Protocol for testing pro- and anti-angiogenic substances
Proliferation assay with HUVEC/TERT2 cells
Version: February 2017

<table>
<thead>
<tr>
<th>Material</th>
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<tr>
<td>• HUVEC/TERT2 cells (Evercyte, Cat# CHT-006-0008)</td>
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<td>• Cell culture medium: EndoUp2 (Evercyte, Cat# MHT-006-2)</td>
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<td>• Basal medium: M199 Earle’s (Biochrom, Cat# F0613)</td>
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<td>• VEGF165 (Evercyte, Cat# A-001-1005)</td>
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<td>• Gelatin solution (2%, Sigma Aldrich Cat# G1393)</td>
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<td>• MTT Thiazolyl Blue Tetrazolium Bromide (Sigma Aldrich Cat# M5655)</td>
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<td>• Fetal calf serum (FCS)</td>
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<td>• 96 well plate, sealing film for plates (Carl Roth, Cat# EN76.1)</td>
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Preparation of assay solutions
• Starvation medium: supplement M199 Earle’s with 1x GlutaMAX-I, 0.5% FCS
• Gelatin solution: dilute 2% gelatin solution to 0.1% with PBS
• MTT solution: prepare a 5 mg/mL solution of MTT in PBS, dissolve MTT protected from light overnight at 4°C, filter sterilize and aliquot
• STOP-solution: 10% SDS in 0.01M HCl

Preparation of 96 well plate
• Fill outer wells with 200 µL PBS (no cells are seeded due to uneven evaporation)
• Add 50 µL of a 0.1% gelatin solution into each well
• Incubate for 10 minutes at 37°C
• Take off the gelatin solution (just prior to seeding of cells)
• Do not let dry out

Practical application, read-out and interpretation of data

Day -4 / Seeding of cells
• Harvest HUVEC/TERT2 cells
• Determine the cell numbers using standard procedures
• Prepare a cell suspension in complete medium with 1. 5 × 10^4 cells per mL
  200 µL cell suspension corresponding to 3 ×10^3 cells are required for each well
• Add 200 µL of the cell suspension into each well using a multi-channel pipette
• Incubate plate in an incubator at 37°C (5% CO₂, ambient oxygen) for 48 hours
Practical application, read-out and interpretation of data

Day -2 / Starvation of cells
- Cells should have reached about 70-80% confluence
  *If a lower cell density is observed, let cells grow for another 24 hours (during starvation some cell death will occur)*
- Carefully take off the supernatant (complete medium)
- Wash the cells once with 200 µL PBS per well to remove residual medium
- Add 200 µL starvation medium per well
- Incubate plate in an incubator at 37°C (5% CO₂, ambient oxygen) for 48 hours

Day 0 / Treatment
- Prepare dilutions of substances to be tested in starvation medium with a final volume of 100 µL test solution per well
- A minimum of three replicates per condition to be tested is recommended
- To test anti-angiogenic properties of a substance mix it with VEGF165
- A concentration of 15 ng/mL VEGF165 is a good starting point
- Use 15 ng/mL VEGF in starvation medium as positive control to induce proliferation
- Include wells with complete medium and starvation medium as reference
- Incubate plate in incubator at 37°C (5% CO₂, ambient oxygen)

Day 3 / MTT assay
- 72 hours after treatment add 10 µL of MTT solution (5 mg/mL) per well
- Incubate for 4 hours at 37°C (5% CO₂, ambient oxygen)
- To stop reaction and solubilize crystals add 100 µL SDS/HCl solution per well
- Seal plate with sealing film and incubate at 37°C (5% CO₂, ambient oxygen) overnight for another 20 hours

Day 4 / Read-out
- For measurement on a plate reader remove seal and lid from plate
- Measure absorbance at 570 nm and 690 nm on a plate reader
- Subtract absorbance 690 nm from absorbance 570 nm
- Evaluate results with a suitable software e.g. GraphPad Prism

Related products

- HUVEC/TERT2 cell line (Evercyte, Cat# CHT-006-0008)
  - Human hTERT immortalized umbilical vein endothelial cells
- EndoUp2 medium (Evercyte, Cat# MHT-006-2)
  - Cell culture medium used for propagation of HUVEC/TERT2 cells
- VEGF165 (Evercyte, Cat# A-001-1005/5 µg, A-001-1020/20 µg, A-001-1100/100 µg)
  - Recombinant human vascular endothelial cell growth factor 165
- VEGF121 (Evercyte, Cat# A-002-1005/5 µg, A-002-1020/20 µg, A-002-1100/100 µg)
  - Recombinant human vascular endothelial cell growth factor 121