

Grade 1,0

Protocol for the experiment  
**Transfection of cells (THG Lab)**

May 04, 2022

# 1 Introduction

Cell therapy is the introduction of autologous or allogeneic cell material into a patient's body. By administering foreign cells in the form of injections, infusions or scaffolds, their attachment or activating properties can restore endogenous functions, repair tissues or detect and target specific cells. Therefore, cell therapy is used in regenerative medicine, immunotherapy and cancer therapy [1]. In nucleic acid therapies, also known as transfection, foreign nucleic acids are introduced into living cells, enable gene products to be produced and gene functions to be studied. A distinction is made between stable transfection, in which e.g., foreign DNA is integrated into the nuclear genome of the host cell and long-term expression of a transgene occurs, and transient transfection, in which e.g., RNA is stable for only a limited period of time [2]. One form of transient transfection is the introduction of in vitro mRNA. Since this is inserted into the cytoplasm and not permanently integrated into genomic DNA, the risk of insertional mutations is eliminated, vectors for insertion into the nucleus are not necessary, and transcription of DNA during gene expression is not required, making it faster and independent of promoters. However, transfected mRNA is relatively unstable in the host cell and degrades within a few hours, making it unsuitable for long-term treatments. Repeated treatments with mRNA to achieve a long-term effect of gene expression proved to be difficult, as this often led to inflammatory reactions in the cell. In addition, exact predictions about transfection efficiency and kinetics cannot yet be made for mRNA transfection, thus further research and mathematical models are required [3].

In the scope of this labwork, the influence of mRNA content during transfection on transfection efficiency and cell viability was investigated [4].

# 2 Methods

In the first step of the labwork, medium surrounding neonatal human foreskin fibroblasts was first aspirated and the remaining cells were washed with 10 ml of phosphate buffered saline (PBS) to remove dead cell material. Then 5 ml of trypsin was added and the cell-trypsin mixture was incubated for 3 minutes at 37° in an incubator so that the cells detached from the bottom of the vessel, which was checked with a microscope. Subsequently, 5 ml of medium were added to inactivate trypsin, which would be lethal to the cells in the long-term. The mixture was then placed in a centrifuge for pelleting at 300 x g for 5 minutes and the resulting supernatant was removed leaving only the fibroblasts at the bottom of the tube, to which 10 ml of medium was added. To count the cells, 50 µl of the prepared mixture was mixed with 50 µl of Trypan Blue, and 27 bright-appearing living cells were counted using a

hemocytometer. According to the following formula 1, the cell concentration  $C$  was determined based on the number of cells  $n$ , the number of squares  $Q$  and the dilution factor  $d$  (see results):

$$C = \frac{n}{Q} \cdot d \cdot 10^4 \quad (1)$$

Using the determined cell concentration, the volume of 0.22 ml, which contained approximately 30,000 cells, could be determined using a rule of three (see results). This volume was placed in each of three wells, 1.5 ml medium was added and the whole was incubated overnight. In the second part of the laboratory work, the cells were transfected. For this purpose, the transfection mixtures were first prepared in three tubes, which was done twice, using a different GFP mRNA content (see table 1).

*Table 1: Composition of the different samples*

| <b>Samples</b>              | <b>Sample Set 1</b>                              | <b>Sample Set 2</b>                              |
|-----------------------------|--------------------------------------------------|--------------------------------------------------|
| <b>1 (negative control)</b> | 1 ml Opti-MEM                                    | 1 ml Opti-MEM                                    |
| <b>2 (negative control)</b> | 1 ml Opti-MEM+2 µl Lipo. 2000                    | 1 ml Opti-MEM+4 µl Lipo. 2000                    |
| <b>3 (mRNA sample)</b>      | 1 ml Opti-MEM+2 µl Lipo. 2000<br>+ 1 µg GFP mRNA | 1 ml Opti-MEM+4 µl Lipo. 2000<br>+ 2 µg GFP mRNA |

The mixtures were incubated for 15 minutes and subsequently added to the cells prepared the previous day, where the medium had previously been aspirated. All samples were incubated at 37° for three to four hours. In the third part of the laboratory work, the amount of expressed protein was first measured using a fluorescence microscope. One brightfield, one fluorescence (max. excitation wavelength: 488 nm, excitation filter 485 nm) and one overlay image were recorded of the 1 µg mRNA and the 2 µg mRNA sample set for evaluation. The second analysis method involved staining the samples with Presto Blue. For this purpose, 400 µl of Presto Blue was mixed with 3.6 ml of medium and 1 ml of this mixture was added to each of the samples where the medium had previously been aspirated. As blank, a well was filled with the last millilitre of Presto Blue mixture and all samples were incubated for 45 minutes, so that the Presto Blue could be metabolized by the cells. Afterwards, a black 96-well plate was filled with triplicates from each of the four samples, for which 100 µl was added to each well. This was done for both sample sets. The plate was subsequently placed in the Mithras spectrometer (excitation wavelength: 535 nm) for measurement of cell viability, and the resulting data was stored in tabular form. For the third measurement, the cell medium was aspirated, the remaining cells were washed with 1 ml of BPS and 1 ml of trypsin was added to loosen the cells from the bottom. After an incubation time of 3 minutes, the

