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Enhancing Chinese Hamster Ovary Cell Phenotype:

Opportunities of microRNA Engineering and Cell Sorting

Master Thesis

Submitted by
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“Research is to see what everybody else has seen, and to think what nobody else has thought.”

Albert von Szent-Györgyi Nagyrápolt

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Abstract

Since Chinese Hamster Ovary cells are extensively used for the production of therapeutic proteins, it is important to improve the performance of the producer cell lines. We tried to enhance the efficiency of the cells by microRNA engineering and by a combination of sorting and dye dilution. An artificial and an endogenous miR221-222 cluster were transfected to examine the impact on the cell phenotype. The results showed that no enhanced cell phenotype was obtained, but the mature MicroRNA levels were much lower in the artificial cluster than in the endogenous one, presumably due to different processing. The overexpression of the endogenous miR23b~24-2~27b cluster lead to an increased specific productivity and the knockdown of miR21 induced better cell performance under suboptimal conditions.

To increase cellular growth rates a new method for dye dilution was implemented for heterogeneously stained cells populations. The addition of an initial sorting step led to a homogeneously dyed cell population from which it was possible to monitor cell division over a limited period of time (48 – 72 hours). Although sorting for cells with the highest division numbers did not improve the growth rate, this procedure can be applied to many other investigations that start out with heterogeneously stained cells.

Zusammenfassung

Seitdem Chinese Hamster Ovary Zellen im großen Umfang für die Herstellung von therapeutischen Proteinen verwendet werden, ist es wichtig, die Leistung der Produktionszellinien zu verbessern. Wir haben versucht, die Effizienz der Zellen durch microRNA Engineering und durch eine Kombination von Sortierung und Farbstoffverdünnung (engl.: Dye dilution) zu verbessern. Ein artifizielles und ein endogener miR221-222-Cluster wurden transfiziert, um die Auswirkungen auf den Zell-Phänotyp zu untersuchen. Die Ergebnisse zeigten, dass kein verbesserter Zell-Phänotyp entstanden ist, jedoch war die Anzahl der reifen MicroRNAs im artifiziellen Cluster sehr viel niedriger als im endogenen, vermutlich aufgrund unterschiedlicher Reifung. Die Überexpression des endogenen miR23b~24-2~27b Cluster führen zu einer erhöhten Produktivität und der spezifische Knockdown von miR21 führte zu einer besseren Leistung der Zelle unter suboptimalen Bedingungen.

Zur Verbesserung der zellulären Wachstumsraten wurde eine neue Methode zur Farbstoffverdünnung für heterogen gefärbten Zellpopulationen entwickelt. Das Einfügen einer anfänglichen Sortierung führte zu einer homogen gefärbten Zellpopulation aus der es möglich war, die Zellteilung über einen begrenzten Zeitraum (48 – 72 Stunden) zu beobachten. Obwohl die Isolation von Zellen mit der höchsten Teilungsrate keine verbesserte Wachstumsrate hervorbrachte, kann diese Prozedur für viele andere Untersuchungen, welche von heterogen gefärbten Zellen ausgehen, angewendet werden.

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1. Introduction

Chinese hamster ovary (CHO) cells were isolated from *Cricetulus griseus* in 1957 by Theodore T. Puck (*1*). Nowadays they are the most commonly used mammalian cell factory for the production of recombinant therapeutic proteins (*2*). The advantages compared to microbial cells are correct protein folding and human-like post-translational modifications (*3*). The main drawback is the time-space yield, which is at least tenfold lower than in microbial host systems (*4*). The factors with the highest impact on the overall production are the growth rate, the specific production rate, the stress resistance and the stability (*5*).

There are two different approaches to increase the volumetric yield of the desired protein. One is to optimize the process. This can be done by supplementing nutrients to the media (*4*) or by using different process control strategies, such as a biphasic cultivation system with an initial growth phase followed by a production phase, which is initiated by a process parameter shift (*6*). Today's most common process strategy is the fed-batch with a temperature shift, by which fresh medium is added to the culture to compensate consumed nutrients. This leads to higher cell densities and allows longer operating times (*2*).

Alternatively, the properties of the cell line are improved using either cell selection technologies or cell engineering. In subcloning and sorting, two cell selection procedures which assist isolation of desired phenotypes by targeted selection, cells are isolated to find subcultures with appropriate characteristics. In limiting dilution, which is a labour and time consuming technique, cells get separated by dilution to generate single cell populations (*7*). Many such clones have to be screened to find one with the required properties. Fluorescent activated cell sorting (FACS), an optical sorting method, is an often used technique to separate cells (*8*) (*9*) (*7*). In this technology, cells are excited by a laser light and depending upon the light scattering and the fluorescent characteristics, electrically charged droplets are produced. The droplets, containing the cell of interest, are then deflected into a collection tube by an electrical field. These cells are expanded and their characteristics are analysed to find the most suitable candidates. Though the clones derived from these methods often display enhanced behavior, they are often unstable which can be caused by intraclonal heterogeneity (*7*).

Alternatively, many cell line engineering strategies were developed to overcome the disadvantages of mammalian cell cultures like overexpression of anti-apoptotic genes, for

instance *Akt* (*10*), *Bcl-2* (*11*) (*12*) and *Bcl-x*$_L$ (*13*) (*14*), or combinations of these (*15*) (*16*), and suppression of pro-apoptotic proteins, for example, Bax and Bak by RNAi (*17*) or Zinc-finger nucleases (*18*). Another approach is to engineer CHO cells using microRNAs (miRNA). MiRNAs are small, non-coding RNAs, which play an important role in gene regulation. Previous studies showed that many different cellular mechanisms, for instance, cell cycle arrest (*19*) (*20*), proliferation (*21, 22*), apoptosis (*23*) (*24*) and stress resistance (*25*) (*26*) are affected by microRNAs. The biggest advantage in using miRNAs instead of functional proteins is that they do not require the translational machinery (*5*). After transcription, processing and transportation from the nucleus to the cytoplasm, one of the two miRNA strands, the guide miRNA, is embedded into the RNA-inducing silencing complex (RISC), while the second strand, the passenger miRNA is mostly degraded. The guide microRNA then binds to the 3' untranslated region (UTR) of specific mRNAs (target mRNAs) by the 5' bases 2 – 7 of the mature miRNA, which is called the seed region (*27*). This causes either degradation or repression of one or more target mRNAs (*28*) by the RISC. MiRNAs can be overexpressed with plasmids expressing the miRNA of interest or silenced by antagomirs, which are chemically modified complete complementary sequences to the target miRNA (*20*). Another silencing method is to use microRNA sponges. Sponges are transcripts consisting of multiple binding sites for the miRNA of interest (*29*), thus reducing its binding to target mRNAs.

In this thesis, two different approaches to enhance CHO performance were pursued: a new sorting method was tested to sort for cells with faster division rates; and engineering of specific microRNAs was used to improve cell specific productivity.

In the first approach, cells were stained with 5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester (5(6)-CFDA, SE). 5(6)-CFDA, SE enters the cell, where the acetate groups are removed by intracellular esterases. This converts the dye to carboxyfluorescein succinimidyl ester (CFSE), which covalently binds to intracellular molecules. After staining, the cells were sorted depending upon the signal intensity to gain a homogeneously stained population. The CFSE fluorescence approximately halves at each cell division, so that after some period of incubation the cells with the highest numbers of division since seeding will have the lowest fluorescence intensity. These cells were sorted again to collect cells with increased growth and higher division rates.

The second approach was to investigate the impact of different miRNA-constructs on CHO cells. The miR-221~222 was chosen for overexpression as the up-regulation of this cluster

leads to proliferative enhancement in different types of cancer (*30, 31*). The endogenous clusters miR-23b~24-2~27b and a sponge against miR-21 were chosen for overexpression or knockdown of these miRNAs in antibody producing CHO cells, as they were shown to be differentially regulated in high producing cell lines. Both overexpression and knock-down strategies were pursued, alone or in combination to test synergistic effects. In addition, the initially used artificial hairpin and flanking sequences of miR-221~222, that were necessary as no genomic information was available at the begin of the thesis (*32*), were tested against the endogenous hairpin structures of miR-221~222 when they became available (*33*) to reveal differences in expression levels probably due to improved processing of the endogenous forms.

2. Material and Methods

For reagent preparations, pipetting schemes, temeperature profiles, primer and sponge sequences see section 7.

2.1. Cell Culture

Chinese hamster ovary cells lines were cultivated in 125 ml Erlenmeyer flasks (Corning® Life sciences, NY, USA) at 140 revolutions per minute (rpm) and 37 °C in humidified atmosphere including 7 % CO_2.

CHO-S cells were cultivated in CD CHO medium (Gibco, Invitrogen, Carlsbad, CA, USA) containing 8 mM L – glutamine, HT-supplement (Gibco, Invitrogen, Carlsbad, CA, USA) and 1 ml Anti – clumping agent (Gibco, Invitrogen, Carlsbad, CA, USA) per 500 ml medium. Cells in multiple well plates were cultivated without shaking.

The recombinant production cell line CHO DUKX-B11 EpoFc 14F2 (*34*), which was adapted to glutamine-free growth, was kept in CD CHO medium supplemented with 0.19 µM Methotrexate and 1 ml Anti – clumping agent per 500 ml medium.

2.2. Cloning of the endogenous MicroRNA – Cluster miR221-222

1 µg of pcDNA6.2-GW/EmGFP-miR vector (Life Technologies, Carlsbad, CA, USA) containing the miR221, was digested with SalI (Fermentas, Waltham, MA, USA) and XhoI (Fermentas,Waltham, MA, USA). A calf intestinal phosphatase (New England Biolabs® Inc., Ipswich, England) was also added to prevent self-ligation. 500 ng of the polymerase chain reaction (PCR) product of the endogenous miR221-222 cluster were digested with the same restriction enzymes. The digested vector was run on an agarose gel to remove the cutout. The insert and the vector were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, USA) according to the manufacturer's protocol with modifications. In step 5, the centrifugation was shortened to 1 min., and in step 6, the centrifugation was extended to 5 min. The purified products were eluted in 15 µl nuclease-free water (NFW).

70 ng of the vector and 40 ng of the insert were ligated for 2 – 3 h at room temperature by the T4 DNA ligase (New England Biolabs® Inc., Ipswich, England). After ligation,

chemically competent *E. coli* cells were thawed on ice, 25 ng of DNA were added and after 5 min. of incubation on ice, the cells were heat-shocked for 45 sec. at 42 °C. After a second incubation step on ice for 10 min., 250 µl of SOC-medium were added and transformed cells were incubated for 45 min. at 37 °C and 300 rpm. Then the cell suspension was plated on LB-Agar plates containing spectinomycin (0,05 mg/ml).

A colony PCR of 8 chosen colonies was performed using the primers miRNA_RP and EmGFP_FP. The colonies were picked with a sterile pipette tip, submerged into the PCR reaction mix and incubated in 1 ml of LB-medium including spectinomycin (0,05 mg/ml). An agarose gel electrophoresis was run with the colony PCR products. For the 2 clones with a proper insert inside the vector, 800 µl of the culture were mixed with 200 µl of 85 % Glycerol and stored at -80 °C. The rest of the cultures were incubated overnight in 10 ml of LB-medium with spectinomycin (0,05 mg/ml) at 37 °C and 180 rpm.

The plasmid minipreps of the overnight cultures were done using the FavorPrep® Plasmid Extraction Mini Kit (Favorgen Biotech Corporation, Ping, Taiwan) according to the manual. Step 6 was extended to 10 min. and the columns were allowed to dry 5 minutes by centrifugation. A second elution step with 20 µl elution buffer was included. An additional restriction digestion with the same restriction enzymes as above was performed.

The purified plasmids with the inserts were sent for sequencing to Eurofins MWG operon, Ebersberg, Germany.

After sequence confirmation, a maxiprep was performed using the EndoFree® Plasmid Maxi Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

2.3. Cloning of the endogenous MicroRNA – Cluster miR23b~24-2~27b

Genomic DNA (gDNA) was isolated from 5 x 10^6 CHO cells using the QIamp® DNA Blood Mini Kit (QIAGEN, Hilden, Germany). A PCR was performed with 100 ng of the gDNA and the primer pair cgr-24-23b_FP and cgr-24-23b_RP_BglII.

2 µg of pcDNA6.2-GW/EmGFP-miR – vector containing the miR221, were digested with SalI and BglII (Fermentas,Waltham, MA, USA). A calf intestinal phosphatase was also added to prevent self-ligation. 500 ng of the PCR product of the endogenous miR23b~24-2~27b cluster were digested with the same restriction enzymes. The digested vector was then

run on an agarose gel to remove the cutout. The insert and the vector were purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol with the same modifications as in section 2.2. The purified products were eluted in 2 x 25 µl NFW.

50 ng of the vector and 42 ng of the insert were ligated for 2 – 3 h at room temperature by the T4 DNA ligase. After ligation, chemically competent *E. coli* cells were transformed by heat-shock transformation with 25 ng of DNA as mentioned in section 2.2. Then the cell suspension was plated on LB-Agar plates containing spectinomycin (0,05 mg/ml).

A colony PCR of 8 chosen colonies was performed using the primers EmGFP_FP and miRNA_RP. The colonies were picked with a sterile pipette tip, submerged into the PCR reaction mix and incubated in 2 ml of LB-medium including spectinomycin (0,05 mg/ml). An agarose gel electrophoresis was done with the colony PCR products. For 2 clones with the proper insert inside the vector, 800 µl of the culture were mixed with 200 µl of 85 % Glycerol and stored at -80 °C. The rest of the selected cultures were incubated overnight in 10 ml of LB- medium with spectinomycin (0,05 mg/ml) at 37 °C and 180 rpm.

The plasmid minipreps of the overnight cultures were done using the FavorPrep® Plasmid Extraction Mini Kit according to the manual with the same modifications as mentioned in section 2.2. The purified plasmids with the inserts were sent for sequencing to Eurofins MWG operon, Ebersberg, Germany.

After sequence confirmation, a maxiprep was performed using the EndoFree® Plasmid Maxi Kit according to the manufacturer's protocol.

2.4. Cloning of the artificial MicroRNA – Cluster miR221-222

3 µg of pcDNA6.2-GW/EmGFP-miR – vector containing the miR221, were digested with BglII and XhoI. A calf intestinal phosphatase was added to prevent self-ligation. 2.4 µg of the pcDNA6.2-GW/EmGFP-miR – vector containing the miR222, were digested with BamHI (Fermentas, Waltham, MA, USA) and XhoI. The digested products were then run on an agarose gel. The digested vector with the miR221, and the miR222 were cut out and purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol with the same modifications as mentioned in section 2.2. The purified products were eluted in 15 µl NFW.

50 ng of the purified pcDNA6.2-GW/EmGFP-miR with miR221 and 18 ng of the miR222 were ligated for 2 – 3 h at room temperature by the T4 DNA ligase. After ligation, chemically competent *E. coli* cells were heat-shock transformed with 25 ng of the DNA as mentioned in section 2.2. Then the cell suspension was plated on LB-Agar plates containing spectinomycin (0,05 mg/ml).

A colony PCR of 3 chosen colonies was performed using the primers EmGFP_FP and miRNA_RP. The colonies were picked with a sterile pipette tip, submerged into the PCR reaction mix and incubated in 1 ml of LB-medium including spectinomycin (0,05 mg/ml). An agarose gel electrophoresis was done with the colony PCR product. For the clones with a proper insert inside the vector, 800 µl of the culture were mixed with 200 µl of 85 % Glycerol and stored at -80 °C. The rest of the cultures were incubated overnight in 10 ml of LB-medium with spectinomycin (0,05 mg/ml) at 37 °C and 180 rpm.

The plasmid minipreps of the overnight cultures were done using the FavorPrep® Plasmid Extraction Mini Kit according to the manual with the same modifications as mentioned in section 2.2. A second elution step with 20 µl elution buffer was included. The purified plasmids with the inserts were sent for sequencing to Eurofins MWG operon, Ebersberg, Germany.

After sequence confirmation, a maxiprep was performed using the EndoFree® Plasmid Maxi Kit according to the manufacturer's protocol.

2.5. Cloning of the miR-21 sponges

Four different sponge sequences were designed for the knockdown of miR-21. The 11AA4JGP_SEQ_miR-21_SPCC_pMA with a complete complementary sequence, the 11AA4JHP_SEQ_miR-21_SPBG_pMA-RQ with a small mutation that causes a bulge, the 11AA4JFP_SEQ_miR-21_SPMT_pMA with a mutated seed region and the 11AA4JEP_SEQ_miR-21_SPSS_pMA with a shuffled sequence were synthesized and delivered by Life Technologies, Carlsbad, CA, USA. They all contain seven binding sites for the designated microRNA. 1µg of each of these plasmids and 2,8 µg of the psiCheck^{TM} – 2 vector (Promega Corp., Madison, WI, USA) were digested by XhoI and NotI (Fermentas, Waltham, MA, USA). After the restriction digest, the four digests were run on an agarose gel. The inserts of the four plasmids and the digested psiCheck^{TM} – 2 vector were cut out and

purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol with the same modifications as mentioned in section 2.2. The purified products were eluted in 1 x 20 µl and 1 x 15 µl NFW.

For each insert, 50 ng of the purified psiCheck^{TM-}2 vector and 8 ng of the insert were ligated for 2 – 3 h at room temperature by the T4 DNA ligase. After ligation, chemically competent *E. coli* cells were heat-shock transformed with 25 ng of DNA as mentioned in section 2.2. Then the cell suspension was plated on LB-Agar plates containing ampicillin (0,1 mg/ml).

A colony PCR of 6 chosen colonies for each transformation approach was performed using the primers psiCHECK2seq_sense and psiCHECK2seq_as. The colonies were picked with a sterile pipette tip, submerged into the PCR reaction mix and incubated in 2 ml of LB-medium including ampicillin (0,1 mg/ml). An agarose gel electrophoresis was done with the colony PCR products. For 2-3 clones with a proper insert inside the vector, 800 µl of the culture were mixed with 200 µl of 85 % Glycerol and stored at -80 °C. The rest of the cultures were incubated overnight in 10 ml of LB- medium with ampicillin (0,1 mg/ml) at 37 °C and 180 rpm.

