RNA Interference - optimizing a protocol for HC11 cells

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Abstract

RNA interference is a naturally occurring process of gene silencing that reduces the expression of the gene from which the RNA sequence is derived, when added in a double-stranded form. RNA interference works as defense against viruses and transposons.

The aim of this study was to optimize a protocol for RNA interference in mouse mammary epithelial HC11 cells by using different controls, and finally to silence the drug transporter BCRP to do an uptake-study with the BCRP-substrate mitoxantrone.

The optimization of the protocol was performed with four different lipids, as well as sicontrol tox, negative control and a positive control Cyclophilin B. Sicontrol tox and the negative control were assessed with ATP determination. Real time PCR was performed to check the down-regulation of Cyclophilin B after RNAi treatment of the HC11 cells.

The results of the optimization protocol showed that transfection for 48 h in the presence of lipid nr 1 diluted 1:4 times works best for transfection in HC11 cells. Cyclophilin B was down-regulated about 90 % with this protocol, and BCRP was down-regulated 80 %. The uptake-study with mitoxantrone showed that the BCRP gene silenced cells accumulated the double amount of mitoxantrone as compared to controls.

In conclusion, the obtained results provide a promising model for screening BCRP substrates, which can be used to examine other transporters in mammary epithelial cells, as well.

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RNA interference – background

History

RNA interference (RNAi) is a naturally occurring process of "gene silencing" in animal cells, and it reduces the expression of the gene from which the RNA sequence is derived, when added in a double-stranded form. It is a reversible form of gene silencing since there is no change in the DNA, only at the RNA-level, and the concentration of a target mRNA can be reduced up to 90%. RNAi works as a defense against viruses and accumulation of transposons in both animal and plant cells (Glick & Pasternak, 2003). The effect of RNAi is temporary and dosage-dependent (Timmons et al, 2003).

RNAi was discovered by plant scientists who reported an unexpected outcome in experiments with petunias. The scientists tried to intensify the purple color of the flower by introducing additional copies of a gene encoding a key enzyme for flower pigmentation in petunias. What they did not expect was that instead, the flowers became totally or partially white. They then discovered that the genes in question had been turned off, a case of posttranscriptional inhibition of gene expression that increased the rate of mRNA degradation. In that time, it was called "co-suppression of gene expression" (Wikipedia).

Craig C. Mello and Andrew Fire were investigating the regulation of muscle protein production in *Caenorhabditis elegans* (*C.elegans*) (Fire et al, 1998) and they observed that neither mRNA nor antisense RNA injections had an effect of protein production, but in a double-stranded form, the RNA effectively silenced the targeted gene. They named the process RNAi, and were in 2006 awarded the Nobel Prize (see figure 1) in Physiology or Medicine for their accomplishment (Wikipedia).
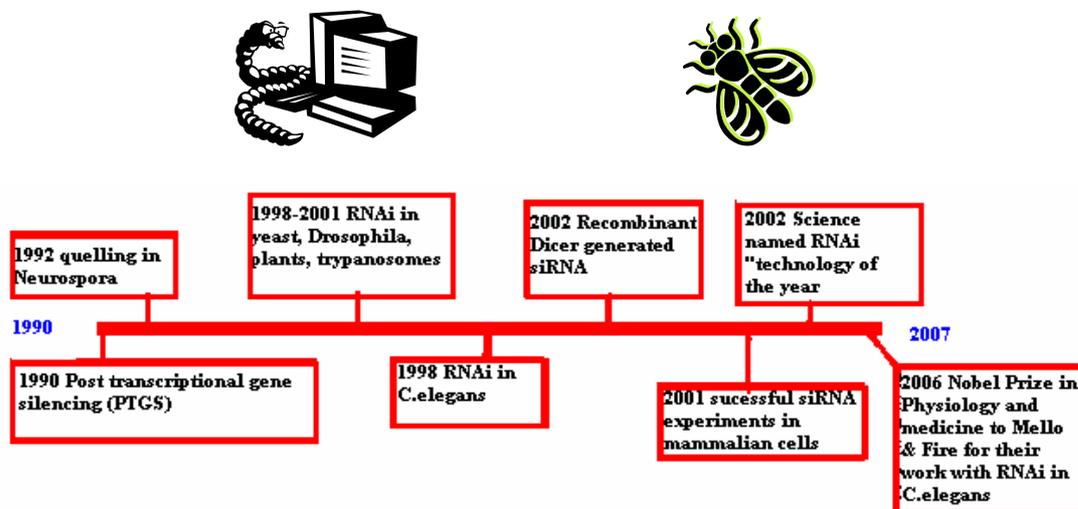

Figure 1. Timeline of RNAi History

Antisense oligonucleotides (ODN) were first introduced in 1978, as a tool for specific inhibition of gene expression, and in the early 1980s the ribozymes (catalytically active RNAs) were discovered. This finding showed that RNA molecules also can act as enzymes independent of the presence of proteins. On the other hand, ribozymes are quite instable molecules and difficult to distribute into the target tissue/cell (Aigner, 2005).

Although described under different names initially (Post transcriptional gene silencing (PTGS), co-suppression etc.), RNAi seems to exist in most eukaryotic organisms, and are not restricted to any specific organism (Aigner, 2005).

PTGS by dsRNA is conserved in both plants, *Neurospora, Drosophila, C.elegans* and mammals (Leung & Whittaker, 2005).

Mechanism

The short double-stranded RNA molecules involved in RNAi is called small interfering RNAs (siRNAs). The siRNA molecule incorporates into the multi-protein RISC (RNA-induced Silencing Complex), catalyzing the cleavage of a complementary mRNA molecule. The siRNA distinguishes from antisense oligonucleotides and ribozymes by its potency and specificity of gene silencing (Dharmacon, 2005).

The endogenously regulatory, non-coding microRNAs (miRNAs),which are naturally occurring and synthesized in the nucleus in long precursor forms, are processed into shorter pre-miRNAs and exported to the cytoplasm. Then an RNAse called Dicer cleaves them into siRNAs, which will be incorporated into the RISC (Dharmacon, 2005).

The protein Dicer cleaves the dsRNA molecule into short 21-23 duplexes with a symmetric 2nt overhang at the 3´-end and a 5´-phosphate and 3´-hydroxyl group referred to as siRNAs, see figure 2. RISC turns into an active state by an RNA helicase, which mediates the loss of one strand of the siRNA duplex. The remaining siRNA strand binds to a complementary target mRNA strand, and makes it possible to induce endonucleolytic cleavage of the same, which leads to a rapid degradation of the mRNA molecule as a whole, after the generation of unprotected RNA ends (Aigner, 2005).
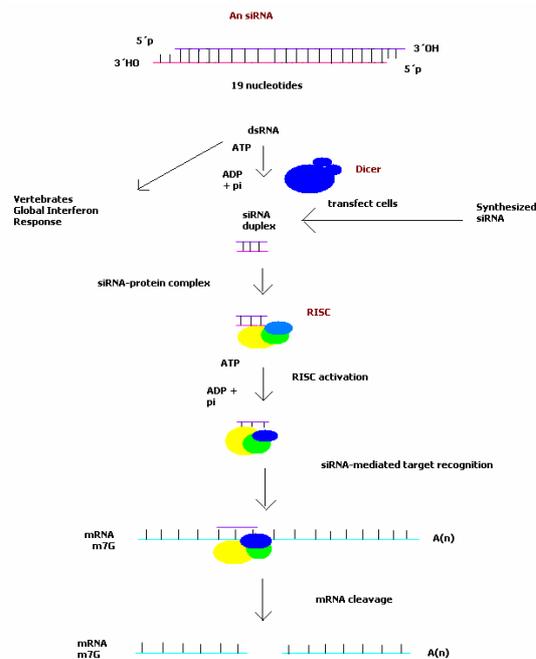
Figure 2. The mechanism of RNAi. An dsRNA molecule is cleaved by Dicer, and the remaining siRNA duplex is incorporated into RISC, catalyzing the cleavage of complementary mRNA.

RNAi phenotypes have generally been elicited in *Caenorhabditis elegans* by using dsRNA molecules that are more than 100 base pairs long, which then will be cleaved inside the cell into siRNAs (Timmons et al, 2003). Fire & Mello found that an injection of dsRNA in any site of C.elegans leads to systemic responses, indicating that it spreads in the body autonomously and does not depend on the site of injection. This also results in an affected offspring, suggesting that the RNA silencing signals is transferred to the germ line (Timmons et al, 2003).
The induction of RNAi can be achieved in mammalian cells by the introduction of siRNAs, plasmid and viral vector systems that express ds short hairpin RNAs (shRNAs) that will be processed to siRNAs by the cellular machinery (Leung & Whittaker, 2005).
When it comes to in vivo applications, RNAi can also lead to activation of the interferon system, due to defense against viruses, leading to non-specific effects (Aigner, 2005). Non-specific responses are elicited in mammalian cells when using dsRNA molecules longer than 30nt, with RNAi. But siRNA duplexes, 21 nt long, are to short to trigger the non-specific dsRNA responses, but still effectively inhibits endogenous genes in a sequence-specific way (Yu et al, 2002).

The activation of the interferon system can in the end lead to inhibition of cellular protein synthesis and induction of apoptosis. But this applies mostly for long dsRNA molecules, and not so much the synthetic siRNAs, which seems to be too small for this purpose (Aigner, 2005).
RNAi in *C.elegans* and *D. melanogaster* can be accomplished by using long (>500bp) dsRNAs, and when it comes to worms like the first, dsRNA can be directly injected, giving them bacteria expressing dsRNA as "food" or by soaking the worms in a solution of dsRNA. On the other hand, it is not that easy in mammalian systems, since as indicated earlier, dsRNAs (>30bp) trigger the γ- interferon pathway, a part of the immune system.

Therefore, one has to use siRNAs, enzymatic cleavage or expression systems when it comes to mammals. These molecules avoid the immune response by mimicking Dicer products and entering the RNAi pathway further downstream. Moreover, the siRNA duplexes have to be transfected into mammalian cells using lipid-based formulations or electroporation for example (Leung & Whittaker, 2005).

SiRNAs may cross-react with targets of limited sequence similarity when there are regions of partial sequence identity between them, so called off-target effects (Aigner, 2005).

Non-specific effects are as mentioned earlier a generalized toxic cellular response, off-target effects on the other hand, occurs when one or a few genes that are not specifically targeted for siRNA pools show loss of gene function. The siRNA sense strand may cause a loss of function from an unrelated gene, or it can be the secondary effect caused of the antisense strand that could knockdown the expression of another related gene where it has the same sequence (Invitrogen).

Several studies implies that members of the AGO protein family (Argonautes) are key components of RISC (see figure 3), and in *C.elegans* is the AGO protein RDE-1 required for gene silencing in response to experimentally introduced dsRNA. AGO proteins have also been associated with this process in fungi, plants, protozoans and metazoans including humans (Yigit et al, 2006).

