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Cultrex® HA-R-Spondin1-Fc 293T Cells

Cat# AMS.RSPO1-CELLS

Stably transfected 293T cells that express murine Rspo1 with an N-terminal HA epitope tag and fused to a C-terminal murine IgG2a Fc fragment.

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I. 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. R-Spondins positively regulate Wnt/beta-catenin signaling, most likely by acting as a ligand for LGR4-6 receptors and an inhibitor for ZNRF3. R-Spondin-1 induces proliferation of intestinal crypt epithelial cells, increases intestinal epithelial healing, and supports intestinal epithelial stem cell renewal [1-5].
The 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.

II. Precautions and Limitations

1. Successful and consistent results are dependent upon the quality and degree of characterization of the cells under investigation. Highly passaged cells may undergo both genotypic and phenotypic changes that render them an inadequate *in vitro* model for specific investigations. We recommend for all studies that highly qualified low passage number cells are used to ensure reliable and reproducible results.
2. For Research Use Only. Not for use in diagnostic procedures.
3. This cell line is not known to harbor any agent known to cause disease in healthy adult humans. Handle as a potentially biohazardous material under at least a Biosafety Level 1 containment. This cell line has NOT been screened for Hepatitis B, human immuno-deficiency viruses or other adventitious agents. We recommend that appropriate safety procedures be used when handling all cell lines, especially those derived fromhuman or other primate material. We assume no liability for damage resulting from handling or contact with these products.

III. Materials Supplied

Component	Quantity	Storage	Catalog #
HA-R-Spondin1-Fc	1 Vial (10^6 Cells)	Liquid Nitrogen***	AMS.RSPO1-CELLS
293T Cells			

***Shipped on Dry Ice, immediately thaw for use, or for long term storage place in vapor phase of liquid nitrogen.

IV. Materials/Equipment Required But Not Supplied

Equipment

1. 1 - 20 µl, 20 - 200 µl, and 200 - 1000 µl pipettors
2. Class 2 Biosafety Hood
3. 37°C CO_2 incubator
4. 37°C Water Bath
5. Hemocytometer or other means to count cells

6. Inverted standard or phase microscope
7. Pipette aid
8. Liquid Nitrogen Storage
9. Low speed swinging bucket centrifuge and tubes for cell harvesting
10. Cell freezing container that allows for slow freezing of cells (e.g. Fisher Scientific cat#15-350-50)

Reagents

1. Cell Culture Medium: DMEM High Glucose (Thermo Fisher Scientific cat# 11965-092 or equivalent)
2. Conditioning Medium: Advanced DMEM/F12 (Thermo Fisher Scientific cat# 12634-010)
3. Cell Harvesting Reagent (Trypsin, Dispase, or equivalent)
4. Fetal Bovine Serum
5. 100X Penicillin-Streptomycin supplement for Media (Thermo Fisher Scientific cat# 15140-122 or equivalent)
6. 100 mg/ml Zeocin (Thermo Fisher Scientific cat# 250-01)
7. GlutaMAX (Thermo Fisher Scientific cat# 35050061)
8. CD293 Medium (Thermo Fisher Scientific cat# 11913-019)
9. PBS (Mg^{2+}, Ca^{2+} free) or HBSS, tissue culture grade
10. Trypan blue or equivalent viability stain
11. DMSO, tissue culture grade
12. 70% Ethanol
13. Sterile ddH_20
14. Protein A Agarose Purification Kit (KPL cat# 553-50-00 or equivalent)

Disposables

1. Cell culture flasks, 25 cm^2, 75 cm^2, or 185 cm^2
2. 525 cm^2 3-layer flasks (Corning cat# 353143)
3. 15 ml tubes
4. 0.22 µm Filter Unit
5. 1 - 200 µl and 200 - 1000 µl pipette tips
6. 2, 5 and 10 ml serological pipettes
7. gloves

V. Reagent Preparation

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination.

1. Basal Growth Medium

For 500 ml of Medium:

DMEM Medium	440 ml
Fetal Bovine Serum	50 ml
100X Penicillin-Streptomycin	5 ml
GlutaMAX	5 ml

Filter sterilize medium and store at 4°C for one month. Ensure medium is at room temperature or 37°C prior to use.

2. Selection Growth Medium (containing 300 µg/ml Zeocin)

For 50 ml of Medium:

Basal Growth Medium	50 ml
100 mg/ml Zeocin	150 µl

Prepare immediately before use. Scale as needed.

