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# Examining proteasome assembly with recombinant archaeal proteasomes and non-denaturing PAGE: the case for a combined approach.

--Manuscript Draft--

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| **Corresponding Author:** | Andrew R Kusmierczyk<br>Indiana University Purdue University at Indianapolis<br>Indianapolis, Indiana UNITED STATES |
| **Corresponding Author Secondary Information:** | |
| **Corresponding Author E-Mail:** | akusmier@iupui.edu |
| **Corresponding Author's Institution:** | Indiana University Purdue University at Indianapolis |
| **Corresponding Author's Secondary Institution:** | |
| **First Author:** | Dilrajkaur Panfair |
| **First Author Secondary Information:** | |
| **Other Authors:** | Dilrajkaur Panfair |
| **Order of Authors Secondary Information:** | |
| **Abstract:** | Proteasomes are found in all domains of life. They provide the major route of intracellular protein degradation in eukaryotes, though their assembly is not completely understood. All proteasomes contain a structurally conserved core particle (CP), or 20S proteasome, containing two heptameric β subunit rings sandwiched between two heptameric α subunit rings. Archaeal 20S proteasomes are compositionally simpler compared to their eukaryotic counterparts, yet they both share a common assembly mechanism. Consequently, archaeal 20S proteasomes continue to be important models for eukaryotic proteasome assembly. Specifically, recombinant expression of archaeal 20S proteasomes coupled with non-denaturing polyacrylamide gel electrophoresis (PAGE) has yielded many important insights into proteasome biogenesis. Here, we discuss a means to improve upon the usual strategy of coexpression of archaeal proteasome α and β subunits prior to non-denaturing PAGE. We demonstrate that although rapid and efficient, a coexpression approach alone can miss key assembly intermediates. In the case of the proteasome, coexpression may not allow detection of the half-proteasome, an intermediate containing one complete α-ring and one complete β-ring. However, this intermediate is readily detected via lysate mixing. We suggest that combining coexpression with lysate mixing yields an approach that is more thorough in analyzing assembly, yet remains labor non-intensive. This approach may be useful for the study of other recombinant multi-protein complexes. |
| **Author Comments:** | Good Afternoon,<br>While I do not have a grant deadline requirement by which I would like to see the article "in press", I do have an upcoming tenure deadline of June 1. Hence if JoVE is able to make the same consideration for faculty submitting tenure dossiers as they do for faculty submitting grant proposals, that would be much appreciated.<br>Sincerely,<br>Andrew Kusmierczyk |
| **Additional Information:** | |

| Question | Response |
| --- | --- |
| If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter. | |

IUPUI
DEPARTMENT OF BIOLOGY
SCHOOL OF SCIENCE
A Purdue University School
Indianapolis

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Ronald Myers, PhD
Science Editor
JoVE
1 Alewife Center, Suite 200
Cambridge, MA
02140

Dear Dr. Myers:

Pursuant to your invitation to contribute an article to *JoVE*, we would like to submit the revised manuscript (**JoVE54860R1**) entitled, *"Examining proteasome assembly with recombinant archaeal proteasomes and non-denaturing PAGE: the case for a combined approach".*

We thank the reviewers for their efforts and time. We have addressed all of their comments and our responses are described in point-by-point detail in a separately uploaded PDF document "Kusmierczyk Reviewer Comments Addressed". Some of the more notable changes to the manuscript that resulted from the reviewer comments include:

- A new Figure 1 which presents a model of proteasome assembly, as requested by a reviewer. The previous Figure 1 now becomes Figure 2.

- Reformatted Tables 1 and 2 in Excel format. A reviewer commented that the tables did not display correctly so we ensured that they now fit onto a single page width.

- An updated materials list in Excel format.

- An added reference.

All changes to the manuscript text that resulted from reviewer comments are highlighted in red via the Track Changes feature of Word.

We hope the revised version has addressed all of the reviewer comments satisfactorily and we look forward to hearing back from you.

Thank you for your consideration and time

Science and Engineering, SL 306 723 W. Michigan Street Indianapolis, IN 46202-5153 (317) 274-0577 fax (317) 274-2846

Indiana University – Purdue University Indianapolis

Sincerely,

Andrew Kusmierczyk, PhD
Assistant Professor
Department of Biology
Indiana University-Purdue University Indianapolis
723 West Michigan Street, SL306
Indianapolis, IN 46202
tel: (317) 274-1048
fax: (317) 274-2846
akusmier@iupui.edu
http://biology.iupui.edu/people/andrew-kusmierczyk

**TITLE:** Examining proteasome assembly with recombinant archaeal proteasomes and non-denaturing PAGE: the case for a combined approach.

**AUTHORS:**
Panfair, Dilrajkaur
Department of Biology
Indiana University-Purdue University Indianapolis
Indianapolis, IN
United States of America
dpanfair@iupui.edu

Kusmierczyk, Andrew R
Department of Biology
Indiana University-Purdue University Indianapolis
Indianapolis, IN
United States of America
akusmier@iupui.edu

**CORRESPONDING AUTHOR:**
Andrew R. Kusmierczyk (akusmier@iupui.edu)

**KEYWORDS:**
Proteasome; protein assembly; archaea; recombinant protein; non-denaturing polyacrylamide gel electrophoresis; *in vivo* assembly

**SHORT ABSTRACT:**
This protocol uses both subunit coexpression and post-lysis subunit mixing for a more thorough examination of recombinant proteasome assembly.

**LONG ABSTRACT:**
Proteasomes are found in all domains of life. They provide the major route of intracellular protein degradation in eukaryotes, though their assembly is not completely understood. All proteasomes contain a structurally conserved core particle (CP), or 20S proteasome, containing two heptameric β subunit rings sandwiched between two heptameric α subunit rings. Archaeal 20S proteasomes are compositionally simpler compared to their eukaryotic counterparts, yet they both share a common assembly mechanism. Consequently, archaeal 20S proteasomes continue to be important models for eukaryotic proteasome assembly. Specifically, recombinant expression of archaeal 20S proteasomes coupled with non-denaturing polyacrylamide gel electrophoresis (PAGE) has yielded many important insights into proteasome biogenesis. Here, we discuss a means to improve upon the usual strategy of coexpression of archaeal proteasome α and β subunits prior to non-denaturing PAGE. We demonstrate that although rapid and efficient, a coexpression approach alone can miss key assembly intermediates. In the case of the proteasome, coexpression may not allow detection of the half-proteasome, an intermediate containing one complete α-ring and one complete β-ring. However, this intermediate is readily detected via lysate mixing. We suggest that

combining coexpression with lysate mixing yields an approach that is more thorough in analyzing assembly, yet remains labor non-intensive. This approach may be useful for the study of other recombinant multi-protein complexes.

