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Production of R-Spondin 1 conditioned medium using Cultrex® Rspo1 cells.

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Introduction

Roof plate-specific Spondin-1 (R-Spondin 1 or Rspo1), also known as CRISTIN3, is a 27 kDa secreted activator protein that belongs to the R-Spondin family. Rspo1 positively regulate Wnt/beta-catenin signaling, most likely by acting as a ligand for LGR4-6 receptors and an inhibitor for ZNRF3. Rspo1 induces proliferation of intestinal crypt epithelial cells, increases intestinal epithelial healing, and supports intestinal epithelial stem cell renewal. Cultrex Rspo1 293T cell line is stably transfected to express murine Rspo1 with an N-terminal HA epitope tag and fused to a C-terminal murine IgG2a Fc fragment. This cell line is used to produce either purified Rspo1 or Rspo1 conditioned media.

The **murine** Rspo1 protein has been used extensively in organoid culture to maintain Lgr5+ stem cells (both in **human and murine** organoid cultures), and the Fc and HA tags make it easy to purify or characterize. Trevigen Organoid Resource Lab (ORL) has optimized a protocol to produce Rspo1 conditioned medium using Cultrex Rspo1 cells, as well as a functional assay to determine the biological activity of each batch of Rspo1 conditioned medium.

Protocol

A more detailed protocol on how to culture Rspo1 cells is included as part of the Cultrex Rspo1 Cells product manual (HA-R-Spondin1 293T cell line). *Note: we recommend to perform a trial run before producing the first high-volume conditioned medium batch (e.g. to an 80% confluent T25 flask add 5 ml of conditioning medium, and evaluate the expression of Rspo1 by western blot).*

Cultrex Rspo1 cells
Catalog No: 3710-001-K
Size: 0.5 ml, 1x10⁶ cells

1) Production of R-Spondin1 Conditioned Medium.

1.1) Thaw a vial of Cultrex Rspo1 cells and expand them to two T75 flasks. Keep cells for at least five days in Selection Growth Medium (DMEM, 10% FBS, 300 µg/ml Zeocin™).

1.2) Transfer cells to multi-layer flasks:

a) When the cells are 80% confluent, passage cells into three 3-layer (525 cm2) flasks. *Note: these multi-layered vessels are designed to conveniently culture more cells in less time. A 3-layer flask has a surface area of 525 cm2 and can produce 150-200 ml of conditioned medium, choose the right flask according to your own applications. Other options include 5- and 10-layer flasks.*

b) Aspirate medium from T75 flasks and rinse each flask with 10 ml of room temperature PBS, aspirate, and dispose.

c) Rinse T75 flask with 1 ml of Trypsin-EDTA, aspirate and dispose.

d) Add 600 µl of Trypsin-EDTA and incubate for a few minutes at 37 °C. Check for cell detachment.

d) Once cells have detached, add 2 ml of warm (37 °C) Selection Growth Medium. Hold the T75 flask upright, and rinse the culture surface with medium three times using a serological pipet.

e) Divide the medium containing cells into three 3-layer flasks.

f) Add 100 ml of warm (37 °C) Basal Growth Medium (DMEM, 10% FBS) to each flask, and pipet up and down to disperse cells, and transfer to the 37 °C tissue culture incubator.

1.3) Change medium to Conditioning Medium.

Inspect the cells in the multilayer flasks, and once the cells are 80% confluent, add conditioning medium.

a) Aspirate medium from the 3-layer flasks.

b) Add 100 ml of warm (37 °C) Conditioning medium (Advanced DMEM/F12 with glutamine) to each flask.

d) Culture cell for 10 days at 37 °C. After approximately 7 days, the cells will detach from the cell culture vessel and grow in suspension. They will continue to produce Rspo1.

e) Collect the supernatant, and centrifuge at 3,000 rpm for 15 minutes at 4 °C to remove cells and debris.

f) Filter the supernatant through 0.22 µm filter at 4 °C.

g) Verify the presence of HA-R-Spondin1-Fc in the medium by western blot, see Figure 1.

h) To assess the activity of the conditioned medium perform a TOPFlash assay, see Figure 3.

h) Concentrate Rspo1 Conditioned Medium if necessary and store at -80 °C.

