# Chapter 8

# Measuring NLR Oligomerization I: Size Exclusion Chromatography, Co-immunoprecipitation, and Cross-Linking

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## Abstract

Oligomerization of nod-like receptors (NLRs) can be detected by several biochemical techniques dependent on the stringency of protein–protein interactions. Some of these biochemical methods can be combined with functional assays, such as caspase-1 activity assay. Size exclusion chromatography (SEC) allows separation of native protein lysates into different sized complexes by fast protein liquid chromatography (FPLC) for follow-up analysis. Using co-immunoprecipitation (co-IP), combined with SEC or on its own, enables subsequent antibody-based purification of NLR complexes and associated proteins, which can then be analyzed by immunoblot and/or subjected to functional caspase-1 activity assay. Chemical cross-linking covalently joins two or more molecules, thus capturing the oligomeric state with high sensitivity and stability. Apoptosis-associated speck-like protein containing a caspase activation domain (ASC) oligomerization has been successfully used as readout for NLR or AIM2-like receptor (ALR) inflammasome activation in response to various pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) in human and mouse macrophages and THP-1 cells. Here, we provide a detailed description of the methods used for NLRP7 oligomerization in response to infection with *Staphylococcus aureus* (*S. aureus*) in primary human macrophages, co-immunoprecipitation and immunoblot analysis of NLRP7 and NLRP3 inflammasome complexes, as well as caspase-1 activity assays. Also, ASC oligomerization is shown in response to dsDNA, LPS/ATP, and LPS/nigericin in mouse bone marrow-derived macrophages (BMDMs) and/or THP-1 cells or human primary macrophages.

**Key words** Cross-linking, Size exclusion chromatography, Co-immunoprecipitation, Protein–protein interaction, Oligomerization, Caspase-1 activity assay, Nod-like receptor, NLR, Inflammasome

## 1 Introduction

Nod-like receptors (NLRs) assemble into large oligomeric signaling complexes, such as inflammasomes and nodosomes [1, 2]. Several biochemical methods, which are based on the detection of protein–protein interactions, are established to detect and quantify the conversion of monomeric proteins into active oligomeric protein complexes [3–5].

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Size exclusion chromatography (SEC) is based on the separation of native protein complexes according to their size by migration through a gel matrix, which consists of spherical beads containing pores of a specific size distribution [6]. When molecules of different sizes are included or excluded within the matrix, it results in separation of these molecules depending on their overall sizes. Small molecules diffuse into pores and are retarded depending on their size, whereas large molecules, which do not enter the pores, are eluted with the void volume of the column. As the molecules pass through the column, they are separated on the basis of their size and are eluted in the order of decreasing molecular weights. Here we describe SEC of the oligomerized NLRP7 complex in response to *S. aureus* infection of human primary macrophages [7].

Immunoprecipitation (IP) and co-immunoprecipitation (co-IP) are routinely used techniques to study protein–protein interactions and to identify novel members of protein complexes [3, 8, 9]. Both techniques use an immobilized antibody specific to the antigen/protein of interest. While IP is designed to purify a single antigen, co-IP is suited to isolate the specific antigen/protein as well as to co-purify any other associated proteins, which are then separated by SDS/PAGE and detected by immunoblotting. Interacting proteins might include complex partners, cofactors, signaling molecules, etc. The strength of the interaction between proteins may range from highly transient to very stable interactions. While studying these interactions by co-IP, there are a number of factors which should be taken under consideration, e.g., specificity of the antibody, optimization of the binding and wash conditions, posttranslational modifications, etc. Here, we describe a co-IP protocol for the endogenous ASC–NLRP3 complex from THP-1 cells and BMDMs and the ASC–NLRP7 complex from human primary macrophages, as the recruitment of ASC to these NLRs is a readout for inflammasome assembly. A particularly useful approach is the combination of SEC with co-IP to allow the analysis of complexes within a specific size fraction, for example, for analyzing NLR-containing complexes within high molecular weight fractions.

