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

In 1 collection

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1 Works for me

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SUBMIT TO PLOS ONE

ABSTRACT

Protocol associated with "DPP9 sequesters the NLRP1 C-terminus to repress inflammasome activation" by Hollingsworth*, Sharif*, Griswold* et al., Bachovchin, and Wu. Please address any questions to Bobby Hollingsworth (bobbyh11@vt.edu).

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PROTOCOL CITATION

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COLLECTIONS

DPP9, NLRP1, CARD8 Collection

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DPP9, protein purification

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PARENT PROTOCOLS

Part of collection

DPP9, NLRP1, CARD8 Collection

MATERIALS 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

Bacmid production 1d 7h 5m

1 Obtain pFastBac HTB plasmids encoding DPP of interest (DPP9 short or long isoform, WT or catalytically-inactive) from Addgene (https://www.addgene.org/Hao_Wu/). DPP8 (cDNA also on Addgene) can be subcloned into this same vector, or the insect expression construct can be obtained from Drs. Breyan Ross and Robert Huber (doi.org/10.1073/pnas.1717565115).

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_e4XHtCvMgIbuIPH4HqW6BKIascmX9YjJ4aAkRDEALw_wcB

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.

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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

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,

8 Miniprep the bacmid. I generally use this protocol:

http://people.virginia.edu/~owp3a/docs/protocols/Bacmid%20miniprep.pdf

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

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) + ~ 2600 bp (DPP8/9) = ~6000 bp PCR product.

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://www.sciencedirect.com/science/article/pii/S1046592821000164?dgcid=author 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.

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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

Sf9 cells are maintained in suspension at 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 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 (autoclaved) 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

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

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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

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

Replace medium 1 construct/well at a time to avoid drying out the 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

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 autoclaved 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

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

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

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25

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

Cells infected with virus should be noticeably larger than untransfected cells under a light microscope. 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

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 are higher, dilute down to 2 million cells/mL.

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.

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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

35 Harvest cells by centrifugation. Sterile technique is no longer necessary. **2500 rpm, 4°C, 00:20:00** 20m

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:

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)

> 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. Imidazole should be pH adjusted to 8.0 before addition to any buffer. Avoid protease inhibitors, as DPP9 is a protease.

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

> 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 get away with 40 mL for a 2L expression as well, which would fit into 1 ultracentrifuge tube.

40 Transfer resuspended cells to a small glass 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.

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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) 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.

> See schematic on protocol, "Purification of the NLRP1-DPP9 Complex from Expi293F Cells", step 15

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. 30m
00:30:00

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.

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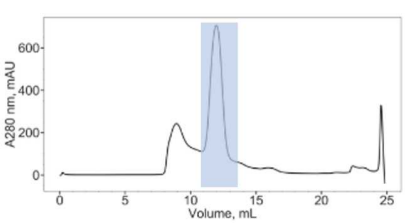
51 Elute protein from the column and collect it in an Amicon Ultra 100 kDa cutoff spin concentrator (Millipore, UFC910096). Use ~1 CV buffer to wash the remaining protein off of the column after flow has stopped. 15m

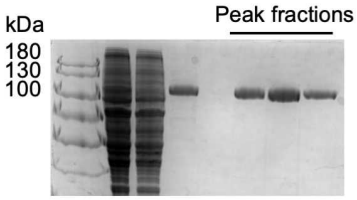
52 Spin concentrate to ~0.5 mL. **4000 rpm, 4°C** , transfer to a microcentrifuge tube. 30m

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

54 Separate protein on a Superdex 200 increase 10/300 GL size exclusion column (Cytiva). Use size exclusion buffer as the running buffer.

55 Collect peak fractions, concentrate, aliquot, and freeze as necessary.





Best to avoid freeze/thaw cycles. For structural studies, fresh (never-frozen) protein is preferable. For cryo-EM, DPP9 has a severe orientation bias--collect data at a 30 degree tilt to get all views. Data processing should be very straightforward.

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

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