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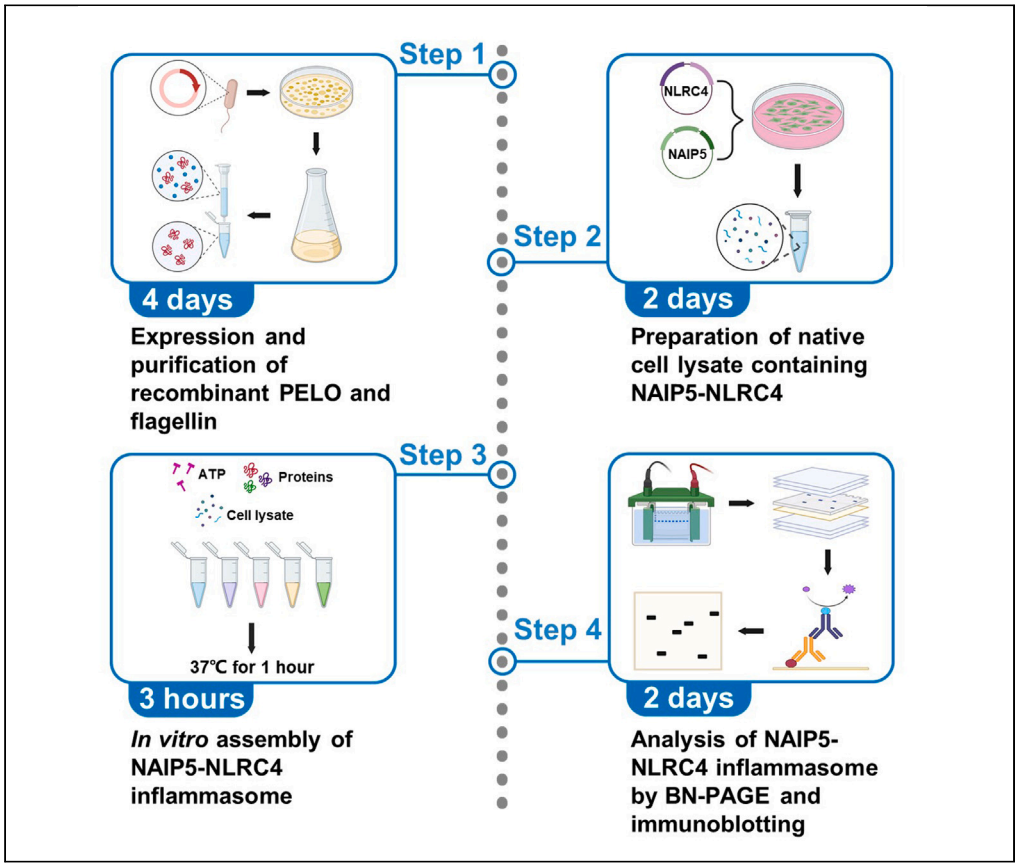
Protocol for reconstitution of oligomeric assembly of NAIP5-NLRC4 inflammasome *in vitro*



Inflammasomes are multimeric protein complexes that have crucial functions in innate immunity. Here, we present a protocol to reconstitute the PELO-driven assembly of NAIP5-NLRC4 inflammasome *in vitro*. We describe steps for expression and purification of recombinant PELO and flagellin, preparation of native cell lysate containing NAIP5-NLRC4, and *in vitro* assembly of NAIP5-NLRC4 inflammasome. We then detail analysis of NAIP5-NLRC4 inflammasome by blue native polyacrylamide gel electrophoresis and immunoblotting. This protocol can be adapted to monitor the oligomeric assembly of other inflammasome types.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Production and purification of recombinant PELO and flagellin proteins in *E. coli*

In vitro reconstitution of oligomeric assembly of NLRC4 inflammasome

Procedures to analyze the oligomeric NLRC4 inflammasome by BN-PAGE

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Protocol for reconstitution of oligomeric assembly of NAIP5-NLRC4 inflammasome *in vitro*

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SUMMARY

Inflammasomes are multimeric protein complexes that have crucial functions in innate immunity. Here, we present a protocol to reconstitute the PELO-driven assembly of NAIP5-NLRC4 inflammasome *in vitro*. We describe steps for expression and purification of recombinant PELO and flagellin, preparation of native cell lysate containing NAIP5-NLRC4, and *in vitro* assembly of NAIP5-NLRC4 inflammasome. We then detail analysis of NAIP5-NLRC4 inflammasome by blue native polyacrylamide gel electrophoresis and immunoblotting. This protocol can be adapted to monitor the oligomeric assembly of other inflammasome types.

For complete details on the use and execution of this protocol, please refer to Wu et al. (2023).[1]

BEFORE YOU BEGIN

The NOD-like receptors (NLRs) are all featured with a highly conserved nucleotide binding NACHT domain.[2,3] The current model proposes that, after ligand recognition, the key process to activate a specific NLR is its assembly into an oligomeric structure, which is facilitated by the NACHT domain.[3] The NLR family apoptosis inhibitory protein 5 (NAIP5) binds conserved bacterial flagellin and recruits the NLR family CARD-containing protein 4 (NLRC4), which acts as an inflammasome adapter to assemble into a high molecular-mass multiprotein complex that activates innate immunity.[4] Our recent study reveals that PELO, an evolutionarily conserved component of the ribosome-associated quality control machinery to rescue the stalled ribosomes, drives the homo-oligomerization of all cytosolic NLRs through boosting their ATPase activity.[1]

This protocol outlines the steps to monitor the *in vitro* assembly of flagellin-dependent NAIP5-NLRC4 inflammasome catalyzed by PELO. Therefore, flagellin, NAIP5, NLRC4 and PELO proteins should be ready before you begin.