This analysis further enables the detection of caspase-1 within inflammasomes and allows quantification of its activity, when combined with caspase-1 activity assays. Caspase-1, also known as interleukin (IL)-1β-converting enzyme (ICE), is a cysteine protease and is the downstream effector molecule that becomes activated within inflammasomes subsequent to the activation of several NLRs [10]. The active 20 kDa and 10 kDa hetero-tetrameric caspase-1 is derived from the auto-proteolytically cleaved 45 kDa proenzyme (zymogen) [11, 12]. Subsequently, the caspase-1 substrate pro-IL-1β (35 kDa) is converted into the biologically active form (17 kDa) [13–15]. Here we describe two assays that determine

caspase-1 activity, which are routinely used in our laboratory [7]. First, a sensitive fluorometric assay that quantifies caspase-1 activity within the NLRP7 inflammasome, where the preferential recognition of the tetrapetide sequence YVAD by caspase-1 is utilized in combination with the detection of the fluorescent substrate 7-amino-4-trifluoromethyl coumarin (AFC) [16]. YVAD-AFC emits blue light (400 nm), but once the substrate is cleaved by caspase-1, the free AFC emits yellow-green fluorescence (505 nm), which can be quantified in a plate reader with fluorescence capabilities and the appropriate filter sets. Second, the caspase-1 substrate pro-IL-1β is converted into mature IL-1β, which can be detected by Western blot analysis [7].

Chemical cross-linking covalently joins two or more molecules [17]. Cross-linking reagents (or cross-linkers) consist of two or more reactive ends. This enables cross-linkers to chemically attach to specific functional groups (e.g., sulfhydryls, primary amines, carboxyls, etc.) on proteins or other molecules. Cross-linker-mediated attachment between groups on two different protein molecules leads to intermolecular cross-linking. This cross-linking results in the stabilization of protein–protein interactions. Cross-linkers can be selected on the basis of their chemical reactivity and properties, like chemical specificity, water solubility, membrane permeability, etc. [17]. Here, we describe the cross-linking of nucleated and polymerized ASC molecules using the membrane-permeable, nonreversible cross-linker disuccinimidyl suberate (DSS), which contains an amine-reactive N-hydroxysuccinimide (NHS) ester at each end of an 8-carbon spacer arm (*see* **Note 1**). ASC oligomerization has been successfully used as a readout for NLR or ALR inflammasome activation in response to various PAMPs and DAMPs in primary macrophages as well as monocytes [18, 19]. We describe ASC oligomerization in response to poly(dA:dT) (dsDNA) as well as LPS/ATP or LPS/nigericin in mouse BMDM and THP-1 cells [20].

## 2 Materials

All solutions should be prepared using ultrapure water (sensitivity of 18 MΩ·cm at 25 °C) and analytical grade reagents.

### 2.1 Cell Culture and Antibodies

1. Appropriate culture medium for THP-1 cells, primary human macrophages, BMDMs, and HEK293 cells.
2. Lipofectamine 2000 (Invitrogen) or transfection reagent of choice.
3. Phosphate-buffered saline (PBS) solution.
4. 100/60 mm tissue culture dishes.
5. 1.5 and 2 ml microcentrifuge tubes.

6. 15 ml centrifuge tubes.
7. Cell scrappers.
8. Refrigerated tabletop centrifuge with 1.5 ml microcentrifuge tube rotor.
9. Tabletop centrifuge with 15 ml tube rotor.
10. Heat block.
11. 10× protease inhibitor cocktail (we use the cocktail from Roche, but other cocktails could be also used).
12. 0.1 M phenylmethylsulfonyl fluoride (PMSF) in ethanol.
13. Poly(dA:dT).
14. Ultrapure *E. coli* lipopolysaccharide (LPS), serotype 0111:B4.
15. ATP.
16. Nigericin.
17. Laemmli sample loading buffer: 60 mM Tris–HCl, pH 6.8, 2 % sodium dodecyl sulfate (SDS), 100 mM dithiothreitol (DTT), 10 % glycerol, and 0.01 % bromophenol blue.
18. SDS/PAGE and blotting equipment and materials.
19. Anti-ASC antibody (we use Santa Cruz, sc-22514-R).
20. Anti-NLRP3 antibody (we use Adipogen, Cryo-2, AG-20B-0014).
21. Anti-NLRP7 antibody (we use Imgenex, IMG-6357A).