3. Freeze Medium

For 10 ml:

Basal Growth Medium	5 ml
FBS	3 ml
DMSO	2 ml

4. Expression Medium

For 1000 ml of Medium:

CD293 Medium	1,000 ml
GlutaMAX	10 ml

5. Conditioning Medium

For 500 ml of Medium:

Advanced DMEM/F12 Medium	500 ml
GlutaMAX	5 ml

VI. Protocol

These procedures should be performed in a biological hood utilizing aseptic technique to prevent contamination. Vessels should be sprayed down with 70% ETOH before placing in Tissue Culture Hood.

A. Thawing HA-R-Spondin1-Fc 293T Cells:

1. Pre-warm Basal Growth Medium (Section V.1) to 37°C by placing in H_2O bath or in Tissue Culture Incubator.
2. Immediately before use, remove the vial of cryopreserved cells from liquid nitrogen freezer and thaw quickly (3-4 minutes) in a 37°C H_2O bath. Ensure

cells are completely thawed before proceeding and do not leave cells at 37°C past thawing.

3. Aseptically, transfer the thawed cells to an empty 15 ml conical tube. Wash ampoule with 1 ml of warm Basal Growth Medium and add to thawed cells. Add 1 ml of warm Basal Growth Medium to 15 ml conical tube containing cells, gently swirling to mix between drops. Total Volume should be 3 ml.
4. Centrifuge cells at 200 x g for 3 minutes.
5. Remove supernatant gently to avoid disturbing cell pellet and resuspend cell pellet in 6 ml of fresh Basal Growth Medium.
6. Transfer cell suspension to a sterile T25 (25 cm^2) Tissue Culture Flask.
7. Place Tissue Culture Flask/Dish in 5% CO_2 Tissue Culture Incubator at 37°C.
8. Change medium in flasks using freshly prepared and pre-warmed Selection Growth Medium (Section V.2) the next day.

B. Passaging HA-R-Spondin1-Fc 293T Cells:

Note: HA-R-Spondin1-Fc 293T Cells may be sequentially passaged under selection into larger culture vessels to expand cell numbers. We recommend that cultures be maintained at densities between 40 – 90% confluent for optimal growth and survival.

1. Selection Growth Medium should be changed every 2-3 days. Cells should be passaged when 80-90% confluent for optimal growth rate/efficiency. We recommend splitting cells at a density of 1:4 to 1:6. Cells have a doubling time of approximately 24 hrs.
2. Prepare Selection Growth Medium on day of use. Warm Basal Growth Medium to 37°C by placing in 37°C H_2O bath or in Tissue Culture Incubator. In a sterile container, add the required volume (10-12 ml/flask) of Basal Growth Medium and Zeocin (final concentration of 300 µg/ml).
3. Remove medium from T25 flask containing the HA-R-Spondin1-Fc 293T Cells.
4. Gently wash flask with 5 ml of sterile 1X PBS (Ca^{2+} and Mg^{2+} free) and remove PBS.
5. Add 1 ml of warmed Trypsin to the flask and place at 37°C for 3-5 minutes until cells are no longer attached to plate. Add 3 ml of Basal Growth Medium to flask to inactivate Trypsin and transfer to 15 ml conical tube.
6. Centrifuge cells at 200 x g for 3 minutes.
7. Remove supernatant gently to avoid disturbing cell pellet and resuspend cell pellet in 2 ml of fresh Selection Growth Medium.
8. Add 1 ml of cell suspension to 11 ml of Selection Growth Medium and transfer to one T75 flask.

C. Freezing Cells

1. In general, one confluent T75 flask will provide cells for 10-12 vials of 1 x 10^6 cells per vial.
2. Prepare 2X Freeze Medium, (see Section V.3) according to volume required. Typically, 0.5 ml of 2X Freeze Medium is mixed with 0.5 ml of 2x10^6 cells.
3. To prepare cells remove medium from T-75 flask containing HA-R-Spondin1-Fc 293T Cells.
4. Gently wash flask with 5-10 ml of sterile 1X PBS (Ca^{2+} and Mg^{2+} free) and remove PBS.

5. Add 2 ml of pre-warmed Trypsin to each flask, and place at 37°C for 2-3 minutes until cells are no longer attached to the plate. Add 4 ml of Basal Growth Medium to flask to inactivate Trypsin and transfer to 15 ml conical tube.
6. Centrifuge cells at 200 x g for 3 minutes.
7. Remove supernatant gently to avoid disturbing cell pellet and resuspend cell pellet in 2 ml of Basal Growth Medium.
8. Count cells on hemocytometer (per standard protocol) and dilute cells to 2 x 10^6 cells/ml in Basal Growth Medium.
9. Add equal volume of 2X Freeze Medium to the cells, mix gently and aliquot 1 ml of cells into labeled cryovials.
10. For initial storage, first place cryovials on ice for 15-30 minutes. Transfer cells to specialized cell freezing container and place in -80°C freezer overnight.
11. For long term storage, transfer cells (next day) to liquid nitrogen freezer to ensure long-term viability.