**INTRODUCTION:**

Multi-protein complexes carry out numerous critical cellular activities[1]. For many of these complexes, much more is known about their structure and function than about their assembly[2,3]. The proteasome is one such complex and is found in all domains of life. In eukaryotes, this molecular machine is at the core of the ubiquitin-proteasome system (UPS) and provides the major route of intracellular protein degradation[4]. The eukaryotic proteasome (referred to as the 26S proteasome) is comprised of two major sub assemblies: a 20S proteasome, or core particle (CP)[5], that can be capped on one or both ends by a 19S regulatory particle (RP)[6].

The 20S proteasome, or CP, is a large compartmentalized protease. Its quaternary structure is absolutely conserved across all domains of life and consists of a stack of four seven membered rings containing two types of structurally related subunits, $\alpha$ and $\beta$[5,7,8]. In eukaryotes, the two outer rings are each comprised of seven distinct $\alpha$ subunits and the two inner rings are each comprised of seven distinct $\beta$ subunits; proteolytic activity resides within three of the $\beta$ subunits. By contrast, the CP rings of archaea and bacteria are usually comprised of only one type of $\alpha$ and one type of $\beta$ subunit. Archaeal proteasomes have provided an important model system to study proteasome assembly due to both their compositional simplicity and their sharing a common assembly mechanism with their eukaryotic counterparts[9-13]. In brief, $\alpha$ subunits assemble into $\alpha$ rings first, which serve as a scaffold onto which $\beta$ subunits assemble. The resulting half-proteasomes ($\alpha_7\beta_7$) dimerize, giving rise to fully assembled CP ($\alpha_7\beta_7\beta_7\alpha_7$). During dimerization, the propeptides present on $\beta$ subunits are autocatalytically removed, exposing the catalytic N-terminal threonines. The use of archaeal proteasomes to model assembly frequently takes advantage of the production of recombinant archaeal proteasome proteins in *Escherichia coli*. This is a worthwhile approach because it enables the subunits to be produced in various combinations, as both wild-type and mutant versions, in a host organism that does not produce its own proteasomes.

Monitoring the assembly of multi-protein complexes biochemically requires some kind of fractionation method that separates fully assembled complexes from assembly intermediates and precursors. Due to its superior resolving capacity, non-denaturing polyacrylamide gel electrophoresis (PAGE) has proven to be especially useful in the fractionation of various large multi-protein complexes[14-17]. The combination of recombinant archaeal proteasome production and non-denaturing PAGE has become a powerful approach in dissecting proteasome assembly[9,11,12,18]. However, the usual method by which this approach is applied (*i.e*. via the recombinant coexpression of $\alpha$ and $\beta$ subunits) has an important drawback. Assembly reactions are cooperative and strongly concentration dependent[3]. Given that the protein concentration inside cells is very high[19], due to excluded volume effects, assembly reactions proceed rapidly *in vivo*. Hence it is possible to miss important assembly intermediates when $\alpha$ and $\beta$ subunits

are coexpressed.

Here, we argue for a combined approach in the study of proteasome assembly using recombinant archaeal proteasome subunits. In this approach, both coexpression and lysate mixing methods are employed. The former allows for rapid analysis of assembly because coexpression is less labor intensive. The latter depends on separate expression of α and β subunits, followed by mixing. Though this requires a bit more effort than coexpression, it is more than compensated for by the ability to detect intermediates that are missed during coexpression. Together, these two methods can provide a more complete picture of proteasome assembly.

**PROTOCOL:**

**1. Bacterial expression.**

**Note:** Expression plasmids used in this study are described in **Table 1**. Solutions, media, and buffers used in this study are described in **Table 2**. The cloning of archaeal proteasome subunit genes and the generation of expression plasmids are described elsewhere[18,20]. 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]. The expression parameters listed below were empirically determined to be optimal for the proteasome subunits in this study. It may be necessary to optimize expression for other proteasome mutants, for proteasomes from other archaeal species, or for other recombinant protein complexes (see Discussion).

1.1) Transform expression plasmid of interest into chemically competent *E.coli* BL21(DE3) cells.

1.1.1) Add 1–2 μl of plasmid (typically 50 to 100 ng) to an aliquot of BL21(DE3) cells that were freshly thawed on ice, and continue to incubate on ice for 20 min.

1.1.2) Heat shock cells at 42 °C for 45 sec and return tube to ice for 2 min.

1.1.3) Add 1 ml of LB medium and incubate at 37 °C with shaking (150 rpm) for 1 hr.

1.1.4) Spread 100–200 μl of the transformation mixture onto LB-kan plates (plates containing solid LB media, supplemented with kanamycin (all plasmids used in this study encode resistance to this antibiotic). Incubate plates at 37 ºC overnight.

1.2) Next morning, inoculate a single colony from the LB-kan plate into 3 ml of liquid LB-kan media in a glass culture tube. Incubate at 37 ºC with shaking (150 rpm) on a platform shaker for approximately 2.5–3 hr, or till turbidity is observed.

1.3) Measure the optical density of the culture at 600 nm ($OD_{600}$) in a spectrophotometer. Dilute the cell culture with an appropriate amount of prewarmed LB-kan media to an $OD_{600}$ of 0.4 in a final volume of 6 ml. Return to platform shaker at 37 ºC for 40 min.

1.4) Induce protein expression in the liquid cultures by adding IPTG from a stock solution to a final concentration of 1 mM. Incubate at 37 ºC with shaking (150 rpm) on a platform shaker for approximately 6–7 hr.

1.5) Harvest bacterial cell cultures in their entirety into 1.5 ml microcentrifuge tubes.

1.5.1) Add 1.5 ml of a culture into microcentrifuge tube. Centrifuge at 10,000 × *g* for 1 min.

1.5.2) Discard the supernatant and add another 1.5 ml of the same culture to the pellet. Centrifuge at 10,000 × *g* for 1 min.

