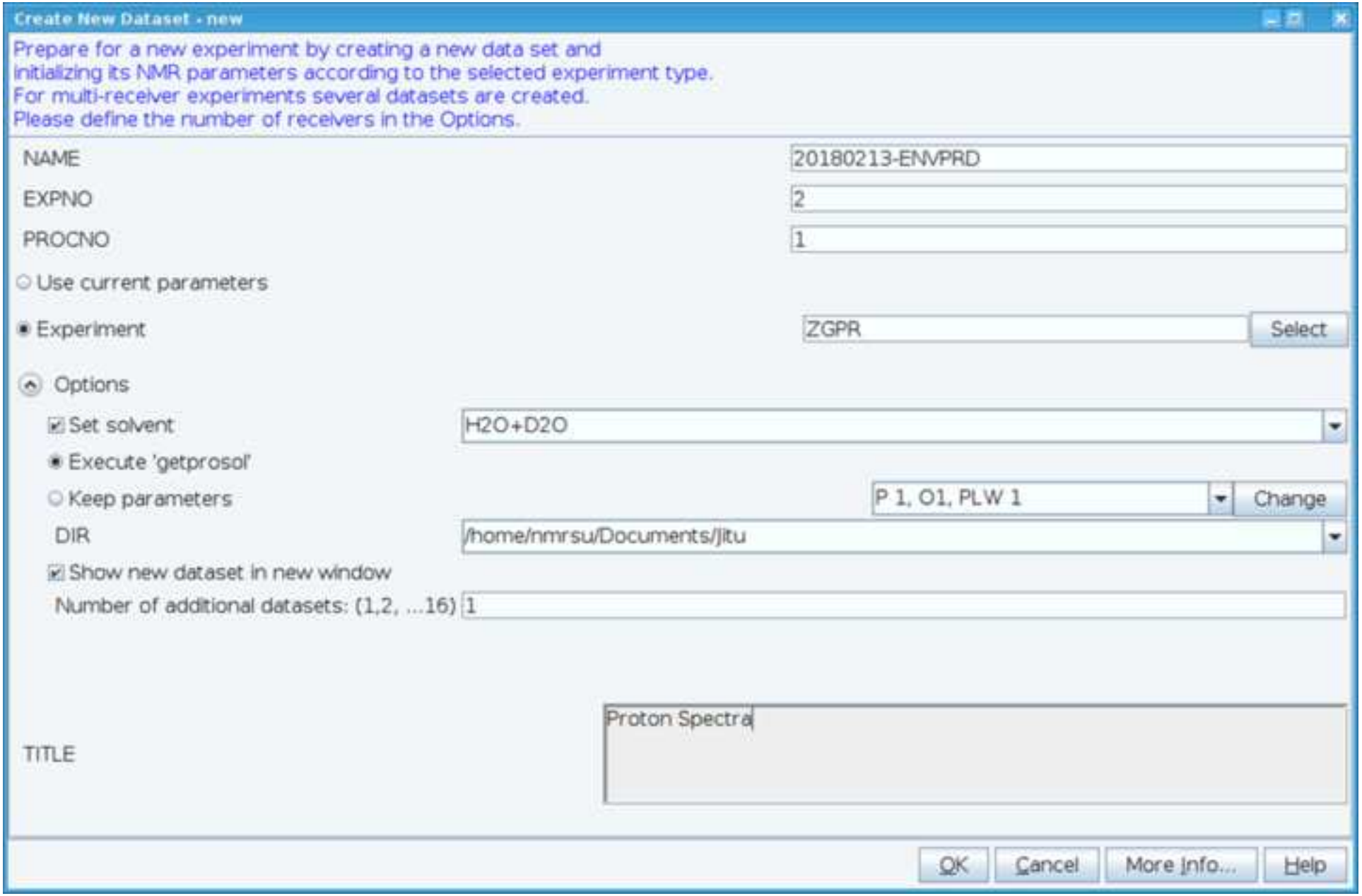

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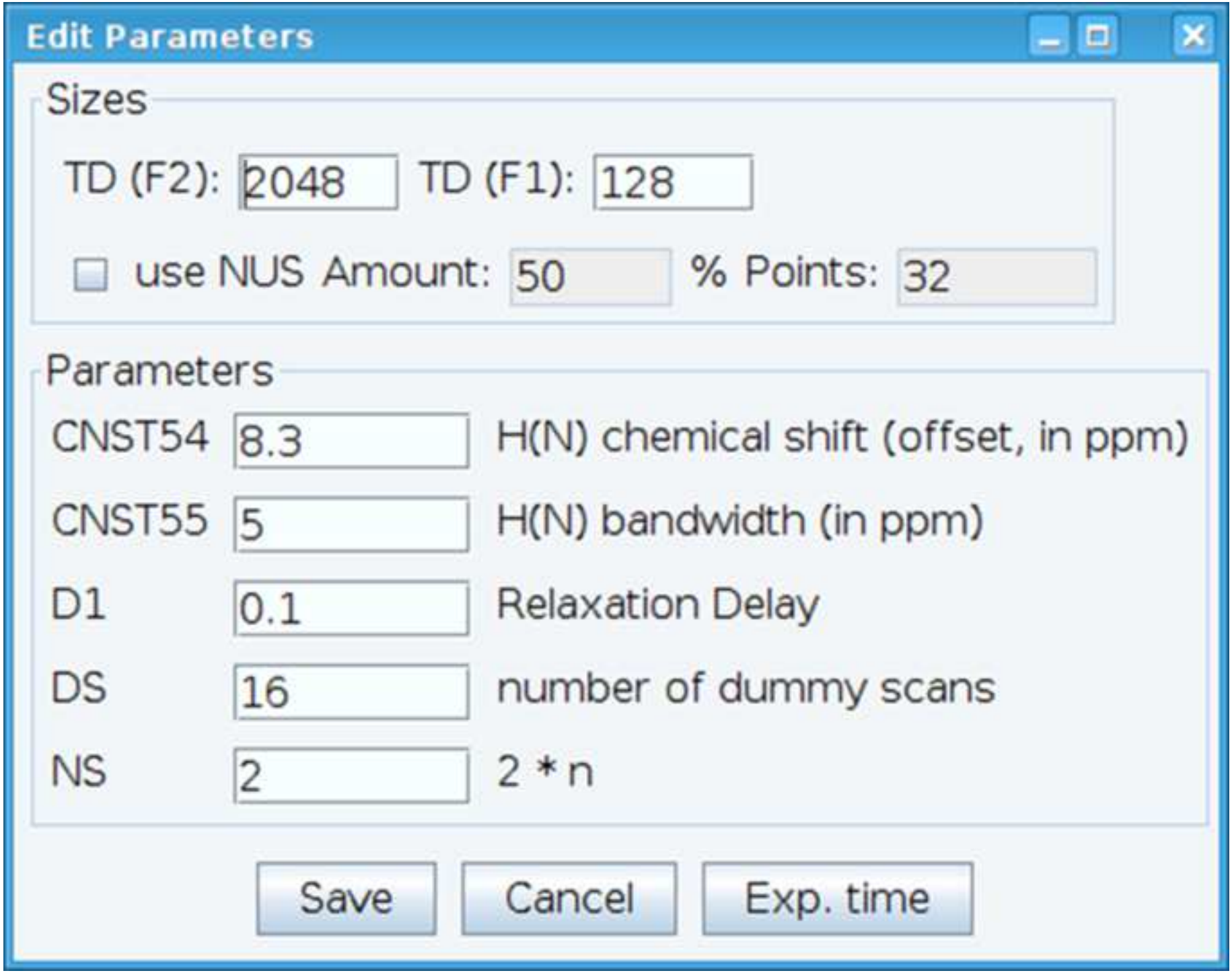

