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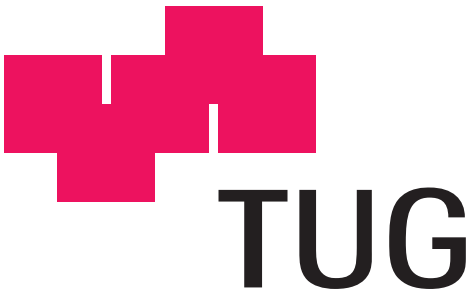
Master Thesis

Adipogenesis Cell Line Model: Transfection Process Optimisation and Validation of Microarray Data via qPCR



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For My Parents

Abstract

The progress on the field of RNA interference makes the sequence-specific knockdown of genes at larger scale feasible. Three pivotal methods are needed to conduct RNAi experiments: Knockdown of a specific gene (via siRNA or shRNA transfections), verification of knockdown (via reporter system or qPCR) and finally expression profiling for a selected knockdown (via microarrays).

The objective of this work was to establish the basic techniques for a silencing system and therefore focuses mainly on two points: firstly the transfection of 3T3L1 mouse fibroblasts with different methods, including on-slide reverse transfections and secondly readout with LUX™Primer-qPCR.

This work shows that 3T3L1 cells are hard to transfect with plasmids using standard transfection methods (cationic lipid, activated dendrimere, calcium phosphate coprecipitation) applied to adherent undifferentiated and differentiated cells.

Reverse transfections using gelatin-embedded plasmid DNA activated with Effectene® or TransMessenger™ on GAPS- or poly-L-lysine coated glass slides were successful. During the experiments the combination of TransMessenger™ Transfection Reagent and poly-L-lysine slides showed most stable results.

The qPCR was able to verify data obtained from a microarray experiment using RNA of 8 timepoints during a 3T3L1 fibroblast to adipocyte differentiation study. Although the use of LUX™Primers on the AbiPrism7000 realtime PCR machine was possible, the primers couldn't show their full potential on this specific machine.

Keywords: fibroblast, transfection, qPCR, reverse transfection, microarray

Kurzfassung

Die Fortschritte auf dem Gebiet der RNA-Interferenz machen das sequenzspezifische Herunterregulieren von Genen in größerem Umfang möglich, es müssen dazu aber verschiedste Methoden angewandt werden: Das Herunterregulieren eines Genes durch Transfektion von siRNA oder shRNA, das Auffinden interessanter Knockdowns (mit qPCR oder einem Reportersystem) und schließlich die Messung des Genexpressionsprofils mittels Microarraytechnologie.

Das Ziel der Arbeit war es, die für ein Silencingsystem notwendigen Methoden zu etablieren, wobei hauptsächlich folgende zwei Punkte bearbeitet wurden: Erstens, die Transfektion der 3T3L1-Mausfibroblasten mit verschiedenen Methoden, darunter auch reverse Transfektionen und zweitens das Messen der Expression via LUX™Primer-qPCR.

Es hat sich gezeigt daß sowohl undifferenzierte als auch differenzierte adhärente 3T3L1-Zellen unter Verwendung von Standardmethoden (kationische Lipide, aktivierte Dendrimere und Calciumphosphat-DNA-Fällung) nur sehr schwer mit Plasmiden zu transfizieren sind.

Reverse Transfektionen mit Effectene® bzw. TransMessenger™ aktivierter Plasmid-DNA in Gelatine auf GAPS- oder Poly-L-Lysin beschichteten Objektträgern waren möglich; die Kombination aus TransMessenger™-aktivierter DNA auf Poly-L-Lysin-beschichteten Slides zeigte die besten reproduzierbaren Ergebnisse.

Mit qPCR konnten Microarraydaten verifiziert werden, die aus Hybridisierungen für ein Differenzierungsexperiments zur Adipogenese in 3T3L1 Zellen gewonnen wurden. Die Messungen mit LUX™Primern auf einer AbiPrism7000 Realtime-PCR-Maschine bestätigten zwar die Resultate der Microarrayexperimente, es konnte allerdings das Potential der LUX™Primer auf diesem speziellen Gerät nicht voll ausgeschöpft werden.

Schlüsselwörter: Fibroblasten, Transfektion, qPCR, Reverse Transfektion, Microarray

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Many thanks also to my colleagues at the institute for their scientific and unscientific discussions. It has been a pleasure.

Contents

Glossary

DEPC Diethyl pyrocarbonate

DEX Dexamethasone

DMEM Dulbecco's Modified Eagle Medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

ds double stranded, e.g. dsDNA

EDTA Ethylenediaminetetraacetic acid

EGFP Enhanced green fluorescent protein

FAM 5́-Fluorescein Phosphoramidite

GAPS Gamma Amino Propyl Silane

GFP Green fluorescent protein

GLP Good Laboratory Practise

HEPES 2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansulfonic acid

HTML Hypertext markup language

IBMX 3-Isobutyl-1-Methylxanthine

LUX Light upon extension

PBS Phosphate Buffered Saline

PCR Polymerase chain reaction

qPCR Quantitative Real Time Reverse Transcription PCR

qRT-PCR Quantitative Reverse Transcription PCR

RNA Ribonucleic acid

RNAi RNA interference

shRNA Short hairpin RNA

siRNA Small Interfering RNA

SOP Standard Operating Procedure

ss single stranded, e.g. ssDNA

X-Gal 5-Brom-4-chlor-3-indolyl-beta-D-galactopyranosid

Chapter 1

Introduction

The aim of classical genetics is the linking of phenotypes to genes that created them by studying the rules of inheritance. This approach was the only one possible in the times prior to the discovery of nucleic acids. That method yields good results in cases where one gene produces one protein and this causes visible changes but interpretation is difficult when trying to reveal regulatory networks or complex pathways. Another disadvantage is that one relies on crossing experiments and random mutations to yield new phenotypes. By this means it is difficult to perform high throughput genetics.

Fortunately the availability of DNA sequencing and sequence-based analytical methods (e.g. PCR and microarrays) made the transition to genomics possible. The knowledge of complete genome sequences allows to study the genes much faster but on a very low level, hence it is difficult to link a gene sequence to a function or phenotype. But there are some ways to find out about function when the DNA sequence is available.

One method is to destroy a gene with unknown function (knock-out) and look at the differences in the cell's metabolism or phenotype, a strategy termed *reverse genetics*. Although the method is very powerful, there are some severe disadvantages:

- Knock-outs are stable (inheritable) modifications of the genome where a gene is removed or altered, so that it is not functional any more. If a gene of great importance for the cell survival is targeted, the cell may die from the consequences of the knock-out or undergo irreversible growth arrest. In both cases cultivation will not be possible. The experimenter may overcome the problems by introducing a copy of the gene with an externally triggerable promoter to switch the gene off after expanding of the cells, but this method is very laborious.