Both siRNAs and miRNAs are loaded onto an argonaute containing RISC. The mammalian Argonaute protein families comprised of eight members, four is expressed ubiquitously and four is expressed only in the germ line (Schmitter et al, 2006). *Drosophila* has five, *Arabidopsis* ten and *C.elegans* 27 argonaute protein families. The argonaute protein is involved in the endonucleolytic cleavage of the target mRNA, and is the core enzyme in RISC (Steiner & Plasterk, 2006).
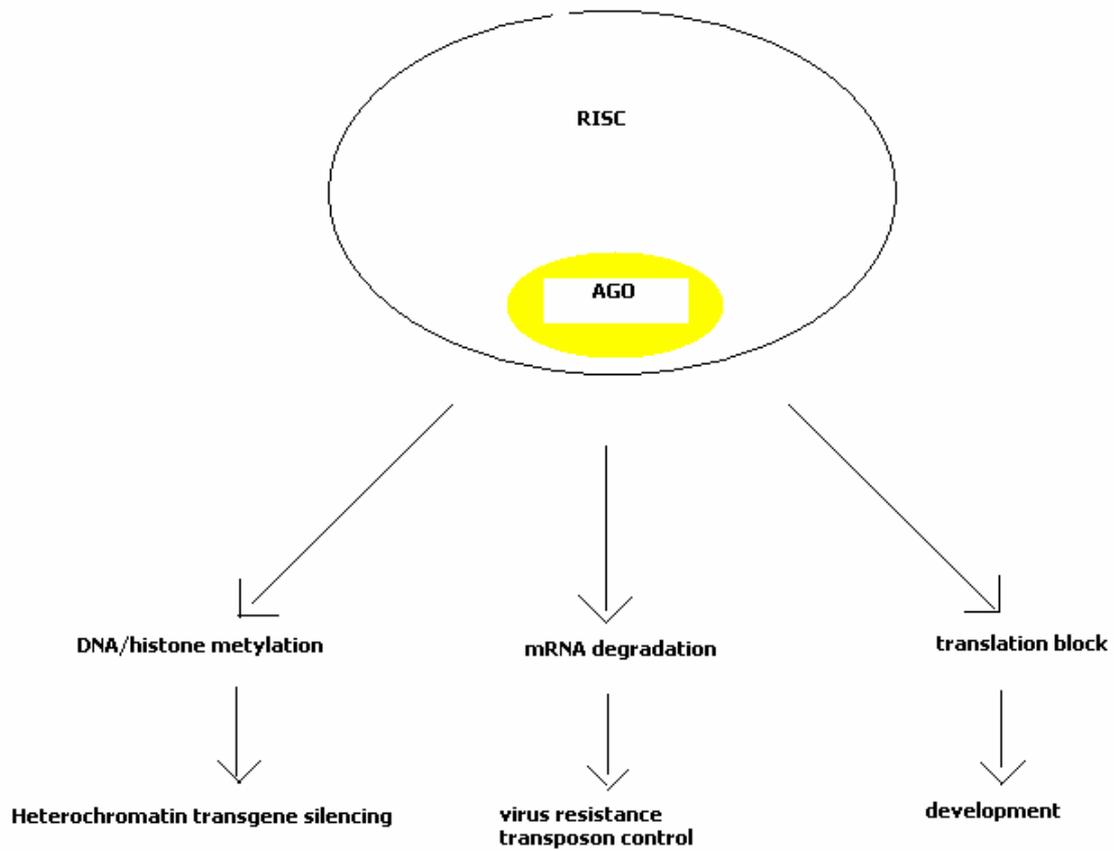
Figure 3. The AGO protein, an important part of RISC (RNA-induced Silencing Complex). RISC is a multi-protein complex that catalyzes the cleavage of complementary mRNA, when bound to a siRNA.

Applications

RNAi technology seems promising, when it comes to quick analyzes of functions of a number of genes in many different organisms, see figure 4. In *C.elegans* have chromosomes I and III been screened by RNAi to identify the genes involved in cell division and embryonic development. Gene knockdown-related functional studies in plants are being performed efficiently with transgenes in the form of RNAi constructs. For example, RNAi constructs targeted against the toxin biosynthesis genes can remove plant endotoxins. Because of the high gene-specificity, it is most likely that RNAi will play an important role in therapeutic applications, as for example down regulate the expression pattern of mutant genes in diseased cells and drug validation. It has also been possible to silence the mutant allele of a diseased gene but not affect the wild-type allele of the same gene. SiRNAs have been shown to inhibit infection by HIV, polio hepatitis C virus in cultured cell lines. Treatment with siRNA has also been shown to cause apoptosis in different cancer cell lines, such as leukemia and lymphoma and seems to have a good potential in other cancer forms to (Agrawal et al, 2003).
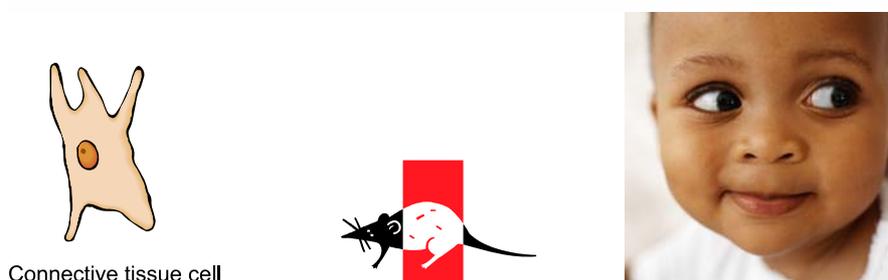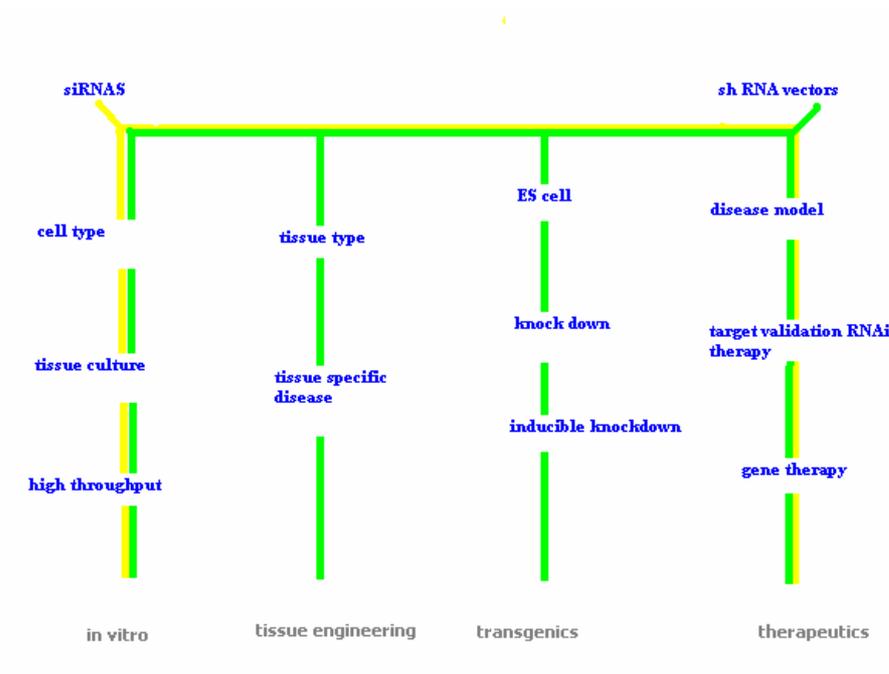
Figure 4. RNAi and gene therapy. RNAi can be used in more than one way.

Optimizing protocol for RNAi

In the present study, a mouse model of mammary epithelial HC11 cells was used to optimize an RNAi protocol.
The HC11 cells are proliferating upon confluence, and can be readily differentiated by adding prolactin to the medium. This gives the opportunity to study the uptake and secretion of different compounds in the cells and into milk, by interfering with known transporters such as DMT1, PGP and BCRP. In this case we chose to examine BCRP, but first we made an effort to silence the house-keeping gene Cyclophilin B, which is a non-essential, abundantly expressed house-keeping gene in human, mouse and rat cells (Dharmacon).

HC11 cells

The HC11 cells are descended from the COMMA-1D mouse mammary epithelial cell line, and exhibits several properties specific for normal mammary gland function (Danielson et al, 1984). This cell-line was tested for the ability of induction of the β-casein gene by prolactin, and a number of clones were isolated, and the one who had a 4-fold higher response to prolactin than the parental COMMA-1D cell line was chosen, clone HC11 (Ball et al, 1988). The HC11 cells can be rapidly induced in vitro by prolactin, to produce β-casein as well as other cellular proteins, and does not require an extra-cellular matrix or co-cultivation with other cell types (Ball et al, 1988).

Cyclophilin B

To optimize a protocol for RNAi, transfection efficiency has to be tested, as well as cell viability during transfection. To achieve this, we used an RNA-based reagent called sicontrol tox that is cytotoxic only when transfected into cells, and causes cell-death that can be correlated with transfection efficiency. For cell viability, a negative control consisting of a pool of non-targeting siRNAs was used, to distinguish between sequence specific silencing and non-specific effects. Both transfection efficiency and viability was measured with ATP determination.
Following optimization of transfection efficiency, RNAi was performed on Cyclophilin B in the HC11 cells. Cyclophilin B is abundantly expressed in all mammalian cells and it has previously been demonstrated that RNAi on Cyclophilin B in various cell lines does not cause cytotoxic effects (Dharmacon).

BCRP

After successful transfection of siRNAs for Cyclophilin B, the same protocol was performed on BCRP that is expressed in mammary epithelial cells, implicated in the transport of a variety of toxic compounds into milk.

BCRP, the breast cancer resistance protein, also called ABCG2 is a member of the ATP-binding cassette family of drug transporters, which actively export many drugs and toxins from cells. BCRP transport various anticancer drugs such as topotecan, mitoxantrone and doxorubicin, and this can cause multidrug resistance in cancer cells. On the other hand, the absence of BCRP in mice has shown to give sensitivity to for example the dietary chlorophyll catabolite pheophorbide A that made the mice extremely photosensitive. In addition, the mice also displayed a type of metabolic disorder also associated with photosensitivity, called protoporphyria. BCRP is physiologically important in processes involving porphyrins, and a deficiency for BCRP may contribute to several porphyrin-related photo toxicities in humans and animals (Jonker et al, 2002).

Overexpression of BCRP leads to reduced intracellular drug concentration and decreased toxicity, and thereby takes part in causing multidrug resistance (MDR) in tumor cells, interfering with chemotherapy. To circumvent this problem, inhibitors of BCRP transport can be used, for example fumitremorgin C, novobiocin and GF120918. Another strategy that came up quite recently is siRNAs targeted against the BCRP gene, knocking out the gene with RNA interference. Ee et al (2004) showed that siRNAs effectively down-regulated endogenous BCRP in BeWo cells with changes in the MDR phenotype, decreasing mRNA and protein levels of the gene in question. In this way the BeWo cells were also sensitized to BCRP substrates such as mitoxantrone and topotecan (Ee et al, 2004).