Preparation of *E. coli* carrying plasmid expressing PELO or flagellin

Timing: 1+ days

Mammalian genes might possess codons that are infrequently employed in bacteria such as *E. coli*, which may hinder protein expression. Thus, we recommend using bacterial strains which encode rare codon tRNA genes, for example, Rosetta (DE3).

Alternatives: We have obtained good yields by using regular Luria Broth (LB) media for the expression cultures, but the Terrific Broth (TB) media can also be used.

1. Introduce into *E. coli* (strain: Rosetta (DE3)) the pET28a plasmid encoding mouse PELO or pET15b LFn-FlaA plasmid (Addgene plasmid #84871) encoding flagellin.
   a. Take bacterial competent cells out of −80°C and thaw on ice.
   b. Add 50 ng plasmid into 50 μL competent cells in a microcentrifuge and mix gently by pipetting up and down.
   c. Place the competent cells/DNA mixture on ice for 30 min.
   d. Heat shock the transformation tube at 42°C water bath for 45 s.
   e. Put the tubes back on ice for 2 min.
   f. Plate the transformed cell suspension onto an LB agar plate containing the appropriate antibiotic.
2. Streak *E. coli* on a LB agar plate with sterile operation and incubate at 37°C for 12–16 h.
3. Next day, take out the LB plate from the incubator. Store it at 4°C to avoid *E. coli* overgrowth. The plate could be stored for up to 1 month.

Preparation of HEK293T cells expressing HA-NLRC4 and FLAG-NAIP5

Timing: 2 days

As PELO is a catalyzer for NLR oligomerization and PELO is highly conserved between human and mouse,[1] *PELO* knock-out (KO) HEK293T cells are used in this step to exclude the potential interference from the endogenous PELO. This cell line is generated by using the standard CRISPR-Cas9 method as described.[1]

4. Seed and culture *PELO* KO HEK293T cells in complete medium in 6-well tissue-culture plates at 1×10^6 cells per well.
5. Transfect cells the next day with plasmids encoding HA-NLRC4 (2 μg) and FLAG-NAIP5 (2 μg) in combination by using the regular calcium phosphate transfection method.
   a. Mix NLRC4 and NAIP5 plasmids (2 μg each) and 100 μL of 0.25 M $CaCl_2$ in a sterile 1.5 mL microtube.
   b. Add 100 μL 2×HBS solution dropwise to the DNA/$CaCl_2$ mixture with continuous mixing.
   c. Incubate the resulting solution at 25°C for 5 min and then add dropwise to the cells.

Note: The plasmids encoding NLRC4 and NAIP5 are in pBOBI backbone with a CMV promoter. Plasmids encoding NLRC4 and NAIP5 with other regular tags also can be used, but we have achieved good results by using the HA and FLAG tag in the subsequent western-blot. Plasmids are prepared by using the EndoFree Plasmid Maxi Kit from QIAGEN.

6. Change culture medium 12 h post-transfection.
7. Collect cells 36 h after transfection.
   a. Discard the culture supernatants and wash cells once with 1 mL cold PBS.
   b. Use 1 mL PBS to transfer cells from the dish to a 1.5 mL tube and pellet at 4°C, 500 × *g*, for 5 min.

Pause point: The cell pellets can be kept at −80°C for several weeks to months before proceeding with lysis buffer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-PELO (Dilution: 1/1,000)	Proteintech	Cat# 10582-1-AP
Mouse anti DYKDDDDK-Tag (3B9) (Dilution: 1/2,000)	Abmart	Cat# M20008M
Rabbit anti HA-Tag (Dilution: 1/2,000)	Affinity	Cat# T0050
Bacterial and virus strains		
Rosetta (DE3) chemically competent *E. coli*	Merck	Cat# 71397
Chemicals, peptides, and recombinant proteins		
Tris	Sangon Biotech	Cat# A600194-0500
Bis-Tris	Sangon Biotech	Cat# A610079-0100
Tricine	Sangon Biotech	Cat# A600546-0500
Bicine	Acmec	Cat# N86990-500
EDTA	Sangon Biotech	Cat# A500895-0500
ATP	Sigma	Cat# A2383
SDS	Sangon Biotech	Cat# A600485-0500
Glycerol	Sangon Biotech	Cat# A501745-0500
Triton X-100	Sangon Biotech	Cat# A110694-0500
NP-40	Sangon Biotech	Cat# A100109-0100
Tween 20	Sangon Biotech	Cat# A600560-0500
Kanamycin	Lablead	Cat# K9316-100G
Ampicillin	Sangon Biotech	Cat# A610028-0025
IPTG	Sangon Biotech	Cat# A600168-0025
2-Mercaptoethanol	Amresco	Cat# 0482-250ML
Imidazole	Sangon Biotech	Cat# A600277-0500
Ponceau S	Sangon Biotech	Cat# A100860-0025
Coomassie brilliant blue G-250	Sangon Biotech	Cat# A600038-0025
Digitonin	Sigma	Cat# D141
Protease Inhibitor Cocktail	MedChemExpress	Cat# HY-K0010
NativeMark Unstained Protein Standard	Invitrogen	Cat# LC0725
180 kDa Prestained protein marker	Vazyme	Cat# MP102-02
Lipofectamine 2000	Invitrogen	Cat# 11668-019
Critical commercial assays		
BCA Protein Assay Kit	Pierce	Cat# 23225
3%–12% NativePAGE	Thermo	Cat# BN1003BOX
Experimental models: Cell lines		
Human: *PELO* KO HEK293T	Wu et al.[1]	N/A
Recombinant DNA		
pET28a-PELO	Wu et al.[1]	N/A
pET15b-LFn-Fla	Addgene	Cat# 84871
pBOBI-HA-NLRC4	Wu et al.[1]	N/A
pBOBI-FLAG-NAIP5	Wu et al.[1]	N/A
Software and algorithms		
ImageJ	NIH	https://imagej.nih.gov/ij/index.html
BioRender	BioRender	https://biorender.com
Equipment		
Ultrasonic homogenizer	Scientz	Scientz-IID
FPLC system	GE HealthCare	AKTA Purifier 10
Other		
Ni-NTA Sepharose resin	GE HealthCare	Cat# 17057502
Superdex G200 column	GE HealthCare	Cat# 17517501
Amicon Ultra Centrifugal filter units, (10 kDa)	Merck	UFC801024
PVDF membrane	Merck	IPVH00010
Endofree Plasmid Maxi Kit	Qiagen	Cat# 12362