D. HA-R-Spondin1-Fc Expression and Purification

1. After at least five days in Selection Growth Medium, HA-R-Spondin1-Fc cells may be expanded in Basal Growth Medium until they reach 80% confluence in the desired culture vessel. We recommend using a 3-layer flask (Corning cat# 353143) to express and purify HA-R-Spondin1-Fc. A 3-layer flask has a surface area of 525 cm^2, accommodates 150-200 ml of medium, and can produce up to 300 µg of HA-R-Spondin-1-Fc.
2. Once the cells were passaged to the 3-layer flask and reach 80% confluence, change the medium to CD293 medium containing GlutaMAX, and continue to culture for 7-10 days. The cells will detach from the cell culture vessel and grow in suspension after several days.
3. Collect the supernatant, and centrifuge at 3,000 x g for 15 minutes at 4 °C to remove cells and debris.
4. Filter the supernatant through 0.22 µm filter at 4 °C.
5. Purify HA-R-Spondin1-Fc using the Fc tag via Protein A Agarose Purification.

E. HA-R-Spondin1-Fc Conditioned Medium

1. After at least five days in Selection Growth Medium, HA-R-Spondin1-Fc cells may be expanded in Basal Growth Medium until they reach 80% confluence in the desired culture vessel. We recommend using a 3-layer flask (Corning cat# 353143) to produce HA-R-Spondin1-Fc conditioned medium. A 3-layer flask has a surface area of 525 cm^2, and can produce 150-200 ml of conditioned medium.
2. Once the cells were passaged to the 3-layer flask and reach 80% confluence, change the medium to Advanced DMEM/F12 containing GlutaMAX, and continue to culture for 7-10 days. The cells will detach from the cell culture vessel and grow in suspension after several days.
3. Collect the supernatant, and centrifuge at 3,000 x g for 15 minutes at 4 °C to remove cells and debris.
4. Filter the supernatant through 0.22 µm filter at 4 °C.

5. Verify the presence and titrate HA-R-Spondin1-Fc in the medium by western blot using an anti-R-Spondin1 and/or an anti-HA tag antibody, the expected size should be approximately 70-75 kDa.

Note: If desired, the activity of the conditioned medium can be determined by performing a TopFlash assay (protocol available at www.trevigen.com)

VII. References

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2. Barker, N., et al., *Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro.* Cell Stem Cell, 2010. **6**(1): p. 25-36.
3. Sato, T., et al., *Single Lgr5 stem cells build crypt–villus structures in vitro without a mesenchymal niche.* Nature, 2009. **459**(7244): p. 262-265.
4. Sato, T. and H. Clevers, *Growing Self-Organizing Mini-Guts from a Single Intestinal Stem Cell: Mechanism and Applications.* Science, 2013. **340**(6137): p. 1190-1194.
5. Jung, P., et al., *Isolation and in vitro expansion of human colonic stem cells.* Nat Med, 2011. **17**(10): p. 1225-7.

VIII. Troubleshooting

PROBLEM	CAUSE	ACTION
Poor viability from initial freeze	Improper thawing of cells	Ensure medium is added slowly to re-equilibrate the cells from freeze medium Ensure cells were removed from freeze medium immediately after vial has been thawed Ensure vial of cells was thawed at 37°C Fresh medium was warmed to 37°C
Poor proliferation	Fetal Bovine Serum not optimal for cell growth	Try alternative lot/source of FBS
	Media not optimal for cell growth	Ensure medium is of the proper formulation
	Frequency of medium change	Ensure medium is changed every 2-3 days
		Ensure pH of medium fresh medium has not changed
	CO_2 incubator not humidified	Add sterile water to CO_2 incubator per manufactures instructions
	No gas exchange is allowed by flask	Ensure cap is loosened to allow air gas or use vented flask
Contamination of Cells	Contaminated Medium	To prevent contamination, filter medium through a 0.22 µm filter before use *Never use contaminated medium once cloudy or after microorganisms are visible under the microscope*
	Improper aseptic technique	Spray down hands, reagents and hood with 70% ethanol before opening any flasks
	Hood is working improperly	Check to make sure blower is on and functioning Ensure hood is currently certified Wipe down hood with 70% ethanol
	Contaminated CO_2 Incubator	Ensure CO_2 incubator is free of microbial growth
Loss of HA-R-Spondin-1-Fc expression	Not growing cells in Zeocin	Ensure Zeocin was added to basal medium just before addition to cells
	Frequency of Medium Change	Ensure medium is being changed every 2-3 days.

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