1.5.3) Repeat step 1.5.2 until the entire culture is harvested into the microcentrifuge tube.

1.6) Store the induced pellets at – 80 °C until lysis.

**2. Bacterial lysis and lysate mixing.**

**Note:** For samples studied via coexpression, follow section 2.1 (and its subsections). For samples requiring lysate mixing, follow section 2.2 (and its subsections). The TSP (total, soluble, pellet) analysis that is included in the protocol is useful in optimizing protein expression which can help ensure that approximately equal amounts of $\alpha$ and $\beta$ subunits are combined during lysate mixing (see Discussion). It also provides an important control if downstream results are not as expected (*i.e.* it verifies that protein was expressed and soluble). Thus, we highly recommend including TSP analysis initially. Once an optimal protocol has been achieved for a particular subunit combination, TSP analysis is not strictly required which is why it is described as optional below.

2.1) Lysis of bacterial cells and preparation of soluble fractions.

2.1.1) Thaw the induced cell pellet on ice for 5 min. Resuspend the pellet in 600 µl of lysis buffer.

2.1.2) Incubate the suspension at 30 ºC with shaking (150 rpm) for 30 min to generate total crude lysate.

**Note:** The following step and the subsection that follows are optional.

2.1.3) Remove two 25 µl aliquots from the total crude lysate into separate 1.5 ml microcentrifuge tubes and carry out TSP analysis as follows.

2.1.3.1) To one of the two 25 µl aliquots, add 5X SDS-sample buffer to a final concentration of 1X. Label the tube with “T” for “total crude lysate”.

2.1.3.2) Incubate the "T" sample at 100 ºC for 5 min to completely denature proteins.

2.1.3.3) Meanwhile, take the second 25 μl aliquot and centrifuge it at 10,000 × *g* for 10 min. Carefully remove the supernatant without disturbing the tiny pellet, and transfer it to a new 1.5 ml microcentrifuge tube. Label this new tube "S" for "soluble fraction".

2.1.3.4) To the tiny pellet left behind in the previous step, add 25 μl of lysis buffer and vortex to resuspend. Label this tube "P" for "pellet fraction".

2.1.3.5) Add 6 μl of 5X SDS-sample buffer to the "S" and "P" tubes and incubate at 100 ºC as described above.

**Note:** If the TSP samples will not be used that day to analyze expression, they may be frozen at –20 ºC until needed. Once thawed, they must be reincubated at 100 ºC, as described above, immediately prior to loading on a 12% and/or 15% standard SDS-PAGE gel.

2.1.4) While the TSP analysis is carried out, centrifuge the remaining 550 μl of total crude lysate (or the entire 600 μl of total crude lysate if TSP analysis is not carried out) at 10,000 × *g* for 10 min. Collect the supernatant in a fresh 1.5 ml microcentrifuge tube. This is the soluble lysate that will be used for purification.

2.2) Lysate mixing.

2.2.1) Carry out lysis as described in steps 2.1.1 and 2.1.2 above.

2.2.2) Mix 600 μl of total crude lysate from bacteria expressing the desired α subunit with 600 μl of total crude lysate from bacteria expressing the desired β subunit. Incubate at 37 ºC with slow shaking for 30 min.

**Note:** It may be necessary to optimize incubation time to achieve maximum assembly during lysate mixing (see Discussion). The time and temperature presented here are optimal for the recombinant proteins in this study and were determined elsewhere[18].

2.2.3) Centrifuge the mixed lysate at 10,000 × *g* for 10 min to separate soluble material from insoluble pellet. Transfer the supernatant to a new 1.5 ml microcentrifuge tube. Use this mixed soluble lysate for protein purification.

**3. Protein purification via immobilized-cobalt affinity resin (ICAR).**

3.1) Equilibrate the resin.

3.1.1) Thoroughly resuspend the resin by inverting the bottle and transfer 50 μl of the slurry to a 1.5 ml microcentrifuge tube. Centrifuge at 700 × *g* for 2 min to pellet resin. Carefully aspirate supernatant.

**Note:** We carry out pipette aspiration using a blue 1 ml pipette tip to remove the bulk of the liquid. A white 2 μl tip is used for fine control of removal of the remaining supernatant, leaving a very thin layer of liquid covering the beads.

3.1.2) Add 1 ml of Buffer A. Mix gently to resuspend resin and centrifuge at 700 × *g* for 2 min to pellet resin. Carefully aspirate the supernatant and repeat this wash step one more time.

3.2) Apply soluble lysate obtained previously in section 2.1 or 2.2 to the equilibrated resin. Incubate the tube with gentle rotation at 4 ºC for 60 min. Centrifuge the tube at 700 × *g* for 5 min and carefully aspirate the supernatant.

3.3) Wash the resin as follows to remove non-specifically bound proteins.

3.3.1) Resuspend resin with 1 ml of Buffer A and incubate with gentle rocking at 4 ºC for 10 min. Centrifuge the tube at 700 × *g* for 5 min and carefully aspirate the supernatant. Repeat this wash step one more time.

3.3.2) Resuspend resin with 1 ml of Buffer B (Buffer A with 5 mM imidazole) and incubate with gentle rocking at 4 ºC for 5 min. Centrifuge the tube at 700 × *g* for 5 min and carefully aspirate the supernatant. Repeat this wash step one more time.

3.3.3) Resuspend resin with 1 ml of Buffer C (Buffer A with 10 mM imidazole) and incubate with gentle rocking at 4 ºC for 5 min. Centrifuge the tube at 700 × *g* for 5 min and carefully aspirate the supernatant.

3.4) Elute the protein by adding 400 μl of Buffer E (Buffer A with 200 mM imidazole) to the resin. Incubate with gentle rocking at 4 ºC for 5 min. Centrifuge at 700 × *g* for 5 min.

3.5) Transfer supernatant containing purified protein to a new 1.5 ml centrifuge tube.

3.6) Desalt the purified protein by serial centrifugation as follows.

3.6.1) To 400 μl of purified protein, add 100 μl Buffer A (this reduces imidazole concentration from 200 mM to 160 mM). Apply purified protein to 0.5 ml ultracentrifugal filters with a 10 kDa molecular weight cut-off. Centrifuge at 14,000 × *g* for 5 min.