MATERIALS AND EQUIPMENT

LB liquid media		
Reagent	Amount	Final concentration
Yeast extract	5.0 g	0.5%
Tryptone	10.0 g	1%
NaCl	10.0 g	1%
ddH_2O	1,000 mL	N/A
Total	**1,000 mL**	**N/A**

Dissolve components in water, then autoclave for 15 min at 121°C. Store at 25°C for months. Add antibiotics into the media before use.

1 M IPTG

Dissolve 2.38 g IPTG into 10 mL ddH_2O, then sterilize using 0.22 μm syringe filter. Store at −20°C for more than 3 months.

LB agar plates with ampicillin or kanamycin		
Reagent	Amount	Final concentration
Yeast extract	5.0 g	0.5%
Tryptone	10.0 g	1%
NaCl	10.0 g	1%
Agar	15.0 g	1.5%
Kanamycin (30 mg/mL)	1 mL	30 μg/mL
Ampicillin (100 mg/mL)	1 mL	100 μg/mL
ddH_2O	Up to 1,000 mL	N/A
Total	**1,000 mL**	**N/A**

Dissolve components in water and sterilize the mixture through autoclaving for 15 min at 121°C. When the media has cooled down below 56°C, add antibiotics before putting them into plates. Stored at 4°C for up to 1 month.

Protein lysis buffer		
Reagent	Amount	Final concentration
Tris-HCl (1 M, pH 7.4)	50 mL	50 mM
NaCl (5 M)	30 mL	150 mM
Imidazole (2 M, pH 8.0)	10 mL	20 mM
Triton X-100	10 mL	1%
2-Mercaptoethanol	1 mL	0.1%
ddH_2O	Up to 1,000 mL	N/A
Total	**1,000 mL**	**N/A**

Store the buffer at 4°C for up to 1 month. Add 2-Mercaptoethanol to the buffer right before use.

Protein wash buffer		
Reagent	Amount	Final concentration
Tris-HCl (1 M, pH 7.4)	50 mL	50 mM
NaCl (5 M)	60 mL	300 mM
Imidazole (2 M, pH 8.0)	10 mL	20 mM
ddH_2O	Up to 1,000 mL	N/A
Total	**1,000 mL**	**N/A**

Store at 4°C for up to 1 month.

Protein elution buffer		
Reagent	Amount	Final concentration
Tris-HCl (1 M, pH 7.4)	1 mL	50 mM
NaCl (5 M)	1.5 mL	150 mM
Imidazole (2 M, pH 8.0)	10 mL	400 mM
ddH_2O	Up to 50 mL	N/A
Total	**50 mL**	**N/A**
Store at 4°C for up to 1 month.		

Protein storage buffer		
Reagent	Amount	Final concentration
Tris-HCl (1 M, pH 7.4)	2.5 mL	50 mM
NaCl (5 M)	1.5 mL	150 mM
Glycerol	5 mL	10%
ddH_2O	Up to 50 mL	N/A
Total	**50 mL**	**N/A**
Store at 4°C for up to 1 month.		

5 × Native lysis buffer		
Reagent	Amount	Final concentration
Bis-Tris (1 M, pH 7.2)	2.5 mL	250 mM
NaCl (5 M)	0.5 mL	250 mM
Glycerol	5 mL	50%
1% Ponceau S	50 µL	0.005%
5% Digitonin	2 mL	1%
ddH_2O	Up to 10 mL	N/A
Total	**10 mL**	**N/A**
Store at 4°C for several months. Dilute 1:5 with ddH_2O before use. Add protease inhibitor cocktail and 1% digitonin to the buffer right before use.		

20 × Native running buffer (pH 6.8)		
Reagent	Amount	Final concentration
Bis-Tris	209.2 g	1 M
Tricine	179.2 g	1 M
ddH_2O	Up to 1,000 mL	N/A
Total	**1,000 mL**	**N/A**
Store at 25°C for several months. Dilute 1:20 with ddH_2O before use.		