The plasmid minipreps of the overnight cultures were done using the FavorPrep® Plasmid Extraction Mini Kit according to the manual with the same modifications as mentioned in section 2.2. A second elution step with 20 µl elution buffer was included. The purified plasmids with the inserts were sent for sequencing to Eurofins MWG operon, Ebersberg, Germany.

Maxipreps were performed after sequence confirmation using the EndoFree® Plasmid Maxi Kit according to the manufacturer's protocol.

After the dual luciferase reporter assay (see section 2.8), 5 µg of the psiCheck^{TM-}2 vector with the complete complementary MiR21 sequence and 5,8 µg of the same vector with the shuffled sequence were digested with XhoI and BglII. The different DNA parts were separated by agarose gel electrophoresis and the inserts were cut out. They were purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol with the same differences as mentioned in section 2.2. The purified products were eluted in 2 x 20 µl NFW.

7 – 10 ng of the purified inserts and 50 ng of pcDNA6.2-GW/EmGFP-miR, digested by SalI and BglII, were ligated for 2 – 3 h at room temperature by the T4 DNA ligase. After ligation, chemically competent *E. coli* cells were heat-shock transformed with 25 ng of DNA with the same procedure as mentioned in section 2.2. Then the cell suspensions were plated on LB-Agar plates containing spectinomycin (0,05 mg/ml).

A colony PCR of 8 colonies for each transformation approach was performed using the primers EmGFP_FP and miRNA_RP. The colonies were picked with a sterile pipette tip, submerged into the PCR reaction mix and incubated in 2 ml of LB-medium including spectinomycin (0,05 mg/ml). An agarose gel electrophoresis was done with the colony PCR products. For each construct, 2 clones with a proper insert inside the vector, 800 µl of the culture were mixed with 200 µl of 85 % Glycerol and stored at -80 °C. The rest of the cultures were incubated overnight in 10 ml of LB- medium with spectinomycin (0,05 mg/ml) at 37 °C and 180 rpm.

The plasmid minipreps of the overnight cultures were done using the FavorPrep® Plasmid Extraction Mini Kit according to the manual with the same modifications as mentioned in section 2.2. A second elution step with 20 µl elution buffer was included. The purified plasmids with the inserts were sent for sequencing to Eurofins MWG operon, Ebersberg, Germany.

After sequence confirmation, a maxiprep was performed using the EndoFree® Plasmid Maxi Kit according to the manufacturer's protocol.

2.6. Determination of DNA Concentration and Quality

After each PCR/Gel purification, miniprep and maxiprep, DNA concentrations were determined by measuring the absorption at 260 nm using UV-VIS spectrophotometery (Nanodrop 1000 spectrophotometer, Thermo Fisher Scientific Inc., Waltham, MA, USA).

$$c\,(DNA) = Abs_{260} * 50\ \mu g/ml$$

Figure 1: Formula to calculate the DNA-concentration.

The quality of the DNA was designated by the A260/280 and the A260/230 ratio. For pure DNA, the A230/260/280 is about 1:1,8:1.

2.7. Transfection

10^7 CHO DUKX-B11 EpoFc 14F2 cells in exponential growth phase were centrifuged for 10 min. at 200 g. The supernatant was removed completely and the pellet was resuspended in 82 µl of cell Nucleofection solution V (Amaxa cell line nucleofector kit V, Lonza Group Ltd., 4002 Basel, Switzerland) including 18 µl of supplement I (Amaxa cell line nucleofector kit V) and 10 – 15 µg of the respective endotoxin-free plasmid. Then the cell suspension was transferred into a cuvette and transfected by the Amaxa Nucleofector I (Lonza Group Ltd., 4002 Basel, Switzerland) / program H-14. After the transfection, cells were transferred to a conical flask containing 50 – 60 ml of pre-warmed medium. Then the suspension was immediately split into two flasks to generate two technical replicates and cells were allowed to recover for 2 hours at 37°C and humidified air containing 7 % CO_2 without shaking.

2.8. Dual Luciferase Reporter Assay

8 h, 24 h and 48 h after transfection with the psiCheck™ – 2 vector containing the different MiR21-sponges, 1 ml of cell suspension was centrifuged for 10 min. and 200 g. The supernatant was removed and the cells were washed with Phosphate buffered saline (PBS). Then the pellet was dissolved in 250 µl of lysis buffer (Promega Corp., Madison, WI, USA) and 2 x 100 µl were transferred into a black 96 well plate (Cellstar, Greiner bio-one international AG, Kremsmünster, Austria). 100 µl of LARII (Promega Corp., Madison, WI, USA) were added and the luminescence was measured by a 96 well plate reader (Infinite M200, TECAN Group Ltd., Männedorf, CH). Afterwards, 100 µl of the Stop & Glo solution were added to the wells and the luminescence was measured again. The i-control™ (TECAN Group Ltd.) software was used to obtain the data.

2.9. Batch

Cells were incubated for 7 days after transfection. The viable cell density (VCD) and the viability were determined using the ViCell analyzer (Beckman Coulter, USA) at day 0, 1, 2, 3, 4 and 7. The ViCell analyzer uses the trypan blue exclusion method to discriminate between dead and viable cells. Trypan blue cannot pass an intact membrane and so only dead cells are dyed.

$$Viability\ [\%] = \frac{viable\ cells}{total\ cells} * 100$$

Figure 2: Formula to calculate the viability

Out of the VCD, the growth rate was calculated using following formula:

$$Growth\ rate\ [\frac{1}{d}] = \frac{ln(\frac{X_1}{X_0})}{d}$$

Figure 3: Formula to calculate the growth rate. x_0 is the initial viable cell density, x_1 is the final viable cell density and d is the time in days

Supernatant and RNA samples were taken at day 0, 2, 4 and 7. After 48 h at batch 1, at least 1 ml of cell suspension was spun down, resuspended in PBS and analyzed by a flow cytometer (Gallios Cytometer, Beckman Coulter, USA) to determine the amount of Green Fluorescence Protein (GFP) – expressing cells. A forward scatter / side scatter plot was used to determine the living cells. The cells were excited by a 488 nm argon laser and the emitted GFP signals were collected via a 525/40 BP filter (Fl1).

2.10. RNA Isolation

5 x 10^5 – 1 x 10^6 cells were harvested and resuspended in 500 µl of TRIzol® reagent (Life Technologies, Carlsbad, CA, USA). 100 µl of chloroform were added, mixed and incubated for 2 – 3 min. at room temperature. Then the solution was centrifuged for 15 min. at 4 °C and 12000 g. The upper, aqueous phase was collected. 250 µl of isopropanol were added, mixed, incubated at room temperature for 10 min and again centrifuged for 10 min. at 4 °C and 12000 g. The supernatant was removed and the RNA pellet was washed with 1 ml of 70 % ethanol and spun down for 5 min. at 12000 g and 4 °C. The supernatant was removed completely and the RNA was resuspended in 30 µl of NFW. The absorption at 260 nm, the A260/280 and the A260/230 ratio were measured by UV-VIS spectrophotometry to determine the yield and the purity of the RNA.

$$c(RNA) = Abs_{260} * 40\ \mu g/ml$$

Figure 4: Formula to calculate the RNA – concentration

For pure RNA, the A260/280 and the A260/230 ratios lie around 2.

2.11. MiRNA Quantification

2.11.1. Reverse Transcription

1 – 10 ng of RNA were reverse transcribed via the Taqman® MicroRNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA), which includes the Multiscribe™ Reverse Transcriptase and a specific stem-looped primer against the mature MicroRNA to generate a cDNA. The reaction volume was decreased to 10 µl.

2.11.2. Quantitative PCR (qPCR) Amplification

Quadruplets of each cDNA were used to analyze the expression level of the different mature MicroRNAs. The amplification was done using the Taqman® MicroRNA assay with a primer against the generated cDNA. The volume of the assay was reduced to 10 µl. The cycling conditions were performed with the Rotor-Gene-Q (QIAGEN, Hilden, Germany). The expression levels were normalised against miR-185-5p, an endogenous control (*32*) and compared to each other using the $2^{-\Delta\Delta C}{}_{T}$ method (*35*). This method calculates the difference between two samples in comparison to the endogenous control.

$$\Delta C_{T,sample} = C_{T,sample} - C_{T,control}$$

Figure 5: Formula to calculate the difference in threshold cycles between the sample and the control

$$2^{-\Delta\Delta C_T} = 2^{\left(-\Delta C_{T,sample\ 1} - \Delta C_{T,sample\ 2}\right)}$$

Figure 6: Formula to obtain the fold difference between two samples

2.12. GFP - mRNA Quantification

2.12.1. DNA Digestion with DNase

1 µg of the RNA samples were treated with RNase-free DNase I (Fermentas, Waltham, MA, USA) for 30 min at 37 °C. Then EDTA was added and the enzyme was inactivated for 10 min at 65 °C.

2.12.2. Standard Preparation

PCR reactions with the primer pairs against GFP-mRNA (EGFP_emGFP_FP1 and EGFP_emGFP_RP1) and ß-Actin-mRNA (cho-actb_2_FW and cho-actb_2_REV) were performed. The amplified target was run on an agarose gel to check the length of the fragment. Then the desired band was cut out and purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol with the same modifications as in section 2.2. The purified product was eluted in 1 x 20 µl and 1 x 15 µl NFW. The copy number was calculated and the stock was diluted to a final concentration of 10^{10} copies / µl.

$$copy\,number = \frac{(amount\ of\ DNA * 6.022 * 10^{23})}{(length * 650)}$$

Figure 7: Formula to calculate the copy number of the DNA template. The amount of DNA [g] was determined by UV-VIS spectrophotometry. Length represents the template length in basepairs. 650 is the average molecular mass of a basepair in Dalton.

2.12.3. cDNA Synthesis

The DNA-free RNA was reverse transcribed using the DyNAmo™ cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), which includes the M-MuLV RNase H^+ reverse transcriptase and a random hexamer primer set.

2.12.4. qPCR Amplification

Quadruplets of each cDNA were used to analyse the expression levels of the GFP - mRNA. The amplification was done using SensiMix SYBR Hi-ROX (Bioline, London, UK) and the same primers as in section 2.12.2. The cycling conditions were performed with the Rotor-Gene-Q (QIAGEN, Hilden, Germany). The copy numbers were calculated via a standard curve in the range of 10^3 to 10^8 copies and the expression levels were normalized against ß-Actin, a housekeeping gene (*36*), which was quantified by the same procedure as GFP.

2.13. Enzyme-linked Immunosorbent Assay

The plates (Nunc-Immuno 96 MicroWell Solid Plates Maxisorp™, Thermo Fisher Scientific Inc., Waltham, MA, USA) were coated with a 1 : 1000 diluted capture antibody (Goat Anti Human IgG gamma chain specific, Sigma-Aldrich, St. Louis, MO, USA) in coating buffer,

and incubated overnight at 4 °C. Then the coated plates were washed twice with wash buffer and 50 µl of the supernatant of the culture samples and a standard, which were serially diluted in dilution buffer, were transferred to the coated plates. After 1 h of incubation at room temperature, the plates were washed twice with wash buffer and 50 µl of the conjugated antibody (Goat Anti Human IgG-HRP, Life Technologies, Carlsbad, CA, USA), which was previously 1 : 1000 diluted in dilution buffer, were transferred to the wells. After another hour of incubation at room temperature, the plates were again washed twice and 100 µl of staining solution were added per well. The reaction was stopped with the addition of 100 µl 2,5 M H_2SO_4 and the absorbance at 492 nm and 620 nm as reference was determined using a 96 well plate reader (Sunrise, TECAN Group Ltd.). The Magellan™ (TECAN Group Ltd.) software was used to analyse the absorbances. The titers were determined using a standard curve. Out of the growth data from the batches and the titers, the specific productivities (qP) were calculated with the following equation:

$$qP\,[pg * cells^{-1} * days^{-1}] = \frac{p_{i+1} - p_i}{\sum_{i=1}^{n} \frac{(x_{i+1} - x_i) * (t_{i+1} - t_i)}{(ln(x_{i+1}) - ln(x_i))}} * 1000$$

Figure 8: Formula to calculate the specific productivity. x_{i+1} and x_i are viable cell densities at the timepoints t_{i+1} and t_i. p_{i+1} and p_i are the titers in µg * L^{-1} at the timepoints t_{i+1} and t_i.

2.14. Dye Dilution with initial sorting

2.14.1. Dye Evaluation

1 x 10^6 CHO-S cells were stained with 5 µM, 10 µM and 15 µM 5-(and-6)-Carboxyfluorescein Diacetate, Succinimidyl Ester (5(6)-CFDA, SE; CFSE) - Mixed Isomers (Invitrogen, Carlsbad, CA, USA) or Oregon Green® 488 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (Oregon Green® 488 DHPE, Invitrogen, Carlsbad, CA, USA) to determine the appropriate initial dye concentration and the proper dye.

2.14.2. Staining

After dye evaluation, 6 x 10^6 CHO – S cells were stained with 5(6)-CFDA, SE. 10 µM dye / 10^6 cells according to the CellTrace™ CFSE Cell Proliferation Kit (C34554) protocol. After the last washing step, cells were re-suspended in medium containing penicillin and streptomycin at a final concentration of 1 x 10^6 cells /ml to avoid bacterial contaminations.

2.14.3. Fluorescence Activated Cell Sorting

3 x 10^6 cells were analysed and sorted into a 12 well plate (Cellstar, Greiner bio-one international AG, Kremsmünster, Austria) containing 1 ml of medium supplemented with penicillin and streptomycin using the FACS Vantage (Becton Dickinson, Franklin Lakes, NJ, USA), which features pulse processing and a sort enhancement module. A forward scatter / side scatter plot was used to determine the living cells. Cells were excited by a 488 nm argon laser (Coherent, Inc., California, USA) and the emitted CFSE signals were collected with a 530/30 BP filter (Fl1) . Sorted cells were incubated for 48 – 72 h.

2.14.4. Flow Cytometry

On day 0, 1, 2 (and 3), at least 10000 cells were taken from the culture and analysed by the Gallios Cytometer. A forward scatter / side scatter plot was used to determine the living cells. The cells were excited by a 488 nm argon laser and the emitted CFSE signals were collected via a 525/40 BP filter (Fl1).

2.14.5. Batch

Unsorted, two times and three times sorted CHO - S cells were seeded into 30 ml medium at an initial concentration of 1,5 x 10^5 cells/ml. The Viable cell densities and the viabilities were measured via Vicell analyzer at day 0, 1, 2, 3 and 4. Out of this data, the growth rates were obtained.

3. Results

After sequence confirmation of the designed miRNA - constructs, described in section 2.2 – 2.5, maxipreps were performed to gain high amounts of endotoxin-free plasmids. Only plasmids with a A230/260/280 ratio around 1:1,8:1 were used for the transfections.

Two individual batches were run and the viabilities and the viable cell densities were obtained to investigate the impact of overexpression and knockdown of the chosen miRNAs.

3.1. Comparison between the Endogenous and the Artificial MiR221-222 Cluster

The vector containing the miR-21_SPSS – insert was used as negative control for the two batches.

3.1.1. Batch 1 - Growth and Viability after Transfection

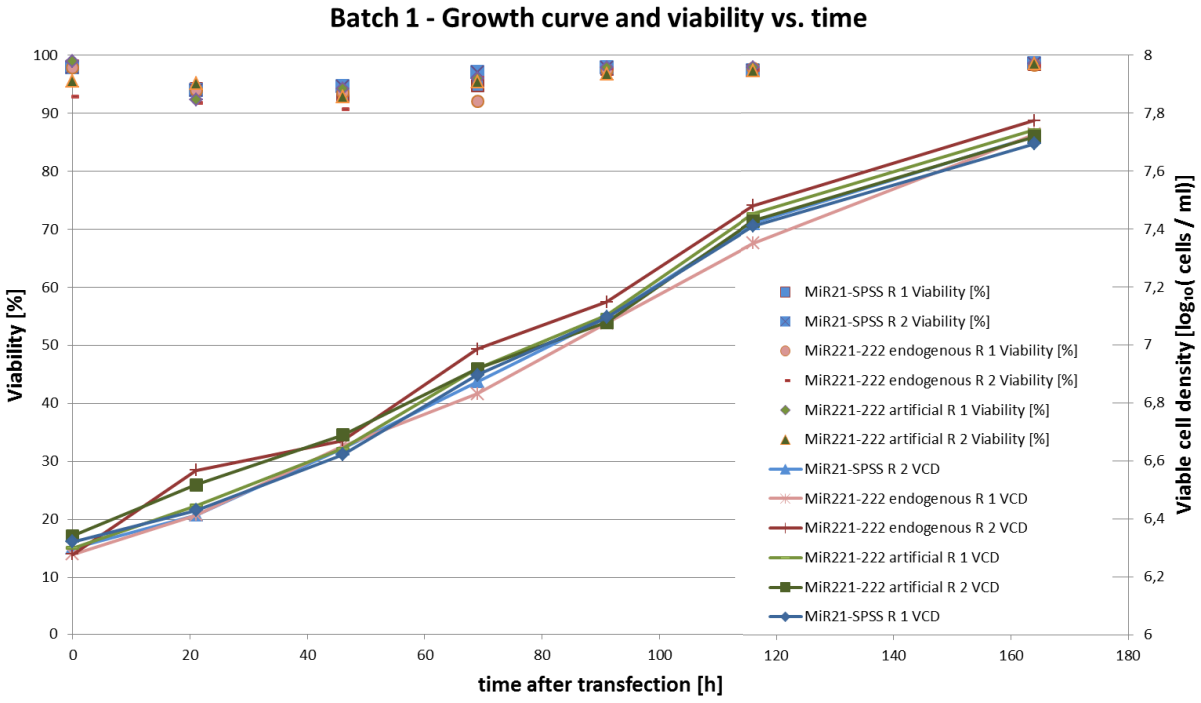


Figure 9 : Viable cell density (VCD) and viability over time

The growth curves and the viabilities of the different samples (Figure 9) are very similar. The growth rates were calculated using the viable cell densities of batch 1 at different timepoints and the equation of Figure 3.