3.6.2) Discard the filtrate and add 400 μl Buffer A to dilute the retentate and centrifuge again. Continue the cycles of centrifugation/dilution until imidazole concentration falls below 4 mM. As an example, if each centrifugation concentrates 500 μl down to ~70 μl (or approximately 7-fold), then two cycles will reduce the starting 160 mM imidazole concentration (7×7 = 49-fold) to approximately 3.3 mM.

3.6.3) Measure the protein concentration of the desalted sample using the BCA assay[21].

**Note:** Desalting is required to reduce imidazole levels below the tolerance limit for the BCA assay, as described in the manufacturer's instructions. Our lab prefers the BCA assay because it is sensitive, has a large dynamic range, and exhibits much less protein-to-protein variation. However, other methods to determine protein concentration can be substituted for the BCA assay. The key is to be aware of each method's advantages and limitations.

3.7) Add 5X native sample buffer to a final concentration of 1X and proceed to electrophoresis. Or, store samples at –20 ºC for later analysis.

## 4. Non-denaturing polyacrylamide gel electrophoresis (PAGE).

**Caution:** Unpolymerized acrylamide is a neurotoxin. Wear appropriate protective equipment.

4.1) Prepare the non-denaturing PAGE gel as follows.

4.1.1) Prepare the gel cassette for casting using clean glass plates and casting stand. Place gradient maker on top of a small magnetic stirrer and place a tiny stir bar into each chamber.

4.1.2) Using 40% (w/v) acrylamide and 2% (w/v) bisacrylamide stock solutions, prepare 5% and 10% (w/v) acrylamide gel solutions in native resolving buffer. Ensure that the ratio of total acrylamide to bisacrylamide is 37.5:1. Chill on ice prior to pouring.

**Note:** The non-denaturing PAGE system used here is essentially identical to the Laemmli SDS-PAGE system, with SDS omitted[22].

4.1.3) Add ammonium persulfate (from a 10% (w/v) stock solution prepared in water) to the acrylamide solutions to initiate polymerization. Final concentration of ammonium persulfate is 0.1% (w/v).

4.1.4) Pour the acrylamide solutions into the two chambers of the gradient maker. With the outlet tubing inserted between the plates of the gel cassette, activate the magnetic stirrer and open the gradient maker valves. Pour the 5–10% non-denaturing gradient gel.

4.1.5) Once poured, overlay the gel with a thin layer of isopropanol and allow the gel to polymerize for 30 min. During this time, prepare fresh 5% acrylamide gel solution in native resolving buffer. After the gel sets, pour off isopropanol and rinse the top of the gel with deionized water from a squirt bottle.

4.1.6) To the 5% gel solution prepared above, add ammonium persulfate (exactly as described in 4.1.3) and pour on top of the polymerized gel until glass plates are full. Insert gel comb. Allow the overlaid gel to polymerize for an additional 30 min.

4.1.7) Once gel is polymerized, assemble gel cassette into electrophoresis apparatus. Alternatively, store gel at 4 ºC wrapped in moistened paper towels and plastic wrap until ready for use.

4.2) Once non-denaturing PAGE gel is assembled into electrophoresis apparatus, fill the tank with 1X native running buffer prepared fresh from 10X native running buffer stock.

4.3) Load 10 μg of purified protein, obtained at the end of section 3, into each well using a glass syringe.

4.4) Load 2 μl of high molecular weight native protein standard (diluted in 1X native running buffer) into one of the wells.

4.5) Run gel at 55 V and 4 ºC until the dye front runs off the gel (approximately 4 to 4.5 hours).

4.6) In addition to the non-denaturing gel, analyze aliquots of the purified protein by standard SDS-PAGE[22].

4.6.1) Mix aliquots (10 μg) of the purified protein samples, obtained at the end of section 3, with 5X SDS-sample buffer to a final concentration of 1X.

4.6.2) Incubate at 100 ºC for 5 min and load onto standard 12% and/or 15% SDS-PAGE gels[22].

4.6.3) Run at 80V for 20 min and then at 120 V until the dye front runs off the gel (this is approximately an additional 75 min for a 12% gel, and 120 min for a 15% gel).

**5. Visualizing activity and protein staining.**

5.1) Following electrophoresis, carefully separate glass plates and transfer the non-denaturing gel to a gel tray containing 50 ml of deionized water. Rinse gel with gentle rocking for 5 min. Discard water and repeat this wash step three times.

5.2) Perform substrate overlay assay as follows.

5.2.1) Add 1 ml of developing buffer containing the fluorogenic peptide substrate Suc-LLVY-AMC and spread uniformly over the gel. Incubate at 37 ºC for 30 min.

**Note:** A glass rod or a gel releaser (small wedge of plastic used to separate glass plates) can be used to spread the liquid over the gel.

5.2.2) Carefully transfer the gel onto the UV transilluminator of the gel imaging system and observe fluorescence due to the cleaved peptide substrate. Record image. Carefully transfer the gel back to the gel tray.

5.3) Rinse the gel twice with 50 ml of deionized water for 5 min with gentle rocking.

5.4) Stain the gel with 10 ml of a colloidal coomassie stain reagent for 60 min with gentle rocking. Destain gel with 50 ml water until background becomes clear. This staining step applies to the standard SDS-PAGE gels as well.

**REPRESENTATIVE RESULTS:**

Proteasome assembly (**Figure 1**) begins when α subunits combine to form rings[9]. This can be illustrated when α subunits from the archaeon *Methanococcus maripaludis* S2 are expressed in *E. coli* as C-terminally hexahistidine tagged (his-tagged) derivatives (**Table 1**). When the recombinant α-his protein was purified by immobilized-cobalt affinity resin (ICAR) and analyzed by non-denaturing PAGE, two bands were observed (**Figure 2A,** lane 1). We have previously demonstrated that these correspond to single α-rings (SR) and double α-rings (DR)[18]. The DR is a dead-end species that is not productive for subsequent assembly[9,18].

