

Extracellular Vesicle Generation from Mesenchymal Stromal Cells Using MesenCult™-ACF Plus

This technical bulletin describes a protocol for generating extracellular vesicles using MesenCult™-ACF Plus Medium (Catalog #05448) which enables extracellular vesicle (EV)-free culture of mesenchymal stromal cells (also known as mesenchymal stem cells; MSCs). MesenCult™-ACF Plus is animal component- and EV-free and is optimized to derive human MSCs from multiple sources, including bone marrow and adipose tissue. The small EVs generated using this medium are fully functional as indicated by the endothelial tube formation assay. The lack of contaminating EVs in this media leads to reduced experimental variability and results in homogeneous samples when generating MSC-EVs.

MesenCult™-ACF Plus: An EV-Free MSC Culture Medium

To confirm that MesenCult™-ACF Plus is EV-free, the complete medium particle concentration and properties were investigated using several methods. MesenCult™-ACF Plus particle concentration was compared to fetal bovine serum (FBS)- or human platelet lysate (hPL)-containing media (Figure 1). Nanoparticle tracking analysis of different batches from each medium detected at least 94% fewer nanoparticles in MesenCult™-ACF Plus compared to FBS- and hPL-containing media. MesenCult™-ACF Plus was also tested for EV-specific markers using Western blotting. The commonly used EV markers, CD9, CD63, and CD81, were not present in MesenCult™-ACF Plus at detectable levels (Figure 2).

MesenCult™-ACF Plus was also compared to EV-depleted FBS. Nanoparticle tracking analysis indicated that MesenCult™-ACF Plus had a similar particle concentration to EV-depleted FBS-supplemented media (Figure 3A) and led to superior MSC expansion when compared to FBS-containing medium or medium supplemented with EV-depleted FBS (Figure 3B).

Why Use MesenCult™-ACF Plus?

HIGH PERFORMING. Achieve superior cell expansion compared to serum-containing or EV-depleted serum-containing media.

FUNCTIONAL. Use cultured MSCs to generate functional EVs capable of enhancing angiogenesis.

OPTIMIZED. Derive MSCs directly from primary human tissue without the addition of animal-derived components.



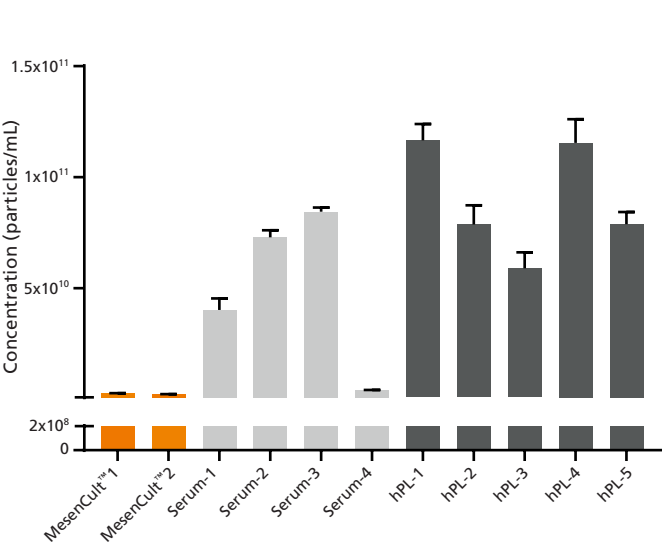


Figure 1. Complete MesenCult™-ACF Plus Medium Contains at Least 90% Fewer Nanoparticles than Serum- and hPL-Containing Media

Nanoparticle tracking analysis was used to demonstrate particle concentration in complete media made with 10% v/v of 4 different lots of serum (FBS), 5 different lots of hPL, or 2 different lots of MesenCult™-ACF Plus. Media containing serum or hPL had high concentrations of nanoparticles with high batch-to-batch variability. MesenCult™-ACF Plus had a significantly lower number of particles/mL when compared to serum- or hPL-containing media. When quantified, particle concentration in each MesenCult™-ACF Plus lot had less than 6% of average serum- or hPL-containing medium nanoparticle concentration. Error bars represent standard error of mean (SEM; n = 3).