2) Detection Rspo1 in Conditioned Medium by Western blot.

a) Transfer 1 ml of Rspo1 Conditioned Medium to a 15 ml conical tube.

b) Prepare sample by pipetting into a 1.7 microtube (to a final concentration): 1X SDS-PAGE loading buffer, 100 mM DTT; Rspo1 Conditioned Medium to amount for 50% of the final volume; add ddH20 to complete volume.

c) Mix samples and boil for 2 minutes.

d) Spin to collect the volume.

e) Load 4-10 µl of a pre-stained protein marker, 15 µl of each sample and negative control (Conditioning medium) into a 1 mm thick Tris-Glycine 4-20% pre-cast gel. Run samples.

f) Transfer proteins onto a PVDF membrane.

g) Block membrane for 1 hour at room temperature with LI-COR® Odyssey® Blocking Buffer.

h) Dilute primary antibody in Odyssey Blocking Buffer. Dilute anti-HA / Rspo1 / Fc to the appropriate concentration.

i) Incubate membrane with primary antibody overnight at 4°C.

j) Wash 3 times for 10 minutes with PBST.

k) Dilute IR secondary LI-COR antibody in Odyssey Blocking Buffer to the appropriate concentration.

l) Incubate membrane with secondary antibody for one hour at room temperature on an orbital shaker.

m) Wash 3 times with PBST.

n) Wash twice with PBS.

o) Visualize using LI-COR Odyssey. The expected size for the Rspo1 (HA-R-spondin1-Fc) protein is approximately 70-75 kDa, see Figure 1.

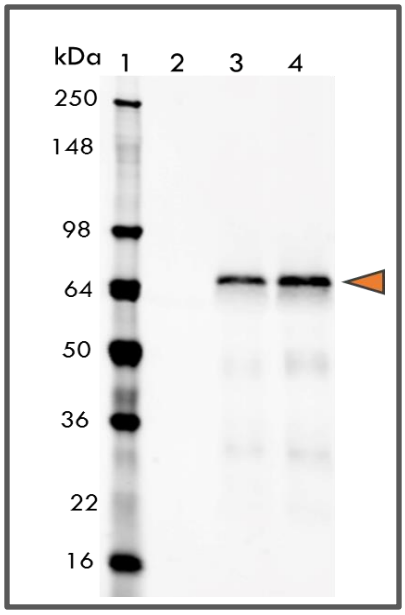

Figure 1. Western blot for HA-R-Spondin1-Fc. The image shows a Western blot against HA-tag (Cell Signaling Technology, catalog number 3724P, rabbit). The primary antibody was diluted 1 to 5000 and incubated overnight at 4°C, while the secondary anti-rabbit IR 680 was diluted 1 to 10000 and incubated for one hour at room temperature. LI-COR Odyssey infrared imager was used to visualize protein bands. Lane 1: Negative control, Conditioning Medium (Advanced DMEM/F12); lane 2: Rspo1 Conditioned Medium batch# 1; lane 3: Rspo1 Conditioned Medium batch# 2. Rspo1 is detected in two independent preparations of Rspo1 Conditioned Medium in lanes 2 and 3. The band corresponding to Rspo1 (orange arrow) has a relative molecular weight of 70-75 kDa.

3) TOPFlash Assay. The TOPFlash assay is a luciferase reporter assay, and it can be used to monitor the concentration of both Wnt and R-spondin in cell culture media. Two luciferase expressing plasmids (one of inducible and the other of constitutive expression) are first 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 previously known source as reference. 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. The construct used in this protocol is called pSTF (STF: SuperTOPFlash) and includes 8 of these TCF binding sites which control the expression of a firefly luciferase. Meanwhile, a renilla luciferase expressing plasmid (pRL) is used as a normalizer. A number of companies (as well as non-profit repository sites) offer both plasmids, along with a mutant control (also called FOPFlash).

