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# Methods to measure NLR oligomerization: size exclusion chromatography, co-immunoprecipitation and cross-linking


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## Summary

Oligomerization of 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 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 crosslinking covalently joins two or more molecules, thus capturing the oligomeric state with high sensitivity and stability. ASC oligomerization has been successfully used as readout for NLR/ALR inflammasome activation in response to various PAMPs and 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 *Stapylococcus 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.



### Keywords

cross-linking; size exclusion chromatography; co-immunoprecipitation; protein-protein interaction; oligomerization; caspase-1 activity assay; Nod-like receptor; NLR; inflammasome


## 1. Introduction

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).

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


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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, co-factors, 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 number of factors which should be taken under consideration, e.g., specificity of the antibody, optimization of the binding and wash conditions, post-translational 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 pro-enzyme (zymogen) (11, 12). Subsequently, the caspase-1 substrate pro-IL-1β (kDa) is converted into the biologically active form (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 AFC (AFC : 7-amino-4-trifluoromethyl coumarin) (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 crosslinking covalently joins two or more molecules (17). Crosslinking reagents (or crosslinkers) consist of two or more reactive ends. This enables crosslinkers to

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chemically attach to specific functional groups (e.g., sulfhydryls, primary amines, carboxyls, etc.) on proteins or other molecules. Crosslinker-mediated attachment between groups on two different protein molecules leads to intermolecular crosslinking. This crosslinking results in the stabilization of protein-protein interactions. Crosslinkers can be selected on the basis of their chemical reactivities and chemical properties, like chemical specificity, water solubility, membrane permeability, etc (17). Here we describe the crosslinking of nucleated and polymerized ASC molecules using the membrane permeable, non-reversible cross-linker DSS (Disuccinimidyl suberate), 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/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.

Appropriate culture medium for THP-1 cells, primary human macrophages, BMDMs and HEK293 cells

Lipofectamine 2000 (Invitrogen) or transfection reagent of choice

PBS

100 mm/60 mm tissue culture dishes

1.5 and 2 ml microcentrifuge tubes

15 ml centrifuge tubes

Cell scrappers (can be obtained from BD Falcon, Corning, or Greiner Bio-one)

Refrigerated table top centrifuge with 1.5 ml microcentrifuge tube rotor

Table top centrifuge with 15 ml tube rotor

Heat block

10x Protease inhibitor cocktail (Roche)

phenylmethylsulfonylfluoride (PMSF), 0.1 M in ethanol

poly(dA:dT) (Sigma)

ultra-pure *E. coli* LPS (0111:B4) (Invivogen)

ATP (Sigma)

[1]This approach can be modified with application of a reversible crosslinker, such as DSP (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.

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Nigericine (Invivogen)

Laemmli sample loading buffer: 60 mM Tris-HCl, pH 6.8, 2 % SDS, 100 mM dithiothreitol (DTT), 10 % glycerol, and 0.01 % bromophenol blue

SDS/PAGE and blotting equipment and materials

Anti-ASC antibody (for example: Santa Cruz, sc-22514-R)

Anti-NLRP3 antibody (for example: Adipogen, Cryo-2, AG-20B-0014)

Anti-NLRP7 antibody (for example: Imgenex, IMG-6357A)

### 2.1. Size Exclusion Chromatography (SEC)

Fast protein liquid chromatography (FPLC) equipment (we use a Bio-Rad Biologic LP Chromatography System)

Gel filtration column: GE Healthcare HiPrep 16/60 Sephacryl S-300HR High Resolution Column (matrix: 50 μm Allyl dextran and N, N′-methylenebisacrylamide, bed dimension: 16 × 600 mm, bed volume: 120 mL)

System tubing: 1.6 mm ID

Fraction collector

5 ml round bottom tubes

Separation buffer: 50 mM Tris pH 7.4, 150 mM NaCl

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 1x Roche Protease Inhibitor Cocktail

Trichloroacetic acid (TCA)

Acetone

Ethanol (20 %)

Gel Filtration Standard (Bio-Rad 151-1901)

Dounce homogenizer

Sonicator

3 ml syringe using with 18½ gauge needle

0.45 μM syringe filter

### 2.2. Co-immunoprecipitation (co-IP)

IP Lysis buffer : 50 mM Hepes pH 7.4, 150 mM NaCl, 10 % glycerol, 2 mM EDTA, 0.5 % Triton X-100, 1x Roche protease inhibitor mix

Specific antibody against NLR of interest

Protein A/G-Sepharose 4B (Invitrogen)

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### 2.3. Caspase-1 activity assay

#### 2.3.1. Fluorometric Caspase-1 Activation Assay

Ac-YVAD-AFC (Calbiochem), 10 mM stock in DMSO (protect from light)

Caspase-1 lysis buffer: 150 mM NaCl, 20 mM Tris, pH 7.5, 0.2 % Triton X-100, **add freshly:** 1 mM DTT

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

#### 2.3.2. In vitro Caspase-1 Activation Assay

pro-IL-1β cDNA

Anti-IL-1β antibody (for example: Cell Signaling, 3A6, 12242)

### 2.4. Cross-linking

Disuccinimidyl suberate (DSS), 100 mM stock prepared in dimethyl sulfoxide (DMSO) **(prepare freshly just before adding to the lysates)**

Lysis Buffer : 20 mM Hepes-KOH, pH 7.5, 150 mM KCL, 1 % NP-40, 0.1 mM PMSF, 1x of protease inhibitor cocktail, 1 mM sodium orthovanadate. (PMSF should be added to the lysis buffer just before cell lysis)

## 3. Method

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 hrs (for cross linking, *see* Note 2). To activate the NLRP3 inflammasome with LPS/ATP, treat cells with 100 ng/ml of LPS for 4 hrs 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 gentamycin (50 mg/ml) for a total time of 90 min (7) (*see* Note 3).