cells were checked with a microscope to see if they had detached. The trypsin was inactivated with 1 ml medium, the three samples were placed in FACS tubes and were centrifuged at 600 x g for 3 minutes. The resulting supernatant in the tube was removed, leaving only the cells at the bottom of the tube. To these, 1 ml of PBS was added. A FACS-scan was performed for both sample sets, where the fluorescence of 10,000 cells was measured [4].

### 3 Results

During the experiment, the required volume was calculated, which was added to the individual wells, so that during the transfection process there were 30,000 cells in each well. With a hemocytometer square number of 4, a counted cell number of 27 and a dilution rate of 2, the cell concentration amounted to 135,000 cells/ml (see formula 1).

The volume in which 30,000 cells were located was calculated according to formula 2:

$$V = \frac{1 \text{ ml} \cdot 3 \cdot 10^4}{C} = \frac{1 \text{ ml} \cdot 3 \cdot 10^4}{135 \cdot 10^3} = 0.22 \text{ ml/well} \quad (2)$$

For evaluating the data, images acquired using the fluorescence microscope were described qualitatively, while the Presto Blue assay and the FACS graph were analysed quantitatively. Figure 1 shows the brightfield, fluorescence and overlay images of the 1 µg GFP mRNA sample acquired with the green channel of the fluorescence microscope. The brightfield image shows the branched structures of the fibroblasts (see figure 1(a)), whereas the fluorescence image displays similar shaped greenish glowing structures (see figure 1(b)). By overlaying the two images, it can be seen in figure 1(c) that this green fluorescence derives from the cells, where some areas appear brighter than others.



Figure 1: Fluorescence microscope images of the 1 µg mRNA sample, (a) brightfield image, (b) fluorescence image, (c) overlay of the brightfield and fluorescence image

Figure 2 shows the fluorescence microscope images of the sample with the mRNA content of 2 µg. As in figure 1(a), the cell structures can be seen in the brightfield image, but this time they appear minimally blurred. As before, the fluorescence image shows green glowing cell-shaped structures (see figure 2(b)). In the overlay image 2(c) it can be seen that

the origins of the green fluorescence are the cells, with the intensity of the fluorescence being almost identical to Figure 1(c).

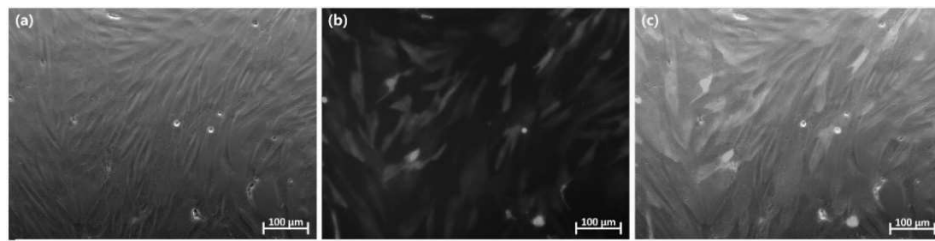


Figure 2: Fluorescence microscope images of the 2 µg mRNA sample, (a) brightfield image, (b) fluorescence image, (c) overlay of the brightfield and fluorescence image

Using a Presto Blue assay, cell viability was measured by the metabolic activity of the cells in each triplicate. Every sample was set in relation to the Opti-MEM control sample and from the resulting ratios the mean and standard deviation of each triplicate were calculated with GraphPad PRISM. In addition, ANOVA and a t-test were performed to check whether there was significance between the samples. For this purpose, the significance level was set to 0.1. The results are shown in Figure 3. In the first data set, it can be seen that the viability of 58 % of the 1 µg mRNA sample is significantly lower than that of the Opti-MEM negative control sample (see figure 3A). The negative control sample containing Opti-MEM and Lipo. 2000 also has a significant difference in cell viability of 135 % compared to the control sample containing only Opti-MEM. In the second data set, it can be seen that both control samples have similar cell viability of approximately 100 %, so they do not show significance, whereas the cell viability of 64 % of the 2 µg mRNA sample is significantly lower in comparison (see figure 3B). Compared to the 1 µg mRNA sample of the first data set, the 2 µg mRNA sample has a significantly higher cell viability (see figure 3C).