Aim

The purpose of the study was to optimize a protocol for RNAi in HC11-cells.
The products that were used to perform RNAi included sicontrol tox, negative control, positive control and a kit of four different lipids for transfection, 1, 2, 3 and 4(Dharmacon). Sicontrol tox and sicontrol non-targeting siRNA pool were used to optimize transfection. As a positive control, On-target plus sicontrol Cyclophilin B pool for mouse was used. Cyclophilin B is a non-essential, abundantly expressed house-keeping gene in human, mouse and rat cells. This control was used to confirm efficiency of siRNA delivery into mouse cells by measuring silencing of the target gene. Four different lipids (Dharmafect 1-4, Dharmacon) were used for transfection, and each one of the lipids was tested in different concentrations to evaluate their transfection efficiency.
After optimization of protocol for RNAi on Cyclophilin B, the purpose was to use the same protocol to silence BCRP, to perform a functional uptake-study with the BCRP-substrate mitoxantrone.
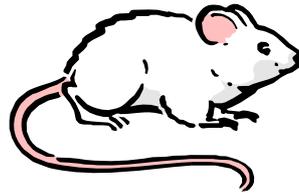
Strategy of the exam-project

Optimization of RNAi: Transfection efficiency control (sicontrol tox) and negative control (sicontrol non-targeting siRNA pool) will be used to see if viability is influenced by non-specific effects. As a positive control, Cyclophilin B from mouse will be used, for silencing efficiency control. Cell viability/transfection efficiency after RNAi treatment as well as untreated cells will be tested with ATP Determination. Cells will be cultured in 96-wellplates.
RNAi: HC11-cells will be transfected in 6-wellplates according to results from optimization.
After 24- 48h incubation, RNA will be isolated with Nucleospin-columns.
Quantification of RNA with Ribogreen to get equal amounts of RNA for PCR.
Primer design of mouse-specific cyclophilin B to see that it works, for PCR.
Use real time PCR with existing cDNA from mouse mammary tissue
Estimate the size of the PCR-product by electrophoresis, and sequence it to compare to the existing sequence in Genbank (NCBI).
Cyclophilin B gene expression in transfected HC11-cells and untreated cells will be quantified with Real time PCR.
The same protocol as for Cyclophilin B can then be used for BCRP to perform a functional uptake-study with the BCRP substrate mitoxantrone.

Material and Methods

Cell culture

The HC11-cells, frozen at -70 ° C, has to be thawed as quickly as possible (in the hand) and after centrifugation, discarding of supernatant containing DMSO, the cells are re-suspended in fresh medium and transferred to a T25 culture bottle.

Medium 1L:

- DDH$_2$O 800 ml
- RPMI 1640 8g (Gibco, Invitrogen, Carlsbad, USA)
- Gentamicin (50mg/L) 1ml (Gibco, Invitrogen, Carlsbad, USA)
- 7,5 % sodium bicarbonate 29,3 ml
- Bovine insulin (10 mg/ml) 250 µL (Sigma-Aldrich, St Louis,USA)
- EGF (10 µg/L) 1ml
- Set the pH to 7,3-7,4 with HCL or NaOH
- Add DDH$_2$O up to 900 ml
- Add 100 ml thawed foetal bovine serum (Gibco, Invitrogen, Carlsbad, USA), heat-inactivated, 10%, during sterile filtration (0,2 µm pore size) of the medium to a sterile bottle, and keep in refrigerator.

The cells were incubated in 37 ° C, 5 % CO$_2$, and at 80-90 % confluency, they were ready to be passaged.

Protocol for passage of HC11 cells:

- Remove medium, and wash with 10 ml of PBS
- Remove the PBS and add 3-5 ml of cell dissociation solution, incubate in 37 ° C, 5 % CO$_2$ for 10-15 minutes
- Rinse with 10-20 ml medium, pipett up and down over 10 times
- Transfer all of it to a falcon tube and centrifuge it in a GS-GR centrifuge (Beckman Coulter, Fullerton, USA), 1500 rpm (450 G), 4 ° C for 5 minutes
- Remove the supernatant carefully and resolve the pellet thoroughly in a suitable amount of medium, pipetting up and down, and take an appropriate amount (for example 1/10) and add to a new T25 culture bottle, pre-filled with 25 ml medium. Incubate in 37 ° C, 5 % CO$_2$.

Medium was changed or refilled every 2-3 days and the cells were passaged once or twice a week.

Primer design

With the help of the accession number for Cyclophilin B in mouse (NM_011149), the cDNA sequence could be found, and checked in UCSC Genome Browser, BLAT to compare mRNA and the genome, looking for the position of introns (http://genome.ucsc.edu/). Then the primers were created at Primer_3, with a product range of minimum 100bp, maximum 300bp, and optimum 150bp. The melting point were also specified, minimum 59,5 ° C, maximum 60,5 ° C and optimum 60,0 ° C. Primer sequences: forward GCGCAATATGAAGGTGCTCT Reverse GAAGTCTCCACCCTGGATCA (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Then the PCR-product was checked at UCSC to see that the primers give the right product (http://genome.ucsc.edu/cgi-bin/hgPcr?command=start). The primers were then ordered from CyberGene AB (http://www.cybergene.se/). The ordered primers were then put to the test by using them in real time PCR with cDNA from mouse mammary tissue, estimate the size of the PCR-product by electrophoresis, and finally send it to KiSeq* for sequencing (http://www.cgb.ki.se/cgb/kiseq/). The purified mRNA from mouse mammary tissue was converted to cDNA with Reverse Transcription Core kit from Eurogentec, Seraing, Belgium. The real time PCR was implemented with qPCR Mastermix SYBR Green I from Eurogentec. The PCR was performed with Rotor-Gene 3000.
The obtained sequence was as follows:
CCCCCNCTATCGTGGGCTCCGTCGTCTTCCTTTTGCTGCCCGGACCCTCCGTGGCC AACGATAAGAAAGAAGGGACCTAAAGTCACAGTCAAGGTATACTTTGATTTACA AATTGGAGATGAATCTGTAGGACGAGTCGTCTTTGGACTCTTTGAAAGACTGTTC CAAAAAACAGTGGATAATTTTGTAGCCTTAGCTACAGGAGAGAAAGGATTTTGGC TACAAAAAACAGCAAGTTCCATCGTGTCATCAAGGACTTCATGATCCNGGGNNNT CAAANNNNNNNNNNNNNN. The sequence was blasted on NCBI and at the highest score and the lowest E-value, the right accession number was found, NM_011149 (http://www.ncbi.nlm.nih.gov/).

* DNA sequence analysis at the Center for Genomics and Bioinformatics, Karolinska institutet.

RNA interference

To confirm transfection efficiency and cell viability, sicontrol tox and negative control was used, and the lipids 1, 2, 3 and 4 were tested in different dilutions to see which lipid and concentration that was best suited for transfection of the HC11 cells.
The conditions that gave rise to as low ATP as possible for sicontrol tox, and as high as possible when it comes to the negative control, was chosen to investigate RNAi for Cyclophilin B. In this case, six-well plates were used instead of 96-well plates (sicontrol tox & negative control) to get more RNA and thereby be able to measure the gene expression of Cyclophilin B.
RNA from the cells was isolated with the help of Nucleospin, and the RNA was quantified with Ribogreen to get exact concentration, to be able to do Real-time PCR, checking the down-regulation of Cyclophilin B. The same protocol was then used for BCRP as well. Finally, an uptake-study with mitoxantrone, and mitoxantrone labeled with ^{3}H as tracer, was performed on RNAi for BCRP treated HC11 cells.

Sicontrol tox, negative control and positive control were carried out according to protocol from Dharmacon, Inc. Colorado, USA. The transfection mix, consisting of mastermix, Opti-MEM and one of the four different lipids in varying concentrations, was incubated in room temperature for 30 minutes. Following incubation, antibiotic-free RPMI medium was added to the mix and 100 µl was added in each well in a 96-well plate, except for the outer wells. After 48 hours of incubation in 37 ° C, 5 % CO_2 the plate was treated with ATP Determination kit (see below) and scanned in a Wallac workstation 1420 VICTOR multilabel counter, "Victor"(Perkin Elmer, Wellesley, USA).

Protocol for transfection of HC11-cells

1. Wash the cell culture with sterile phosphate buffered saline (PBS) and then add 3-5ml cell dissociation solution and incubate in 37°C for 10-15 minutes to release the cells from the culture plate.
2. Add RPMI-medium and then transfer the cells to a sterile centrifuge tube (falcon tube), and save a small aliquot in an eppendorf tube, for cell counting with a hemacytometer. Pellet the cells by centrifugation, and resuspend them based on the cell count in antibiotic-free growth medium (RPMI without gentamycin).
3. Transfer 100 µL of the cell resuspension (10000 cells/well resp. 25000 cells/well) into 60 wells of the 96-well plate, leaving out the outer wells, which will only be filled with medium.
4. Incubate cells in a humidified incubator at 37°C with 5% CO_2 overnight.
5. Sicontrol tox. According to protocol on p. 89 (RNA Interference Technical Reference & Application Guide) with minor modifications as follows:
   60 wells, five wells/treatment. Prepare master mix (sicontrol tox /negative control) and combine with Opti-MEM. Prepare two-fold dilutions of your lipid reagent, 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64, see figure B2.The transfection mixes will be prepared as follows:
   - Only Opti-MEM 200 µL
   - Opti-MEM + Mastermix 100+ 100 µL
   - Opti-MEM 90 µL, Mastermix 100 µL, Lipid 10 µL

   In the first row in the 96 well plate, only Opti-MEM will be added, in the second row half Opti-MEM and half master mix (sicontrol tox/negative control) will be added. In the following rows, OptiMEM, master mix and four different dilutions of lipid will be added, beginning with 1:8, then 1:16, 1:32 and finally 1:64. See table B3. (Other dilutions series were used also, for example 1:4 and 1:8 for all four lipids.)
   Incubate for 30 minutes at room temperature, and then add 450 µL antibiotic-free culture medium to each tube, to prepare transfection mixes. Mix gently. Remove the culture medium from each of the 60 wells of the culture plate and add transfection mix as described above. Incubate cells in a humidified incubator at 37°C with 5% CO_2 for 48 hours. For Cyclophilin B, 6-well plates was used, total amount per well 2 ml. Three times more of everything in the transfection mix was added, per well: 600 µL Opti-MEM, Opti-MEM + Mastermix 300+ 300 µL and Opti-MEM 270 µL, Mastermix 300 µL, Lipid 30 µL. Antibiotic-free medium was added after incubation up to 2 ml.

ATP Determination

This kit from Molecular Probes, Invitrogen makes it possible for quantitative determination of ATP, as a bioluminescence assay with recombinant firefly luciferase and its substrate D-luciferin. Luciferase requires ATP to produce light, as shown in the following reaction (in the presence of luciferase and Mg^{2+}):

$$luciferin + ATP + O_2 \rightarrow oxyluciferin + AMP + pyrophosphate + CO_2 + light$$
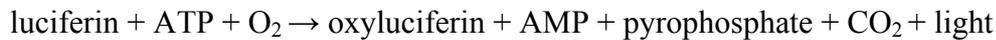
The kit consists of five different components:
- D-luciferin, 5x3mg of lyophilized powder
- Luciferase, firefly recombinant, 40 µL of a 5mg/mL solution in 25 mM Tris-acetate, pH 7,8, 0,2 ammonium sulfate, 15% glycerol and 30% ethylene glycol
- Dithiothreitol (DTT), 25 mg
- Adenosine 5´-triphosphate (ATP), 400 µL of a 5mM solution in TE buffer
- 20X Reaction Buffer, 10 mL of 500 mM Tricine buffer, pH 7,8, 100 mM $MgSO_4$, 2mM EDTA and 2mM sodium azide

To perform the experiment, a standard reaction solution has to be made (10mL), as follows:
- 8,9 mL dH_2O
- 0,5 mL 20X Reaction Buffer
- 0,1 mL 0,1 M DTT
- 0,5 mL of 10 mM D-luciferin
- 2,5 µL of firefly luciferase 5mg/ml stock solution

We used 96-wells plates with 2500, 5000, 10000 and/or 25000cells/well. The cell medium was removed and 100 µL of the standard reaction solution was added per well, plus 10 µL of TE- buffer. The solution and the plate have to be protected from light. The bioluminescence then was measured in Victor. With ATP determination it is possible measure the cells viability, in controls the ATP levels are 100 %. Transfected cells have a lower ATP level than the untransfected cells, for example 21 % of the control. Since the transfection efficiency (sicontrol tox) measures the amount of transfected and therefore also dead cells, the transfection efficiency is in this case 79 %. See table 2.