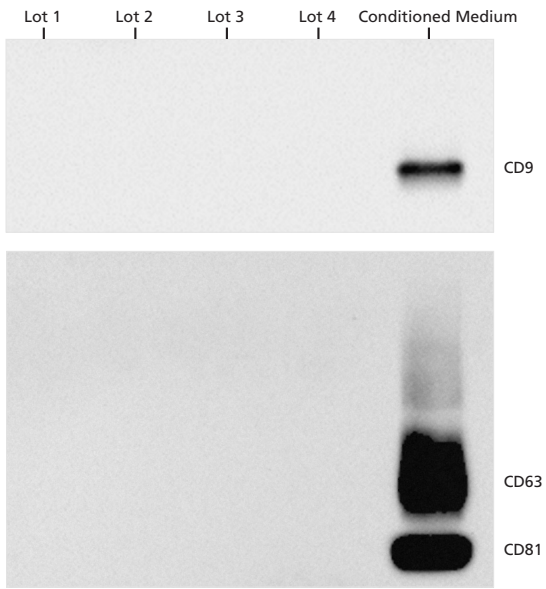


Figure 2. EV Markers Are Undetectable in Complete MesenCult™-ACF Plus Medium by Western Blot Analysis

Western blot analysis was used to investigate 4 different lots of MesenCult™-ACF Plus complete medium for the presence of EVs. Conditioned MSC-cultured MesenCult™-ACF Plus Medium was used as positive control. EV markers, CD63, CD81, and CD9 were only observed in conditioned medium and not in any of the unconditioned MesenCult™-ACF Plus lots, suggesting that this medium is EV-free. 2.3×10^7 , 1.9×10^8 , 2.7×10^7 , 2.5×10^8 , and 4.6×10^8 nanoparticles/well were loaded for MesenCult™-ACF Plus lots 1, 2, 3, 4, and the conditioned medium sample respectively.

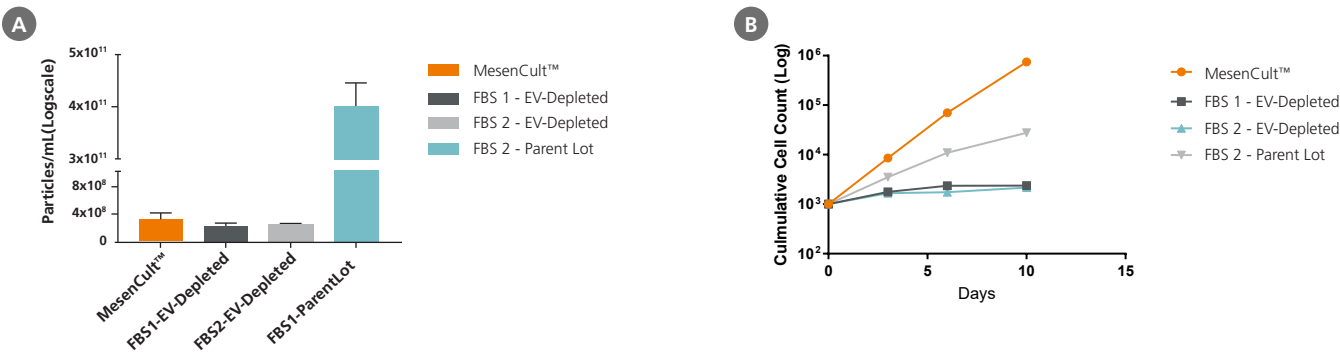


Figure 3. Complete MesenCult™-ACF Plus Medium Performs Similarly or Better than EV-Depleted FBS-Supplemented Medium

Commercial EV-depleted FBS (FBS 1 and FBS 2) was compared to MesenCult™-ACF Plus complete medium. FBS 1 parent lot (non-EV-depleted) was also used for comparison. (A) Particle concentration was measured in complete MesenCult™-ACF Plus and the FBS-supplemented media using nanoparticle tracking analysis. (B) MSC proliferation in MesenCult™-ACF Plus Medium was compared to media supplemented with EV-depleted FBS-supplemented media. The complete MesenCult™-ACF Plus medium had similar nanoparticle concentration to the FBS-depleted media and led to superior cell proliferation compared to FBS-containing or EV-depleted FBS-supplemented media. Error bars represent SEM (n = 3).