20 × Native transfer buffer (pH 7.2)		
Reagent	Amount	Final concentration
Bis-Tris	104.8 g	500 mM
Bicine	81.6 g	500 mM
EDTA	6 g	20 mM
ddH_2O	Up to 1,000 mL	N/A
Total	**1,000 mL**	**N/A**
Store at 4°C for several months. Dilute 1:20 with ddH_2O before use.		

5% Coomassie G-250 additive:

Dissolve 2.5 g of G-250 in 50 mL ddH_2O. Store at 25°C for several months.

5% Digitonin:

Add 0.05 g of digitonin into 1 mL ddH_2O. Heat to 95°C for 5 min and vortex slowly to dissolve. Cool the solution to 25°C prior to use. After cooling, the solution should remain practically clear for at least 24 h. Store at −20°C for several weeks. Next time before use, you may reheat the solution to get clear solution.

10% SDS buffer:

Dissolve 50 g of SDS in 500 mL ddH_2O. Store the buffer at 25°C for more than 6 months.

1× Dark-blue cathode buffer		
Reagent	Amount	Final concentration
20×Native running buffer (pH 6.8)	10 mL	1×
5% Coomassie G-250 additive	800 μL	0.02%
ddH_2O	Up to 200 mL	N/A
Total	**200 mL**	**N/A**
Prepare prior to use.		

1× Light-blue cathode buffer		
Reagent	Amount	Final concentration
20×Native running buffer (pH 6.8)	10 mL	1×
5% Coomassie G-250 additive	80 μL	0.002%
ddH_2O	Up to 200 mL	N/A
Total	**200 mL**	**N/A**
Prepare prior to use.		

10× SDS running buffer		
Reagent	Amount	Final concentration
Glycine	720 g	1.9 M
Tris	150 g	250 mM
SDS	50 g	1%
ddH_2O	Up to 5 L	N/A
Total	**5 L**	**N/A**
Store at 25°C for more than 6 months. Dilute 1:10 with ddH_2O before use.		

10× SDS-PAGE transfer buffer		
Reagent	Amount	Final concentration
Glycine	720 g	1.9 M
Tris	150 g	250 mM

(*Continued on next page*)

Continued		
Reagent	Amount	Final concentration
ddH_2O	Up to 5 L	N/A
Total	**5 L**	**N/A**

Store at 25°C for several months. Dilute 1:10 with ddH_2O before use and add 10% methanol.

10 × TBST		
Reagent	Final concentration	Amount
Tris	200 mM	242 g
NaCl	1.37 M	800 g
Tween 20	1%	100 mL
ddH_2O	N/A	Up to 10 L
Total	**N/A**	**10 L**

Mix thoroughly and adjust the pH to 7.4. Store at 25°C for several months. Dilute 1:10 with ddH_2O before use.

10× PBS (pH 7.4)		
Reagent	Amount	Final concentration
NaCl	800 g	1.37 M
KCl	20 g	27 mM
KH_2PO_4	24 g	18 mM
$Na_2HPO_4 \cdot 7\ H_2O$	268 g	100 mM
ddH_2O	Up to 10 L	N/A
Total	**10 L**	**N/A**

Store at 25°C for several months. Dilute 1:10 with ddH_2O before use.

STEP-BY-STEP METHOD DETAILS

Expression of recombinant PELO protein

Timing: 2+ days

This section outlines procedures to express recombinant PELO protein in *E. coli*.

1. Prepare LB media containing 30 μg/mL kanamycin.
   a. Inoculate 5 mL of LB media in a 50 mL tube with a single colony by using a sterile pipette tip.
   b. Make sure the tube lid is attached loosely to ensure adequate air exchange of the culture.
2. Grow the pre-culture at 37°C for 8–12 h with shaking at 220 rpm.
3. Add 1 mL of the culture to 50 mL of LB media in a 250 mL conical flask.
4. Cultivate the culture at 37°C with continuous shaking at 220 rpm and regularly monitor OD600 of the culture.
5. Take the flask out of the shaking incubator and transfer it onto ice for 10 min once the culture attains 0.8–1.0 (typically within 3–5 h).

⚠ CRITICAL: The OD600 of culture should not exceed 2.0 before adding IPTG.

6. Add IPTG to the chilled culture (25 μL of 1 M stock for 50 mL culture, final concentration 0.5 mM).
7. Return the culture flask to the incubator and enable the expression to continue for 12–18 h at 25°C with shaking at 200 rpm.
8. Harvest bacteria.
   a. Collect cultures to a 50 mL tube and centrifuge at 6,000 × rpm for 5 min.

b. Remove the supernatant and wash the cell pellets with 10 mL 1× PBS.
c. Repeat the centrifugation step (6,000 × rpm for 5 min). Remove supernatants.

Pause point: Bacterial pellets can be frozen down and stored at −80°C for several months before proceeding with lysis.

Purification of recombinant PELO protein

Timing: 2 days

This step describes the details of how to purify recombinant PELO protein.

9. Use 10 mL of protein lysis buffer to resuspend bacterial pellet (~2 mL of lysis buffer is needed per 10 mL of expression culture).

⚠ CRITICAL: Perform all steps below at 4°C and with chilled reagents.

