# Gene Expression Profiling with qPCR
## Applicable for Cells Cultivated in Channel-Slides

Gene expression profiling provides information about the transcriptome of a living cell. The DNA is transcribed into mRNA, when the specific gene is active. This can be influenced by stimuli like chemical compounds, internal signals of the cell or mechanical stress.

This Application Note describes the preparation of cell lysates, directly in the ibidi channel-µ-Slides, and the subsequent quantification of mRNA in HUVEC. The mRNA is isolated from cells and analyzed afterwards with quantitative PCR (qPCR). In this example, the detection of Claudin-5 in HUVEC is presented. The protocol consists of four main steps as seen in Figure 1.

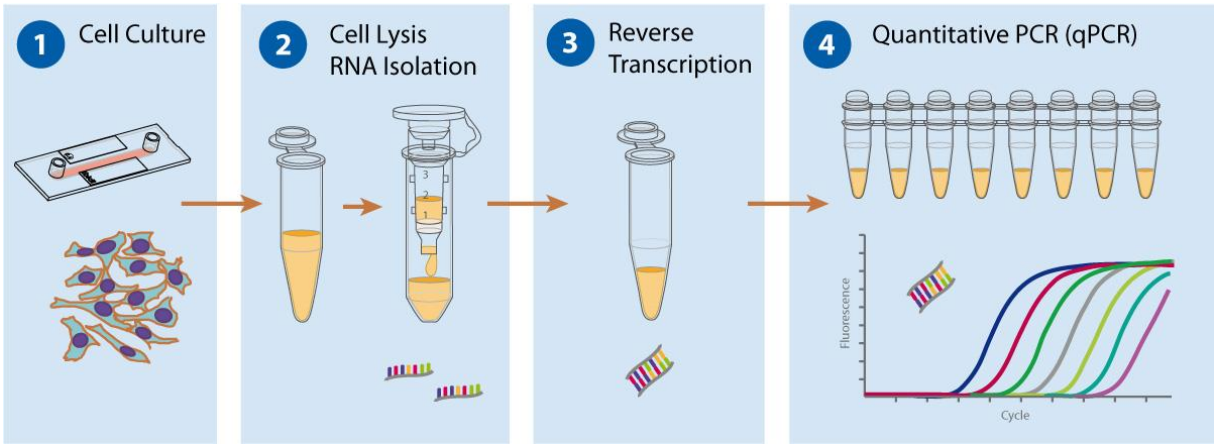


**Figure 1:** Four main steps for gene expression profiling.

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# 0. Material

## Cell Culture

- HUVEC (ca. 2 x $10^5$ cells)
- µ-Slide I $^{0.4}$ Luer (one slide)
- Optional: ibidi Pump System for flow conditioning of cells
- Appropriate cell culture medium and reagents for cell splitting

## Cell Lysis and Homogenization

- Lysis Buffer (RLT Buffer from the Kit)
- PBS
- 20-gauge needle and syringe

## RNA Extraction

- RNeasy Micro Kit (Qiagen)
- Ethanol (70% and 96%)
- Ethanol 80% with RNAse-free water

## Reverse Transcription

- QuantiTect Reverse Transcription Kit (Qiagen)
- Optional: gene-specific primer

## Quantitative PCR

- Light Cycler 480 SYBR Green (Roche)
- Gene-specific primer and GAPDH primer

## Equipment

- Centrifuge
- Vortex
- Sterile, RNAse-free pipet tips
- Reaction cups (2 ml / 200 µl)
- Microplate Spectrometer
- Heating block

## 1. Cell Cultivation

Cultivate the cells inside a channel slide, and then treat them to meet your special experimental needs.
In our example, we show the gene expression profiling with flow-cultivated HUVEC in µ-Slides I $^{0.4}$ Luer. The cells were seeded in the Slides and connected to the flow conditions after the time period needed for cell attachment. At several time points, samples were disconnected and prepared following the steps of this protocol, to determine the time response of the gene expression. As gene of interest we chose a tight junction protein (Claudin-5), which is only present in endothelial cells, and which is strongly regulated by flow conditions.
The setup of an experiment to cultivate HUVEC under shear stress conditions is described in detail in **Application Note 13**.