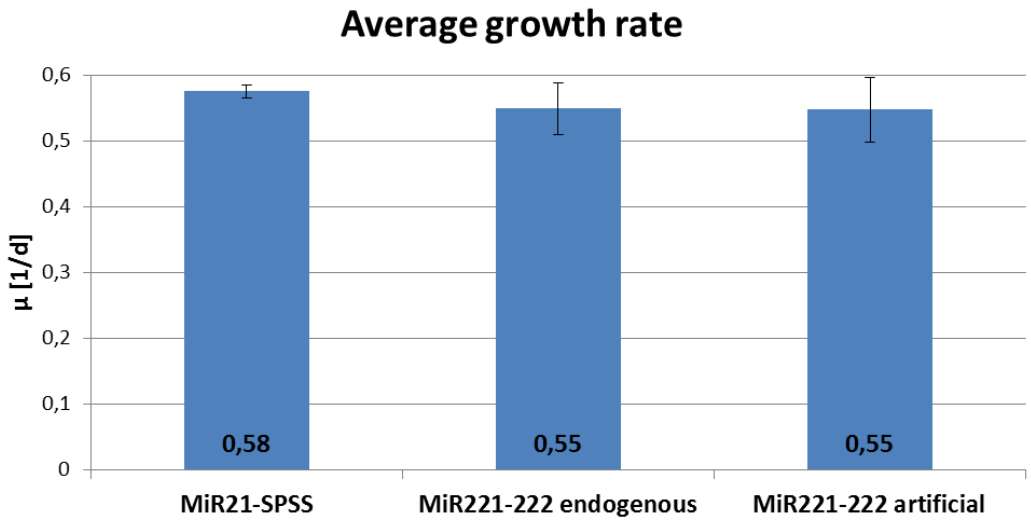


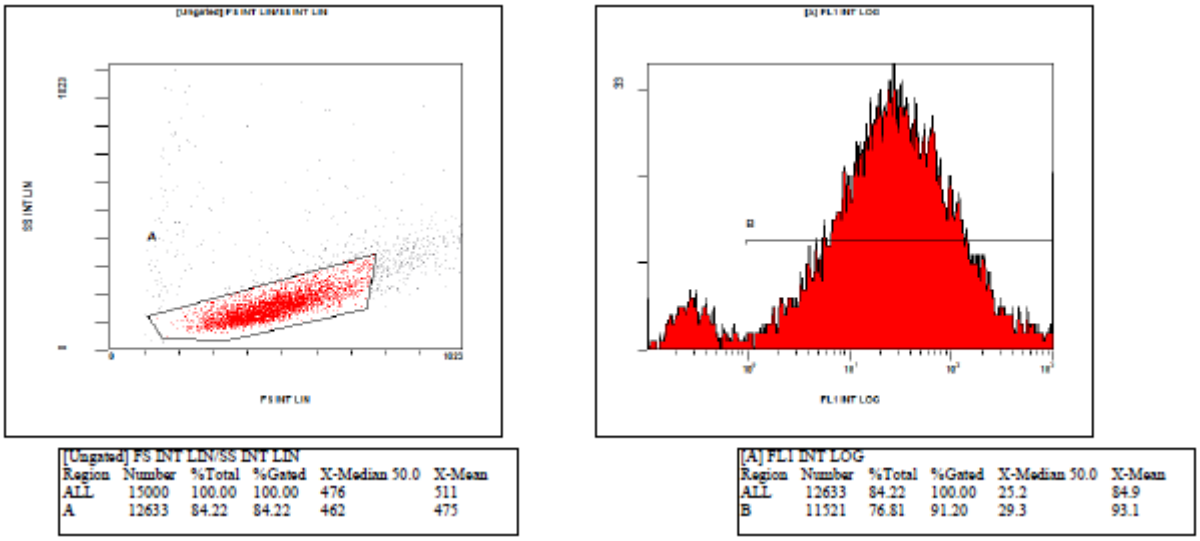
Figure 10: Average growth rate (µ) of the 2 replicates of each transfection

There is no difference in growth characteristics between the different samples.

3.1.2. Batch 1 – GFP Measurement

At least 10000 events were analysed 48 h after transfection by flow cytometry to determine the fraction of functional GFP expressing cells.

A)



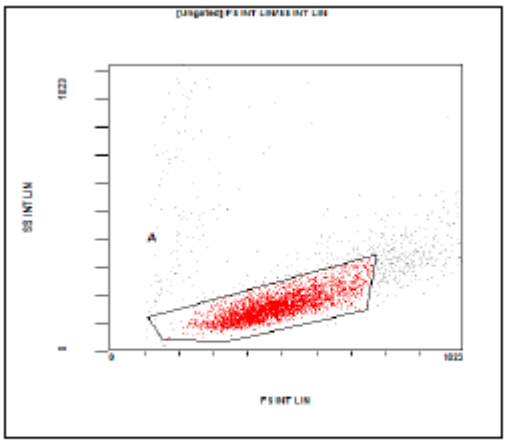
[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	476	511
A	12633	84.22	84.22	462	475

[A] FL1 INT LOG

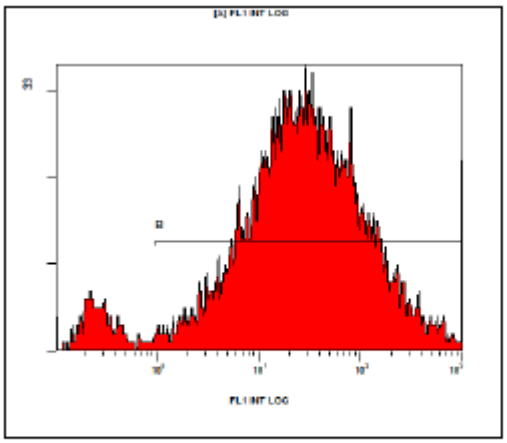
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12633	84.22	100.00	25.2	84.9
B	11521	76.81	91.20	29.3	93.1

B)



[Ungated] FS INT LIN/SS INT LIN

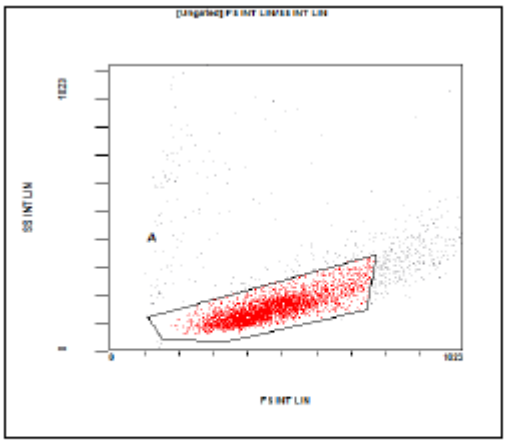
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	482	516
A	12543	83.62	83.62	467	479



[A] FL1 INT LOG

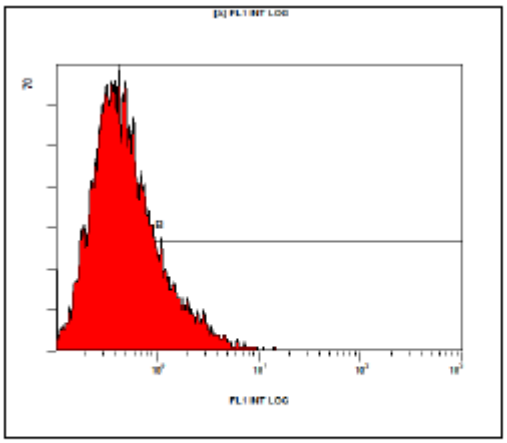
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12543	83.62	100.00	25.4	86.4
B	11532	76.88	91.94	29.6	94

C)



[Ungated] FS INT LIN/SS INT LIN

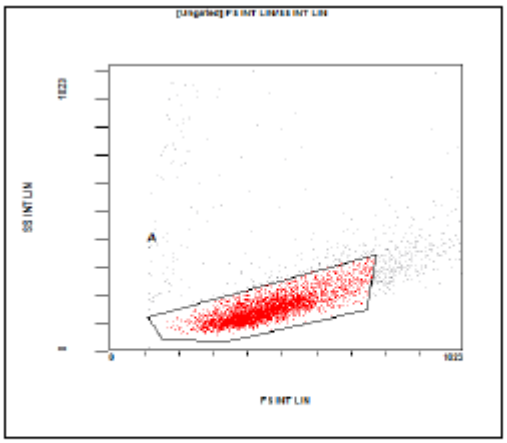
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	466	500
A	12838	85.59	85.59	454	466



[A] FL1 INT LOG

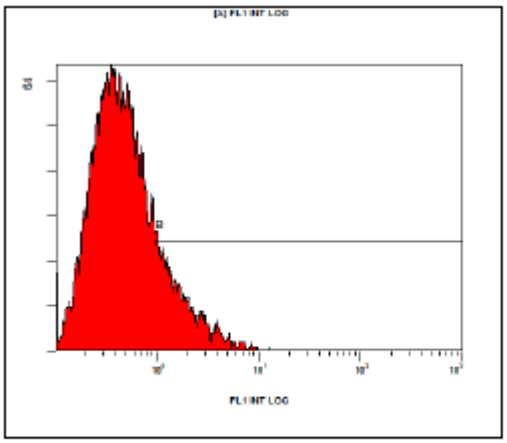
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12838	85.59	100.00	0.46	1.58
B	2702	18.01	21.05	1.79	5.91

D)



[Ungated] FS INT LIN/SS INT LIN

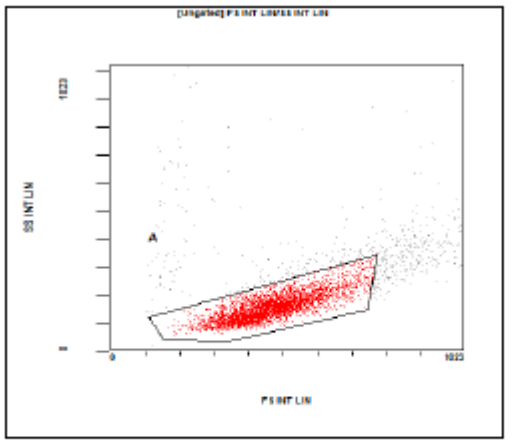
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	460	497
A	12766	85.11	85.11	447	461



[A] FL1 INT LOG

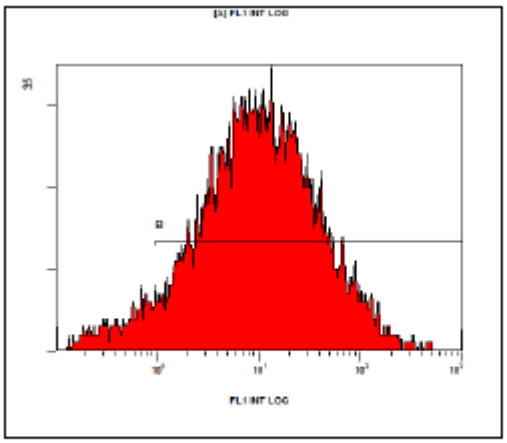
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12766	85.11	100.00	0.46	1.83
B	2687	17.91	21.05	1.74	7.12

E)



[Ungated] FS INT LIN/SS INT LIN

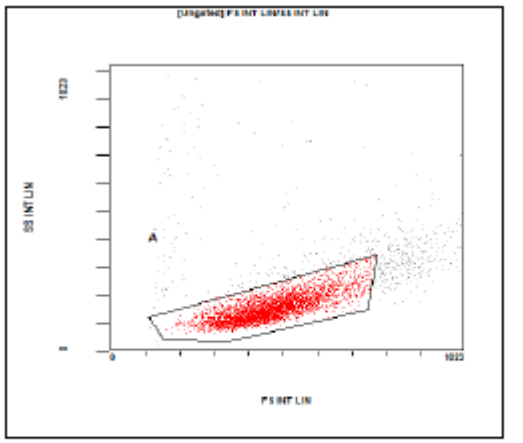
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	473	508
A	12734	84.89	84.89	457	469



[A] FL1 INT LOG

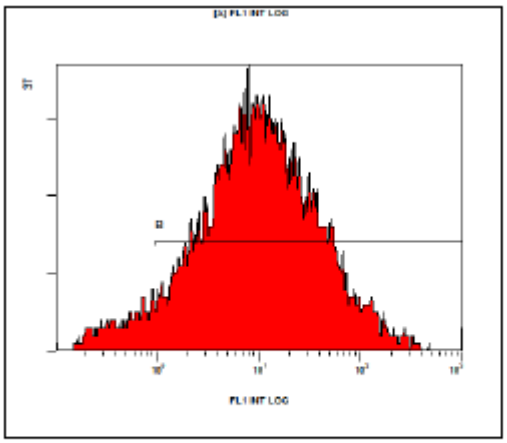
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12734	84.89	100.00	9.62	32.6
B	11608	77.39	91.16	11.3	35.7

F)



[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	474	508
A	12747	84.98	84.98	458	469



[A] FL1 INT LOG

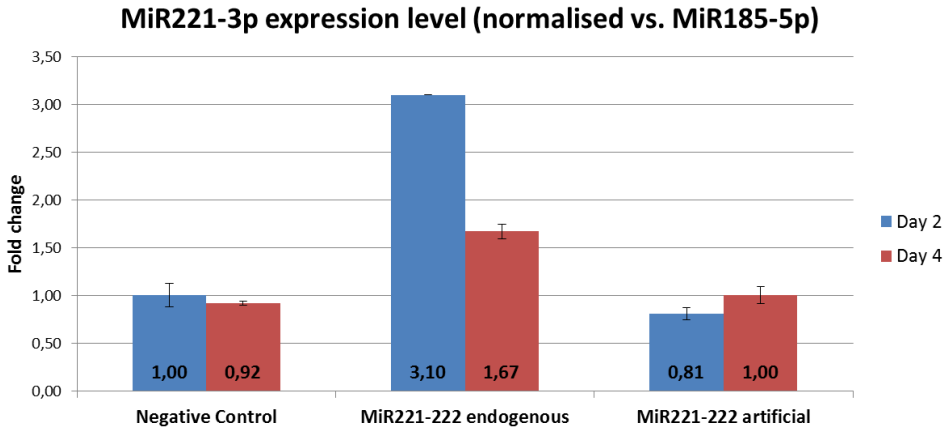
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12747	84.98	100.00	9.62	34
B	11620	77.47	91.16	11.2	37.3

Figure 11: Flow cytometry analysis of the samples 48 h after transfection. Living cells were chosen by gate A on the forward scatter (FS) / side scatter (SS) plot. Transfected cells were selected by region B on the FL1 histogram.
A) MiR21_SPSS replicate 1
B) MiR21_SPSS replicate 2
C) MiR221-222 endogenous replicate 1
D) MiR221-222 endogenous replicate 2
E) MiR221-222 artificial replicate 1
F) MiR221-222 artificial replicate 2

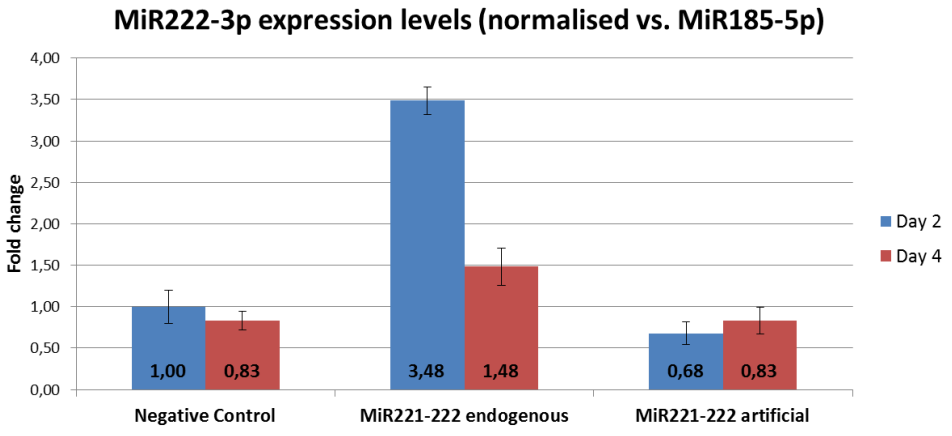
CHO DUKX-B11 EpoFc 14F2 cells, which were not transfected, were analyzed to determine region B. More than 90 % of the cells, which were transfected with the artificial miR221-222 construct or the negative control, and selected by gate A, contained GFP (region B). Only about 21 % of the cells, which were transfected with the endogenous miR221-222 construct, and selected by gate A, expressed GFP.

3.1.3. Batch 1 – Quantitation of mature MicroRNAs

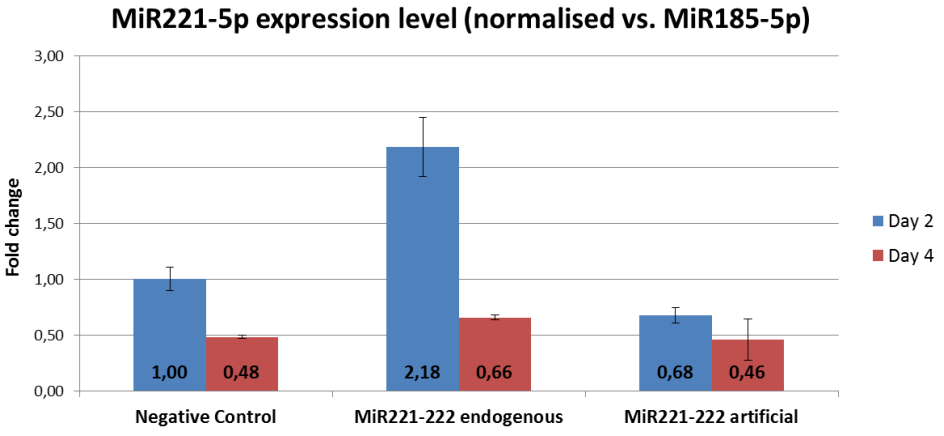
The isolated RNA samples of day 2 and 4 were reverse transcribed and the expression levels of the transfected miRNAs were analysed via qPCR.