Picogreen

The reagent in Picogreen displays a large fluorescence enhancement after binding to dsDNA, and this enhancement is much less significant when bound to RNA or ssDNA. In this way, it is possible to quantify dsDNA. After 2-5 minutes of incubation of the samples, the fluorescence enhancement is measured in Victor, and the results can be compared with a standard curve.
Picogreen dsDNA Quantitation Kit from Molecular probes; Invitrogen, USA, was used in this assay, and was carried out according to their protocol with minor modifications: The standards that were used were 2,5 , 25 and 250 ng/ml.

RNA purification

RNA from the transfected/untreated HC11 cells was purified with the Nucleospin RNA II purification kit from BD Biosciences, according to their protocol.

Ribogreen

The Ribogreen RNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating RNA. Although it is insensitive to proteins and salts for instance, it is sensitive to both RNA and DNA. This problem is circumvented by treating the samples with DNAse.
The experiment was applied according to the experimental protocol from Molecular probes, Invitrogen, high-range assay. DNAse-treatment was not needed, since the RNA was treated with DNAse during purification with nucleospin before.
Eight RNA standards were used, three of each, in the following concentrations: 0, 20, 50, 100, 250, 500, 750 and 1000 ng/ml.
To each well in 96-wellplate (not all wells were used), 20 µL TE-buffer pH 8 was added, and 20 µL sample or standard. 160 µL Ribogreen reagent were added to each well, and then the plate was incubated in 37 ° C for 30 minutes, protected from light. Finally the absorbance was measured in Victor, the Pico/Ribogreen protocol.

PCR

RT-PCR (Reverse transcriptase PCR) is used to synthesize cDNA from purified mRNA with the help of the enzyme reverse transcriptase.
Real time PCR, or quantitative PCR is a method used to measure the amount of mRNA in for example a group of cells. The amount is measured in relation to a universal reference transcript that exists in many different cell types, a so called housekeeping gene. In this case, we used Cyclophilin B. The template of interest is amplified during the PCR and a fluorescent molecule binds specifically to DNA and emits light of a specific wavelength that can be detected and so the amount of DNA can be determined (Wikipedia).
To see if Cyclophilin B gene expression was downregulated in the different samples, Real time RT-PCR was used (QuantiTect SYBR Green RT-PCR, one step, from Qiagen). The advantage with this kind of PCR-kit is that reverse transcription of the RNA to cDNA, and the real time PCR for quantification is done at the same time (and in the same tube). The real-time cycler used in this experiment was Rotor-gene 3000.
The mastermix was prepared as follows (per reaction):
- 2X QuantiTect SYBR Green 12,5 µL
- Forward primer 2 µL
- Reverse primer 2 µL
- QuantiTect RT mix 0,25 µL
- DEPC (RNase free water) 3,25 µL

Template and DEPC was mixed according to the calculated amount of RNA in the different samples, see results for Ribogreen, and consisted of 500 ng of RNA. The final volume was 5 µL in all samples, plus 20 µL mastermix = 25 µL in each tube. The standards that were used were in the range between 10^{-4} and 10^{-8}. Two blanks were used, two standards of each concentration and four of each of the samples.

Uptake-study of mitoxantrone in HC11 cells

The cells were cultured in a 12 well-plate, and four wells were treated with RNAi for BCRP. After completed RNAi treatment (48h), the cells were cultured in ordinary cell culture medium for an additional three days and there after washed with 37 ° C HBSS (Hank´s Balanced Salt Solution) containing 10mM HEPES (N-(2-Hydroxyethyl) piperazine-N´-(2-ethanesulfonic acid) hemisodium salt) 2x2ml/well. The HBSS was discarded and 2ml fresh 37 ° C HBSS was added to controls and RNAi-treated cells, and HBSS containing 1μM GF120918 (inhibitor) were added to the remaining four control wells.
Following 30 minutes of pre-incubation in 37 ° C, the HBSS was discarded and fresh HBSS containing 5 μM Mitoxantrone, radiolabeled with ^{3}H as tracer, was added to the controls and RNAi treated wells. To the remaining four wells, the same HBSS was also added, but it also contained 1 μM of the BCRP inhibitor GF120918. After 1 hour of incubation at 37 ° C, the cells were dissolved in 1 ml NaOH per well.10 ml Hionic Fluor (liquid scintillation solution) was added to aliquots of the cell lysates. The following day, all the samples were measured in a liquid scintillation analyzer (1900CA TRI-CARB from PACKARD), with a blank containing only NaOH, and controls from the working solutions of HBSS.
An aliquot from each sample was saved to do a protein determination, with BCA (Bicinchoninic acid solution) and Copper (II) sulfate pentahydrate, 19,6 ml BCA to 400 μl $CuSO_4$. This was performed in 96-well plate with only RIPA-buffer as blank, and with protein standards 0,025 , 0,05 , 0,1 , 0,2 , 0,3 , 0,4 , 0,5 and 1,0 mg/ml. 10 μl of sample or standard was added to each well, together with 200 μl of prepared BCA. This was measured on VICTOR.

Results
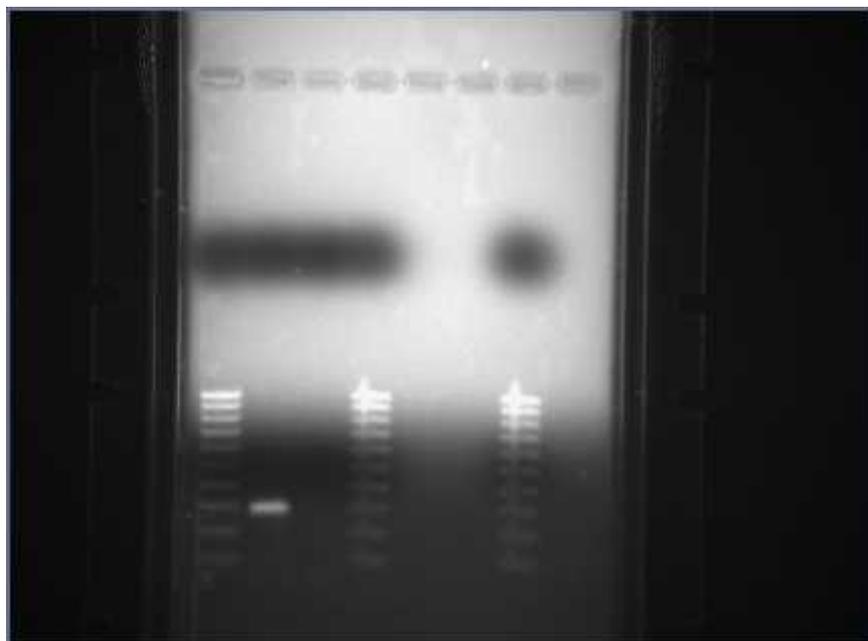
Figure 5. Primer-control of Cyclophilin B. We used real time PCR and the melt curve analysis gave one melting point, and to check the length of the product, we used electrophoresis. The result is shown above with one band next to the ladder to the left, 304 bp in length. Blank PCR product gave nothing. The PCR product was purified and then sent away for sequencing. More information in material and methods, Primer design.

The ATP determination kit was tested in the following way: The cells was sown out in a 96-well plate, and incubated in 37 ° C, 5% CO_2 for 24 hours. Five wells were used as control, with untreated cells, and five wells with cells were treated with formalin for a few minutes. With the help of the ATP determination kit, fluorescence was measured in Victor and the results was clear:

Table 1. ATP determination test.

Sample	Fluorescence, mean value (counts per second) and std
Untreated cells	11277 ± 4569
Formalin treated cells	505 ± 133
Background (empty wells)	94

These results shows that in the formalin treated cells, only 4,5 % fluorescence from ATP is measured, compared to the untreated cells, and demonstrates that ATP-determination can be used to check viability of the HC11- cells in the optimization of transfection efficiency as described in Materials and Methods.

Table 2. RNAi (sicontrol tox) treated cells, measured with ATP Determination kit in Victor. ATP-levels in controls are 100 %, therefore transfection efficiency is calculated as 100%- % of control value. For example lipid 1 diluted 1:2, 2500 cells/well, see table below. Opti-MEM is the control, and the ATP value from our sample is compared to the control, in percent. The ATP level is in this case 21 % of control value; therefore transfection efficiency is 79 %.

Sample (treatment)	% transfection efficiency 2500 cells/well (mean value of luminescence)	% transfection efficiency 5000 cells/well (mean value of luminescence)
opti-MEM	0	0
opti-MEM + Mastermix 1:1	-	-
Lipid 1 diluted 1:2	79	76
Lipid 1 diluted 1:4	69	76
Lipid 1 diluted 1:8	61	75
Lipid 1 diluted 1:16	58	59

Table 2 and figure 6 show that, when using the Opti-MEM treated cells as a control, Lipid 1 dilution 1:2 and 1:4 acquires the highest transfection efficiency of these four different dilutions.
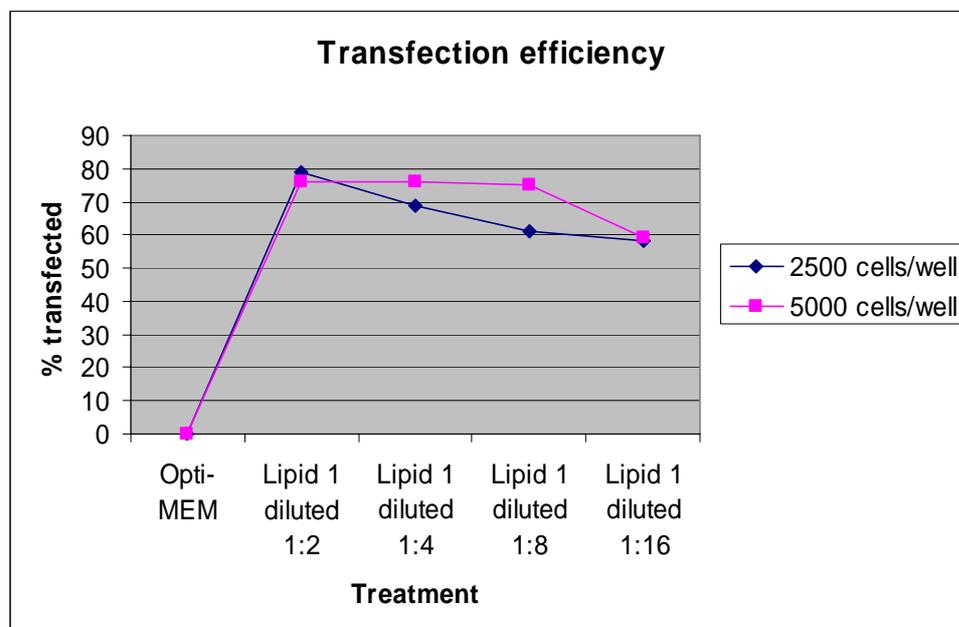
Figure 6. RNAi (sicontrol tox) treated cells, measured with ATP Determination kit in Victor.

Table 3. RNAi (negative control) treated cells, measured with ATP Determination kit in Victor.

