Journal of Visualized Experiments

Extraction and Visualization of Protein Aggregates after treatment of Escherichia coli with a Proteotoxic Stressor

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62628R1
Full Title:	Extraction and Visualization of Protein Aggregates after treatment of Escherichia coli with a Proteotoxic Stressor
Corresponding Author:	Illinois State University, Ph.D., M.Sc Illinois State University Normal, Illinois UNITED STATES
Corresponding Author's Institution:	Illinois State University
Corresponding Author E-Mail:	jdahl1@ilstu.edu
Order of Authors:	Sadia Sultana
	Greg M. Anderson
	Kevin Pierre Hoffmann
	Jan-Ulrik State Dahl, Ph.D., M.Sc
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Normal, Illinois, USA
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Extraction and Visualization of Protein Aggregates After Treatment of *Escherichia coli* With A Proteotoxic Stressor

AUTHORS AND AFFILIATIONS:

Sadia Sultana, Greg M. Anderson, Kevin Pierre Hoffmann, Jan-Ulrik Dahl

School of Biological Sciences, Illinois State University, Campus Box 4120, Normal, IL 61790, USA

Email addresses of co-authors:

Sadia Sultana	(ssulta7@ilstu.edu)
Greg M. Anderson	(gmande1@ilstu.edu)
Kevin Pierre Hoffmann	(kevin.hoffmann@tu-braunschweig.de)

Corresponding author:

Jan-Ulrik Dahl	(jdahl1@ilstu.edu)

KEYWORDS:

Stress response; protein aggregation; protein unfolding; bacteria; protein quality control; oxidative stress; antimicrobials; proteostasis

SUMMARY:

This protocol describes the extraction and visualization of aggregated and soluble proteins from *Escherichia coli* after treatment with a proteotoxic antimicrobial. Following this procedure allows a qualitative comparison of protein aggregate formation *in vivo* in different bacterial strains and/or between treatments.

ABSTRACT:

The exposure of living organisms to environmental and cellular stresses often causes disruptions in protein homeostasis and can result in protein aggregation. The accumulation of protein aggregates in bacterial cells can lead to significant alterations in the cellular phenotypic behavior, including a reduction in growth rates, stress resistance, and virulence. Several experimental procedures exist for the examination of these stressor-mediated phenotypes. This paper describes an optimized assay for the extraction and visualization of aggregated and soluble proteins from different *Escherichia coli* strains after treatment with a silver–ruthenium-containing antimicrobial. This compound is known to generate reactive oxygen species and causes widespread protein aggregation.

The method combines a centrifugation-based separation of protein aggregates and soluble proteins from treated and untreated cells with subsequent separation and visualization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. This approach is simple, fast, and allows a qualitative comparison of protein aggregate formation in different *E. coli* strains. The methodology has a wide range of applications, including the possibility to investigate the impact of other proteotoxic antimicrobials on *in vivo* protein

aggregation in a wide range of bacteria. Moreover, the protocol can be used to identify genes that contribute to increased resistance to proteotoxic substances. Gel bands can be used for the subsequent identification of proteins that are particularly prone to aggregation.

INTRODUCTION:

Bacteria are inevitably exposed to a myriad of environmental stresses, including low pH (e.g., in the mammalian stomach)[1,2], reactive oxygen and chlorine species (ROS/RCS) (e.g., during oxidative burst in phagocytes)[3–5], elevated temperatures (e.g., in hot springs or during heat-shock)[6,7], and several potent antimicrobials (e.g., AGXX used in this protocol)[8]. Proteins are particularly vulnerable to any of these stressors, and exposure can provoke protein un-/misfolding that then seeds aggregation. All organisms employ protective systems that allow them to cope with protein misfolding[9]. However, severe stress can overwhelm the protein quality control machinery and disrupt the secondary and/or tertiary structure of proteins, which ultimately inactivates proteins. As a consequence, protein aggregates can severely impair critical cellular functions required for bacterial growth and survival, stress resistance, and virulence[10]. Therefore, research focusing on protein aggregation and its consequences in bacteria is a relevant topic due to its potential impact on infectious disease control.

Heat-induced protein unfolding and aggregation are often reversible[7]. In contrast, other proteotoxic stresses, such as oxidative stress, can cause irreversible protein modifications through the oxidation of specific amino acid side chains resulting in protein un-/misfolding and, eventually, protein aggregation[4]. Stress-induced formation of insoluble protein aggregates has been extensively studied in the context of molecular chaperones and their protective functions in yeast and bacteria[11–13]. Several protocols have been published that utilize a variety of biochemical techniques for the isolation and analysis of insoluble protein aggregates[14–17]. The existing protocols have mainly been used to study bacterial protein aggregation upon heat-shock and/or identification of molecular chaperones. While these protocols have certainly been an advancement to the field, there are some major inconveniences in the experimental procedures because they require *(i)* a large bacterial culture volume of up to 10 L[14,17], *(ii)* complicated physical disruption processes, including the use of cell disruptors, French press, and/or sonication[14,15,17], or *(iii)* time-consuming repeated washing and incubation steps[15–17].

This paper describes a modified protocol that aims to address the limitations of the previous approaches and allows the analysis of the amount of protein aggregates formed in two different *Escherichia coli* strains after treatment with a proteotoxic antimicrobial surface coating. The coating is composed of metal—silver (Ag) and ruthenium (Ru)—conditioned with ascorbic acid, and its antimicrobial activity is achieved by the generation of reactive oxygen species[8,18]. Herein is a detailed description of the preparation of the bacterial culture after treatment with the antimicrobial compound and a comparison of protein aggregation status upon exposure of two *E. coli* strains with distinct susceptibility profiles to increasing concentration of the antimicrobial. The described method is inexpensive, fast, and reproducible and can be used to study protein aggregation in the presence of other proteotoxic compounds. In addition, the protocol can be modified to analyze the impact that specific gene deletions have on protein aggregation in a variety of different bacteria.

PROTOCOL:

1. Stress treatment of *E. coli* strains MG1655 and CFT073

1.1. Inoculate 5 mL of lysogeny broth (LB) medium with a single colony of commensal *E. coli* strain MG1655 and uropathogenic *E. coli* (UPEC) strain CFT073, respectively, and incubate for 14–16 h (overnight) at 37 °C and 300 rpm.

NOTE: *Escherichia coli* CFT073 is a human pathogen. Handling of CFT073 must be performed with appropriate biosafety measures in a Biosafety Level-2 certified lab.

1.2. Dilute each strain into a 500 mL flask containing 70 mL of 3-(*N*-morpholino)propanesulfonic acid (MOPS)-glucose (MOPS-g) (**Table 1**) medium to an optical density at 600 nm (OD_{600}) value of 0.1. Incubate at 37 °C and 300 rpm until mid-log phase is reached (OD_{600} = 0.5–0.55).

1.3. Transfer 20 mL of each culture into three prewarmed 125 mL flasks and incubate at 37 °C and 300 rpm for 2 min.

NOTE: As timely processing of the samples is required, handle no more than 6 cultures at a time.

1.4. Prepare an antimicrobial compound solution in MOPS-g medium at a concentration of 2 mg/mL. Add the antimicrobial to each culture to reach the indicated concentrations. For the untreated control, add the required volume of MOPS-g medium.

NOTE: Vortex the 2 mg/mL antimicrobial solution to allow an even distribution of the compound particles and avoid sedimentation.