A)



B)



C)

Figure 12: Fold changes of MicroRNAs compared to the negative control at two different timepoints.
A) Expression levels of miR221-3p
B) Expression levels of miR222-3p
C) Expression levels of miR221-5p

In Figure 12, it can be seen that 48 h after transfection, miR221-3p, miR222-3p and miR221-5p are clearly upregulated in the cells which were transfected with the endogenous miR221-222 cluster while none of these are upregulated in the cells which were transfected with the artificial cluster. The miR221-3p and the miR222-3p are still upregulated four days after transfection in the cells containing the endogenous cluster. The miR221-5p level decreases from day 2 to day 4 in all samples.

3.1.4. Batch 1 – Quantitation of GFP – mRNA

Because the amount of functional GFP expressing cells were only about 21 % at the endogenous transfection, the isolated RNA was also used to determine the GFP-mRNA levels by reverse transcription and qPCR.

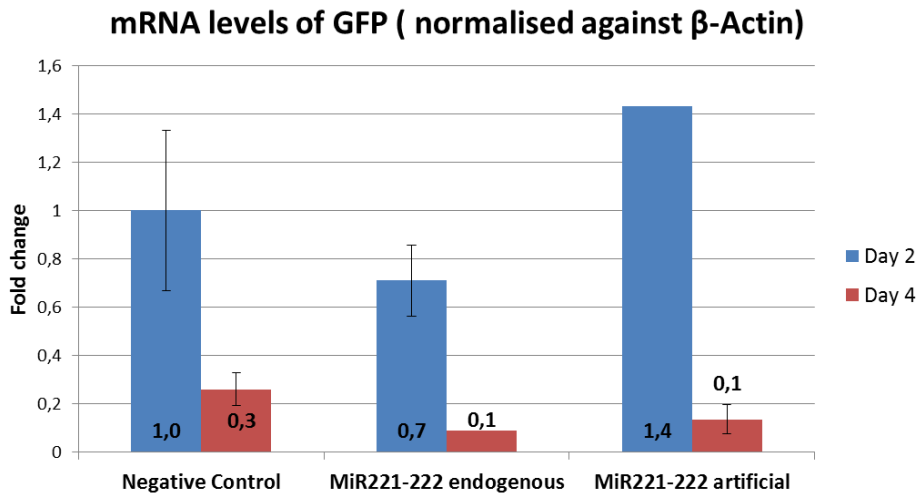


Figure 13: Fold changes of the GFP-mRNA compared to the negative control at two different timepoints.

The analysis in StatGraphics Plus 5.0 indicates that there is no correlation between the amount of active GFP (Figure 11) and the mRNA level (Figure 13).

3.1.5. Batch 2 – Growth and Viability after Transfection

It was decided to include a second transfection with the endogenous construct, because the amount of functional GFP containing cells which were transfected with this cluster was lower than the others.

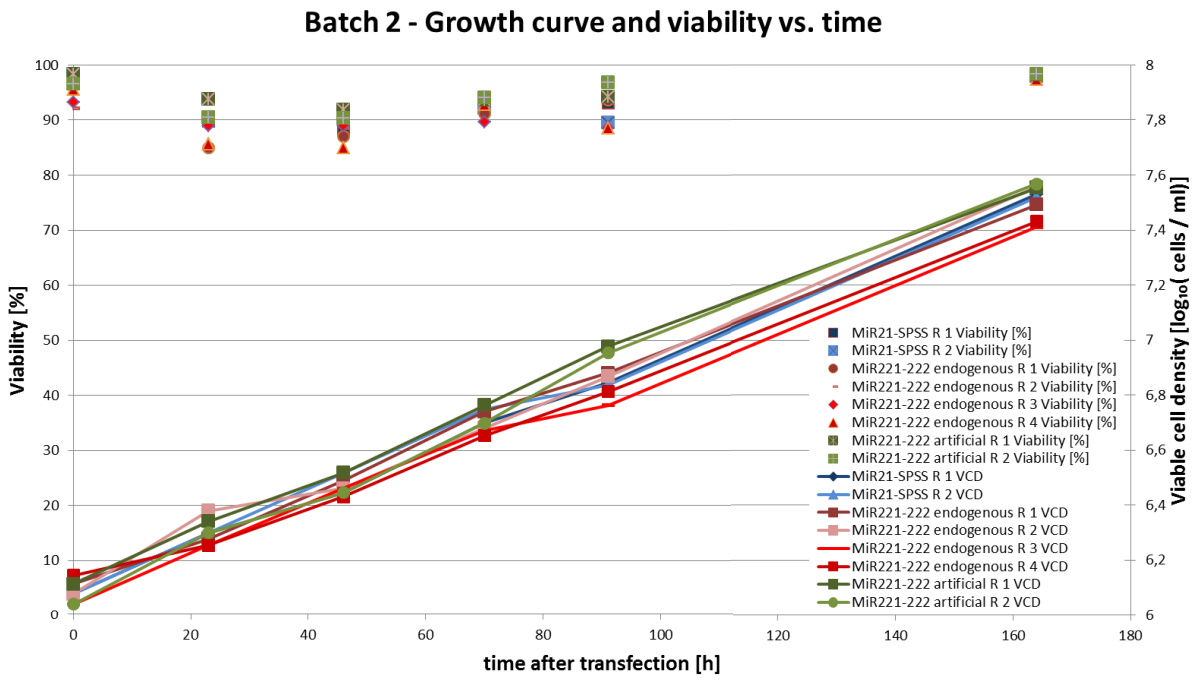


Figure 14: Viable cell density (VCD) and viability over time

The growth curves and the viabilities of batch 2 (Figure 14) vary more than the ones from batch 1 (Figure 9). The growth rates of the second batch were calculated using the viable cell densities at different time points.

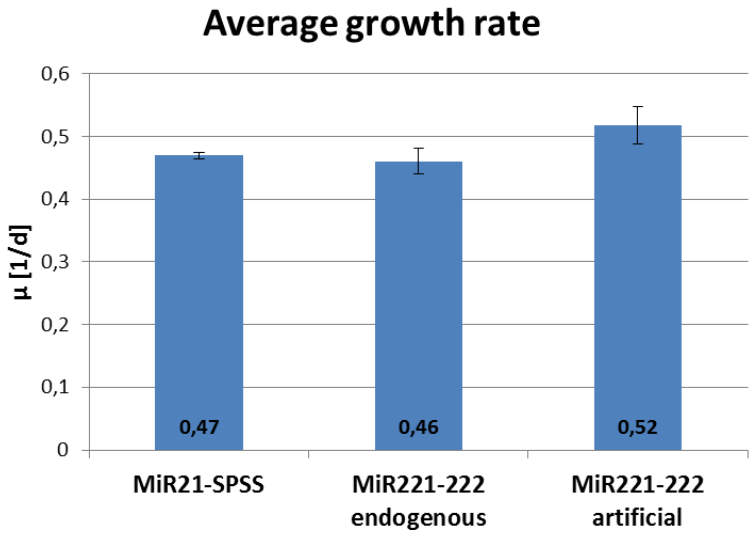


Figure 15: Average growth rate (μ) of the 2 - 4 replicates of each transfection

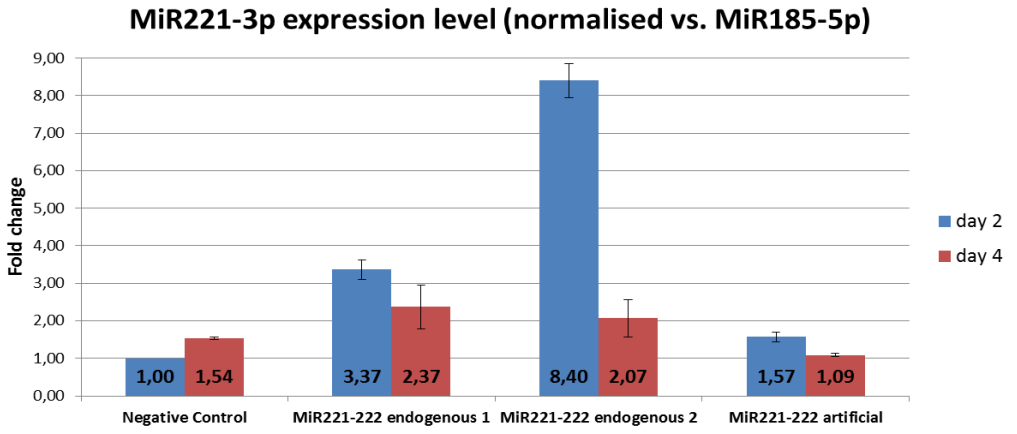
Results shown include the two independent transfections performed with the endogenous cluster. There is no statistically significant difference between the three average growth rates of batch 2. All growth rates and viabilities were lower than the ones from batch 1, presumably

due to higher stress, due to insufficient control of the CO_2 concentration and thus pH during this culture.

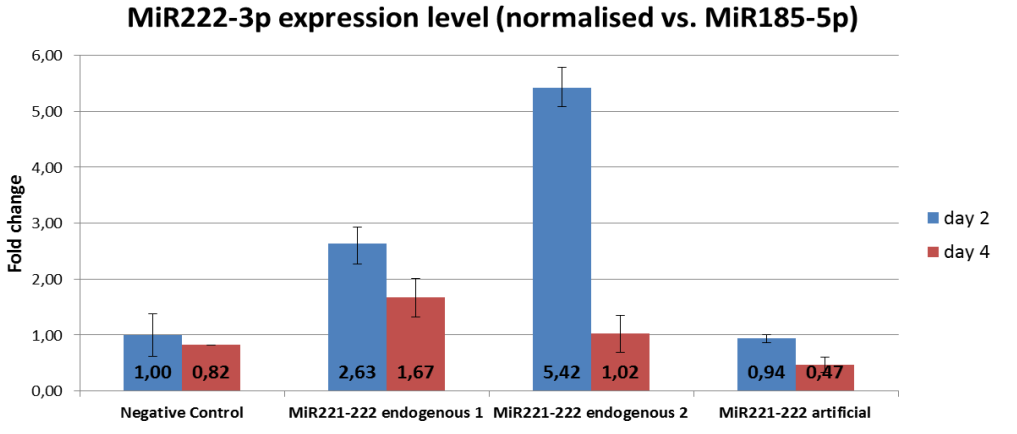
3.1.6. Batch 2 – Quantitation of Mature MicroRNAs

The isolated RNA samples of day 2 and 4 were reverse transcribed and the expression levels of the transfected miRNAs were analysed via qPCR.

A)



B)



C)

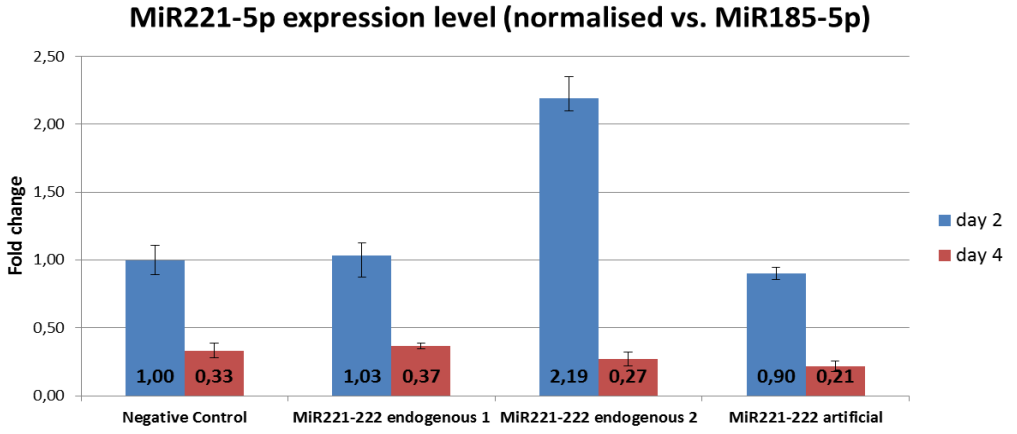


Figure 16: Fold changes of MicroRNAs compared to the negative control at two different timepoints.
A) Expression levels of miR221-3p
B) Expression levels of miR222-3p
C) Expression levels of miR221-5p

In Figure 16, it can be seen that 48 h after transfection, miR221-3p and miR222-3p are up-regulated in the first transfection with the endogenous cluster. In the second transfection, the fold changes are much higher than in the first one and also miR221-5p is higher expressed compared to the negative control. None of the analysed miRNAs are upregulated in the cells which were transfected with the artificial cluster. The miR221-3p is still higher expressed four days after transfection in the cells containing the endogenous cluster. The miR221-5p level decreases from day 2 to day 4 in all samples.

Because no correlation was found between the amount of active GFP and the mRNA level in batch 1, GFP was not analysed in the second batch.

3.2. Dual Luciferase Reporter Assay

The four miR21-sponge constructs were first cloned into psiCHECK™-2 to check the functionality. This vector consists of two different luciferases. The sponge sequences are inserted in the 3' UTR of one luciferase, the other one serves as a reference. If a miRNA binds to the sponge, the translation of the first luciferase is repressed. See section 7.6 for further information.

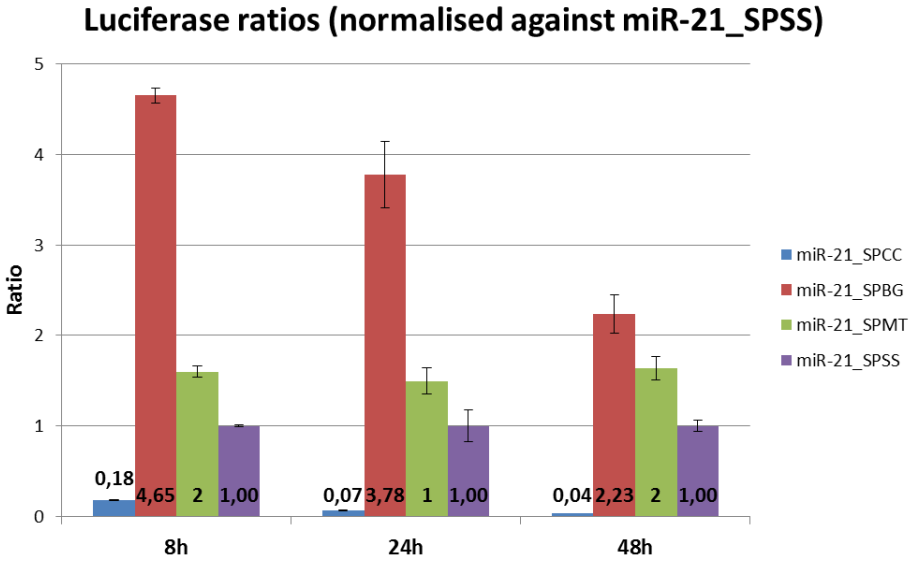


Figure 17: Luciferase ratios of miR-21_SPCC, miR-21_SPBG and miR-21_SPMT normalised against miR-21_SPSS at different timepoints.

Figure 17 depicts the luciferase ratios of the different miR-21 sponges. The miR-21_SPBG and miR-21_SPMT cause the opposite effect. Only miR-21_SPCC causes a lower ratio than

miR-21_SPSS. These two were chosen to clone into the pcDNA6.2-GW/EmGFP-miR – vector for further investigations.

3.3. Effects of miR23b~24-2~27b and miR-21_SPCC on CHO DUKX-B11 EpoFc 14F2

Two batches were run after transfection with 15 µg of miR-21_SPSS (negative control), miR23b~24-2~27b and miR-21_SPCC. A co-transfection with 7,5 µg of the miR23b~24-2~27b cluster and 7,5 µg of miR-21_SPCC was also included into the batches.

3.3.1. Batch 1 – Growth and Viability after Transfection

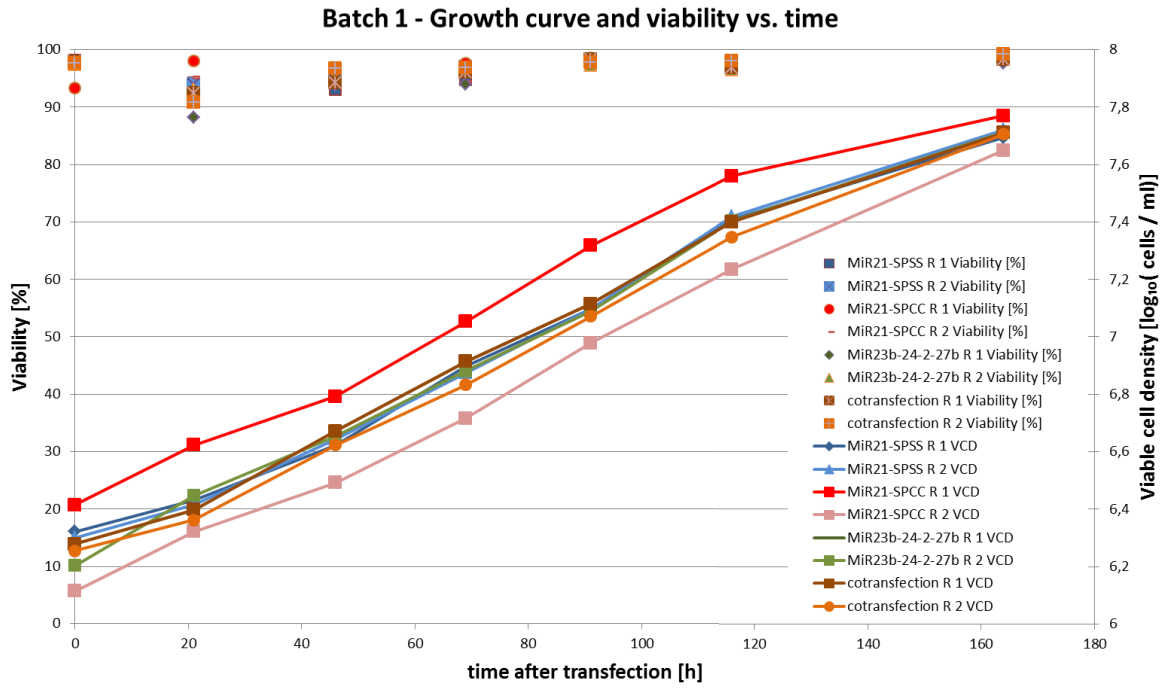


Figure 18: Viable cell density (VCD) and viability over time

The growth curves and the viabilities of the different samples (Figure 18) are not very different. The differences in the growth curves of the miR21-SPCC transfections derive from different initial VCD. The viable cell densities at different timepoints were used to calculate the growth rates.