3.1) Thaw and expand HEK293 cells. Passage cells at least once before transfection.

3.2) Transfection of HEK293 cells with pSTF and pRL plasmids.

a) Prepare enough cells to seed all data point in triplicate. Count cells and seed 1.25 x 10^5 cells per well of a 24-well plate, each well contains 0.5 ml of medium. *Note: avoid using antibiotics in growth medium as they interfere with transfection.*

b) After 24 hours prepare transfection mix:

Quantity per well:
50 µl serum free DMEM.
400 ng pSTF
25 ng pRL
0.8 µl Turbofect™

Pipette first serum-free DMEM, then pSTF and pRL plasmids and mix well. Add Turbofect and mix vigorously immediately after. Incubate the transfection mix for 15 minutes at room temperature.

c) Without changing medium, dispense 50 µl in each well drop by drop. Gently, shake plate from side to side to mix medium and transfection complexes together.

d) Transfer cells to the tissue culture incubator.

e) Change transfection medium to Growth medium (DMEM, 10% FBS) after 4-6 hours. *Note: transfection complexes can be left in cell culture medium overnight.*

3.3) Measure luciferase activity and analyze results.

a) 24 hours post-transfection, inspect cells and change the medium to conditioned medium. Prepare the different conditioned media to be tested. *Note: it is usually useful to evaluate conditioned media by making several serial dilutions.*

b) Remove Growth medium and replace with 0.5 ml of each conditioned media tested per well.
c) Transfer plate to the 37 °C tissue culture incubator for 48 hours.
d) Discard medium in each well and wash gently with 0.5 ml of 1X PBS, discard.
e) We recommend using Dual-Glo® Luciferase Assay System (Promega) to measure both firefly and renilla luciferase activities.
f) Analyze data: normalize firefly counts with renilla counts. Normalize once more with basal controls and titrate by comparing your conditioned medium to a known source of each growth factor. See Figures 2 and 3 for representative data analysis.