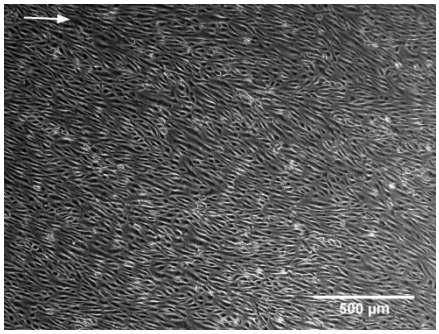


**Figure 2:** Flow cultivated HUVEC at 30 dyn/cm² after a period of one day. The arrow indicates the direction of flow.

## 2. RNA Extraction

In the first step, RNA is extracted from the cells of interest. The total mRNA represents the spectrum of genes which is active at the actual time point.

The RNeasy Micro Kit from Qiagen was used to isolate total RNA from flow-cultivated HUVEC. This kit is easy to handle and enables the selective binding of RNA to a silica-based membrane.

Differing from the manufacturer's instructions, some improvements to the protocol have been made to get the best results, related to the quality and quantity of RNA. For further information about the kit, please read the RNeasy Micro Kit Handbook ([1] Qiagen).

Important Note!
This Application Note is optimized for the RNA extraction of HUVEC.

## 2.1. Cell Lysis and Homogenization

It is important to perform all of the following steps at room temperature (15–25°C) and to work as fast as possible. Homogenized cell lysates can be stored at –70°C. For further instructions, please read the RNeasy Micro Kit Handbook [1] (Qiagen).

1. Aspirate the cell culture medium from the channel slide.
2. Wash the cells four to five times with 120 µl of PBS.
3. Remove the PBS from the channel.
4. Add 100 µl of RLT Buffer to the channel and plug the syringe into one Luer adapter (Figure 3A).
5. 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 empty Luer adapter of the slide.
6. Repeat Steps 4 and 5 one time.

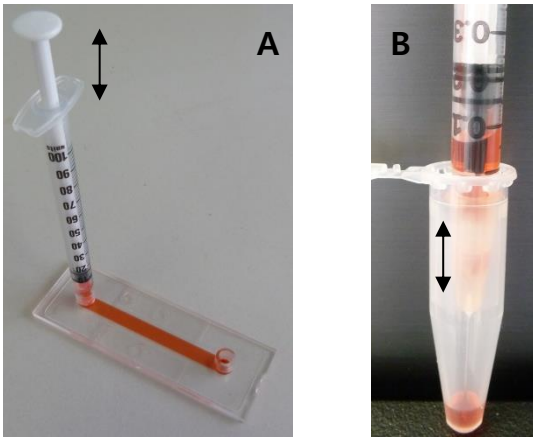


**Figure 3: A)** µ-Slide I$^{0.4}$ Luer filled by the pipette. Lyse cell layer in channel slide by moving the plunger up and down. **B)** Homogenize the cells with syringe and needle by moving the plunger up and down.

7. Mix the disrupted cells with 150 µl of RLT Buffer, to achieve a total volume of 350 µl.
8. Slip a needle onto a new syringe and pass the lysate through the needle at least 10 times (figure 3B).
9. Vortex the solution for 30 seconds to homogenize the lysate completely.

## 2.2. Extraction (According to the Qiagen protocol [1])

***Bind total RNA***

1.) Add 350 µl of 70% ethanol to the lysate and mix by pipetting.

2.) Transfer the sample to an RNeasy MinElute spin column. Close the lid and centrifuge for 15 seconds at ≥8000 x *g*. Discard the flow-through.

### *Digest DNA*

3.) Add 350 µl of RW1 Buffer to the column. Close the lid and centrifuge for 15 seconds at ≥8000 x *g* to wash the column. Discard the flow-through.

4.) Add 10 µl of DNase I stock solution to 70 µl RDD Buffer and mix gently.

5.) Add the DNase I incubation mix directly to the column membrane and place it for 15 minutes at room temperature.

6.) Add 350 µl of RW1 Buffer to the column. Close the lid and centrifuge for 15 seconds at ≥8000 x *g* to wash the column. Discard the flow-through.

### *Wash*

7.) Place the spin column in a new 2 ml collection tube.

8.) Add 500 µl of RPE Buffer, then close the lid and centrifuge for 15 seconds at ≥8000 x *g* to wash the membrane. Discard the flow-through.

9.) Repeat Step 8.

10.) Add 500 µl of 80% ethanol to the column, close the lid and centrifuge for 2 minutes at ≥8000 x *g*. Discard the flow-through and the collection tube.

