Conditioned Medium Secreted Cell line Culture Protocols

Materials:

<table>
<thead>
<tr>
<th>Reagent name</th>
<th>Supplier</th>
<th>Cat No.</th>
<th>Stock solution</th>
<th>Final Conc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Advanced DMEM/F12</td>
<td>Invitrogen</td>
<td>12634-028</td>
<td></td>
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<tr>
<td>DMEM</td>
<td>Invitrogen</td>
<td>11995-073</td>
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<td>FBS</td>
<td>Invitrogen</td>
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<tr>
<td>GlutaMAX-I</td>
<td>Invitrogen</td>
<td>35050-061</td>
<td>100X</td>
<td>1X</td>
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<tr>
<td>G418</td>
<td>Invitrogen</td>
<td>10131035</td>
<td>50mg/ml</td>
<td>0.4mg/ml</td>
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<td>Zeocin</td>
<td>Invitrogen</td>
<td>45-0430</td>
<td>100mg/ml</td>
<td>300ug/ml</td>
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<td>Puromycin</td>
<td>Invitrogen</td>
<td>A11138-03</td>
<td>10mg/ml</td>
<td>10ug/ml</td>
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<td>Trypsin-EDTA</td>
<td>Invitrogen</td>
<td>25200-056</td>
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</table>

Equipment

Flask T75 (Corning #430641U)

Centrifuge

Medium

Growth Medium

L-Wnt 3a: DMEM+10% FBS +G-418 (stock is 50mg/ml, add 400ul into 50ml medium, final 0.4mg/ml)

R-Spondin: DMEM+10% FBS +Zeocin (stock is 100mg/ml, add 150ul into 50ml medium, final 300ug/ml)

Noggin: DMEM+10% FBS +puromycin (stock is 10mg/ml, add 50ul into 50ml medium, final 10ug/ml)

Medium for making conditioned medium

Advanced DMEM/F12+1xGlutamax+10%FBS
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**ATCC L-Wnt-3A Conditioned Medium:**

1. Thaw a vial of ATCC L-Wnt3A cell from liquid nitrogen storage.

2. Divide one vial of frozen cells into 4 x T75 flasks each containing 15ml of L-Wnt3a growth medium. After 2-3 days, when cells are confluent, split 1:10, add 15ml **Growth Medium** in 1xT75 and 10ml **medium for conditioned medium** in 9xT75

3. For 9 x T75 flasks, harvest medium after 4 days culture. Take off the medium, centrifuge at 1000g and sterile filter (0.22uM filter). This is the first batch of medium.

4. Add 10ml fresh **medium for conditioned medium** to the 9 flasks and culture for another 3 days.

5. Take off the medium, centrifuge at 1000g and sterile filter. This is the second batch of medium. Discard the cells, because they will be overgrown.

6. Mix the first batch and second batch of medium 1:1. This is the Wnt3A conditioned medium.

7. Aliquot 25ml per 50ml conical tube and store at -80c.

**R-Spondin Conditioned Medium:**

1. Thaw a vial of 293T-HA-Rspol-Fc cells (originally generated from Dr. Calvin Kuo’s lab at Stanford U. Medical School - MTA required) from liquid nitrogen storage

2. Divide one vial of frozen cells into 4 x T75 flasks containing 15ml of growth medium for R-Spondin. After 2-3 days, when cells are confluent, split 1:5. Add 15ml media containing zeocin to 1 x T75 flask and 15 ml media without zeocin to 4 x T75 flasks. Culture for another 2-3 days until confluent.

3. Trypsinize 4 x T75 flasks without Zeocin with 1:3 diluted Trypsin-EDTA in PBS (from 0.25% Trypsin-EDTA, at room temperature for approximately 30 second or until cell start detach from the flask) neutralize with DMEM+10% FBS and centrifuge at 200g. Remove the trypsin and medium, divide the cell pellet into 10 x T75 flasks each containing 20ml medium for making conditioned medium.

4. Harvest after one week. Pool medium from all flasks into centrifuge tube, centrifuge at 1000g for 10 minutes and filter supernatant through .22uM filter.

5. Aliquot 10ml per 15ml tube and store at -80c.
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6. 1xT75 with media containing Zeocin from step 2, passage as described in steps 3-5.

**Note:** when you trypsinize 293T-HA-Rspol-Fc cells, use original (0.25%) trypsin 1:3 diluted with PBS (-Mg, -Ca).

Noggin Conditioned Medium:


2. Divide one vial of frozen cells into 4 x T75 flasks containing 15ml of growth medium for Noggin. After 2-3 days or when cells reach confluency, split 1:5. Add 15ml media containing puromycin to 1 x T75 flask and 15 ml media without puromycin to 4xT75. Culture for another 2-3 days until confluent.

3. Trypsinize 4 x T75 flasks without puromycin with 1:3 diluted Trypsin-EDTA in PBS (from 0.25% Trypsin-EDTA, at room temperature for approximately 30 second or until cell start detach from the flask) neutralize with DMEM+10% FBS and centrifuge at 200g. Remove the trypsin and medium, divide the cell pellet into 10 x T75 flasks each containing 20ml medium for making conditioned medium.

4. Harvest after one week. Pour all medium into centrifuge tube, centrifuge at 1000g for 10 minutes, and filter the medium through .22uM filter.

5. Aliquot at 10ml per 15ml tube and store at -80c.

6. 1xT75 with medium containing puromycin from step 2, passage as described in steps 3-5.

**Note:** when trypsinizing 293 Noggin cells, use original (0.25%) trypsin 1:3 diluted with PBS (-Mg, -Ca), otherwise the original 0.25% trypsin is too strong for cell and the cells won’t grow well.