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Quantification of Proteins and Genes Associated with Endothelial Cell Function after Different Shear Stress Intensities in vitro V.2

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We use this protocol and it's working

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Disclaimer

The authors have nothing to disclose

Abstract

This protocol describes the steps necessary to culture human umbilical vein endothelial cells (HUVEC), subculture the cells into Ibidi slides, induce endothelial shear stress (ESS) using the Ibidi Pump System, and detect proteins and gene expression associated with endothelial function using immunocytochemistry and qRT-PCR respectively.

Guidelines

Use only 70% ethanol as a cleaning agent.

Avoid repeated freeze-thaw cycles.

Avoid exposing fluorescent secondary antibodies or stained slides to direct sunlight.

Figures

Figure 1. **The cell suspension is directly added to the slide luer.** HUVEC cell in growth media must have a density of 1 x 10^6 cells/mL for optimal adhesion to the slide surface. When adding the cell suspension to the slide, make sure the pipet tip is tilted toward the channel to facilitate media movement. Once the cell suspension fills the channel, cover the luers with the plastic caps included and check the cells using an inverted microscope.

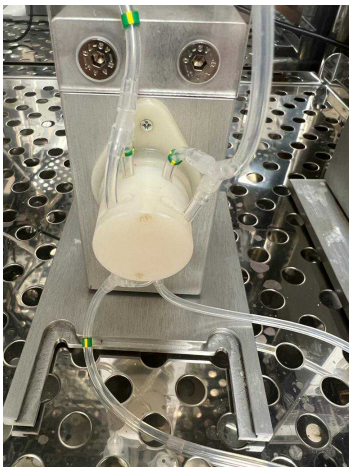

Figure 2. **Perfusion set set-up.** When the perfusion set is installed in the first fluidic unit (to create the unidirectional flow), the tubing marked with a green/yellow ring must be placed in the spaces closer to the fluidic unit. For the second fluidic unit (to create the pulses), install the tubing in the spaces closer to the fluidic unit.

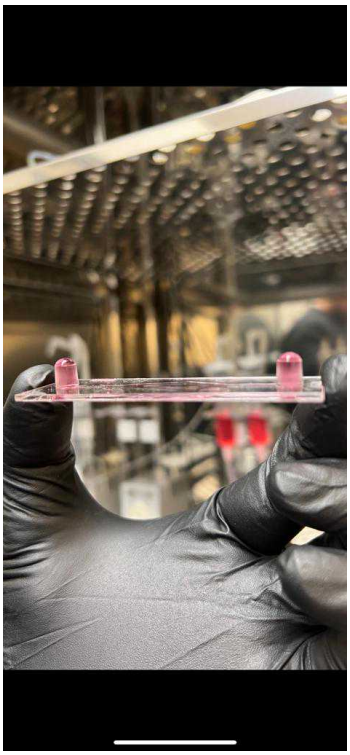

Figure 3. **Attachment of the slide to the fluidic unit.** Add approximately 50 microliters to one of the slide's luer. The media should overfill the luer, this will prevent the formation of air bubbles during the flow experiments.

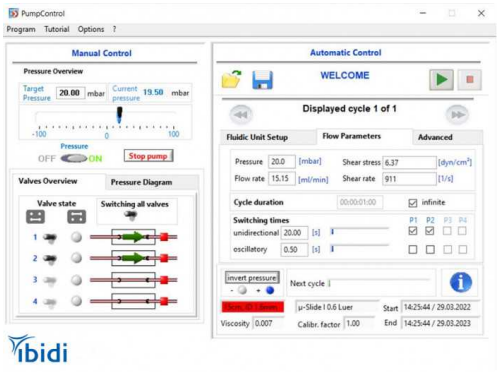

Figure 4. **Ibidi pump control software setup. A.** To set up the correct perfusion set, select the fluidic unit setup tab, select the perfusion set and slide for the experiment and click on apply changes. **B.** To set up the flow intensity, select the flow parameters tab, input the target shear stress, set P1 as unidirectional and P2 as oscillatory, and input the target oscillatory value, all other parameters are calculated automatically.

