Generation of RSPO1 Conditioned Media

Introduction

R-Spondin 1 (RSPO1) is a secreted protein within the R-Spondin family that modulates Wnt/beta-Catenin signaling. In vitro, RSPO1 is widely used to support stem cell maintenance, proliferation, and epithelial tissue homeostasis. In organoid and advanced 3D culture systems, RSPO1 is a critical component of niche-mimicking media formulations, particularly for intestinal, gastric, and other epithelial-derived models. It promotes expansion of $LGR5^+$ stem cells, enhances clonogenicity, and supports long-term culture by sustaining Wnt signaling activity in combination with Wnt ligands.

Previous studies indicate that conditioned media is preferred over lyophilized material when comparing organoid growth and viability.[1] 293T-RSPO1 (ATCC® CRL-3797™) is an adherent epithelial-like cell line derived from HEK293T cells and genetically engineered to express the mouse RSPO1 protein. This cell line can be used with the following protocol to generate RSPO1 conditioned media.

Table 1: Recommended reagents

Material	Supplier	Catalog number
293T-RSPO1	ATCC®	CRL-3797™
Dulbecco's Modified Eagle's Medium (DMEM)	ATCC®	30-2002™
Fetal Bovine Serum (FBS)	ATCC®	30-2020™
Dulbecco's Phosphate Buffered Saline (D-PBS)	ATCC®	30-2200™
Zeocin	Thermo Fisher Scientific	R25001
Advanced DMEM/F12	Gibco	12634-028
HEPES (1M)	Gibco	15630-080
GlutaMAX Supplement	Gibco	35050-061

Table 2: Suggested materials and equipment

Material	Supplier	Catalog number
5-layer CellSTACK Culture Chambers	Corning	3319
*Filtration capsules, Acropak	VWR	28143-969
*Platinum-lined tubing	Cole-Parmer	96420-17
*2L PETG Bottle	VWR	89132-054
*Peristaltic Pump	Argos Technology	77922-38
*Support stand	Fisher Scientific	14-675BQ
*Clamp	Fisher Scientific	02-300-211

Note: We recommend using the suggested equipment. Usage of a 0.2 µm PES bottle-filter system is likely acceptable, but we recommend performing a trial run in small scale prior to producing large volume conditioned media.

Preparing RSPO1 conditioned media for use in cell culture

Determine the specific volume of RSPO1 conditioned media (RSPO1 CM) necessary for culture. Specific media formulations and the required volume of RSPO1 CM can be found on product-specific pages on the ATCC website. The following protocol generates 2.2 L of conditioned media. **Note:** An optimal schedule diagram is shown in Figure 1.

Week 1				
Mon	Tues	Wed	Thurs	Fri
		Seeding, Selection		
Week 2				
Mon	Tues	Wed	Thurs	Fri
Rinse, Conditioning 1				Batch 1, Conditioning 2
Week 3				
Mon	Tues	Wed	Thurs	Fri
Batch 1, Conditioning 2				

Figure 1: Optimal schedule diagram.

Thawing, seeding, and antibiotic selection of 293T-RSPO1 (ATCC® CRL-3797™) cells

1. Thaw a vial of 293T-RSPO1 (ATCC® CRL-3797™) and expand to at least 16.08 x 10^6 cells following the cell handling information in the product page.
2. In a biological safety cabinet (BSC) under strict aseptic conditions, transfer 66 mL of DMEM (ATCC® 30-2002™) into a full 500 mL bottle of DMEM. Transfer 64 mL FBS (ATCC® 30-2020™) into the bottle containing 566 mL DMEM.
3. Warm the medium by leaving it at ambient temperature for 30 minutes to 2 hours, or by placing it in a 37°C water bath for 30 minutes to 1 hour.
4. Harvest expanded cells and transfer to a 15 mL conical containing 10 mL DMEM. Gently pipette the cells to homogenize the suspension. **Do not centrifuge**.

