Nanostring Counter Sample Collection SOP:

Objective: Consistent culture and treatment of human aortic endothelial cells (HAECs) and to reduce the contribution of extraneous factors in expression analysis between experimental conditions.

## I. Culture prep:

1. Pre-warm MCDB-131 media containing 10% FBS inside sterile gas permeable vessel (filter flask or culture plate) inside incubator. Wait until media is pH'd (orange in color) before using with cells.
2. Into a 10cm tissue culture plate add 10mL gelatin to coat plate for HAEC culture (2mL for 6-wells).
3. Leave to incubate at 37°C with 5% $CO_2$ for a minimum of 10 minutes.
4. Wash twice with 5mL Versene (1-2mL for 6-wells)
5. Leave in the last wash or fill with media for culture.

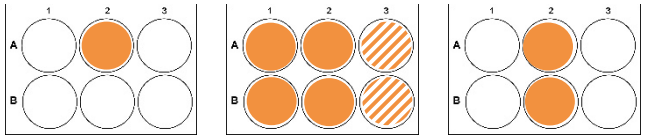
## II. Thawing HAEC seed:

1. Have a gelatin coated 10cm plate with 9mL pre-warmed MCDB-131 +10% FBS media ready (see culture prep)
2. Remove seed from liquid nitrogen storage and place on ice to transport to cell culture room.
3. Immediately proceed to thaw vial in 37°C water bath
4. When only a small shard of ice remains, transfer to culture hood with aforementioned culture plate.
5. Using a 5mL pipette collect cell slurry from cryovial and add one drop at a time to the culture dish at varying locations.
6. Label plate and transfer to 37°C with 5% $CO_2$ incubator.
7. Before leaving to settle, rotate plate two times in the motion of a forward and then reversed figure-8.
8. Leave cells to settle between 3-6hrs and then give full media change with pre-warmed MCDB-131 +10% FBS media.
9. Subsequent media changes should be performed approximately every 24-48 hours.

## III. Passaging HAECs:

### *A. Passaging Preparation:*

1. Pre-warm MCDB-131 media containing 10% FBS inside sterile gas permeable vessel (filter flask or culture plate) inside incubator. Wait until media is pH'd (orange-red in color) before using with cells.
2. Pre-warm Versene in 37°C water bath
3. Allow 1xTrypsin to come to room temperature.
    a. If culturing for flow into 6 well plates, coat wells to be cultured with 2mL gelatin and wash twice with at least 1mL Versene after 10 min incubation. Wells should also not be left dry.
        i. Culture configurations:
            1. Note: for siPort – wells = n +1 for each condition (need extra cells post-trypsinization)




Single Flow  Si-Port (4 or 6)  Single Flow w/ static

    b. A typical confluent 10cm plate will contain just 1.5-2 million cells – plan cultures accordingly (see C.a. for possible combinations):
        i. IBIDI µ-slide = 200k/100µL
        ii. 6-well seeding density = 350-400k in max 2mL
        iii. 10cm plate = up to 800K-1 million cells

### *B. Trypsinizing cells:*

1. Transfer 10cm dish containing cells, Versene, MCDB-131 media, and 1xTrypsin into hood along with several normal and aspirating pipettes and a 15mL conical
2. Wash cells twice with 5mL Versene
3. Add 2mL 1xTrypsin and transfer plate to incubator.
4. After approximately 30 seconds, tap plate gently to lift cells and observe under microscope.
5. If the majority of cells are still attached, repeat step 4 until cells are mostly lifted (typically 30 sec is more than enough time – better to under than over trypsinize)

6. Transfer plate into hood, tilt and add 8mL media down the side of the dish to wash down and collect cells to the side.
7. Transfer cell suspension to 15mL conical.
8. Wash plate once with 2-5mL of media and add to the same 15mL conical
9. Spin cells at 850-950rpm
10. Aspirate supernatant. If passaging solely for flow, using a 5mL pipette add 3-4mL media. If passaging for IBIDI µ-slides, using a 5mL pipette add 1-2mL media (desired concentration should be 2 million cells/mL). carefully break the cell pellet by pipetting up and down.
11. Transfer 10µL of cell suspension into hemocytometer and count cells.