Tables

Component	Volume (µL)
10X RT Buffer	2.0
25X dNTP Mix (100 mM)	0.8

10 X RT Randon Primers	2.0
Multiscribe Reverse Transcriptase	1.0
Nuclease-free Water	4.2
Total for One Reaction	**10.0**

Table 1. Master Mix Volumes for the cDNA

Component	Volume (µL)
TaqMan Fast Advanced Master Mix (2X)	10
TaqMan Assay (20 X)	1.0
Nuclease-free Water	7.0
Total for One Reaction	**18.0**

Table 2. Master Mix Volumes for RT-qPCR

Materials

Materials

MesoEndo cell growth medium – Cell Applications (211-500)

HUVEC – Cell Applications (200-05n)

T-75 cell culture Flasks – Fisher Scientific (FB012937)

HBSS – Cell Application (062-100)

DPBS – Life technologies (14040-133)

Trypsin EDTA – Cell Applications (070-100)

TNS – Cell Applications (080-100)

Ibidi µ-Slide – Ibidi (80166)

Perfusion set – Ibidi (10964)

Paraformaldehyde – Thermo Scientific (J19943K2)

Triton X-100 – Millipore Sigma (T8787)

BSA – Fisher Scientific (BP1600-100)

eNOSp – Thermo Scientific (PA517917)

Alexa fluor 488 – Invitrogen (A11094)

DAPI – Thermo Scientific (62248)

RNeasy Micro Kit – Qiagen (74004)

High-Capacity cDNA Reverse Transcription Kit – Applied Biosystems (43-688-14)

TaqMan Fast Advanced Master Mix – Applied Biosystems (44-445-56)

Equipment

Ibidi Pump – Ibidi (10902)

Zeiss LSM 700 Confocal Microscope – Zeiss

Thermo Cycler – Applied Biosystems (4339386)

StepOne Plus – Applied Biosystems (4376600)

Safety warnings

Refer to the Safety Data Sheets (SDS) for safety and environmental hazards.

Ethics statement

The protocol needs to be approved by the University's (or institution's) Institutional Review Board (IRB) or the equivalent ethics committee(s) before it can be implemented.

Before start

Make sure that the appropriate ethics committee has approved the protocol.

Determination of Exercise Intensities for Exercise-Induced Endothelial Shear Stress Measurements

1 Notes: Two exercise tests must be performed on a cycle ergometer. The first test determines maximal oxygen uptake (VO2max), maximal heart rate (HRmax), and lactate threshold (La). The second test determines the three exercise intensities for measuring endothelial shear stress (ESS). There must be at least 48 hours between exercise tests. ESS is expressed in dynes/cm^2.

2 Obtain the weight and height of the participants using a standard weighing scale and stadiometer.

3 Adjust the height of the bike's seat to allow a 5° to 15° knee flexion.

4 Have the participant quietly sit on the bike for 10 minutes to ensure sympathetic activity does not alter blood pressure readings.

4.1 Measure the participant's blood pressure at the end of the 10 minutes and repeat the measurements three times.

4.2 Average the blood pressure readings.

5 Perform the VO_2max test following the American Heart Association (AHA) and the American College of Sports Medicine (ACSM) guidelines.

5.1 Have the participants complete 2-minute stages until voluntary exhaustion.

5.2 Increase the workload by 25 Watts after each stage.

5.3 Increase the workload by 25 Watts after each stage.

6 Measure VO_2, HR, and La at the end of each two-minute stage.

6.1 VO_2 is measured with a metabolic cart (i.e., TrueOne 2400, Parvomeics Inc., Sandy, UT).

6.2 HR is measured with a cardiac stress system (i.e., Quinton, Q-Stress Cardiac Stress System, Mortara Instrument, Milwaukee, WI).

6.3 La is measured using a micro-sample of blood from the earlobe and an automated blood lactate analysis (e.g., Lactate Plus, Nova, Inc., Boston, MA).

7 At the end of each test, determine three exercise intensities by determining three La curves as follows: Low-intensity (0-2 mmol/L), Moderate-intensity (2-4 mmol/L), and High-intensity (>4 mmol/L).