- The permanent knock-out does not allow the monitoring of transient

effects when reducing the protein concentration

- In diploid organisms, both alleles have to be knocked-out

Although the method does not allow high throughput analysis, the knock-out organisms are valuable objects of studies for the function of selected genes.

In the last years another technique emerged: the progress on the field of RNA interference makes the sequence-specific knock-down of genes at larger scale feasible. RNAi can be used to destroy the mRNA of selected genes without the need to modify the genome directly. At least 4 techniques are required to utilise the power of the new method:

- Cultivation of cells
- Efficient transfection of cells with siRNA or shRNA constructs
- Monitoring of interesting genes via reporter system or real-time RT-PCR (qPCR)
- Expression profiling with microarrays

1.1 Cell Culture

In the beginning of the 20th century first attempts were made to cultivate animal cells *ex vivo*. This kind of culture is nowadays called *tissue culture* because it used undisaggregated tissue fragments. A modern definition of the term cell culture is the cultivation of dispersed cells taken from original tissue (primary culture) or from a cell line.

Nowadays research has gone further than just cultivating the cells: by changing the media's composition it is possible to influence the metabolism or trigger differentiation. The cell strain used during this thesis is the 3T3-L1 mouse fibroblast cell strain which is available since the 1970's when Green *et al.* [6] established immortal fibroblast lines that readily differentiated into adipocytes when appropriate hormonal inducers were added. These lines were isolated from Swiss albino mice and are already committed (or determined) to the adipocytic lineage. When treated with an empirically derived prodifferentiative regimen they can undergo differentiation to mature fat cells within a 4-6 day period.

In vivo adipocytes arise from a pluripotent mesenchymal stem cell (MSC) population that is also capable of osteogenic, myogenic, or chondrogenic differentiation. MCSs become adipoblasts when unidentified mechanisms trigger a commitment to the adipocyte lineage. Growth arrest gives the signal

for the progression to preadipocytes, which can consequently differentiate into mature adipocytes under appropriate conditions. This multi-step process occurs throughout life in mammals. The hormonal factors that trigger adipocyte differentiation have been well described, particularly for rodent cell lines. This physiological conversion requires regulation of numerous genes, mediated mainly by increased expression of the transcription factors peroxisome proliferator-activated receptor-γ (PPAR-γ), CCAAT/enhancer binding protein- (C/EBP), and ADD1 sterol regulatory element binding protein ADD1/SREBP1, and down-regulation of other genes such as Pref-1 [19].

Cell culture has proven in some cases to be superior to animal experiments. This is mainly because the culturing of only a single cell type eliminates the interactions with other tissues as they would be present in animal models.

1.2 Transfections

Transfection is the introduction of DNA into eukaryotic cells by chemical or physical means and is distinguished from transduction which brings DNA into cells by the use of viruses. What makes transfections difficult is that the organisms developed sophisticated mechanisms during evolution to prevent the entry of DNA into cytoplasm and subsequent transition to the nucleus. The different methods to overcome these barriers can be divided into two subgroups:

- Chemical methods use chemical carriers (like DEAE-Dextran, calcium phosphate, cationic lipids or dendrimers)
- Physical methods utilise direct transfer of DNA into cytoplasm (with micro-injection, electroporation, bombardment with nucleic-acid-coated carrier particles)

In the beginning the main purpose of transfections was to assay the infectivity of extracted viral DNA but efficiencies were very low and the reproducibility was rather poor. The accidental discovery of the calcium phosphate precipitate transfection method (published 1973 [5]) gave a boost to the emerging field of gene transfer to eukaryotic cells because from then on it was much easier to assay newly developed vectors. Another positive effect was that the possibility to introduce bigger amounts of DNA into the cells made it easier to trace the DNA on its way to the nucleus and to try to unveil the underlying mechanisms. However, very often coprecipitation method is a source of intense frustration since it is extremely pH-dependent, does not

work with many cell types, and can be very toxic, particularly to primary cells.

Another step forward was the development of liposome mediated transfection methods since 1980 [4] and the improvement by applying cationic lipids. One of the first papers describing the method was written in 1987 [3], since then the mechanism of the transfection method was analysed and the method further improved.

1.2.1 Transfection Mechanism

For a long time the assumption was kept up that the negatively charged cell membrane was the main barrier against DNA entry into cells and that the DNA entered the cytoplasm directly. Further investigations revealed that nearly all chemical methods only prepared the DNA for endocytosis. Even for liposomal transfers, the contact of the vesicle with the cell does not lead to fusion releasing it's content to the cytoplasm, the small vesicles also have to go through the endocytotic machinery.

Although this mechanism imports 10^3 to 10^6 plasmids per cell, only very few of them will express the coded proteins. This is mainly because the endosomes are directed towards fusion with lysosomes. The lysosomes with their numerous hydrolases working at the lowered pH degrade the DNA rapidly to small pieces (around 100bp) and the degraded mixture is released into cytoplasm. Therefore one major enhancement is to help the DNA to escape the endosomal compartment undamaged. Some methods try to prevent the fusion with the lysosome, others increase the pH after fusion to render the nucleases nonfunctional, others protect the DNA by coating it. However with any of these methods DNA-loss is still great at this stage. Some virus proteins mediate the escape of DNA from the late endosomes, hence addition of these virus proteins to the transfection increases the transfection efficiency.

However, the release of plasmids into cytoplasm alone does not lead to transcription, since the plasmid has also to enter the nucleus to get access to the cell's transcription machinery, a process that is also very hard to manipulate on a low chemical level. Some researchers overcame the problem by introducing T7-RNA polymerase into the cytoplasm what allows DNA-sequences with a T7-promoter sequence to be transcribed in the cytoplasm.

However, at the moment the transfection methods using chemical carriers are highly inefficient and only work through a mass action effect.

1.2.2 High Throughput Reverse Transfections

The current state of the art is that transfections in eukaryotic cells are only possible with very high amount of work for transferring one type of plasmid to the cell. The main reason is that for every plasmid a unique transfection mix containing the plasmid has to be prepared. Thus it made large scale transfection experiments very costly.

The pioneering work of Sabatini *et al.* showed that it was possible to do transfections in parallel by reverting the standard way [1] [23]: Instead of preparing a transfection mix with one kind of DNA and adding it to the cells, his group prepared slides holding spots, each with DNA of another kind, and then seeded the cells onto the slides in a media containing transfection reagents. The cells that sat down over a spot of DNA took up the DNA and hence expressed the coded protein. In combination with RNA interference using siRNA-expressing plasmids even loss of function studies can be performed this way.