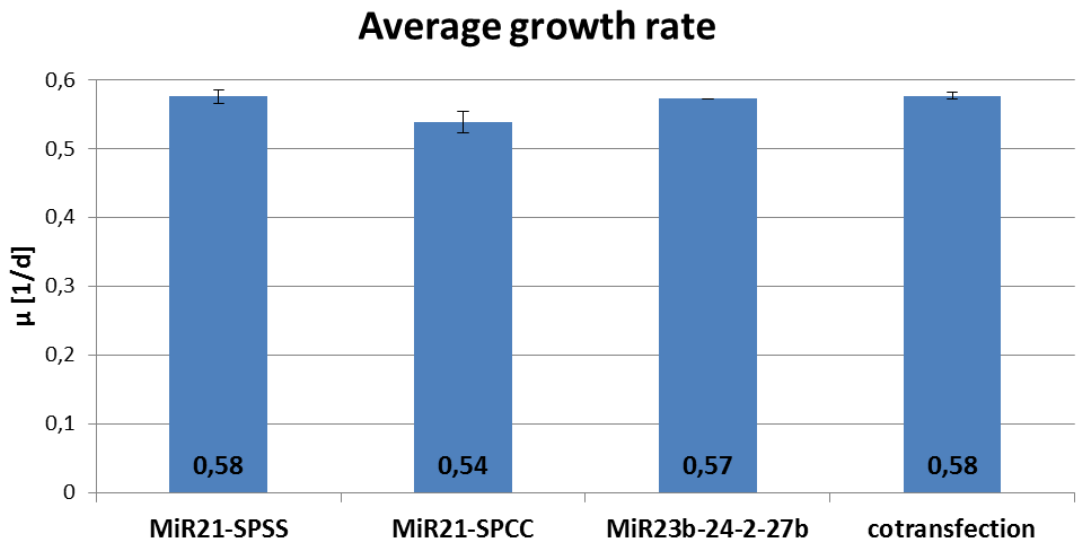


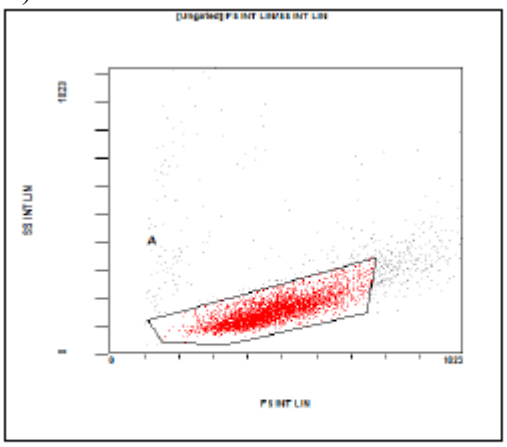
Figure 19: Average growth rate (µ) of the 2 replicates of each transfection

There was no significant effect on growth rates in any of these transfections.

3.3.2. Batch 1 – GFP Measurement

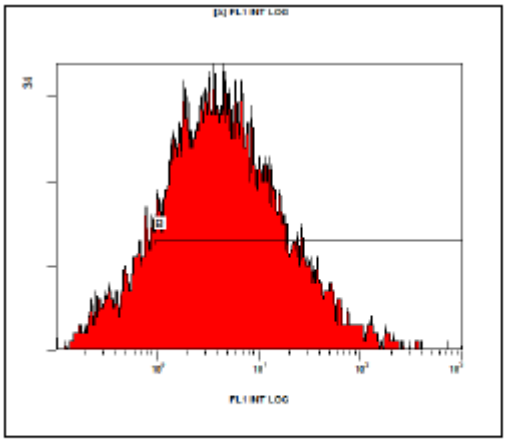
At least 10000 events were analysed 48 h after transfection by flow cytometry to determine the fraction of functional GFP containing cells.

A)



[Ungated] FS INT LIN/SS INT LIN

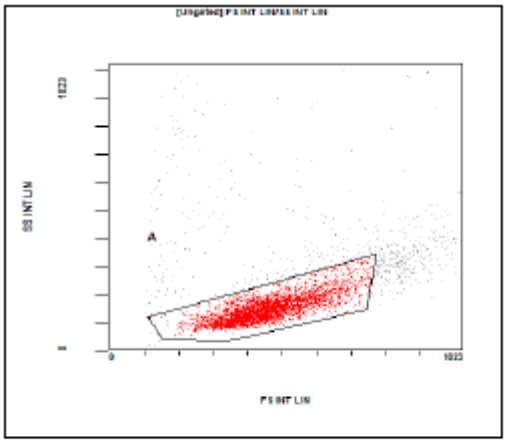
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	474	506
A	12731	84.87	84.87	459	472



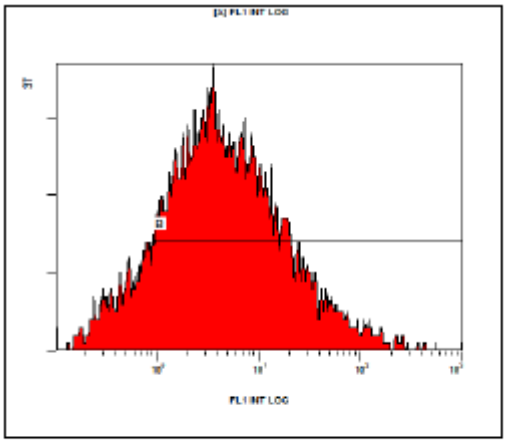
[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12731	84.87	100.00	4.28	21.1
B	10826	72.17	85.04	5.66	24.7

B)

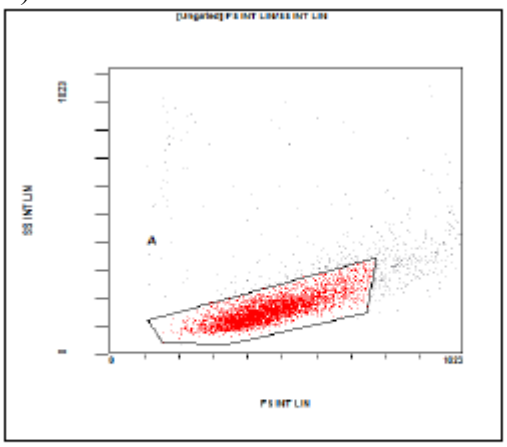


[Ungated] FS INT LIN/SS INT LIN					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	469	502
A	12678	84.52	84.52	455	467

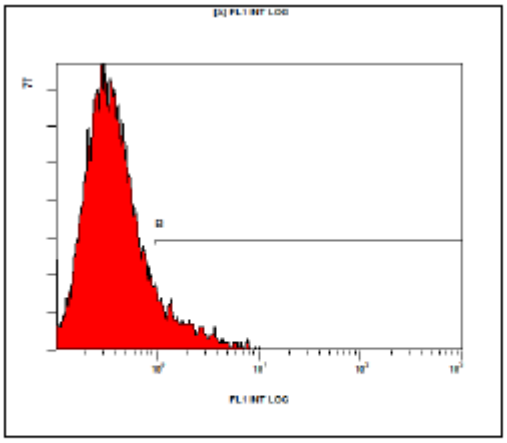


[A] FL1 INT LOG					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12678	84.52	100.00	4.13	21.7
B	10780	71.87	85.03	5.61	25.4

C)

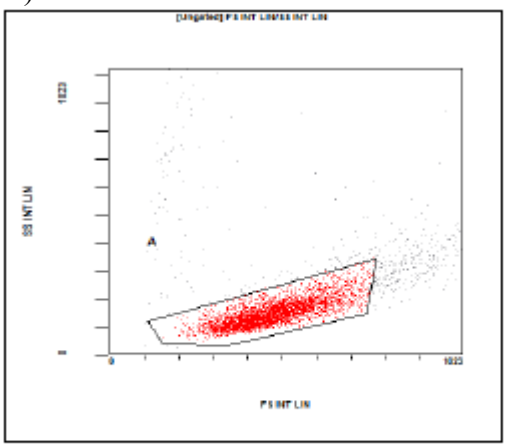


[Ungated] FS INT LIN/SS INT LIN					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	469	508
A	12777	85.18	85.18	452	465

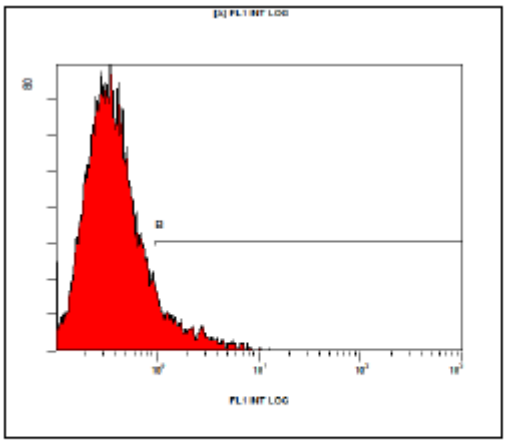


[A] FL1 INT LOG					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12777	85.18	100.00	0.364	0.854
B	1853	12.35	14.50	1.9	3.7

D)

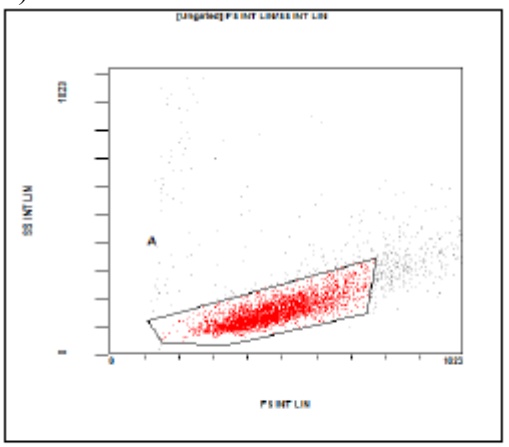


[Ungated] FS INT LIN/SS INT LIN					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	472	506
A	13005	86.70	86.70	458	471



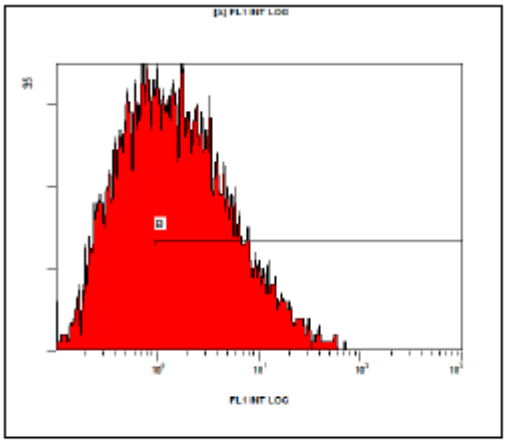
[A] FL1 INT LOG					
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	13005	86.70	100.00	0.364	0.839
B	1755	11.70	13.49	1.97	3.81

E)



[Ungated] FS INT LIN/SS INT LIN

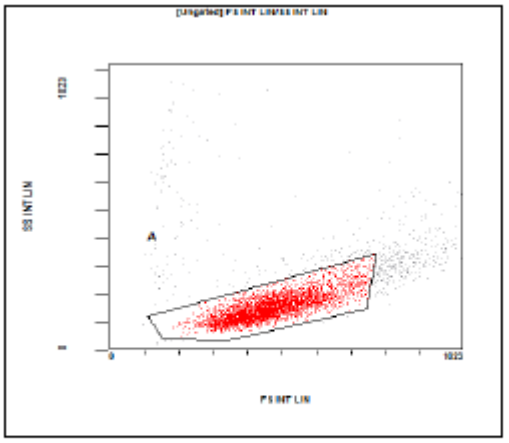
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	474	511
A	12743	84.95	84.95	456	470



[A] FL1 INT LOG

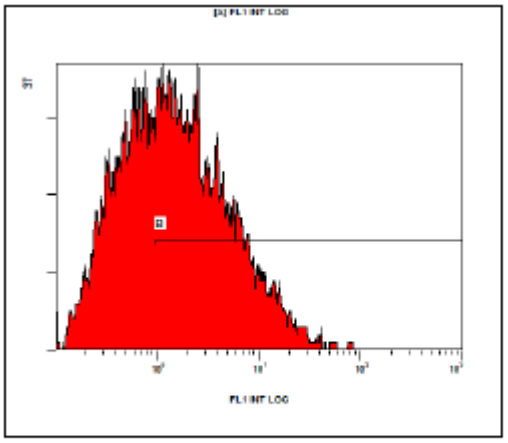
Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12743	84.95	100.00	1.42	5.99
B	7794	51.96	61.16	2.96	9.47

F)



[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	15000	100.00	100.00	471	504
A	12854	85.69	85.69	458	470



[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Median 50.0	X-Mean
ALL	12854	85.69	100.00	1.39	5.22
B	7900	52.67	61.46	2.83	8.18

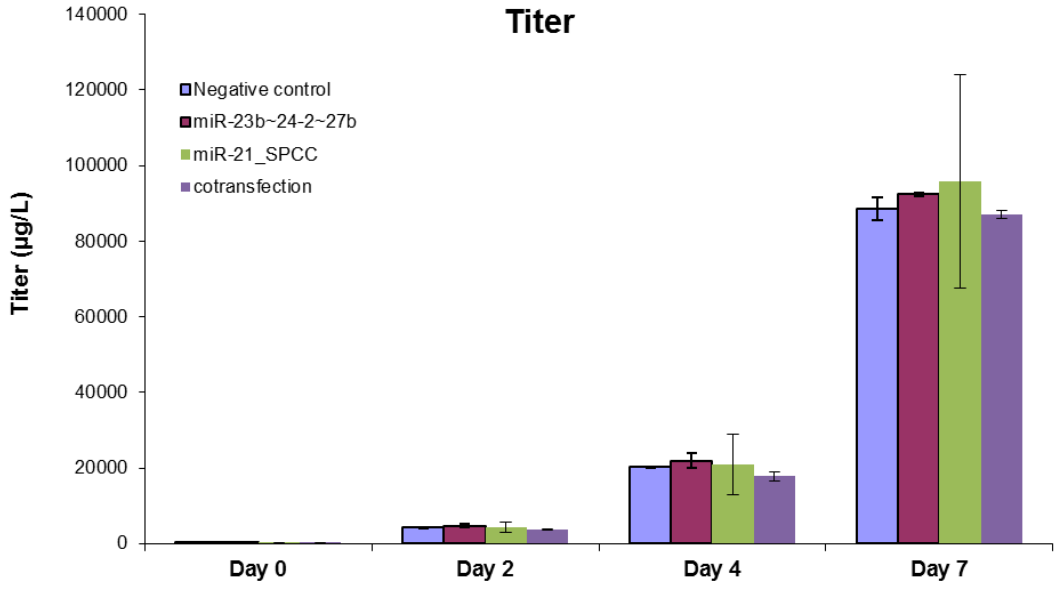
Figure 20: Flow cytometry analysis of the samples 48 h after transfection. Living cells were chosen by gate A on the forward scatter (FS) / side scatter (SS) plot. Transfected cells were selected by region B on the FL1 histogram.
A) miR-23b~24-2~27b replicate 1
B) miR-23b~24-2~27b replicate 2
C) miR21_SPCC replicate 1
D) miR21_SPCC replicate 2
E) miR-23b~24-2~27b / miR21_SPCC replicate 1
F) miR-23b~24-2~27b / miR21_SPCC replicate 2

The negative control is depicted on Figure 11 A and B. About 85 % of the cells which were transfected with the miR23b~24-2~27b cluster express GFP. Only about 15 % of the cells which were transfected with the sponge contain functional GFP. 61 % of the co-transfected cells express GFP. These values indicate that the transfections and also the knockdowns are functional.

3.3.3. Batch 1 – ELISA

The collected supernatant samples were analysed by ELISA to check the impact of the miRNAs on the productivity and the product titer.

A)



B)

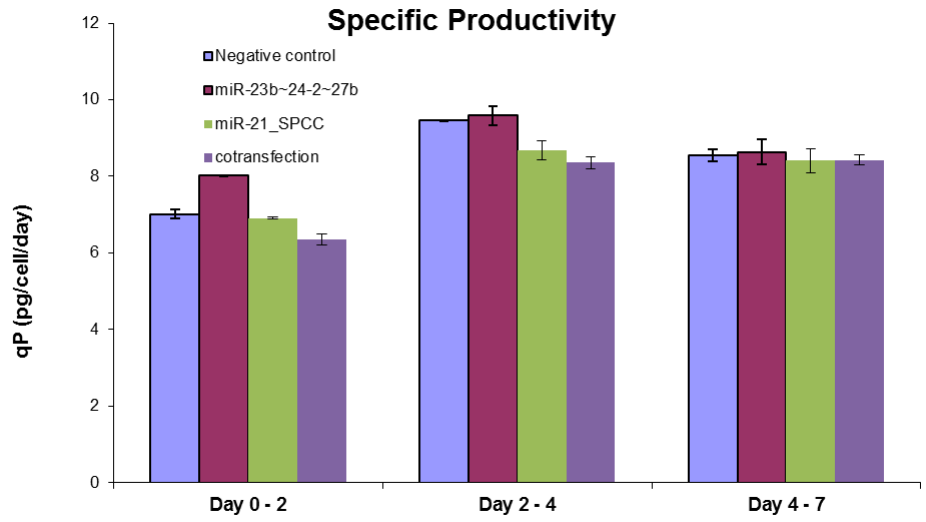


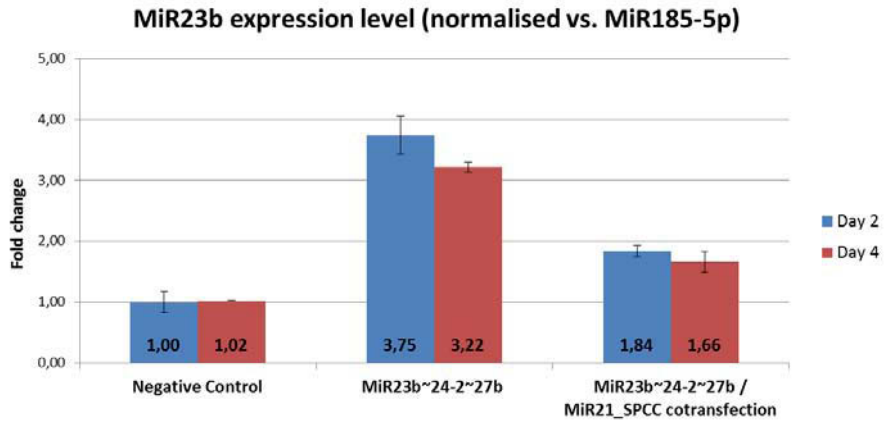
Figure 21: Product Titer (A) and specific productivity (B) at different timepoints after transfection. The titer is pictured in a logarithmic scale.