Exercise-Induced Endothelial Shear Stress Measurements

8 Before the start of the exercise test, determine the participant's hematocrit from 2 earlobe capillary blood samples.

8.1 Collect the blood using microhematocrit capillary tubes and seal them.

8.2 Centrifuge and determine hematocrit using a semi-automated hematocrit measuring device (i.e., HemataStat II Hematocrit Analyzer, Separation Technology Inc.).

9 Have the participant exercise for 5 minutes at each predetermined intensity.

9.1 Monitor VO_2 and HR throughout the exercise session.

9.2 Assess La at minutes 2 and 4 of each exercise intensity to confirm that the exercise intensity corresponds to the La expected at each intensity.

10 Measure blood flow patterns in the carotid and/or brachial artery using a 12 to 18 Hz ultrasound transducer (i.e., LA435, Esaote, Firenze, Italy).

10.1 Brachial artery – 5 cm proximal to the antecubital fossa. Arm placed on a flat surface, maintaining a ~80° of shoulder abduction and a 35° to 45° flexion.

10.2 Common carotid artery – lateral aspect of the neck.

11 Adjust the ultrasound settings to 12 Hz frequency and 3 cm depth for the brachial artery and 12 to 18 Hz and 3 cm to 5 cm depth for the common carotid artery.

12 Identify a transverse section of the artery and center it.

13 Rotate the transducer 90° to obtain a longitudinal view of the artery and use a pulse wave Doppler to record blood flow velocity.

13.1 Ensure an insonation angle below 60°.

13.2 For the carotid artery place the Doppler 5 to 10 mm distal to the bifurcation.

13.3 For the blood flow of the internal and external carotid arteries, move the Dopper 5 to 12 mm proximal to the bifurcation.

14 Calculate ESS using the Womersley approximation as follows:

14.1 ESS = μ X SR
SR = 2K X V/D

- μ is blood viscosity
- SR is the shear rate
- V is the peak systolic velocity
- D is artery diameter
- K is the complex factor dependent on the Womersly parameter α

14.2 $\alpha = (D/2) X (\omega/[\mu/\rho])^{1/2}$

- D is artery diameter
- ω is the angular frequency of flow pulsation (ω = freq X 2π)
- ρ is blood density
- μ is blood viscosity

Huvec Cell Seeding

15 Notes: This step aims to increase the number of HUVEC in a T-75 flask before seeding them in an Ibidi μ-Slide I Luer with a channel height of 0.2 mm. The following procedures must be performed inside a biosafety cabinet with HEPA-filtered laminar flow. To avoid contamination inside the biosafety cabinet, spray all materials with 70% ethanol before placing them inside.

16 Place the MesoEndo cell growth medium (Cell Applications, San Diego, CA), and the frozen vial of HUVEC cells (Cell Applications, San Diego, CA.) in a water bath set at 37°C and monitor the cells closely. Once there is only minimal ice, remove the cells and the media and place them inside the biosafety cabinet.

17 Pipette 20 mL of MesoEndo cell growth medium in a T-75 cell culture flask (Fisher Scientific, Hampton, NH.).

18 Resuspend the cells inside the vial by slowly pipetting up and down five times. Pipette the cells into the T-75 cell culture flask containing the MesoEndo cell growth medium.

19 Gently rock the flask for 10 seconds to evenly distribute the cells in the flask.

20 Check for cell viability and distribution across the flask using an inverted microscope.

21 Incubate the cells overnight at 37°C with 5% carbon dioxide (CO2). Check for cell adhesion to the flask, and change the cell growth medium to remove traces of dimethyl sulfoxide (DMSO) used during cell cryopreservation. Use 20 mL of growth medium in every cell growth media change.

22 After the overnight cell growth media change, change the cell growth medium every other day until the cells reach 80% confluence.

Subculture into Ibidi µ-Slide I Luer

23 IBIDI offers different µ-Slide versions. This paper uses the µ-Slide I Luer with ibiTreat surface modification for better cell adhesion since we apply high ESS in our experiments. Alternatively, untreated slides can be coated with an extracellular matrix (i.e., collagen). The slides and media must be placed inside the incubator overnight to avoid the formation of bubbles inside the slides.