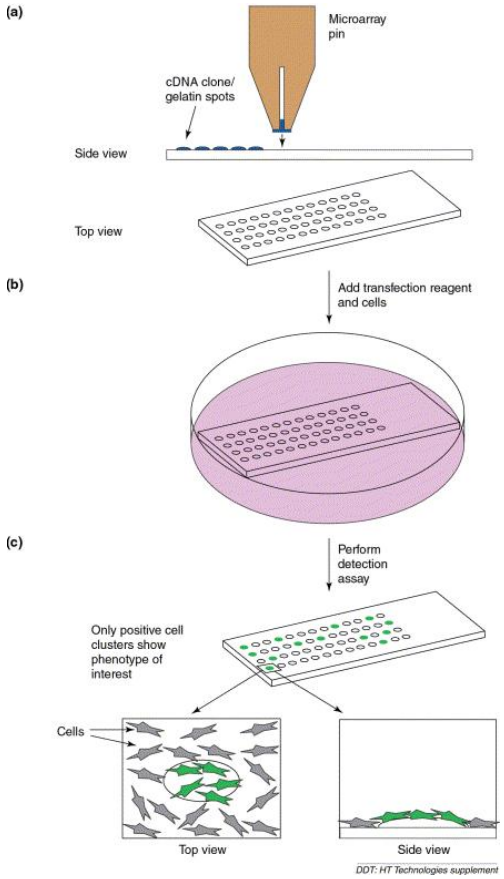


Figure 1.1: Reverse transfection procedure: a) spotting of DNA/gelatin mixture, b) reverse transfection by seeding cells onto the slide surface and c) readout to detect changes in the cells growing on a spot

The crucial step of this method is the data readout. Since every spot can only hold some 10 to 100 cells, the readout has to occur on some per-cell basis reporter system. Some cases will allow the use of cell phenotype information to detect interesting spots, e.g. if the protein triggers cell death or initiates differentiation. But even with clearly distinguishable phenotypes, the readout needs special microscopy equipment and digital image analysis tools to take full advantage of this high-throughput approach.

A different method is the direct assay of protein levels, e.g stainings with specific antibodies. Also the measurement of enzyme activities is possible if a substrate is known whose product can be measured easily, e.g. colourimetrically.

1.3 PCR Methods

1.3.1 From Plain PCR to qPCR

The discovery of the polymerase chain reaction PCR in 1986 by Mullis *et al.* [13] provided the first tool to copy and amplify DNA. Shortly after this discovery researchers realised, that the new method is very practical to speed up DNA sequencing or to verify the existence of a target sequence in a mixture. Years later researchers discovered that the method could not only be used as qualitative tool to detect if a template is there or not, it could also be useful to quantify the copy number. The early methods were very time consuming since they needed coamplification of other sequences, the readout was performed by separating the amplification product on agarose gels or by hybridisation with labelled probes. The quantification was only possible if the reaction was terminated after a certain number of PCR cycles, a number that had again to be determined empirically for each experiment.

The improvement that made the method applicable for standard laboratory use was the development of real-time monitoring of PCR reactions (real-time PCR or RT-PCR), first published by Higuchi *et al.* in 1993 [7]. The basic concept was to add DNA-intercalating dyes to the PCR-reaction that show increased fluorescence when incorporated into dsDNA. The emitted light was detected using a camera. In every PCR cycle the amount of DNA nearly doubles, so the light intensity should increase continuously. The number of cycles needed to reach a certain light intensity is therefore mathematically related to the number of templates in the analysed sample.

With that invention it was only a small step towards real-time PCR quantification of mRNA templates: The mRNA was reversely transcribed to cDNA prior to real-time PCR analysis. Hence the method was termed qRT-PCR (quantitative real-time PCR) or qRT-RT-PCR (real-time reverse-transcriptase PCR or qPCR).

1.3.2 qPCR Mathematics

During the PCR amplification the concentration of a *target* sequence (in most cases derived from a gene) rises in every cycle from initially c_0 to c_n. The perfect case would be a doubling of the concentration in every step ($c_{n+1} = c_n \cdot 2$) but since that does not occur in reality, the increase is slower depending on the efficiency E of the PCR reaction.

$$c_n = c_0 \cdot (1 + E)^n$$

All qPCR methods measure the fluorescence light intensity correlating to

the number of amplicons and not to the amplicon concentration, but since the mix volumes of two PCR reactions are the same, concentration or copy number may be used for all calculations without affecting the result.

$$I = f \cdot c_n$$
$$I = f \cdot c_0 \cdot (1+E)^n$$

Since the correlation factor f between the light intensity and amplicon concentration is machine and dye specific and usually unknown but constant, readout analysis uses the number of cycles needed to reach a certain light intensity threshold (thus the number of cycles is written as Ct and called *threshold cycles*). One possibility is to compare the Ct-value of an unknown sample with the Ct of a solution of known concentration (*standard*), this method is called absolute quantification.

$$\begin{aligned} &\text{From} && I = f \cdot c_{0,sample} \cdot (1+E)^{Ct_{sample}} = f \cdot c_{0,std} \cdot (1+E)^{Ct_{std}} \\ &\text{follows} && c_{0,sample} = c_{0,std} \cdot (1+E)^{Ct_{std} - Ct_{sample}} \end{aligned} \tag{1.1}$$

$c_{0,sample}$, $c_{0,std}$	Initial template concentration in the sample or standard
Ct_{sample}, Ct_{std}	Threshold cycles of sample and standard

Most of the qPCR applications have no need for absolute quantification. This is due to the mRNA processing before quantification: It is nearly impossible to harvest a known (or at least reproducible number) of cells, extract RNA and reverse transcribe it with known efficiency. Thus the absolute amount of RNA is not known precisely, hence the absolute amount of some species of RNA alone has no meaning either.

Beside that, absolute quantification needs a standard substance with known concentration like a plasmid DNA or better RNA from a DNA to RNA *in vitro* transcription, something which in both case needs much additional work to obtain. In addition many experiments focus only on regulatory changes and not absolute concentration, hence an absolute quantification is an overkill.

qPCR for determination of gene regulation does not even try to measure absolute concentrations, the aim is only to measure the change of the concentration of a *target* sequence in a RNA sample compared to a sample showing no regulation. The sample representing the unregulated state is called *calibrator*, therefore the concentration of the target sequence is defined to be 1 there.

Since the efficiency of cDNA-preparation is not known, a reference gene is needed, that is a sequence who's fraction in the sample RNA is believed to be constant in all samples. Hence good references are genes with constitutive expression like 18S-rRNA (RNA part of ribosomes) or GAPDH

(Glyceraldehyde-3-phosphate dehydrogenase, a key enzyme of glycolysis). The reference gene is therefore used to normalise the cDNA amount used in the PCR samples.

$$r_{t,ref,sample} = \frac{c_{t,sample}}{c_{ref,sample}} = \frac{k_t(1+E_t)^{-Ct_{t,sample}}}{k_{ref}(1+E_{ref})^{-Ct_{ref,sample}}}$$

$$r_{t,ref,cal} = \frac{c_{t,cal}}{c_{ref,cal}} = \frac{k_t(1+E_t)^{-Ct_{t,cal}}}{k_{ref}(1+E_{ref})^{-Ct_{ref,cal}}}$$

$$\begin{aligned} R &= \frac{r_{t,ref,sample}}{r_{t,ref,cal}} \\ &= \left(\frac{k_t(1+E_t)^{-Ct_{t,sample}}}{k_{ref}(1+E_{ref})^{-Ct_{ref,sample}}}\right) / \left(\frac{k_t(1+E_t)^{-Ct_{t,cal}}}{k_{ref}(1+E_{ref})^{-Ct_{ref,cal}}}\right) \\ &= \frac{(1+E_t)^{Ct_{t,cal}-Ct_{t,sample}}}{(1+E_{ref})^{Ct_{ref,cal}-Ct_{ref,sample}}} \end{aligned} \tag{1.2}$$