5. Take a sample of the suspended cells for cell count and evaluate cell density and viability. Maintain the cell suspension on ice while performing the cell count and preparing the cell dilution. **Note:** If cell viability is below 80%, do not proceed. If cell count is less than 16.08 x 10^6 cells, thaw an additional vial of cells to supplement the suspension or continue expanding the cell line to reach the desired cell count.
6. Transfer 16.08 x 10^6 cells to the prepared media bottle containing 630 mL of DMEM and FBS. Thoroughly mix by gently inverting the bottle at least four times.
7. Using a P1000 micropipette, add 1.92 mL of 100 mg/mL Zeocin to the bottle containing cells, DMEM, and FBS (~640 mL total). The final concentration of Zeocin should be 300 µg/mL. Thoroughly mix by gently inverting the bottle at least four times.
8. Decant the cell-containing solution into a 5-layer CellSTACK.
9. Tightly recap CellSTACK and distribute the contents evenly.
    9.1. Set the CellSTACK on its side with the filling port in the bottom right corner. Allow the medium to become level.
    9.2. Turn the CellSTACK 90° counterclockwise so that both filling and venting ports are up.
    9.3. Remove the CellSTACK from the BSC carefully, ensuring that medium does not enter the access chamber to prevent uneven distribution of the cell suspension across layers. Gently lower the CellSTACK to its normal horizontal position and place it on the incubator shelf.
    9.4. Gently shuffle the chamber back-and-forth and side-to-side until the surface is completely covered with medium to ensure an even distribution of cells across the growth surfaces.
    9.5. Ensure that the incubator shelves are level and that the medium is distributed evenly in each layer and between the layers.
10. Incubate for 5 days at 37°C, 5% CO_2, in a humidified atmosphere.

End of antibiotic selection and first conditioning

Note: The selection medium contains a selection antibiotic that will have a negative impact on organoid growth. **It is critical that antibiotics are not present in the final conditioned medium.**

1. Make the conditioning medium by combining 1078 mL Advanced DMEM: F12, 11 mL HEPES, and 11 mL GlutaMAX and warm in a 37°C water bath for 30 minutes to 1 hour.
2. Retrieve the CellSTACK from the incubator and assess monolayer confluency. Expected confluency is between 10-30% at this stage.
3. Aspirate the selection medium currently in the CellSTACK.

4. Rinse the flask by decanting approximately 250 mL of D-PBS (ATCC® 30-2200™) into the CellSTACK, distributing it evenly across all flask layers as described in step 9 of "Thawing, seeding, and antibiotic selection of 293T-RSPO1 (ATCC® CRL-3797™) cells." Tilt the CellSTACK gently back-and-forth and side-to-side at least 5 times each direction. Discard the D-PBS.
5. Decant the pre-warmed conditioning medium prepared in step 1 into the CellSTACK and distribute evenly across all layers.
6. Incubate the CellSTACK for 4 days at 37°C, 5% CO_2, in a humidified atmosphere.

Batch 1 collection and second conditioning

Note: If a peristaltic pump is unavailable, bottle-top filtering can be used instead. Make sure the medium is filtered through a 0.2 µm PES filter by using a bottle-top filter system compatible with a standard vacuum method. For example, decant the contents of the CellSTACK into the top of a 0.2 µm filter unit and apply the vacuum to completely filter through to the collection bottles.
Note: After collecting and filtering the medium for each step, it's recommended to test the medium for sterility prior to pooling the material. It is recommended to pool and aliquot batches to minimize batch-to-batch variability.

1. Make the conditioning medium by combining 1078 mL Advanced DMEM: F12, 11 mL HEPES, and 11 mL Glutamax and then warm in a 37°C water bath for 30 minutes to 1 hour.
2. Retrieve the CellSTACK from the incubator and assess monolayer confluency. Expected confluency is between 30-80% at this stage. **Note:** A moderate number of floating cells is expected at this stage.
3. 293T-RSPO1 cells tend to detach from the surface of the flask easily. This can cause filters to clog during harvesting. Centrifugation of the medium prior to filtering prevents this issue. It is recommended to follow steps 4–5 before collecting the medium for filtration.
4. Using a serological pipette, transfer the medium from the CellSTACK to conical tubes of the appropriate volume (ex. 5 × 250 mL conicals).
5. Centrifuge the conical tubes at 400-500 × *g* for 5-10 minutes.
6. While centrifuging the medium, rinse the CellSTACK with approximately 250 mL of D-PBS (ATCC® 30-2200™). Distribute evenly and tilt gently back-and-forth and side-to-side at least 5 times each direction. Aspirate the D-PBS.
7. Decant the pre-warmed conditioning medium prepared in step 1 into the CellSTACK and distribute evenly across all layers.
8. Return the CellSTACK to the humidified 37°C incubator with 5% CO_2.
9. Incubate 4 days prior to second collection.

