Jan 22, 2024

Purification of rat NLRP1, rat DPP9, and rat NLRP1-DPP9 complex from Sf9 Cells for structural and biochemical studies

Forked from Purification of DPP8/9 from Sf9 Cells for structural and biochemical studies

DOI
dx.doi.org/10.17504/protocols.io.n2bvj31zblk5/v1

Louis R R Hollingsworth[1]

[1]Harvard Medical School

Hao Wu Lab

Louis R R Hollingsworth
Harvard Medical School

DOI: https://dx.doi.org/10.17504/protocols.io.n2bvj31zblk5/v1

Protocol Citation: Louis R R Hollingsworth 2024. Purification of rat NLRP1, rat DPP9, and rat NLRP1-DPP9 complex from Sf9 Cells for structural and biochemical studies. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n2bvj31zblk5/v1

Protocol status: Working
We use this protocol and it's working

Created: January 08, 2024

Last Modified: January 22, 2024

Protocol Integer ID: 93039

Keywords: DPP9, protein purification, NLRP1, Lewis rat, nlrp1 inflammasome activation, purification of rat nlrp1, dpp9 complex from sf9 cell, rat nlrp1, host e3 ubiquitin, dpp9 complex, rat dpp9, sf9 cell, nlrp1, autonomous immunity, itch mediate, toxoplasma, purification

Funders Acknowledgements:
NIH
Grant ID: R01-AI080621
NIH
Grant ID: R21-A151081
NIH
Grant ID: R01-AI144149
NIH
Grant ID: R01-AI124491
NIH
Grant ID: R00-AI163285
NIH
Grant ID: T32-GM007726
NIH
Grant ID: F31-AI152267-01A1

Abstract

Protocol associated with "Host E3 ubiquitin ligase ITCH mediates *Toxoplasma gondii* effector GRA35-triggered NLRP1 inflammasome activation and cell-autonomous immunity" by Wang et al. and Saeij. Please address any questions to Bobby Hollingsworth (bobbyh11@vt.edu).

Protocol adapted from Huang* and Zhang* et al., Zhong and Chai 2021 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8081665/)

Materials

Plasmids (see text)
LB agar
Kanamycin
Gentamicin
Tetracycline
Bluo-gal
IPTG
M13 forward primer
M13 reverse primer
Sf9 insect cells
SFX medium
Grace's insect medium, unsupplemented
Cellfectin II
Sterile DMSO
TCEP (Tris(2-carboxyethyl)phosphine hydrochloride)
DTT (1,4-Dithiothreitol)
BME (2-Mercaptoethanol, also β-mercaptoethanol)
Imidazole
Bio-Rad columns 7321010 and 7311550
Amicon Ultra 100 kDa cutoff spin concentrator (Millipore, UFC910096)

Troubleshooting

Bacmid production

1d 7h 5m

1 Obtain plasmids by emailing Dr. Jeroen Saeij (jeroensaeij@gmail.com) or from Addgene (deposition details will be added as a comment once processed). These include:
- Lewis rat NLRP1 (Lew-rNLRP1) allele (allele 5 Uniprot ID D9I2G4) in pFastBac (His-TEV-rNLRP1[Lew]-FLAG)
- Proteolysis-deficient Lew-rNLRP1 His-TEV-rNLRP1[Lew, S969A]-FLAG
- Brown Norway rat NLRP1 (BN-rNLRP1) allele (allele 1, Uniprot ID D9I2F9) in pFastBac (His-TEV-rNLRP1[BN]-FLAG)
- Proteolysis-deficient BN-rNLRP1 (His-TEV-rNLRP1[BN, S969A]-FLAG)
- Rat DPP9 (rDPP9, Uniprot ID M0R781) in pFastBac (His-TEV-rDPP9)
- Catalytically-deficient rDPP9 (His-TEV-rDPP9[S729A])

2 Acquire other reagents and read up on the Bac-to-Bac system prior to continuing.

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fbrochures%2F710_01985_BactoBac_bro.pdf&title=QmFjLXRvLUJhYw==https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fbrochures%2F710_01985_BactoBac_bro.pdf&title=QmFjLXRvLUJhYw==

http://kirschner.med.harvard.edu/files/protocols/Invitrogen_bactobacexpression.pdf

3 Pour (or buy) bacmid LB agar resistance plates prior to proceeding. These are LB agar plates with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG. Cover in foil (because plates contain doxycycline) and store at 4 degrees until needed. These generally last less than 1 month--blue/white discrimination becomes impossible if plates are kept too long.