1.6. Incubate the cultures for 45 min at 37 °C and 300 rpm.

----- Insert Figure 1 -------

2. Collecting bacterial cell samples

2.1. After 45 min of stress treatment, determine the OD_{600} of each culture. For each sample, harvest cells equivalent to 4 mL of OD_{600} = 1 in 15 mL centrifuge tubes by centrifugation for 15 min at 3,000 × *g* and 4 °C.

2.2. Completely remove the supernatant and resuspend the cell pellets in 50 µL of ice-cold lysis buffer (**Table 1**). Incubate the samples for 30 min on ice.

NOTE: This lysis step degrades the peptidoglycan layer. Always use freshly prepared lysis buffer.

2.4. Transfer the samples into 1.7 mL microcentrifuge tubes. Freeze at -80 °C until further use.

----- **Insert Figure 2** -------

3. Extracting the insoluble protein aggregates

3.1. Thaw samples on ice.

NOTE: The freeze-thaw cycle contributes to cell lysis.

3.2. Add 360 µL of ice-cold buffer A (**Table 1**) and mix gently by pipetting.

NOTE: The osmotic shock will also contribute to cell lysis.

3.3. Transfer the sample to a 2 mL microcentrifuge tube containing ~200 µL of 0.5 mm glass beads. Incubate for 30 min at 8 °C in a thermomixer with shaking at 1,400 rpm.

NOTE: This step results in the physical disruption of the cell. A protease inhibitor can be used to minimize protein degradation. The disruption can be performed at 4 °C. Note that the use of glass beads has been reported to induce aggregation of a small subset of proteins in yeast[19].

3.5. Incubate for 5 min on ice without shaking to settle the glass beads. Transfer 200 µL of the cell lysate into 1.7 mL microcentrifuge tubes.

NOTE: Avoid the transfer of the glass beads.

3.6. Centrifuge at 16,000 × *g* and 4 °C for 20 min. Collect the supernatant, which contains soluble proteins, and proceed to section 4.

3.7. Resuspend the pellet in 200 µL of ice-cold buffer A (**Table 1**) using the pipette. Centrifuge at 16,000 × *g* and 4 °C for 20 min. Carefully remove the supernatant altogether.

3.9. Add 200 µL of ice-cold buffer B (see **Table 1** and the **Table of Materials**) and carefully resuspend the pellet by pipetting.

NOTE: The non-ionic detergent solubilizes membrane protein.

3.10. Repeat the centrifugation at 16,000 × *g* and 4 °C for 20 min. Carefully remove the supernatant.

3.11. Resuspend the pellet in 200 µL of cold buffer A (**Table 1**) by pipetting. Centrifuge at 16,000 × *g* and 4 °C for 20 min. Completely remove the supernatant.

3.12. Resuspend the pellet in 100 µL of 1x reducing SDS sample buffer (**Table 1**) and boil for 5 min at 95 °C in a thermomixer.

3.13. Store the sample at -20 °C to proceed later or immediately load on an SDS polyacrylamide gel for separation.

----- Insert Figure 3 -------

4. Soluble protein sample preparation

4.1. Mix 1 volume of 100% trichloroacetic acid (TCA) with 4 volumes of soluble protein sample from step 3.6.

NOTE: Handling of TCA requires a fume hood and personal protective equipment and an approved waste disposal procedure.

4.2. Incubate for 10 min at 4 °C for to allow for protein precipitation.

NOTE: White precipitate will appear very soon.

4.3. Centrifuge to precipitate at 21,000 × *g* and 4 °C for 5 min and remove the supernatant. Wash the pellet with 200 µL of ice-cold acetone to remove cellular debris. Centrifuge at 21,000 × *g* and 4 °C for 5 min and remove the supernatant. Repeat these actions in step 4.3 a total of three times.

4.4. Place the microcentrifuge tubes with open lids in a thermomixer at 37 °C to remove the remaining acetone from the pellet.

NOTE: Incubation of more than 5 min may reduce the solubility of the protein pellet.

4.5. Add 100 µL of 1x reducing SDS buffer (**Table 1**) and completely dissolve the pellet. Boil the sample for 5 min at 95 °C.

4.6. Store sample at -20 °C to proceed later or immediately load on an SDS polyacrylamide gel for separation.

----- Insert Figure 4 -------

5. Separation and visualization of extracted protein aggregates using SDS-PAGE

5.1. Prepare a 12% SDS-polyacrylamide gel.

5.1.1. For two separating gels, pipette 5.1 mL of double-distilled water (ddH_2O), 3.75 mL of Tris-HCl (pH 8.8), 7.5 mL of 20% (w/v) SDS, 6 mL of 30% acrylamide/bisacrylamide (29:1) solution, 75 mL of 10% w/v ammonium persulfate, and 10 mL of tetramethylethylenediamine (TEMED) into

a 15 mL centrifuge tube and mix gently without introducing air bubbles. Pour the gel using a 1 mL pipet within the glass plates, leaving the upper 2 cm free of the mixture. Add 70% ethanol on the top of the separating gel and allow an even interface between the two layers.

5.1.2. After polymerization of the separating gel, prepare the stacking gel by pipetting 1.535 mL of ddH_2O, 625 mL of Tris-HCl (pH 6.8), 12.5 mL of 20% (w/v) SDS, 335 mL of 30% acrylamide/bisacrylamide (29:1) solution, 12.5 mL of 10% w/v ammonium persulfate, and 2.5 mL of TEMED. Remove the ethanol from the separating gels and add the stacking gel solution. Insert a comb with the desired number of pockets without introducing air bubbles. Allow polymerization for 20–30 min.

5.2. Load 4 µL of each sample and protein ladder into separate wells and run the gel(s) in Tris-Glycine running buffer (**Table 1**) at 144 V for 45 min at room temperature.

NOTE: Stop the gel when the bromophenol band is about to migrate out of the gel.

5.3. Stain the gel(s) in a prewarmed Fairbanks solution A (**Table 1**) for 30 min on a rocker.

5.4. Decolor the gel(s) in a prewarmed Fairbanks solution D (**Table 1**) until the desired background (e.g., overnight) on a rocker.

----- Insert Figure 5 -------

REPRESENTATIVE RESULTS:

----- Insert Figure 6 -------

Here, two *E. coli* strains were used that differ in their susceptibility to a proteotoxic silver–ruthenium-containing antimicrobial to demonstrate this protocol. Preliminary survival data revealed that the commensal *E. coli* strain MG1655 is significantly more sensitive to the ROS-generating antimicrobial than the UPEC strain CFT073 (data not shown). Both strains were grown in MOPS-g media at 37 °C and 300 rpm. At the mid-log phase, the cells were either left untreated or treated with 175 µg/mL and 200 µg/mL of the antimicrobial, respectively, and incubated for 45 min. Subsequently, the cells were lysed, and cellular protein aggregates separated from the soluble proteins. Proteins in both fractions were then separated by SDS-PAGE and visualized by Coomassie staining. The insoluble fraction shown in **Figure 6** represents the amount of protein aggregates formed, which was increased when cells were incubated in the presence of the antimicrobial compared to untreated cells. The increase in protein aggregate formation was independent of the strain background, although a much more pronounced increase in aggregate formation was detected in the more sensitive strain MG1655. Conversely, lower amounts of soluble proteins (soluble fraction) were observed after antimicrobial treatment of the cells compared to the untreated counterpart. This result was expected given the preliminary data that showed a substantially higher tolerance of the antimicrobial in CFT073 than MG1655.