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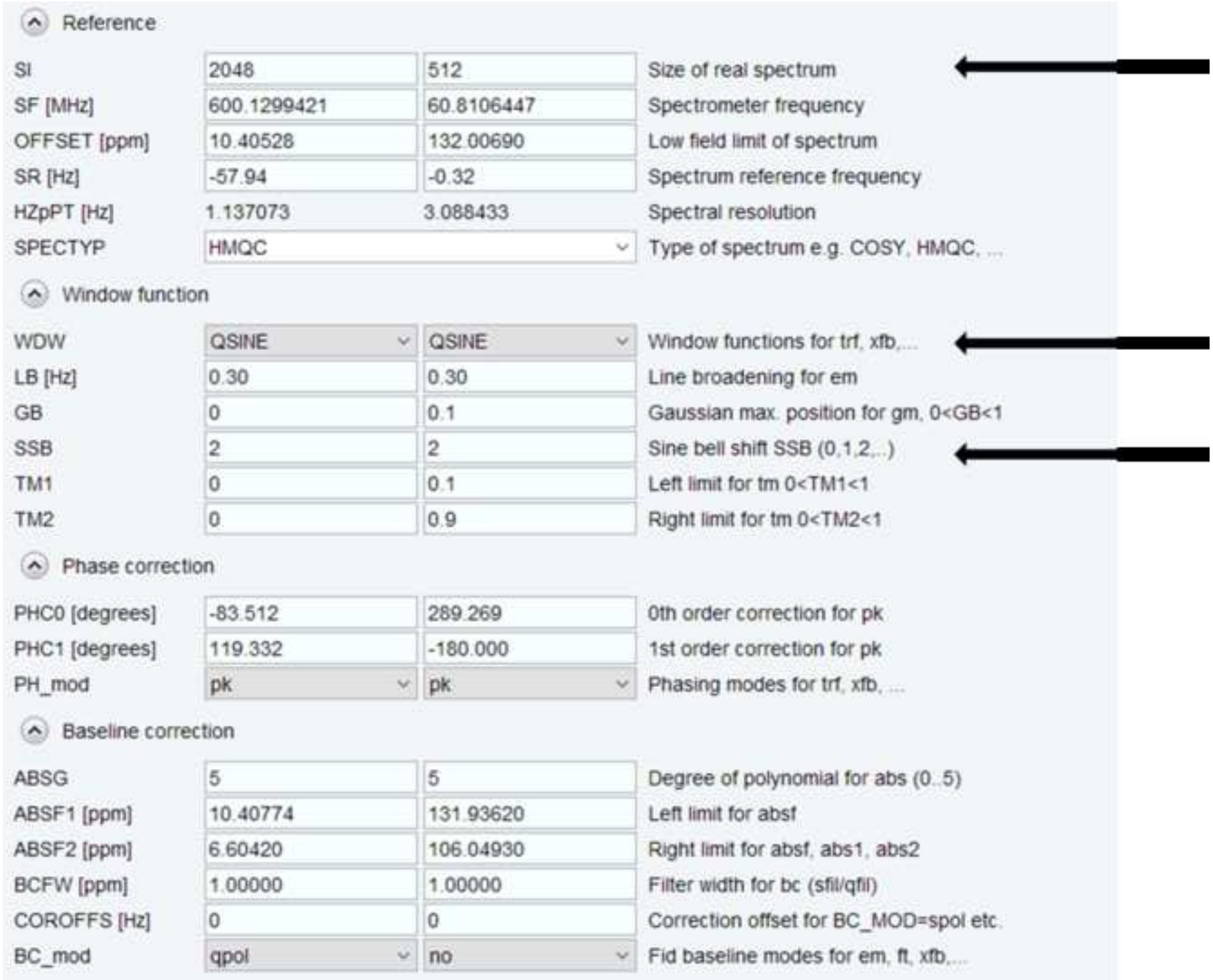

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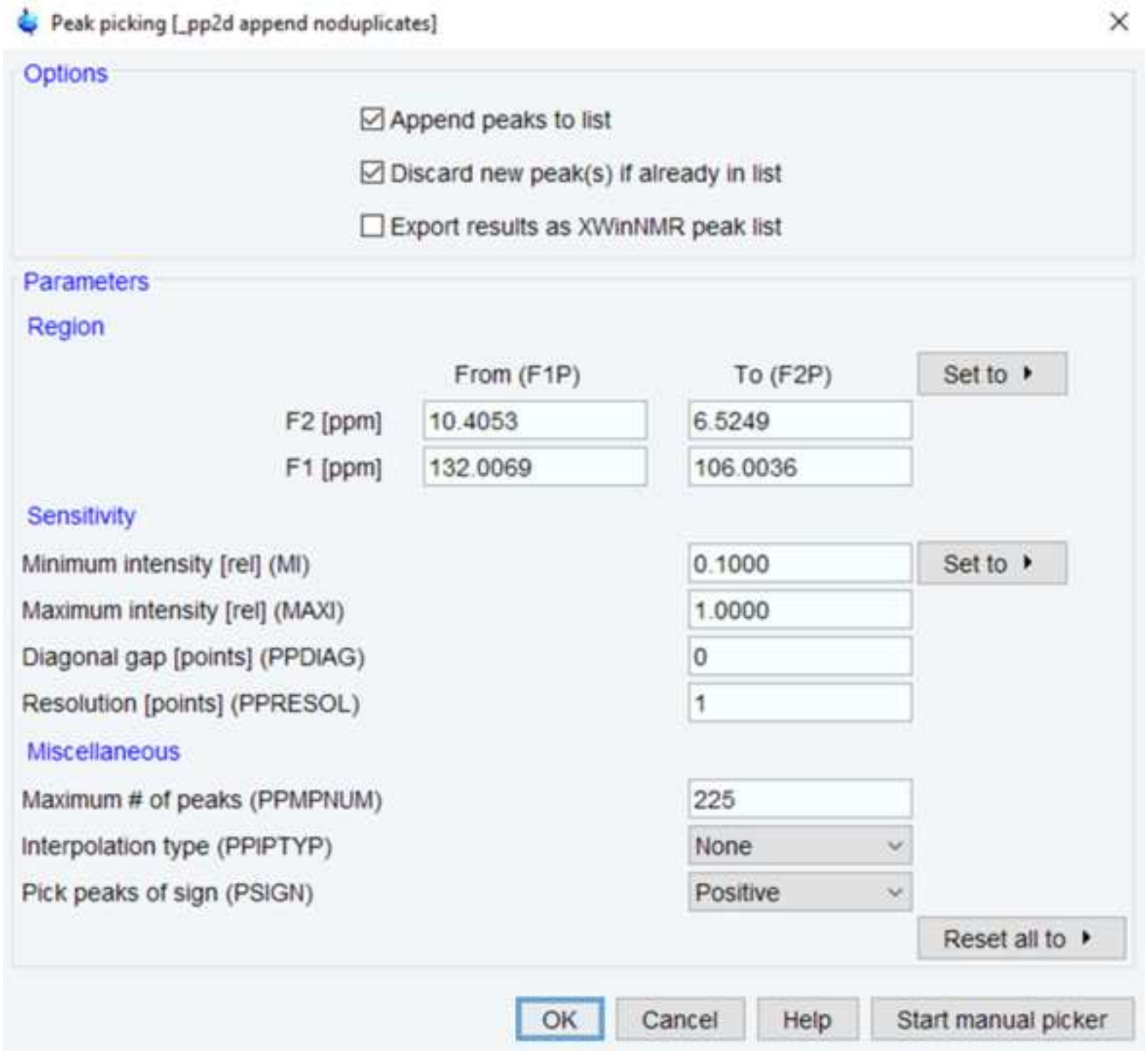