When α-his subunits were coexpressed with β subunits, representing the usual method by which assembly of recombinant archaeal proteasomes is assayed, a novel species was observed migrating near the 670 kDa size standard (**Figure 2A,** lane 3). This species was proteolytically active (**Figure 2B,** lane 3) and contained only completely mature β subunits (mβ) whose propeptides have been removed (**Figure 2C,** lane 3). This species is the fully mature CP. This sample also contained some SR species, which was expected because SR is a known assembly intermediate, but no DR species. The lack of DR when α-his and β subunits are coexpressed suggests that correct assembly was occurring fast enough such that incorporation of β subunits was able to outcompete the non-productive formation of DR (for a more detailed analysis see[18]).

To demonstrate the limitation of the coexpression method, and argue for the utility of a combined approach, α-his and β subunits were expressed separately in *E. coli*. Following lysis, the soluble fractions were mixed and proteins purified by ICAR prior to analysis by non-denaturing PAGE. Fully functional proteasomes were also generated via the lysate mixing approach (**Figure 2A** and **2B,** lane 2) and the SR species was also observed as expected. The reappearance of the DR species in the lysate mixing sample indicates that once formed, β subunits are unable to reversibly disassemble it; this underscores the dead-end nature of the DR. 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]. To illustrate this here, a β subunit mutant (R166W) was employed. This mutation disrupts β-β ring interaction, leading to impaired half-proteasome dimerization[18]. Since half-proteasomes are the immediate precursors to CP, the R166W mutation should lead to both accumulation of half-proteasomes and a decrease in CP formation. When lysate mixing with α-his and β (R166W) subunits was carried out, lower levels of CP and increased levels of the "half" species were observed (**Figure 2A,** lane 4). This is consistent with a precursor-product relationship for these two bands, and confirms the identity of the

"half" species as the half-proteasome. The slightly faster migration of the half-proteasome in the mutant sample (lane 4 versus lane 2) is likely due to the R166W mutation altering the mass-to-charge ratio of the β subunit.

The ability to visualize the half-proteasome during lysate mixing is due mainly to much lower protein concentrations in the lysate as compared to the high protein concentrations inside cells. Lower concentrations result in less efficient (*i.e.* slower) assembly, which enables intermediates to become more populated and thus detectable. Besides the appearance of the half-proteasome, an additional observation highlights the decreased assembly efficiency during lysate mixing: unprocessed β subunits, which retain their propeptides, become detectable (proβ). Since propeptide processing does not occur until half proteasomes dimerize, the appearance of the immature proβ form correlates with the level of half-proteasome accumulation (compare **Figure 2C,** lane 3 versus lane 2 versus lane 4). 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. A more detailed narrative of coexpression versus lysate mixing, as it pertains to recombinant proteasome assembly, can be found here[18].

**Figure Legends:**

**Figure 1: A simplified schematic of core particle (CP) assembly.**
The α subunits can assemble into a single α-ring first (SR) which serves as a template for the incorporation of β subunits. This leads to the generation of the half-proteasome intermediate (half) which quickly dimerizes. Concurrent with dimerization, the β subunit propeptides (not shown) are autocatalytically removed giving rise to the fully functional core particle (CP). Double α-rings (DR) can arise from SR and are not competent for assembly into CP. Their formation represents a non-productive assembly pathway (dashed arrow) in contrast to productive assembly events (solid arrows).

**Figure 2: A combined approach for assaying proteasome assembly.**
Coexpression (C) and lysate mixing (L) were employed to study assembly of recombinant proteasomes from the archaeon *M. maripaludis*. Proteins were purified by immobilized cobalt affinity resin (ICAR). **(A,B)** Purified proteins (10 μg) were loaded onto non-denaturing 5–10% gradient gels. Following electrophoresis, peptidase activity was visualized by overlaying the gel with buffer solution containing the fluorogenic peptide substrate Suc-LLVY-AMC **(B)** prior to staining the gel with colloidal coomassie stain reagent **(A)**. Black arrowheads denote the positions of the assembled 20S core particle (CP), half-proteasome intermediate (half), double α-ring (DR) and single α-ring (SR). The migration of several molecular size standards (in kDa) is indicated at right. **(C)** Purified proteins (10 μg) from **A** were also loaded onto 15% SDS-PAGE gels. Following electrophoresis, gels were stained with a colloidal coomassie stain reagent. Migration of α-his subunit and of fully mature (mβ) and immature (proβ) β subunits is indicated. The position of the 25-kDa molecular size standard is shown at right. Asterisk indicates a truncated α-his subunit fragment resulting from non-specific proteolysis

during lysis.

**Table 1: Bacterial plasmids used in this study.** psmA is the archaeal α subunit gene and psmB is the archaeal β subunit gene.

**Table 2: Solutions, media, buffers used in this study.**

**DISCUSSION:**

We demonstrate the benefit of a combined approach to analyzing proteasome assembly by non-denaturing PAGE using recombinant archaeal proteasomes. The usual method[9,11] of bacterial coexpression of proteasome subunits allows for rapid analysis but may not reveal key assembly intermediates. We suggest combining coexpression with lysate mixing to develop a broader picture of assembly events.

The advantage of this combined approach is that despite requiring separate expression of α and β subunits for lysate mixing, it is still relatively labor-friendly. The results can also be semiquantitative if one ensures comparable and consistent expression levels of the individual proteins. To this end, we recommend carrying out the usual optimization of recombinant protein expression to determine conditions that allow comparable levels of expressed proteins prior to lysate mixing. 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. We also highlight the importance of determining protein concentrations of the ICAR-purified samples prior to loading the non-denaturing PAGE. Even with expression optimization in place, and with the best care taken to ensure that parallel samples are processed through the purification steps in the same way, variance can still be inadvertently introduced. A concentration measurement ensures that the same amount of total protein is loaded per sample well. This makes lane-to-lane comparisons of band intensities for a given migrating species more meaningful.

If comparable and consistent levels of protein expression cannot be achieved for lysate mixing, one can always purify all components beforehand and carry out mixing experiments with pure proteins. This has the advantage of allowing more accurate protein determinations and thus makes the approach quantitative. However, the drawback of full purification is that the process becomes considerably more labor intensive, which can preclude rapid analysis of multiple mutants simultaneously[18]. A final caveat worth mentioning is that sometimes separate expression of α and β subunits may not be possible. This can occur if a mutation causes a subunit to be insoluble in *E. coli* when expressed on its own but allows solubility to be regained when the mutant is expressed with its binding partner. If this occurs, it will limit analysis to coexpression only. However, this is a caveat that can arise during recombinant protein expression in general, and is not specific to archaeal proteasome subunits[24,25].