### 2.2 Size Exclusion Chromatography (SEC)

1. Fast protein liquid chromatography (FPLC) equipment (we use a Bio-Rad BioLogic LP Chromatography System, but other systems are also suitable).
2. Gel filtration column. We use GE Healthcare HiPrep 16/60 Sephacryl S-300 High-Resolution Column (matrix, 50 μm allyl dextran and *N*,*N*′-methylenebisacrylamide; bed dimension, 16×600 mm; bed volume, 120 ml). Other similar columns are also suitable.
3. System tubing of 1.6 mm internal diameter.
4. Fraction collector.
5. 5 ml round bottom tubes.
6. Separation buffer: 50 mM Tris, pH 7.4, 150 mM NaCl.
7. Lysis buffer: 10 mM $Na_4P_2O_7$, 10 mM NaF, 20 mM Tris, pH 7.4, 150 mM NaCl, 1 % octylglucoside, 1 mM PMSF, and 1× protease inhibitor cocktail.
8. Trichloroacetic acid (TCA).
9. Acetone.
10. 20 % ethanol.
11. Gel filtration standard (we use Bio-Rad 151-1901).

12. Dounce homogenizer.
13. Sonicator.
14. 3 ml syringe using an 18½-gauge needle.
15. 0.45 μM syringe filter.

### 2.3 Co-immunoprecipitation (co-IP)

1. IP lysis buffer: 50 mM HEPES, pH 7.4, 150 mM NaCl, 10 % glycerol, 2 mM EDTA, 0.5 % Triton X-100, 1× protease inhibitor cocktail.
2. Specific antibody against NLR of interest (*see* Subheading 2.1).
3. Protein A/G-Sepharose (we use the Protein A/G-Sepharose® 4B from Life Technologies).

### 2.4 Fluorometric Caspase-1 Activation Assay

1. 10 mM Ac-YVAD-AFC. This is a stock solution and should be prepared in dimethyl sulfoxide (DMSO); protect solution from light.
2. Caspase-1 lysis buffer: 150 mM NaCl, 20 mM Tris, pH 7.5, 0.2 % Triton X-100; **add freshly**: 1 mM DTT.
3. Caspase assay buffer: 50 mM HEPES, 7.2–7.4, 50 mM NaCl, 1 mM EDTA, 0.1 % CHAPS, 10 % sucrose; **add freshly**: 10 mM DTT, 100 μM substrate Ac-YVAD-AFC.

### 2.5 In Vitro Caspase-1 Activation Assay

1. pro-IL-1β cDNA.
2. Anti-IL-1β antibody suitable for Western blotting (e.g., we use cell signaling antibody clone 3A6, 12242).

### 2.6 Cross-Linking

1. 100 mM disuccinimidyl suberate (DSS). This is a stock solution and should be prepared in DMSO (**prepare freshly just before adding to the lysates**).
2. Lysis buffer: 20 mM HEPES–KOH, pH 7.5, 150 mM KCL, 1 % NP-40, 0.1 mM PMSF, 1× of protease inhibitor cocktail, 1 mM sodium orthovanadate (PMSF should be added to the lysis buffer just before cell lysis).

## 3 Methods

### 3.1 Cell Culture and Activation

1. Culture THP-1 cells, primary human macrophages, or BMDM in the appropriate culture dish and with your established media.
2. To activate the AIM2 inflammasome, transfect cells with poly(dA:dT) using Lipofectamine 2000 (follow the manufacturer's protocol for the transfection) for 5 h (for cross-linking, *see* **Note 2**). To activate the NLRP3 inflammasome with LPS/ATP, treat cells with 100 ng/ml of LPS for 4 h followed by a pulse with 5 mM ATP for 20 min. or incubation with nigericin (5 μM)

for 45 min. Infect cells with *S. aureus* (MOI = 3) or *Listeria monocytogenes* (MOI = 12) at 37 °C for 45 min to activate multiple inflammasomes, including NLRP3, NLRP7, and AIM2 [7, 20]. Extracellular bacteria will be eliminated with gentamicin (50 mg/ml) for a total time of 90 min [7] (*see* **Note 3**).