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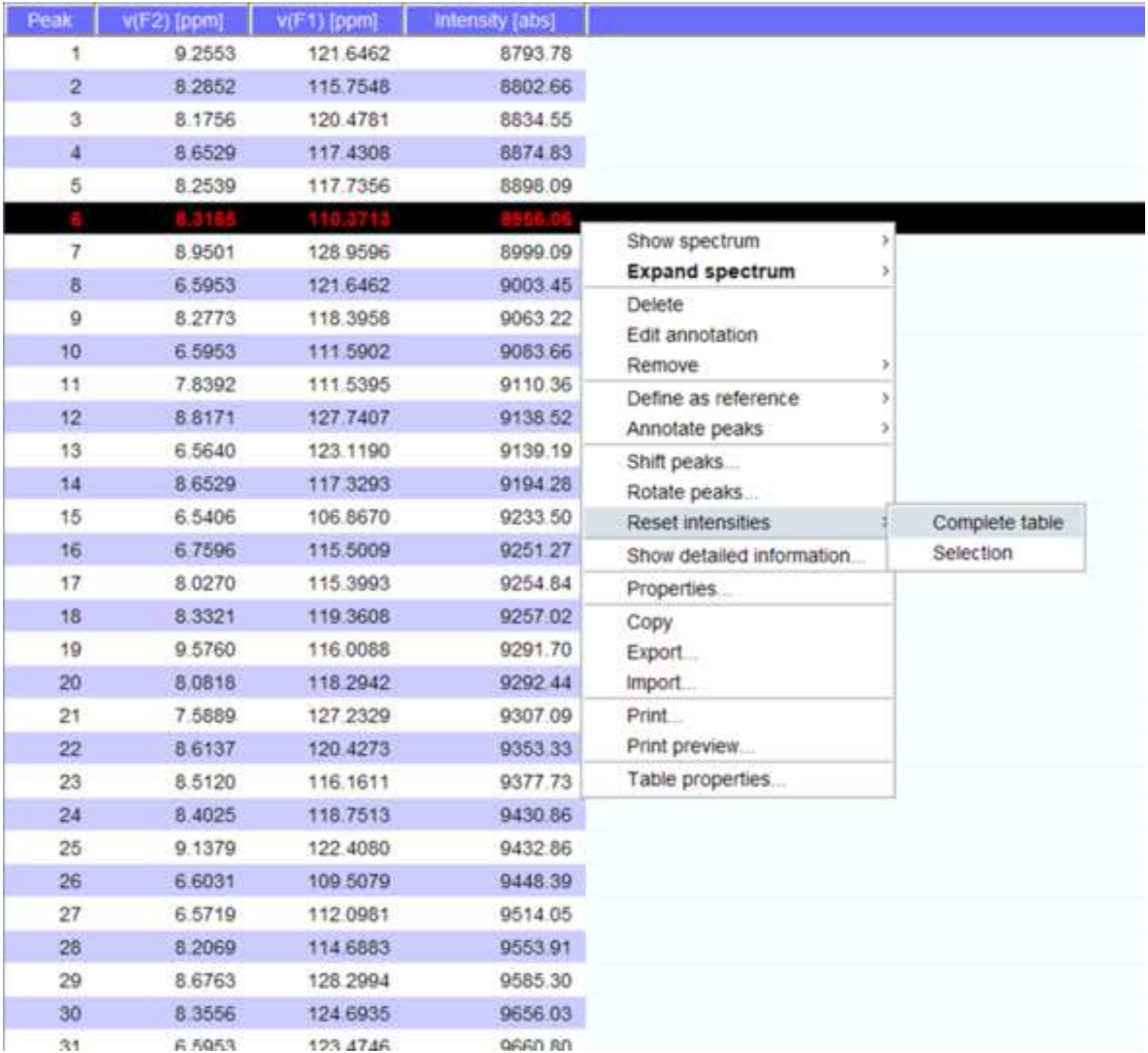