[2]This time point is too long for purifying the complex by co-IP, SEC or to determine caspase-1 activity, but works well for crosslinking. 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 hr to overnight. Similarly, the concentration of ATP and nigericin can be adjusted to cause the appropriate level of activation (usually 3 to 5 mM ATP and 2 to 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.

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### 3.1. 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) $ddH_2O$ (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 x $10^7$ cells in 60 mm culture dishes containing 5 x $10^6$ macrophages per plate for 90 min) (*see* Note 4).
4. Aspirate the culture medium and wash cells twice with ice-cold 1x 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, pre-chill a glass 2 ml Dounce tissue grinder on ice, and 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 microfuge tube and spin at 12,000xg 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 a 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 and collect 9 min/fraction (4.5 ml) for a total of 24 fractions.

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[4]The large number of cells is required to detect low expressing NLRs, when separated nto 24 fractions.

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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 (step 4 below) or continue for TCA precipitation and Western blot analysis below.
15. Divide each fraction into three 1.5 ml aliquots in 2 ml microcentrifuge tubes.
16. TCA-precipitate 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 14,000xg 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 14,000xg 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 minutes to evaporate any remaining acetone (*see* Note 5).
19. Resuspend protein pellets in 50 μl 1.5x 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 vitamine B12 (MW range from 1,350–670,000 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.), 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.

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[5]Do not over dry 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).

### 3.2. 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 1500 rpm 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 12,000 rpm 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 above).
5. Pre-clear lysates with 1 μg control IgG and 5 μl of Protein A/G-sepharose beads
6. Centrifuge the samples at 5000 rpm 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 hr (*see* Note 9).
9. Add 5 μl of Protein A/G-sepharose beads to the lysates-antibody mix, and incubate at 4°C with rotation for at least 2 hrs or overnight.
10. Centrifuge the samples at 5000 rpm 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 5000 rpm 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 immunobloting using specific antibodies.

*An alternative approach may be suitable as well, which we used to analyze the ASC-NLRP3 and ASC-NLRP7 interactions (*7, 20, 21):

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[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.



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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 hrs posttransfection, lyse the cells (120 mM NaCl, 20 mM Tris pH 7.4, 10 % glycerol, 0.2 % Triton X-100, and 1x protease inhibitor.
3. Proceed for the coIP using protein/tag specific antibody as described above (*see* Note 12).

### 3.3. Caspase-1 activity assay

#### 3.3.1. Fluorometric Caspase-1 Activation Assay

1. Pellet cells (1–5x$10^6$) at 2000 rpm for 3 min in a centrifuge (*see* Note 13). Wash 1x with ice-cold PBS.
2. 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.
3. Spin for 5 min in refrigerated centrifuge (full speed) and transfer SN into fresh, pre-chilled 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.
4. Assay equal volume of extract (determined by cell number), keep some lysates to determine protein concentration for normalization of the relative fluorescent units (RFU) (although preferably, you determine it upfront). Alternatively, one can use immobilized proteins purified by co-IP experiments using immobilized anti-NLRP7, such as above step 10 (3.2) 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).
5. Turn on the fluorescent plate reader, add the correct excitation and emission filters (AFC excitation: 400 nm, emission: 505 nm) and warm up the reader to 37°C.
6. Prepare 100 μl caspase assay buffer/sample with fresh DTT and substrate (10 mM Ac-YVAD-AFC stock in DMSO).
7. Add same volume of adjusted lysates, including a negative control (max: 15 μl) into white or black 96-well plates.

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[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 we also 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 use for detection 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).

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8. Quickly add 100 μl of assay buffer to each well (if necessary remove air bubbles quickly with a syringe needle.)
9. Measure caspase-1 activity over time for 1 hr at 37°C at 400/505 nm (*see* Note 14).

#### 3.3.2. In vitro Caspase-1 Activation Assay

1. Transfect a 100 mm tissue culture dish of HEK293 cells with a pro-IL1β cDNA (*see* Note 15). 24 to 36 hrs post-transfection, lyse cells in caspase-1 lysis buffer, and centrifuge the lysates at high speed 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 (3.2.), 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 hrs at 37°C.
4. Stop reaction by adding 2x Laemmli buffer and analyzing the conversion of pro-IL-1β to mature IL-1β by western blot (*see* Note 17).

### 3.4. Cross-linking

1. Plate $4x10^6$ cells in a 60 mm tissue culture plate.
2. Transfect or treat cells as described above
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 2000 rpm for 10 min. Remove 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. Remove 50 μl of lysate for Western blot analysis.
7. Centrifuge the lysates at 5000 rpm for 10 min at 4°C.
8. Transfer the supernatants to fresh tubes. Resuspend the pellets in 500 μl PBS. Add 2 mM disuccinimydyl suberate (DSS) (from a **freshly prepared**

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[14]Prolonged incubation times for several hrs 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 pro-domain 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.

100 mM stock) to the re-suspended pellets, and the supernatants. Incubate at RT for 30 min with agitation on a rotator or nutator.

**9.** Centrifuge the samples at 5000 rpm for 10 min at 4°C.

**10.** Remove the 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).

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[18]Consider running a gradient SDS/PAGE to properly resolve the multiple-sized oligomers.



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