Sample (treatment)	% viability 2500 cells/well (mean value of luminescence)	% viability 5000 cells/well (mean value of luminescence)
opti-MEM	100	100
opti-MEM + Mastermix 1:1	80	78
Lipid 1 diluted 1:2	15	24
Lipid 1 diluted 1:4	69	40
Lipid 1 diluted 1:8	69	55
Lipid 1 diluted 1:16	83	50

In table 3 and figure 7, it seems like that although dilution 1:2 had the highest transfection efficiency; it is also quite toxic for the cells. Dilution 1:4 on the other hand does not seem to be that toxic, at least in 2500 cells/well.
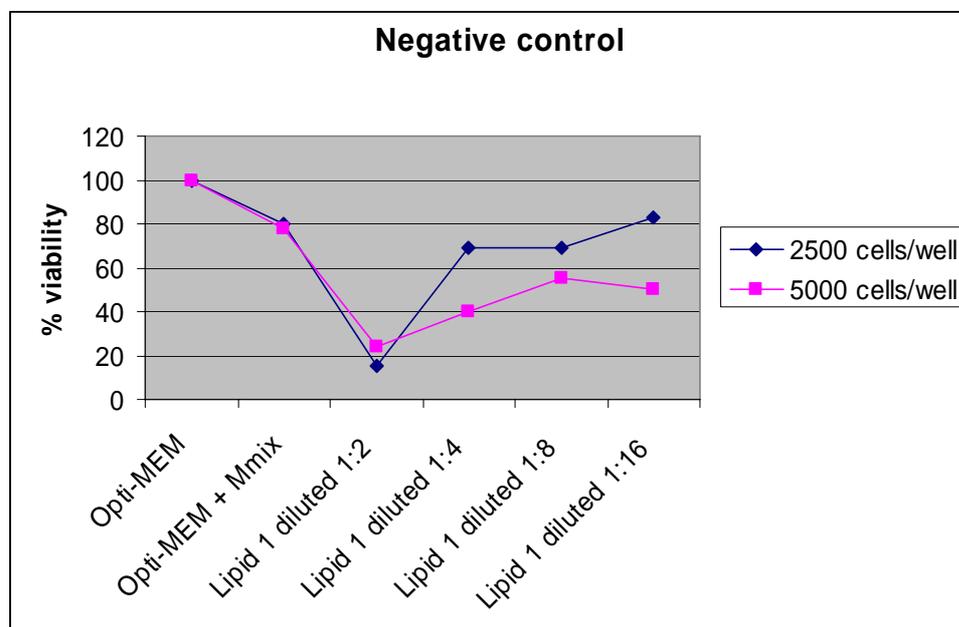
Figure 7. RNAi (negative control) treated cells, measured with ATP Determination kit in Victor.
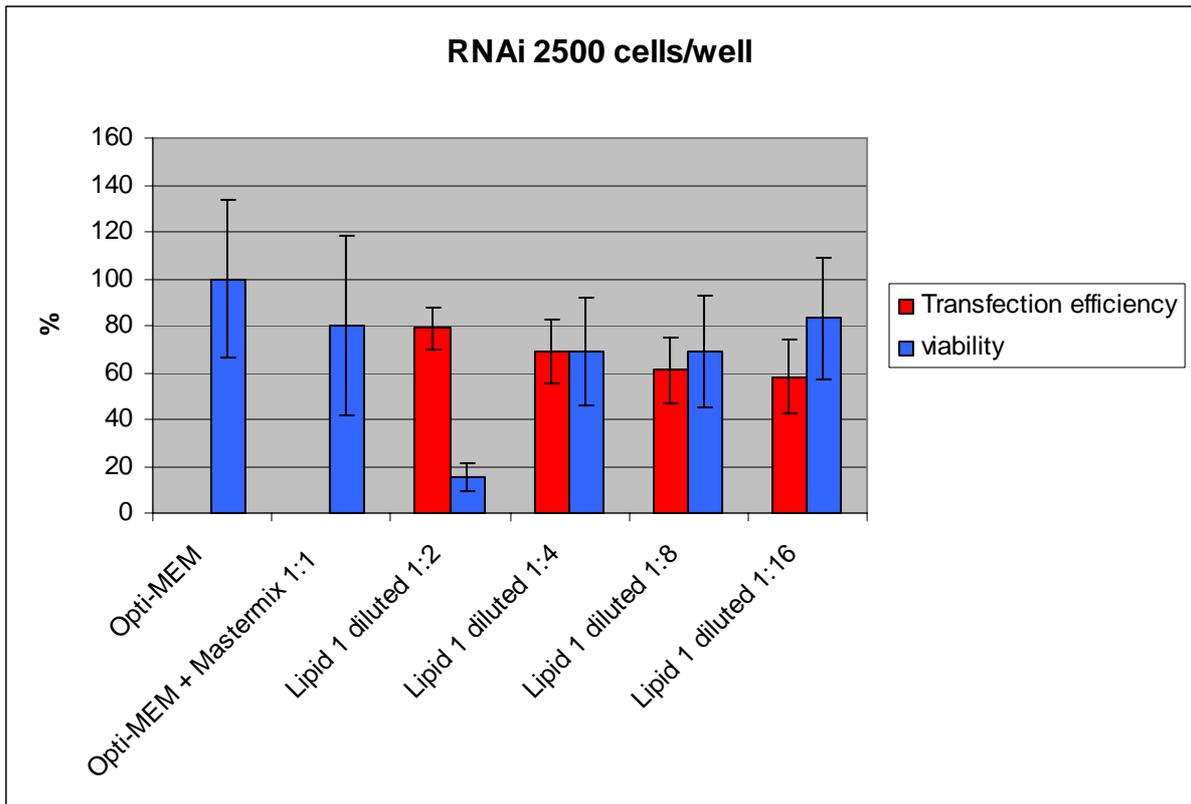
Figure 8. Comparison between sicontrol tox and negative control. When using sicontrol tox, low ATP-values are desirable, for negative control it is the opposite, high values are desired.
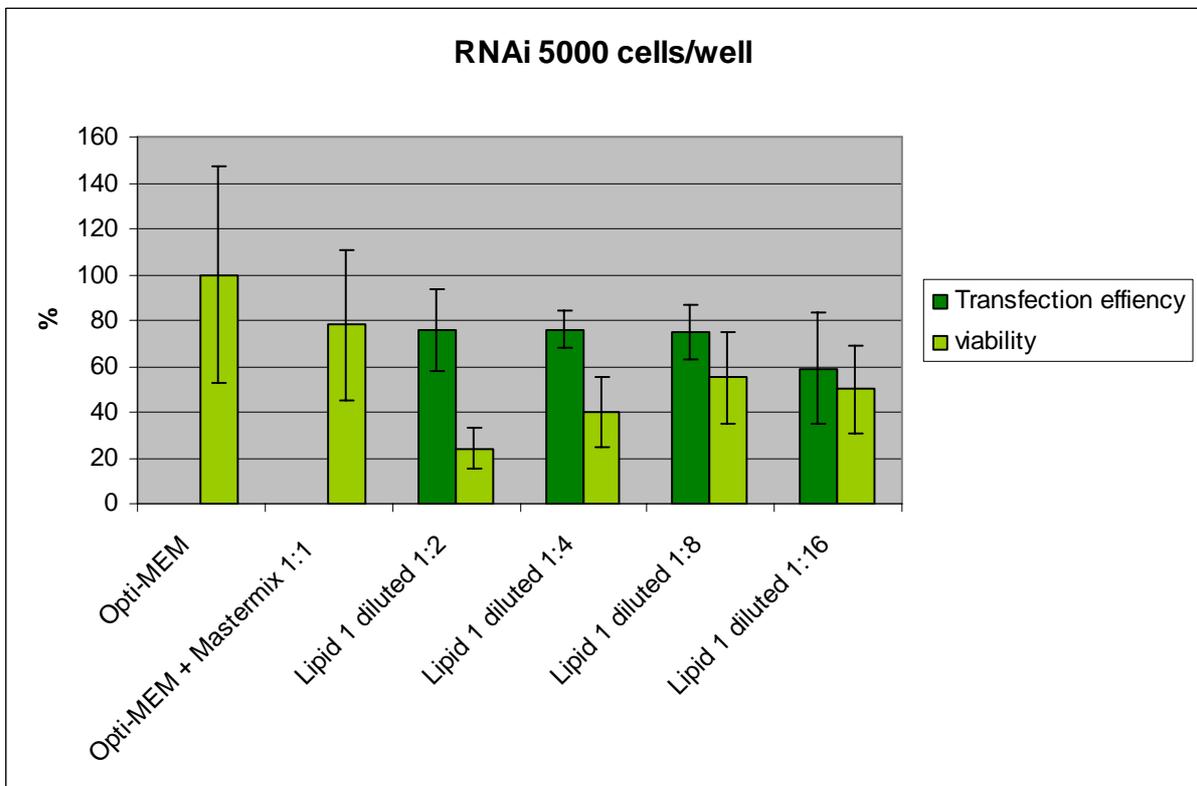

Figure 9. Comparison between sicontrol tox and negative control.

Table 4. RNAi, mean value of luminescence, measured in Victor. Lipid dilution 1:4. 2500 cells/well.

Sample (treatment)	sicontrol tox % transfection efficiency	negative control % viability
Opti-MEM	0	100
Opti-MEM + Mastermix 1:1	46	76
Lipid 1	74	57
Lipid 2	78	47
Lipid 3	66	59
Lipid 4	85	32

Lipid 2 shows the highest transfection efficiency, but lipid 1 is almost as high, and has a less toxic effect on the cells. Lipid 4 has high transfection efficiency, but is also very toxic. See table 3 and diagram 3.
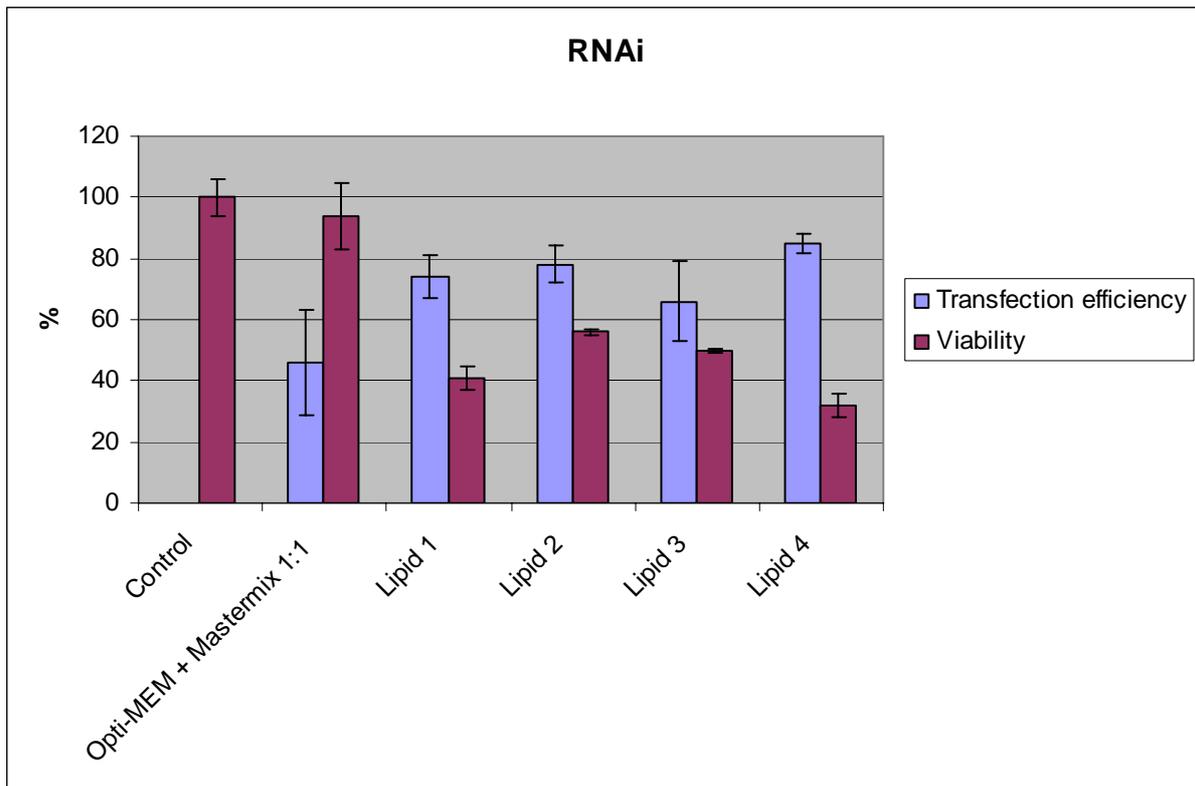
Figure 10. RNAi, mean value of luminescence, measured in Victor. Lipid dilution 1:4. 2500 cells/well.

