TOPFLASH ASSAY

THIS PROTOCOL PROVIDES THE PROCEDURE FOR ASSESSING THE BIOLOGICAL ACTIVITY OF R-SPONDIN1 CONDITIONED MEDIUM. BASED ON HTTP://WWW.BIO-PROTOCOL.ORG/E1183

EQUIPMENT
1. Cell culture incubator
2. Biological hood
3. Pipette-aid
4. 2-20 µl pipette
5. 20-200 µl pipette
6. 200-1000 µl pipette
7. 37˚C water bath
8. Inverted microscope
9. BioRad TC10 Automated cell counter
10. Swinging bucket centrifuge for conical tubes 15-50ml
11. Plate reader for luminescence absorbance
12. Orbital shaker

MATERIALS
1. T25 flask
2. T75 flask
3. DMEM, high glucose medium (Thermo Fisher, cat# 11965084)
4. Advanced DMEM/F12 medium (Thermo Fisher, cat#12634-010)
5. FBS
6. GlutaMAX supplement (Thermo Fisher, cat# 35050061)
7. 0.05% Trypsin-EDTA
8. 1X PBS (Tissue Culture Grade)
9. Trypan Blue
10. 15 and 50 ml conical tubes
11. 1.7 ml microtubes
12. 500/250ml, 0.1 µm Filter Unit
13. Gloves
14. P200 and P1000 µl tips
15. 2. 5 ml, 10 ml, 25 ml pipettes
16. HEK293 cells (ATCC, CRL-1573)
17. HA-R-spondin1-Fc conditioned medium (HA-R-Spondin1-Fc HEK293T cell line, Trevigen, cat# 3710-001-01)
18. L Wnt-3A conditioned medium (ATCC, CRL-2647)
19. Turbofect (Thermo Fisher, cat# R0532)
20. Dual-Glo Assay System (Promega, cat# E2920)
21. pSTF (super top flash plasmid, available on Addgene https://www.addgene.org/12456/; and Millipore cat# 21-170)
22. pRL (renilla luciferase, any renilla expressing vector for mammalian cells should work)
PROCEDURE

Background:

The TopFlash assay is a luciferase reporter assay, and it can be used to monitor the concentration of both Wnt and R-spondin in conditioned media. The assay begins when 2 luciferase expressing plasmids (one of inducible and the other of constitutive expression) are transfected into HEK293 cells. Then cells are exposed to medium containing either Wnt or R-spondin alone or a combination of both. After 48 hours of induction, luciferase activity is read and the amount of growth factors present in the conditioned media is compared to a known source of protein. Endogenously, and upon Wnt activation, beta-catenin is stabilized and joins TCF inside the nucleus. Together they bind to TCF sites and induce the expression of Wnt target genes. pSTF plasmid (STF: SuperTopFlash) includes 8 of these TCF binding sites which control the expression of a firefly luciferase. On the other hand, pRL contains the coding sequence of the renilla luciferase and is used as a normalizer. With this assay you should be able to test both Wnt and R-spondin activity.

Thaw HEK293 cells.

1. Prepare growth medium (DMEM 10% FBS + glutamine) cell culture medium and filter sterilize through a 0.1µm filter.
2. Remove 1 vial of HEK293 cells.
3. Thaw frozen cells for 2 minutes in a 37°C water bath.
4. Transfer the thawed cells to a 15 ml conical tube.
5. Rinse cryovial with 1 ml of growth medium, and transfer to the 15 ml conical tube containing cells. Gently pipet up and down three times to suspend cells.
6. Centrifuge cell suspension at 200 X g for 3 minutes.
7. Aspirate supernatant gently to avoid disturbing cell pellet.
8. Resuspend cell pellet in 6 ml of fresh Complete Growth Medium and transfer it into T-25 cell culture flask.
9. Transfer the T25 Flask the 37°C tissue culture incubator.
10. Passage cells at least once before transfection.

Transfection of HEK293 cells with TopFlash plasmids.

Day 0.
1. Discard growth medium and wash cells with 5 ml of 1X PBS.
2. Discard PBS and detach cells using 1 ml of 0.05% Trypsin-EDTA. Stop reaction by adding 1 ml of growth medium.
3. Centrifuge cell suspension at 200 X g for 3 minutes. Discard supernatant and resuspend cell pellet in 1 ml of growth medium.
4. Count cells and seed 1.25 x 10^5 cells per well of a 24-well plate. Dilute cells in growth medium if necessary. The final volume in each well should be 0.5 ml. Note: avoid using antibiotics in growth medium as they interfere with transfection.

Day 1. Transfection.
5. Calculate volume needed to transfect all wells (See Table 1). Pipette first serum free DMEM, then pSTF and pRL plasmids and mix well. Add Turbofect and mix vigorously immediately after. Incubate the transfection reaction mix for 15 minutes at room temperature.
6. Dispense 50 µl in each well drop by drop. Gently, shake plate from side to side to mix medium and transfection complexes together.
7. Transfer cells to cell incubator.
8. Change transfection medium to growth medium after 4-6 hours. Note: transfection complexes can be left in cell culture medium overnight.

Table 1. Transfection reaction mix for one well of a 24-well plate

<table>
<thead>
<tr>
<th>Materials</th>
<th>Amount needed for one well of a 24-well plate</th>
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<tbody>
<tr>
<td>DMEM</td>
<td>60 µl</td>
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<tr>
<td>pSTF (SuperTopFlash)</td>
<td>400 ng</td>
</tr>
<tr>
<td>pRL (Renilla luciferase)</td>
<td>25 ng</td>
</tr>
<tr>
<td>Turbofect</td>
<td>0.8 µl</td>
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Evaluate conditioned medium performance by measuring luciferase activity.

Day 2.
1. 24 hours post-transfection, change the medium to conditioned medium. Prepare the different conditioned media to be tested.
2. Remove growth medium and replace with 0.5 ml of each conditioned media tested per well.
3. Transfer plate to the 37 °C tissue culture incubator for 24-48 hours.

Day 5.
4. Discard Medium in each wells and wash gently with 0.5 ml of 1X PBS, discard.
5. Use Dual-Glo luciferase kit and follow manufacturer’s instructions to measure both firefly and renilla luciferase activities.
6. Analyze data: normalize firefly counts with renilla counts. Normalize with basal controls and titrate comparing your conditioned medium with a known source of each growth factor.