$c_{t,sample},\ c_{ref,sample}$	Concentration of target/reference template in sample
$c_{t,cal},\ c_{ref,cal}$	Concentration of target/reference template in calibrator
$k_t,\ k_{ref}$	Correlation factor Ct to gene concentration
$Ct_{t,cal},\ Ct_{ref,cal}$	Threshold cycles of target and reference gene in calibrator
$Ct_{t,sample},\ Ct_{ref,sample}$	Threshold cycles of target and reference gene in sample
$E_t,\ E_{ref}$	PCR Amplification efficiency of target and reference gene
$r_{t,ref,cal},\ r_{t,ref,sample}$	Target gene/reference gene concentration ratio in calibrator and sample
R	Expression ratio of target gene between sample and calibrator

The formula 1.3.2 is sufficient to calculate expression ratios if the PCR efficiencies for target and reference gene are known. Two possibilities exist if this is not the case.

- Firstly one could optimise the primers to get efficiencies near one and then use a constant efficiency of 1 for all calculations. This kind of analysis is called $\Delta\Delta$Ct because it only takes into account the different threshold cycle values ignoring the efficiency of the reaction.
- The other possibility is to measure the efficiency of the PCR. That can be done with a *standard curve*. A standard curve consists of a series of standard samples with known dilution factors between the samples. After measuring of the samples the efficiency of the PCR reaction can be calculated.

1.3.3 qPCR Advantages

There are two methods to look at regulatory changes in gene expression at mRNA levels that are utilised to produce most of the expression data: Microarrays and quantitative PCR. The main differences between the methods are

- Microarrays give an overview across thousands of genes while qPCR can only be used to evaluate some. Therefore qPCR is faster and cheaper if only a few genes should be assayed.
- qPCR yields data with higher precision because it is possible to calibrate the method and to run samples in replicates easily.
- qPCR is more sequence specific than microarrays because it uses two specific primers to select a target gene while microarray spots will not only bind target cDNA but also related sequences.

Additionally the PCR-product melting curve analysis allows some verification if really the target sequence was amplified or if primer dimers or random products were generated.

1.4 Objectives

The goal of this thesis was to establish the basic techniques for a silencing system Therefore the work focused mainly on two points:

- Firstly the transfection of the 3T3-L1 cells with different methods, including on slide reverse transfections and
- secondly readout with qPCR using LUX™Primers.

Chapter 2

Materials and Methods

2.1 qPCR

All the quantification procedures, starting with RNA extraction and ending with the qPCR run readout are based on the protocol recommendations from the manufacturer of the material or equipment.
All sample preparation steps, the mixing of the reaction, the configuration of the run and the data evaluation are all laid down in a SOP proposal (see A.5).

2.1.1 RNA Extraction

Two sets of RNA samples were used: one set of samples was prepared by Thomas Burkhard during his masters thesis [2] following the standard TRIzol®-Reagent protocol [8]. The qPCR should verify the microarray experiments done with these samples, therefore these samples were used for the final qPCR measurements.

For the first experiments RNA samples were prepared from a small adipocyte differentiation study in 6-well plates. RNA was harvested at day 0 (induction), 1, 7 and 14. For each timepoint two wells were washed two times with prewarmed PBS before addition of 1ml TRIzol®-reagent per well. The two cell lysates were pooled and stored at $-80°$C.

After the differentiation study all TRIzol® cell lysates were thawed and left for 5 minutes at room temperature before transferring them to centrifugation tubes. To each tube 0.2 mL chloroform per mL TRIzol®-reagent were added and the content was mixed for 15 seconds. The tubes were left for 2 minutes at room temperature before 15 minutes of centrifugation at 12000 g and 4°C.

The colourless aqueous supernatant was then transferred to empty centrifugation tubes, 0.5 mL isopropanol per mL TRIzol®-reagent were added and the mixture was left at room temperature for 10 minutes. After 10 minutes of centrifugation at 12000 g and 4°C the supernatant was discarded and 1 mL ice-cooled 75% ethanol per mL TRIzol®-reagent was added to the RNA-pellet, thoroughly mixed and put into the centrifuge for 5 minutes at 7500 g and 4°C. Afterwards the supernatant was replaced by 1 mL 100% ethanol per mL TRIzol®-reagent but the pellet wasn't resuspended in that solution. After 5 minutes of centrifugation at 7500 g and 4°C the supernatant was discarded. The centrifugation tubes were lain down to air-dry the pellet for 10 minutes, afterwards the RNA was dissolved in 10 μL DEPC-treated water per mL TRIzol®-reagent.

To measure the RNA-concentration, the RNA-extract was diluted 1:100 with DEPC-treated water and measured in a 1cm cuvette on a Beckman DU 520 Spectrophotometer. All aqueous RNA-solutions were stored in the -80°C freezer.

2.1.2 Reverse Transcription

For the reverse transcription step the SuperScript™III First-Strand Synthesis System for qPCR (Cat. No. 1080-051, Lot No. 175801, *Invitrogen Corp., Carlsbad, 92008 CA*) was used. The RNA samples for the main experiments were prepared by Thomas Burkhard during his masters thesis. The reaction was performed according to the kit's standard protocol using 2.5 μg RNA per reaction with random hexamere primers (included in kit). The optional RNase H digest after the first strand synthesis was carried out.

2.1.3 Primer Selection

All real-time RT-PCR assays were performed with LUX™Primers. The reference gene chosen was the 18S rRNA. Invitrogen offers a presynthesised primer pair: Human/Mouse/Rat 18S rRNA - Certified LUX™Primer Set, Cat. No. 115HM01. The remaining primers were designed using Invitrogen's LUX™Primerdesign web interface [1] and ordered from Invitrogen (see section A.1 for sequences). Suitable primers lying across exon-exon boundaries were extracted from the output of Invitrogen's primer design page using ExonCheck (see A.3).

[1]The interface is reachable from Invitrogen's main page at `http://www.invitrogen.com/` or directly at `https://pf.invitrogen.com/primerf/pages/Default.cfm?cc=169`

2.1.4 Plate Preparation

The reaction was prepared in a 96-well plate (96-Well Optical Reaction Plate, Barcoded, Part No. 4306737, Applied Biosystems, Foster City, 94404 CA), using Invitrogen's Platinum qPCR Supermix - UDG (Cat. No. 11730-025, Lot 1177046). The final volume per well was 25 μL. The precise protocol for all steps is written down in a SOP proposal in A.5.