2h

4 Transform ~100 ng plasmid into ~50 uL of DH10Bac cells. Proceed w/ normal transformation protocol, but incubate for 4 hours shaking at 37 before plating instead of the normal hour. This allows recombination and production of resistance proteins.

5h

Helpful general transformation protocol:
https://www.addgene.org/protocols/bacterial-transformation/?gclid=Cj0KCQiA6t6ABhDMARIsAONIYyxEfw6FVU1ul6ajQQhP8_e4XHtCvMglbuIPH4HqW6BKlascmX9YjJ4aAkRDEALw_wcB

Note

Different DH10Bac cells will have various competencies, particularly if the cells are homemade. It's important to try serial dilutions here--for example, transform 10, 50, 100 ng of plasmid into three separate batches of cells. There's a sweet-spot for blue/white selection.

5 Plate all cells from an individual transformation onto bacmid plate(s) from step 3. 5m

6 Wrap the bacmid plate in foil (because plates contain doxycycline) and keep in a 37 degree plate incubator for 2 days.

7 Pick at least 2 completely white colonies per construct and 1 negative control blue construct and grow in 5 mL of LB containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline overnight. 1d

Note

If plates are overcrowded, then a lower concentration of pfastBac is likely more optimal for transformation. If little/no white colonies appear, then a higher plasmid concentration is needed. If blue/white colonies are indistinguishable, place the plates in 4 degree for several days, the color will continue to develop.

8 Miniprep the bacmid. I generally use this protocol from Dr. Owen Pornillos' lab (UVA):

Bacmid miniprep.pdf

Note

The concentration of the purified bacmid should likely be between 1-30 ug/uL.

IMPORTANTLY: At the ethanol precipitation step, handle everything as sterile in a cell culture hood to avoid contamination--insect cells are particularly prone to fungal contamination due to the growth temperature. Let the bacmid air dry in a cell culture hood before re-suspending with sterile TE buffer; can an aliquot for nanodrop concentration determination outside of the hood, and dilute to a linear range if necessary.

9 PCR validate the insert on the bacmid. I've had limited success with Taq polymerases, so I generally do small (5-10 uL) Q5 polymerase reactions. Use M13 forward and reverse primers, which anneal to the backbone--there's a clear shift between the negative control (blue colony) and colonies that properly incorporated. With M13 primers, the PCR product should be ~2430 bp (HT vectors) + the size of the insert for a total of well over 5 kB.

Note

M13 f: 5'-GTTTTCCCAGTCACGAC-3'

M13 r: 5'-CAGGAAACAGCTATGAC-3'

10 Dilute the verified bacmid(s) to 1000 ng/uL in endotoxin-free/sterile TE buffer. Keep in the 4 degree short-term and freeze at -20 for long-term storage. Avoid freeze-thaw cycles.

Virus production

1h

11 Below, I detail a fairly generic an unoptimized protocol for generating virus. For maximum protein yield, careful consideration of virus MOI is required. This recent protocol from Dr. Mark Gorrell's lab also has some nifty tricks for optimizing sf9 expression: https://doi.org/10.1016/j.pep.2021.105833

1h

Plate 0.8 million cells from a suspension culture in a 6-well dish--1 well for each construct in addition to 1 well for the negative control. Use room-temperature complete SFX to bring the total volume between 1-2 mL in each well.

Wait 1 hr for cells to attach. 01:00:00

https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FMAN0007821_Cellfectin_II_Reagent_UG.pdf&title=VXNlciBHdWlkZTogQ2VsbGZlY3RpbiBJSSBSZWFnZW50

Note

Sf9 cells are maintained in suspension near room temperature (~27 degrees) with SFX supplemented with antibiotic-antimycotic, but adhere strongly when plated. Transfection efficiency is much higher with these plates cells. Adherence can be checked by examining plate(s) under a light microscope--cells should stay put when the plate is moved.

The virus production protocol depends on the type of cells you use and the transfection reagent. This one works well, but there are other quicker protocols, too.

All insect cell culture and virus manipulation should be done in a cell culture hood using proper sterile technique. Sterile spin steps can be done outside of the hood in tightly shut autoclaved/sterile containers. Insect cells are prone to fungi infection because they are grown at 27 degrees, which is evident from a strong smell and discoloration/particulates in the medium. Contaminated cells should be removed and bleached promptly.