MSCs Cultured in MesenCult™-ACF Plus Medium Generate Functional EVs

Since MesenCult™-ACF Plus is animal component- and EV-free, it is an ideal medium for generation of EVs from MSCs. The EV-free nature of MesenCult™-ACF Plus ensures the absence of contaminating EVs. MSC expansion and EV generation is not compromised using this medium. To confirm this, the EVs derived using MesenCult™-ACF Plus were assayed for functionality using a human umbilical vein endothelial cell (HUVEC) tube formation assay (Figure 4). Bioactive molecules, including miRNAs, produced by MSCs and packaged into EVs have been shown to promote angiogenesis in endothelial cultures^{1,2}, making this assay a suitable

method to test MSC-EV functionality. HUVECs treated with EVs isolated from MSCs cultured in MesenCult™-ACF Plus exhibited enhanced angiogenesis with an increased number of branch points. In contrast, HUVECs not treated with MSC-EVs formed fewer branch points and at slower rates (Figure 4). The cargoes carried by MSC-EVs produced in MesenCult™-ACF Plus were analyzed by qPCR. A high abundance of EV-specific markers, let7, miR21, and miR26a, was observed in EVs derived in MesenCult™-ACF Plus (Figure 5).

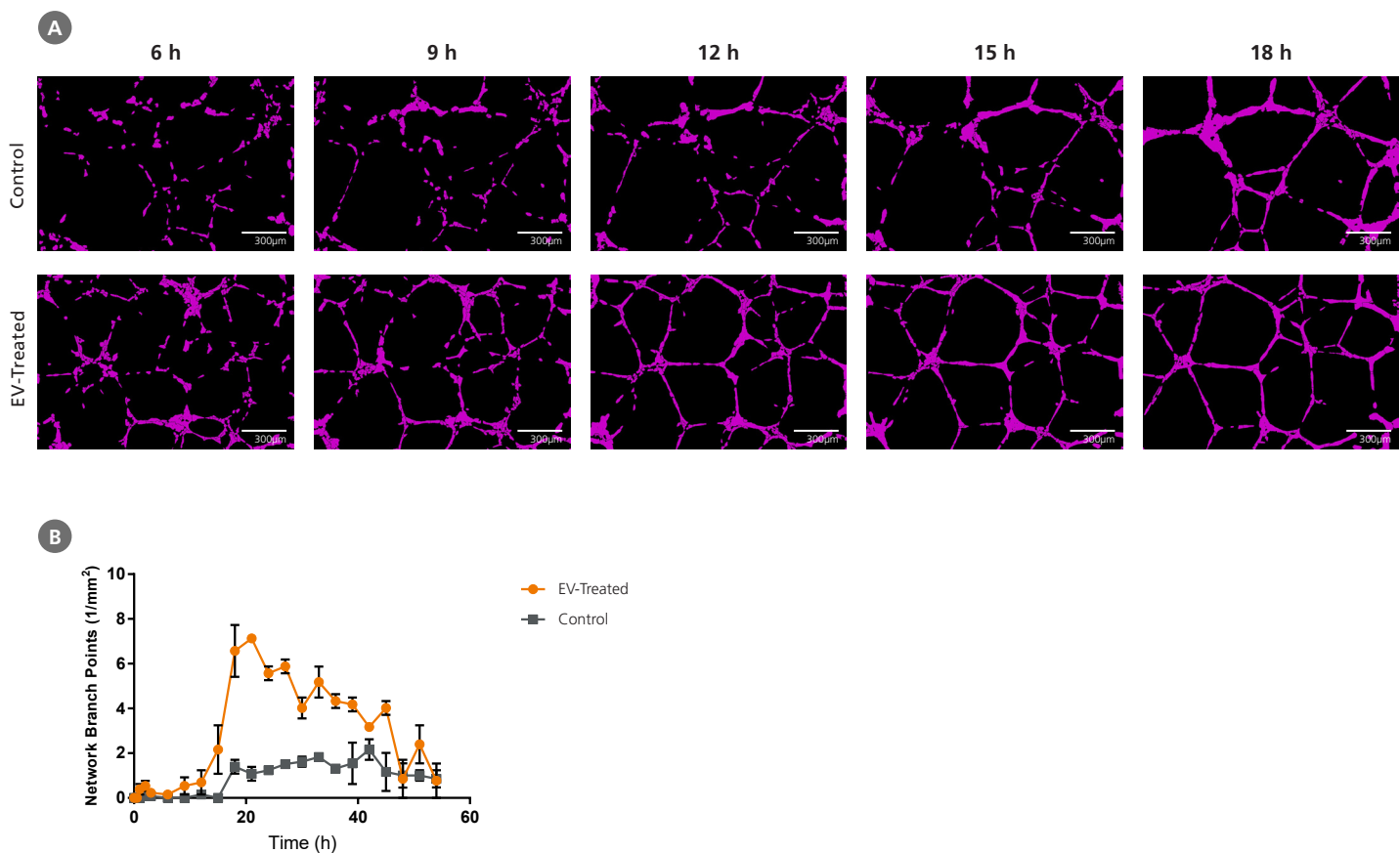


Figure 4. MesenCult™-ACF Plus-Generated EVs Enhance Angiogenesis in HUVECs

