We thank all of the reviewers for their time, effort, and constructive comments on our manuscript. Our responses to the specific comments appear below.

Responses to Reviewer #1

Reviewer comment 1: The authors do not provide sufficient data that allow this reviewer to assess the identity of "half" and "DR" species on the native gel. Couldn't these bands represent disulfide bonds formed by some proteasome subunits? The authors do mention that they have previously shown the identity of all observed species. Regarding the CP, the fluorogenic peptide assay suggests that CP assembly has indeed occurred (is the kDa of the band in 1B at 670?). Since the relevant data for assignment of the composition of "half" and "DR" species is not shown, the authors must include a statement how the identity of each species, especially "half" and "DR" can be further analyzed.

Response: Thank you for your comments. The focus of this manuscript is to describe a useful protocol for the analysis of proteasome assembly using recombinant proteins and non-denaturing PAGE. The data presented here is a representative result illustrating the utility of this approach in identifying proteasome assembly intermediates. These intermediates (SR, DR, half, etc) have been characterized in considerable detail in a recent publication from our lab[1] (reference 18 in the manuscript). We specifically point out in this manuscript that these intermediate species have been characterized before, for example:

new line numbering 380-381: "We have previously demonstrated that these correspond to single α-rings (SR) and double α-rings (DR)[18]."

and

new line numbering 401-403: "Interestingly, a new species also appeared in the lysate mixing sample, migrating just below the CP (termed "half"). Recently, we showed that this species corresponds to the half-proteasome ($\alpha_7\beta_7$)[18]."

The readers can choose to explore the cited reference and evaluate the evidence provided there for the identity of these species. We do not feel it is necessary to present all of this evidence again here.

[1]Panfair et al. (2015) "Alpha-ring Independent Assembly of the 20S Proteasome". *Scientific reports* 5, 13130. doi:10.1038/srep13130

Reviewer comment 2: Long abstract: … alpha and beta subunit of the proteasome as well as half-proteasome are not defined. Please edit the abstract, briefly introducing proteasome components and assembly intermediates before using these terms. Please also add a sentence that the described protocol may be useful for the analysis of other multi-subunit protein complexes as well.

Response: Thank you for your suggestion. We have amended the abstract to define the quaternary structure of the proteasome and the half-proteasome intermediate. The suggested sentence was also added.

Reviewer comment 3: *Discussion: The discussion could use some editing for grammar and more concise sentence structure.*
Response: Where appropriate, grammatical changes were made to the discussion.

Reviewer comment 4: *115: LB-Kan plates … please indicated that the antibiotic used is specific for the expression plasmid.*
Response: A sentence was added to this effect immediately following the first mention of LB-kan plates in step 1.1.4 of the protocol.

Reviewer comment 5: *120: … or until*
Response: *Till* and *until* are both correct according to most style manuals, and either may be used. We have left the original as written.

Reviewer comment 6: *128: Briefly note (here as well) that induction conditions as well as IPTG concentration may have to be worked out for individual plasmids and cells.*
Response: Thank you for your suggestion. The protocol already alerts the reader that expression parameters are optimized for this study (new line numbering 109-110, "The expression parameters listed below were empirically determined to be optimal for the proteasome subunits in this study"). However, to make this more explicit, we have added a new sentence immediately following this one which states that additional optimization may need to be carried out for other conditions (new line numbering 110-112).

Reviewer comment 7: *195: Questions for the authors … how will you know that equal amounts of alpha and beta subunits are combined? Wouldn't you have to complete the TSP analysis first? Please clarify.*
Response: Thank you. This is another good suggestion and we have amended the Note at the start of Section 2 in the methods to clarify the usefulness of TSP analysis initially until an optimal expression protocol is developed (new line numbering 160 to 166).

Reviewer comment 8: *207: is this a 50% slurry of the resin? This is important to know how many beads are used!*
Response: Yes, this is a slurry and we have amended this line in step 3.1.1 to reflect this.

Reviewer comment 9: *297 and 308: approximately # hours for a minigel?*
Response: Changes made to 4.5 and 4.6.3 to insert the requested information.

Reviewer comment 10: *318: to clarify please write: Suc-LLVY-AMC, a fluorogenic peptide substrate.*
Response: The words "fluorogenic peptide substrate" were added as requested in 5.2.1.

Reviewer comment 11: *322: observe activity? Do you mean "fluorescence due to proteolysis of the substrate?"*
Response: The reviewer is correct. See 5.2.2 for an amended version.