24 Remove the cell medium from the flask and wash the cells twice using Hank's Buffered Saline Solution (HBSS; Cell Applications, San Diego, CA.) or Dulbecco's Phosphate Buffered Saline (DPBS; Life Technologies, Carlsbad, CA.)

25 After the last wash, completely remove HBSS, add 5 mL of Trypsin/EDTA (Cell Applications, San Diego, CA.), rock the flask until all the cells are covered, and immediately remove 4.5 mL of the Trypsin/EDTA from the flask.

26 Monitor the trypsinization of the cells under the microscope. Once the cells become round, tap the flask lightly to detach the cells completely.

27 Add 5 mL of trypsin neutralizing solution (TNS; Cell Applications, San Diego, CA.) and transfer the cell suspension to a 50 mL conical tube (Fisher Scientific, Hampton, NH.). Rinse the T-75 flask with 5 mL of TNS and transfer it to the 50 mL conical tube containing the cells.

28 Centrifuge the conical tube for 5 minutes at 220 x g to pellet the cells. After pelleting the cells, carefully remove the supernatant without disturbing the pellet.

29 Loosen the pellet by flicking the tip of the tube, add 2 mL of cell growth media, and carefully resuspend by pipetting up and down.

30 Count the cells and make a suspension with a density of 1 x 106 cells/mL. Dilute the cells with cell growth media if necessary.

31 Add 150 µL of the cell suspension directly into the channel inlet of an Ibidi µ-Slide I Luer (Ibidi USA, Fitchburg, WI.) using a 200 µL pipette (Figure 1).

32 Incubate the slide at 37°C with 5% carbon dioxide (CO2) and change the media daily until the cells reach 80% confluence.

32.1 Add the media slowly to prevent the formation of air bubbles inside the slide.

Setting up the Ibidi Pump system for ESS

33 The perfusion set and media must be placed inside the incubator to avoid the formation of bubbles during the ESS experiments. Make sure to use the appropriate perfusion set. For this experiment, we used the yellow-green perfusion set.

34 Place two fluidic units inside the incubator. One unit is used to produce the unidirectional flow, and the second unit creates the pulses. Attach the yellow/green perfusion set (Ibidi USA, Fitchburg, WI.) to the fluidic units, ensuring that the tubing with the yellow/green marks is against the fluidic unit (Figure 2).

35 Pinch the line with the plastic clip, add 5 mL of media to each syringe, and connect the two ends of the perfusion set with the plastic connector.

36 In the Flow Control Software (Ibidi USA, Fitchburg, WI.), load the remove air bubbles demo set-up, remove the plastic clip, and press the play button to remove the bubbles.

36.1 Under the fluidic unit set-up, select the appropriate perfusion set.

36.2 Under slide selection, select without any slide.

37 Once the bubbles are removed, pinch the line, remove the plastic connector, add approximately 50 µL of media to one of the channel inlets of the Ibidi µ-Slide I Luer, and connect the perfusion set (Figure 3.)

38 Set the ESS conditions in the flow parameters tab using the pump control software. Make sure that fluidic unit 1 (P1) is set as unidirectional and fluidic unit 2 (P2) is set as oscillatory.

38.1 Shear stress – 10 dyn/cm^2.

38.2 To achieve 60 pulses per minute, set the oscillatory value to 1 (s). For higher pulses, divide 60 over the target pulses per minute, for example 100 pulses per minute 60/100 = 0.6.

38.3 All other parameters are calculated automatically (Figure 4).

38.4 If you are running two experiments simultaneously, set P3 as unidirectional and P3 as oscillatory.

Protein Detection Using Immnocytochemistry

39 Notes: All the dilutions for IHC are made using DPBS, and incubations are performed at room temperature unless otherwise indicated. If two proteins are detected simultaneously, primary antibodies must be from different species (e.g., mouse and rabbit) with corresponding secondary antibodies. For this experiment, we targeted the phosphorylated enzyme endothelial nitric oxide synthase (eNOSp; Thermo Scientific, Ward Hill, MA.) and used the fluorescent secondary antibody Alexa fluor 488 (Invitrogen, Carlsbad, CA.).