12 Meanwhile, heat up grace's insect cell medium (much like Opti-MEM for the mammalian-cell transfection savvy) to RT.

13 Add 100 uL grace's media to two microcentrifuge tubes (sterile) per construct.

14 Add 6 uL CellFectin II to one of the microcentrifuge tubes.

15 Add 1000 ng of bacmid to the other 100 uL microcentrifuge tube.

16 Transfer the ~100 uL medium containing the bacmid to the tube containing CellFectin II. Pipette up and down gently, incubate 30 min. 00:30:00 30m

17 Once Sf9s have attached (step 11), exchange medium with unsupplemented Grace's insect cell medium. Wash once with 2 mL of Grace's medium, remove, then add 1 mL of medium to keep on top of the cells. Return to incubator, or leave in the hood if step (16) is complete.

18 Remove medium from sf9 plated cells, and immediately proceed to step 19

Note

Proceed from step 18 to 19 one construct/well at a time to avoid drying out the cells.

19 Add 800 uL Grace's medium to each microcentrifuge tube from step 16, bringing the total volume slightly above 1 mL. Quickly but gentle pipette up and down to mix and gently transfer to the sf9 well. Return the plate(s) to the 27 degree incubator for 4-5 h. 04:00:00 4h

Note

Be sure to label all of the wells. Different constructs. particularly point mutants, will be hard to distinguish from one another.

20 Remove medium from each well and add 2-mL complete sf9 medium (pre-warmed to room temperature). Return to the incubator and wait 3 days. 72:00:00 3d

Note

Replace medium 1 construct/well at a time to avoid drying out the cells. Be gentle as to not lift cells.

Note: For sub-optimal transfection efficiencies, this step can be extended to 5 days instead of 3.

21 Plate 1.5 million cells from a suspension culture in a 6-well dish--1 well for each construct in addition to 1 well for the negative control. Use room-temperature complete SFX to bring the total volume between 1-2 mL in each well. Return the plate to the incubator for at least 1 h. 01:00:00 1h

Note

Virus can be stored in the 4 degree, and proceeding through virus generation can be halted for weekend/event timing, etc.

22 Harvest virus from the plate in step 20, which is in the cell culture supernatant. Transfer supernatant into labeled microcentrifuge tubes and spin at 2000g for 10 min to pellet cell debris. 2000 rpm, 4°C, 00:10:00 15m

23 Transfer supernatant to fresh sterile microcentrifuge tube(s). This is considered the P1 virus (some call it P0, but for the sake of this protocol I will refer to it as P1). 10m

Note

Extra virus-containing medium can be stored in the 4 degree for several weeks, and used as a fallback if there's contamination during future passages/amplification of the virus.

24 Remove medium from the plate in step 21, and add 1 mL of fresh complete medium to each well. Transfer 700 uL - 1 mL of P1 virus-containing medium into each well. Return to incubator and wait 3 days. 72:00:00 3d

25 Harvest P2 virus as in steps 22 and 23. 15m

26 Attach 15 million cells to a 15-cm plate, and allow them to attach for at least 1 h at 27 degrees. 1h

27 Add 1-mL of P2 virus to the 15 cm plate, and incubate for 3 days. 72:00:00 3d

28 Harvest P3 virus as in steps 22-23. Instead of a microcentrifuge tube, use falcon tubes, as there should be ~15 mL of virus-containing medium. 15m

Note

Cells infected with virus should be noticeably larger than untransfected cells under a light microscope; this can be quantified with automated cell counters as well (you'll have to use fresh medium to lift a sample of cells after harvesting virus-containing medium). Some cell death should also be apparent with floating cells.

29 Add 5-mL of P3 virus to 200-mL suspension culture (at 2 million cells/mL). Store extra P3 virus-containing medium in the 4 degree. Wait 2 days. 48:00:00 2d

30 Harvest baculovirus-containing cells (BIICs). Centrifuge medium in sterile bottle(s) or several falcon tubes 1000 x g, 00:10:00 20m

Note

Harvesting here takes some time, and protein expression and purification also take quite a bit of time. Inspect cells to ensure they are larger than uninfected control cells (i.e. your passage cells), essentially because they will be almost bursting with virus.

31 Prepare 20 mL freezing medium (at RT or 4 degrees) per construct. Freezing medium: Complete SFX medium with 10% DMSO and 10% FBS 5m

32 Working in a cell culture hood, remove medium from bottles and resuspend BIICs in freezing medium. Aliquot resuspended BIICs into sterile microcentrifuge tubes (1 mL each). Label, and transfer them to -80 degree storage. 15m

Protein expression 2d

33 Grow suspension sf9 cells to 1 L at 2 million cells/mL. If cells more confluent, dilute down to 2 million cells/mL.

Note

You can scale up the volume, but I would recommend a 1 L expression to test the virus titer before larger-scale purifications.

