Cell Culture Transfection for Production and Purification of Wnt Ligands

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[Abstract] Wnt ligand proteins are extremely difficult to purify and enrich *in vitro*. This protocol uses Wnt11r protein as an example to illustrate how to use 293T cells to produce secreted Wnt11r and collect it *in vitro* for additional downstream biochemical experiments.

Materials and Reagents

1. 293T cells
2. 0.1 M glycine-HCl (pH 3.5)
3. 0.5 M Tris-HCl
4. Fetal bovine serum (FBS) (Life Technologies, Invitrogen™, catalog number: 10438-026)
5. Dulbecco's modified eagle medium (DMEM) (high glucose) (Life Technologies, Invitrogen™, catalog number: 11965)
6. Trypsin EDTA (Life Technologies, Invitrogen™, catalog number: 15050-065)
7. PBS buffer (Life Technologies, Invitrogen™, catalog number: 14040-182)
8. Anti-FLAG M2 (Sigma-Aldrich, catalog number: A2220)
9. Pen/Strep (Sigma-Aldrich, catalog number: P4333)
10. Effectene transfection regent (QIAGEN, catalog number: 301425)
11. Media (see Recipes)

Equipment

1. Standard tabletop centrifuges
2. 37 °C, 5% CO_2 incubator
3. Water bath
4. 50 ml conical tubes
5. Nitrocellulose membrane
6. 100 mm Petri dishes

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Procedure

A. Split 293T cells:
1. Warm media and PBS buffer in 37 °C water bath and 0.05% Trypsin EDTA at RT. Get one 50 ml conical tube, and a 100 mm dish to pass cells.
2. Remove old medium by aspiration. Add 10 ml PBS buffer to rinse the cells. Gently shake the dish. Remove Hass buffer by aspiration.
3. Add 1 ml 0.05% Trypsin-EDTA to 100 mm dish confluent with 293T cells. Incubate at 37 °C for 1 min. Gently rock dish. Cells should come off from the dish.
4. Add 9 ml media and gently pipet up and down to dissociate cells from plate.
5. Once dissociated, pour into 50 ml conical tube.
6. Centrifuge cells at 1,000 rpm for 3-4 min and then remove the supernatant with vacuum-attached Pasteur pipet.
7. Add 10 ml medium and pipet up and down to resusupend cells.
8. Add 9 ml fresh media and 1 ml of resuspended cells to a fresh dish. This dish will become confluent by day 5.
   Note: You can also do a 1:5 and 1:20 dilution, in which the cells will become confluent at day 3 and day 7 respectively.
9. Grow cells in 37 °C, 5% CO_2 incubator. Check the media 3 days later, if the media turns yellowish, change the media.

B. Seed the cells and transfection:
1. Split the cells at 1:10 ratio as steps 1-6 in 100 mm dish.
2. Grow cells for 16-24 h. The cells will become 30-40% confluent.
3. Warm media and Hass buffer at 37 °C. Mix DNA (Wnt11r expression construct with a FLAG tag) in the hood. Follow the transfection protocol from Qiagen:
   a. Mix 8 μg DNA in 300 μl EC buffer.
   b. Add 64 μl enhancer and mix well by pipetting. Incubate in the hood for 5 min.
   c. Add 60 μl for effectene and mix well and incubate for 10 min.
   d. Add 3,000 μl media to the mix.
      Note: The amounts above are for transfection in one 100 mm dish.
   e. During the incubation, remove the old media from the cells and rinse once by 10 ml PBS buffer.
   f. Add 7 ml fresh media to the cells. When the incubation is over, add the mix (total 3,424 μl: 300 μl+64 μl+60 μl+3,000 μl) to the dish. Gently shake the dish.
   g. Grow the cells in 37 °C, CO_2 incubator.
4. Day 2: Remove transfection media from dish.

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5. Add another 10 ml fresh media (slowly pipet media along edge; cells will dislodge by addition of media by pipet). Grow for 3-4 days.
   Note: This step is completely optional. The cells grow very well in effectene transfection media.

C. Collection of Wnt11r-FLAG supernatant:
1. After 4 days of growth in transfection media collect media in 15 ml conical tube. Spin 5 min at 1,000 rpm.
2. Filter through a nitrocellulose membrane (0.2 mm). According to literature, wnt ligands can be stored in medium for several months at 4 °C without loss of activity. Not clear if this is true for wnt11r.
   Optional: Aliquot supernatant in 1 ml aliquots and store in -80 °C.
3. Pull-down the wnt11r-FLAG proteins by anti-FLAG beads:
   a. Thoroughly susupend the anti-FLAG M2 gel in the vial. The ratio of suspension to packed gel volume is 2:1 (*i.e.* if you want to get 20 μl packed beads, take 40 μl out from the vial using the wide-end tips).
   b. Clean the anti-FLAG beads following the manufacture's directions.
   c. Wash the beads 4 times using cold TBS buffer (500 μl for 40 μl of gel suspension).
   d. Centrifuge the beads at 5,000-8,000 *x g* for 30 sec. Wait for 1-2 min before handling the samples. Remove the supernatant with a narrow-end pipet tip. Be careful not to transfer any beads.
   e. Wash the beads with 0.1 M glycine-HCl pH 3.5 to reduce the traces of unbound anti-FLAG antibody from the suspension.
      Note: Do not leave the beads in glycine HCl for longer than 20 min.
   f. Wash the beads again with TBS buffer for total 3 times.
   g. Add wnt11r-containing medium to the beads. Leave the tubes on the wheel at 4 °C O/N.
      Optional: You can add proteinase inhibitors to the medium before adding them to the beads.
      Note: Every step above should be performed on ice or at 4 °C.
4. Elute the wnt11r-FLAG proteins using glycine-HCl buffer:
   a. Centrifuge the beads and remove the medium with a narrow-end pipet tip.
   b. Wash the beads 3 times with TBS buffer. Make sure all the supernanant is removed.
   c. Elute wnt11r-FLAG proteins with 0.1 M glycine-HCl.
   d. Add 100 μl of 0.1 M glycine-HCl buffer to the beads. Incubate the samples with gentle shaking at RT for 5 min.
   e. Centrifuge at 5,000-8,000 *x g* for 30 sec.

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   f. Transfer the supernatant to a fresh tubes containing 10 µl of 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. Be careful not to transfer any beads.
   g. For immediate use, store the supernatant at 4 °C. Store at -20 °C for long-term storage.
5. Determine the amount of protein in the elution buffer:
   a. Take 10 µl of eluted proteins and add 10 µl 2x SDS sample buffer. Boil for 5 min and then run a SDS-PAGE gel. For markers, run BSA with different amounts in each lane. Stain the gel with G-blue and then determine the amount of protein present in 10 µl elution.
   b. The size of wnt11r-FLAG is 44 kDa.

Recipes

1. Media
   DMEM
   10%FBS
   1%Pen/Strep

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References

1. Jing, L., Lefebvre, J. L., Gordon, L. R. and Granato, M. (2009). Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/MuSK receptor. *Neuron* 61(5): 721-733.

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