Purification of the NLRP1-DPP9 Complex from Expi293F Cells

In 1 collection

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1 Works for me dx.doi.org/10.17504/protocols.io.833hyqn

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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).

General Expi293F expression protocol adapted from Dr. Andrew Kruse's protocols.

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PROTOCOL CITATION

Louis R Hollingsworth 2021. Purification of the NLRP1-DPP9 Complex from Expi293F Cells. **protocols.io** https://dx.doi.org/10.17504/protocols.io.833hyqn

COLLECTIONS

DPP9, NLRP1, CARD8 Collection

KEYWORDS

Innate immunity, cryo-EM, protein purification

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PARENT PROTOCOLS

Part of collection
DPP9, NLRP1, CARD8 Collection

MATERIALS TEXT

MATERIALS

Opti-MEM™ I Reduced Serum Medium, no phenol red **Thermo Fisher Catalog #11058021**

Expi293™ Expression Medium **Thermo Fisher Catalog #A1435101**

Expi293F™ Cells **Thermo Fisher Catalog #A14527**

BEFORE STARTING

Cells should be >95 % viable and doubling every 24 hr. Mantain cells with constant shaking at 100 RPM, 37°C, 5% CO_2.

Need maxi-prepped DNA, ideally using an endotoxin-free kit. For 1-L expression, up to 1 mg of DNA is needed.

Protein Expression 5d 1h 26m

1 Grow Expi293F cells (ThermoFisher, A14527) in Expi293™ Expression Medium (ThermoFisher, A1435101) to 2-3 million cells/mL at the desired culture volume, such as 1L. Choose the appropriate flask for the desired volume of cell culture to ensure appropriate gas exchange--I use a 2L Fernbach flask for a 1L batch of cells. If cells are grown slightly above 3 million cells/mL (up to 6 million cells/mL), dilute to 3 million cells/mL using fresh medium day-of. Otherwise, dilute below 1.5 million cells/mL and try again another day. Put cells back in the incubator once prepared, for now.

> Cells should be >95 % viable (e.g. with trypan blue, Thermofisher 15250061) and doubling every 24 h. There are many strategies to grow cells to the appropriate volume/density. For example, I often grow 500-750 mL of cells to ~5-6 million cells/mL, then dilute the appropriate amount of cells with fresh medium in a new flask.

2 Warm Opti-MEM to RT. In the hood, transfer RT Opti-MEM **100 mL per 1L cells** to a small sterile, autoclaved flask. For smaller culture volumes, falcon tubes can be used. 1m

3 Transfer endotoxin-free DNA encoding His-TEV-DPP9 (0.3 mg/1L cells) and DNA encoding NLRP1ΔΔ-TEV-GFP-FLAG (0.7 mg/1L cells) into the Opti-MEM flask. Swirl to mix. Plasmids are available on Addgene: https://www.addgene.org/Hao_Wu/ 5m

4 Add polyethylenimine (PEI, **3 mL per 1L cells** at **1 mg/ml** or **1.5 mL per 1L cells** at **2 mg/ml**). Swirl to mix. Incubate in the hood 30-45 min **00:30:00** . See the following resource for preparing PEI: https://www.addgene.org/protocols/transfection/ 30m

5 In the cell culture hood, slowly pour Opti-MEM-DNA-PEI mixture into the flask containing cells (from step 1) while gently shaking. Return flask to the incubator, and incubate 24 hr. **24:00:00** 1d

6 While gently swirling the flask, slowly transfer sterile glucose (9 mL, 45%, Sigma G8769 or in-house preparation) and sterile-filtered valproic acid (VPA, 10 mL, 300 mM, prepared from Sigma P4543) into the flask with the transfected cells. This step boosts protein production. At this stage, you could also take a small sample of cells and check for fluorescence in the green channel. ~50% of the cells or more should be GFP-positive. Return to the shaker for 4 d. **96:00:00** . 4d

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7 Take a small sample of cells and check for fluorescence in the green channel--50% of the cells or more should be GFP-positive. If the cells are not green under a fluorescent microscope, something went wrong with transfection or the cells were contaminated. Harvest cells by centrifugation **2000 rpm, 4°C, 00:20:00** . Sterile technique is no longer required. 25m

8 Resuspend cell pellet(s) with ice-cold PBS, combine and transfer all resuspended cells to 2-50 mL falcon tubes (or a different size if appropriate). This step is important, as it removes proteins and other components in complete medium. Tubes will be frozen in aliquots for individual protein preps, so budget accordingly. 25m

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

9 Gently remove PBS. 10m

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

Protein Purification 5d 1h 26m

10 Prepare and pre-chill the following buffers:

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

On-column cleavage buffer (5.5 mL, 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM ADP, 1 mM TCEP, 0.2 mg TEV protease)