2.1.5 Readout

The real-time PCR run was performed on a AbiPrism7000 Sequence Detection System (Applied Biosystems). qPCR runs with an on-plate standard curve allow two different methods to determine the threshold value of the calibrator:

- The triplicate method used the average of calibrator sample triplicate.
- The regression line method used the threshold value at concentration 1 (undiluted calibrator sample) derived from the semilogarithmic standard curve regression line as reference threshold to compare the samples. This was only possible because the standard curve samples were prepared using calibrator cDNA samples.

The data analysis was done using equation 1.3.2 with primer efficiencies derived from a relative standard curve [14] [16].

2.2 Cell Culture

For all experiments 3T3-L1 cells were used (ATCC# CL-173). The cryotubes with cells in the 5^{th} passage were prepared by Thomas Burkhard during his master's thesis (see [2]).

2.2.1 Cultivation

The cells were cultured in round cell culture treated dishes with a diameter of 35mm (6-well), 60mm and 100mm, respectively. The cells were incubated using standard conditions for 3T3-L1, this is at a temperature of 37°C with air containing 20% oxygen, 5% carbon dioxyde and nitrogen. The humidity is stabilised by evaporation of water from a bowl. The cell culture media was replaced at least every third day using $DMEM^{+}$.

Component	Concentration	Amount
DMEM (Cat. No. 41966-029, *Gibco™ Invitrogen*)	89.3%v/v	500 mL
Foetal Bovine Serum heat inactivated Cat. No. 10500-064, *Gibco™ Invitrogen*	8.9%v/v	50 mL
Penicillium-Streptomycin 10000 U/mL Cat. No. 15140-122, *Gibco™ Invitrogen*	89 U/mL	5 mL
L-Glutamine Solution 200 mM (100x) Cat. No. 25030-024, *Gibco™ Invitrogen*	1.79 mM	5 mL

Table 2.1: DMEM$^+$ media formulation

The media was mixed in the 500 mL DMEM bottle (max. volume 700 mL). The additives nor the mix was sterile filtered because all components were initially sterile and handled according to GLP.

Some transfection methods needed a special transfection media consisting only of DMEM and glutamine.

Component	Concentration	Amount
DMEM (Cat. No. 41966-029, *Gibco™ Invitrogen*)	90.9%v/v	50 mL
L-Glutamine Solution 200mM (100x) Cat. No. 25030-024, *Gibco™ Invitrogen*	1.82 mM	0.5 mL

Table 2.2: Transfection media formulation

2.2.2 Subcultivation

The cells were subcultivated at 50-70% confluence due to the fact that cells harvested above 70% confluence needed a longer trypsinisation time, showed a 1 to 2 day lag-phase after reseeding and yielded lower transfection efficiencies and general bad condition of the cells.
The cells were washed with PBS pH 7.4 (Cat. No. 10010-015, *Gibco™ Invitrogen*) and then trypsinisated with 10x (0.5% Trypsin, 5.3 mM EDTA, 8.5g NaCl, Cat. No. 15400-054, Gibco Life Products, *Invitrogen*) for 2 to 5 minutes at 37°C. Reseeding was done at 1000 cells/cm^2 density for standard cultivation and at higher densities for transfection experiments.

2.2.3 Adipocyte Differentiation

Differentiation using an established protocol [2] was needed to test transfection methods on differentiating cells. The differentiation media was prepared using the media composition noted in table 2.3:

Component	Concentration	Amount
DMEM^{+}	98.8%v/v	100 mL
Insulin stock 1 mg/mL	988 ng/mL	100 μL
Biotin stock 3.3 mM	3.26 μM	100 μL
Panthotenic acid stock 1.7 mM	1.68 μM	10 μL
IBMX-Stock 10 mM*	9.88 μM	1 mL
DEX Stock 0.5 mM*	0.49 μM	50 μL

Table 2.3: Differentiation media composition

For the first media change (induction of differentiation) all the media additives noted in table 2.3 were used. For the following changes of media 2 and 4 days after start of induction a media lacking the components marked with a star was used (maintenance of induction).

2.3 Transfections

Since 3T3-L1 cells are hard to transfect, 5 different transfection methods were tested. The experiments were all carried out in 6-well plates (*Corning Inc.*) with plasmid DNA coding for β-galactosidase (pcDNA4/HisMax©/lacZ, *Invitrogen*) or EGFP (pEGFP-C1, Cat. No. 6081-1, *BD Biosciences Clontech*).

The pcDNA4/HisMax©/lacZ-transfected cells were detected 24h after transfection using the X-Gal staining (see 2.4.1), the pEGFP-C1-transfected under a fluorescence microscope (*Olympus*). In both cases 4-6 photos were taken per culture to count the cells. The number of cells and the number of transfected cells was then used to assess different measures to evaluate the transfection reactions:

- The transfection rate is the ratio of transfected cells to the total number of cells. Hence it is a measure for the probability that any given cell was transfected.
- The transfection density is the number of transfected cells per area.
- The death rate compares a transfected culture with an untransfected one, that was grown in parallel. The death rate is the number of cells the transfected culture has less than the untransfected divided by the number of cells in the untransfected culture.

2.3.1 Superfect® Transfection Reagent

The transfections were performed using the Superfect® Transfection Reagent Kit (Cat. No. 301305, *Qiagen GmbH*). This method describes the transfection in 6-well plates but scale-up to other dish formats was not problematic. The basic handling of the kit and the cells is described in the handbook [15] but the amount of DNA and superfect was changed during the optimisation process:

- The cells were allowed to reach 60% confluence but this value should not be exceeded to avoid growth inhibition.
- Transfection mixes were prepared by dilution of 4 μg pcDNA4/HisMax©/lacZ or pEGFP-C1 in 100 μL in transfection media (see table 2.2). After mixing, 20 μL Superfect® Transfection Reagent were added and the mix was incubated for 10 minutes at room temperature.
- During incubation the cells were washed with PBS.
- After incubation 0.6 mL DMEM$^+$ were added to the transfection mix using the sampler and the whole mixture was then transferred to the cell culture dish immediately.
- After a 3 hour incubation using standard cell culture conditions, the cells were washed with PBS once and the wells were then filled with 1 mL DMEM$^+$. GFP fluorescence was evaluated 5 hours and 24 hours after transfection, X-Gal staining was performed 24 hours after addition of the transfection mix.

2.3.2 Effectene® Transfection Reagent

Transfection of 3T3-L1 cells were also carried out with Effectene® Transfection Reagent from the Transfection Reagent Selector Kit (Cat. No. 301399, Lot 11549318, Qiagen) in a 6-well culture dish. The best results were obtained with following method:

- $1.2 \cdot 10^4$ Cells/cm^2 were seeded the day prior to transfection, the transfection procedure itself was performed when the culture had reached 60% confluence.
- 0.4 μg pcDNA4/HisMax©/lacZ were diluted in buffer EC (from the kit) to a final volume of 87.2 μL. After addition of 12.8 μL enhancer

from the kit and mixing, the solution was incubated for 5 minutes at room temperature (this step should condense the DNA).