Reviewer comment 12: *326: colloidal coomassie stain not provided - is this the same as GelCode Blue? Please specify.*

Response: JoVE manuscript instructions require omission of commercial brand names. Hence this was left as originally written.

Reviewer comment 13: 400: the table (plasmid/genotype/source) appears a bit misformatted in the reviewer's copy.
Response: Thank you for pointing this out. We have readjusted the column widths in both tables so that the columns fit on a single page as opposed to overlapping onto two pages. Hopefully this resolves the issue.

Reviewer comment 14: 451: The discussion should include a statement regarding the usefulness of the described protocol for the analysis of other (non-proteasome) multi-subunit protein complexes.
Response: Thank you. See new line numbering 512-513 for the added statement.

Reviewer comment 15: Figure legend: source (and visualization) of native gel ladder is missing in reagents list.
Response: The extended materials list that JoVE requires for submission includes the source of the native gel ladder. Visualization of protein standards is not required, especially when this information is readily available on the vendor website.

Responses to Reviewer #2

Reviewer comment 1: A major concern would be that the technique is described somewhat narrowly focused on archaeal proteasomes, which have a more select group of interested researchers. Related to this, the authors do not early on discuss the option of purifying both alpha and beta separately, which would be a logical question for readers. This might be worth to mention earlier (it is now discussed to some extend in the discussion) in particular because that has been done for bacterial 20S, something that might be worth a reference as well.

Response: Thank you for your comment. We respectfully disagree with the reviewer's view that focusing the technique on archaeal proteasomes is somehow limiting the scope of this article. Yes, it is true that comparatively fewer labs study archaea, especially in vivo. Part of this has to do with ease of experimentation because many archaea are strict anaerobes and/or obligate extremophiles. However, recombinant protein production in bacteria makes archaeal protein biology accessible to any lab and this opens up a treasure trove of potential experiments for those who wish to pursue them. Because "information transfer systems" (DNA replication and repair, transcription, translation, protein quality control, etc) in archaea are highly similar to eukaryotes, yet often on a simpler scale, many important insights into these processes have been gleaned from in vitro studies with recombinantly produced archaeal proteins[1]. And this is before mentioning the importance of archaea to the ongoing discussion on the origins, and evolution, of life.

With regards to discussing the option of separately purifying α and β subunits earlier, the focus of this manuscript is to present a technique for "rapid" and "labor friendly" analysis of proteasome assembly. This is of particular interest for labs pursuing structure-function studies where the generation of dozens of mutants quickly may be important. While it is possible to purify each mutant separately and determine its effect (if any) on assembly, one can obtain an answer much faster with the approaches described here and decide if a mutant is worth following-up. The follow-up can then be much more rigorous and involve the generation of purified protein. JoVE requests that the Discussion features commentary on alternate approaches to the presented method. We agree with the reviewer that purification of proteins to homogeneity is an acceptable and useful approach, and we feel that the Discussion is the proper place to present it as an alternative.

[1]Jarrell et al., (2011) "Major players on the microbial stage: why archaea are important". *Microbiology*. 157(Pt 4):919-36. doi: 10.1099/mic.0.047837-0.

Reviewer comment 2: The authors argue that co-expression leads to in vivo assembly as a result of very high concentration. I do not necessarily disagree with that notion, but I think it might not be the only factor at play and it is hard to known what the real reason is. E.g. in vivo, there is also much more time for the assembly to occur and there is a continuous production of the subunits. Also, page 9 top induction of coexpression is 7 hours, while lysates are mixed for 30 minutes. so besides concentration time might be a factor? Did the authors optimize the 30 min lysate incubation? Some comment on timing of the assembly would be insightful.

Response: Thank you for your comments. The reviewer raises important points, all of which we have discussed in detail in a preceding manuscript[1]. We have added a sentence to the results section directing readers to this manuscript should they desire a more detailed treatment of coexpression versus lysate mixing (new line numbering 427-428: "A more detailed narrative of coexpression versus lysate mixing, as it pertains to recombinant proteasome assembly, can be

found here[18]"). We have also added a new note in the methods section, below step 2.2.2, mentioning that the timing has been optimized for this study (new line numbering 214-216).