We chose to generate his-tagged derivatives of our proteasomal proteins due to

ease of purification and affordability of the ICAR resin. Other epitope tags are possible, including those for antibody-based purification, and we have successfully expressed and purified Flag-tagged versions of our proteasome subunits (not shown). However, if purification to homogeneity (or increasing the scale of production) is required, the his-tagged versions provide the fastest and most cost-effective means of doing so.

It is a given that results obtained with recombinant proteins, be they archaeal or eukaryotic proteins produced in bacteria, will gain further meaning when followed up with *in vivo* observations. However, an *in vivo* approach may not always be immediately accessible experimentally. This is especially true when the subject of study is a large, multi-subunit complex such as the proteasome. Recombinant protein approaches provide an important launch point for future experiments. In the case of the proteasome, pairing recombinant archaeal proteasome production with non-denaturing PAGE will continue to be very effective in elucidating key features of proteasome assembly, which are shared between archaeal and eukaryotic species[9,11,12,18]. Such an approach is likely to be useful for studying the assembly of other large multi-protein complexes as well. Recently, we used this strategy to demonstrate that archaeal proteasomes can assemble via more than one pathway, and that $\alpha$-rings are not the obligate intermediates during assembly that they were thought to be[18]. It remains to be determined if the same holds true for eukaryotic proteasomes.

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**DISCLOSURES:**
The authors have nothing to disclose.