MSCs were cultured in MesenCult™-ACF Plus complete medium and EVs were isolated subsequently. Human umbilical vein endothelial cells (HUVECs) were plated at 10,000 cells/well (Matrigel®-coated 96 well plate) in EC-Cult™-XF Culture Medium (Catalog #08000) and treated with 2.6 x 10⁹ particles/well. Formation of tubular networks was then (A) imaged and (B) quantified over the course of 54 hours. The EV-treated group formed a larger number of tubular networks compared to the untreated control group. Error bars represent SEM (n = 3).

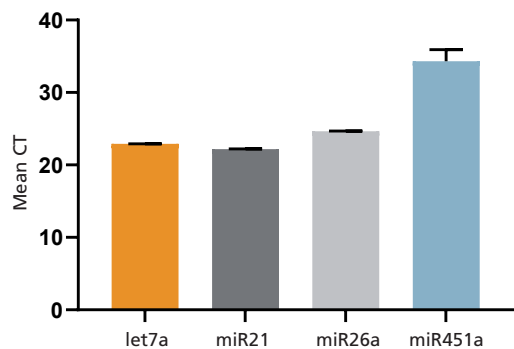


Figure 5. Cargo Analysis of EVs Generated in MesenCult™-ACF Plus Medium

EVs isolated from MSCs cultured in MesenCult™-ACF Plus were analyzed using RT-qPCR and EV-specific mRNA and miRNA cargoes were detected. Error bars represent SEM (n = 3).

Protocol

EV Isolation From MSCs

MSCs may be derived from bone marrow, umbilical cord, fat, and other tissues. It is recommended to use an EV-free medium for MSC derivation and expansion to avoid external EV contaminations. Following derivation and expansion of MSCs, EVs can be isolated from MSCs using the methods described below. For details on the maintenance and expansion of MSCs, refer to the product information sheet (PIS) for MesenCult™-ACF Plus Medium (Document #10000003462), available at www.stemcell.com. A schematic overview of the isolation process can be found in Figure 6.