C. *Cell counting*:

1. Cover slip should be centered over the slide brackets before adding the cell suspension. Reference Fig.1 for technique.
2. If cell viability assessment is need, dilute the sample 1:2 in 25% trypan blue. Count blue stained cells as dead (for flow experiments viability is not needed if culture techniques are well established).
3. Count cells within squares identified in Fig.2
4. Average the counts, multiply by dilution factor, and multiply by total cell suspension volume to obtain total cell count
5. Determine culture layout and adjust cell suspension volume as necessary:
    a. Sample layouts –
        1. Final count 1.5 million cells:
            i. 2 x 10cm dishes (750k each)
            ii. 2 x single flow (350-400k/well) + 1 x 10cm dish (700-800k)
            iii. 4 x single flow OR 2 x flow w/ static (375k/well)
        2. Final count 2 million cells:
            i. 2-3 x 10cm dishes
            ii. 2 x single flow (350-400k/well) + 2 x 10cm dish (600-650k each)
            iii. 4 x single flow OR 2 x flow w/ static (350k/well) + 1 x 10cm dish (500k)

Figure 1: Hemocytometer loading guide.

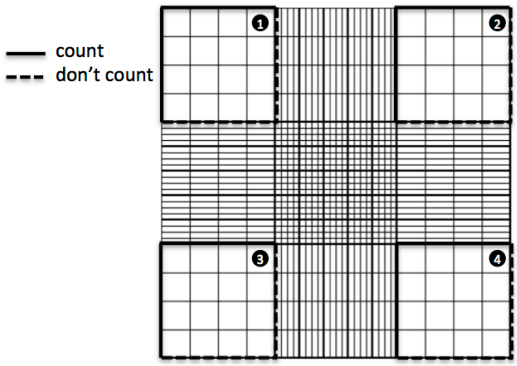



Figure 2: hemocytometer slide counting area.

D. *Passaging cells:*

1. After determining the culture layout, bring cell suspension volume up as necessary for easy distribution.
2. Subtract the cell suspension volume from the total volume to be distributed to culture vessel and add this remainder of media to the culture vessel before adding the cell suspension.
3. Label culture vessels with Donor number, passage number, date, initials, and any other information before transferring to incubator.
4. Before leaving to settle rotate the culture vessel in a figure 8 motion two times then in the reverse direction two times.
5. Cells should be given full media changes every 24-48 hours until confluent and ready to be passaged again.

## IV. Cone-and-Plate Device:

*A. Programing*

a. Connect the device power and micro-USB to USB connection to the computer terminal
b. Computer terminal should have updated versions of Arcus Drivemax and Drivers and Tools Setup installed. Available at http://www.arcus-technology.com/support/downloads/
c. To set the parameters for **20 dynes/cm$^2$**:
    a. Run "SOFT-EXE-DMX-UMD-236C" and select the option for detecting USB connections
    b. The device currently connect via USB to the computer should appear, select this device.
    c. In the control panel "Time Control" box, select "C" to clear default program.
    d. Set speed to 4400 at time index 0 and select "set time index."
    e. Set speed to 4400 at time index 999 and select "set time index."
    f. Hit "Calculate time control" and then "Store" (lightning bolt).

g. Disconnect and reconnect device power.
h. To ensure proper speed has been set, reopen the device control panel, check the enable box and START. The running speed should be 4400 in the Status box.