10. Sonicate to lyse the bacteria on ice by using Ultrasonic Homogenizer (Scientz-IID, Scientz) (35% intensity, 3 s pulses on/off, for 5 min).
11. Remove the cell debris by centrifugation (30,000 × *g*, 30 min, 4°C).
12. Transfer the clarified supernatants to a 15 mL tube.
13. Transfer 0.5 mL of Ni-NTA agarose beads (~ 1.0 mL of slurry) into a 15 mL tube.
14. Wash beads using 10 mL of lysis buffer three times.
15. Introduce the Ni-NTA beads into the clarified lysate and incubate at 4°C for 2 h with gentle rotary agitation. The Ni-NTA agarose beads will capture the His-tagged target protein.
16. Collect the beads (4°C, 1,000 × *g*, 3 min) and discard the supernatant. Wash beads with 10 mL of protein wash buffer three times.
17. Elute the bound proteins from the beads by incubation with 0.5 mL elution buffer for 10 min. Repeat this step three times.
18. Collect eluates and further purify the protein by standard size-exclusion chromatography with Superdex 200 10/300 GL prepacked filtration column.
    a. Flush the column with 1.5 column volumes of ddH2O to remove the storage solution. Set flow rate to 0.5 mL/min. For Superdex 200 10/300 GL, the column volume is 24 mL and the maximum pressure limit is 1.5 MPa.
    b. Equilibrate the column with 1.5 column volumes of protein wash buffer at 0.5 mL/min.
    c. Set the 1.0 mL sample loop state to "Load" in the FPLC system. Inject protein wash buffer, whose volume is equivalent to twice of the sample loop, into the sample loop. Repeat the injection twice.
    d. Centrifuge the protein elute at 10,000 g and 4°C for 10 min. Inject the protein solution into the sample loop.
    e. Set sample loop state to "Inject" in the FPLC system. Elute with 1 column volume of protein wash buffer and collect the fractions with the expected volume. We collected the eluates ranging from 7.85 to 23.85 mL at 1 mL per fraction.
19. Run SDS-PAGE and stain gel using Coomassie blue R-250 to identify fractions that contain target protein. The targeted protein is mainly eluted between 13.85 to 15.85 mL (Fraction 7&8).
20. Pool peak fractions containing target protein.
21. Use Amicon Ultra centrifugal filter unit with a 10 kDa size cutoff to concentrate pooled fractions (4°C, 5,000 × *g*, several 5 min spins).
22. Determine the protein concentration by using the BCA method.

Note: In case of low protein concentration (< 0.2 mg/mL), you can return to step 21 to further concentrate the protein solution.

23. Aliquot concentrated protein solution into PCR tubes (5 μL or 2 μg aliquot). Snap freeze in liquid nitrogen and store the solution at −80°C.

Pause point: The purified recombinant PELO protein can be kept at −80°C for several months.

Expression and purification of recombinant flagellin protein

Timing: 2 days

The role of purified recombinant flagellin protein as a ligand is indispensable for the *in vitro* NAIP5-NLRC4 inflammasome reconstitution; in this specific protocol, recombinant *L. pneumophila* flagellin fused to the *B. anthracis* lethal factor (LF) can be produced and purified following the same procedures outlined above for recombinant PELO protein.

Preparation of native cell lysate containing NAIP5-NLRC4

Timing: 2 h

This step elaborates on the details of preparing NAIP5-NLRC4 containing native cell lysate.

24. Resuspend NAIP5-NLRC4 expressing HEK293T cell pellet in 200 μL 1× Native lysis buffer containing 1% digitonin and fresh protease inhibitor cocktail.

⚠ CRITICAL: Cool all the reagents before use and all the following steps should be performed on ice.

⚠ CRITICAL: The volume of native lysis buffer required to lyse the cells may vary depending on different NLRs expression levels. In our case, the amount of NLRC4 protein in the whole cell lysate from one well of HEK293T cells is about 1 μg. High concentration (> 10 μg/mL) of NLRC4 in the whole cell lysate will lead to ligand-independent formation of NLRC4 self-aggregate.

Note: Detergents used in native lysis buffer should be determined empirically. Digitonin (0.5–1.0%) works well, but other regular detergent also can be used: Triton X-100 (0.1%–0.5%), dodecylmaltoside (0.1%–0.5%), Brij 96 (0.1%–0.5%).[5]

25. Use 200 μL pipette to triturate per sample 10 times. Repeat 3 times over 30 min at 4°C.
26. Centrifuge samples at 20,000 × *g* for 30 min at 4°C.
27. Collect the clarified supernatants to fresh cold 1.5 mL tubes with caution and discard the cell pellet.
28. Determine the total protein concentration per sample by using BCA method and adjust the samples with 1× Native lysis buffer to equalize the total protein between samples.

⚠ CRITICAL: Whenever feasible, utilize native lysates on the same day when they are collected to prevent freeze and thaw cycles.

Perform *in vitro* assembly of NAIP5-NLRC4 inflammasome

Timing: 2 h

Here we provide procedures for conducting an *in vitro* assembly of flagellin-dependent NAIP5-NLRC4 inflammasome.

29. Remove one aliquot of recombinant PELO and flagellin protein from the freezer and allow it to thaw on ice.

⚠ CRITICAL: Avoid freeze and thaw cycles of the recombinant protein to maintain its activity. To ensure reliable outcomes, use a new portion of the protein for each experiment.

Note: Ensure the protein activity remains during the process of assay preparation by keeping protein solutions and buffers on ice.

30. Prepare a water bath in advance to a steady temperature of 37°C.
31. Mix 10 μL of NAIP5-NLRC4 lysates with ATP (0.2 mM) + Mg^{2+} (2 mM), 200 ng flagellin, 100 ng PELO in various combinations in a total volume of 20 μL.