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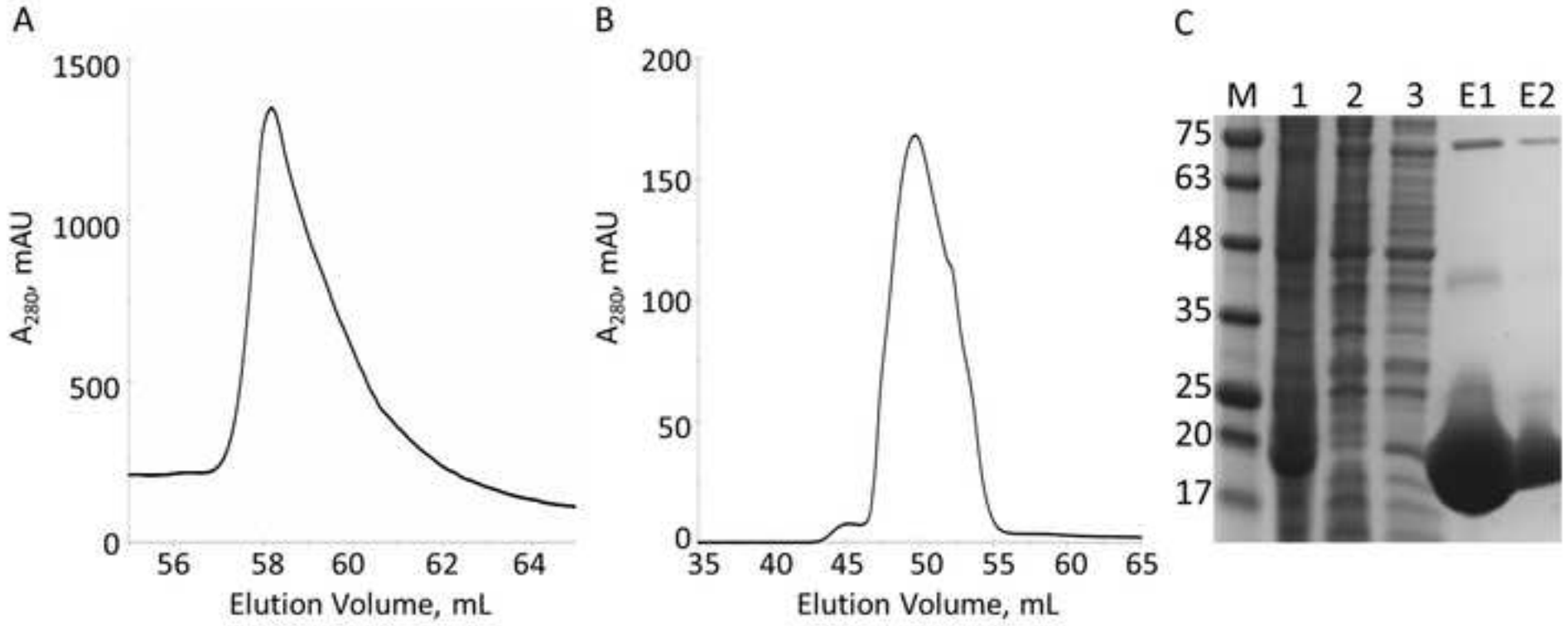

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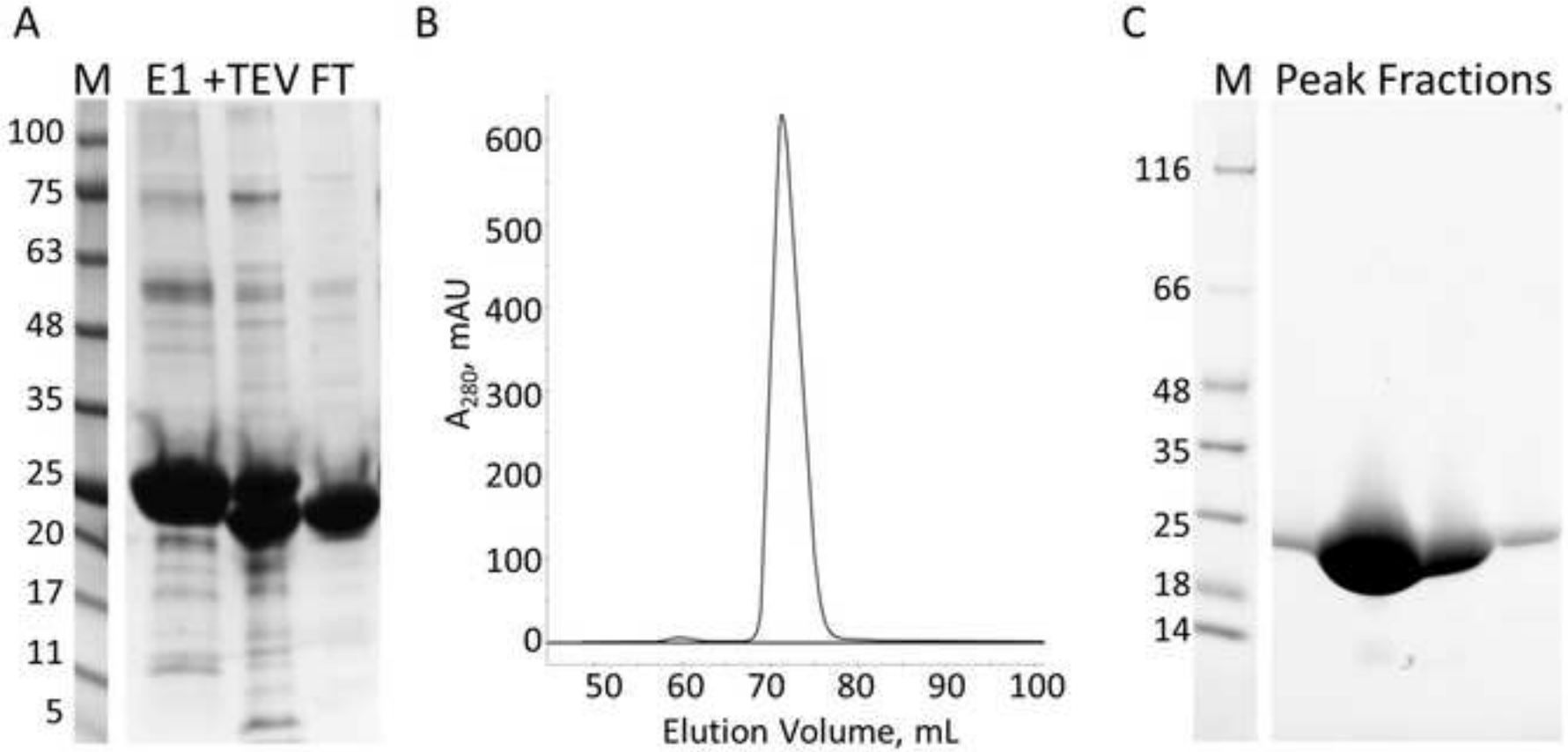