1. Culture MSCs in MesenCult™-ACF Plus according to the PIS (Document #10000003462). Once 100% confluent, change medium and allow 3 - 4 days for EV production by MSCs.
2. Transfer conditioned medium to a 50 mL centrifuge tube.
3. Centrifuge for 10 minutes at 2,000 x g at 4°C to separate debris and dead cells.
4. Transfer the supernatant into an ultracentrifuge tube.
Note: Always use a pipette rather than decanting the supernatant and leave behind some liquid above the pellet to avoid contamination.
5. Ultracentrifuge for 30 minutes at 10,000 x g, 4°C.
6. Making sure not to disrupt the pellet, transfer the supernatant to new tubes using a pipette. You will probably not have a visible pellet at this step.
Note: For fixed-angle rotors, the pellet is on the side of the tube facing out from the centre of the rotor, near the bottom of the tube. Pour off supernatant rather than use a pipette when working with this setting. For swinging-bucket rotors, the pellet is in the centre of the bottom of the tube. When removing the supernatant,

hold the tube at an angle so that the pellet is always covered and stop removing supernatant when half a centimeter of liquid is still covering the pellet location.

7. Ultracentrifuge for 70 minutes at 100,000 x g, 4°C.
8. Remove and discard the supernatant. You will most likely not have a visible pellet at this step.
9. Resuspend the pellet in 1 mL of PBS by pipetting up and down with a P1000 micropipette. This will be your EV wash step.
Note: If spinning replicate tubes to increase yield, you can pool the pellets at this step.
10. Add PBS to fill the tube completely.
11. Ultracentrifuge for 60 minutes at 100,000 x g, 4°C.
12. Remove and discard the supernatant, leaving about 200ul in the vial and add and 200 - 400 µl of PBS for a total volume of ~500 µl, resuspend the pellet and keep on ice. For further purification of your EVs, refer to the next section: EV Purification Following Differential Centrifugation. This additional step is optional, you can store or use your EVs without it. Keep in mind that EVs may have more contaminants if these additional steps are not performed.

EV Purification Following Differential Centrifugation

EVs isolated by differential ultracentrifugation can be further purified using size-exclusion columns and sterilized by filtration (Figure 6). These extra purification steps lead to small-EV samples with minimal protein contamination (Figure 7).

NOTE: Perform all steps at 4°C

1. Add the resuspended pellet from the ultracentrifugation step to an EV size-exclusion column.
2. Add PBS gradually (up to 20 mL) to the column and collect 500 µL fractions.
3. Combine 5 fractions from the range of 7 - 12 fraction numbers for a total of ~2.5 mL and discard others.
4. Pre-wash a 0.2 µm polyethersulfone (PES) membrane syringe filter with 2 mL of PBS and discard flow-through.
5. Pass the 2.5 mL EV solution from step 6 of the previous protocol (EV Isolation From MSCs) through the syringe filter.
6. Wash filter with 2.5 mL of PBS to collect retained EVs and add to EV solution from step 5 for a total of 5 mL.
Note: Skip this step to achieve a more concentrated EV sample for assays. If you intend to measure the total EV recovered, this step is required.
7. Analyze small-EVs and use them immediately, or store them at 4°C for short-term and -80°C for long-term storage. Avoid repeated freeze-thaw cycles.