In figure 10, lipid 4 shows the highest transfection effiency, but is also most toxic for the cells. Lipid 3 displays the highest viability, but instead the lowest transfection efficiency. Lipid 1 and 2 seems to be best choice for transfection in HC11 cells, according to this result.

Table 5. Results of RT-PCR for Cyclophilin B 061221

Sample	% expression
opti-MEM	100
opti-MEM + Mastermix	83,3
Lipid 1 diluted 1:4	11,8
Lipid 1 diluted 1:8	68,5

Table 5 and diagram 11 show a strong down regulation of gene expression for Cyclophilin B, in the 1:4 dilution of lipid 1. Dilution 1:8 does not seem to have much effect.
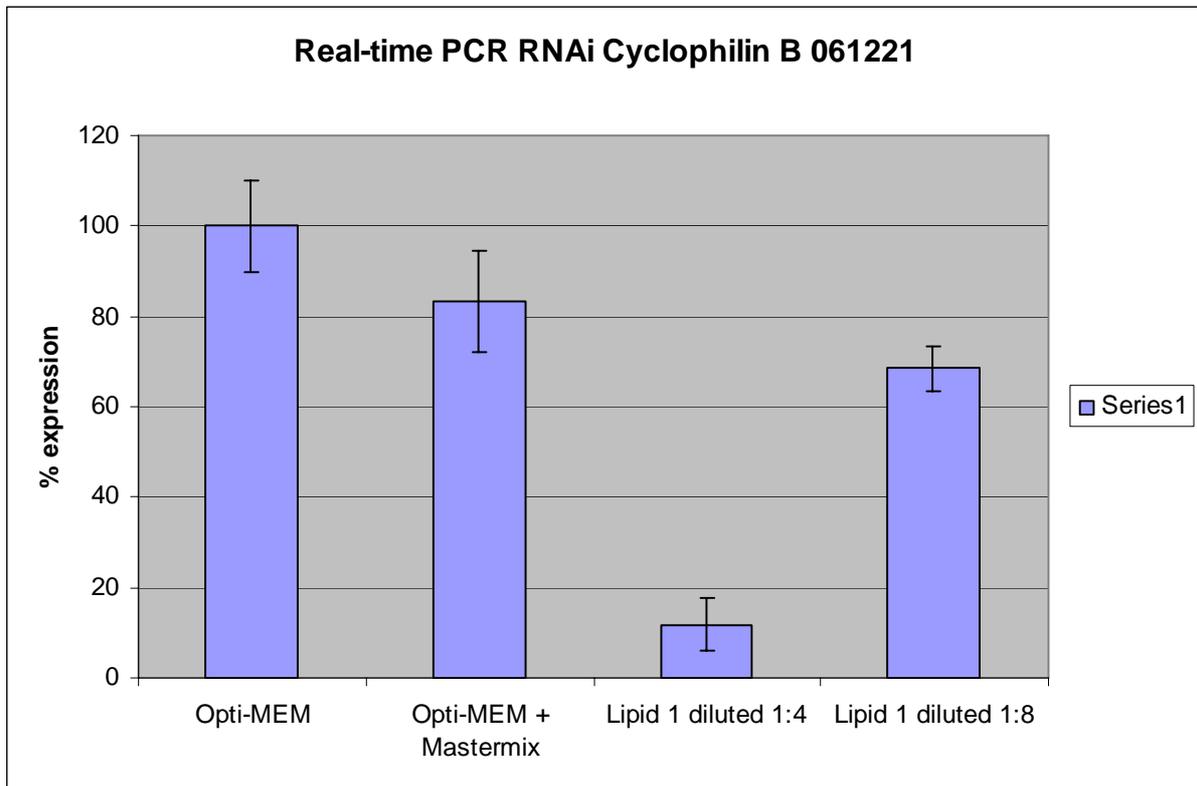

Figure 11. Lipid 1 diluted 1:4 shows the highest down-regulation, compared to the dilution 1:8.
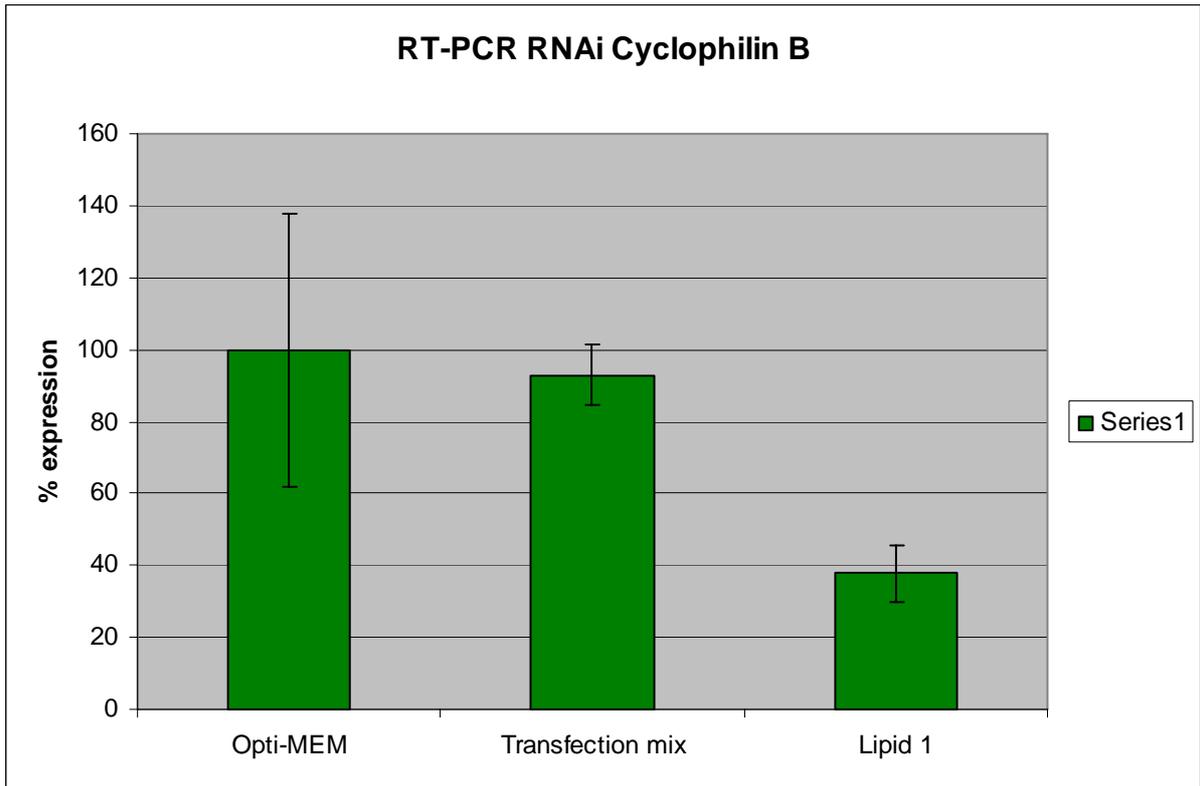
Figure 12. Transfection with lipid 1, diluted 1:4 reduces expression of Cyclophilin B.
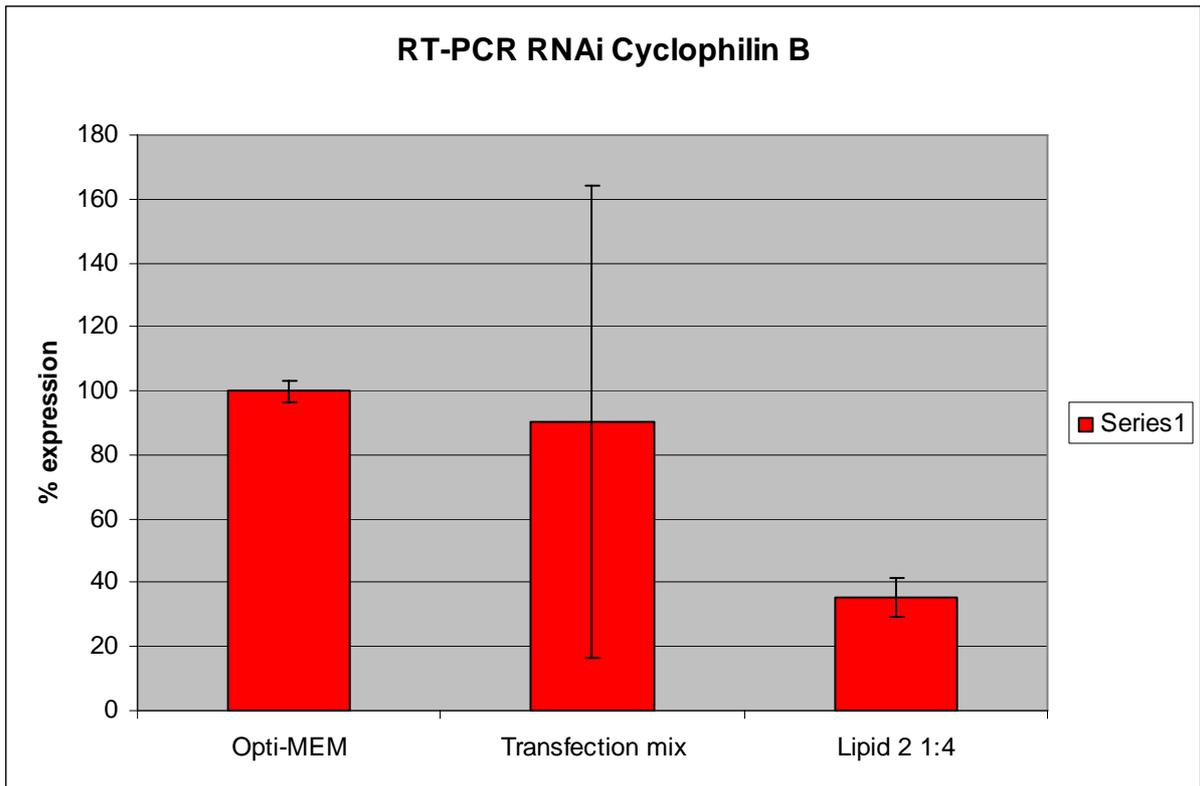
Figure13. The expression of Cyclophilin B is reduced with lipid 2.

Lipid 1 and 2 displays similar transfection efficiency in diagram 12-13.