Note: We usually prepare 10×solution: ATP (2 mM)+Mg^{2+} (20 mM), aliquot and store it at −20°C. Take out and thaw it before use.

32. Incubate the mixture at 37°C water bath for 1 h.

Note: The incubation period may vary depending on the concentration of the NLRs in the reaction mixture.

33. Centrifuge samples at 4°C (20,000 × *g*, for 10 min) to eliminate potential protein aggregates.
34. Transfer the supernatant carefully to pre-chilled PCR tubes on ice.

⚠ CRITICAL: Native samples should be run the day they are collected and freeze–thaw of samples should be avoided.

Analysis of the NAIP5-NLRC4 inflammasome by blue-native PAGE and immunoblotting

Timing: 2 days

Blue native polyacrylamide gel electrophoresis (BN-PAGE) is a powerful method to separate intact protein complexes by non-denaturing polyacrylamide gel electrophoresis.[5,6] The protein mixtures can be separated based on the molecular size of polyacrylamide gradient gels by using the dye Coomassie blue G-250, which imparts a net negative charge to proteins and at the same time preserves native protein conformation and interactions.[7] Previous reports and our results have successfully used BN-PAGE to assess the NAIP-NLRC4 inflammasome complex in stimulated cells.[1,4]

This step describes how to perform BN-PAGE to analyze the assembled NAIP5-NLRC4 inflammasome *in vitro*.

35. Mix 1.5 μL of 2.5% Coomassie blue G-250 sample additive with 13.5 μL of the final reaction mixture from step 34 to prepare samples for Native PAGE.

Note: The concentration of G-250 should be 1/4th the detergent concentration. For example, if the digitonin concentration is 1% in the samples, the final concentration of G-250 required is 0.25%.

36. Remove the white tape near the bottom of the 3%–12% NativePAGE minigel cassettes and carefully extract the comb to avoid any harm to the lane dividers. Flush gel wells twice with 1× Native running buffer before loading samples.

⚠ CRITICAL: Invitrogen offers NativePAGE minigels as precast gels with gradients of 3%–12% and 4%–16%. Choose the proper gels for analysis of the complex of interest. For example, the 4%–16% gels cannot achieve efficient separation of ~1,200 kDa complexes such as NAIP5-NLRC4 inflammasome. In contrast, the 3%–12% gels allow for separation of protein complexes in such a size. So, in this protocol, 3%–12% gel should be used to analyze NAIP5-NLRC4 inflammasome.

37. Put the gels in the gel running tank, and load 1× Native dark-blue cathode buffer into the gel wells.
38. Load 10 μL of prepared samples into each well and load 5 μL of NativeMark protein standard as marker. Denature the reminding samples with SDS sample buffer and run SDS-PAGE for control.

Note: To facilitate the observation of the sample wells, load samples into sample wells before filling the cathode chamber. Invitrogen NativePAGE minigels are offered as precast in 10-well and 15-well format and 1.0 mm thickness. The loading volume should not exceed 15 μL for 15-well gels.

39. Fill the inner buffer chamber with ~200 mL 1× Native dark-blue cathode buffer, and fill the outer chamber with ~500 mL 1× Native running buffer.
40. Run the gel at 150 V for 20 min. The initial current should be ~ 18 mA per gel.
41. Pause the run and carefully replace the dark-blue cathode buffer with 1× light-blue cathode buffer.

⚠ CRITICAL: For western blotting applications, as excessive dye will block the PVDF membrane during protein transfer, the cathode buffer should be changed from "dark" to "light" after the dye front has migrated about 1/3 of the way through the gel to flush excess dye from the gels.

42. Continue the run at 150 V for another 90 min. The current should end at ~2 mA per gel.
43. Disassemble the gel apparatus. Use metal spatula to remove the plastic casing from the gel. Carefully trim the gel ~1 mm below the well bottom and cut off the gel foot.

Note: The marker lane can be cut off from the gel and visualized by Coomassie blue staining. Alternatively, the 1,246 and 1,038 kDa markers can be observed through background reaction with secondary antibody during exposure.

44. Transfer the trimmed gel to a plastic tray. Equilibrate the gel in 10% SDS solution with shaking for 10 min, and then wash with Native transfer buffer twice (1 min each).

⚠ CRITICAL: To avoid tearing the gel, ensure to lift it from the 12% foot, not the 3% wells.

Note: Protein complexes can be completely denatured by SDS prior to transfer, releasing all available epitopes for immunodetection by antibody.

45. Transfer proteins to PVDF membrane using standard wet transfer protocol.
    a. Activate the PVDF membranes with 100% methanol for 1 min and rinse with transfer buffer.
    b. Disassembly the gel and construct the gel "sandwich" in the transfer cassette.
    c. Clamp tightly together to avoid air bubbles between any of the layers.
    d. Load a cassette into the wet/tank transfer apparatus.
    e. Transfer at 100 V for 1 h at 4°C.
46. Rinse the membrane with pure methanol 3 times (5 min each) to destain the Coomassie blue G-250, then wash the membrane with TBST buffer three times, 5 min each.

