Western Blot Analysis with Cell Samples Grown in Channel-µ-Slides

Polyacrylamide gel electrophoresis (PAGE) and subsequent analyses are common tools in biochemistry and molecular biology. This Application Note describes a protocol for preparing cell lysates directly in the ibidi channel-µ-Slides and the subsequent separation in an SDS-PAGE. The proteins are blotted to a nitrocellulose membrane and the species of interest are analysed with immunochemistry. In our example, we show the detection of VE-cadherin in HUVECs.

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1. Material

Cell Culture	
HUVEC	Minimum 1 x 10^6 cells
µ-Slide $I^{0.4}$ Luer	4 pieces
Endothelial Cell Growth Medium	
Supplement Mix (containing 2% FCS)	
PBS 1x	
Accutase	
Optional: ibidi Pump System for flow conditioning of the cells	
Cell Lysis	
Cell Lysis Buffer	Cell Signaling (#9803)
PMSF (proteinase inhibitor)	1 mM PMSF in lysis puffer
PBS 1x	
Fine dosage syringe with displacement spike (1 ml) + 25-gauge needle	
Reaction tubes 1.5 ml	
Sonicator	
BCA Protein Assay	
BCA Protein Assay Kit (Thermo Scientific)	
96 well plate	
Plate reader (562 nm)	

SDS-PAGE	
Loading Buffer (Roti®-Load 1)	K929.1; Carl Roth
Heating Block with Shaker (90°C)	
Gel (12% SDS)	
Gel electrophoresis apparatus with power supply	
Protein standard	
Electrophoresis running buffer	
Blotting	
Blotting apparatus	
Blotting buffer	
Nitrocellulose membrane	
Filter paper	
Ponceau S solution	20 ml
PBST	0.075% Tween20 in PBS
Antibody Labeling and Scan	
Compact Rocker	For membrane incubation
Milk powder	
PBST	0.075% Tween20 in PBS
Primary antibody	
Secondary antibody	
Immunofluorescence reader	

2. Cell Cultivation

Before the lysis of the cells cultivate them in a ibidi µ-Slide according to your experimental setup. This Protocol was established with flow-cultivated HUVECs. Therefore, µ-Slides $I^{0.4}$ Luer are filled with a cell suspension of $2*10^6$ cells/ml. The µ-Slides $I^{0.4}$ Luer with seeded HUVECs are placed in an incubator for two hours to assure that most of the cells are adherent before the start of the flow cultivation. The setup of a flow experiment to cultivate HUVECs under shear stress conditions is described in Application Note 13. Four µ-Slides, which were flow-cultivated over a period of two days with a shear stress of 10 dyn/cm² (Figure 1), are used for producing one cell lysate.

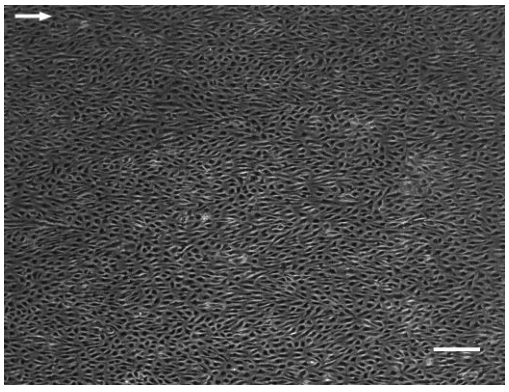

Figure 1: Flow-cultivated HUVECs at 10 dyn/cm² after a period of two days. The arrow indicates the direction of the flow (Scale bar: 200 µm).

3. Cell Lysis

Work on ice to avoid degradation of proteins. Put PBS, cell lysis buffer, needle, syringe, and 1.5 ml reaction tubes on ice. Just prior to use, add 1 mM of PMSF to the lysis buffer. For each cell sample, use a minimum of four µ-Slides $I^{0.4}$ Luer with a confluent cell layer.
In order to minimize the volume of the resulting protein solution, a volume of 200 µl lysis buffer is used for the first Slide to lyse the cells, and then reused for the subsequent Slides.

1. Put the four µ-Slides on ice.
2. Wash every channel Slide three to four times with 120 µl PBS each, in order to remove the cell culture medium.
3. Start with one µ-Slide; keep the other slides filled with PBS on ice, while lysing the cells of the first Slide.
4. Remove the PBS from the channel.
5. Draw up 200 µl of the lysis buffer with the syringe (Figure 2 A). Do not use the needle here!
6. Plug the syringe into one Luer adapter of the empty channel Slide and fill up the channel with the lysis buffer (Figure 2 B).
7. Incubate with the lysis buffer for five minutes.
8. Shear the cells out of the channel by moving the plunger quickly up and down. Be careful not to squeeze out the cell lysate at the free Luer adapter of the slide. Check under the microscope to see if the cell layer is completely washed out.
9. Fully remove the PBS from the next channel slide.
10. Draw up the entire cell lysate with the syringe and fill the next PBS-free channel with the lysate. The residual lysate can be pipetted directly into a precooled 1.5 ml reaction tube.
11. Repeat Steps 6 through 10 for all Slides.
12. After the treatment of the last Slide, fill the lysate into the previously used 1.5 ml reaction tube.
13. Slip the precooled needle onto the syringe and pass the lysate through the needle 10 to 15 times. For a better cell disruption, you can also use a sonicator.
14. Spin the extract in a microcentrifuge at 14,000x g for 10 minutes at 4°C.
15. Transfer the supernatant into a new, precooled 1.5 ml reaction tube. For future processing, the lysate can be stored at -20°C.