- After incubation 10 μL of Effectene® Transfection Reagent Transfection reagent was added and mixed with the sampler. The mix was left to incubate at room temperature for another 5 minutes.

- During incubation the cells were washed once with PBS, the well was then filled with 1600 μL DMEM$^+$. After incubation 600 μL DMEM$^+$ were added to the transfection mix and the whole mix was added dropwise to the DMEM-filled well.

- After 24 hours incubation at standard conditions (see 2.2.1) the cells were assayed for β-galactosidase activity using the X-Gal staining method.

2.3.3 Lipofectamine™ Reagent

The transfections were carried out using reagents from the Lipofectamine™ Reagent kit (Cat. No. 18324-111, Lot 1141919, *Qiagen*).

- Cells were seeded at $1.2 \cdot 10^4$ Cells/cm^2 density in a 6-well dish one day prior to transfection, the transfection procedure itself started when the culture had reached 60% confluence.

- 10 μL of a pcDNA4/HisMax©/lacZ plasmid solution (100 μg/mL) were diluted with 100 μL transfection media (see table 2.2) and also 7 μL Lipofectamine™ Reagent were diluted in 100 μL transfection media. Both solutions were mixed and incubated for 30 minutes at room temperature.

- During that time the media in the culture dish was replaced by 0.8 mL transfection media.

- DNA-transfectant complexes were diluted with 150 μL transfection media and added to the culture.

- After incubating the dish for 5 hours at standard cell culture conditions, the media was replaced by standard DMEM$^+$.

- Transfection efficiency was assayed 48 hours after addition of the transfection mix.

2.3.4 Lipofectamine™2000 Reagent

The procedure was carried out using the standard transfection media (see table 2.2) and the Lipofectamine™2000 Reagent (Cat. No. 11668-027, Lot 1171551, *Qiagen*) kit. For transfection with the Lipofectamine™2000 Reagent higher cell densities at the time of transfection are recommended compared to the Lipofectamine™ Reagent.

- Cells were seeded at $2.4 \cdot 10^4$ Cells/cm^2 density in a 6-well dish the day before transfection and reached more than 90% confluence when the transfection procedure started.
- 5 μg of a pcDNA4/HisMax©/lacZ plasmid and 10 μL Lipofectamine™2000 Reagent were diluted in two different tubes containing 250 μL transfection media each.
- After 5 minutes incubation at room temperature both solutions were mixed and incubated for another 20 minutes and added to the well.
- During the incubation time the media in the culture dish was replaced by 2 mL transfection media.
- 12 hours later, the media was replaced by 1 mL standard DMEM$^+$
- Transfection efficiency was assayed 48 hours after addition of the transfection mix.

2.3.5 Calcium-phosphate-DNA-Coprecipitation

There are a dozen different protocols for the calcium phosphate transfection method, but only one that yielded reproducible results in our application. The method is mainly based on a paper about the precipitate formation kinetics [10]. Since the contact between the cells and the precipitate alone yielded very low transfection efficiencies, an additional glycerol shock step was applied. Tables 2.4 to 2.7 show the composition of liquids needed to accomplish the method, after preparation they were stored at at -20°C.

Component	Concentration	Amount
$CaCl_2 \cdot H_2O$ p.A. (Cat. No. 2382.1000, *Merck*) ddH_2O	2.5 M	0.735 g 1.63 mL
2.5 M $CaCl_2$ solution		2 mL

Table 2.4: 2.5 M $CaCl_2$ solution

The solution was filtered through a 0.2 μm filter (Part No. 431219, *Corning Inc.*).

Component	Conc.	Amount
HEPES (Cat. No. 1.10110.0250, *Merck*)	50 mM	596 mg
NaCl (Art. 7530.1 *Carl Roth GmbH, Karlsruhe, 76185 DE*)	140 mM	409 mg
ddH_2O		45 mL
Adjust pH 7.05 with 1 M NaOH		$\approx$ 0.9 mL
Adjust volume to 50 mL with ddH_2O		$\approx$ 4 mL
HEPES 2x Buffer, pH 7.05		50 mL

Table 2.5: 2x HEPES buffer composition

The solution was filtered through a Corning 0.2 μm filter into 1.5 mL tubes.

Component	Conc.	Amount
$Na_2HPO_4 \cdot 2H_2O$ (Cat. No. 1.06580.0500, *Merck*)	195 mM	178 mg
$NaH_2PO_4 \cdot H_2O$ (Cat. No. 1.06345.0500, *Merck*)	105 mM	75 mg
ddH_2O		5.2 mL
Phosphate stock solution 300 mM		5.2 mL

Table 2.6: Phosphate stock solution composition

The solution was filtered through a Corning 0.2 μm filter into empty 1.5 mL tubes.

Component	Conc.	Amount
Glycerol (Art. 7530.1, *Carl Roth GmbH, Karlsruhe, 76185 DE*) PBS pH 7.4 (Cat. No. 10010-015, *Gibco™ Invitrogen*) Adjust volume to 50 mL with PBS	15%v/v	7.5 mL 40 mL
15%v/v Glycerol solution		50 mL

Table 2.7: Glycerol shock solution

The solution was filtered through a Corning 0.2 μm filter into an empty bottle.

The procedure to transfect one well on a 6-well plate included the following steps:

- The culture media was replaced by 1 mL standard DMEM$^+$ 1 hour before transfection of the cells
- Two solutions were prepared: Solution A is a dilution of 2.5 μg pcDNA4/HisMax©/lacZ plasmid DNA and 5 μL $CaCl_2$-solution (2.5 M) in 45 μL water. Solution B contained 50 μL HEPES 2x and 0.25 μL phosphate stock (300 mM). Both solutions were prewarmed to 37°C.
- After the tubes reached 37°C, 50 μL solution A were transferred to one tube containing solution B, mixed quickly with the sampler and put onto the heater immediately.
- After 60 seconds of precipitate formation at 37°C the mix was added to one well, after that the mix preparation for the next well began.
- After finishing of the last well the cells with the precipitate were put into the incubator for 5 hours at standard conditions.
- To increase transfection efficiency a glycerol shock was needed. Therefore the media was replaced by 1 mL 15% glycerol solution. After 4 minutes incubation, 1 mL DMEM$^+$ was added to stop the reaction and the glycerol/media mix was aspirated. After filling of the wells with 1mL DMEM$^+$ the dish was put into the incubator for 24 hours.
- After that time X-Gal staining (see 2.4.1) was performed to detect the transfected cells.

2.4 Detection

2.4.1 X-Gal Staining

The X-Gal staining was used to visualise the cells transfected with the β-galactosidase coding pcDNA4/HisMax©/lacZ-plasmid. The preparation of the reagents needed is noted in the tables 2.8 to 2.10.