40 Remove all the cell media and wash the cells with 150 µL of DPBS (Life Technologies, Carlsbad, CA.) and add 150 µL of 2% paraformaldehyde (Thermo Scientific, Ward Hill, MA.) to fix the cells and incubate them for 10 minutes at room temperature.

41 Remove the paraformaldehyde and wash the cells three times with DPBS. Add 150 µL of 0.1% Triton X-100 (Millipore Sigma, St Louis, MO) and incubate for 10 minutes at room temperature.

42 Remove the Triton X-100 and wash the cells two times with DPBS. Add 150 µL of 1% bovine serum albumin (BSA, Fisher Scientific, Waltham, MA.), and incubate overnight at 4°C.

43 Remove the BSA, add 150 µL of 1:100 primary antibody diluted in 1% BSA, and incubate for 60 minutes at 37°C.

44 The following steps must be performed in a dark environment to protect the cells and secondary antibodies from light.

44.1 Remove the primary antibody and wash the cells three times with DPBS, add 150 µL of 1:1000 secondary antibody, and incubate for 60 minutes.

44.2 Remove the secondary antibody and wash the cells three times with DPBS, add 150 µL of 1:1000 4’,6-diamino-2-phenylindole (DAPI; Thermo Scientific, Ward Hill, MA.) and incubate for 5 minutes.

44.3 Remove DAPI and wash the cells three times with DPBS. Add 150 µL of DPBS and store the cells at 4°C protected from light.

45 Visualize the proteins and nucleus using a confocal microscope. The representative images were taken using the Zeiss LSM confocal microscope (Zeiss, White Plains, NY.).

mRNA Extraction and qRTPCR

46 Note: A minimum of four Ibidi µ-Slide I Luer must be used for optimal mRNA concentration. The mRNA extraction uses the Qiagen column-based RNeasy Micro Kit (Qiagen, Hilden, Germany) and TaqMan fast advanced components and the StepOnePlus real time System (Applied Biosystems, Waltham, MA.) for gene expression assessment.

47 Remove all the cell media, wash the cells five times with DPBS, and add 100 µL of RLT buffer to lyse the cells.

48 Attach a 1 mL syringe to one of the channel inlets and move the plunger quickly up and down several times to shear out the lysed cells.

49 Add an additional 100 µL of RLT buffer and shear out any remaining the cells in the slide. In a 1.5 mL microcentrifuge tube mix the 200 µL of cell lysate with 150 µL of RLT buffer to achieve a total volume of 350 µL.

50 Using a new syringe with a 20-gauge needle, pass the entire cell lysate 15 times. Completely homogenize the lysate by vortexing for 30 seconds.

51 Add 350 µL of 70% ethanol to the homogenized cell lysate and mix by pippeting up and down.

52 Bind the total RNA to a MinElute spin colum, by transferring up to 700 µL of the cell lysate. Centrifuge the column at 8,000 x g for 15 seconds and discard the flow through from the collection tube.

52.1 Repeat this step for any remaining cell lysate.

53 To digest DNA, add 350 µL of RW1 buffer, centrifuge at 8,000 x g for 15 seconds and discard the flow through. Add DNase I incubation mix and incubate for 15 minutes. Add 350 µL of RW1 buffer, centrifuge at 8,000 x g for 15 seconds and discard the flow through.

54 To wash the spin column, use a new collection tube. Add 500 µL of RPE buffer and centrifuge at 8,000 x g for 15 seconds. Repeat this step. Add 500 µL of 80% ethanol, centrifuge at 8,000 x g for 2 minutes and discard the flow through and collection tube.

55 To elute the RNA, place the spin column in a new collection tube and centrifuge at >12,000 x g for five minutes and discard the flow through and collection tube. Place the spin column into a 1.5 mL tube, add 14 µL of RNA free water, incubate for 3 minutes and centrifuge at >12,000 x g for one minute.