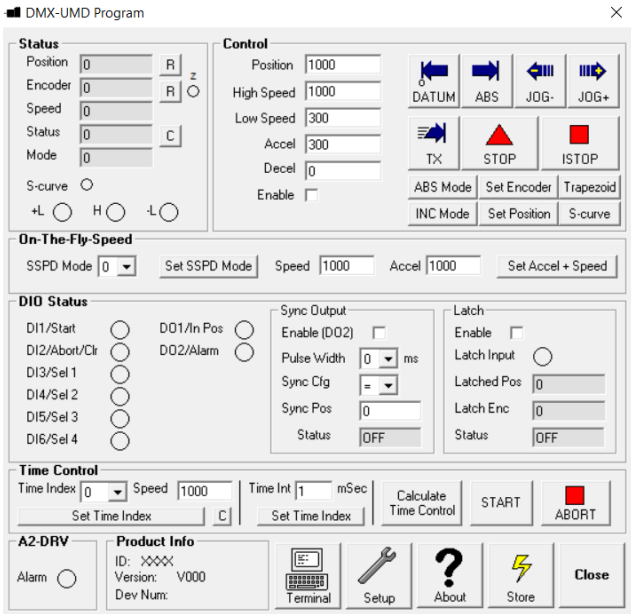


Figure 3: Arcus SOFT-EXE-DMX-UMD-236C control panel

B. *Sterilizing the cone*:

1. Move device to center of sterile hood. Wipe with alcohol if transferring into hood.
2. Balance the cone upright on device stage so that the flat surface faces upward toward the UV light.
3. Lay the plate covers, so that the interior will be exposed to UV.
4. Close the sterile hood sash and turn on UV for a minimum of 15 min.
5. After UV, turn on light and open the sash. Allow air flow to return and circulate for a minute or two.

C. *Assembling the device:*

1. The micrometer should be set to 0.
2. To keep the cone-face sterile, use a sterile petri dish to cover the cone and preserve the sterile surface while attaching to rotor.
3. Align the flat portion of the piston to the loosened screw on the cone and slide the cone into place.
4. Push the cone all the way up before tightening the screw with the Allen wrench.

D. *Calibrating cone depth:*

1. Mix a solution of dye and water
2. Pipette 1µL of the solution in the center of a 6-well.
3. Secure the plate in place on the device stage and lower the cone until almost touching the droplet
4. Looking through the bottom stage cutout, slowly lower the cone until you can see the droplet just adhere to the cone.
5. Repeat steps 2-4 two to three times.
6. Record the average height on device as the calibrate level (the height of 1µL hemisphere is ~700µm).

E. *Cleaning cone-and-plate device*:

1. Ensure the micrometer is at position 0, and using the Allen wrench remove the cone from the rotor.
2. Rinse briefly with di$H_2O$.
3. In plastic container place the cones face down and fill with di$H_2O$ so that it just covers the cone.
4. Place on horizontal shaker and leave for several minutes (i.e. leave while harvesting cells).
5. Repeat steps 2-4 at least once to ensure removal of viscous dextran media.
6. Wipe dry with 70% EtOH and leave in sterile hood.

## V. Cell Treatments:

A. *SiRNA (6-well plate):*

1. Vortex siPort Amine for a few seconds then centrifuge briefly to collect everything to the bottom.
1. Add volumes of Opti-MEM and siPort Amine in a sterile Eppendorf. Vortex for a few seconds, spin to collect everything to the bottom and let the tube(s) sit at RT for 30 min.
2. Add siRNA, invert several times to mix, spin to collect everything to the bottom and let the tube(s) sit at RT for 20 min.
3. Near the end of the 20 min incubation, wash cells twice with 1.5mL serum-free DMEM (without antibiotics) per each well and then add 1.5mL DMEM + 1% FBS (without antibiotics). Incubate at 37°C with 5% $CO_2$ for the remainder of the 20 min.
4. After, add 300µL of the siRNA mixture to each well, and rock gently to mix.
5. Incubate the cells for 4 hours at 37°C with 5% $CO_2$. Avoid disturbing the plate as much as possible
6. Wash twice with 1.5mL serum-free DMEM (without antibiotics) per each well and then leave cells in 2.5mL MCDB-131+10% FBS (without antibiotics).