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Figure 1



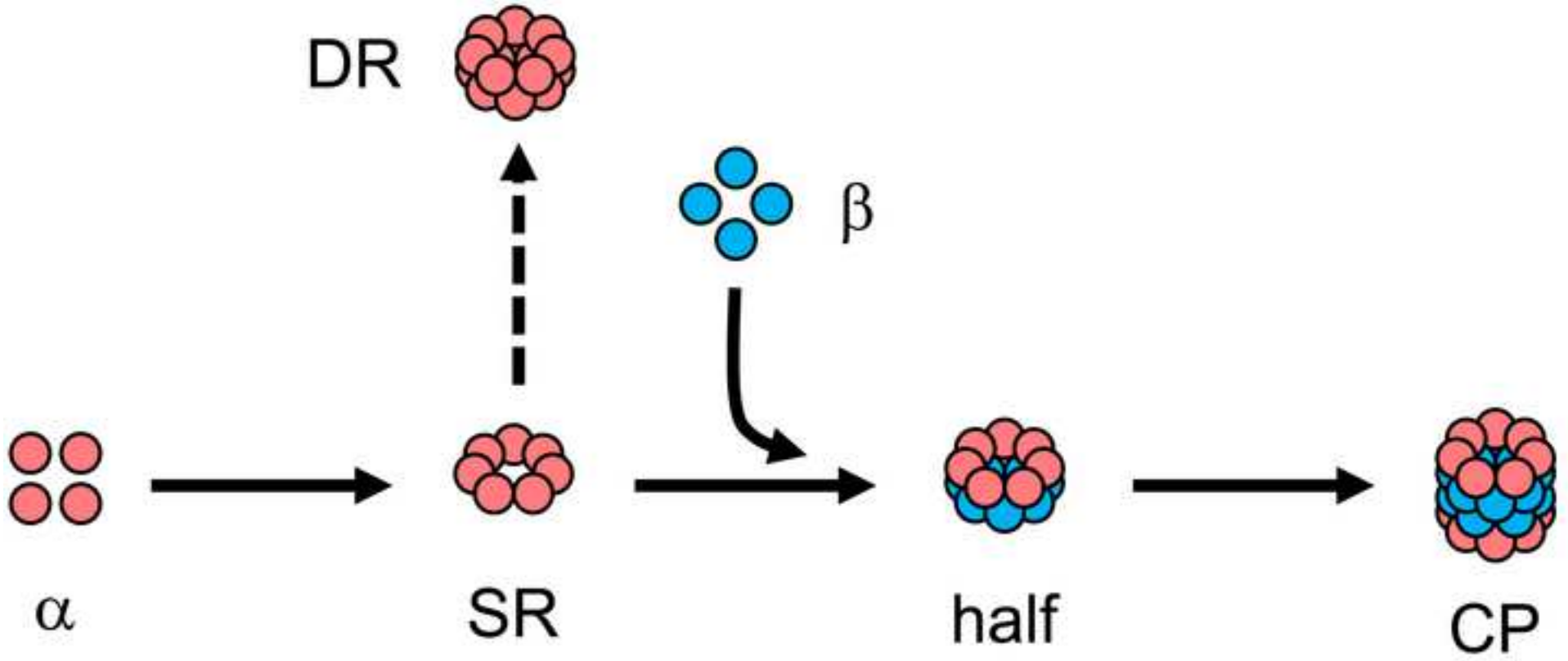

Figure 2



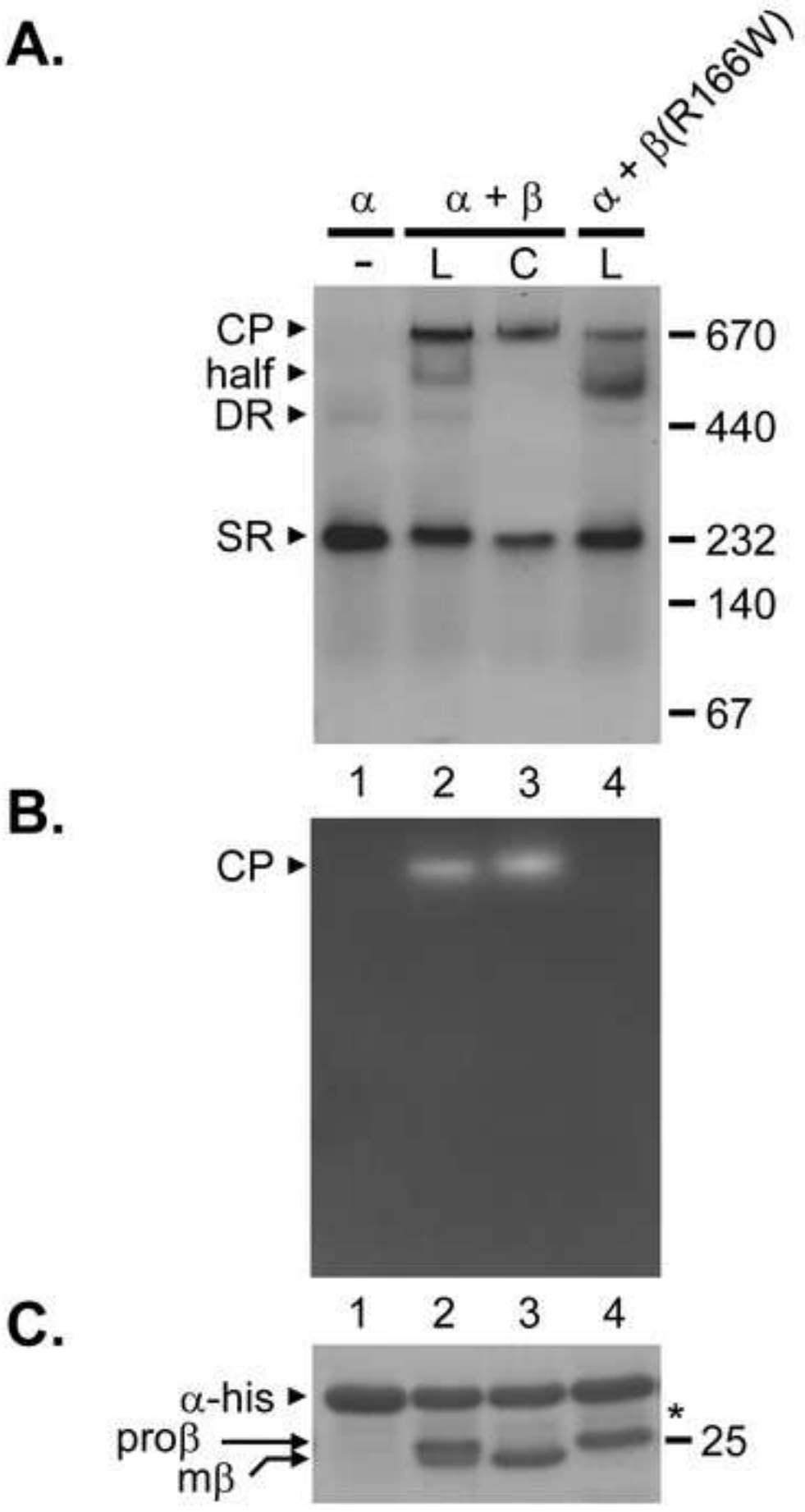

Table 1



| Plasmid | Genotype | Source |
|---|---|---|
| AKB191 | pET42 psmA-his | Kusmierczyk *et al* ., (2011) |
| AKB464 | pET42 psmA-his psmB | Kusmierczyk *et al* ., (2011) |
| AKB946 | pET42 psmB | Panfair *et al* ., (2015) |
| AKB952 | pET42 psmB (R166W) | Panfair *et al* ., (2015) |

Table 2 Click here to download Table Kusmierczyk Table 2.xlsx

| Solution, media, buffer | Components | Notes |
|---|---|---|
| LB (lysogeny broth) media | Per liter: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 2 pellets NaOH. For solid media (plates) add 20 g agar. | Sterilize by autoclaving. For LB-kan media, add kanamycin sulfate to a final concentration of 50 μg/ml. This antibiotic can be prepared as a 50 mg/ml stock solution in water. |
| Lysis buffer | 50 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$, 2 mM pefabloc, 0.3 mg/ml lysozyme, 10 μg/ml DNase I, 0.1% (v/v) Triton X-100. | |
| Buffer A | 50 mM HEPES-NaOH pH 7.5, 300 mM NaCl, 5 mM $MgCl_2$. | Buffers B, C, and E, are derivatives of Buffer A containing imidazole. It is useful to add imidazole from a 2 M stock prepared in water and stored in the dark. |
| Native resolving buffer | 375 mM Tris-HCl pH 8.8, 0.1% (v/v) tetramethylethylenediamine (TEMED) and 0.1% (w/v) ammonium per sulfate (APS). | Prepared fresh no more than an hour before gel is to be polymerized and kept on ice. When preparing acrylamide gel solutions in native resolving buffer, it is useful to add the Tris-HCl from a 4X stock (1.5 M Tris-HCl, pH 8.8). The APS is added immediately prior to polymerization. |
| Native running buffer 10X stock | 250 mM Tris, 1.92 M glycine, do not adjust pH. | |
| 5X native sample buffer | 0.5 M Tris-HCl pH 8.8, 50% (v/v) glycerol, traces of bromophenol blue. | Traces refers to a very small amount, usually a few grains transferred via spatula. |
| 5X SDS-sample buffer | 0.3 M Tris-HCl pH 6.8, 600 mM dithiothreitol (DTT), 10% (w/v) SDS, 50% (v/v) glycerol and traces of bromophenol blue. | Traces refers to a very small amount, usually a few grains transferred via spatula. |
| Developing buffer | 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 50 μM Suc-LLVY-AMC. | Suc-LLVY-AMC is a fluorogenic peptide substrate used to assay proteasome activity. |