[1]Panfair et al. (2015) "Alpha-ring Independent Assembly of the 20S Proteasome". *Scientific reports* 5, 13130. doi:10.1038/srep13130

Reviewer comment 3: *Step 2.2.2. Would it not make sense to add a comment on the stoichiometry here? The ratio of alpha to beta is very important in assembly analyses and is something that is hard to control in coexpression. Using separate lysates will allow you to correct (or introduce variation) in that ratio. The discussion mentioned something about proteins need to be expressed at similar levels. Why would it not be possible to use the TSP analyses to compare alpha and beta expression levels and correct the amounts of soluble alpha and beta by using different amounts of crude lysates and thus ensure 1:1 levels of alpha and beta or whatever variant one might like to test.*

Response: Thank you for a very useful comment. Another reviewer also suggested expanding commentary on the usefulness of the TSP analysis in ensuring equal protein levels are mixed. We have done this by revising a Note at the start of section 2 which now mentions the usefulness of including TSP analysis initially (see new line numbering 160 to 166).

Reviewer comment 4: *Similarly, in the representative results lane 3; do the authors know here is the SR ring is not there as a consequence of limited amounts of beta or is it an equilibrium? free beta would not be observed as it is not co purified with alpha.*

Response: Perhaps we misunderstand the reviewer's comment here, but lane 3 represents coexpression of wild-type α and β subunits and SR is clearly present as indicated by the arrowhead. Because the α and β subunits are expressed from an engineered bicistronic operon, expression levels of the two subunits are nearly identical thus the levels of β are not limiting. The protocol already directs readers to two previous manuscripts that describe the cloning of expression plasmids (eg. new line numbering 105-107: "The cloning of archaeal proteasome subunit genes and the generation of expression plasmids are described elsewhere[18,20]"). However, to make it more explicit and encourage readers to further explore these manuscripts, we added a sentence that specifically mentions the operon strategy and its effect on ensuring comparable levels of coexpressed proteins (eg. new line numbering 107-109: "In brief, plasmids for recombinant coexpression of subunits employ a bicistronic operon strategy which helps in obtaining comparable expression levels of individual subunits[18,20]").

Reviewer comment 5: *As intended with the protocol, many simple steps are included. Considering the level of detail at specific steps, I would argue some other important details are lacking: e.g. step 1.1.1 BL21 should be thawed gentle on ice as these cells are fragile. Current description does not clearly mention this. after step 1.1.4 It should be mentioned cells are incubates at 37 degrees overnight.*

Response: Thank you for the useful comments. The suggested changes were made in the referenced steps.

Reviewer comment 6: *3.1.1. I assume video will show details on how resuspending is achieved (vortex, invert, pipet?).*

Response: We are not sure what the production team will decide. Consequently, we clarified in 3.1.1 that resuspension was achieved by inversion.

Reviewer comment 7: *Step 4.1.2. What are the recipes for the 5% and 10% solution? The products indicate a 40% acrylamide solution is used and 5% or 10% in native resolving buffer ends up with different concentration. It also says in the table 2 has "native resolve buffer" that already contains APS and TEMED. and then in Step 4.1.3. It says to add APS to the solutions. While, researchers familiar with the techniques will understand this, considering the details at other steps in the protocols, this step 4 does not seems to be clearly explained for the intended audience.*

Response: Thank you for bringing what could be a confusing description to our attention. We have altered step 4.1.2 to make it more clear, and have added a note immediately below it (new line numbering 300-301) to inform the reader that the non-denaturing PAGE system we use is identical to the well-known Laemmli SDS-PAGE system, except with SDS omitted. The reader can then consult the reference for more details, if necessary. As to Table 2, the native resolve buffer explicitly states to add APS immediately prior to gel polymerization

Reviewer comment 8: *Is step 4.1.4 described in sufficient detail? no stir bar being used?*

Response: The reviewer is correct. A tiny stir bar is used. We have amended steps 4.1.1 and 4.1.4 to make this clearer.

Reviewer comment 9: *The authors argue that mixing lysate is (a bit more) laborious as compared to coexpression. Isn't this a bit exaggerated as both are easy and straightforward? Also, when comparing various beta mutants it might be better to have separate lysates as it introduces less variation?*

Response: Thank you for your comment. The reviewer correctly pointed out earlier the difference between “researchers familiar with the techniques” and the “intended audience” of this manuscript. We do feel that coexpression and lysate mixing are pretty straightforward but that’s because we are well-practiced in these techniques. However, someone attempting this for the first time might find that it does take them somewhat longer to do lysate mixing properly. So we would like to leave the language as is. As to comparison of various beta mutants, we do use separate lysates as shown in the data Figure.