*Table 1: siRNA transfection reagent volumes for each well of 6-well plate*

| Reagent | Volume (µL/well) |
|---|---|
| Opti-MEM (-abs) | 289.45 |
| siPort Amine | 8.75 |
| siRNA (20µM stock) | 1.8 |
| Total | 300 |

7. Give the cells approximately 48h to recover.
8. Repeat the transfection: steps 1-7 (“day 3”).
9. Give the cells approximately 24h after the second transfection to recover. If using for flow, cells should be trypsinized and re-plated in new 6-wells to kept time in culture consistent with other treatments (12-18h).

B. *DMSO/DAPT (6-well plate):*

1. Before starting, cells in which treatments are added should be in culture for 12-18h.
2. Aliquot 2.5mL per well of pre-warmed (reference I.1) MCDB-131 with 2% dextran (1g/25mL).
3. Thaw DAPT (25mM stock) by briefly swirling in 37°C water bath.
4. Using a 1mL syringe, pierce the septum and remove desired amount to obtain a final concentration of 50μM.
   a. i.e. 2 wells will require a total of 5mL dextran media → adding 10μL of stock will produce 50μM solution.
   b. Any excess removed DAPT stock can be stored light protected and reused up to 24h.
5. Add an equal amount of DMSO in control media.
6. To each well add 2mL of desired media (either DMSO or DAPT media) and incubate in 37°C with 5% $CO_2$ incubator for 30 min before culturing under flow.

**VI. Culturing under flow:**

1. After cells have been in culture for 12-18h, aspirate media and replace with 2mL MCDB-131 with 2% dextran (1g/25mL).
2. Leave in 37°C with 5% $CO_2$ incubator for a minimum of 30 min before initiating flow
3. Transfer plate(s) to hood.
4. Remove plate lid to fit onto stage.
5. If there are bubbles in the well, use EtOH vapor to burst them.
6. Secure the plate with putty, so that the cone hovers over the desired well.
7. Carefully lower the cone via the micrometer, just so the cone is level with the well opening. Use this opportunity to center the cone directly in the middle of the well. (look from both front and side to ensure the cone is properly centered before lowering any further.
8. Prop the device up so that it tilts backward slightly to easy the cone into the media without creating bubbles.
9. Lower the micrometer to where it is just submerged, make motion swift and steady to avoid forming bubbles.
10. Cover plate with aluminum brackets and remove from hood to look through bottom stage window to ensure no bubbles are present.
    a. If there are bubbles, try to gently tap to get them out.
    b. If they are too large the cone can be lifted again, but this will create bubbles as it leaves the media. They can be burst using EtOH vapor before attempting cone submersion again, but this will require removing the brackets and possibly removing the plate from the stage.
11. Lower the cone further to calibration point and lock into place by tightening the screw on the opposite side of the device.
12. Transfer flow devices to incubator and plug in power and USB connections (wiped with 70% EtOH and fed through the incubator).
13. Device speed should be pre-programmed, see IV.A., Run “SOFT-EXE-DMX-UMD-236C” and select the option for detecting USB connections
14. The device currently connect via USB to the computer should appear, select this device.
15. Check the enable box and hit START. Status box should show speed at 4400
16. Close the control panel and repeat steps 13-15 as necessary for each device.
17. Avoid opening the incubator as much as possible while running to avoid creating unnecessary disturbances to the cell environment and run for the prescribed amount of time.

**VII. Harvesting Cells:**

1. Pre-warm HBSS 1x (with calcium and magnesium) in 37°C water bath.
2. At a maximum of 30 min before collection aliquot 400μl RLT buffer per sample (from Qiagen Miniprep Kit) into a 1.5mL Eppendorf. To this Eppendorf in a fume hood add 2-Mercaptoethanol at 1:100 dilution (i.e. into 400μL add 4μL). Place this solution on ice until ready to use.
3. At the predetermined time, terminate flow by disconnecting the device power source. Also disconnect the USB if still attached.
4. Transfer the device(s) to the sterile hood.
5. Loosen the micrometer break screw and lift the cone out of the well.
6. Once clear, remove the plate and cover with 6-well plate lid. Proceed to IV.E. steps 1-4 and then continue.