Figure 21 A compares the titers of the different samples. There is no difference between the product yields. In Figure 21 B it can be seen that the specific productivity is about 14 % higher in cells containing the miR23b~24-2~27b cluster from day 0 to day 2. Cells which were transfected with the miR21-SPCC had an 11 % lower qP between day 2 and day 4. The co-transfected ones also show a decline in the productivity of 10 – 12 % between day 0 and day 4.

3.3.4. Batch 1 – Quantitation of mature MicroRNAs

The collected RNA samples were reverse transcribed and analysed by qPCR to determine the expression levels of the transfected miRNAs in comparison to the negative control.

A)



B)

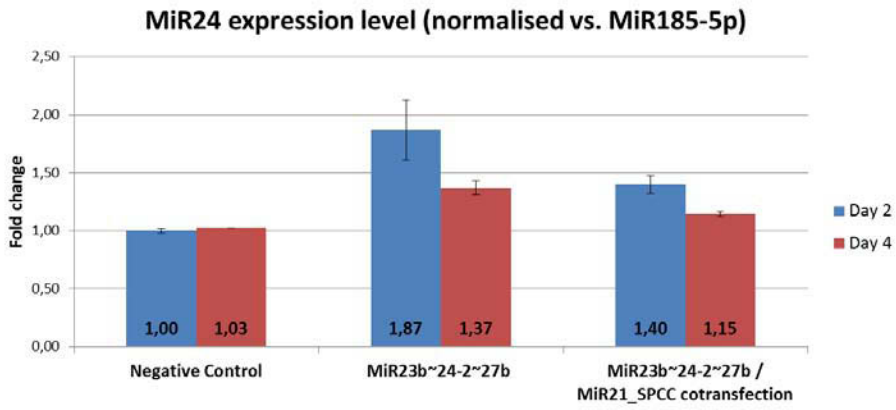


Figure 22: Fold changes of MicroRNAs compared to the negative control at two different timepoints
A) Expression levels of miR23-3p
B) Expression levels of miR24-3p

In Figure 22, it can be seen that 48 h after transfection, miR23b-3p and miR24-3p are up-regulated in all transfections compared to the negative control, although the expression levels of the co-transfected cells are lower than the ones which were transfected with the miR23b~24-2~27b cluster only. All expression levels except the ones from the negative control are decreasing from day 2 to day 4.

3.3.5. Batch 2 – Growth and Viability after Transfection

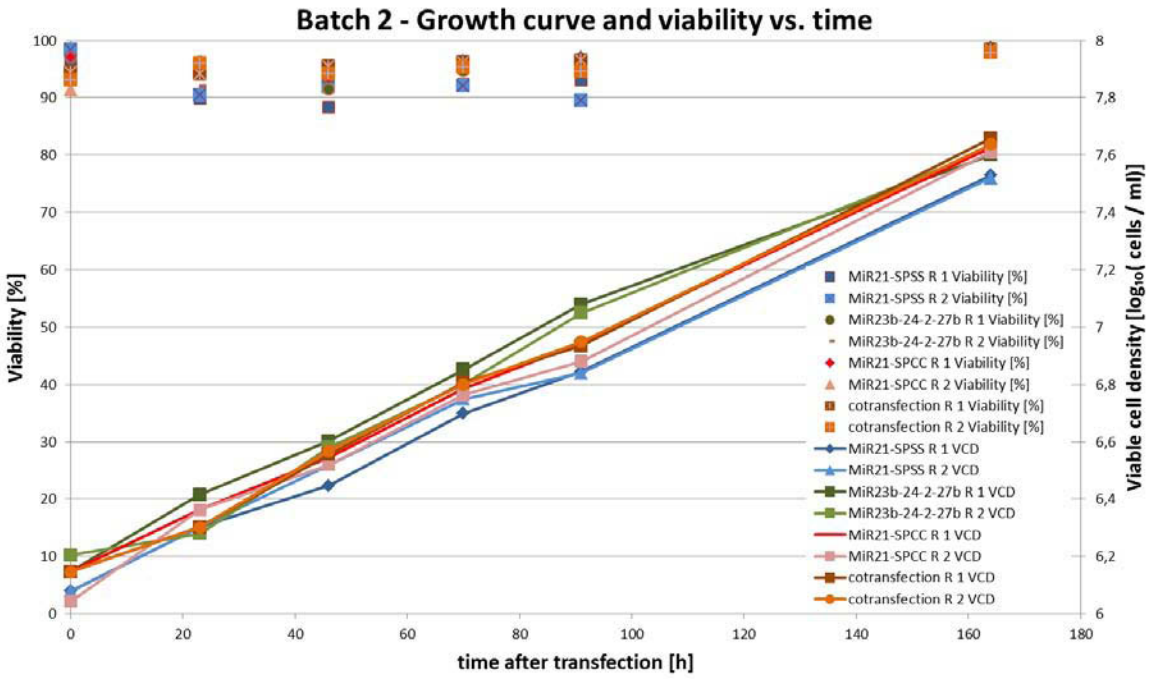


Figure 23: Viable cell density (VCD) and viability over time

The growth curves and the viabilities of the different samples (Figure 23) are not very different. All samples had a higher VCD than the negative control at day 7. The viable cell densities at different timepoints were used to calculate the growth rates.

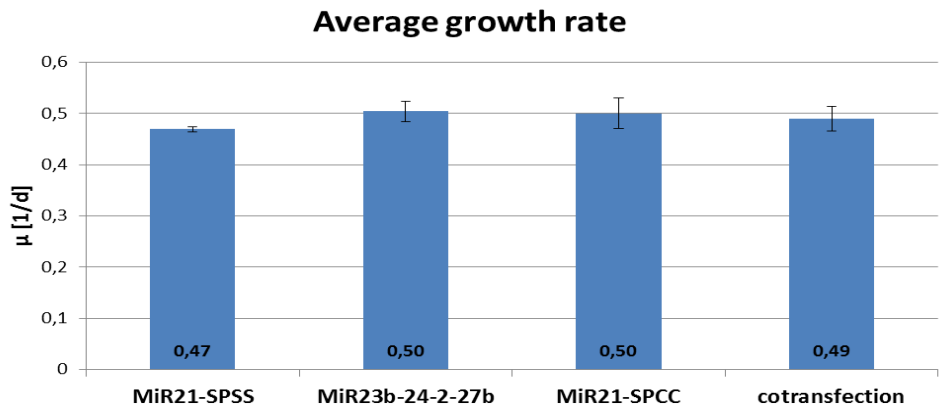


Figure 24: Average growth rate (μ) of the 2 replicates of each transfection

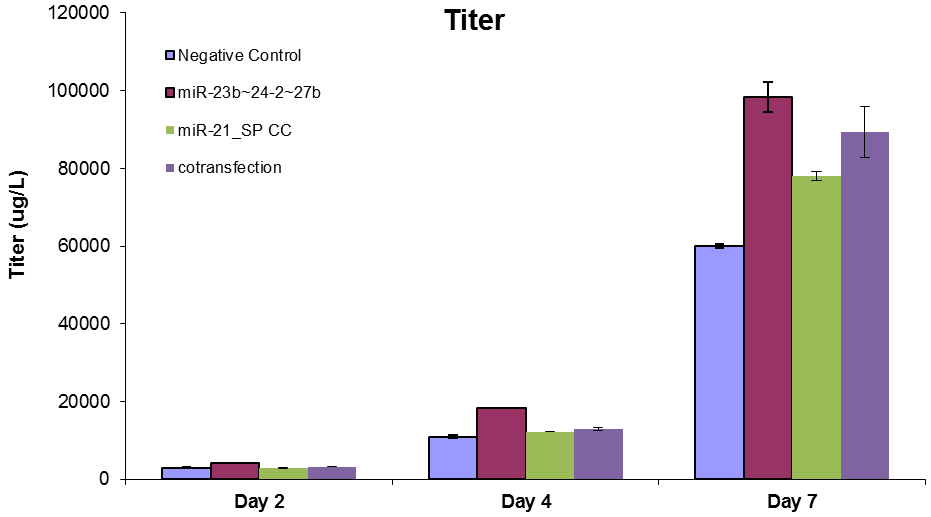
The average growth rates did not vary much between the different samples.

All of the samples in batch 2 had worse growth characteristics than the ones from batch 1. The cells of batch 2 were more stressed, because the CO_2 concentration was lower than 7 % and the shaker was often stopped during the batch.

3.3.6. Batch 2 – ELISA

The collected supernatant samples were analysed by ELISA to check the impact of the miRNAs on the productivity and the product titer.

A)



B)

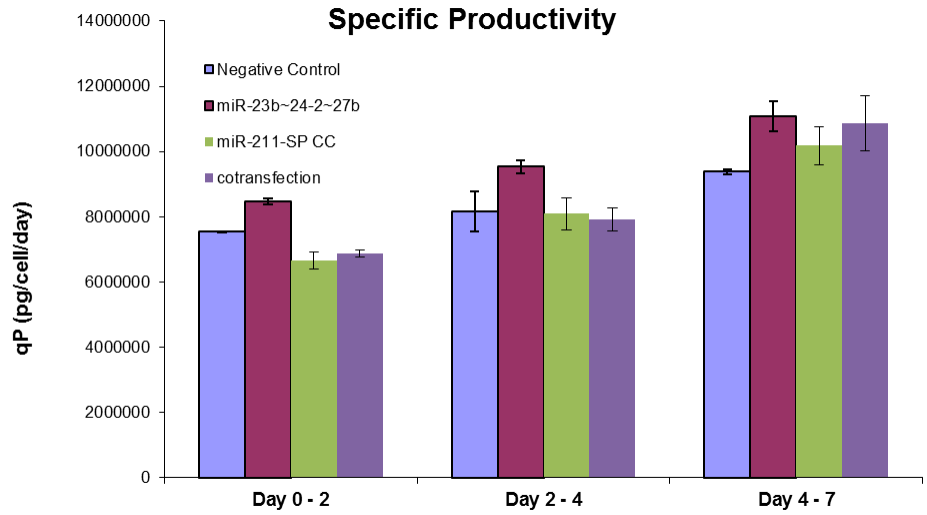


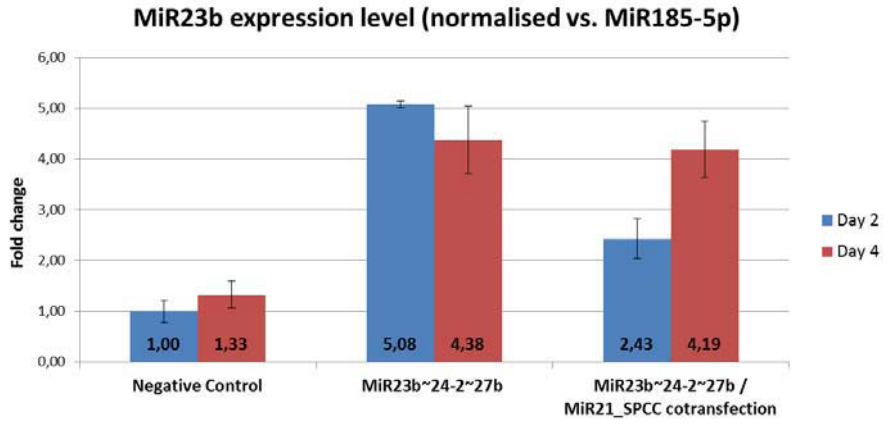
Figure 25: Product Titer (A) and specific productivity (B) at different timepoints after transfection The titer is pictured in a logarithmic scale.

Figure 25 A compares the titers of the different samples. It can be seen that the amount of product (+40 % on day 2 and +60 % on day 4 and 7) and also the specific productivity (+12 % from day 0 to day 2, +18 % between day 2 and day 7) is higher throughout the whole batch of cells which were transfected with the miR23b~24-2~27b construct. The cells which were transfected with the miR21_SP CC or co-transfected with the two vectors have a 10 % lower qP in the beginning (Day 0 – 2) but an increased one from day 4 to day 7 (miR21_SP CC: +10 %, co-transfection: +19 %). Also the titers are enriched at day 7 compared to the negative control (miR21_SP CC: +30 %, co-transfection: +49 %).

3.3.7. Batch 2 – Quantitation of Mature MicroRNAs

Also at the second batch, the expression levels of the transfected miRNAs in comparison to the negative control were determined using the collected RNA samples, which were reverse transcribed and analysed by qPCR.

A)



B)

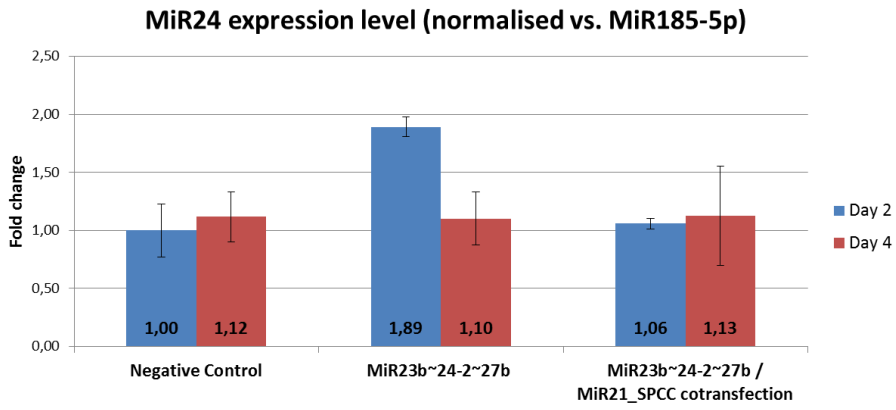


Figure 26: Fold changes of MicroRNAs compared to the negative control at two different timepoints
A) Expression levels of miR23-3p
B) Expression levels of miR24-3p

In Figure 26, it can be seen that miR23b is always higher expressed in the cells transfected with the miR23b~24-2~27b cluster in relation to the negative control. Though the content of the microRNA is decreasing from day 2 to day 4 at the miR23b~24-2~27b – transfected cells, the amount is increasing between day 2 and day 4 in the co-transfected cells.

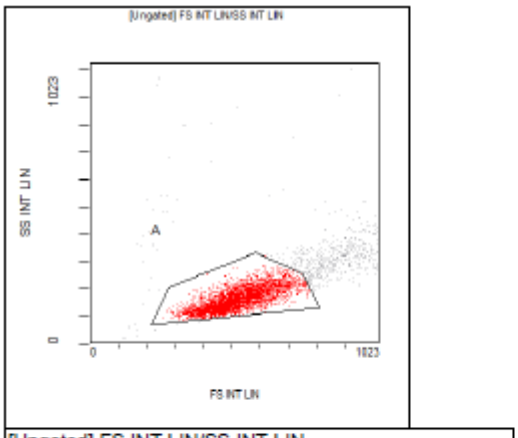
MiR24 shows only an increase on day 2 in the cells which were solely transfected with the cluster.

3.4. Dye Dilution with Initial Sorting

3.4.1. Initial Sorting

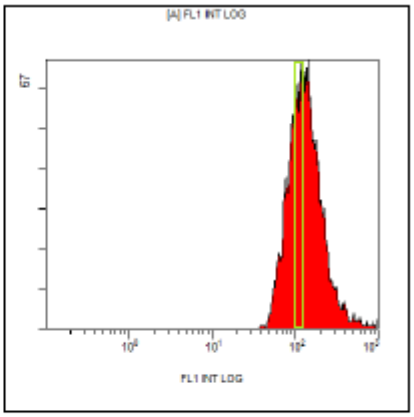
In an alternative approach to improve the growth rate of cells, CHO-S cells were stained with CFSE. A small fraction of these cells were then sorted to gain a uniformly stained population.

A)



[Ungated] FS INT LIN/SS INT LIN

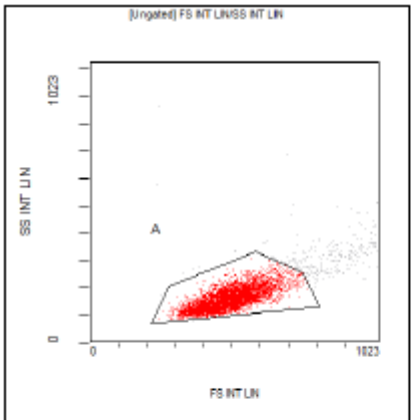
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	100.00	580	205
A	8369	83.69	83.69	521	159



[A] FL1 INT LOG

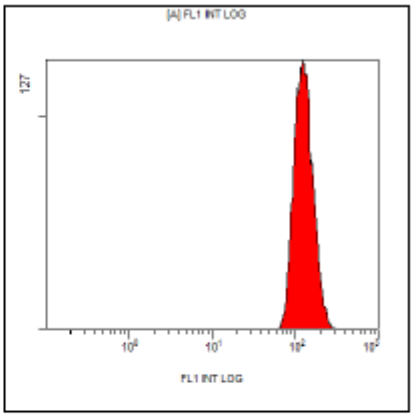
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	8369	83.69	100.00	162	###

B)



[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	100.00	521	170
A	9347	93.47	93.47	497	159



[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	9347	93.47	100.00	131	###

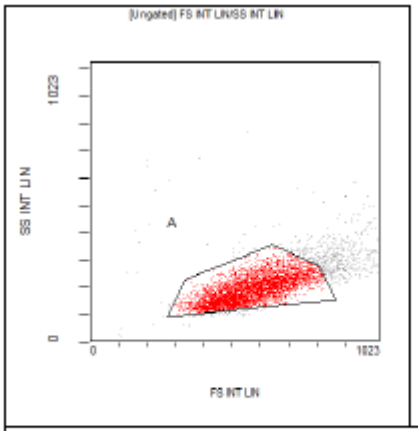
Figure 27: Flow cytometry analysis of the cells after staining with 10 µM CFSE. Living cells were chosen by gate A on the forward scatter (FS) / side scatter (SS) plot. FL1 depicts the stain intensity.
A) Stained cells before initial sorting. The green square depicts the sorting range
B) Stained cells after initial sorting

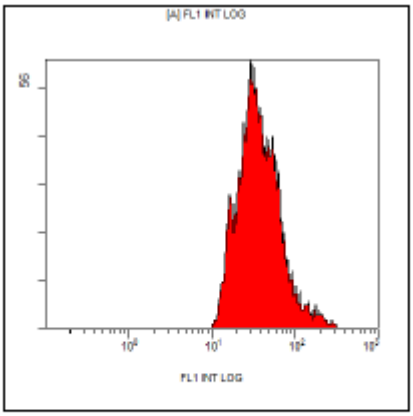
Figure 27 shows that the CFSE peak narrows when cells are sorted after staining.