Responses to Reviewer #3

Reviewer comment 1: Although the data are fully consistent with what is known for the specific assembly step demonstrated in this article, this Reviewer felt that the article may have one slightly misleading point to an uninformed reader. On pg. 9, in the final paragraph prior to the Figure Legends, the authors argue that decreased protein concentration (due to cell lysis and mixing) is responsible for the decreased assembly efficiency. Although this Reviewer agrees, other factors, such as changes in the amount of soluble but incorrectly folded protein that forms in the absence of a stabilizing cofactor, or the pre-formation of misassembled species prior to the initiation of assembly, could be, and likely are, also contributing.

Response: Thank you for your insightful comments. We couldn't agree more that additional reasons, besides dilution, can contribute to decreased assembly rates in lysate mixing versus coexpression. However, dilution is the greatest contributor by far in this study. A ~50 µl bacterial cell pellet (typical for a ~6 ml culture used here) lysed in ~600 µl of lysis buffer results in at least a 12 fold dilution of the protein contents because the ~50 µl pellet is not all cytoplasm. The alternate suggestions offered by the reviewer for the lowered assembly rates during lysate mixing (misfolded protein and/or pre-formed species that are misassembled) have the same root mechanism: decreasing the starting concentration of assembly-competent species. To have the same effect on assembly rates as pure dilution, these alternate suggestions would need to lower the concentration of assembly-competent species by the same amount (i.e. at least 12 fold). Were this to occur, one would observe a profound decrease in the amount of assembled proteasomes during lysate mixing i.e. less assembly-competent material (due to misfolding and/or pre-formed misassembled species) = less assembled proteasome. But our data clearly indicates that lysate mixing produces comparable amounts of assembled CP; it just does so less efficiently. Even the small amount of DR formed in the lysate mixing sample, which we know is not assembly-competent, does not appreciably affect the amount of CP formed. Consequently, we believe that lysis does not significantly alter the amounts of assembly-competent species and that dilution is the largest contributor to observed effects following lysis.

Keeping all of the above in mind, what the reviewer proposes can become much more important as one introduces mutations that may perturb folding. We already allude to this in the Discussion (eg. new line numbering 474-479: "Optimization can include varying induction time, induction temperature, optical density at induction, bacterial expression strain, and so on, until desired levels of soluble protein are achieved. This is especially important when comparing wild-type and mutant versions of a protein because sometimes the mutant may exhibit comparable expression levels, but decreased solubility"). In short, we'd like to keep the focus of the manuscript on the technique itself and the usefulness of lysate mixing as a complement to coexpression. However, to acknowledge the valid points raised by the reviewer, we altered the language in the manuscript to imply that dilution need not be the sole reason for decreased assembly rates post-lysis (new line numbering 414). The new text now reads: "The ability to visualize the half-proteasome during lysate mixing is due mainly to much lower protein concentrations…" (added text is underlined).

Reviewer comment 2: Second, an additional point that is overlooked, is that at the time of lysis in a coexpression culture, there has already been significant assembly taking place, such that a continuum of assembly intermediates containing any and all proteins can be present. In contrast, in a lysate-mixing experiment, it is impossible for some intermediates to be formed until its

cognate components are brought together by mixing. Thus, there is an element of synchronization that is possible in a lysate-mixing experiment that is not possible to control in coexpression studies. These alternative interpretations should be presented to the reader.
Response: Thank you for your comments. The reviewer is correct that lysate mixing affords more control over timing (what the reviewer calls synchronization). However, if the assembly pathways are the same, the same continuum of assembly intermediates will be populated during lysate mixing as during coexpression. The major difference is that this will happen less efficiently in lysate mixing, allowing you to visualize these intermediates. Indeed, just because we don't observe the half-proteasome intermediate during coexpression (lane 3) doesn't mean it is not there. In fact, we have shown that it is possible to make it appear during coexpression if we make assembly during coexpression less efficient[1]. In that paper, we spend considerably more time discussing the differences between coexpression and lysate mixing which are (in our opinion) not relevant to the purpose of this manuscript. However, to provide the readers the opportunity to explore this topic further, we have added a new line to this manuscript which directs them to our previous paper (new line numbering 427-428: "A more detailed narrative of coexpression versus lysate mixing, as it pertains to recombinant proteasome assembly, can be found here[18]").