| Name | Company | Catalog # | Comments |
|---|---|---|---|
| Acrylamide (40%) solution | Biorad | 1610104 | Unpolymerized acrylamide is a neurotoxin. Wear proper protective equipment |
| Amicon ultra 0.5ml centrifugal filters | EMDMillipore | UFC501024 | |
| Ammonium persulfate | Sigma | A3678 | |
| ATP | Sigma | A7699 | |
| BCA assay kit | Pierce | 23225 | |
| Bisacrylamide (2%) solution | Biorad | 1610142 | |
| Bromophenol blue | Sigma | B8026 | |
| DNaseI | Sigma | DN25 | |
| Dithiothreitol (DTT) | Thermo Fisher | BP172 | |
| *E.coli* BL21 competent cells | EMD Millipore | 69450 | |
| GelCode Blue | Thermo Fisher | 24592 | Colloidal coomassie stain reagent for gels |
| Gel doc EZ system | Biorad | 1708270 | Gel documentation system |
| Gel releasers | Biorad | 1653320 | Wedge shaped plastic used to separate gel plates; useful for spreading liquid. |
| Glass rod | Thermo Fisher | 11-380B | |
| Glycerol | Sigma | 49767 | |
| Glycine | Thermo Fisher | BP3865 | |
| Hamilton syringe | Thermo Fisher | 14-813-38 | Glass syringe for loading gels |
| HEPES | US Biologicals | H2010 | |
| HMW Native calibration kit | GE Healthcare | 170445-01 | High molecular weight protein standards |
| Hoefer SG30 | Thermo Fisher | 03-500-277 | Gradient maker |
| Imidazole | US Biologicals | 280671 | |
| IPTG | US Biologicals | I8500 | For induction of protein expression |
| Isopropanol | Thermo Fisher | BP26181 | |
| Kanamycin sulfate | US Biologicals | K0010 | |
| Lysozyme | Sigma | L6876 | |
| $MgCl_2$ | Fluka analytical | 630680 | |
| Mini Protean Tetra Cell | Biorad | 1658002EDU | Gel electrophoresis apparatus |
| NaCl | Thermo Fisher | S640-3 | |
| NaOH | Thermo Fisher | S318-1 | |
| Pefabloc SC | Roche | 11429876001 | Protease inhibitor |
| pET42 | EMD Millipore | 70562 | Expression plasmid |

| | | | |
|---|---|---|---|
| Precision plus all blue standard | Biorad | 1610373 | Molecular protein standard for SDS-PAGE |
| Quickchange mutagenesis kit | Agilent technologies | 200521 | |
| Sodium dodecyl sulfate (SDS) | Thermo Fisher | BP166 | |
| Suc-LLVY-AMC | Enzo lifesciences | BML P802-0005 | Fluorogenic substrate |
| Talon Metal Affinity Resin | Clontech | 635502 | Immobilized-cobalt affinity resin |
| TEMED | Sigma | T7024 | |
| Tris | US Biologicals | T8600 | |
| Triton-X100 | Sigma | 93426 | |
| Tryptone | Bacto BD | 211699 | |
| UV sample tray | Biorad | 1708271 | For UV imaging of gels |
| Yeast extract | Bacto BD | 212720 | |

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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.**

May 9, 2016

RE: Editorial revisions for the manuscript entitled *"Examining proteasome assembly with recombinant archaeal proteasomes and non-denaturing PAGE: the case for a combined approach"*.

Dear Dr. Zaman:

As per your emailed instructions, I enclose a list of the changes made to the manuscript in response to the editorial comments (see below). An uploaded revised manuscript file, and revised Excel materials file, has been uploaded into the online system.

I thank the Editor for their comments and suggestions for improving the manuscript.

Please let me know if there is anything else I can provide at this stage. Thank you in advance for your consideration and time.

Sincerely,

Andrew Kusmierczyk

Andrew Kusmierczyk

*1. Editor modified the formatting of the manuscript and made minor copy-edits. In keeping with the JoVE format, a few of your protocol steps may have been revised to the imperative tense and any step that could not be written in the imperative tense may have been added as a "Note". Please maintain the current formatting throughout the manuscript. You can find the updated manuscript attached to this e-mail.*

**Action taken:** Current formatting maintained as changes were made using "track changes" feature in Word.

*2. Please revise the Short Abstract so that it clearly states the goal of the protocol within 50 words. For example, "This protocol/manuscript describes…"*

**Action taken:** Short abstract rewritten as suggested.

*3. Please add more details to the following protocol steps*

*a) 1.1 – how is the transformation done? How much of the transformation mixture is plated on the LB-Kan plates?*

**Action taken:** More detail is provided about the transformation by adding protocol steps 1.1.1 to 1.1.4. These were not highlighted for video production.

*b) 1.5 – please clarify what is meant by "repeated centrifugation".*

**Action taken:** To clarify this, the term "repeated centrifugation" was removed. In its place, an expanded version of step 1.5 was included (see steps 1.5.1 to 1.5.3).

*c) 2.1.3.5 – how much of the SDS-sample buffer is added?*

**Action taken:** The appropriate amount is now listed.

*d) Please provide a reference for BCA assay.*

**Action taken:** Reference provided (see new reference 21).

*4. If step 3.3 is to be filmed, please highlight the relevant sub-steps.*

**Action taken:** None. The narrator can say "wash the resin to remove non-specifically bound proteins" (as is currently highlighted). But filming the actual wash steps, which are described in 3.3.1 to 3.3.3 is not necessary. The reader can refer to the protocol to see what is exactly meant by washing the resin.

*5. Please re-write the "Note" following step 4.5 in the imperative tense (as if you are telling someone how to do the technique i.e. "Do this", "Measure that" etc.) and add it as a numbered protocol step.*

**Action taken:** Note rewritten as step 4.6 (including substeps 4.6.1 to 4.6.3). These are not highlighted for filming because SDS-PAGE is a fairly common procedure (much like PCR) which does not require detailed instruction.

*6. After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is a 10-page limit for the protocol text, and a 3- page limit for filmable content. If your protocol is longer than 3 pages OR if there steps in protocol that will NOT be filmed, please highlight 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.*

**Action taken:** Protocol section evaluated and relevant sections highlighted. Despite additions to the protocol text following revision, the length of the filmed section remains at about 2.75 pages.

*7. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove the following commercial sounding language from your manuscript: "Hamilton" and "GelCode". All commercial products should be sufficiently referenced in the table of materials/reagents.*

**Action taken:** "Hamilton" changed to "glass syringe" and "GelCode" changed to "colloidal coomassie stain reagent". Accompanying changes were made to the materials Excel file and the revised version uploaded.

*8. Please minimize use of the pronoun "we" throughout the manuscript.*

**Action taken:** In several places throughout the manuscript, the text was altered so that "we" was removed (see page 2, several instances on page 8, and page 10).

*9. The legend for Figure 1 shows considerable overlap with your previously published work. Please re-word the legend to reduce the text overlap. Alternatively, if you are using a previously published figure you may re-use the figure legend as well but you must have permission to re-use the figure from the previous publisher and you must cite the original work in the figure legend.*

**Action taken:** Legend rewritten to remove overlap. Figure 1 was created specifically for this manuscript.

*10. In the Discussion, please use superscripted reference numbers to cite comparable methods when discussing the significance of your technique with respect to existing methods.*

**Action taken:** References 9, 11, 12 were cited on page 10 in the discussion.