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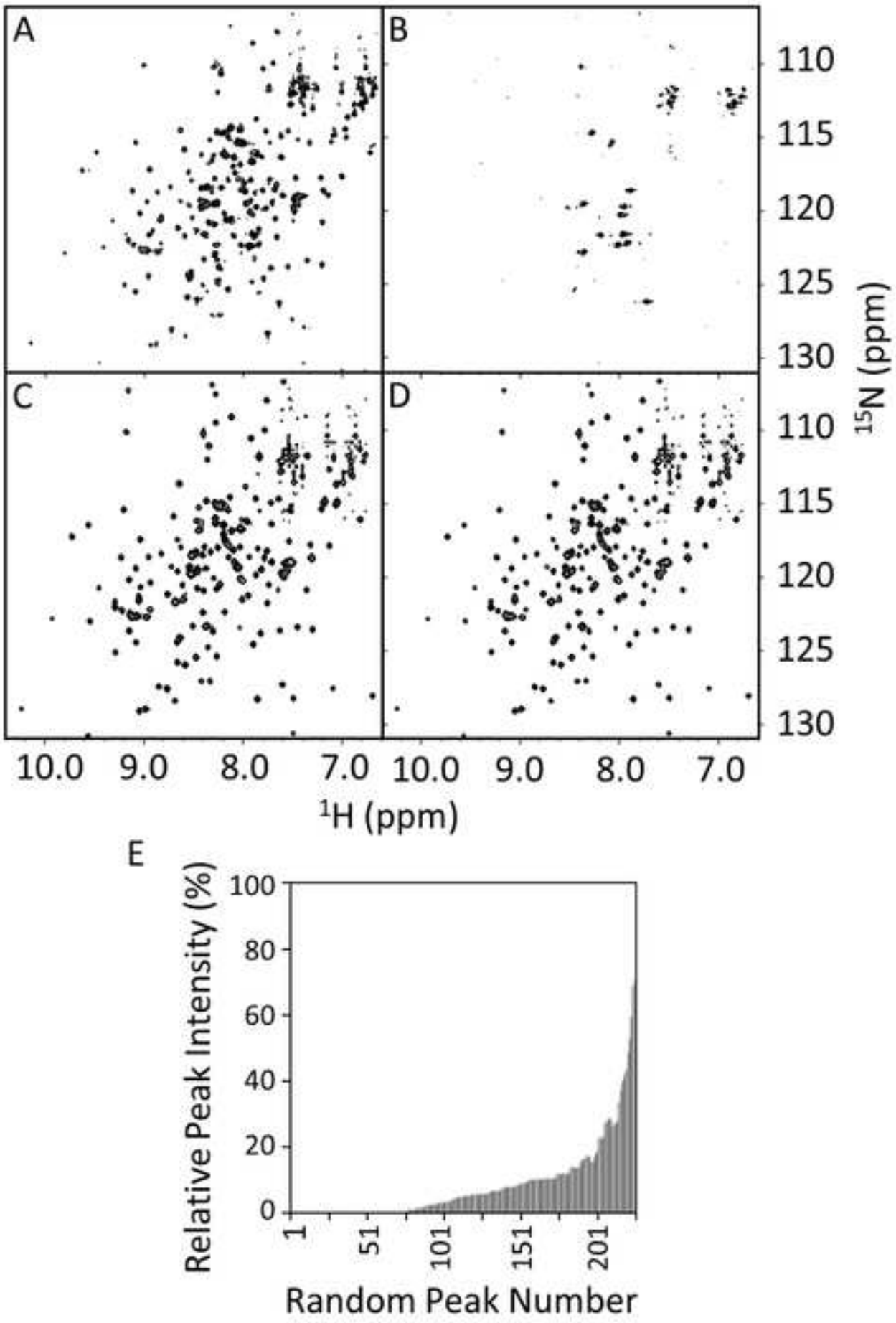

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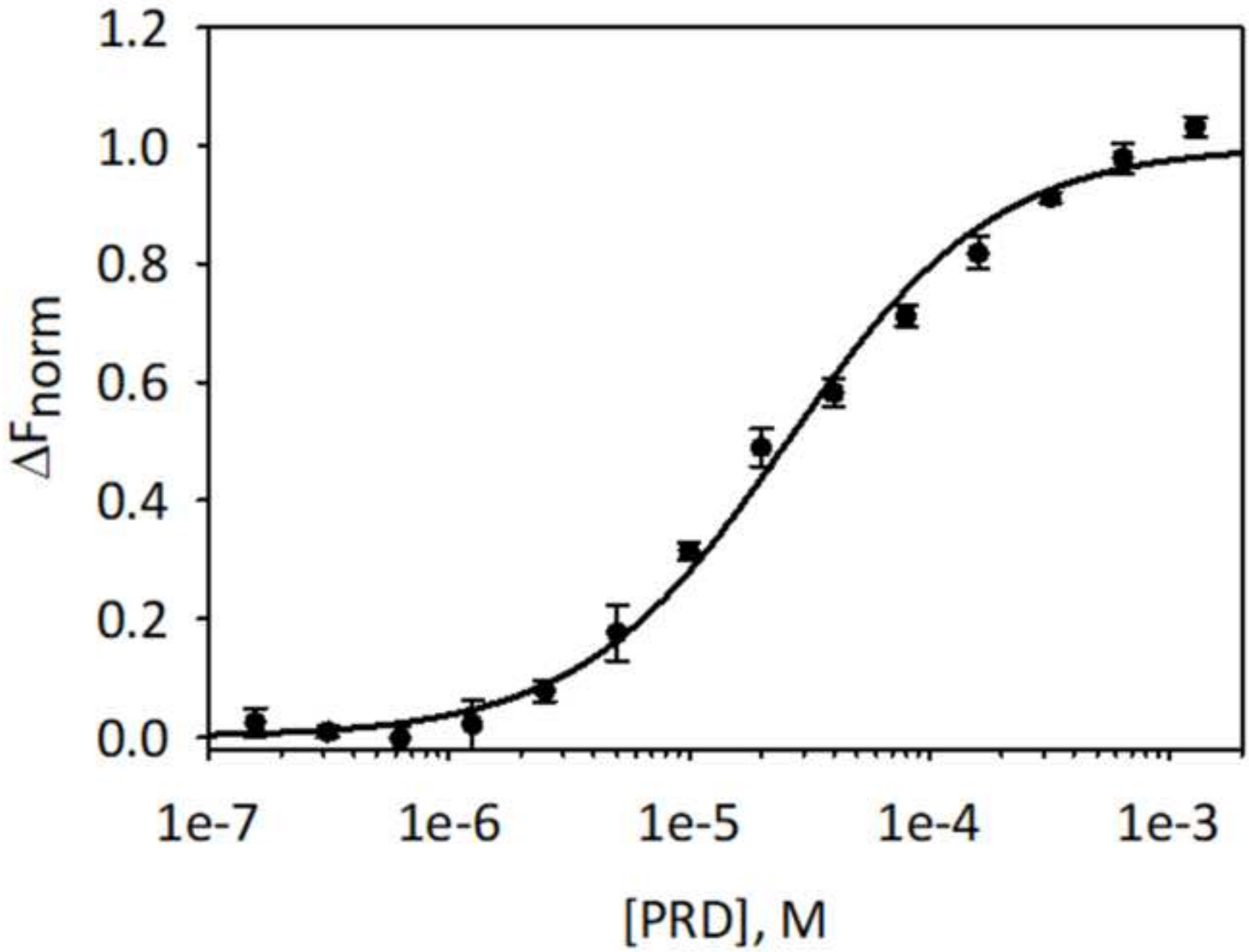

Reagent	**Quantity**
Sodium phosphate, dibasic (anhydrous)	6.0 g
Potassium phosphate, monobasic (anhydrous)	3.0 g
Sodium Chloride	0.5 g
H_2O	Up to 950 mL

Reagent	Quantity
$^{15}NH_4Cl$	1.0 g
Glucose (or ^{13}C-glucose)	2.0 g
1 M $MgSO_4$	2 mL
50 mM $CaCl_2$	4 mL
20 mg/mL Thiamine	1.0 mL
3 mM $FeCl_3$	400 µL
Metal Mix (Table 3)	500 µL
H_2O	Up to 50 mL

Reagent	Quantity
4 mM $ZnSO_4$	323 mg
1 mM $MnSO_4$	75.5 mg
4.7 mM H_3BO_3	145 mg
0.7 mM $CuSO_4$	55.9 mg
H_2O	Up to 500 mL