[1] Panfair et al. (2015) "Alpha-ring Independent Assembly of the 20S Proteasome". *Scientific reports* 5, 13130. doi:10.1038/srep13130

Reviewer comment 3: One other small concern is that no controls are suggested for the lysate-mixing experiments, such as simple dilution of the two components with buffer; some species present in the lysate-mixing experiments could also be disassembly products resulting from diluting lysates below the Kd for the complex. Whereas it is not likely necessary to include such data for the purpose of this manuscript, it is worth including some text recommending such controls to otherwise uninformed reviewers.
Response: Thank you for your comment. It has been demonstrated for many protein complexes that their disassembly (dissociation) pathway is merely the reverse of its assembly (association) pathway[1,2]. This is based in part on identical intermediates being encountered during induced disassembly and subsequent reassembly. Hence, even if there were disassembly occurring during lysate mixing of proteasome subunits, the disassembly products would only serve to help identify key assembly intermediates. However, under the experimental conditions used (proteasome concentrations in the 10^{-7} to 10^{-6} M range), CP disassembly is not likely to be a significant factor. If it were, then proteasomes generated by coexpression (lane 3), which are comparable in abundance to those generated by lysate mixing (lane 2), should have fallen apart upon lysis to give rise to stable intermediates such as the half-proteasome. Yet this does not occur. Hence, while the reviewer is correct that dilution below the K_d of a complex can lead to its disassembly, experimental conditions here do not warrant this concern.

[1]Levy, et al.(2008) "Assembly reflects evolution of protein complexes". *Nature*. 453(7199): 1262-5. doi:10.1038/nature06942.
[2]Marsh, et al. (2013) "Protein complexes are under evolutionary selection to assemble via ordered pathways". *Cell*. 153(2):461-70. doi: 10.1016/j.cell.2013.02.044.

Reviewer comment 4: In the short abstract, the wording makes it sound like the lysate-mixing is a subsequent step to the coexpression analysis. It would be helpful to clarify the wording to make it clear that these are distinct approaches.

Response: Thank you for your helpful suggestion. Changes to the short abstract were made and hopefully any confusion has been mitigated.

Reviewer comment 5: In line 106, the authors indicate the use of bacterial strain BL21. This should be BL21(DE3), or another strain that contains an integrated copy of the T7 RNA polymerase under control of an inducible promoter.
Response: The reviewer is correct and the change has been made in section 1.1.

Reviewer comment 6: The term "till" in line 120 should be changed to "until."
Response: *Till* and *until* are both correct according to most style manuals, and either may be used. We have left the original as written.

Reviewer comment 7: Lines 208, 211, and 216, the phrase "aspirate off the supernatant" should be adjusted to "aspirate the supernatant."
Response: Thank you for pointing this out. The phrasing has been changed in the indicated sections (3.1.1, 3.1.2, and 3.2).

Reviewer comment 8: Line 249, would it be possible to use (or recommend) other protein assays, such as Bradford, to bypass the requirement for dilution and re-concentration?
Response: As a lab, we prefer the BCA assay because it exhibits less variation between proteins than Coomassie-based methods (i.e. with the latter, there is more variation between standard curves depending on what protein you use as your standard). It also has a higher dynamic range than Coomassie-based methods, enabling the measurement of more concentrated solutions without needing to dilute them first. But other methods of protein concentration can be used. We have expanded the Note that immediately follows step 3.6.3 in the protocol to clarify this (new line numbering 276-280)

Reviewer comment 9: The sentence beginning "The reappearance of the..." on lines 355-356 is a bit awkward. A suggested edit would be"The reappearance of the DR species in the lysate mixing sample indicates that once formed, DRs cannot be reversibly disassembled by beta subunits."
Response: Thank you, it is awkward indeed. We have modified the phrasing (new line numbering 400).

Reviewer comment 10: In the recipe for native resolving buffer, the concentration of tris-HCl is listed as 375 mM. This stikes the reviewer as an awfully high concentration of tris for a 1x resolving buffer. Is this a typo?
Response: No, this is correct.

Responses to Reviewer #4

Reviewer comment 1: It would be useful to the reader to have a model figure depicting the assembly pathway(s) for the proteasome. This could be either a separate figure or a panel in the existing figure. Such an image would facilitate interpretation of the data in what is now Figure 1 (and may also be helpful in the video production).
Response: Thank you for the suggestion. We think it is a good one. We have created a new Figure 1 that summarizes the salient features of CP assembly.