FIGURE AND TABLE LEGENDS:

Figure 1: *Escherichia coli* stress treatment. Bacterial cultures are grown in MOPS-g and treated with the indicated concentrations of the silver–ruthenium-containing antimicrobial when the mid-log phase is reached. Abbreviations: LB = lysogeny broth; Ag-Ru = silver-ruthenium; MOPS-g = 3-(*N*-morpholino)propanesulfonic acid (MOPS)-glucose.

Figure 2: Bacterial sample collection. Cell samples are harvested by centrifugation and resuspended in lysis buffer followed by storage at -80 °C.

Figure 3: Extraction of insoluble protein aggregates. The extraction of protein aggregates involves a series of steps including cell disruption, the separation of protein aggregates from soluble proteins, the solubilization of membrane proteins, and washing. Abbreviation: SDS = sodium dodecyl sulfate.

Figure 4: Preparation of soluble proteins. The preparation of soluble protein involves a precipitation step with trichloroacetic acid and repeated washing with ice-cold acetone. Abbreviations: TCA = trichloroacetic acid; SDS = sodium dodecyl sulfate.

Figure 5: Protein separation and visualization. The samples are separated by SDS-PAGE and visualized by Coomassie staining. Abbreviation: SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Figure 6: Representative results of antimicrobial-induced protein aggregation in commensal *Escherichia coli* strain MG1655 and UPEC strain CFT073. *E. coli* strains MG1655 and CFT073 were grown at 37 °C and 300 rpm to OD_{600}= 0.5–0.55 in MOPS-g media before they were treated with the indicated concentrations (-, 0 mg/mL; +, 175 mg/mL; ++, 200 mg/mL) of the antimicrobial for 45 min. Soluble and insoluble protein samples were prepared as described in the protocol and **Figure 1, Figure 2, Figure 3,** and **Figure 4** and visualized on a 12% SDS polyacrylamide gel (**Figure 5**). Protein aggregate formation (insoluble fraction) was increased in both strains in the presence of the antimicrobial while the amounts of soluble proteins were decreased. Overall, the antimicrobial had a much more potent effect on MG1655 than on CFT073. Abbreviations: M= Protein marker; UPEC = uropathogenic *E. coli;* MOPS-g = 3-(*N*-morpholino)propanesulfonic acid (MOPS)-glucose; SDS = sodium dodecyl sulfate.

Table 1: Buffer, Media, and Solutions. Recipes for buffer, media, and solutions used in this protocol.

DISCUSSION:

This protocol describes an optimized methodology for the analysis of protein aggregate formation after treatment of different *E. coli* strains with a proteotoxic antimicrobial. The protocol allows the simultaneous extraction of insoluble and soluble protein fractions from treated and untreated *E. coli* cells. Compared to existing protocols for protein aggregate isolation from cells[14–16,20], this method has several advantages: *(i)* only small culture volumes (4–8 mL) are needed; *(ii)* the cell disruption process does not rely on special equipment such as a French press,

cell disruptor, or sonicator; and *(iii)* the protocol is easy to follow even for relatively early-career scientists in the field.

Bacteria encounter a myriad of stresses in their natural environment, and many of them represent a threat, particularly to proteins, the most abundant macromolecule in the cell[21]. The described methodology offers many potential applications. One of them is the possibility to investigate the efficacy by which a wide range of stresses (e.g., elevated temperatures, reactive oxygen species, reactive chlorine species) and chemical compounds affect protein homeostasis in bacteria, archaea, and even eukaryotic cells[5,12,22,23]. Our extraction was performed with the two distantly related *E. coli* strains, MG1655 and CFT073. However, it has also been successfully applied to study the role of specific gene products for protein homeostasis by comparing protein aggregate formation in wild-type and mutant strains[11,12].

After considering appropriate modifications and troubleshooting of growth conditions and stressor concentrations, this protocol can also be used to determine protein aggregate formation in other gram-negative[5] and gram-positive bacteria[8]. Notably, the gel bands separated after SDS-PAGE can be densitometrically analyzed (i.e., using ImageJ). This approach can also be used to analyze the impact of additional therapeutic compounds such as inhibitors of molecular chaperones[24]. Most intriguingly, it can be combined with mass spectrometric approaches to provide information on the type of protein species that are aggregation-sensitive under a specific stress condition, simply by determining their presence or absence in the insoluble protein fraction[5,15].

The success of this protocol requires careful consideration of the stressor concentrations that cells are exposed to and the exposure time; hence, the critical step of the assay. We, therefore, recommend performing a preliminary survival assay with increasing stressor concentrations to determine the proteotoxic concentration. Moreover, the stressor solutions should be prepared freshly before each experiment, and sample collection time be kept consistent. One limitation of this method is the low number of samples that can concurrently be processed. This is mainly due to the time-sensitive handling of samples in section 1, which involves significant vortexing steps of the antimicrobial solution in between the addition of the compound to the cultures to avoid sedimentation. However, this may not be such an issue when different soluble stressors are applied. Moreover, the described procedure does not provide any time-resolved information on protein aggregate location and trajectory, which would require more advanced techniques such as fluorescence microscopy in combination with time-lapse microscopy[25]. In summary, the improved methodology is simple, easy to follow, inexpensive, and offers the potential for additional modification that allows a tailored approach for identifying proteotoxic compounds or bacterial stress response genes.

ACKNOWLEDGMENTS:

This work was supported by the Illinois State University Undergraduate Research Support Program (to G.M.A.) and the Illinois State University New Faculty Initiative Grant (to J.-U.D.). K. P. H. was supported by a RISE fellowship provided by the German Academic Exchange Service (DAAD). The authors thank Dr. Uwe Landau and Dr. Carsten Meyer from Largentech Vertriebs

GmbH for providing the AGXX powder. **Figures 1, Figure 2, Figure 3, Figure 4,** and **Figure 5** were generated with Biorender.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Dahl, J.-U. et al. HdeB functions as an acid-protective chaperone in bacteria. *Journal of Biological Chemistry.* **290** (1), 65–75 (2015).
2. Foit, L., George, J. S., Zhang, B. W., Brooks, C. L., Bardwell, J. C. A. Chaperone activation by unfolding. *Proceedings of the National Academy of Sciences of the United States of America.* **110** (14), E1254–1262 (2013).
3. Sultana, S., Foti, A., Dahl, J.-U. Bacterial defense systems against the neutrophilic oxidant hypochlorous acid. *Infection and Immunity.* **88** (7), e00964-19 (2020).
4. Dahl, J.-U., Gray, M. J., Jakob, U. Protein quality control under oxidative stress conditions. *Journal of Molecular Biology.* **427** (7), 1549–1563 (2015).
5. Groitl, B., Dahl, J.-U., Schroeder, J. W., Jakob, U. *Pseudomonas aeruginosa* defense systems against microbicidal oxidants. *Molecular Microbiology.* **106** (3), 335–350 (2017).
6. Casadevall, A. Thermal restriction as an antimicrobial function of fever. *PLoS Pathogens.* **12** (5), e1005577 (2016).
7. Richter, K., Haslbeck, M., Buchner, J. The heat shock response: life on the verge of death. *Molecular Cell.* **40** (2), 253–266 (2010).
8. Loi, V. Van, Busche, T., Preuß, T., Kalinowski, J., Bernhardt, J. The AGXX ® antimicrobial coating causes a thiol-specific oxidative stress response and protein *S*-bacillithiolation in *Staphylococcus aureus*. *Frontiers in Microbiology.* **9**, 3037 (2018).
9. Anfinsen, C. B., Scheraga, H. A. Experimental and theoretical aspects of protein folding. *Advances in Protein Chemistry.* **29**, 205–300 (1975).
10. Schramm, F. D., Schroeder, K., Jonas, K. Protein aggregation in bacteria. *FEMS Microbiology Reviews.* **44** (1), 54–72 (2020).
11. Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., Bukau, B. Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the *Escherichia coli* cytosol. *Molecular Microbiology.* **40** (2), 397–413 (2001).
12. Gray, M. J. et al. Polyphosphate is a primordial chaperone. *Molecular Cell.* **53** (5), 689–699 (2014).
13. Weids, A. J., Ibstedt, S., Tamás, M. J., Grant, C. M. Distinct stress conditions result in aggregation of proteins with similar properties. *Scientific Reports.* **6**, 24554 (2016).
14. Mogk, A. et al. Identification of thermolabile *Escherichia coli* proteins: prevention and reversion of aggregation by DnaK and ClpB. *EMBO Journal.* **18** (24), 6934–6949 (1999).
15. Fay, A., Glickman, M. S. An essential nonredundant role for mycobacterial DnaK in native protein folding. *PLoS Genetics.* **10** (7), e1004516 (2014).
16. Schramm, F. D., Heinrich, K., Thüring, M., Bernhardt, J., Jonas, K. An essential regulatory function of the DnaK chaperone dictates the decision between proliferation and maintenance in *Caulobacter crescentus*. *PLoS Genetics.* **13** (12), e1007148 (2017).
17. Maisonneuve, E., Fraysse, L., Moinier, D., Dukan, S. Existence of abnormal protein