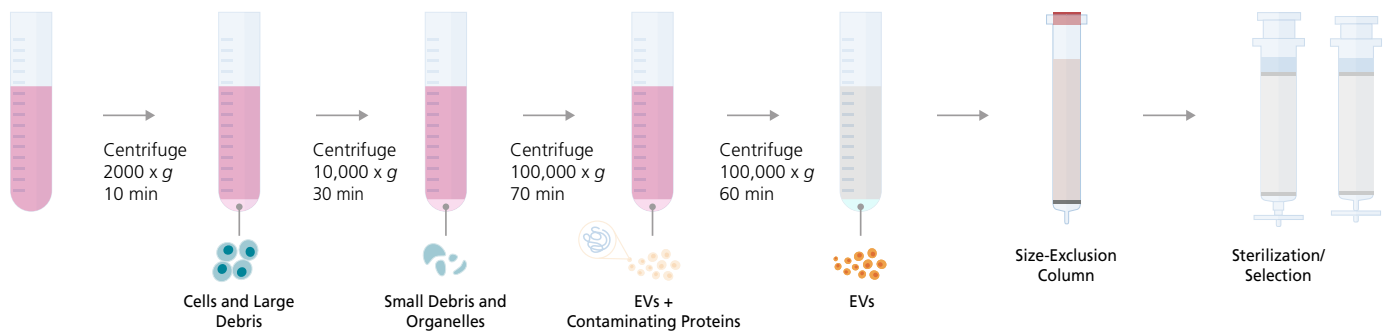


Figure 6. A Schematic Overview of EV Isolation and Purification Method

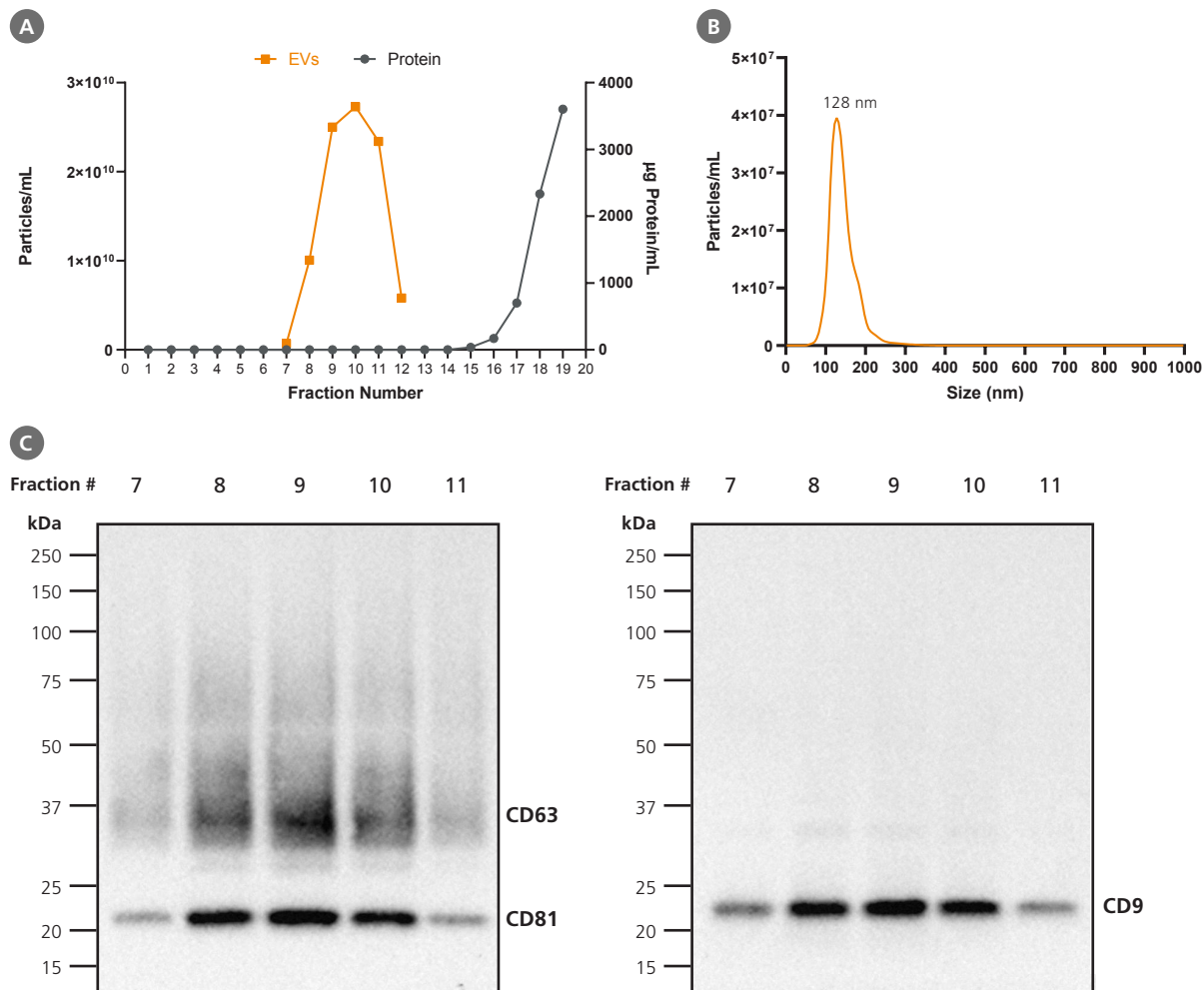


Figure 7. Additional Size Exclusion Chromatography Removes Contaminating Proteins And Provides Purer EV Samples

EVs were isolated from MSCs following a 3 - 4 day culture period using differential chromatography and were further purified using size-exclusion chromatography as previously described. (A) Fractions highly enriched in EVs can be selected and pooled to achieve EV samples with (B) characteristic EV size and (C) EV markers, CD9, CD63, and CD81.

HUVEC Tube Formation Assay

The tube formation assay in this technical bulletin was performed using HUVECs that stably express green fluorescent protein (HUVEC-GFP). For details on maintenance and expansion of MSCs, refer to the product PIS for MesenCult™-ACF Plus Medium (Document #10000003462). For culture of endothelial cells (HUVECs) used in the tube formation assay, refer to the PIS for EC-Cult™-XF Culture Kit (Document #DX20709). Both information sheets are available at **www.stemcell.com**.

1. Culture MSCs in MesenCult™-ACF Plus according to the PIS (Document #10000003462). Once 100% confluent, change media and allow 3 days for EV production by MSCs.
2. Thaw Corning Matrigel® Matrix the night before the assay on ice in the cold room.
Note: Keep Matrigel® on ice at all times to prevent polymerization.
3. Isolate MSC-EVs from MesenCult™-ACF Plus after 3 days by differential ultracentrifugation using the EV isolation protocol above.