Sample	1 mM E-PRD in buffer A[1] (µL)	1mM VimRod in buffer A (µL)	Buffer A (µL)
E-PRD alone	50	0	50
E-PRD + VimRod	50	50	0

[1]Buffer A: 20 mM Tris-HCl, 1 mM DTT, pH 7

[2]Buffer B: 23 mM Tris-HCl, 1.14 mM DTT, pH 7

200 µM DSS in D_2O (µL)	Buffer B^2 (µL)	Total Volume (µL)
50	350	500
50	350	500

Name of Material/ Equipment	Company	Catalog Number
Monolith NT.115, includes control and analysis software	NanoTemper Technologies	MO-G008
His-Tag Labeling Kit RED-Tris-NTA	NanoTemper Technologies	MO-L008
Standard capillaries	NanoTemper Technologies	MO-K022
HisTrap HP, 5 mL	GE Healthcare	17524801
HisPur Ni-NTA resin, 100 mL	Thermo Fisher Scientific	88222
HiLoad 16/600 Superdex 75 pg	GE Healthcare	28989333
TEV protease	Sigma Aldrich	T4455
BL21(DE3) Competent *E. coli*	New England Biolabs	C2527H
cOmplete Protease Inhibitor Cocktail Tablets EDTA-Free	Sigma Aldrich	11873580001
TCEP, Tris(2-carboxyethyl)phosphine hydrochloride	Sigma Aldrich	C4706
Reagents (HEPES, NaCl, etc)	Sigma Aldrich	various
Ammonium chloride (^{15}N, 99%)	Cambridge Isotope Laboratories	NLM-467
D_2O, Deuterium Oxide (D, 99.8%)	Cambridge Isotope Laboratories	DLM-2259
DSS, Sodium 2,2-dimethyl-2-silapentane-5-sulfonate-D6 (D,98%)	Cambridge Isotope Laboratories	DLM-8206
Precision 5 mm NMR Tubes, 7” long	SJM/Deuterotubes	BOROECO-5-7
NMR spectrometer (14.1 Tesla)	Bruker	
TCI 5mm z-PFG cryogenic probe	Bruker	
Software		
Bruker TopSpin 4.0.1	Bruker	
MO.Control	NanoTemper Technologies	
MO.Affinity Analysis	NanoTemper Technologies	

Comments/Description
Instrument for microscal thermophoresis
RED-tris-NTA dye for MST
capillaries for MST
IMAC column
IMAC resin
SEC column
cleavage of his tag from E-PRD
cells for protein expression
protease inhibitors for protein purification by IMAC
reducing agent for protein purification
Preparation of media and buffers
isotope labelling for NMR
NMR sample preparation
reference for NMR
NMR tubes
acquisition of NMR data
acquisition of NMR data
processing of NMR data
included with Monlith NT.115
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Article Title:	Measuring interactions of globular and filamentous proteins by nuclear magnetic resonance spectroscopy
Signature:	Michael Overduin
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Authors Response to Reviewers
Changes to the manuscript and figures by the Authors to address the reviewers' comments are shown in red text.

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. The authors have double checked for spelling and grammar issues.
2. Figures 6 and 7: Please change "ml" to "mL". Please provide units for the numbers in the SDS-PAGE figure. Figure 6 and 7 units have been changed to mL, the units for the MW markers (kDA) have been added to the figure legends.
3. Figure 8: The unit for 15N chemical shift should be ppm, not pm. This has been changed as requested.
4. Tables 1-3 are mentioned in the manuscript but are not uploaded. Please upload each table individually to your Editorial Manager account as an .xls or .xlsx file. These have now been uploaded.
5. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …" This has been rephrased.
6. Please use x g instead of xg for centrifuge speeds. Spaces have been inserted between x and g.
7. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Now corrected throughout text.
8. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s, 8000 x g; etc. Now corrected throughout text.
9. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Falcon, GE healthcare, HiLoad 16/60 Superdex, Bruker, NanoTemper Technologies, Tween-20, etc. Company names and product names have been moved to the Table of Materials.
10. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). Instances of "we", "you" and "our" have been removed.
11. 1.1.3: Please break up into sub-steps. This step has been split into sub-steps.
12. 1.2.6: What volume is considered to be small here? Small volume was replaced with 20 mL in 1.2.6.
13. 2.1.4: What does pH 7.5 refer to? Is the column equilibrated with these solutions separately or together as a mixture? How is the flow rate controlled? Please specify throughout. Buffers are now clearly defined, FPLC was used and the flow rates are indicated for each step.
14. 2.1.8: What do peak elution fractions refer to? Section 2.1.8-9 refer to identifying the fractions to pool.
15. 2.1.9 and 2.1.10: What is the flow rate here? Flow rates indicated throughout.
16. 2.1.10: Is the concentrated cleaved protein taken from step 2.1.8? This is now 2.1.11 and the concentration protein to be loaded is indicated as being from step 2.1.9 in updated protocol
17. 2.2.1: Please describe how to determine the protein content using A280. Alternatively, add references to published material specifying how to perform the protocol action. Reference added in step 2.1.8.
18. 2.2.4: Please specify where to load the cleaved E-PRD protein. Section reworked to add more details. The cleaved E-PRD in added to the equilibrated resin in 2.2.4 as indicated.
19. 3.2.4, 3.2.13, 3.3.2, 3.4.3, 3.4.4, 3.4.7, 4.5.4: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "Note." These have been reworded.
20. 3.2.13: Please mention how these parameters are optimized. The parameters are optimized using the constants are CNST54 and CNST55, extracting the first FID and looking for observed signals to define bandwidth and center of the spectrum. This section has been updated.
21. 4.1.2, 4.1.3: What happens after centrifugation? Is the supernatant kept or discarded? Please specify. The insertion of text makes it clear that the supernatant is transferred to a new tube.
22. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-

highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. The NMR section 3 has been highlighted for filming.

23. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. These steps will take the user through collecting and analysing an NMR sample.
24. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion is now reworked to focus on the methods, their applications and troubling shooting.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors describe with great details their approach in characterizing the interactions of globular and filamentous proteins by nuclear magnetic resonance and microscale thermophoresis. The article is well structured and clearly written. But there are some issues and details, which must be improved before the manuscript can be accepted for publication. I recommend the acceptance of this manuscript after major revision.

Major Concerns:
1. the authors explain nicely the protein purification. But when it comes to the characterization of interactions, it is less clear how the procedure took place. For example, NMR sample preparation (3.1.1): there is no information on the concentration of the proteins used for the experiments. This information must be added, so that the sample preparation is clear. The protein concentrations are now stated in 3.1.2 and Figure 8.
2.1 Similarly as above I recommend rephrasing the Preparation of the Ligand (4.1). At this point the ligand should be defined. Which protein was used as a ligand? The ligand is now defined as “the protein ligand E-PRD”.

