Protocol for *in vitro* Co-culture Angiogenesis Assay Using Angio-***Ready***™ Angiogenesis Assay Kit (ATCC® ACS-2001-2™ and ACS-2001-10™)

Angiogenesis is a multi-step physiological process. Angiogenesis is also involved in a large number of disease state processes, such as, tumor formation and metastasis. *In vitro* angiogenesis models provide very useful tools to study these processes, one of which is the analysis of tubule formation. Tubules formed in co-culture assays were significantly more heterogeneous and more closely resembled capillaries than matrigel tubules[1]. The ATCC® Angio-***Ready***™ Angiogenesis Assay Kit utilizes hTERT immortalized mesenchymal stem cells and hTERT immortalized aortic endothelial cells, which eliminates donor variability, and reduces the lot to lot variations seen with primary cells, while offering the advantage of larger lot size and assay consistency.
This protocol describes an *in vitro* co-culture assay system using cell lines that are immortalized by hTERT alone and the cell mixture is ready-to-use for compound screening or other angiogenesis applications.

General Considerations for using the Angio-*Ready*™ Angiogenesis Assay Kit:

- All steps should be performed in a biosafety cabinet using proper aseptic technique.
- Do not leave the vial in the 37ºC water bath longer than 5 minutes when thawing the frozen cells. Do not pellet the cells using a centrifuge speed higher than 250 X g.
- Re-suspend the cells uniformly in the complete Angio-***Ready***™ Angiogenesis Medium and use a multi-channel pipet when plating, to ensure an equal number of cells in each well.
- The complete Angio-***Ready***™ Angiogenesis Medium should be stored in 2-8°C and used within one month after preparation. It is recommended that the complete medium be aliquoted in one use volumes to avoid repeated exposure to heat in the water bath.

Materials

Materials included in this kit	ATCC® No.
The Angio-***Ready***™ Angiogenesis Assay Kit-2 assays	ACS-2001-2™
The Angio-***Ready***™ Angiogenesis Assay Kit-10 assays	ACS-2001-10™
Angio-***Ready***™ Cells	
Angio-***Ready***™ Angiogenesis Medium with VEGF Supplement	ACS-2008
Angio-***Ready***™ Angiogenesis Medium	ACS-2008B
rh VEGF	PCS-999-024

Materials required but not provided
Tissue culture plates and supplies
Fluorescent microscope or live cell imaging system capable of reading λ 123 nm
Angiogenesis tubular length analysis software

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Protocol:
The following protocol describes how to establish angiogenesis tubular formation assay using Angio-***Ready***™ Angiogenesis Assay Kit in **a single 96 well plate.** The assay can be scaled up as needed. Please refer to Table 1 for recommended seeding conditions for other plate sizes.

Table 1: Recommended Cell Seeding Density for various culture vessels

Culture Vessel	96 well plate	24 well plate	12 well plate	6 well plate
Surface area	0.3 cm^2	1.9 cm^2	3.8 cm^2	9.6 cm^2
Volume Cell suspension to add[1]	150 µL	0.5 mL	1.0 mL	2.5 mL
Total number of wells per vial	96	16	8	4

1 As prepared in Step B.2. below

A. Prepare the complete Angio-*Ready*™ Angiogenesis Medium

1. Thaw Angio-***Ready***™ Angiogenesis Medium (ATCC® ACS-2008B) in the refrigerator overnight.
2. Add 0.2 mL of rhVEGF (ATCC® PCS-999-024) to 200 mL Angiogenesis Medium.

B. Setting up the angiogenesis assay

1. Place the frozen cell vials in 37ºc water bath for about 5 minutes; when thawed, transfer the cells to a 50 ml tube using a serological pipette. Add 10 mL of complete Angio-***Ready***™ Angiogenesis Medium, and pellet the cells at 150 g for 4-6 minutes.
2. Carefully move the medium using a 10 ml serological pipette, taking care not to disturb the cell pellet. Re-suspend the cell pellet in 15 mL of fresh complete Angio-***Ready***™ Angiogenesis Medium by ***gently*** pipetting up and down with a 10 ml pipettor.
3. Immediately transfer the cell suspension to a 50mL reagent reservoir.
4. Transfer 150 µL of the cell suspension to each well of the 96 plates using multi-channel pipet; avoid introducing bubbles to the cells.
5. Place the 96 well plate in a 37ºC incubator with 5% CO_2 or a live cell imaging system.
6. Compound screening can begin within 18 hours post-seeding. It is recommended to run the assay in triplicate for each condition and to include a no compound control as one of the conditions. If a positive control is desired, please talk to ATCC technical service for your individual needs.

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7. Change the complete medium every 2-3 days. Tubules should be visible within 72 hours after plating and will increase thereafter for the no compound control.
8. Analyze the results 7-8 days after the initial plating, the tubules can be analyzed live or fixed in 4% paraformaldehyde for additional markers staining.

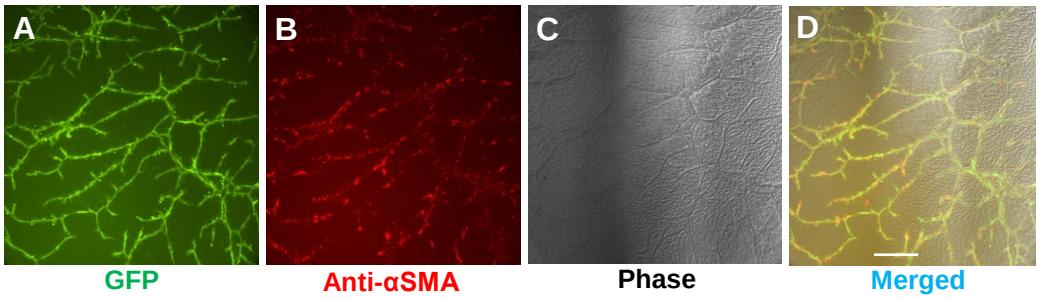

Figure1. Establishment of angiogenesis co-culture assay using the ATCC® Angio-*Ready*™ Angiogenesis Assay Kit (ATCC® ACS-2001-2™ and ACS-2001-10™)**.** TeloHAEC-GFPs co-cultured with hTERT-MSCs for 7 days in the optimized angiogenesis medium displayed a long branching organization (A) and exhibited immuno-reactivity to an αSMA antibody (Sigma)(B), which co-localized with the TeloHAEC-GFPs(D) . Phase contrast microscopy indicated the 3-dimensional structure of the tubes(C), (scale bar: 40 μm).

Reference

1. Donovan D, *et al.* Comparison of three *in vitro* human 'angiogenesis' assays with capillaries formed *in vivo*. *Angiogenesis* 4:113-121, 2001.

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