3.4.2. Dye Dilution

After initial sorting, the cells were cultivated and the fluorescence intensity was measured at different timepoints. As a control, the unsorted stained cells were also analysed.

A)





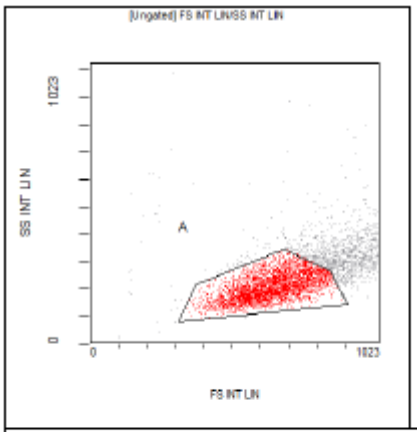
[Ungated] FS INT LIN/SS INT LIN

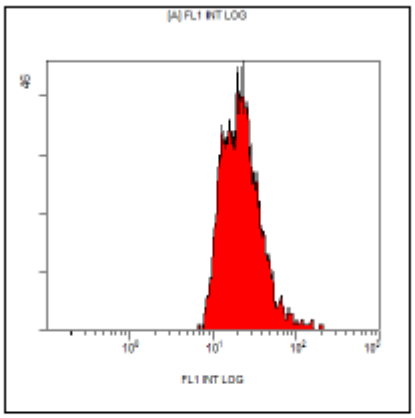
Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	100.00	617	224
A	8217	82.17	82.17	565	188

[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	8217	82.17	100.00	48.6	###

B)





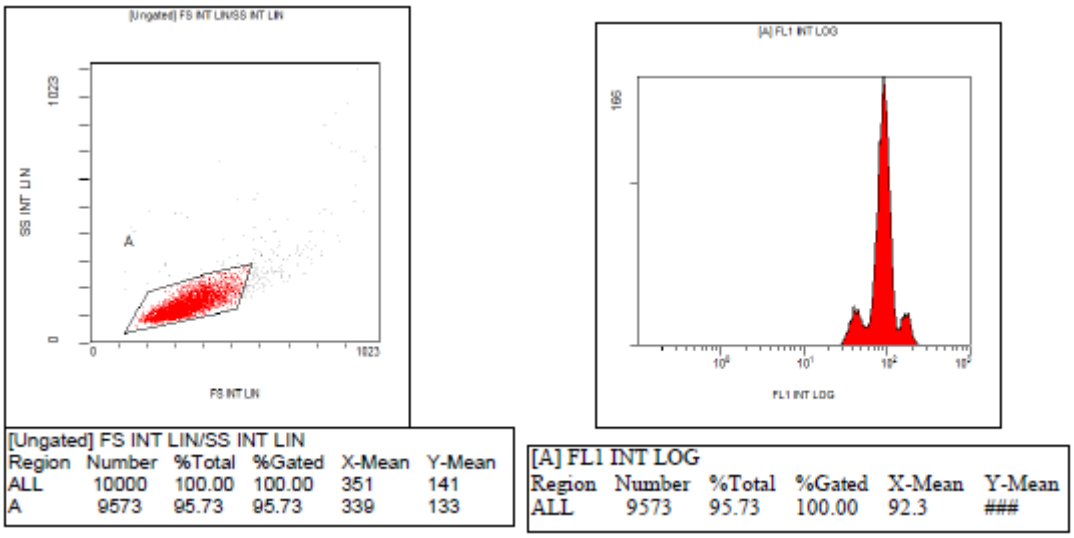
[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	100.00	745	270
A	6382	63.82	63.82	643	205

[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	6382	63.82	100.00	29.7	###

C)



[Ungated] FS INT LIN/SS INT LIN

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	10000	100.00	100.00	351	141
A	9573	95.73	95.73	339	133

[A] FL1 INT LOG

Region	Number	%Total	%Gated	X-Mean	Y-Mean
ALL	9573	95.73	100.00	92.3	###

D)

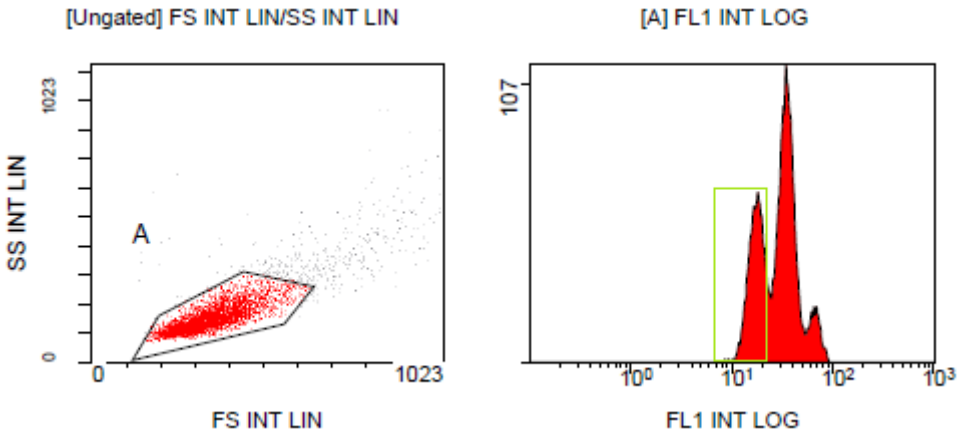


Figure 28: Flow cytometry analysis of the cells after staining with 10 µM CFSE at different timepoints. Living cells were chosen by gate A on the forward scatter (FS) / side scatter (SS) plot. FL1 depicts the stain intensity.
A) 24 h after staining without initial sorting.
B) 48 h after staining without initial sorting.
C) 24 h after staining with initial sorting.
D) 48 h after staining with initial sorting. The green square depicts the sorting range for the second sort

In Figure 28, it can be seen, that the cells which were cultivated without initial sorting, show one broad peak while cells which were sorted after staining, give rise to more distinct peaks, each of which represents cells that have undergone a distinct number of divisions.

After 48 h the cells with the lowest fluorescence intensity were sorted again, cultivated and the procedure was repeated two times.

3.4.3. Batch

After three of these sorting cycles, a batch was performed to compare the growth rates.

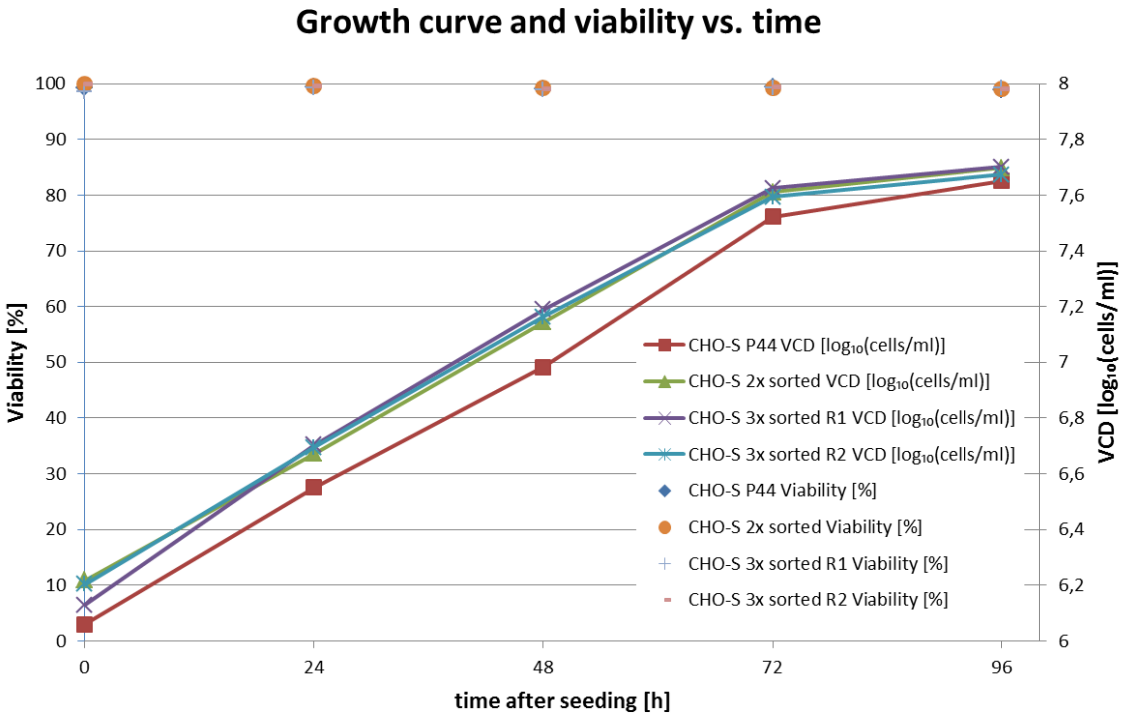


Figure 29: Viable cell density (VCD) and viability over time

The viabilites of the different samples remained above 95 % for the whole batch. The shapes of the growth curves are also nearly the same. The growth rates of the batch were calculated using the viable cell densities at different timepoints. The lower seeding concentration of the unsorted cells (CHO-S P44) lead to a lower VCD throughout the whole batch.

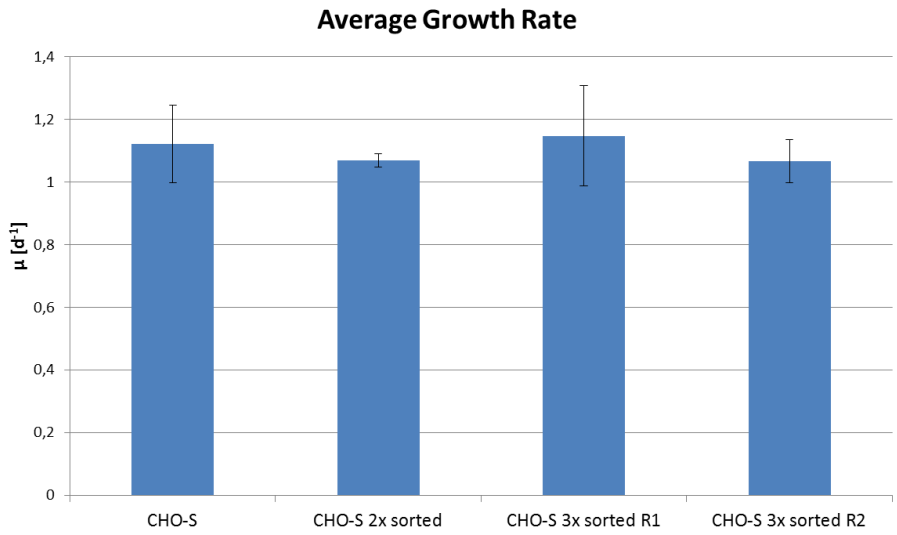


Figure 30: Average growth rate (μ) of the different samples from day 0 to day 3

Figure 30 shows that there is no enhancement in growth after two to three times sorting of faster dividing cells. The data from day 3 to day 4 was not included, because the growth rate was already decreasing.

4. Discussion

The aim of the project was to enhance the CHO phenotype. We tried to achieve this by transfecting a recombinant producing cell line with different miRNA-constructs, which led to a better performance in other organisms or which emerged from previous experiments. Because the sequence of the endogenous cluster became available during the thesis, a comparison between an artificial and an endogenous construct was included. We also tried to increase the growth rate by repeated cycles of dye dilution with initial sorting.

4.1. MicroRNA Transfections

Although up-regulation of miR221-222 leads to proliferative enhancement in different types of cancer, the growth rates and viabilities of the two batches did not differ from the negative control. This could be caused by different reasons. One possibility is that transient transfection is too short to develop a different phenotype. Another cause may be that the effect of the miR221-222 cluster is species specific so that this miRNA does not have any or another effect on CHO cells. The amounts of active GFP, GFP-mRNA and of the mature miR221 and miR222 were determined for comparison. The quantity of active GFP was very low in cells containing the endogenous cluster, but the mRNA levels of GFP did not differ correspondingly. Though the same amount of plasmid was used for all transfections, the mature guide miR221-3p and miR222-3p (http://www.mirbase.org) of the endogenous cluster were two- to threefold higher expressed in two out of three replicates. In the third one the expression levels were even five to eightfold higher compared to the negative control. The expression levels of the mature MicroRNAs of the artificial construct, which consists of the flanking and loop sequences of the mmu-miR-155 and of the mature miRNAs of CHO, did not show an increase. Because in mouse, the miR-155-5p is the guide MicroRNA, another qPCR was included to check the levels of miR221-5p. However, there was also no overexpression of the miR221-5p in the cells with the artificial construct and in two out of three replicates containing the endogenous cluster, it was approximately twofold overexpressed. Overall, the levels of the overexpressed MicroRNAs and the GFP-mRNA

levels decrease from day 2 to day 4. Because the stem loop primers are sequence and end specific, a slight change in processing of the miRNAs could lead to an incorrect reverse transcription. It could be possible that the artificial construct was processed differently than the endogenous one. An additional northern blot would indicate whether there is a difference in processing or whether the endogenous cluster is processed much more efficiently than the artificial one. A transfection with a higher amount of the artificial cluster would also be helpful to see that the amount of plasmid was not too low. Another possibility would be to analyse the amount of predicted targets for the miR221-3p and miR222-3p. If the expression of the targets in cells transfected with the artificial cluster would be higher compared to those transfected with the endogenous cluster, then the artificial cluster would have performed worse. From the comparison of miR221-3p and miR221-5p presence, it can be seen that the expression levels of the passenger microRNA are more than 200 fold lower than the levels of the guide microRNA (data not shown).

Previously performed internal, unpublished studies demonstrated that the miR23b~24-2~27b cluster and miR21 are good candidates to investigate their influence on productivity. The growth characteristics of the cells which were transfected with the miRNAs of interest did not differ significantly from the negative control. The lower overexpression of miR24-3p in the transfected cells is caused by the already high expression level of miR24-3p, which is about 400 times higher than the amount of mature miR23b-3p in the negative control.

The overexpression of the miR23b~24-2~27b cluster leads to an improvement in productivity during the initial two days after transfection in batch 1 and over the whole batch in batch 2. The co-transfected cells and the ones which were transfected with the miR21_SPCC have a worse performance at the first days in the two batches. Only between day 4 and day 7 in batch 2, did they perform better than the negative control. However, the miR21_SPCC and the co-transfection produced more in batch 2, which suffered from a lower CO_2 concentration and frequently stopped shaking. This may be an indicator that the knockdown of MiR21 leads to a better productivity under stressful conditions.

4.2. Dye Dilution with Initial Sorting

CFSE staining is a useful tool to monitor dividing cells over time (*37, 38*). The dye content after staining should be equally distributed between the cells. Because the variability between the stained cells was too high, no distinct peaks can be seen by flow cytometry analysis. An initial sorting step was included to derive a homogenously stained cell population. After some

days, the cells which divided most often were sorted again. Then they were cultivated again to gain a sufficient amount of cells. This procedure was repeated three times to minimize random effects. The growth analysis demonstrates that there is no enhancement in growth or viability at the sorted cells. The cells which are faster dividing may already outgrow the ones which are not or slower dividing without assisted evolution. If this happens, then this method is without success. Maybe the fraction of the sorted cells was too large and so also slower dividing cells were included. Alternatively, increases in growth rate are randomly distributed within a population, without any connection to genomic differences. They may be controlled by random changes in gene transcription that occur in cells. In this case any cell, whatever its actual recent division rate, would revert to any of the random growth rates within the range possible, so that sorting would indeed not have any effect. The examined cell line has also a very high growth rate compared to other CHO cell lines. This might already be the upper limit which can be reached by CHO cells.

5. Conclusion and Outlook

5.1. MicroRNA cluster cloning

It seems that there is a big difference between the processing of a MicroRNA cluster with endogenous derived sequences and an artificial one which consists of sequences from different species. The specific miRNA content should also be analysed by other methods to ensure that the artificial cluster is also expressed but differently processed. Another interesting aspect would be to look at predicted targets for the mature miRNA to see whether the different processing and/or expression level has also an effect on target knockdowns.

The miR23b~24-2~27b cluster is a good candidate for cell line engineering to enhance the specific productivity. A stable transfection would be helpful to investigate the long-term effect of the overexpressed cluster on the phenotype of producer cell lines. A miR21 knockdown could be useful for cells which should produce high amounts of therapeutic proteins under harsh conditions. The knockdown should also be confirmed by other methods in addition to the previously described ones. Also a stable transfection with cultivation under suboptimal conditions would clarify if this is only a transient effect or whether the better performance can be achieved over a prolonged period of time.

5.2. Dye Dilution with initial sorting

The initial sorting was an appropriate method to obtain a homogenously stained cell population. Also, the monitoring of the cell divisions by dye dilution was successful. But the growth rate of the sorted cells was not affected by this method. Various factors can be critical for the uninfluenced growth rate at this method as described in section 4.2.

