**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 (KD) 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.