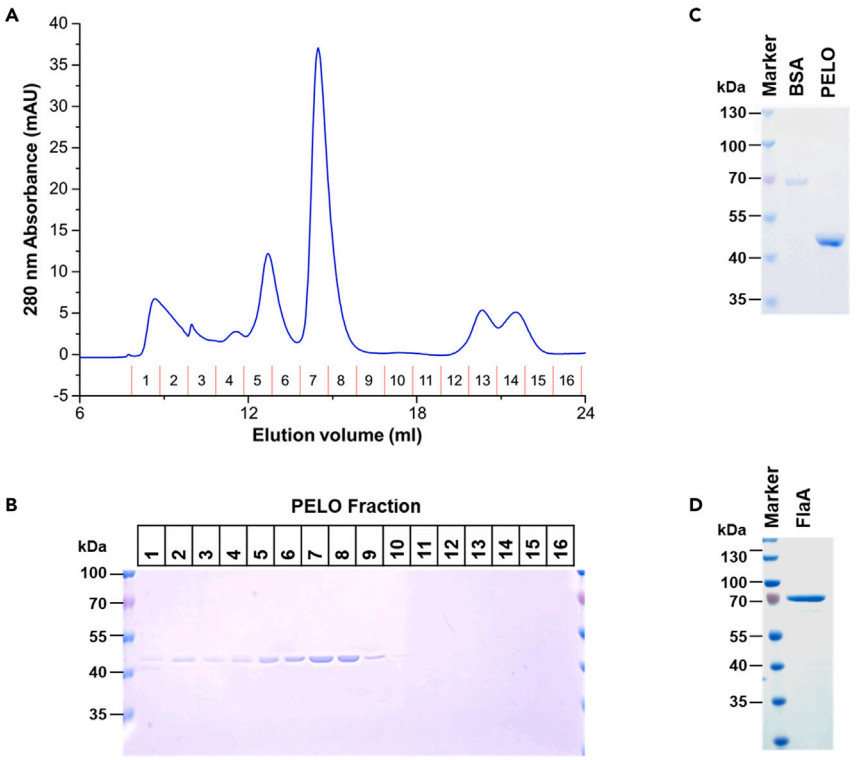

Figure 1. Expression and purification of recombinant PELO and flagellin protein from bacteria

(A) Example of a size-exclusion chromatography purification of the protein. The blue line indicated the 280 nm absorbance of the eluate. The annotated numbers separated by red lines are corresponding to the collected fractions.

(B) SDS-PAGE analysis of the annotated fractions in (A).

(C and D) Recombinant PELO (C) and LFn-flagellin (FlaA) (D) proteins were separated by SDS-PAGE and analyzed by Coomassie staining. BSA = bovine serum albumin.

⚠ CRITICAL: Destaining is very important since Coomassie blue G-250 might interfere with antibody recognition in the following western-blot.

47. To block the PVDF membrane, incubate it in TBST + 5% BSA for 1 h at RT while gently shaking, followed by standard western-blot protocol. We probe the membrane with anti-HA antibody at RT for 3 h to detect the monomeric and oligomeric NLRC4.

Alternatives: TBST + 5% milk is also suitable for blocking.

EXPECTED OUTCOMES

With this protocol, we typically obtain a yield of 0.5–2 mg of pure, recombinant mouse PELO protein (Figures 1A–1C), and 0.2–1 mg of recombinant flagellin protein from a 50 mL expression culture (Figure 1B).

For the *in vitro* experiments, we used cell extracts derived from *PELO* KO HEK293T cells co-expressing HA-NLRC4 and FLAG-NAIP5 as the source of NLRC4 and NAIP5 monomer protein. We mixed and incubated HA-NLRC4 and FLAG-NAIP5 with or without recombinant PELO, along with recombinant flagellin and ATP/$Mg2^{+}$. In the absence of PELO, we did not detect the NAIP5-NLRC4 oligomer using BN-PAGE (Figure 2A). In the presence of PELO, we observed efficient formation of a complex (~1200 kDa) with a similar molecular weight to the NAIP5-NLRC4 complexes from cells

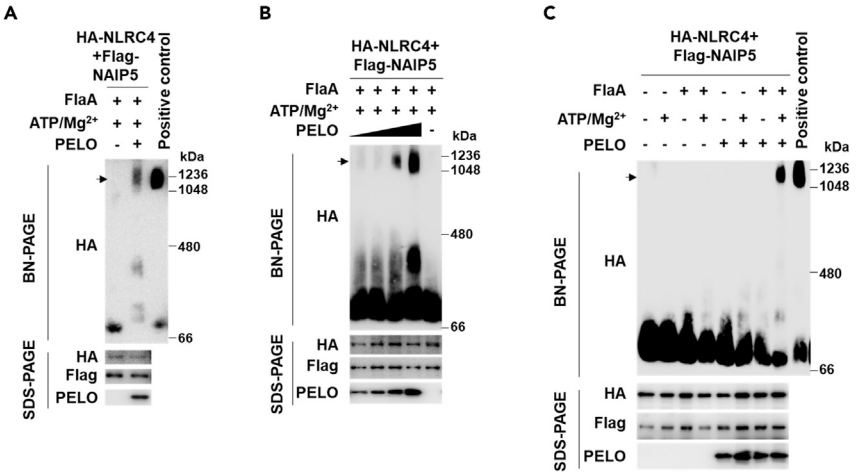

Figure 2. *In vitro* NLRC4 inflammasome complex reconstitution

(A) We incubated cell lysates containing HA-NLRC4 and FLAG-NAIP5 in the presence of purified recombinant LFn-flagellin (FlaA) and ATP/Mg^{2+} with or without recombinant PELO. Then, we separated the mixtures using BN-PAGE or SDS-PAGE and analyzed the mixtures by immunoblotting as indicated.