MonoQ Low-Salt Buffer (250 mL, 150 mM NaCl, 25 mM Tris-HCl pH 8.0, 1 mM TCEP, filtered)

MonoQ High-Salt Buffer (250 mL, 1 M NaCl, 25 mM Tris-HCl pH 8.0, 1 mM TCEP, filtered)

Dialysis Buffer (50 mL, 25 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.2 mM ADP, 1 mM TCEP)

> Buffers can be prepared ahead of time, but add ADP and TEV protease day-of to pre-chilled buffers. Ideally, do not keep reducing agent (+TCEP) buffer for more than 1 week.
>
> Buffers do not contain protease inhibitors because they might inhibit DPP9 and affect NLRP1 binding. Work quickly, and always with cold buffers and/or on ice (unless otherwise specified)!
>
> TEV protease needs to be validated for high activity prior to use. Add more if necessary.
>
> All subsequent purification steps assume a pellet from 1L of cells. Scale accordingly.
>
> For purification of the VbP-bound complex, add 10 µM to all purification buffers.

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

> 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.

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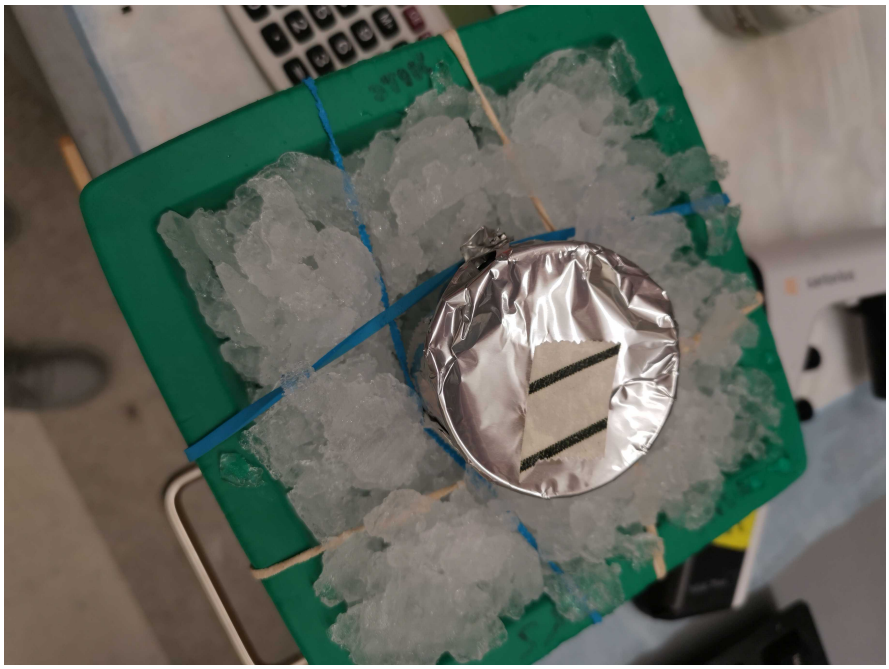
12 Transfer resuspended cells to a small glass beaker. Place beaker with cells in an ice-water bath and sonicate with a probe sonicator. 15m

For a 1L pellet: 2 s on 8 s off, 3.5 min total on, 40% power, Branson Sonicator.

Cell lysis can be checked for completion under a microscope.



Example of an ice-water bath for probe sonication.

13 Transfer to ultracentrifuge tubes and carefully balance the rotor. 1h

40,000 RPM for 1 h (45 Ti fixed-angle rotor, Beckman) or similar. **40000 rpm, 4°C, 01:00:00**

14 While centrifuging, pre-equilibrate M2 flag beads with lysis buffer (1 mL bed volume beads per 1L expression volume) in cold lysis buffer. 15m

CV = column volumes

15 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.

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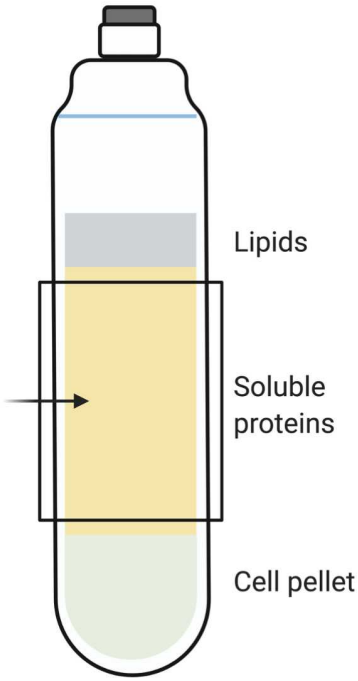

16 Transfer M2 flag beads between all 50-mL falcon tube(s). 1m

17 Fill falcon tubes with cold lysis buffer to avoid bubbles while rocking. Nutate/gently rock in the cold room for 4 h. 4h

4 °C .