2.2Please, rename also the MST buffer into assay buffer. NanoTemper namely provides MST buffer, which has different composition than the buffer used by authors. Replacing the "MST buffer" with "assay buffer" will guarantee, that the readers who are familiar with "MST buffer" provided by NanoTemper, do not get confused. Thus, exchange the "MST buffer" with "assay buffer" thought the manuscript. The ligand is defined as E-PRD throughout the text. MST buffer was replaced with assay buffer and defined as 20 mM HEPES, pH 7.5, 10 mM NaCl, 0.015% Tween-20 in 4.1.3.
3. Line 339: "PBS is an alternate recommended buffer for MST." Please, rephrase or omit this sentence. The meaning of it is not clear. Sentence removed
4. The title: Preparation of labelled target compound (4.2) is misleading. The authors labeled the protein. Please, state which protein was labeled at this point. The labeled protein is defined as VimRod in 4.2
5. The official name of the dye is RED-tris-NTA and not RED-NTA. Please, correct the name thought the manuscript. Corrected throughout.

6. Why there is no free dye removal step with this dye? Please, elucidate the reasons behind and the way how RED-tris-NTA labels the proteins. Explanation added to 4.2.2 and to the discussion, binding analysis paragraph 5.

7. Preparation of the dilution series (4.3): name the ligand directly to prevent confusion. Also, briefly mention why the factor 1.17x. Ligand named throughout and the dilution factor explained in 4.3.2
8. Point 4.3.3: Why changing the tips each time when aliquoting the buffer in empty tubes? This is quite unusual, so please, add a comment on that. Also add the information on the final labeled target concentration and the dye concentration. Pipetting accuracy is affected by reusing tips. Sentence added as well as a recommendation to consult manufacter's guides such as the Gilson link given

9. Data Analysis 4.5.3: The error of the data fit (Kd) is not returned as a standard deviation, it is calculated as Kd confidence. Please, correct. This has been corrected in the Fig 9 legend where it is mentioned.
10. Representative results: Explain why is the removal of the His-tag from the E-PRD construct essential? This is explained in results, paragraph one.
11. In Discussion you have an opportunity to present and discuss the uniqueness of RED-tis-NTA (specificity of labeling, no need for removal of free dye, need to remove His-tag from the ligand etc.). Please, expand the discussion with this information. This is now added to the discussion, binding analysis paragraph 5.

12. Last paragraph of the discussion: in this paragraph should be clear that the observation relates only to this specific interaction pair. At present it is written as it would be a general issue with MST. The reader must understand the difference between the uniqueness of tested system and the specific characteristics of MST. Additionally, some citations are recommended. The statements at present are too vague. The discussion has been changed to reflect more troubleshooting of the method and indicate that the results herein are specific to this system and other binding interactions would require optimization.

Reviewer #2:
Manuscript Summary:
In this method article, Overduin and colleagues provide a combined method, using NMR spectroscopy and microscale thermophoresis, to establish filamentous and globular proteins interactions with ligands. In addition, this methodology serves to identify solution conditions for the interactions as well as screening of mutations that alter protein-ligand interactions.
The manuscript is well written and well descriptive. Approaches on how to proceed and identify protein-ligand interactions by solution NMR spectroscopy are provided in detail. The MST experiments are presented as alternative and complementary methods to quantify interactions in solution using minute amounts of protein. The manuscript should be accepted after answering the following comments:

Major Concerns:
1. Given that the major limitation of the proposed studies is the size of the protein, the author need to provide the size for the envoplakin PRD. Also, are both PRD and VimRod from human origin? The sizes are now included in the results, first paragraph as is the human origin.
2. What plasmids are being used to express the recombinant proteins? Plasmids are indicated in results, paragraph one.
3. I do not see Tables 1, 2, and 3 in the current submission Will be uploaded
4. How is His-tagged VimRod cleaved? What are the cleavage conditions? It is not cleaved. The tag needs to be present to bind the RED-tris-NTA
5. Figure 3: why is "BC_mod" labeled with an arrow? No description of this step is indicated in the text. The arrows have been removed.

6. Why is Tween-20 added in MST experiments? Sentence "Tween-20 is added to the assay buffer to prevent adsorption to the capillaries." added to 4.1.3
7. The authors should provide a description of what the RED-NTA dye does. This is now added to the discussion, binding analysis paragraph 5.
8. The authors should discuss the result shown in Figure 8E. The result shown here is now described in more detail in the results section.

Minor Concerns:
1. Please provide full name for MST in the summary section Added microscale thermophoresis (MST) to summary.
2. Section 3.1.1: What is the range of protein concentration recommended? What is the sample volume recommended? This is now indicated in section 3.1.1.
3. Please provide the name of the Bruker (and version) software described in the manuscript. The software and instrument are indicated in the Materials Table.
4. Figure 3: the spectrum type selected is HMQC. Please correct to HSQC. HMQC is correct for the Spectrum type as the experiments were SOFAST-HMQC. This change does not affect processing and is required for the file to be read by addon analytical software like Dynamics Center by Bruker.
5. 3.3.2: Qsine should be capitalized. Qsine is now capitalized.
6. 3.3.5: is this referring to ABSG? The abs2 is a run line command for baseline correction of 2D spectra as is now indicated in the text and its use is described in this section.
7. To make readable for nonexperts, please identify what F1 and F2 are in section 3.4 These are now defined in 3.3.1.

8. Section 4.1.2.: do the authors meant multiple spins may be required if less than 500 uL of ligand need to be concentrated? This sentence has been removed.
9. Provide full name for KD KD is no defined as binding dissociation constant (K_b) in 4.2.2
10. Line 390: what is step 7? Reworked this section to make clear and removed step 7 reference.

Reviewer #3:
Manuscript Summary:
In this manuscript the authors describe a protocol for measuring protein:protein interactions using NMR chemical shift perturbation mapping supported by microscale thermophoresis (MTS). Details of the sample preparation are given, along with general parameters for NMR and MTS data collection and analysis. Presented is a rather idealized analysis that does not mention limitations of the methodology.

Major Concerns:
-Figure 8 is problematic. The peak intensities for the wildtype E-PRD protein are not very consistent in terms of intensity (panel A). The differences in peak intensity for the wild type must be justified in particular compared to the relatively uniform intensities of the mutant (panel C). Is this protein known to be monomeric? (The oligomeric state of the binding partner is reported to be very dependent on osmolyte concentration and pH.) The E-PRD protein is monomeric as shown in ref4 and fig 7B and this in now stated in Results, paragraph 1. The spectrum for panel A (wild type E-PRD) have been plotted to a contour level which shows comparable intensities to the mutant form in panel C.

-Panel E must be described better in the legend. Is the x-axis a random peak number or is it really a residue number? Peak numbers were arbitrarily assigned as is stated now in the legend of Figure 8.

-No mention is made of backbone assignments (a limitation of the method) in particular of those in the bound state. Some general discussion of backbone assignments is warranted with additional discussion of how the bound state was assigned (if it was). At the least, mention of how backbone assignments would improve interpretation of the method would be useful. Backbone assignments are out of scope for this study but are briefly mentioned in the Discussion under Binding Analysis, paragraph 1.