Component	**Conc.**	**Amount**
PBS pH 7.4 (Cat. No. 10010-015, *Gibco™ Invitrogen*)		9.98 mL
Glutaraldehyde 25% (Cat. No. G-6257, *Sigma Corp.*)	0.05%v/v	20 μL

Table 2.8: Glutaraldehyde 0.05% fixation solution

The solution has to be freshly prepared before use.

Component	**Conc.**	**Amount**
$K_4Fe(CN)_6 \cdot 3H_2O$ (Cat. No. P-9387, *Sigma Corp.*)	5 mM	2.11 g
$K_3Fe(CN)_6$ (Cat. No. P-8131, *Sigma Corp.*)	5 mM	1.65 g
$MgCl_2$ (Cat. No. M-8266, *Sigma Corp.*)	1 mM	95 mg
PBS pH 7.4 (Cat. No. 10010-015, *Gibco™ Invitrogen*)		1000 mL
Staining Solution A		1000 mL

Table 2.9: X-Gal staining solution A

The solution was stored in the dark at $-20°C$.

Component	**Conc.**	**Amount**
X-Gal (Cat. No. B-4252, *Sigma Corp., St. Louis Mo, 63178 USA*)	20 mg/mL	20 mg
DMSO		1 mL
20x X-Gal Stock Solution		1 mL

Table 2.10: 20x X-Gal stock solution composition

The solution is very sensitive to light and therefore has to be stored in the dark at $-20°C$.

The procedure to stain 6-well plates was as follows:

- The media was replaced by the 1 mL 0.05% glutaraldehyde solution per well.

- After 10 minutes incubation at room temperature (fixation) the fixative was removed, each well was washed with 1 mL PBS pH 7.4 and filled with another 1 mL of PBS. An incubation step of 10 minutes duration followed.
- During incubation the staining solution was prepared by mixing 4.6 mL solution A and 240 μL 20x X-Gal stock at room temperature.
- After removal of the PBS solution the cells were covered with 0.8 mL staining solution per well. The blue colour developed during a 1 day incubation at standard 3T3-L1 cell culture conditions.
- After incubation the results were analysed immediately. For later analysis the dish was sealed with parafilm and stored in the dark at 4°C.

2.4.2 Oil-Red O Staining

This method visualises lipid droplets in cells by colouring them deep red. The solutions needed are shown in table 2.11 to 2.13.

Component	**Conc.**	**Amount**
37.5% aqueous formalin	10%	107 mL
PBS pH 7.2 (Cat. No. 20012-050, *Gibco™ Invitrogen*) [a]		293 mL
Formalin fixation solution	10%	400 mL

[a]PBS pH 7.4 should also have worked but was not available at that time

Table 2.11: Formalin fixation solution

Component	**Conc.**	**Amount**
Oil-Red O (Cat. No. 155984, *ICN Biomedicals Inc., Aurora Ohio, 44202 USA*)	0.5%w/v	1.25 g
2-propanole		250 mL
Oil-Red O Stock Solution		250 mL

Table 2.12: Oil-Red O stock solution composition

Component	Conc.	Amount
Lightgreen (Cat. No. 9174B, *ICN Biomedicals Inc., Aurora Ohio, 44202 USA*)	2%w/v	2g
ddH_2O		100 mL
Light Green Solution 2%(w/v)		100 mL

Table 2.13: Lightgreen staining solution composition

- The Oil-Red O working solution was prepared shortly before the staining by mixing 6 mL Oil-Red O Stock solution and 4 mL ddH_2O. The solutions was kept at room temperature.
- The cells were washed with pre-warmed PBS and fixated 30 minutes with the fixation solution. During that time the Oil-Red O working solution was filtered.
- The fixation solution was removed from the cells and the cell layer was covered with filtered Oil-Red O solution for 1 hour.
- The cells were washed with PBS and afterwards a counter staining was applied by covering the cells with lightgreen 2% for 3 minutes.
- After removing the green stain with PBS the dry cell layer was observed with transmission light microscopy.

2.5 Reverse Transfections

2.5.1 Slide Handling

Since not all steps can be performed in a sterile environment and sterilisation of the slides is impossible, careful handling is of great importance. Several measures were taken to keep the number of germs as low as possible:

- All surfaces that had contact with the slides were cleaned with cleaning ethanol (solution of 70% ethanol in water). The plastic slide-box was washed also with cleaning ethanol before use.
- Slides were only handled with a cleaned forceps and transferred into the slide box immediately after use to minimise the amount of dust on the slide surface.
- The slides were cleaned with an airduster before transferring them into the laminar flow for the transfection procedure.

2.5.2 Slide Preparation - Manual Spotting

Two different slide types were tested in the experiments. The first ones were CMT-GAPS2™Coated Slides (Cat. No. 40005, Lot. 27501000B, *Corning Inc., Corning NY 14831*).
Another set of slides were glass slides for microscopy (Art. Nr. H896, *Carl Roth GmbH, Karlsruhe, 76185 DE*) that were coated with poly-L-lysine solution 0.1% w/v (Cat. No. P-8920, Sigma-Aldrich Co., St Louis) according to a protocol for poly-L-lysine slide preparation [18].

For the purpose of spotting, different gelatin and gelatin-DNA solutions had to be prepared. A gel-stock solution with 2% w/v gelatin (Type B from bovine skin, 225 bloom, Cat. No. G9391, Sigma) in water was prepared by solving the powder at 60°C. This solution was then used to prepare solutions with 0.2% gelatin and 10 to 100 ng/μL plasmid DNA. The solution was spotted by reverse pipetting 0.2 μL manually with a $0.1 - 2.5$ μL-sampler onto a 4mm x 3mm grid. The spotted slides were stored overnight in a slide-box at 4°C.

2.5.3 Slide Activation

The process of converting the DNA on the slide into transfectable complexes was named *slide activation* to distinguish it from the transfection itself. Two different transfection agents were used: The Effectene® Transfection Reagent included in the Transfection Reagent Selector Kit (Cat. No. 301399, Lot 11549318, *Qiagen*) and the TransMessenger™ Transfection Reagent (Cat. No. 301525, Lot 11552430, *Qiagen*).

- **Effectene® Transfection Reagent:** 8 μL enhancer were mixed with 75 μL Buffer EC and incubated for 5 minutes at room temperature. After addition of 12.5 μL Effectene® Transfection Reagent and mixing, the solution was immediately transferred onto a DNA/Gel-slide by distributing it dropwise across the slide. The slide was covered with a 24x60 mm coverslip (Deckglas #1 Art. Nr. H878, *Carl Roth GmbH, Karlsruhe, 76185 DE*) and left to incubate for 20 minutes at room temperature.

- **TransMessenger™ Transfection Reagent:** Per slide 100 μL transfection mix were prepared by diluting 7 μL enhancer ER in 77 μL buffer ER and incubating the mixture for 5 minutes at room temperature. After the incubation 16 μL of TransMessenger™ Transfection Reagent were added, mixed shortly and transferred immediately onto the slide.