Do not stop here and leave the protein overnight. It will aggregate and it's prone to nonspecific protease digestion.

18 Wash a MonoQ column with 10 mL water, 10 mL high salt buffer (pump B), then 10 mL low salt buffer (pump A) to equilibrate it for later. Keep column and buffers refrigerated.

19 Centrifuge falcon tubes gently to pellet resin. 5m

800 x g, 4°C, 00:05:00

20 Remove supernatant. Add 20 CV lysis buffer and centrifuge again. 5m

800 x g, 4°C, 00:05:00

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The flag beads should be very green at this stage or there were expression issues.

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

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

23 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. 1h

Cleave off the TEV-GFP-FLAG tag by incubating the resin with 5-CV (5 mL) on-column cleavage buffer as soon as the column is stopped up. Gently agitate the resin within the column then let stand. 1 h @ RT **01:00:00** . Save a little buffer to rinse the column after elution to get as much protein off of the column as possible.

You should add the freshly-thawed ADP and TEV protease to the buffer immediately before on-column cleavage.

The resin will stay green (TEV-GFP-FLAG left behind). I recommend mixing everything, taking a 10-uL sample, and immediately quenching the recovered sample with SDS-PAGE buffer. You can mix and take samples in this fashion every ~15 minutes. Run out the full protein time course to on an SDS-PAGE gel to confirm complete cleavage by ~1 h, and adjust amount of TEV or the cleavage time in future protein preparations.

24 Elute from the column and collect in 1.5 mL microcentrifuge tubes. Use the remaining ~0.5 mL buffer to wash the remaining protein off of the column. 10m

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

26 Take a 10 uL sample and add to 4X SDS sample buffer for a later SDS-PAGE gel. 1m

27 ⚠ 20m

Load all protein onto a MonoQ 5/50 GL column and begin anion exchange. Use a 5-mL loop, or inject multiple times with smaller loops.

Program: 5 mL load, 5 CV wash, 0 to 100% pump B (high salt buffer) over 15 CV, final wash with 5 CV. Collect 0.5 mL fractions.

Note: NLRP1 aggregates on all size exclusion columns I have tried (Superose 6, Superdex 200). Anion exchange

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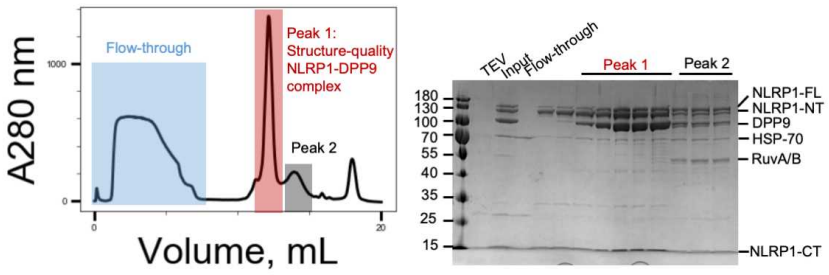
yields very clean, homogenous protein that is not aggregated, check yourself under negative stain!

28 Take the fractions with major peaks and run an SDS-PAGE to confirm protein identity. 1h



Expected anion exchange profile and SDS-PAGE gel. Some HSP70 contaminantion is standard and was not resolved with ATP washing on-column. RuvA/B contamination in the second peak occured in every NLRP1-DPP9 purification (N>10). Proteins were identified by western blot and/or mass spectrometry.

29 Measure the concentration (nanodrop A280) of fractions of note and record. Concentrate or dilute desired fractions. For cryo-EM, 0.5-0.6 mg/mL works well. 20m

30 Add 0.2 mM ADP and 5 mM $MgCl_2$ (final concentration)--ideally, use concentrated stocks to avoid sample dilution. 1m

Nanodrop concentration will not be accurate after ADP addition.

Note: For structure, I pool the 2 peak fractions, which are generally around the same concentration. If one peak fraction is much higher concentration/A280 than the other, proceed with this single fraction without combining.

31 Dialyze protein into Dialysis Buffer using a Slide-a-lyzer (ThermoFisher 66384). NOTE: this is only necessary if crosslinking is required for downstream applications, e.g. for cryo-EM. 6h

This requires a buffer change after 2 hr followed by further dialysis (4 h to overnight). However, it's usually late at this point so I generally dialyze overnight and change the buffer in the am. I'll give it 4 h following buffer change before making cryo-EM grids or using the protein for other purposes. Be careful with the buffer exchange, particularly when handling the membrane.

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In vitro assays

32 Carefully remove dialyzed protein, proceed with downstream application.

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