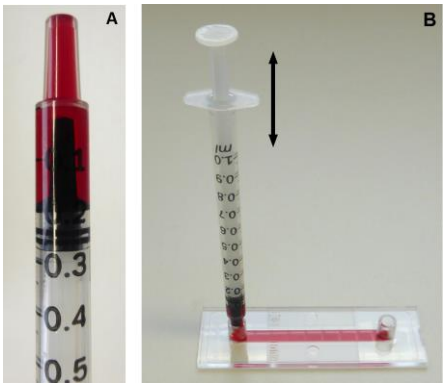

Figure 2: A) Fine Dosage syringe with the displacement spike filled with 200 µl. **B)** µ-Slides $I^{0.4}$ Luer filled by the syringe. Lyse cell layer in channel slide by moving the plunger up and down.

4. BCA Protein Assay

The Pierce BCA Protein Assay Kit (Thermo Scientific) is used to determine the total amount of protein. The total protein is quantified by a formulation that is based on bicinchoninic acid (BCA) for a colorimetric detection. The assay should be performed as described in the manufacturer's instructions, and using a µ-Plate 96 well.

5. SDS PAGE and Blotting

A general protocol for lysate preparation, SDS PAGE, as well as membrane blotting, is described below. The optimal protocol for your protein of interest might differ from the one given below according to the characteristics of your protein.

1. Mix the desired protein amount with the sample buffer (at least 15 µg of total protein is recommended).
2. Heat the sample at 90-100°C for 5 minutes and then cool down the sample afterwards.
3. Load the complete sample onto an SDS PAGE gel. Add a (prestained) protein ladder into an extra lane.
4. Set an appropriate constant voltage, depending on how many gels you run and the gel's thickness.
5. Stop the electrophoresis run when the dye front reaches the bottom of the gel. The running time depends on the applied voltage.
6. Place the gel and nitrocellulose membrane separately in the blotting buffer for 15 minutes.
7. Prepare the transfer stack (the membrane should be on the cathode and the gel on the anode side).
8. Transfer the proteins at a constant current. The duration and the current's height will depend on the blotting method.
9. Stain the membrane with Ponceau S to check the transfer quality.
10. Rinse the membrane three times for 10 minutes with PBST to remove the Ponceau S staining.

6. Antibody Labeling and Scan

To detect the protein bands on the membrane a suitable labelling must be done. The detection of the analyzed protein band was performed using fluorophore-conjugated secondary antibodies. Figure 3 shows an example of a fluorescence-labeled VE-cadherin band.

During the mentioned incubation times of step 1 to 5 the membrane has to be shaked gently.

1. Block the membrane for 1 hour at room temperature using a blocking solution (5% milk powder in PBST).
2. Incubate the membrane with the recommended dilution of the primary antibody in a blocking solution overnight at 4°C.
3. Wash the membrane three times with PBST for a duration of 10 minutes each time.
4. Incubate the membrane with the recommended dilution of the fluorophore-conjugated secondary antibody in blocking solution for 1 hour at room temperature.
5. Wash the membrane three times with PBST for a duration of 10 minutes each time.
6. Dry the fluorescently labeled membrane between filter papers for at least 1 hour.
7. Scan the dried membrane with an appropriate emission filter. The generated digital image of the western blot allows for further data analysis.

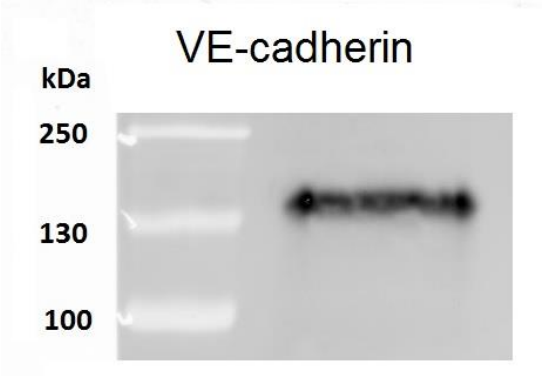

Figure 3: Scan of the fluorophore-labeled VE-cadherin band at the expected size of ~140 kDa.