aggregates in healthy *Escherichia coli* cells. *Journal of Bacteriology.* **190** (3), 887–893 (2008).
18. Heiss, A., Freisinger, B., Held-Föhn, E. Enhanced antibacterial activity of silver-ruthenium coated hollow microparticles. *Biointerphases.* **12** (5), 05G608 (2017).
19. Papnayotou I., Sun B., Roth A. F., Davis N. G. Protein aggregation induced during glass bead lysis of yeast. *Yeast.* **27** (10), 801–816 (2010).
20. Chuang, S. E. & Blattner, F. R. Characterization of twenty-six new heat shock genes of Escherichia coli. *Journal of Bacteriology.* **175** (16), 5242–5252 (1993).
21. Imlay, J. A. The molecular mechanisms and physiological consequences of oxidative stress: Lessons from a model bacterium. *Nature Reviews Microbiology.* **11** (7), 443–454 (2013).
22. Mühlhofer, M. et al. The heat shock response in yeast maintains protein homeostasis by chaperoning and replenishing proteins. *Cell Reports.* **29** (13), 4593–4607.e8 (2019).
23. Chandrangsu, P., Rensing, C., Helmann, J. D. Metal homeostasis and resistance in bacteria. *Nature Reviews Microbiology.* **15**, 338–350 (2017).
24. Stevens, M. et al. HSP60/10 chaperonin systems are inhibited by a variety of approved drugs, natural products, and known bioactive molecules. *Bioorganic and Medicinal Chemistry Letters.* **29** (9), 1106–1112 (2019).
25. Schramm, F. D., Schroeder, K., Alvelid, J., Testa, I., Jonas, K. Growth-driven displacement of protein aggregates along the cell length ensures partitioning to both daughter cells in *Caulobacter crescentus*. *Molecular Microbiology.* **111** (6), 1430–1448 (2019).

