Insect Cell Protocol for LRRK1 and LRRK2 Expression

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Insect Cell Protocol for LRRK1 and LRRK2 Expression

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Protocol for expressing LRRK1 and LRRK2 in insect cells.

As used in Snead, Matyszewski, Dickey et al. 2022.

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Materials

Starting Insect Cell Culture from frozen stock:

- Sf900 cell stock
- SF900-II Serum media

Preparing genomic DNA using chemical transformation to transfect insect cell:

- DH10EmBacY Cells
- Qiagen Miniprep Kit
- Isopropanol; Ethanol

Transfecting insect cell using Fugene Transfection Reagent (generating V0):

- Fugene Transfection Reagent
- insect cell media

Starting Insect Cell Culture from frozen stock 5m

1

- Frozen stocks of sf900 cells contain DMSO, which needs to be removed and replaced with **SF900-II Serum media.** The Cells are started at high-density (~2 x 10^6 cells/ml) until they start to divide. Subsequently, cultures are maintained at **2-4 x 10^6 cells/ml** to keep them in log phase growth. Only split cells that are between **2-5 x 10^6 cells/ml to 1 x 10^6 cells/ml**.
- For growth over the weekend, dilution to 0.7 x 10^6 cells/ml on Friday pm and splitting Monday am can work well. Ideally, cells should double every 24-30 hour. Fresh cultures should be started about every 2 months.

Pre-chill centrifuge to **4 °C** .

2 Retrieve 1 vials containing **1 mL** of ~2x10^7 SF9 cells from liquid N_2 storage.

3 Thaw vial in hands until ice pellet just disappears, then spray the outside of the tube with 70% EtOH and place in biosafety cabinet, keep everything sterile

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4 

Transfer vial of cells (~ **1 mL** total) to 15-ml tube, then add **10 mL** SF900 II media.

5 

5m

Spin at **1000 rpm, 4°C, 00:05:00** .

6 

Remove supernatant, add fresh media to **10 mL** and transfer to 125-ml flask.

7 Move flask to sticky shaker **90 rpm, 27°C** .

8 Let it grow for 3-4 days. Count the cells to check concentration.

9 Once cells have started dividing and reached >2 x 10^6, add fresh medium and split to ~1.5 x 10^6 cells/ml.

10 Transfer cells to 500-mL shaking flask with a volume of at least **100 mL** when splitting.

11 Maintain cells so that they are around 1-2 x 10^6 cells/ml.

Preparing genomic DNA using chemical transformation to transfect insect cell

13h 10m 15s

12 

30m

Transform **50 µL** of DH10EmBacY chemical-competent cells with **100 ng** of your

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miniprepped plasmid DNA. Incubate **On ice** for **00:30:00** .

13 Heat shock at **42 °C** for **00:00:15** . 15s

14 Chill immediately **On ice** for **00:02:00** . 2m

15 

Add **1 mL** of SOC medium (**Room temperature**) and transfer to 14-mL falcon tube.

16  5h 2m

Shake cells at **220 rpm, 37°C** **Overnight** (or at least for **05:00:00**).

17 Plate **12 µL** , **20 µL** and **200 µL** of the transformation on LB-Kan/Gen/Tet +IPTG +BluoGal Plate.

18 Wait 2 - 3 days for WHITE colonies to appear. Color change will not happen until day 2 or so.

19  5h

Screen the white colonies for the presence of all the chains using colony PCR (skip if you are expressing one protein). Grow at least 3 colonies **Overnight** in **6 mL** LB culture containing **50 µg/ml** of kanamycin, **7 µg/ml** of gentamicin and **10 µg/ml** of tetracycline and additional antibiotics in plasmid.

20  5m

Spin down overnight culture in floor centrifuge for **3500 rpm, 00:05:00** .

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21 Resuspend cell pellets in **300 µL** of buffer P1 from the Qiagen miniprep kit, and transfer to a microfuge tube.

22  5m

Add **300 µL** buffer P2 to each tube. Incubate for **00:05:00** .

23  6m

Add **400 µL** chilled buffer P3 to each tube. Incubate **On ice** for **00:06:00** .

24  10m

Centrifuge for **00:10:00** at maximum speed at **4 °C** .

25  10m

Transfer supernatant to a new centrifuge tube and centrifuge for **00:10:00** at max speed at **4 °C** .

26 

While centrifuging, add **800 µL** ice-cold isopropanol to STERILE 2-mL Eppendorf tube.

> **NOTE**: This tube must be sterile since the contents of the tube will be added to insect cells.

27  1h

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After the spin is done, remove supernatant and add into 2-mL Eppendorf tube containing **800 µL** isopropanol. Incubate for **01:00:00** **On ice** .

28  10m

Spin at maximum speed for **00:10:00** at **4 °C** . Remove supernatant.

29 

Wash pellet with **800 µL** cold (**-20 °C**) 70% ethanol, invert tube, spin at maximum speed for minutes at **4 °C** .

30 

Remove ethanol, repeat ethanol wash.

30.1 Wash pellet with **800 µL** cold (**-20 °C**) 70% ethanol. (1/2)

30.2 Wash pellet with **800 µL** cold (**-20 °C**) 70% ethanol. (2/2)

31 After third wash, remove supernatant and transfer microfuge tube containing precipitated DNA to the hood. Leave cap off of tube and let evaporate for > **00:20:00** . 50m

(I usually leave for **00:30:00** just to be extra.)