Reviewer comment 2: Step 1.1.1. I recommend including the phrase "chemically competent" in front of BL21 cells.
Response: The reviewer is correct. Change made in 1.1.

Reviewer comment 3: Line 183. What dictates the decision to use 12% vs 15% gel?
Response: 10%, 12%, and 15% are probably three of the most common gel percentages used in labs. Some labs prepour gels in batches and store them for use. Some purchase prepoured gels. And sometimes you pour a gel and have a few lanes free. In any of these cases is true, if your gel is 12 or 15%, you can run your proteasome samples.

Reviewer comment 4: Step 2.2.2. What is "slow shaking"? Authors should provide numerical speed (in RPM).
Response: Many inexpensive models of rotators, nutators, rockers (what have you), have dials with numbers on them that do not correspond to any RPM values (i.e. 0-10). Precision here is not required. The importance is to ensure some mixing is occurring.

Reviewer comment 5: 3.1.1., 3.1.2., and 3.1.3. "Aspirate off" can be economized to "aspirate" (as authors use the term in later steps) (very minor).
Response: Thank you for your suggestion. Changes have been made in these sections.

Reviewer comment 6: 3.1.2. *Authors should indicate how specifically samples should be gently mixed (pipetting? Inverting?).*
Response: There are several ways of gentle mixing (inversion, pipetting slowly, flicking tube with finger, etc), any of which would be fine here which is why we have not specified further.

Reviewer comment 7: 3.2 and following wash, elution steps: Note to authors and video editors regarding video of this protocol: It will be very instructive for the authors to provide details on (and close up video shots of) removal of supernatant from pelleted beads. Do the authors recommend pipette-aspiration or vacuum-aspiration? My lab members often debate about how to remove the supernatant from IPs and related purifications - should one remove every last drop of liquid possible? Should one allow a certain amount of liquid to remain above the beads, and if so, how much? Should one use a vacuum aspirator to "dry out" the resin (where the resin color detectably changes as moisture is removed)?
Response: Thank you for this comment. We will work with the video team to ensure this can be demonstrated. We have added a note to the materials section, immediately below step 3.1.1, to give more details on how we aspirate liquid (new line numbering 230-232).

Reviewer comment 8: 3.7. Include the word "at" in: "store samples at -20°C".
Response: Correction made as suggested to 3.7.

Reviewer comment 9: 4.1.2. Regarding the pouring of native PAGE gels, do the authors recommend including TEMED in the native resolving buffer as well, or adding at the time of APS addition? I have always added TEMED at the time of APS addition, but if authors have verified that buffer can be prepared (and stored) with TEMED, then no problem. (Note: my experience is primarily with SDS-PAGE, so common practice with native PAGE may vary).
Response: Thank you for your question. TEMED can be added to the native resolving buffer for up to an hour before the gel is ready to be polymerized, as long as the gel solution is kept on ice. We have amended Table 2 to include this information which we hope should clarify the question raised by the reviewer.

Reviewer comment 10: 4.1.5. Do authors recommend overlaying resolving gel with water or isobutanol while it polymerizes? (again: my experience is primarily with SDS-PAGE, so common practice with native PAGE may vary).
Response: Thank you for bringing this to our attention as we overlooked it. Yes, we do perform an isopropanol overlay and have added this information to the protocol in section 4.1.5.

Reviewer comment 11: 4.6. missing period at end of sentence.
Response: Period added to 4.6.

Reviewer comment 12: 5.2.1. How exactly should Suc-LLVY-AMC be "spread" over gel?
Response: A new note immediately below step 5.2.1 was added to explain how this is done.

Reviewer comment 13: 5.2.2. "and observe activity" --> I recommend the following text change "and observe fluorescence (indicative of activity)".
Response: Thank you. Another reviewer made the same comment (correctly). A change has been made to 5.2.2.

Reviewer comment 14: Discussion, Lines 367-369. Authors assert that faster migration of the half-proteasome in the context of the R166W mutation is due to the change in mass-to-charge ratio imposed by the point mutation. I would recommend authors include the word "likely" in this sentence (unless they or others have conclusively demonstrated this, thereby warranting a citation), as one could imagine other (admittedly much less likely) scenarios whereby this mutant subunit results in formation of structures that migrate differently because subunit composition is actually different.
Response: Fair enough. The requested insertion of "likely" has been made (new line numbering 412).