-The concentration of the R1914E mutant is not stated, but should be even if equivalent to the wild type (100 uM). Also, the single amino acid change affects the entire 15N-HSQC spectra, thus this has a very large effect on the overall fold. This and any other information known about the mutant should be

mentioned and discussed more thoroughly. The idea that site directed mutagenesis can be used to identify critical residues needed for binding is not valid in this case and should be explained. Equivalent concentrations of E-PRD and the R1914E mutant were used, as is clarified in the section 3.1.4, Table 4 and the Figure 8 legend. Most of the R1914E peaks retained their chemical shifts in the improved Figure 8 panels, indicating retention of the overall structure. Size exclusion chromatographic analysis showed monomer for wild type and mutant form of E-PRD.

R1914E was chosen as an extreme loss of function point mutant for the purpose of better understanding the method. Other mutants have intermediate effects, as is now mentioned in the results section.

-The MST results were not reliable at anything close to physiological conditions (150 mM NaCl). This must be acknowledged as a limitation. Also, a direct comparison or mention of the MST conditions versus the NMR conditions is needed. (What was the NMR buffer, beyond the addition of 10% D2O and DSS?) Buffer conditions have been updated throughout the method, and this limitation of the VimRod protein is now discussed in the last paragraph of the discussion.

Minor Concerns:
-Detailed information about how the peaks intensity was determined needs to be included along with justification of comparing peak intensity versus peak volume. This is intended to deal with the problem of line broadening, or at least an explanation of what conditions must be met for this not to be considered.

The E-PRD interaction with VimRod leads to the formation of larger molecular weight species and have rapid longitudinal relaxation rates and therefore we see disappearance of the majority of the peaks in Figure 8B. There are only a few peaks observed in the spectrum. We used peak intensity for analysis as it was hard to identify a peak area for volume calculation for a disappeared peak. There was no real difference observed for visible peaks in the inferred binding activity when judged by comparison of relative peak intensity or by comparison of peak volumes. However, by measuring peak intensities, we could analyze peaks that were positioned close to each other and hence obtain more complete coverage. This point is now considered in 3.4.8

-Somewhat related to the backbone assignments, is the structure of E-PRD known? In particular, is the region from approximately residue 205 to the end in Figure 8, panel E structured (if those are residue numbers)? Discussion of the structure (and dynamics) might help in explaining the differences in peak intensity and the presence of observable signals in the bound state. This point is now discussed in the Discussion under Binding Analysis. The peak numbers in 8E are random and not residue numbers. This method is intended to assess binding interactions in the absence of a structure.

Reviewer #4:
Manuscript Summary:
this protocol describes the purification and preparation of a globular and a filamentous protein, and two methods demonstrating their interaction : heteronuclear NMR and microscale thermophoresis.

Major Concerns:
Table 1 to 3 of paragraph 1.2 are not to be found in the manuscript. These are now uploaded. Table 4 for NMR sample preparation is added.

Minor Concerns:
line 221 : please modify as following "direct reference for the 1H and indirect reference for the 15N chemical shifts" This is now clarified in step 3.1.3.
line 254 : please modify as following "of the spectrum on the water resonance" This is now clarified in

step 3.2.7.
line 258 : "FID (?)", please replace the question mark by "free induction decay" This is now clarified in step 3.2.9.
lines 357-339 : Why is dialysis needed to exchange buffer, as the last step of purification is size exclusion chromatography ? Why not performing SEC directly in the appropriate buffer ? Dialysis is required in step 2.2.2 to remove imidazole prior to performing a second round of IMAC chromatography. This is now clarified in this step.
line 390 : "repeat step 7", you meant step 4.3.3 ? This has been corrected.
line 431-432 : "the removal of the His tag...is essential", please add a few words reminding that it is essential only because of your choice of NTA-Dye This is now explained in results paragraph one and in the discussion, binding analysis, paragraph five.
lines 467-473 and 476-480 : please add in the figure legend the expected molecular weights of VimRod and E-PRD This has been added to the results section, paragraph one.
line 571 : typing error on "although" corrected

Reviewer #5:
This is a well written article with a lot of detail and I believe that the authors did a good job in describing the relevant methods. I was able to find only some minor issues which are listed below.

Section:
1.2 Expression of isotopically labeled protein
*1.2.6-1.2.7 There is no mention of the nature of radioactive precursor used to label the protein. It would be important to mention as to what was used to label the protein with15N. The protein is not radioactive, it is isotopically labelled using N15-ammonium chloride as indicated in Table 2 which was missing from the submission and is now uploaded.
2.1 Purification of 6xHis-tagged VimRod

*2.2.2-2.2.4 There is no mention of how the TEV protease was removed after the incubation. Is the TEV protease His-tagged as well? It would be important to provide a description of how the protease was removed from the reaction and the cleaved E-PRD protein. The TEV is his-tagged and removed by the second IMAC column, this is now included in section 2.2.4.

*Protein Purification, third paragraph. Line 553. Size exclusion.

*Please discuss why gel filtration elution is different for two proteins that are very close in size (fig. 6B and 7B). VimRod elution peak is at approximately 50ml while E-PRD is at around 72ml. Given that void volume of that column is around 40ml, is the peak observed for VimRod due to an oligomer (dimer) formation or due to the nature of VimRod being a filamentous protein? It would be important to discuss this point. This point has been addressed in the Results, paragraph one.

4.1 Preparation of the ligand
*4.1.1 It's confusing to mention Tween-20 at the beginning. It's my understanding that the Tween-20 was added only in the last step (4.1.3). Tween-20 was added to the ligand 4.1.3, and the target 4.2.2 and ligand were diluted 4.3.3 in assay buffer containing Tween-20 at a concentration of 0.015% as clarified in the text. All of the preparations used in the MST experiments were in the same buffer. This assay buffer is defined in 4.1.3
*4.2 Preparation of Labelled Target Compound – Compound changed to Protein and defined as VimRod
*4.2.2 According to the protocol there is no mention of removal of unincorporated dye (RED-NTA). Please provide a brief mention in the paper of why this is not required for this specific dye. This is now addressed in 4.2.2 and added to the discussion, binding analysis paragraph 5.
4.3 Preparation of ligand two-fold dilution series

*4.3.3 Did the MST buffer used in this case contained Tween 20 or no Tween 20. From the description, it is my understanding that the labeled target had Tween 20 at that concentration. Was it needed for consistency in the experiments or only for labeling? It may be good to clarify this point because of a vague reference that Tween-20 was used as an additive (target labeling. 4.2.6) Assay buffer is defined in 4.1.3 as 20 mM HEPES, pH 7.5, 10 mM NaCl, 0.015% Tween-20. In 4.2.6 it is indicated that the amount of Tween-20 may be increased to 0.05% if protein adsorption to the capillaries is observed.

In conclusion. Chromatograms and SDS-PAGE gels from the protein purification look good indicating that the protein is more than 95% homogeneous. NMR 2D spectra look very good as well and peaks are very well dispersed, suggesting that the protein is well folded. Binding curves are also impressive.