Figure 1

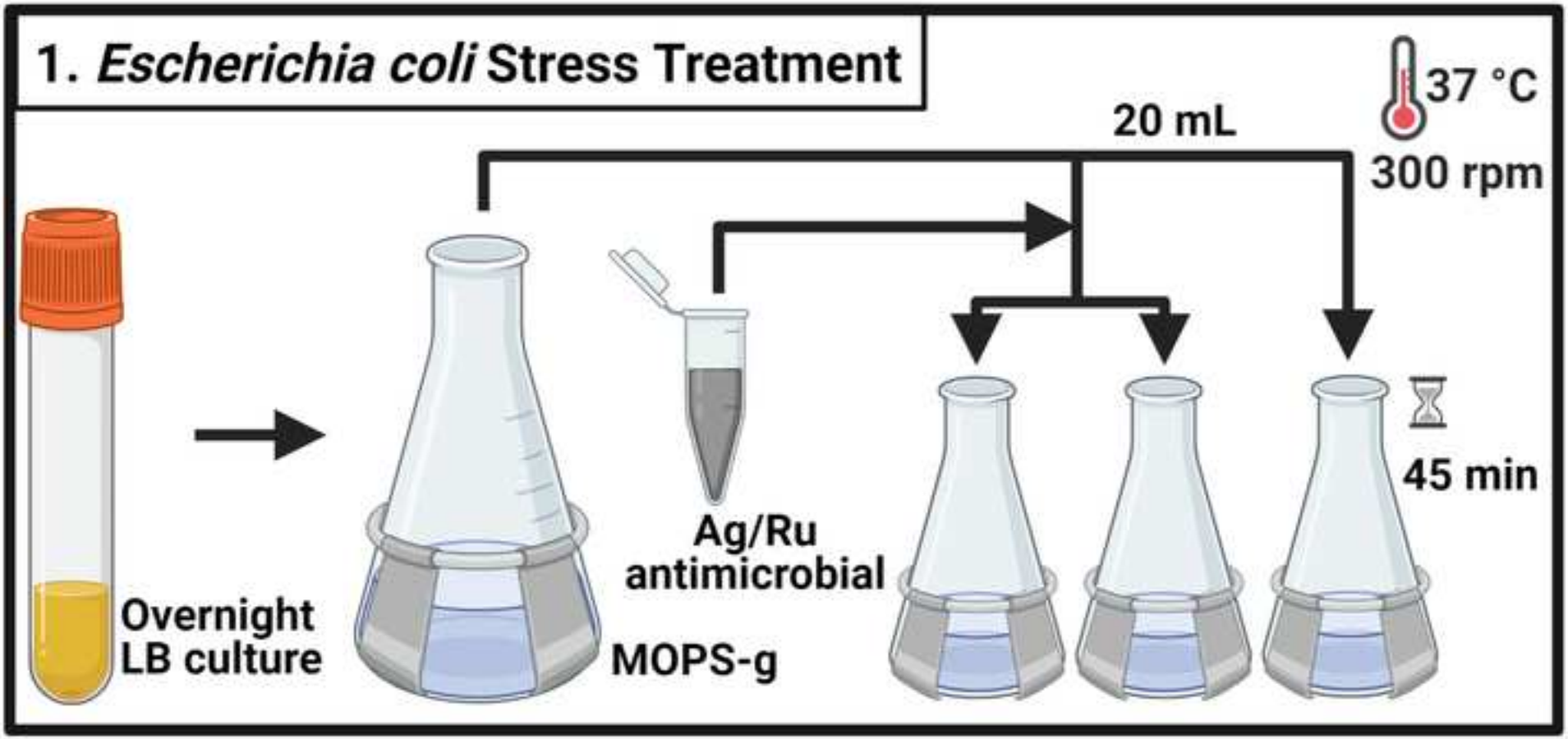

Figure 2

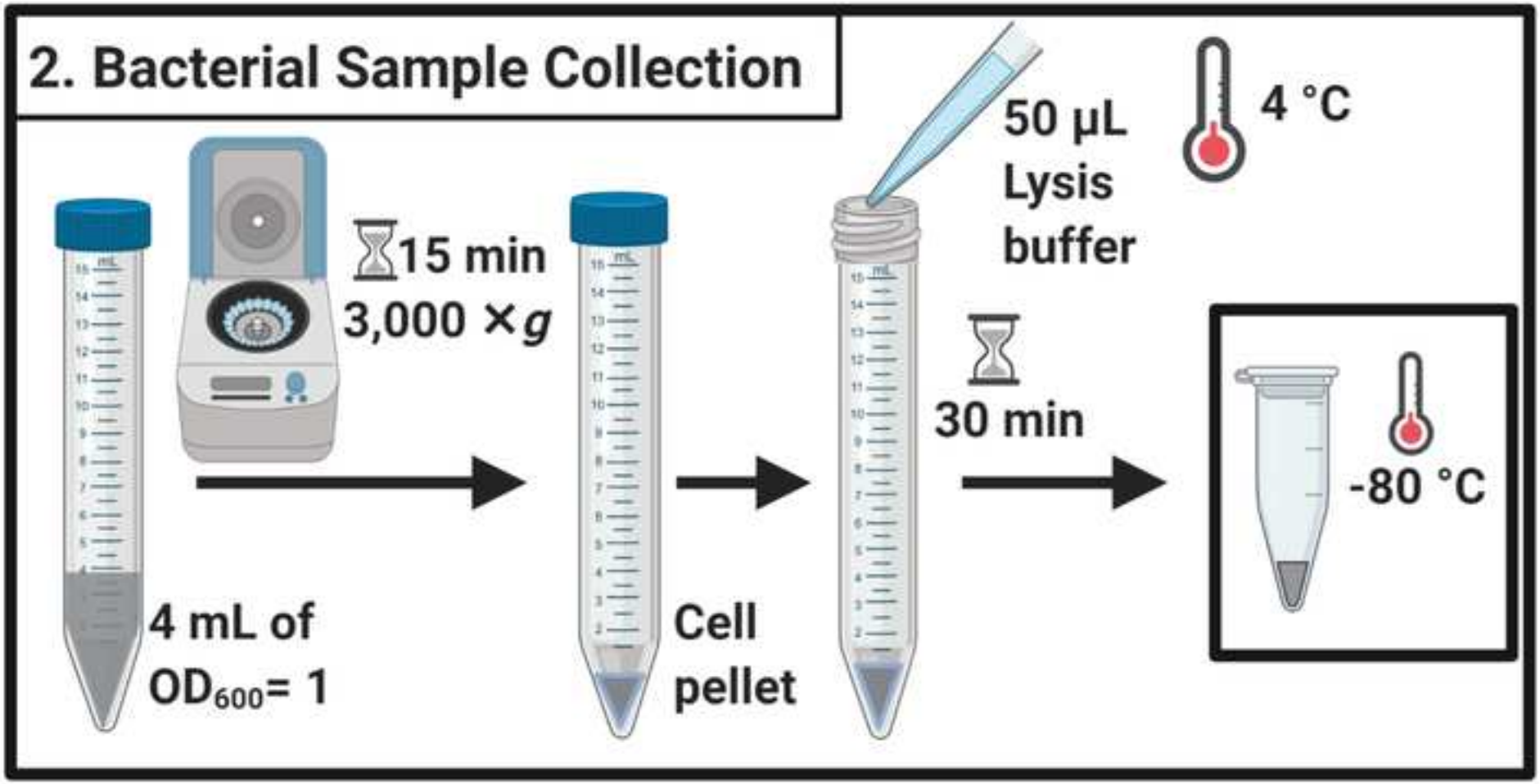

Figure 3

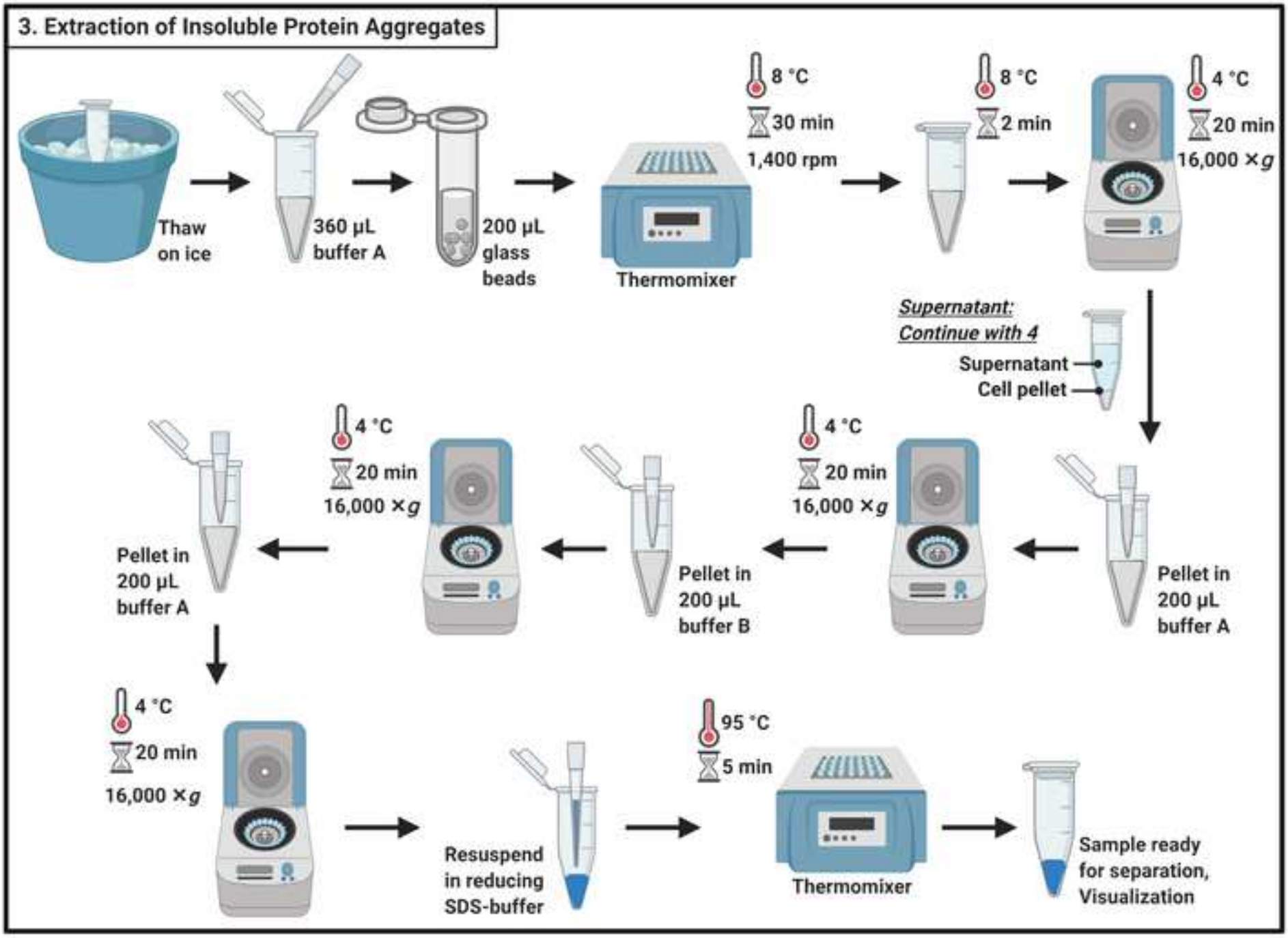

Figure 4

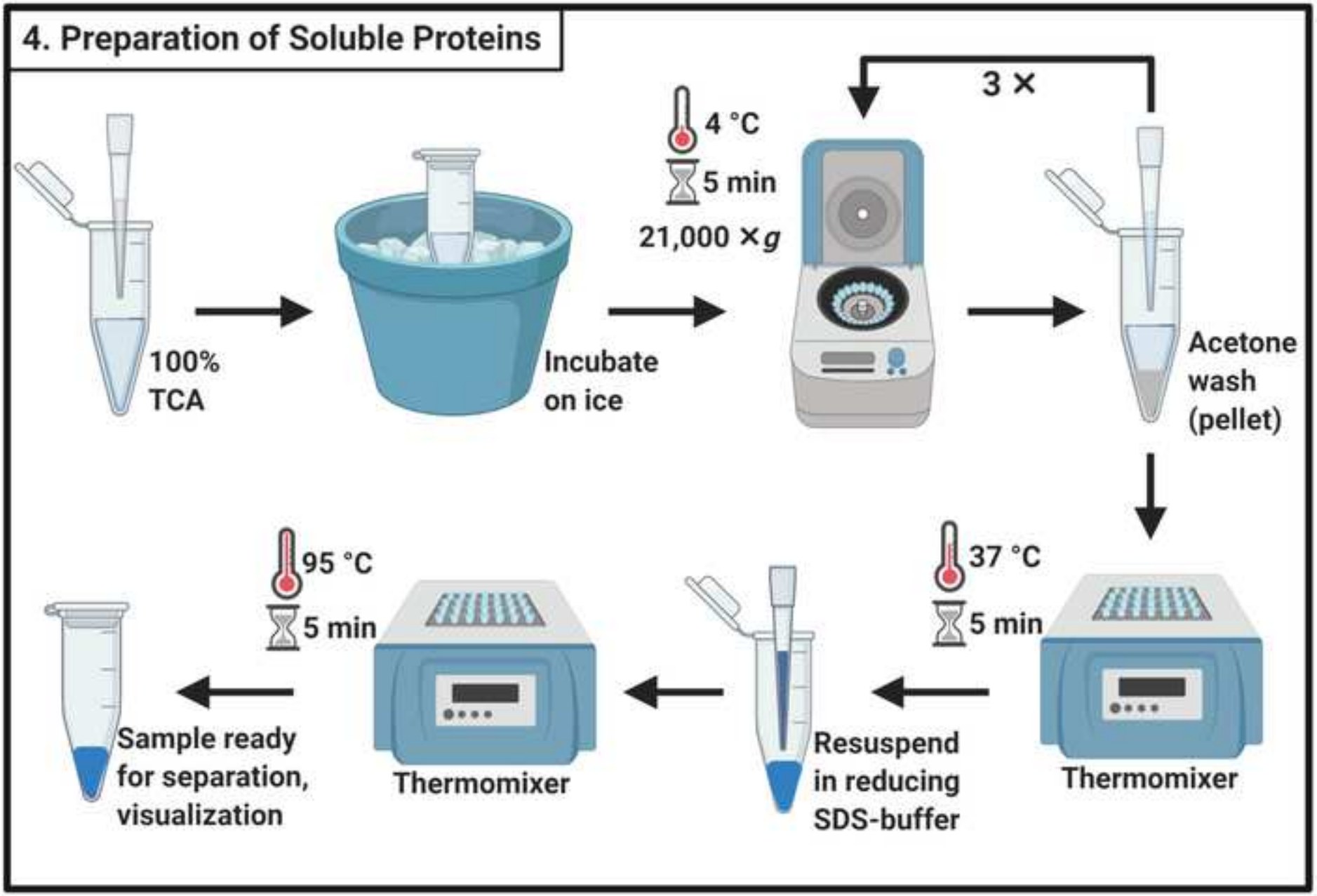

Figure 5

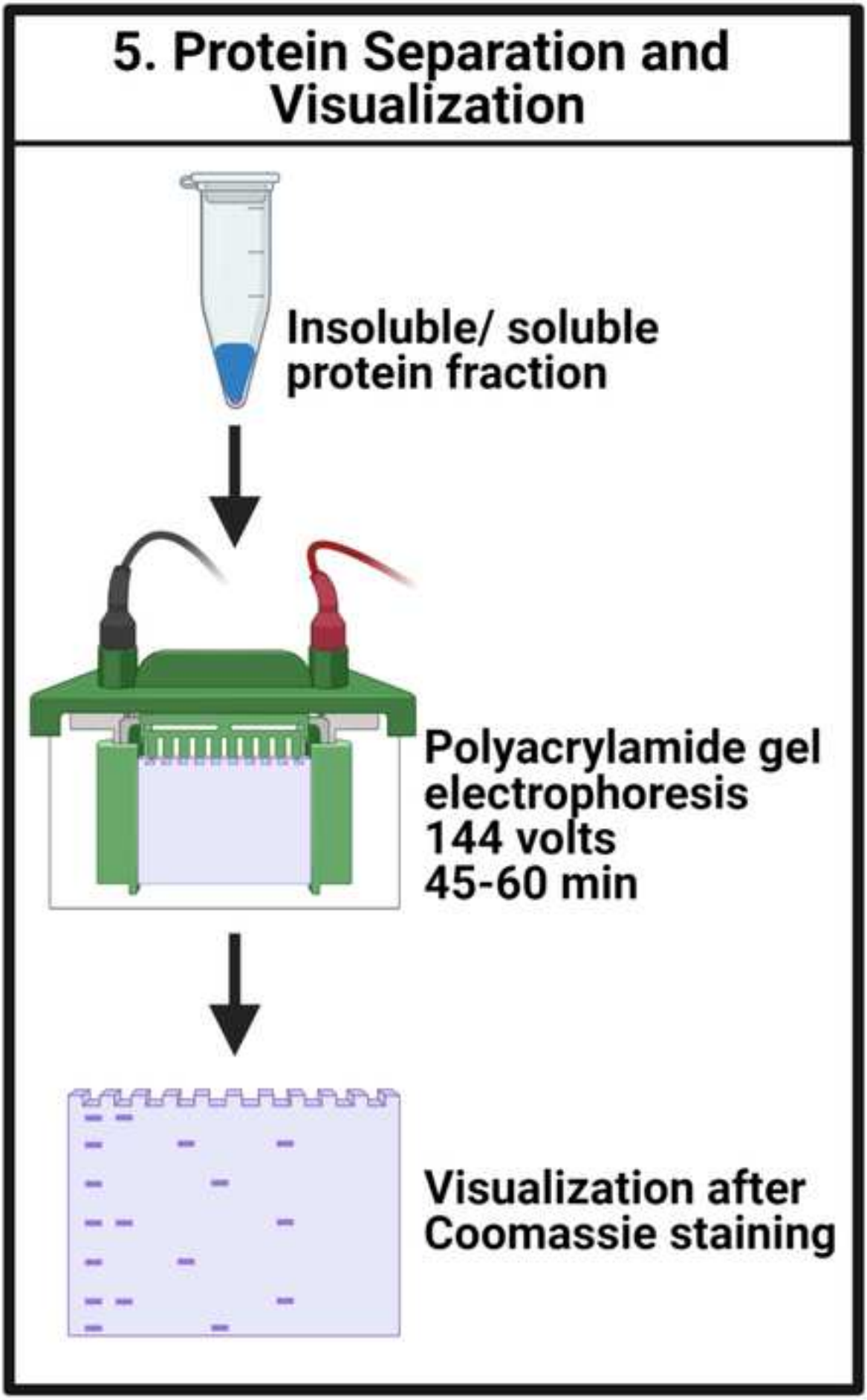

Figure 6

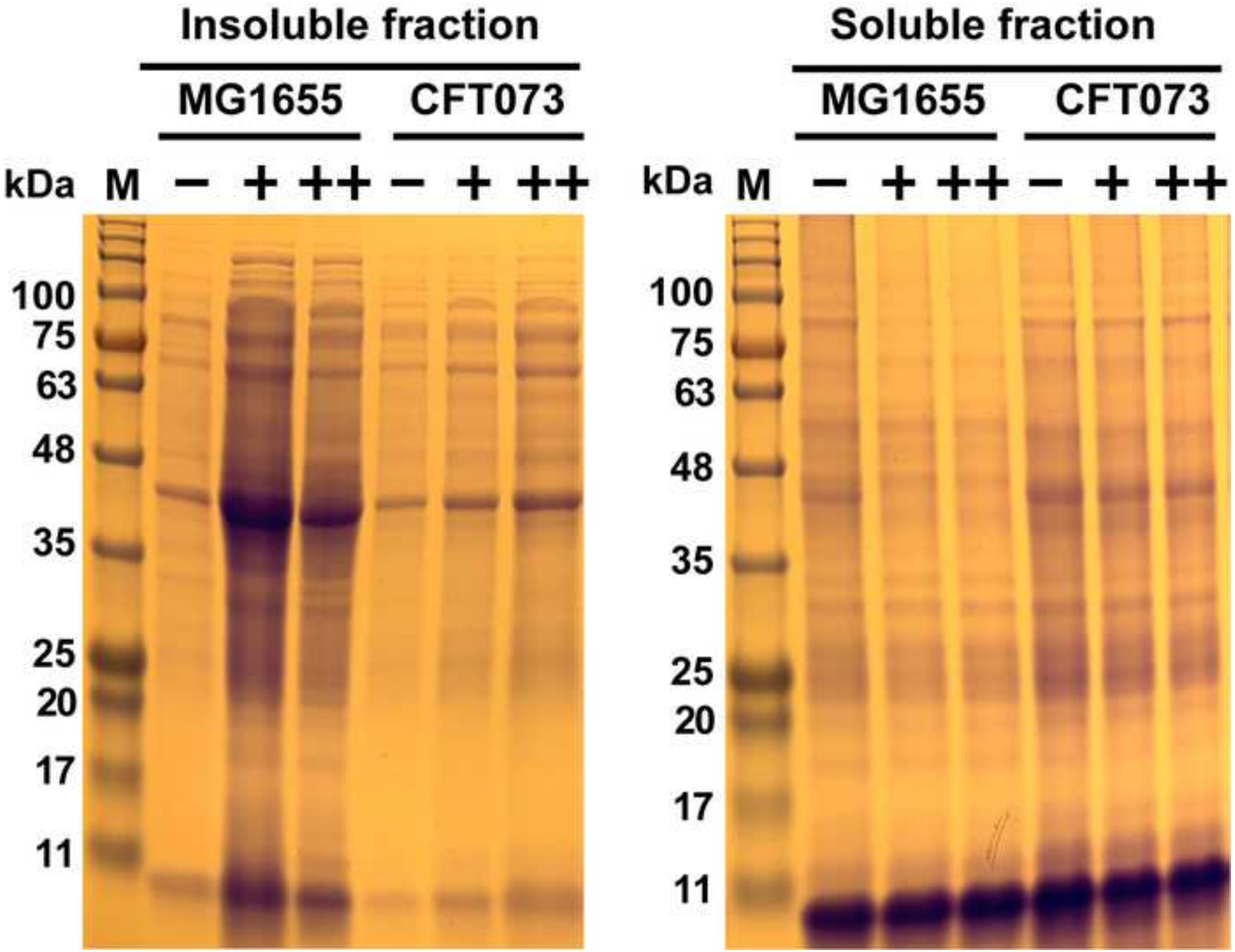

Table 1

Solutions	Recipes
Buffer A	10 mM potassium phosphate (pH 6.5), 1 mM EDTA
Buffer B	Buffer A containing 2% Nonidet P-40. Can be stored in room temperature for later use.
Fairbanks A (Staining solution)	25% isopropanol, 10% Glacial Acetic acid, 1.4 g Coommassie R-250
Fairbanks D (Destaining solution)	10% Glacial acetic acid solution
Lysis buffer	10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 20% sucrose can be prepared and stored at room temperature for long term use. Add 1 mg/mL lysozyme and 50 u/mL Benzonase fresh before use.
MOPS-g media	100 mL 10x MOPS, 10 mL 0.132 M K_2HPO4, 10 mL 20% glucose, 0.5 mL 20 mM thiamine. Fill up to 1 L with ddH_2O and sterile-filter