### 3.2 Size Exclusion Chromatography (SEC)

1. Connect the column according to the manufacturer's instructions and verify proper bubble-free packing of the column.
2. Equilibrate the column with ½ column volume (CV) dd$H_2O$ (60 ml) at a flow rate of 0.5 ml/min, followed by 2 CV (240 ml) separation buffer at 1 ml/min at 4 °C.
3. Activate $5 \times 10^7$ cells in 60 mm culture dishes containing $5 \times 10^6$ macrophages per plate for 90 min (*see* **Note 4**).
4. Aspirate the culture medium and wash cells twice with ice-cold 1× PBS.
5. Lyse cells in 200 μl lysis buffer per plate, pool lysate from all plates into a 15 ml conical tube, and incubate for 20 min on ice.
6. Prior to homogenization, prechill a glass of 2 ml Dounce tissue grinder on ice, transfer lysate to the cooled tissue grinder, and homogenize through 30 strong strokes, carefully avoiding bubble formation.
7. Following homogenization, place the lysate in a 2 ml microcentrifuge tube and spin at 12,000 × *g* for 30 min to clarify the lysate.
8. Transfer the cleared lysates to a fresh tube and then draw it into a 3 ml syringe using an 18½-gauge needle. Perform a second clarification step to remove any remaining debris using a 0.45 μM syringe filter. Draw the final lysate in a new 3 ml syringe for injection into the chromatography system or store it at 4 °C until injection.
9. Carefully examine the lysate within the syringe for any air bubbles prior to injection, and if necessary remove it by gently flicking the syringe.
10. Insert the syringe into the injection valve in the valve controller and inject the lysate into a sample loop, which needs to fit the entire lysate (we usually use a 2.5 ml sample loop) prior to introducing it into the separation column. During injection, the flow of the chromatography system bypasses the sample loop. Adjust the flow rate to 0.5 ml/min, and use the valve selection device to divert the flow into the sample loop, which is subsequently connected to the separation column and the rest of the system. Take care to avoid any introduction of air bubbles into the system.
11. Start an automated collection program:

(a) 0.5 ml/min flow rate for 72 min, diverting flow to disposal (column dead volume).

(b) 0.5 ml/min flow rate for 216 min, diverting flow to fraction collector; collect 9 min/fraction (4.5 ml) for a total of 24 fractions.

(c) Divert flow to waste for another 200 min at 0.5 ml/min to remove any small molecules remaining in the column.

12. Continuously monitor fractions by UV absorbance.
13. Initiate any subsequent injections at this point, repeating **steps 10–12**.
14. At this step, either continue with co-IP (*see* Subheading 3.3 below) or continue for TCA precipitation and Western blot analysis.
15. Divide each fraction into three 1.5 ml aliquots in 2 ml microcentrifuge tubes.
16. TCA precipitates proteins by adding 500 µl 100 % w/v trichloroacetic acid per tube. Mix tubes and incubate on ice for at least 10 min and collect proteins by centrifugation at 14,000 × *g* for 30 min. White protein precipitates are present at the bottom of the tubes. Discard the supernatants and wash pellets with 0.5 ml of ice-cold acetone with vortexing and pool the contents of three tubes corresponding to the same fraction and spin at 14,000 × *g* for 10 min.
17. Aspirate the acetone supernatant and wash the pellets twice with 1 ml of ice-cold acetone as above.
18. Aspirate the acetone and keep the tubes uncapped at 30 °C in a heat block for 10 min to evaporate any remaining acetone (*see* **Note 5**).
19. Resuspend protein pellets in 50 µl 1.5× Laemmli buffer (*see* **Note 6**). Vigorously vortex the samples to fully resuspend any insoluble portion of the pellet. Sonicate samples in a water bath to ensure maximum dissolution of the protein pellet. Boil samples in a 95 °C heat block for 10 min before separating by SDS/PAGE (a 10 % acrylamide gels is usually well suited) and analysis by immunoblotting (*see* **Note 7**).
20. To match fractions to a particular molecular weight, either pre-run or post-run a gel filtration molecular weight standard under the same conditions. The protein standards can be detected by UV absorbance and matched to a particular fraction. We use a lyophilized protein mixture from Bio-Rad consisting of thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B12 (MW range from 1350 to 670,000 Da, pI 4.5–6.9), which we dissolve in lysis buffer.