Reviewer comment 15: Discussion. It would be useful for the authors to include some description about the applicability/utility of this approach to the study of assembly of other protein complexes (or of proteasomes from other species).
Response: Thank you. We have added such a sentence (new line numbering 512-513).

Reviewer comment 16: Discussion. I recommend a brief description about the choice of affinity tag used in this study, and whether others (e.g. immunoaffinity tags) could also be used instead.
Response: We appreciate the suggestion and have added new text in the discussion (new line numbering 498-503) to this effect.

Responses to Reviewer #5

Reviewer comment 1: ln 407: as written implies that ref 12 only co-expressed the proteasome subunits in vivo and does not acknowledge that ref 12 also separately expressed the archaeal alpha rings and pro-beta proteins and demonstrated in vitro assembly of these subunits into 20S proteasomes

Response: The reviewer is correct. We regret the error. It was not intentional. Reference 12 has now been omitted from this group of references.

Reviewer comment 2: ln 62: please confirm that the majority of archaeal proteasomes are predicted to be comprised of only one type of alpha and one type of beta subunit (as implied by use of the word usually)

Response: Many archaeal genomes encode a single α and β subunit of the proteasome. There are species of archaea (eg. halophiles) that encode two α (α1 and α2) and a single β subunit. But even in these cases, the proteasome during normal growth consists of a single α and single β subunit (α1β) whereas a hybrid proteasome (α1α2β) is detectable only during select growth conditions[1]. There are also archaea (eg. *Sulfolobus* and *Pyrococcus* spp.) that encode a single α and two β subunits (β1 and β2) and, at least for some of these species, incorporation of both β subunit types into a single CP occurs in vivo[2]. With few exceptions[3], the limited bacterial lineages that encode proteasomes contain CPs consisting of a single α and single β subunit type. Hence while not all prokaryotic CPs fit the single α and single β subunit mode (recently reviewed here[4]), this quaternary structure appears more common than not and we are comfortable with using the term "usually" in our text.

[1]Kaczowka, et al. (2003) "Subunit topology of two 20S proteasomes from *Haloferax volcanii*." *J. Bacteriol*. 185(1):165-74.

[2]Madding, et al. (2007) "Role of the beta1 subunit in the function and stability of the 20S proteasome in the hyperthermophilic archaeon *Pyrococcus furiosus*." J. Bacteriol. 2007 189(2):583-90.

[3]Tamura, et al. (1995) "The first characterization of a eubacterial proteasome: the 20S complex of Rhodococcus." *Curr. Biol.* 5(7):766-74.

[4]Humbard and Maupin-Furlow. (2013) "Prokaryotic proteasomes: nanocompartments of degradation." J. Mol. Microbiol. Biotechnol. 23(4-5):321-34. doi:10.1159/000351348.

Reviewer comment 3: ln 362-363: please clarify that the R166W amino acid exchange not only impaired 20S assembly but also impacted CP catalytic activity as demonstrated by the lack of Suc-LLVY-Amc peptidase activity (lane 4 panel A vs. B).

Response: Thank you for the comment. The lack of activity in the R166W mutant is actually due to a complete lack of processing of the β subunit propeptide (lane 4, panel C). Given the position of R166 at the β-β ring interface, its conservation among archaea, and its likely involvement in stabilizing salt-bridge interactions, mutation of this residue impairs the correct assembly of the CP (specifically, dimerization of half proteasomes) as we have shown[1]. This is supported by the accumulation of the half-proteasome species as the most abundant species in lane 4. Moreover, the small amount of CP that does form in lane 4, fails to become activated. This again reflects an assembly defect as it is known that autocatalytic activation of the CP occurs only when two half-proteasomes dimerize correctly[2]. Thus, the effect of R166W on activity is really due to its effect on assembly. We have added text to the results, and cited a new reference, which we hope will

clarify this issue (new lines 423-427: “In the case of the R166W mutant, the propeptide processing failure during lysate mixing is absolute even though a small amount of CP does form. This is because propeptide processing not only requires half-proteasome dimerization, but also a properly-formed β-β ring interface[23] which the R166W mutation does not afford”).

[1]Panfair et al. (2015) “Alpha-ring Independent Assembly of the 20S Proteasome”. *Scientific reports* 5, 13130. doi:10.1038/srep13130

[2]Chen and Hochstrasser (1996) “Autocatalytic subunit processing couples active site formation in the 20S proteasome to completion of assembly.” *Cell.* 86(6):961-72.