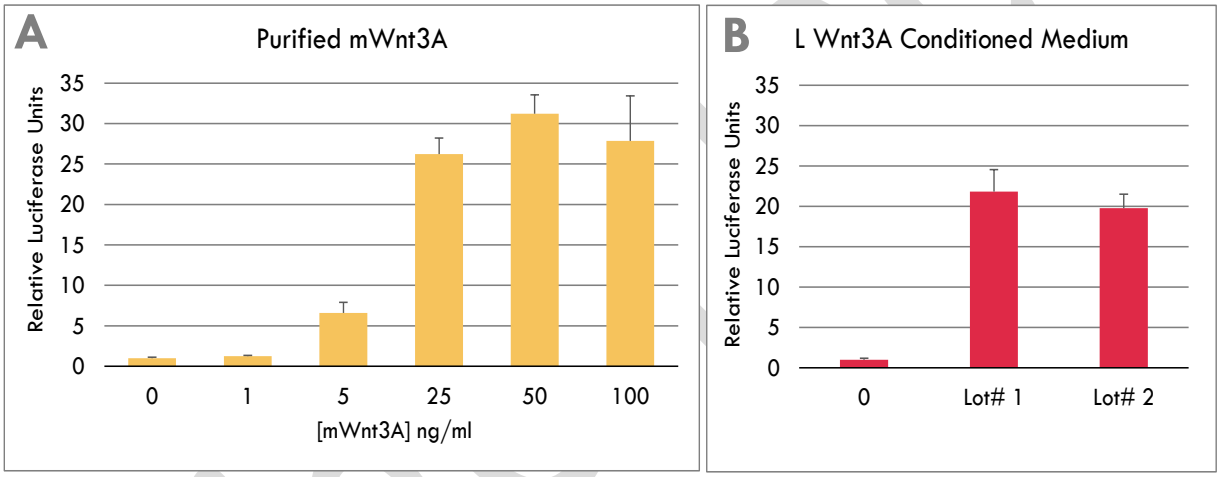

Figure 2. Titration of Wnt Conditioned Medium with the TOPFlash assay. A) Transfected HEK293 cells were exposed to different concentrations of recombinant murine Wnt3a (Time Bioscience), from 100 to 0 ng/ml diluted in Advanced DMEM/F12 with Glutamine. B) Two different preparations of L Wnt3a Conditioned Medium (produced with ATCC® CRL-2647™) were diluted 1 to 2 in Advanced DMEM/F12 with Glutamine. The dose curve presented in panel A serves as a reference to compare the activity of Wnt3A conditioned media.

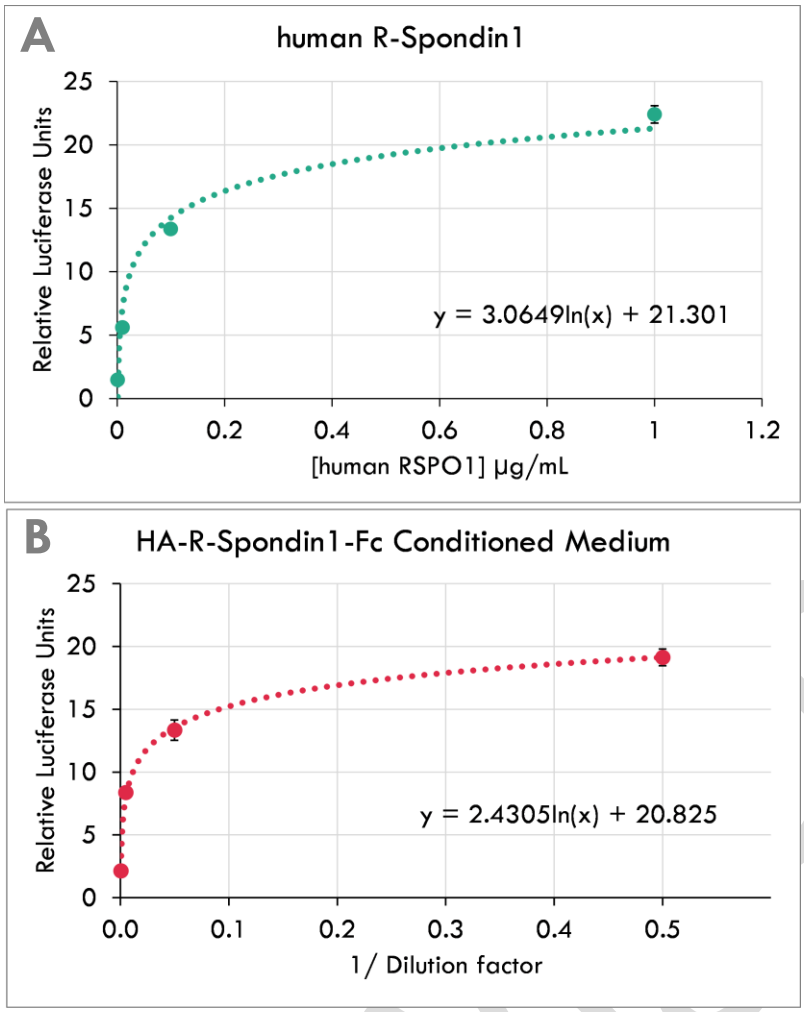

Figure 3. Titration of Rspo1 Conditioned Medium with the TOPFlash assay. A) As a reference and in order to titrate Rspo1 Conditioned Medium, serial dilutions of human R-Spondin1 (Peprotech) were used. Human Rspo1 was added to cell culture medium (Advanced DMEM/F12 with glutamine) containing Wnt3A and diluted in serial dilutions of 1, 0.1, 0.01, 0.001, and 0 µg/ml (the data point corresponding to 0 µg/ml was not included in the graph). B) Rspo1 Conditioned Medium prepared as indicated above and was diluted as in A) serial dilution of 1/2, 1/20, 1/200, and 1/2000.

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