1x SDS sample buffer	6.5 mM Tris-HCl (pH 7), 10% glycerol, 2% SDS, 0.05% bromophenol blue and 2.5% β-mercaptoethanol. Stored at -20 ºC.
12% SDS polyacrylamide gel preparation (for 2 gels)	**Separating gel:** 5.1 mL ddH_2O, 3.75 mL Tris-HCl (pH 8.8), 75 mL 20% w/v SDS, 6 mL 30% Acrylamide/Bisacrylamide solution 29:1 solution, 75 mL 10% w/v ammonium persulfate, 10 mL TEMED **Stacking gel:** 1.535 mL ddH_2O, 625 mL Tris-HCl (pH 6.8), 12.5 mL 20% w/v SDS, 335 mL 30% Acrylamide/Bisacrylamide solution 29:1 solution, 12.5 mL 10% w/v ammonium persulfate, 2.5 mL TEMED
SDS running buffer	25 mM Tris, 192 mM Glycine, 0.1% SDS in ddH_2O. Store in room temperature.

Chemicals/ Reagents	Company	Catalog Number
Acetone	Fisher Scientific	67-64-1
30% Acrylamide/Bisacrylamide solution 29:1	Bio-Rad	1610156
Ammonium persulfate	Millipore Sigma	A3678-100G
Benzonase nuclease	Sigma	E1014-5KU
Bluestain 2 Protein ladder, 5-245 kDa	GoldBio	P008-500
β-mercaptoethanol	Millipore Sigma	M6250-100ML