### *Elute*

11.) Place the spin column in a new 2 ml collection tube. Open the lid and centrifuge at full speed for 5 minutes. Discard the flow-through and collection tube.

12.) Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14 µl of RNAse-free water to the membrane and incubate for 3 minutes. Close the lid and centrifuge for 1 minute at full speed to elute the RNA.

13.) Storage is possible at –20°C in RNAse-free water for several weeks.

The concentration of RNA can be determined by measuring the absorbance at 260 nm directly from the isolated sample. The quotient of the absorbance at 260 nm and 280 nm provides an estimated purity of RNA.

**A maximum yield of 1.5 µg RNA is expected for this amount and type of cells.**

## 3. Reverse Transcription

In the reverse transcription step, the RNA is transferred into complementary DNA (cDNA), which is much more stable for the use in the subsequent PCR.
We used the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. For further information about this kit please read the QuantiTect Reverse Transcription Kit Handbook.

## 4. Quantitative PCR

The quantitative PCR shows the increment of targeted DNA in real time. A DNA-binding dye is used, which only binds to double stranded DNA. This binding induces fluorescence which is then detected in real time and thus can be used as a quantitative marker for the amount of DNA.
However, this method is not capable to detect absolute quantification of the sample. The targeted DNA is compared to a housekeeping gene, which is not affected by the experimental conditions. Like this, a relative quantification of the various experimental conditions can be determined. Sample data are shown in the graph below.

In our protocol, we used the Light Cycler® 480 SYBR Green I Master mix to perform the real-time PCR with the Light Cycler® 480 instrument. For further information please read the Light Cycler handbook ([2] Roche).

In our example, GAPDH is used as a reference gene, because it is not regulated under flow conditions [3]. The gene of interest is a tight junction protein, which is strongly regulated by flow conditions. The primers are given in the table below.

**Table 1:** Primer sequence for Claudin-5 and the reference gene GAPDH.

| Name | Sequence |
|---|---|
| hGAPDH_forward | ATG GGG AAG GTG AAG GTC G |
| hGAPDH_reverse | GGG GTC ATT GAT GGC AAC AAT A |
| Claudin-5_forward | CGG GTG TCA GAC TGA GGA TT |
| Claudin-5_reverse | CCT GCC GAT GGA GTA AAG AC |

The following heating steps were used to amplificate the cDNA template (preliminary data, the settings might still be optimized).

**Table 1:** Light cycler® program.

| Steps | Temperature | Time |
|---|---|---|
| Pre-Incubation: | 95°C | 5 min |
| Amplification: | 95°C | 10 s |
| | 60°C | 10 s |
| | 72°C | 10 s |

## 5. Example Result

As an example we show here preliminary results of the Claudin–5 relative gene expression in HUVEC at several time points during cultivation under flow conditions (internal data).

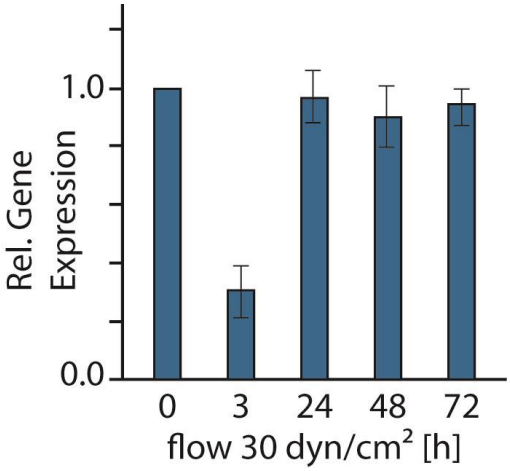


**Figure 4: Relative Gene expression of the tight junction protein Claudin-5**

The gene expression of the cultured cells before applying the flow (0 h) is assumed as 100%. After three hours of flow the gene expression goes clearly down and recovers after 24 hours.

### Sources:

[1] Qiagen, *RNeasy Micro Handbook*, Second Edition, December **2007**.
[2] Roche Diagnostics GmbH, *Light Cycler 480 Instrument, Operator's Manual*, Version 1.5, **2008**
[3] R. D. Barber, D. W. Harmer, R. A. Coleman, B. J. Clark, *GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues*. *Physiological genomics* **2005**, *21 (3)*, S. 389–395.