32 

Resuspend the pellet with **30 µL** to **50 µL** of nuclease free water and gently flick to mix.

Note: Do this in the tissue culture hood.

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33 Measure the concentration using nanodrop.

If you can't proceed to transfection, store the DNA in **4 °C** for up to 2 weeks.

Transfecting insect cell using Fugene Transfection Reagent (generating V0) 10m

10m

34

Note:
- Everything involving insect cells should be done in the tissue culture hood to avoid contamination.
- There is no antibiotics in the media.
- keep everything as sterile as possible.

Prepare **4 mL** of insect cells at 0.5 x10^6 for each construct. Distribute **2 mL** each into two 6-well plate. Leave it at **Room temperature** for at least **00:10:00** for cells to adhere.

35 

Allow the vial of Fugene Transfection Reagent to reach **Room temperature** .
Mix by inverting so that there is no precipitate.

36 

To a total volume of **426.8 µL** , add **4.4 µg** of bacmid DNA (so **2 µg** final in each well). For DNA concentration of **1 µg/µL** ,

- **4.4 µL** of **1 µg/µL** DNA
- **422.4 µL** of insect cell media

37 

15m

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To the above DNA in insect cell media, add **13.2 µL** of **Room temperature** Fugene Transfection Reagent. Add the transfection reagent directly to the middle of media without touching the side the tube. Mix carefully by tapping at least 10 times. Incubate **00:15:00** at **Room temperature** .

38 

10m

About **00:10:00** of incubation Fugene and DNA, remove media from the well. Add fresh of medium to the cells.

Be careful as to not disturb the cells at the bottom of the wells.

39 

Add **200 µL** of solution from Step 35 dropwise to each well containing **0.8 mL** cells for a given baculovirus. This will result in **2 µg** of DNA per well and **6 µL** of Fugene (hence 3:1 Transfection Reagent:DNA ratio). Swirl the plates gently to mix.

Note: This step needs optimization. Vary the amount of DNA and the ratio of transfection reagent:DNA.

40 

1d

Incubate in **27 °C** insect cell incubator for **24:00:00** .

41 After 24 hours, add additional **1 mL** of medium and incubate for 2 more days at **27 °C** .

42 Check the transfection using YFP signal. If cells are more than 30-50% transfected (expressing YFP) , harvest the supernatant within the wells, spin for **1000 rpm, 4°C, 00:05:00** , and store the supernatant (v0) at **4 °C** in the dark.

5m

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Subsequent baculovirus generation (V1 onwards) 20m

43 

For subsequent virus generation beyond V0, previous virus generation is added at 1-2% of the total volume of the culture.

V1 = **50 mL** total: use **1 mL** of V0

V2 = **400 mL** total: use **4 mL** of V1

(Can be used to harvest cells for protein purification from this point)

Add 1-2% the total volume of previous virus generation to the flask with insect cells at $1x10^6$.

44 Also, set up control flask to compare.

45 

Incubate in **27 °C** sticky shaker for **90 rpm, 72:00:00** .

46 After 3 days, *assess quality of baculoviruses by visualizing cells .*

- *The transfected cells should swell ~20-100% larger than the control.*
- *Express YFP signal; at this point, 100% of the cells should express YFP.*
- *Cells that are transfected should be less concentrated than control after 3 days.*

47 Harvest virus only if the cells show YFP signal and swelling. Harvest the supernatant, Spin for 5m **1000 rpm, 4°C, 00:05:00** , and store the supernatant (virus) at **4 °C** in the dark.

Virus can be stored for several months. (about half a year for v0, and a year for v1)

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48 To check for protein production and virus stability, save **100 µL of 1x10^6 culture (for culture, use less volume accordingly) for gel**, spin it down max speed for **00:05:00** , discard supernatant. Resuspend cell pellet in **15 µL** of 4x sample buffer, **6 µL** of 10x reducing agent and **39 µL** water and boil for **00:10:00** at **95 °C** . Load **10 µL** into the gel. 15m

Harvesting Large Insect Cell Culture for Purification 32m

49

This is the V2 stage as described above in 400-mL tubes; For LRRK2 purification, grow 3x 400 mL growths or 2x 600 mL growths. Harvest after 3 days.

Divide the cell culture into appropriate conical tubes (15 mL or 50 mL) or JA10 tube (#355605, max volume 465 mL).

50  22m

Spin at **3500 rpm, 00:05:00** (for conical tubes) or for **00:17:00** (for JA10 tubes) at **4 °C** .

51 Pour off supernatant into a container with bleach.

52 

Add PBS (~ **10 mL** for initial **400 mL** culture) to one of the conical tubes. Resuspend pellets in PBS and store in conical tube.

52.1 

Repeat the above step until all cell pellets are resuspended (we usually do 1x 50 mL conical tube for each **400 mL** growth for ease of protein purification down the road).

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53 10m

Spin at **3500 rpm, 4°C, 00:10:00** .

54 Pour off supernatant.

55 Label the tube with virus numbers, date harvested and initial cell culture volume.

56 Flash liquid nitrogen and store the pellets at **-80 °C** .

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