RT-PCR RNAi Cyclophilin B 070320
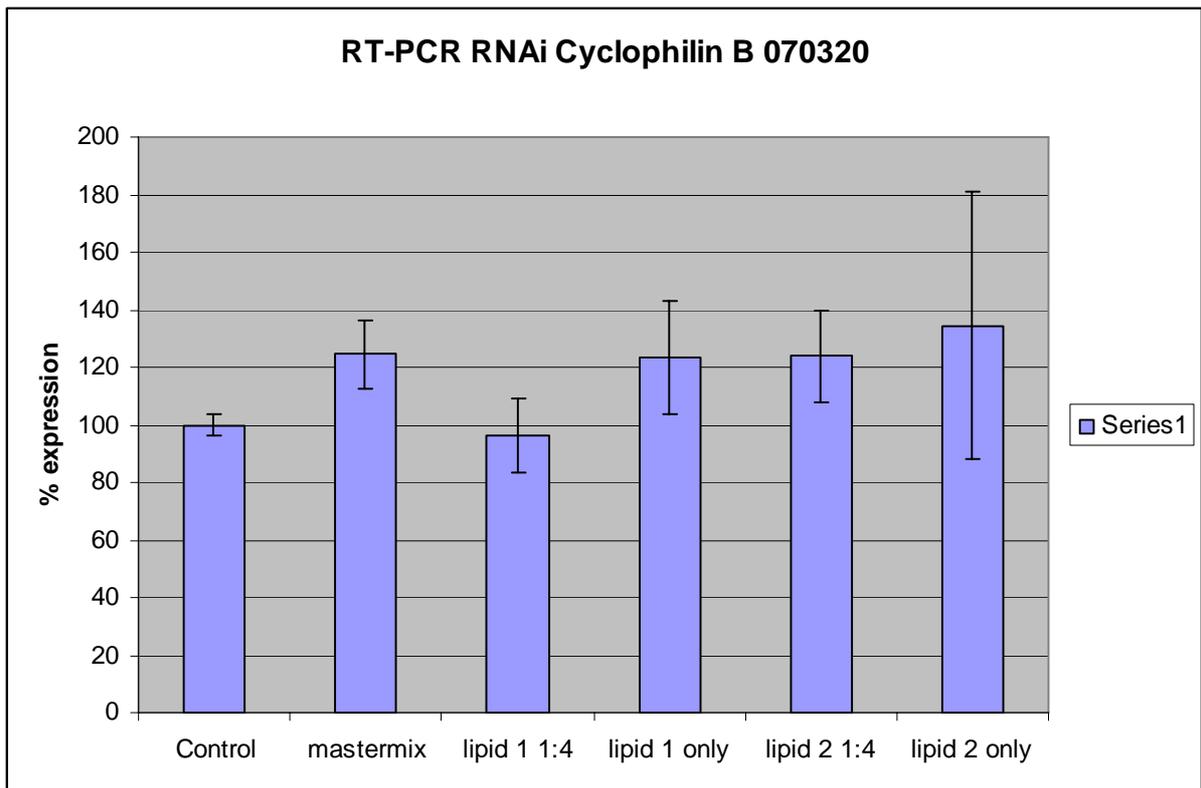
Figure 14. To try to verify previous results, the procedure was repeated but did not succeed. It shows no down-regulation of the targeted gene.

In figure 14, there was no down-regulation at all of Cyclophilin B.
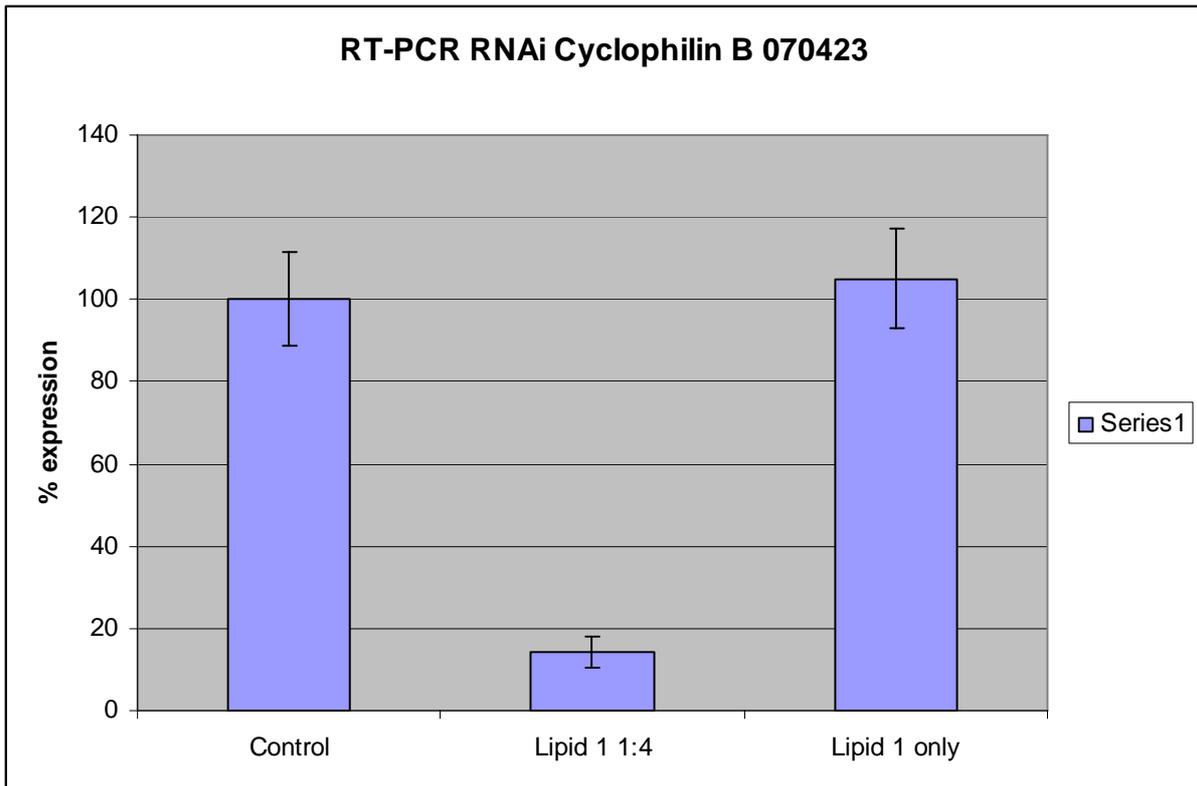
Figure 15. Since we suspected that the lipid was not good in the previous experiment (070320), it was repeated with new lipid (lipid 1 diluted 1:4), and this time it worked out.
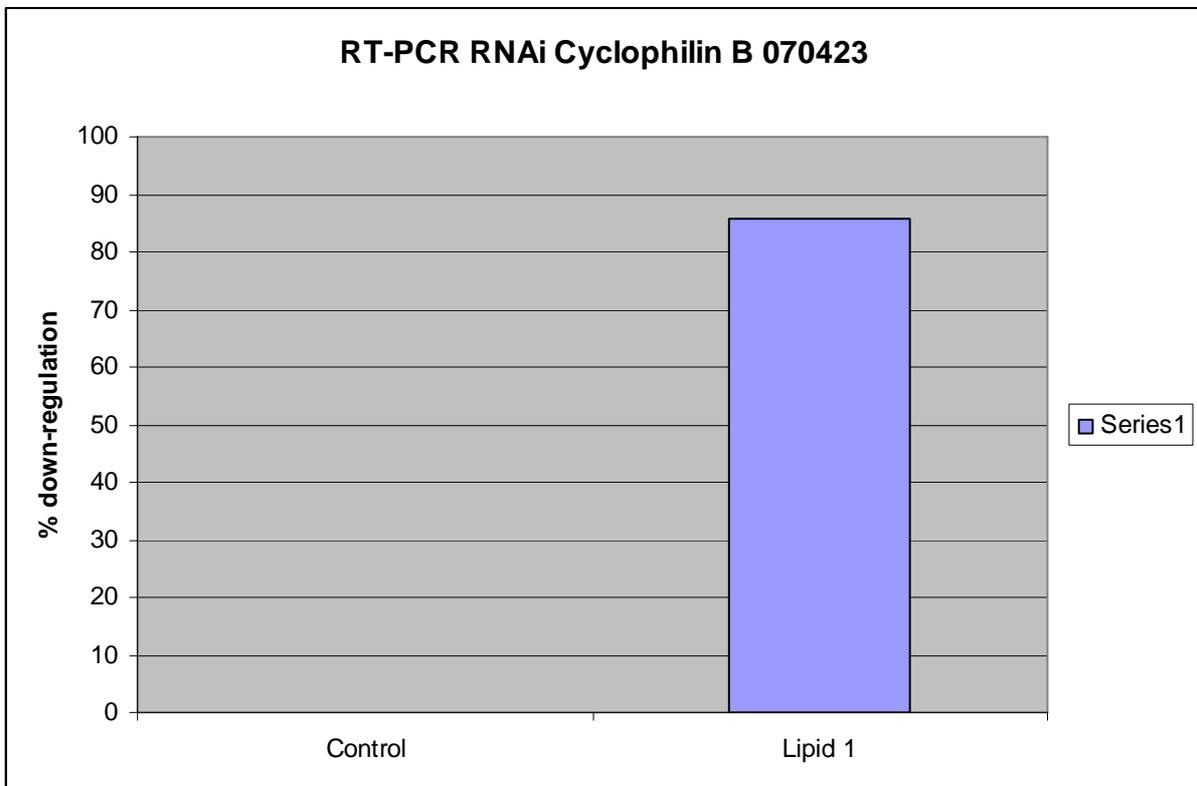