55.1 The eluted RNA can be stored at -20°C for a month.

55.2 The concentration of RNA can be measured using the absorbance at 260 nm. The ratio of absorbance at 260 nm and 280 nm provides a measurement for the purity of the eluted RNA.

56 Prepare a reverse transcription master mix for up to 2 µg of mRNA to cDNA using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA.) following the volumes in Table 1.

57 Load 10 µl of the reverse transcription master mix in a PCR plate or PCR tubes. Add 10 µL of the eluted mRNA and load the plate/tubes in a thermocycler. Set the thermocycler reaction as follows:

57.1 Step 1: 25 °C for 10 minutes.

57.2 Step 2: 37°C for 120 minutes.

57.3 Step 3: 85°C for 5 minutes.

57.4 Step 4: 4°C.

58 Prepare a PCR master mix using the TaqMan Fast Advanced Master Mix (Applied Biosystems, Waltham, MA.) following volumes in Table 2.

59 Load 18 µL of the PCR master mix in a PCR plate or PCR tubes. Add 2 µL of cDNA and assess gene expression on the StepOnePlus PCR system following the following reaction set-up:

59.1 One cycle at 50°C for 2 minutes.

59.2 One cycle at 90°C for 20 seconds.

59.3 40 cycles at 95°C for 1 second, followed by 60°C for 20 seconds.

60 Determine gene expression using the relative standard curve, comparative threshold cycle method, or other methods[1].

Protein Extraction and Western Blot Analysis

61 Add 10 mL of Dulbecco's Phosphate Buffered Saline (DPBS) in a 15 ml conical tube.

61.1 Keep it in ice or refrigerated until use

62 Prepare 1 mL lysis buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF).

62.1 Add the PMSF immediately before use.

62.2 Keep it in ice or refrigerated until use.

63 Pre-cool a syringe, needle, and two 1.5 mL tubes by placing them in ice or the refrigerator (4°) 15 to 20 minutes before starting the extraction.

64 Remove the Ibidi slides from the incubator and immediately place them on ice.

64.1 All the following steps are performed with the slides on the ice.

65 Wash the channels 3 times using cold PBS.

65.1 Remove the cell culture media by aspirating it from one of the luers.

65.2 Pipette about 100 µL of cold DPBS in one luer and remove from the opposite luer.

66 After the third wash, add cold DPBS and keep the slide on ice until lysed.

66.1 Lyse only one slide at a time and keep the rest in ice.

67 Remove the DPBS from the slide, and using the pre-cool syringe, add 200 µL of cold lysis buffer with PMSF.

67.1 Make sure the slide channel is completely covered with lysis buffer.

67.2 Remove bubbles by pumping the syringe's plunger up and down to move the buffer inside the slide back and forth.

68 Incubate for 5 minutes.

68.1 Keep the slide on ice and the syringe attached to the luer.

69 Remove the lysed cells by quickly moving the plunger of the syringe up and down.

69.1 Make sure not to squeeze the cell lysate out of the other luer.

70 Remove all the lysate from the slide and keep it inside the syringe.

71 Remove the DPBS for the next slide, add the lysate from the syringe, and repeat steps 67 - 70.

72 Once all the slides have been completed, collect the lysate in the pre-cooled 1.5 mL tube.

73 Attach the needle to the syringe and pass the entire lysate through it 15 times.

73.1 Do it slowly to avoid theexcessive creation of bubbles.

74 Centrifuge the lyste at 14,000 x g at 4 °C for 10 minutes.

75 Collect the supernatant in the remaining pre-cooled 1.5 mL tube.

76 Proceed to electrophoresis or freeze the sample.

76.1 If used immediately, the sample can be maintained at 4 °C or in ice.

76.2 If not used immediately, store the sample at -20 °C for a few days to up to 2 weeks, or at -80 °C for up to 6 months.