Other insect cell types, like sf21 and Hi5, might have better expression.

34 Thaw an aliquot of frozen BIICs in your hand, and transfer to the insect cell flask to the under the cell culture hood. Return to the incubator for 2 d. 2d

NOTE: Rat NLRP1 and DPP9 can be purified by themselves for biochemical studies. For the DPP9-NLRP1 complex, I co-express these to ensure that N-terminal degradation frees the C-terminal fragment for DPP9 sequestration.

35 Harvest cells by centrifugation. Sterile technique is no longer necessary. 20m

2500 rpm, 4°C, 00:20:00

36 Resuspend cell pellet(s) with ice-cold PBS and transfer them to 50 mL falcon tube(s) (or a different size if appropriate). This step is important, as it removes proteins and other components in the medium. Tubes can be split and aliquoted for individual protein preps, so budget accordingly. 20m

Centrifugation 2000 rpm, 4°C, 00:20:00

37 Gently remove PBS. 10m

Flash-freeze pellet(s) in liquid nitrogen and store at −80 degrees celsius. Alternatively, proceed directly to purification.

Protein purification 5h 6m

38 Prepare and pre-chill the following buffers (NOTE: exclude TCEP for TRX-containing NLRP1 complexes, not discussed here):

Lysis buffer (100 mL, 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine abbreviated as TCEP, 5 mM imidazole)

Wash buffer (500 mL, 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM TCEP, 25 mM imidazole)

Elution buffer (20 mL, 25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM TCEP, 500 mM imidazole)

Size exclusion buffer (500 mL, 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP)

Note

5 mM BME can be used as a reducing agent in lysis buffers and 2 mM DTT can be used in size exclusion buffers; however, avoid DTT for Ni-NTA steps. 1 mM TCEP can be used throughout. Reducing agent should be added fresh to all buffers, unless you intend to do experiments with TRX-containing complexes (see **PMC9850498**). Imidazole should be pH adjusted to 8.0 before addition to any buffer.

Avoid protease inhibitors for DPP9-containing preps, as DPP9 is a protease.

For purifying NLRP1-DPP9 complexes, you might want to purify on FLAG to remove excess DPP9, which usually expresses much better than NLRP1 (unless your DPP9 viral titer is very low). See this protocol for an example of FLAG resin purification (avoid reducing agent prior to eluting from FLAG resin as this will reduce binding capacity for regenerated beads) :https://www.protocols.io/view/purification-of-the-nlrp1-dpp9-complex-from-expi29-q26g7b8p3lwz/v1

39 Thaw insect cell pellet and resuspend in 40 mL of lysis buffer (per L of cells). 15m

Note

I generally don't recommend using less than 40 mL of buffer for probe sonication, even with smaller expression volumes, such as from a 500 mL cell pellet. You can try to get away with 40 mL resuspension buffer for a 2L expression as well, which would fit into 1 ultracentrifuge tube

40 Transfer resuspended cells to a small metal beaker (glass works if you don't have one of these, just do not rest the sonicator against the bottom--this will shatter the beaker). Place beaker with cells in an ice-water bath and sonicate with a probe sonicator. 15m

For a 1L pellet: 3 s on 5 s off, 3.5 min total on, 45% power, Branson Sonicator.

41 Transfer to ultracentrifuge tubes and carefully balance the rotor. 2h

40,000 RPM for 1.5 h (45 Ti fixed-angle rotor, Beckman) or similar.

40000 rpm, 4°C, 01:30:00

42 While centrifuging, pre-equilibrate Ni-NTA resin with lysis buffer (1 mL bed volume beads per 2L expression volume NLRP1) in cold lysis buffer.

CV = column volumes

43 Carefully remove the supernatant from the ultracentrifuge tubes and transfer to 50-mL falcon tube(s) or another appropriate vessel. 5m

Caution: handle delicately as to not disturb the pellet or lipid layer!

Avoid 1) Cloudy liquid at the top of the tube, which contains lipids and other junk, and 2) the cell pellet.

Keep the cell pellet on ice just in case lysis was incomplete.