21. Regenerate the column after each run with one CV (120 ml) separation buffer (at 1 ml/min at 4 °C) and four CV (480 ml) $ddH_2O$, followed by four CV (480 ml) 20 % ethanol, and store in 20 % ethanol at 4 °C in an upright position.

### 3.3 Co-immunoprecipitation (co-IP)

1. Plate $1 \times 10^7$ cells in a 100 mm tissue culture dish (*see* **Note 8**).
2. Wash the adherent cells (primary human macrophages and BMDM) with ice-cold PBS. Add 1 ml of ice-cold lysis buffer. Keep the plate on ice and make sure the lysis buffer is distributed evenly. In case of THP-1 cells (suspension cells), transfer the cells to a 15 ml tube and centrifuge at $400 \times g$ for 10 min at RT. Add 1 ml of ice-cold lysis buffer and transfer the cell suspension to a 1.5 ml tube. Incubate the cells in lysis buffer for 30 min on ice.
3. Centrifuge the cell lysates at $10{,}000 \times g$ for 15 min at 4 °C.
4. Transfer the cleared lysates to a fresh 1.5 ml microcentrifuge tube. Alternatively, use a particular fraction or pooled fractions from SEC as input for the co-IP (from **step 14** of Subheading 3.2).
5. Preclear lysates with 1 μg control IgG and 5 μl of Protein A/G-Sepharose beads.
6. Centrifuge the samples at $2500 \times g$ at 4 °C for 2 min.
7. Transfer the supernatant to a fresh 1.5 ml microcentrifuge tube.
8. Add 1 μg of specific antibody to the NLR of interest or ASC to the cleared lysates and incubate at 4 °C with rotation for 1 h (*see* **Note 9**).
9. Add 5 μl of Protein A/G-Sepharose beads to the lysate–antibody mix and incubate at 4 °C with rotation for at least 2 h or overnight.
10. Centrifuge the samples at $2500 \times g$ at 4 °C for 2 min. Remove the supernatant and wash the beads three times with 1 ml lysis buffer (*see* **Note 10**).
11. Extract the immunoprecipitated proteins by adding 60 μl of Laemmli sample loading buffer and incubating at 95 °C for 10 min.
12. Centrifuge the samples at $2500 \times g$ for 2 min to pellet the Sepharose beads.
13. Run the cleared samples on the SDS/PAGE gel and detect the immunoprecipitated proteins and the potential interacting partners by immunoblotting using specific antibodies.

### 3.4 co-IP of Overexpressing NLR Proteins with ASC

This is an alternative approach which has been previously used to analyze ASC–NLRP3 and ASC–NLRP7 interactions [7, 20, 21].

1. Transfect a 100 mm tissue culture dish of HEK293 cells with myc-ASC, control GFP, GFP-NLRP3, or GFP-NLRP7, adjusted to yield comparable expression (*see* **Note 11**).

2. 36 h post-transfection, lyse the cells in IP lysis buffer.
3. Proceed for the co-IP using protein-/tag-specific antibody as described in Subheading 3.3 (*see* **Note 12**).