Bromophenol blue	GoldBio	B-092-25
Coomassie Brilliant Blue R-250	MP Biomedicals LLC	821616
D-Glucose	Millipore Sigma	G8270-1KG
D-Sucrose	Acros Organics	57-50-1
Ethylenediamine tetra acetic acid (EDTA)	Sigma-Aldrich	SLBT9686
Glacial Acetic acid	Millipore Sigma	ARK2183-1L
Glycerol, 99%	Sigma-Aldrich	G5516-1L
Glycine	GoldBio	G-630-1
Hydrochloric acid, ACS reagent	Sigma-Aldrich	320331-2.5L
Isopropanol (2-Propanol)	Sigma	402893-2.5L
LB broth (Miller)	Millipore Sigma	L3522-1KG
LB broth with agar (Miller)	Millipore Sigma	L2897-1KG
Lysozyme	GoldBio	L-040-25
10x MOPS Buffer	Teknova	M2101
Nonidet P-40	Thomas Scientific	9036-19-5
Potassium phosphate, dibasic	Sigma-Aldrich	P3786-1KG
Potassium phosphate, monobasic	Acros Organics	7778-77-0
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L3771-500G
Tetramethylethylenediamine (TEMED)	Millipore Sigma	T9281-50ML
Thiamine	Sigma-Aldrich	T4625-100G
100% Trichloroacetic acid	Millipore Sigma	T6399-100G
Tris base	GoldBio	T-400-1

