Oct 18, 2023 Version 1

# Quantification of Proteins and Genes Associated with Endothelial Cell Function after Different Shear Stress Intensities in vitro V.1

DOI
https://dx.doi.org/10.17504/protocols.io.ewov1q34kgr2/v1

Daniel Conde[1], Mario Garcia[2], Manuel Gomez[3], Alvaro Gurovich[1]

[1]Physical Therapy and Movement Sciences Department, The University of Texas at El Paso;
[2]Driskill Graduate Program in Life Sciences, Northwestern University Feinberg School of Medicine;
[3]Interdisciplinary Health Sciences Ph.D. Program, The University of Texas at El Paso

Daniel Conde
Physical Therapy and Movement Sciences Department, The Unive...



OPEN ACCESS

**DOI:** https://dx.doi.org/10.17504/protocols.io.ewov1q34kgr2/v1

**Protocol Citation:** Daniel Conde, Mario Garcia, Manuel Gomez, Alvaro Gurovich 2023. Quantification of Proteins and Genes Associated with Endothelial Cell Function after Different Shear Stress Intensities in vitro. **protocols.io** https://dx.doi.org/10.17504/protocols.io.ewov1q34kgr2/v1

**Protocol status:** Working
**We use this protocol and it's working**

**Created:** October 16, 2023

**Last Modified:** October 18, 2023

**Protocol Integer ID:** 89369

**Keywords:** HUVEC, Ibidi, Immunocytochemistry, qRT-PCR, endothelial shear stress, genes associated with endothelial cell function, endothelial cell function, umbilical vein endothelial cell, endothelial cell, endothelial function, endothelial, cells into ibidi slide, gene expression, different shear stress intensity, umbilical vein, ibidi pump system, ibidi slide, quantification of protein

## 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.

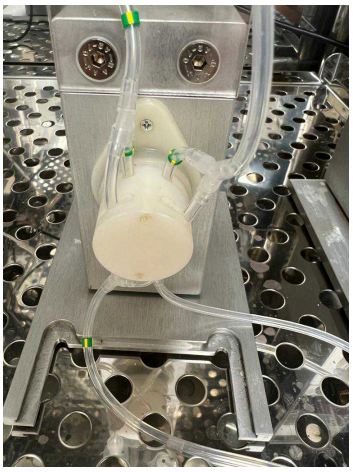

Figure 2. **Perfusion set set-up.** When the perfusion set is installed in the first fluidic unit (to create the unidirectional flow), the tubing marked with a green/yellow ring must be placed in the spaces closer to the fluidic unit. For the second fluidic unit (to create the pulses), install the tubing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

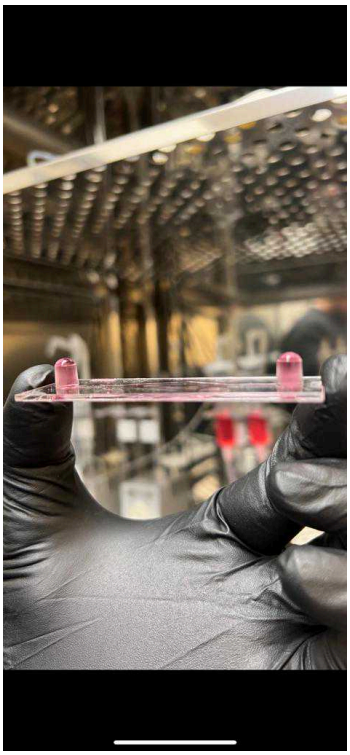

Figure 3. **Attachment of the slide to the fluidic unit.** Add approximately 50 microliters to one of the slide's luer. The media should overfill the luer, this will prevent the formation of air bubbles during the flow experiments.

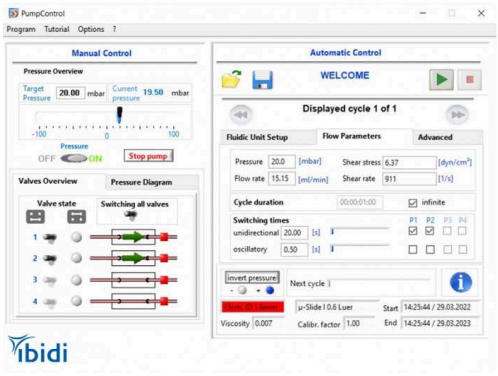


Figure 4. **Ibidi pump control software setup. A.** To set up the correct perfusion set, select the fluidic unit setup tab, select the perfusion set and slide for the experiment and click on apply changes. **B.** To set up the flow intensity, select the flow parameters tab, input the target shear stress, 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 (20X) | 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

N/A.

## Before start

N/A.

## Huvec Cell Seeding

1 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.

2 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.

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

4 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.

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

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

7 Incubate the cells overnight at 37°C with 5% carbon dioxide ($CO_2$). 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.

8 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

9 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.

10 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.)

11 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.

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

13 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.

14 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.

15 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.

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

17 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).

18 Incubate the slide at 37°C with 5% carbon dioxide ($CO_2$) and change the media daily until the cells reach 80% confluence.

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

## Setting up the Ibidi Pump system for ESS

19 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.

20 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).

21 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.

22 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.

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

22.2 Under slide selection, select without any slide.

23 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.)

24 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.

24.1 Shear stress – 10 dyn/cm$^2$.

24.2 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.

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

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

## Protein Detection Using Immnocytochemistry

25 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.).

26 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.

27 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.

28 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.

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

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

30.1 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.

30.2 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.

30.3 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.

31 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

32 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.

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

34 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.

35 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.

36 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.

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

38 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.

38.1 Repeat this step for any remaining cell lysate.

39 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.

40 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.

41 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.

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

41.2 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.

42 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.

43 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:

43.1 Step 1: 25 °C for 10 minutes.

43.2 Step 2: 37°C for 120 minutes.

43.3 Step 3: 85°C for 5 minutes.

43.4 Step 4: 4°C.

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

45 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:

45.1 One cycle at 50°C for 2 minutes.

45.2 One cycle at 90°C for 20 seconds.

45.3 40 cycles at 95°C for 1 second, followed by 60°C for 20 seconds.

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

## 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.