4. Resuspend EVs in PBS to a final concentration of approximately 2.6×10^9 particles/50 µl and keep on ice while preparing HUVECs and assay plates.
5. Pipet 30 µl of Matrigel® into the 96-well plate kept on ice.
Note: Use cold pipettes and tips. To avoid air bubbles when adding Matrigel® to the wells, pipette slowly and carefully.
6. Incubate the culture plate for 10-20 min at 37°C to allow Matrigel® to polymerize.
7. Add 30 µl of EC-Cult™-XF media to the wells to prevent them from drying out, and then return the plate to the incubator.
8. Dissociate HUVEC-GFP cells from flask and add 120 µl of EC-Cult™-XF media containing 10,000 cells/well in the Matrigel®-coated 96-well plate.
9. Immediately add 50 µl of MSC-EVs to the HUVEC-GFP cells.
10. Image and analyze plate using the live cell imaging IncuCyte® instrument running the Angiogenesis Software module.

Product Information

Product	Unit Size	Catalog #
MesenCult™-ACF Plus Culture Kit	1 Kit	05448
EC-Cult™-XF Culture Kit	1 Kit	08000
Extracellular Vesicle SEC Columns	0.5 mL	100-0414
	2 mL	100-0415
	20 mL	100-0416

References

1. Liang X et al. (2016) Exosomes secreted by mesenchymal stem cells promote endothelial cell angiogenesis by transferring miR-125a. J Cell Sci 129(11): 2182–9.
2. Gong M et al. (2017) Mesenchymal stem cells release exosomes that transfer miRNAs to endothelial cells and promote angiogenesis. Oncotarget 8(28): 45200–12.

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