10. Prepare for peristaltic pump filtering by setting up the spray pump, stand, clamp, tubing, sterile 2 L PETG collection bottle, and Acropak filter in the BSC. Use steps 10.1–10.6 for setup information.
    10.1 Remove the cap of the sterile 2 L PETG collection bottle and set it aside.
    10.2 Remove the Acropak filter from the packaging, using care not to touch the inside of the filling bell. Use the clamp and support stand to hold the Acropak filter at an appropriate height. The filling bell should fit around the opening of the sterile 2 L collection bottle.
    10.3 Cut a piece of tubing approximately 2 feet long using a sterile scalpel or scissors. Feed the middle section of tubing into the peristaltic pump.
    10.4 Attach one side of the tubing to the Acropak filter inlet port located at the top of the filter.
    10.5 Set the peristaltic pump to 200–300 rpm.
    10.6 Insert a 5 mL aspirating pipette tip-first into the silicone tubing.

    Note: It is recommended to use a sterile 50 mL conical to place the aspirator pipette in while not in use or between steps.

11. Retrieve the conical tubes from the centrifuge and place in the BSC. Remove the lids from the conical tubes and have them prepared for rapid succession to prevent large volumes of air from entering the filter between each tube. Insert the end of the 5 mL aspirating pipette into the first 250 mL conical just below the surface of the media and turn on the peristaltic pump.
12. Using the peristaltic pump, aspirate the medium from the 250 mL conical, taking care to not disturb the pellet. Vent any air in the pump/Acropak filter system throughout the process using the pressure valve to prevent bubbles from clogging the filter. Repeat for each conical tube.
    Note: Air inside of the peristaltic pump/Acropak filter system will cause a buildup of pressure that could lead to leaks in the system. Air must be released from the filter via the pressure valve. Opening the pressure valve releases some of the medium as well. Use a Kimwipe to absorb the medium and work quickly to minimize its loss through the venting port.
13. Discard the empty 250 mL conical tubes, Acropak filter, and tubing as biohazard waste.
14. Recap the 2 L collection bottle and label appropriately. Store the collected medium at 4°C. It is recommended to test the medium for sterility prior to use.

Figure 2: Peristaltic pump and harvest setup.

Batch 2 collection

1. Retrieve the CellSTACK from the incubator and assess monolayer confluency. The expected confluency is between 80-100% at this stage. **Note:** A moderate to high number of floating cells is expected at this stage.
2. 293T-RSPO1 cells tend to detach from the surface of the flask easily. This can cause filters to clog during harvesting. Centrifugation of the media prior to filtering prevents this issue. It is recommended to follow steps 3–4 prior to advancing to collecting media for filtration.
3. Using a serological pipette, transfer the media from the CellSTACK to conical tubes of an appropriate volume (ex. 5 × 250 mL conicals).
4. Centrifuge at 400-500 × *g* for 5-10 minutes.
5. Repeat section “Batch 1 collection and second conditioning” steps 10–14 for harvesting.
6. Discard the CellSTACK as biohazardous waste.

Batch Pooling

Note: If testing the medium for sterility, it is recommended to complete this step prior to pooling the material. It is recommended to pool and aliquot batches to minimize batch-to-batch variability.

1. Remove all batches from 4°C storage, spray with 70% ethanol, wipe clean, and bring into the BSC.
2. Mix each batch by gently inverting the bottle at least 4 times.
3. Decant all batches into a sterile 5 L bottle.
4. Mix by gently inverting the bottle at least 4 times.
5. Aliquot the material into sterile 500 mL PETG bottles.
6. Label appropriately and store aliquots at 4°C, protected from light.

Reference

1. Wilson S, et al. Optimized Culture Conditions for Improved Growth and Functional Differentiation of Mouse and Human Colon Organoids. Front Immunol 11: 547102, 2021. PubMed: 33643277