Name of Material/Equipment	Company	Catalog Number
Centrifuge tubes (15 mL)	Alkali Scientific	JABG-1019
Erlenmeyer flask (125 mL)	Carolina	726686
Erlenmeyer flask (500 mL)	Carolina	726694
Freezer: -80 °C	Fisher Scientific	
Glass beads (0.5 mm)	BioSpec Products	1107-9105
Microcentrifuge	Hermle	Z216MK
Microcentriguge tubes (1.7 mL)	VWR International	87003-294
Microcentriguge tubes (2.0 mL)	Axygen Maxiclear Microtubes	MCT-200-C
Plastic cuvettes	Fischer Scientific	14-377-012
Power supply	ThermoFisher Scientific	EC105
Rocker	Alkali Scientific	RS7235
Shaking incubator (37 ºC)	Benchmark Scientific	
Small glass plate	Bio-Rad	1653311
Spacer plates (1 mm)	Bio-Rad	1653308

Spectrophotometer	Thermoscientific	3339053
Tabletop centrifuge for 15 mL centrifuge tubes	Beckman-Coulter	
Vertical gel electrophoresis chamber	Bio-Rad	1658004
Vortexer	Fisher Vortex Genie 2	12-812
Thermomixer	Benchmark Scientific	H5000-HC
10 well comb	Bio-Rad	1653359

Editor's comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done!

2. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: AGXX, etc. The term AGXX has been replaced throughout the manuscript with the exception for the acknowledgement and an introduction in the introduction.

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. Please use generic term for AGXX. The term can be introduced once somewhere in the introduction. Done!

4. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without brackets. Done!

5. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, sub step and note in the protocol section. Done!

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Done!

7. Please ensure each step of the protocol answers the how question: How is this step performed. Done!

8. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. All steps listed in the protocol.

9. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. Done!

10. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Done!

11. Please do not abbreviate the journal names in the reference section. Done!

12. Please sort the materials table in alphabetical order. Please make a separate table for solution recipes and upload it separately as .xlsx file. Done!

Reviewers' comments:

Reviewer 1:

We thank the two reviewers for their helpful comments.

* In several places in the text, including step 3.2, parentheses within parenthesis are used. Please replace inner parentheses with brackets. We replaced the inner parenthesis with brackets.

* Step 3.4. How critical is 8°C to the procedure? This temperature is much less convenient than say,4°C. Is it crucial to maintain this precise temperature? We appreciate bringing up this concern. We have performed this step at 8°C, however, 4°C is also possible. We've added this note accordingly.

* Step 3.5, please specify time range rather than indicating "incubate few minutes" We defined the time as 5 minutes.

* Step 4.1 the authors say "waste disposal plant". Did they mean to say "waste disposal plan"? We apologize for this typo, which is now corrected.

* In step 4.3, the authors state "spin down" samples, where in other steps state "centrifuge". Please use consistent language. We changed the term "spin down" to "centrifuge".

* In step 4.7, the authors indicate use of a "thermocycler". However, Figure 1 indicates use of a thermomixer. Please clarify. We apologize for this typo and changed to thermomixer.

Comments on figures:

* Figure 1: The authors could consider separating this figure into multiple figures from the individual panels, which could be then be placed right next the correspond in the text. We are grateful for this feedback and split the content into Figures 1-5.

* Figure 2:

o Marking conditions above the gel (e.g. -/+/++ for the 3 conditions), rather than ramp, could more clearly indicate the zero-stress lane. We appreciate this suggestion and modified Fig. 6 accordingly.

o Indicate kDa of protein marker. We added the information.

o The authors might consider quantifying protein levels in the soluble vs. insoluble samples. This is a good suggestion and could be done, if desired. Given that the scope of this paper is the methodology, we refrained from quantifying the protein level. However, we mention this possibility in the discussion.

Reviewer 2:

- Why use glass beads? Some insoluble proteins attached to the glass and that create make some variation. Also, glass beads increase protein aggregation during lysis step https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3880186/. We thank the reviewer for bringing this concern to our attention. The protocol in this form allows for a qualitative comparison between different samples/treatments, which can be modified to a semi-quantitative approach. The reference also states that the majority of the proteome remains intact and that glass beads-mediated aggregation is only a concern for a small subset of the proteins. In contrast to the reference, we do not vigorously vortex our samples but simply shake them while samples are chilled at 8 C. This should decrease the risk of aggregation. We added a note that glass beads could represent a concern and referenced the paper.

- Protease inhibitors are routinely used to limit the effect of the proteases before they are physically separated from the protein and this step appears to missing from the protocol? We agree with the reviewer that the use of protease inhibitors is certainly a possibility, which we refer to in a special note in the manuscript (Step 3.4). However, we did not used any protease inhibitor but made sure that timely handling of samples occurred on ice throughout steps 2-4.

- The authors focus on AGXX which has a specific effect, it would be more comforting the know that the protocol also worked for other types of inclusion treatments (eg heat) or compounds https://pubmed.ncbi.nlm.nih.gov/29491361/. The protocol has already been used to test a wide range on proteotoxic treatments, including hypochlorous acid, heat etc., which were referenced (Ref 4, 11, 12)

Minor Concerns:

- Why do you use MOPS-glucose medium and LB, any reason? For cultivation of *E .coli,* we use MOPS-glucose medium and LB as the standard minimal and complex media, respectively. However, this protocol is not limited to MOPS-glucose and/or LB medium. Depending on the scope of the experiment, other media can be used.

- What is AGXX vehicle? AGXX is composed of the two transition metals silver (Ag) and ruthenium (Ru), which are conditioned with ascorbic acid. The Ag+ and Ru+ metal ions form a micro-galvanic cell and generate ROS, particularly hydroxyl radicals and hydrogen peroxide, which are proposed to be the main cause of the antimicrobial properties.

- It is mentioned that the AGXX sample is vortexed to avoid sedimentation, implying that is not soluble in the vehicle? Is that not problematic? We agree that the insolubility of AGXX represents a potential issue. However, we have established 2 mg/ml as ideal concentration for the stock solution that allows reproducibility. Important is that the suspension is vortexed vigorously prior to pipetting and that the pipetting occ

- How did the authors confirm that the bacteria have a good stress response at 45min treatment ?!Why not shorter or longer? We utilized this protocol to test a variety of stressors including the proteotoxic oxidant hypochlorous acid (HOCl). While HOCl is extremely fast acting, AGXX appeared to cause protein aggregation at a much slower time frame. We determined that notable amounts of AGXX-induced protein aggregation were only observed after 30-60 min of AGXX exposure.

- How do the authors deal with any lethality due to the treatment? the input number of bacteria is determined, but is there a control for how much actually survives and goes into the extraction? Given JoVE's focus on the methodology and that the data presented here have not yet been published, we decided to only show the SDS gel in this manuscript. We have performed growth curve and survival-based assays to determine the impact of the AGXX concentrations on the strains tested. The two AGXX concentrations chosen were sublethal for CFT073 but had significant negative effect on the survival of MG1655. The formation of protein aggregates is toxic and results in increased killing of the cell.