(B) We incubated HA-NLRC4 and FLAG-NAIP5 containing lysates with increasing doses of recombinant PELO while also adding into the mixture purified recombinant LFn-flagellin (FlaA) and ATP/Mg^{2+}. After incubation, the mixtures were separated by BN-PAGE or SDS-PAGE and analyzed by immunoblotting as indicated.

(C) HA-NLRC4 and FLAG-NAIP5 containing lysates were incubated with LFn-flagellin (FlaA), ATP/Mg^{2+}, and PELO in various combinations as indicated. After incubation, we separated the mixtures using BN-PAGE or SDS-PAGE and analyzed the mixtures by immunoblotting as indicated. The arrow indicates the NLRC4 inflammasome.

stimulated with flagellin (Figure 2A). As reported,[4] the HEK293T cells expressed with NAIP5-NLRC4 were treated with flagellin to induce the formation of NAIP5-NLRC4 inflammasome, and the sample was used as positive control. Moreover, the NLRC4 inflammasome formation *in vitro* was dependent on the concentration of PELO (Figure 2B).

The withdrawal of ATP in the *in vitro* reconstitution assay abolishes the formation of NLRC4 oligomer (Figure 2C), which agrees with the reported essential role of ATPase activity for NLRC4 oligomerization.[4] Furthermore, the assembly of NAIP5-NLRC4 inflammasome relies on the ligand stimulation, as PELO could not drive the formation of NLRC4 inflammasome without flagellin (Figure 2C).

In a previous publication, the current protocol enabled us to confirm the necessity of PELO in initiating the oligomeric assembly of NLRC4 inflammasome. Using this approach, we could analyze the role of ligand, ATP and ATPase activity of NLRC4 in complex assembly, shedding light on the molecular determinants of oligomeric assembly of NLRC4 inflammasome.[1] Other than inflammasome, it is also possible to adjust this protocol to analyze other oligomeric protein complexes, such as apoptosome.

LIMITATIONS

The *in vitro* reconstitution of NLRC4 inflammasome assay is a straightforward and simple process. However, it only allows for the formation of oligomer inflammasome complexes. No information about the exact activity of the assembled inflammasome can be determined, as it requires more laborious measurements of proteolysis of its downstream effector, caspase-1.

The self-oligomerization of NLR proteins following engagement with a ligand is widely recognized as a prevailing model for their activation,[3] but we cannot ensure that our *in vitro* reconstitution protocol will work well for all other NLR oligomeric complexes of interest.

TROUBLESHOOTING

Problem 1

Recombinant protein yield is low (step 22 of "step-by-step method details" section).

Potential solution

Optimize expression conditions. Make sure the OD600 of culture does not exceed 2.0 before adding IPTG. Make sure the cells are sufficiently lysed to release target protein; extend the sonication time and/or extend the binding time of Ni-NTA beads to overnight. Make sure most of the proteins have been successfully eluted from the beads.

Problem 2

Gels break during handling (step 44 of "step-by-step method details" section).

Potential solution

The Native PAGE gels used to analyze NLRC4 inflammasome are the 3%–12% NativePAGE minigel cassettes, which contain a low acrylamide percentage that makes the gels more fragile. Handle the gels with caution by only touching the bottom.

Problem 3

Weak or no western blotting signal (steps 41, 44 and 46 of "step-by-step method details" section).

Potential solution

Given that Coomassie dye can impair the protein binding capacity during protein transfer as it can bind to PVDF membranes, after the dye front has migrated about 1/3 of the way through the gel (usually 20 min later), make sure to replace the blue cathode buffer from "dark" to "light". Another problem that may arise is the nonspecific signal caused by the Coomassie dye. The antibody binding might also be interfered by the high local concentration of dye bound to the target protein. So, make sure the Coomassie blue G-250 has been destained from the membrane.

The success of BN-PAGE depends on recognition of the native conformation of the protein of interest by the immunoblotting antibody. Therefore, different antibodies should be tested. Make sure to denature the protein in the gel with 10% SDS prior to transfer.

Avoid including sodium azide in the secondary antibody solution since it can hamper HRP development. Use more concentrated primary antibodies and incubate longer to overnight.

Problem 4

Weak formation of NLRC4 inflammasome (steps 29 and 32 of "step-by-step method details" section).

Potential solution

Check the expression level of NAIP5-NLRC4 in HEK293T cells by SDS-PAGE. Make sure the native samples have not been heated in the whole process. Alternatively, run the Native PAGE in a cold room and with a lower voltage constant to avoid dissociation of oligomeric-protein complexes during running.

Problem 5

Formation of ligand-independent NLRC4 oligomer (step 24 of "step-by-step method details" section).

Potential solution

NLRC4 protein has high tendency to self-aggregate, especially when its concentration is high in the solution.[8] To monitor inflammasome assembly *in vitro*, it will probably require titrating expression

levels of NAIP5-NLRC4 empirically, to avoid ligand-independent inflammasome assembly. We usually titrate expression level by changing the amount of transfected DNA.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jiahuai Han (jhan@xmu.edu.cn).

Materials availability

Requests for resources and reagents should be directed to and will be fulfilled by the lead contact.

Data and code availability

This study did not generate any datasets or code.

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AUTHOR CONTRIBUTIONS

X.W. conceived the study, performed experiments, and wrote the manuscript. J.H. conceived the study, supervised the work, and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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