Figure 16. Shows the down-regulation of Cyclophilin B.
Figure 10 and 11 shows a significant down-regulation of expression of Cyclophilin B, almost 90 %.
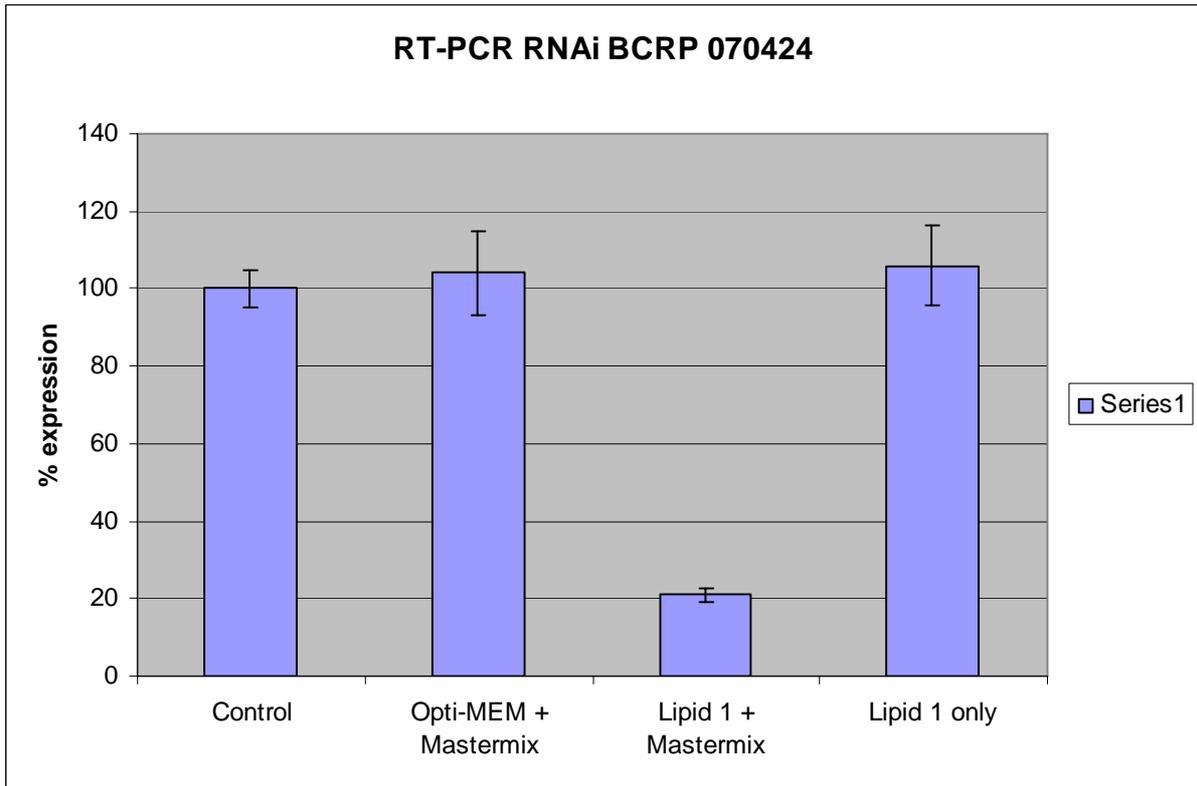
Figure 17. The expression of BCRP is clearly reduced after transfection with lipid 1, dilution 1:4.
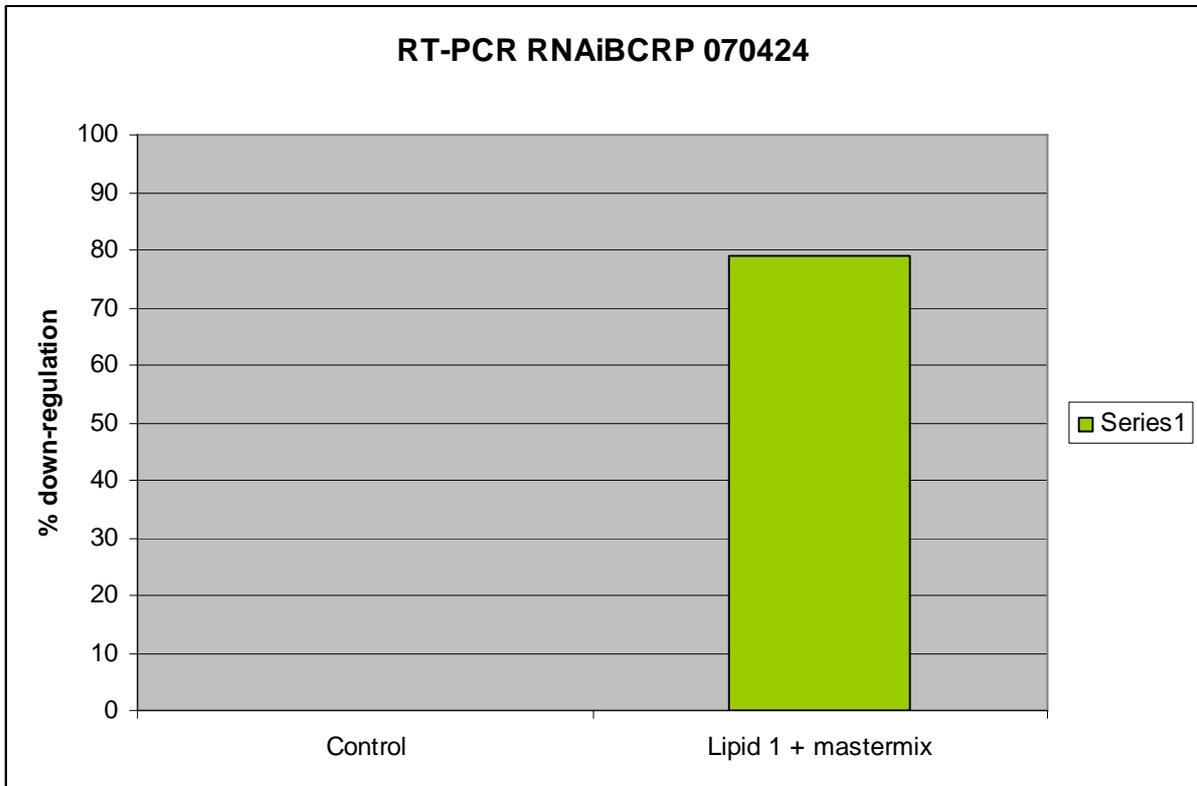
Figure 18. Shows the down-regulation of BCRP after transfection.

BCRP is down-regulated almost 80 %, as shown in figure 17 and 18.

Results of the uptake-study with mitoxantrone. Two controls, two transfection groups to down-regulate BCRP with RNAi and two controls that were also treated with inhibitor (GF120918) against BCRP.
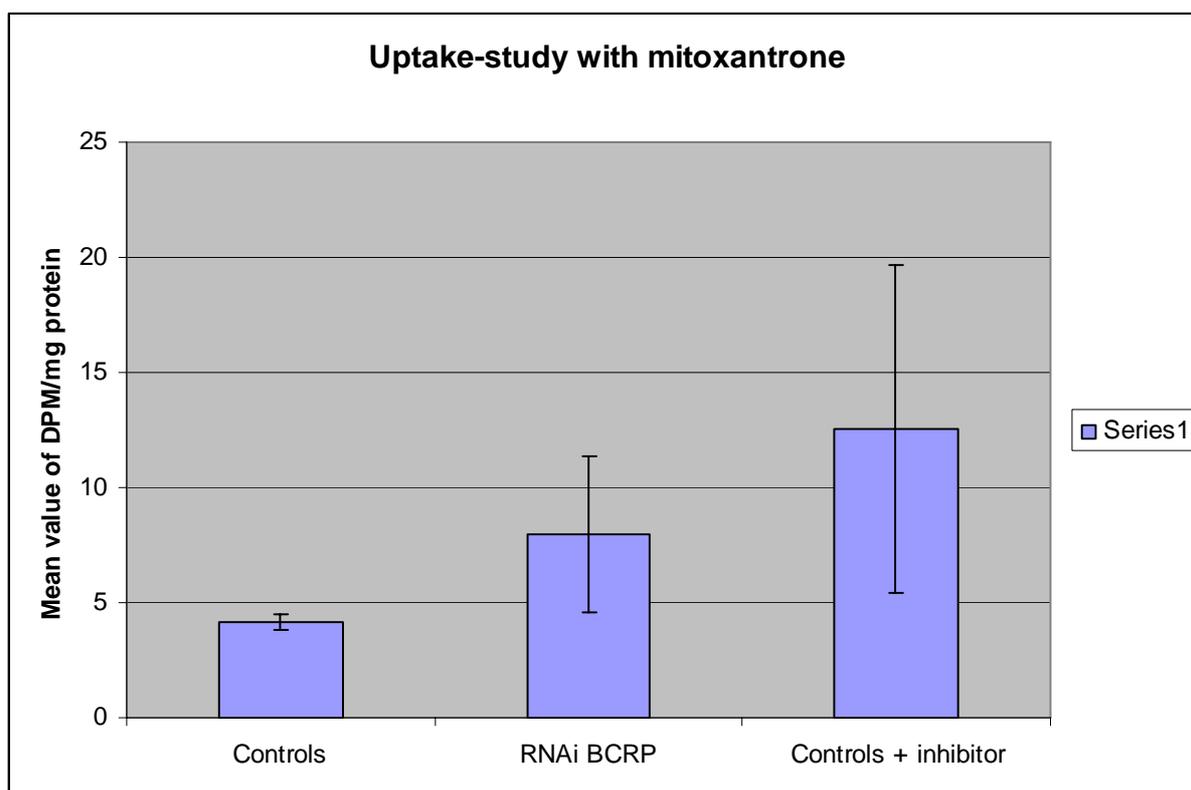
Figure 19. Mean values from the uptake-study with mitoxantrone that show an increase of mitoxantrone in the cells after transfection, but the controls with inhibitor shows even higher levels of mitoxantrone in the cells.

Table 7. Mean values from the uptake-study with mitoxantrone.

Treatment	Mean value (DPM/mg protein)
Controls	$4,15 \pm 0,35$
RNAi BCRP	$8,00 \pm 3,39$
Controls + inhibitor	$12,55 \pm 7,14$

Discussion

The aim of the exam project was to optimize a protocol for RNAi in mammary epithelial HC11 cells. The results obtained in the project provide a promising tool to examine mechanisms involved in the transport of various substances into milk.

Initially, we tried to optimize the transfection control by changing the antibiotic-free transfection medium after 24 hours, to the regular culture medium with antibiotics, but this did not work, since no effects on cellular viability were observed. However, following treatment for 48 hours, cellular viability was affected.

We also examined different numbers of cells per well in a 96-well plate: 25000, 10000, 5000 and 2500 cells/well. No effects were observed in wells with 10000-25000 cells and therefore we did not proceed with them.

The optimization of the transfection protocol with sicontrol tox and negative control show that the best choice for transfection in HC11 cells appear to be Lipid 1 diluted 1:4, or maybe Lipid 2 in the same dilution. 2500 cells/well seems to be the best choice for this transfection, since it is important that the cells are dividing at a quite high level for a successful transfection. In diagram 8, lipid 1 diluted 1:4 shows both high transfection efficiency and viability, approximately 70%, compared to lipid 1, diluted 1:2 that shows high transfection efficiency but very low viability of the cells. Lipid 1 diluted 1:8 shows high viability but lower transfection efficiency than dilution 1:4. This applies for dilution 1:16 as well. In diagram 10, lipid 2 shows the highest transfection efficiency, but also lower viability of the cells, than lipid 1 that shows almost as high transfection efficiency. Lipid 3 shows higher viability, but lower transfection efficiency than the first two. Lipid 4 shows the highest transfection efficiency of the four lipids, but also the lowest viability. Therefore, we chose to continue with lipid 1 and 2.

As it shows in the results, many of the transfections succeeded, with down-regulation of the gene in question (Cyclophilin B or BCRP) at high levels. In table 5, RT-PCR for Cyclophilin B, lipid 1 diluted 1:4 shows the highest effect, only about 12 % expression of Cyclophilin B compared to the control. In diagram 12-13, lipid 1 and 2 shows similar transfection efficiency as showed earlier with sicontrol tox and negative control. Not quite as high efficiency, but still.

Diagram 14 is an example of those transfections that did not go as expected, and this could be explained partly by the use of lipids that already were diluted in Opti-MEM, and not diluted directly before transfection. Another explanation could be that in the sicontrol tox and negative control transfections, about 400 nM of duplexes were used. This was applied also for the Cyclophilin B transfections, but in that case it seems to be better to use not more than 100 nM of siRNA duplexes. So, in the continuing transfections, only newly diluted lipids were used, as well as the right amount of siRNA duplexes. In diagram 15 and 16 the result can be seen quite clearly and the transfection down-regulates Cyclophilin B expression with almost 90 %. When it comes to BCRP, diagram 17 and 18, the down-regulation is almost 80 %.

Finally, we performed an uptake-study with the BCRP-substrate mitoxantrone to see if the down-regulation of BCRP from RNAi would make a difference compared to untransfected cells. Furthermore, we also used an inhibitor of BCRP to compare with transfected cells. The results is shown in table 7 and figure 19, and shows a higher accumulation of mitoxantrone in the cells after transfection compared to the controls, but not as high as those cells treated with the GF120918 inhibitor of BCRP. In the uptake study 1 µM of the inhibitor GF120918 was

used. It can be possible that other transporters than BCRP such as PGP are involved at this GF120918 concentration and that this explains the increased mitoxantrone accumulation as compared to the accumulation in the BCRP-gene silenced cells. Further studies are required to fully optimize and evaluate RNAi conditions in mammary epithelial HC11 cells.

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