77 Perform a bicinchoninic acid (BCA) assay or another method to quantify each extracted sample's protein content.

78 Prepare 1 mL of 4X Laemly with 1M dithiothreitol (DTT).

78.1 Weight 154.25 mg of DTT and add it to the Laemli buffer.

79 Dilute the proteins using the 4X Laemly buffer to achieve 30 µg per sample.

80 Use a Biorad pre-cast gel and assemble the electrophoresis cassette.

80.1 Remove the tape at the bottom of the pre-cast gel before assembly.

81 Place the cassette in the boat and add enough Tris-glycine sodium dodecyl sulfate (TGS) buffer.

81.1 Remove the comb slowly to keep the well walls straight.

82 Load 2 µL of Biorad dual color molecular weight marker in the first lane and 30 µL of sample in the remaining lanes.

82.1 Load the proteins slowly to avoid spills of samples to other wells.

83 Run the electrophoresis apparatus at 50 V for about 2.5 hours until the proteins run to the bottom of the gel and stop the current.

84 Disassemble the cassette, rinse the pre-cast gel with deionized (DI) water, and break the plastic casing to remove the gel.

84.1 Remove one side of the casing, leaving the gel on one side of the casing.

85 Open a Biorad transfer pack to create a transfer sandwich.

85.1 Remove the contents of the side labeled bottom which contains the membrane, and place iton the bottom piece of the transfer cassette

86 Place the gel on top of the membrane.

86.1 Use the roller to remove air bubbles.

87 Remove the contents of the side labeled top and place it on top of the gel.

87.1 Use the roller to remove air bubbles.

88 Close the transfer cassette and place it on the Biorad Trans-Blot Turbo Transfer System.

88.1 Run the preset “turbo” and “1 mini TGX”.

88.2 The system automatically makes the transfer in 3 minutes.

89 When the transfer is completed, remove and open the cassette.

89.1 Dispose of the filters from the sandwich and gel.

89.2 A successful transfer shows the molecular weight marker in the membrane.

90 Place the membrane in an incubation box and add enough LiCor intercept blocking buffer to cover the membrane.

90.1 The membrane should be floating in the box.

90.2 Remove any air bubbles from the bottom of the membrane by rocking the box.

90.3 If bubbles cannot be removed by rocking the box, lift the membrane using forceps and slowly place it back in the box.

91 Place the incubation box on a shaker set at 130 revolutions per minute (RPM) and incubate it for 2 hours at room temperature (22 °C to 25 °C).

92 Prepare 20 µL of the primary antibody by diluting it in blocking buffer according to the vendor's recommendation for western blot.

92.1 Abcam's eNOS primary antibody is recommended as 1:1000.

93 After the incubation in blocking buffer, remove it from the incubation box and add the diluted primary antibody.

93.1 The blocking buffer can be saved at 4 °C and re-used 3 times.

94 Place the incubation box in a shaker set at 130 RPM and incubate overnight at 4 °C.

94.1 It can be placed in a cold room if available or inside a refrigerator.

95 After overnight incubation, remove the primary antibody and wash the membrane 3 times using Tris-buffered saline (TBS).

95.1 Add TBS to the incubation box, place it on the shaker for 10 minutes, and discard the TBS.

95.2 The diluted primary antibody can be saved at 4 °C and re-used approximately 2 or 3 times.

96 Prepare a fluorescent secondary antibody following the vendor's recommendation for western blot.

96.1 LiCor IRDye 800 fluorescent secondary antibody is recommended at 1:15,000.

96.2 The secondary antibody must be from the same species as the primary antibody.

97 Incubate at room temperature for 2 hours, protecting it from light.

98 After incubation, wash the membrane 3 times with TBS.

99 If normalization is required, follow steps 93 - 98, describing the incubation in primary and secondary
antibodies.

100 Scan the membrane using a near-infrared fluorescent scanner such as the Li-Cor Odyssey DLx.

100.1 The membrane can be saved at 4 °C in TBS.

100.2 Alternatively, the membrane can be dried in between two filter papers.

Protocol references

Rao, X., et al., *An improvement of the 2ˆ (−delta delta CT) method for quantitative real-time polymerase chain reaction data analysis.* Biostatistics, bioinformatics and biomathematics, 2013. **3**(3): p. 71.