Note

See schematic on protocol, "Purification of the NLRP1-DPP9 Complex from Expi293F Cells", step 15: https://www.protocols.io/view/purification-of-the-nlrp1-dpp9-complex-from-expi29-q26g7b8p3lwz/v1

44 Transfer Ni-NTA beads between all 50-mL falcon tube(s). 5m

45 Fill falcon tubes with cold lysis buffer to avoid bubbles while rocking. Nutate/gently rock in the cold room for 30 m. 00:30:00 30m

46 Centrifuge falcon tubes gently to pellet resin. 5m

800 x g, 4°C, 00:05:00

47 Remove supernatant. Add 20 CV wash buffer and centrifuge again (batch wash). 5m

800 x g, 4°C, 00:05:00

48 Remove supernatant. Add 5 CV wash buffer and transfer to a gravity column (we like Bio-Rad columns 7321010 and 7311550, depending on the bed volume). 1m

49 Wash the resin on-column with 25 CV ice-cold wash buffer. Be careful not to disturb the resin bed. 30m

50 Let the remainder on top of the resin bed drain out, but do not let the bed dry. Stop-up the column as soon as it stops flowing. 30m

Incubate the resin (on the column) with 3 CV ice-cold elution buffer in the cold room. Gently mix, then wait 30 minutes. 00:30:00 . Save buffer to rinse the column after elution to get as much protein off of the column as possible.

51 Elute protein from the column and collect it in an Amicon Ultra 100 kDa cutoff spin concentrator (Millipore, UFC910096). Use ~1 CV buffer to wash the remaining protein off of the column after flow has stopped. 15m

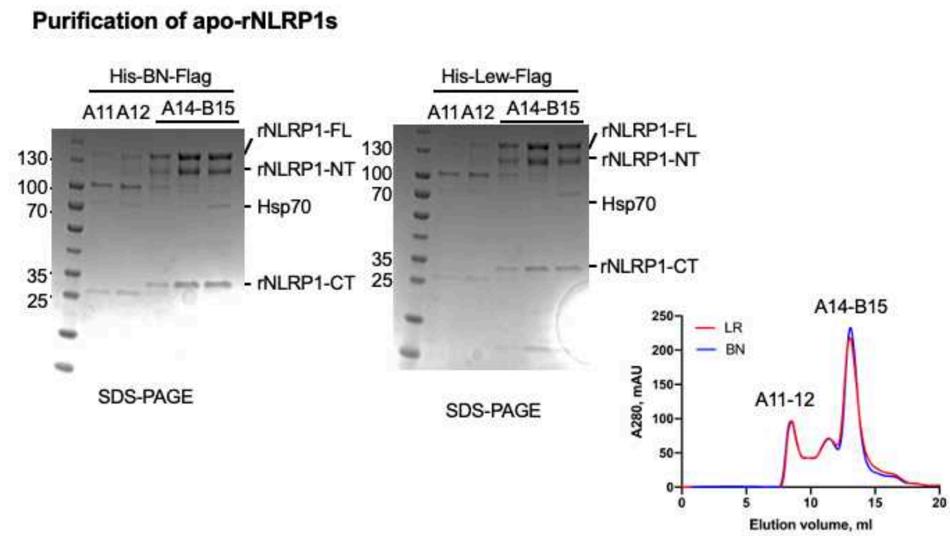
52 Spin concentrate to ~0.5 mL. 4000 rpm, 4°C , transfer to a microcentrifuge tube. 30m

53 Spin microcentrifuge tubes hard (cold) to pellet any aggregated protein.
10000 rpm, 4°C, 00:10:00

54 For apo proteins, separate them from aggregates and contaminants on a Superdex 200 increase 10/300 GL size exclusion column (Cytiva). Use size exclusion buffer as the running buffer.

NOTE: A superose 6 increase 10/300 column is more appropriate for the rDPP9:rNLRP1 complex

55



Superdex 200 size exclusion chromatography of BN and Lew NLRP1.

Collect peak fractions (annotated A14-B15 here for NLRP1 alone), run a quality control SDS-PAGE gel, concentrate, aliquot, and freeze as necessary.

Note

Best to avoid freeze/thaw cycles. For structural studies, fresh (never-frozen) protein is preferable (freezing the pellet is ok, but once you start the prep proceed as quickly as possible to grid making). For cryo-EM, DPP9 has a severe orientation bias--collect data at a 30 degree tilt to get all views.

Protein activity (or lack thereof for catalytic dead mutants) should be validated with a GP-AMC assay (see various DPPIV and DPP8/9 publications).