### 3.5 Fluorometric Caspase-1 Activation Assay

1. Pellet from 1 to $5 \times 10^6$ cells at $400 \times g$ for 10 min in a centrifuge at 4 °C (*see* **Note 13**).
2. Wash the cells once with ice-cold PBS.
3. Add caspase lysis buffer with fresh DTT (use the same volume as the cell pellet, as a very concentrated cell lysate is required: about 50 µl), resuspend and keep on ice for 10 min.
4. Spin for 5 min in a refrigerated centrifuge at $14{,}000 \times g$ and 4 °C (full speed) and transfer supernatant into a fresh, prechilled tube (lysates can be stored at −80 °C). For different time points, snap-freeze the lysates in liquid nitrogen instead of incubating it on ice.
5. Assay equal volume of extract (determined by cell number); keep some lysates to determine protein concentration for normalization of the relative fluorescence units (RFU) (although preferably, you determine it up front). Alternatively, it can use immobilized proteins purified by co-IP experiments using immobilized anti-NLRP7, such as from **step 10** of Subheading 3.3 in combination with SEC or directly from cell lysates, as source for the caspase-1. In this case, equilibrate beads in caspase assay buffer (lacking the substrate Ac-YVAD-AFC).
6. Turn on the fluorescent plate reader, set up the correct excitation and emission filters (AFC excitation, 400 nm; emission, 505 nm), and warm up the reader to 37 °C.
7. Prepare 100 µl caspase assay buffer/sample with fresh DTT and 100 µM of substrate Ac-YVAD-AFC.
8. Add same volume of adjusted lysates, including a negative control (max: 15 µl) into white or black 96-well plates.
9. Quickly add 100 µl of assay buffer to each well (if necessary remove air bubbles quickly with a syringe needle).
10. Measure caspase-1 activity over time for 1 h at 37 °C at 400/505 nm (excitation/emission) (*see* **Note 14**).

### 3.6 In Vitro Caspase-1 Activation Assay

1. Transfect a 100 mm tissue culture dish of HEK293 cells with a pro-IL-1β cDNA (*see* **Note 15**). 24–36 h post-transfection, lyse cells in caspase-1 lysis buffer, and centrifuge the lysates at high speed ($14{,}000 \times g$) for 10 min at 4 °C to obtain the cleared lysate (*see* **Note 16**).
2. Purify the NLRP7 complex and IgG control complex as described above (Subheading 3.3), except maintain the bound proteins on the Sepharose beads (do not elute proteins in Laemmli buffer) and equilibrate beads in caspase-1 assay buffer.

3. Incubate the Sepharose beads containing immobilized NLRP7 and IgG control complex in caspase-1 assay buffer with total cleared lysates from HEK293 for 1–2 h at 37 °C.
4. Stop reaction by adding 2× Laemmli buffer and analyzing the conversion of pro-IL-1β to mature IL-1β by Western blot (*see* **Note 17**).

### 3.7 Cross-Linking

1. Plate $4 \times 10^6$ cells in a 60 mm tissue culture plate.
2. Transfect or treat cells as described in previous Subheading 3.1 or 3.4.
3. Remove the culture supernatants and rinse the cells with ice-cold PBS. Add 1 ml of ice-cold PBS to the plate and remove the cells using a cell scraper and transfer to 1.5 ml microcentrifuge tubes.
4. Centrifuge the cells at $400 \times g$ for 10 min and discard the supernatant.
5. Add 500 μl ice-cold lysis buffer to the cell pellet and lyse the cells by shearing 10 times through a 21-gauge needle.
6. Reserve 50 μl of cell lysate for Western blot analysis.
7. Centrifuge the remaining lysates at $2500 \times g$ for 10 min at 4 °C.
8. Transfer the supernatants to fresh tubes. Resuspend the pellets in 500 μl PBS. Add 2 mM disuccinimidyl suberate (DSS) (from a **freshly prepared** 100 mM stock) to the resuspended pellets and the supernatants. Incubate at RT for 30 min with agitation on a rotator or nutator.
9. Centrifuge samples at $2500 \times g$ for 10 min at 4 °C.
10. Remove supernatants and quench the cross-linking by resuspending the cross-linked pellets in 60 μl Laemmli sample buffer.
11. Boil the samples for 10 min at 95 °C and analyze by running the samples on a SDS/PAGE gel and detect the respective NLR or ASC by Western blotting (*see* **Note 18**).

## 4 Notes

1. This approach can be modified with application of a reversible cross-linker, such as dithiobis(succinimidyl propionate) (DSP) or Lomant's reagent, which contains a disulfide bond in its spacer arm, which is cleaved by reducing agents, such as Laemmli buffer. This approach allows co-IP experiments under stringent conditions of cross-linked protein complexes and

detection of purified proteins by immunoblot according to their monomeric molecular weight.