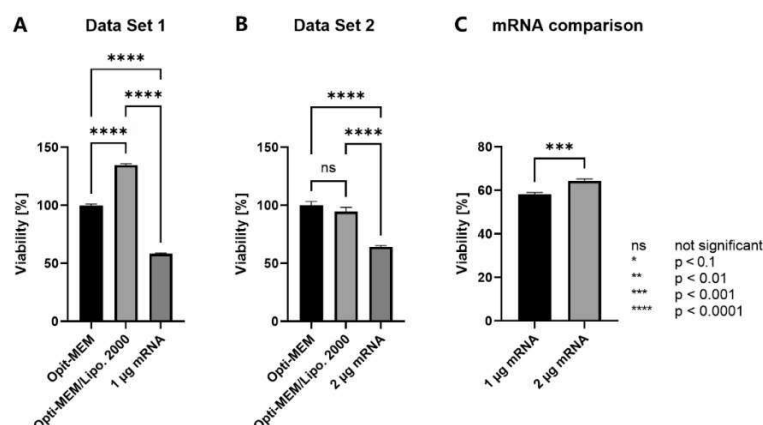


Figure 3: Viability of the cells in relation to the Opti-MEM control sample based on the Presto Blue assay data

Figure 4 shows the histograms of the FACS-scan. It can be seen from the red curves that in the 2 µg mRNA sample in 4(b), more of the 10,000 cells emitted green fluorescence in

the high intensity regions of approximately  $10^3$  than in the 1  $\mu\text{g}$  mRNA sample (see figure 4 (a)). The transfection rate of 81.4 % of the 2  $\mu\text{g}$  mRNA sample is also higher than that of the 1  $\mu\text{g}$  mRNA sample with 69.3 %. A second peak can be seen in the curve of both mRNA samples, which is in the range of  $10^1$ , corresponding to the fluorescence intensity of the negative Opti-MEM/Lipo. 2000 control sample (grey curve). This peak is significantly higher for the 1  $\mu\text{g}$  than for the 2  $\mu\text{g}$  mRNA sample.

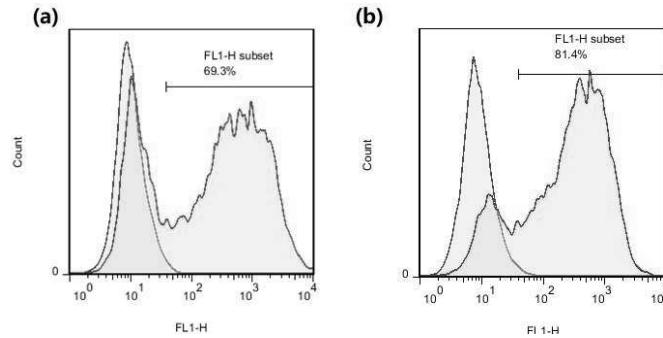


Figure 4: Histograms of flow cytometry where the x-axis represents the fluorescence intensity and the y-axis the number of cells, (a) 1  $\mu\text{g}$  mRNA sample, (b) 2  $\mu\text{g}$  mRNA sample

## 4 Discussion

mRNA transfection proved to be an effective method for introducing foreign genetic material into cells. Using the fluorescence microscope, fluorescence of the GFP protein in the cells could be detected in both mRNA samples, indicating successful transfection of the mRNA. An optical difference between the fluorescence images of the two mRNA samples could not be observed. Quantitative evaluation of cell viability using Presto Blue assays showed that the two mRNA samples had significantly lower viability than the control samples. According to the theory, a higher mRNA content should have led to an equal or higher transfection efficiency and thus to an equal or lower cell viability due to the cytotoxicity of GFP [5] [6]. However, in this experiment, the cell viability of the 1  $\mu\text{g}$  mRNA sample of 58.27 % was significantly lower than the cell viability of the 2  $\mu\text{g}$  sample, which was 64.64 %. In addition, the viability of the Opti-MEM/Lipo. 2000 negative control sample from the first data set was significantly higher than the Opti-MEM negative control sample, although actually both should be around 100 %. Reasons for this may be impurities or inaccuracies during preparation and statistical errors, since only one triplicate of each sample was used for calculation. The histograms recorded during the flow cytometry study showed, that the 2  $\mu\text{g}$  mRNA sample had a higher percentage of transfected cells of 81.4 % than the 1  $\mu\text{g}$  mRNA sample with 69.3 %. This was in line with expectations, as a higher concentration of mRNA should result in equal or more transfection of cells [5].

## 5 References

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