7. Using falcon cell scrapper, occlude the central portion of the well (which due to the device geometry did not receive the same exposure of shear stress).
8. Look at cells under microscope to ensure proper removal of the central portion and to ensure proper cell elongation and alignment consistent with exposure to flow.
9. Return to sterile hood, and wash each well twice with 2mL HBSS.
10. Add 350µL of RLT buffer+ 2-Mercaptoethanol per well.
11. Rock plate to ensure buffer dispersion.
12. Use cell lifter to scrap cells from the bottom of the well.
13. Tilt plate to collect the cell lysate to one side and pipette into fresh labeled Eppendorf tube.
14. Place cell lysate on ice and transfer quickly to -80°C freezer.

**VIII. Isolating RNA**

*A. RNeasy Miniprep Kit:*

1. Thaw cell lysate on ice (recommend performing the following with a maximum of 4 sample tubes at a time).
2. Homogenize by vortexing at medium-high speed for 1 minute.
   a. Protocol allows vortex homogenization for samples containing ≤ 1 x $10^5$ cells. Each 6-well yields ~2 x$10^5$ cells, and considering the center portion and a good portion of the cells are removed, this step should be acceptable.
   b. For larger samples, cell homogenization can be performed using 20g needle and syringe and ejecting 5-10 times.
3. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.
4. Transfer up to 700µL of the sample, including any precipitate, to an RNeasy spin column placed in a 2 mL collection tube. Close the lid and centrifuge for 15 s at ≥8000 x g (≥10k rpm). Discard the flow through.
   c. Flow-through contains Buffer RLT or Buffer RW1 are incompatible with bleach.
   d. After each centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.
5. Add 350µL RW1 buffer to spin column. Close lid and centrifuge for 15 s at ≥8000 x g (≥10k rpm). Discard the flow through.
6. Mix together 5µL DNase I to 35µL ROD buffer per sample in an Eppendorf. Mix well.
7. Add 40µL DNase I mix directly to the column, incubate at RT for 30 min.
8. Repeat step 5.
9. Add 500µL buffer RPE (diluted with 4 volumes of ethanol (96–100%) as indicated on the bottle) to each column. Close lid and centrifuge for 15 s at ≥8000 x g (≥10k rpm). Discard the flow through.
10. Add 500µL buffer RPE to each column. Close lid and centrifuge for 2 min at ≥8000 x g (≥10k rpm). Discard the flow through.
11. Place the spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid and centrifuge at full speed for 1 min.
12. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30µl RNase-free water directly to the spin column membrane. Close the lid and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
13. Keep sample on ice for Nanodrop analysis. Otherwise, store at -80°C.

*B. Nanodrop Analysis:*

1. Select option for Nucleic Acid analysis.
2. Clean the pedestal and lid with Kimwipe dampened with RNase-free water.
3. Add 2µL RNase-free water to the pedestal. Close the lid and initiate the device.
4. Change analysis from DNA to RNA.
5. Wipe down pedestal and lid with clean Kimwipe. Add 2µL RNase-free water to the pedestal. Close the lid and select BLANK.
6. Wipe down pedestal and lid with clean Kimwipe. Add 2µL sample to the pedestal. Close the lid and select Measure.
7. Record numbers given (absorbance, 260nm, 280nm, 260/280, 260/230, ng/µL).
   a. Ratios should be ≥ 2.0 to be pure enough to send for Nanostring analysis.
8. Repeat steps 6-7 for each sample.
9. Clean the pedestal and lid with Kimwipe dampened with RNase-free water. Close the application and leave the lid on the device shut to keep dust out.

10. Samples should then be aliquoted for Nanostring analysis (minimum of 150ng RNA) and stored at -80°C.