2. This time point is too long for purifying the complex by co-IP and SEC or to determine caspase-1 activity, but works well for cross-linking. We used vaccinia virus or MCMV infection for shorter times (90 min) to determine the AIM2–ASC complex in response to viral DNA [7].
3. We use activation of NLRP3 by LPS/ATP and LPS/nigericin and AIM2 by poly(dA:dT) as examples. However, any other inflammasome activator can be used. Crude LPS can be substituted for ultra pure LPS, but it already causes some inflammasome activation. The timing can be adjusted as needed from as short as 1 h to overnight. Similarly, the concentration of ATP and nigericin can be adjusted to cause the appropriate level of activation (usually 3–5 mM ATP and 2–5 μM nigericin is sufficient). Any transfection reagent of choice can be used for poly(dA:dT) transfection. However, the concentration may need to be adjusted based on transfection efficiency and manufacturer's protocol. Poly(dA:dT) conjugated to the cationic lipid transfection reagent LyoVec is sold by InvivoGen for direct cytosolic delivery, but we did not obtain sufficient inflammasome activation in our hands.
4. The large number of cells is required to detect low expressing NLRs, when separated into 24 fractions.
5. Do not overdry the pellets or incubate at higher temperatures, as this will cause problems when resuspending the protein pellet. Incubating at room temperature is also sufficient, but may require longer incubation times.
6. In case the Laemmli buffer changes color to yellow (indicating leftover TCA and insufficient washing), neutralize pH with a drop of 1 M Tris base.
7. You will need more gels, or a wide gel (for example the Bio-Rad Criterion gels can run up to 26 samples).
8. Less cells can be used, but to achieve sensitivity we usually use 60 or 100 mm dishes.
9. For the NLRP3–ASC interaction, we commonly IP with an anti-ASC antibody (Santa Cruz, sc-22514-R) and detect NLRP3 with an anti-NLRP3 antibody (Adipogen, Cryo-2, AG-20B-0014). This NLRP3 antibody also works well for IP and cross-reacts with human and mouse NLRP3. We purify the NLRP7 complex with an anti-NLRP7 antibody (Imgenex, IMG-6357A).

10. This is the step to continue to the caspase-1 activity assay, rather than Western blot analysis. Alternatively, the beads can be divided for both analyses.
11. We established cells stably expressing myc-ASC (HEK293$^{ASC}$) for this purpose and only transfect with the NLR of interest. This helps with the usually poor expression of NLRs. At minimum, adjust expression of ASC and NLRs by transfecting approximately 1/3 ASC and 2/3 NLR. The GFP fusion stabilizes expression of NLRP3 and NLRP7, but additionally we tested HA and Flag tags, which also work.
12. In the case of epitope-tagged proteins, consider pulling down with directly Sepharose-immobilized antibodies, which are widely available and used for detecting directly HRP-conjugated anti-tag antibodies, as ASC runs very close to the antibody light chain band.
13. Keep cells on ice all the time to minimize activation of caspase-1, which can be activated by cell lysing at 30 °C [10].
14. Prolonged incubation times for several hours may be necessary for diluted lysates or low activity.
15. We use HEK293 cells for this assay, as these cells do not express endogenous caspase-1 and therefore pro-IL-1β is not cleaved and is maintained as pro-IL-1β in the lysate. Any other cell type lacking caspase-1 would be equally suited.
16. Alternatively, a pro-IL-18 cDNA could be used, as pro-IL-18 is also cleaved by caspase-1 [13–15, 22]. Several other substrates are less well characterized [23–25]. Helpful is the use of a C-terminally epitope-tagged cDNA, as the N-terminal prodomain is proteolytically removed by caspase-1.
17. The size of pro-IL-1β is 31 kDa and of mature IL-1β is 17.5 kDa. If you use IL-18, pro-IL-18 is 24 kDa and mature IL-18 is 18 kDa.
18. Consider running a gradient SDS/PAGE to properly resolve the multiple-sized oligomers.

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