Although this method is not applicable for growth enhancement of this cell line, it may be useful for other studies. All investigations which are based on dye dilution can now be adapted for heterogeneously stained cell populations. For example, cells which are still dividing under suboptimal conditions can be sorted for analysis or to increase the stress resistance. Another field of application would be to sort cells which divided and cells which did not for comparison to determine the underlying molecular and regulatory mechanisms.

6. References

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7. Appendix

7.1. Primer List

Primer ID	sequence (5' – 3')
emGFP_eGFP_FP_1	GGACGGCAACATCCTGGGGC
emGFP_eGFP_RP_1	CGTCGCCGATGGGGGTGTTC
cgr-24-23b_FP	GGAAGTCGACGTGTGGTAGGCTCAGAGG
cgr-24-23b_RP_BglII	GGAAAGATCTTGGGTAGGCGTTTTGCTC
EmGFP_FP	GGCATGGACGAGCTGTACAA
miRNA_RP	CTCTAGATCAACCACTTTGT
psiCHECK2seq_sense	TACCTTCGGGCCAGCGACGA
psiCHECK2seq_as	GCCAGTTACCACCCGCCGAC
cho_actb_2_fw	TACGTGGGTGACGAGGCCCA
cho_actb_2_rev	AGCCAGGTCCAGACGCAGGA

7.2. MiR-21 Sponge Sequences

Sponge ID	sequence (5' – 3')
miR-21_SPCC	TCAACATCAGTCTGATAAGCTA
miR-21_SPBG	TCAACATCAGAGAATAAGCTA
miR-21_SPMT	TCAACATCAGTCTGATGCAATA
miR-21_SPSS	TCCTATACCAAGTTCGCAATA

7.3. Reagents

LB – agar (pH = 7,0)

	Amount
Bacto-Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g
Reverse osmosis (RO) - water	to 1 l

LB – medium (pH = 7,0)

	Amount
Bacto-Tryptone	50 g
Yeast extract	25 g
NaCl	50 g
RO – water	to 5 l

Coating Buffer (pH = 9,6 – 9,8)

	Amount
$NaHCO_3$	8,4 g
Na_2CO_3	4,2 g
RO – water	to 1 l

Wash Buffer (pH = 7,2 – 7,4)

	Amount
Na_2HPO_4 x 2 H_2O	1,15 g
KH_2PO_4	0,2 g
KCl	0,2 g
NaCl	8 g
Tween 20	1 ml
RO – water	to 1 l

Dilution Buffer

The dilution buffer consists of wash buffer with 1 % bovine serum albumin.

Staining Buffer (pH = 5,0)

	Amount
Citric acid x 2 H_2O	7,3 g
Na_2HPO_4 x 2 H_2O	11,86 g
RO – water	to 1 l

Staining Solution

	Volume
Staining buffer	10 ml
H_2O_2	6 µl
1,2-o-Phenyldiamindihydrochlorid (1 mg/ml)	100 µl

7.4. Pipetting Schemes and Temperature Profiles

Restriction Digestion

	Volume
10 x Fast Digest Buffer / 10 x Fast Digest Buffer Green	5 µl
Restriction enzyme	2 µl each
Calf intestinal phosphatase	1 µl (only at vector digestions)
DNA	x µl
NFW	y µl
Final volume	50 µl

The 10 x Fast Digest Buffer Green was used when the product was run on an agarose gel afterwards.

Time	Temperature
45 min	37 °C
5 min	85 °C

Ligation

	Volume
NEB 10 x Buffer	1 µl
NEB Ligase	1 µl
Vector	x µl
Insert	y µl
NFW	z µl
Final Volume	10 µl

Colony PCR

	Volume
10 x Reaction Buffer Biotherm + 15 mM $MgCl_2$	2 µl
50 mM $MgCl_2$	0,6 µl
10 mM dNTPs	0,5 µl
Forward Primer	0,2 µl
Reverse Primer	0,2 µl
Biotherm Polymerase	0,1 µl
NFW	16,4 µl
Final Volume	20 µl

Time	Temperature	Repeats
5 min	95 °C	
30 s	95 °C	30 x
30 s	57 °C	
30 – 90 s *	72 °C	
10 min	72 °C	

*Depends upon the insert length.

PCR of the MiR23b~24-2~27b cluster and for Standard Preparation

	Volume
5 x HF Phusion Buffer	10 µl
50 mM $MgCl_2$	1 µl
10 mM dNTPs	1 µl
DMSO	1,5 µl
cgr-24-23b_FP	1 µl
cgr-24-23b_RP_BglII	1 µl
Phusion TaqDNA – Polymerase	0,5 µl
gDNA / cDNA*	5 µl
NFW	29 µl
Final Volume	50 µl

*The cDNA was used for standard preparations.

Time	Temperature	Repeats
30 s	98 °C	
10 s	98 °C	30 x
15 s	57 °C	
45 s	72 °C	
10 min	72 °C	

Taqman® MicroRNA reverse transcription

	Volume
10 x Reverse Transcription Buffer	1 µl
100 mM dNTPs (with dTTP)	0,1 µl
RNase Inhibitor, 20 U/µl	0,12 µl
Stem – loop RT Primer	2 µl
MultiScribe™ Reverse Transcriptase, 50 U/µL	0,6 µl
RNA template (1 -10 ng)	1 µl
NFW	5,18 µl
Final Volume	10 µl

Time	Temperature
30 min	37 °C
30 min	42 °C
5 min	85 °C

Taqman® MicroRNA assay for qPCR

	Volume
2 x TaqMan® Universal PCR Master Mix II, no UNG	5 µl
20 x TaqMan® Small RNA Assay	0,5 µl
Product from RT reaction	1,33 µl
NFW	3,17 µl
Final Volume	10 µl

Time	Temperature	Repeats
10 min	95 °C	
15 s	95 °C	40 x
1 min	60 °C	

The fluorescence intensity was determined every cycle after 1 min. at 60°C.

DNA Digestion

	Volume
10 x Reaction Buffer with $MgCl_2$	1 µl
RNase free DNase I (1 U/µl)	1 µl
RNA template (≤ 1 µg)	x µl
NFW	y µl
Final Volume	10 µl

DyNAmo™ cDNA Synthesis

	Volume
2x RT buffer (includes dNTP mix and 10 mM $MgCl_2$)	14,3 µl
Random hexamers (300 ng/µl)	1,4 µl
M-MuLV RNase H^+ reverse transcriptase (includes RNase inhibitor)	2,85 µl
10 µl RNA, DNase I treated	10 µl
Final Volume	28,55 µl

Time	Temperature
10 min	25 °C
30 min	37 °C
5 min	85 °C

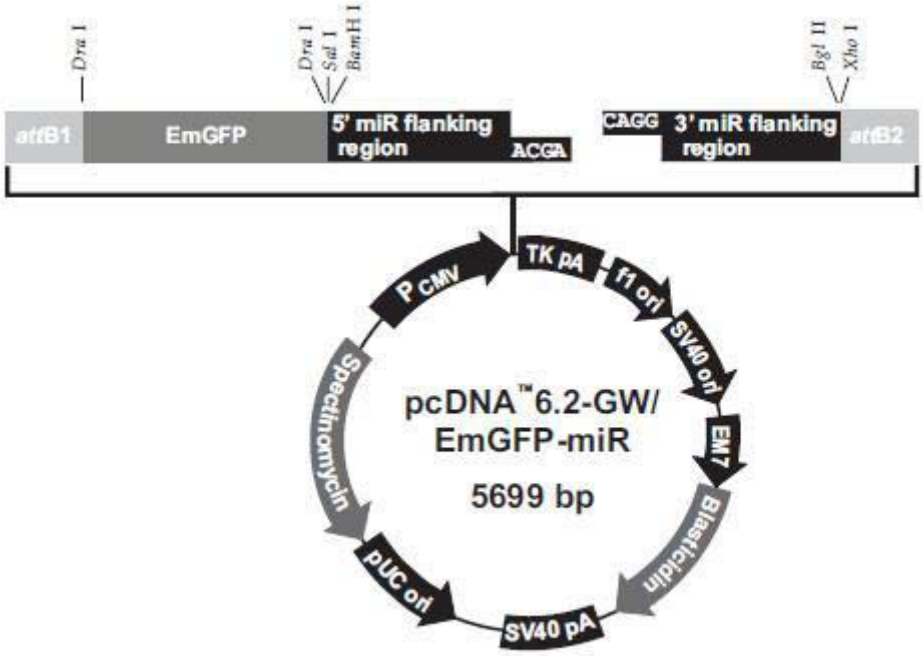
qPCR of the mRNAs

	Volume
2 x Reaction Mix	5 µl
Forward Primer	0,25 µl
Reverse Primer	0,25 µl
Template	1 µl
NFW	3,5 µl
Final Volume	10 µl

Time	Temperature	Repeats
10 min	95 °C	
15 s	95 °C	40 x
15 s	60 °C	
15 s	72 °C	
0 s	80 °C	

The fluorescence intensity was determined every cycle after 15 seconds at 72 °C and again at 80 °C. After the cycling, a melting curve was generated by increasing the temperature from 60 °C to 99 °C, 1 °C per 5 seconds.

7.5. Vector Maps



Comments for pcDNA™ 6.2-GW/EmGFP-miR
5699 nucleotides

CMV promoter: bases 1-588
*att*B1 site: bases 680 - 704
EmGFP: bases 713-1432
EmGFP forward sequencing primer site: bases 1409-1428
5' miR flanking region: bases 1492-1518
5' overhang (C): bases 1515-1518
5'overhang: bases 1519-1522
3' miR flanking region: bases 1519-1563
*att*B2 site (C): bases 1592-1616
miRNA reverse sequencing primer site (C): bases 1607-1626
TK polyadenylation signal: bases 1645-1916
f1 origin: bases 2028-2456
SV40 early promoter and origin: bases 2483-2791
EM7 promoter: bases 2846-2912
Blasticidin resistance gene: bases 2913-3311
SV40 polyadenylation signal: bases 3469-3599
pUC origin (C): bases 3737-4410
Spectinomycin resistance gene (C): bases 4480-5490
Spectinomycin promoter (C): bases 5491-5624

Figure 31: Vector map of pcDNA™6.2-GW/EmGFP-miR, taken from http://tools.invitrogen.com

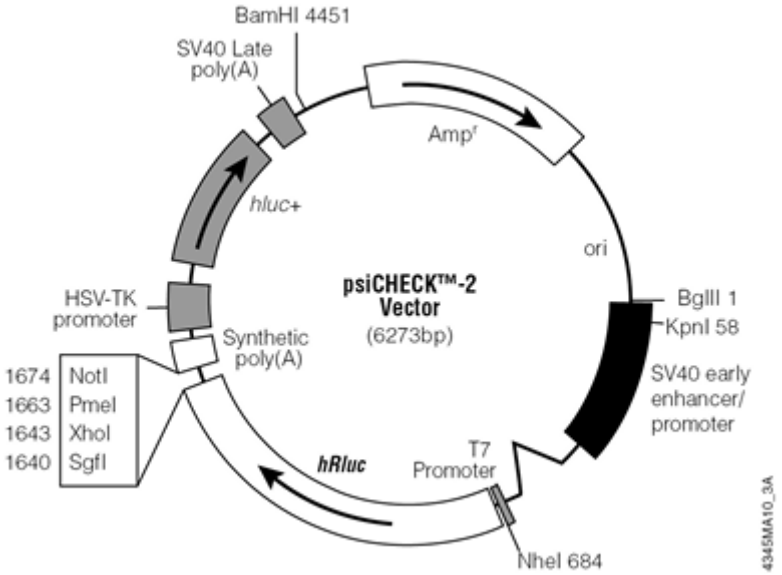


Figure 32: Vector map of psiCHECK™-2, taken from http://www.promega.com

7.6. Additional Information

Mechanism of pcDNA6.2™-GW/EmGFP-miR

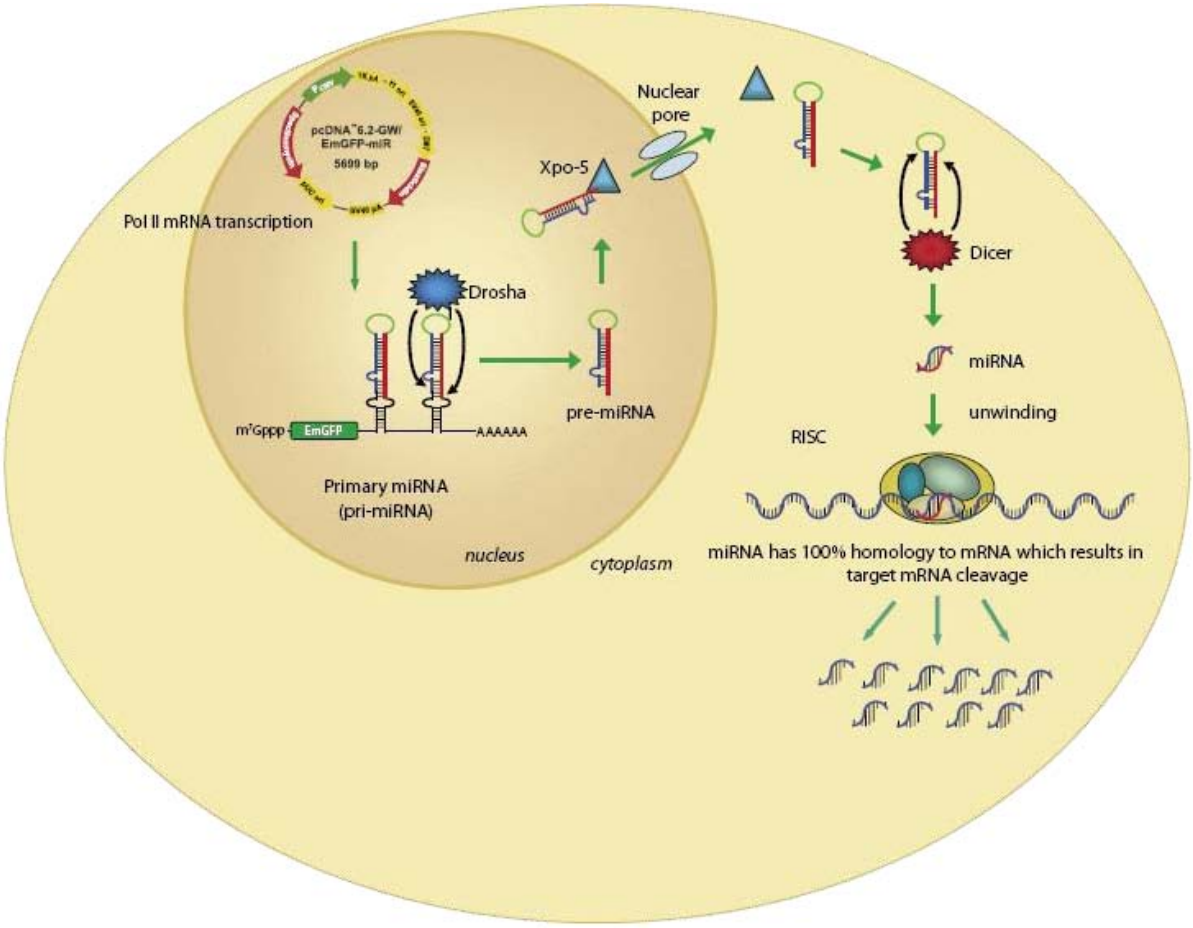


Figure 33: Mechanism of pcDNA6.2™-GW/EmGFP-miR, taken from http://tools.invitrogen.com

Mechanism of psiCHECK™-2

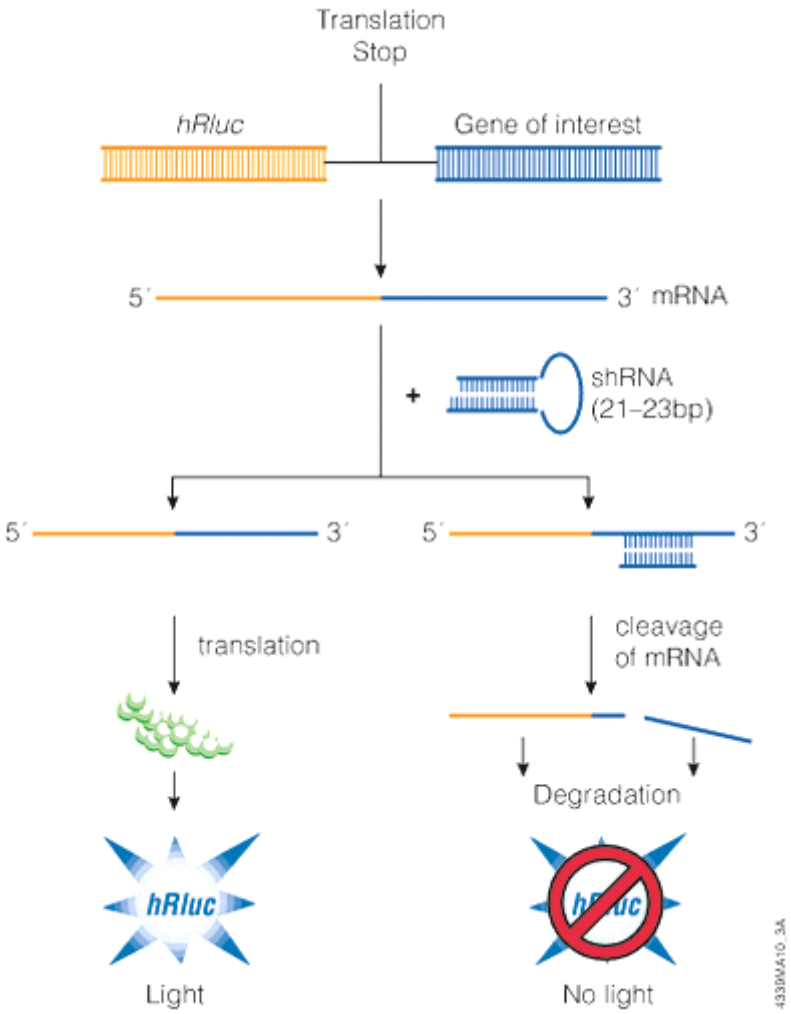


Figure 34: Mechanism of psiCHECK™-2, taken from http://www.promega.com