The slide then was covered with a 24x60 mm coverslip and and incubated for 20 minutes at room temperature.

After activation of the slides the coverslip was removed by dipping the slide into a PBS-filled 50 mL falcon tube. The slides were then lain into a 12x12 cm dish (Petri Dish Square, 120x120x17 mm, Cat. No. 688102, *Greiner Bio-one GmbH., 4550 Kremsmünster*) with the spotted side pointing upwards.

2.5.4 Reverse Transfection

3T3-L1 cells at 30 to 50% confluence were harvested using 10x trypsin (see 2.2.2) and after centrifugation (3 minutes, 400 g at room temperature) resuspended in 10 mL DMEM$^+$. After cell counting, the amount needed to seed $1.5 \cdot 10^4$ cells/cm^2 was taken out and diluted with DMEM$^+$, e.g. for seeding in a 144 cm^2 dish 144 cm^2 $\cdot$ 15000cells/cm^2 = $2.16 \cdot 10^6$cells were diluted to a final volume of 30 mL. The cell suspension was poured into the dish but not directly onto the slides.

2.5.5 Readout of β-Galactosidase Activity Using X-Gal Staining

In order to detect β-galactosidase enzyme activity in the cells, a modified X-Gal staining procedure was used to perform the staining on the slide. The reagents mentioned here are the same as used for the standard X-Gal staining procedure on cell culture dishes (see 2.4.1).

- The slides were washed once with PBS and fixated for 15 minutes with 0.05% glutaraldehyde solution.
- After two PBS washing steps 100 μL of X-Gal staining solution were added to the middle of the slide surface and covered with a 60x24 mm coverslip.
- The slide was incubated in a dark box for 1 day at 4°C.
- Two vessels, deep enough to submerse a slide with the long side pointing upwards and filled with PBS were used to wash off the staining solution. The slide was dipped into the first vessel using a forceps and pulled out after the cover slip came off. The second wash in the other vessel removed remainings of X-Gal staining solution from the slide.
- The slides were dried through centrifugation at 400 g for 2 minutes.

- The slides were then scanned using a GenePix Model 4000B Microarray Scanner (*Axon Instruments Inc., Union City, CA 94587, USA*) at 5 μm pixel size. The excitation frequencies of the two lasers in this model were 532nm and 635nm. Photomultiplier settings were chosen so that the cell nuclei (the brightest spots on the slide) did not overload the reader's input circuit.

2.5.6 Readout of GFP-Fluorescence

The fluorescence properties of pEGFP-C1 (excitation maximum at 488 nm, emission maximum at 507 nm) made it necessary to use another microarray scanner (ArrayWoRx$^{©e}$ Biochip Reader, *Applied Precision, Issaquah, Washington 98027 USA*). The pictures at 480 nm/530 nm and 540 nm/595 nm excitation/emission wavelength were the most suitable for GFP detection.

- Slides with GFP-positive cells were washed 3x with PBS while lying in a cell culture dish.
- The slides were scanned using the ArrayWoRx$^{©e}$ Biochip Reader, the exposure time was 0.250 seconds on every channel, additional signal amplification of the 530 nm picture was disabled and for the 595 nm picture the gain was adjusted to 0.02-fold.
- The 530 nm picture shows the GFP fluorescence and the 595 nm picture an unspecific fluorescence of all cells. The pictures were overlain using linux command line programs from the libtiff, ImageMagick, netpbm-progs, coreutils package and Gimp or alternatively viewed using the software coming with the GenePix Model 4000B Microarray Scanner. In both cases the 530 nm picture was assigned to the green channel, the other one to the red.

Chapter 3

Results

3.1 qPCR

The problems while designing LUX™Primers manually gave the impetus to write a helper program to aid the design process (see A.3). The information gathered during the whole qPCR procedure was used to write a SOP proposal (see A.5).

The 13 primer pairs listed in table A.1 successfully amplified their target sequence and generated products with melting curves clearly distinguishable from primer dimers. One primer pair designed for kif23 did not work, the cause was that the sequence in the database (ensembl build m30) at the time of the design was wrong, the current or future builds may contain the correct sequence.

The LUX™Primers in combination with the AbiPrism7000 machine showed a smaller increase in fluorescence intensity when incorporated into PCR product than expected after reading the LUX™Primer manual [9]. The run performed on January 2, 2004 using 18S/mki67/nur77 primers was examined closely and the increase of fluorescence intensity in the standard curve wells was calculated after fitting of 30 intensity values per well to a sigmoidal fit (see equation 3.1) similar to that from [20]. The term $b \cdot n$ was added to account for the steady linear rise of the fluorescence intensity (baseline) prior to the exponential phase of fluorescence intensity increase.

n	Number of the cycle
I_n	Observed fluorescence intensity in cycle n
$a,\ b,\ c,\ d,\ e$	Fit parameters

$$I_n = a + b \cdot n + \frac{c}{1 + exp(d + e \cdot n)} \tag{3.1}$$

A measure for the fluorescence increase is c/a, that is the fluorescence increase

compared to the base fluorescence level, values calculated from January 2nd run are shown in table 3.1.

Primerpair	**Average Increase**	**Stdev Increase**
18S	0.548	0.137
mki67	0.208	0.018
nur77	0.105	0.052

Table 3.1: Relative fluorescence increase during PCR

This average increase of fluorescence intensity is unexpectedly low, the manual from Invitrogen mentions an increase from 0.4 to 1 between free and incorporated primer. That would correspond to an 1.5-fold intensity increase (without background fluorescence), table 3.1 shows that at most a 0.55-fold increase was observed.

The assessment of the qPCR data quality was done using data from 5 different plates, each containing a standard curve with calibrator cDNA and 8 cDNA samples measured with 18S-rRNA primers. The relative concentrations were calculated using the calibrator threshold value acquired using the triplicate method and the regression line method.

Due to the fact that the identical 8 samples were measured in 5 runs, they should show the same relative concentration in all runs. Hence the standard deviation between the 5 measurements corresponds to the qPCR method's imprecision.

The regression line method was able to reduce the standard deviation in the readout procedure and so reduced the analysis' error. Compared to the triplicate method, the regression line method reduced the standard deviation of the relative sample concentrations on the 5 plates by 42%-78% with an average decrease of 59%, while the average value did not change more than 7.2% (see table 3.2).

Because of these findings all qPCR measurements were evaluated using the regression line method. The values calculated this way were then compared with the data from microarray experiments. For each gene a search for corresponding ESTs on the slide was started using text-search across the EST annotation files and blasts to discover more EST IDs. If more than one EST for a gene was found then the arithmetic mean of the log ratios was used to compare it to the qPCR data.

The correlation of microarray to qPCR data was evaluated using the square of the Pearson product moment (RSQ) applied to the $\log_2$ of microarray analysis and PCR ratios (see table 3.3).

Sample	Triplicate Meth.		Regression Meth.	
	Average	Stdev	Average	Stdev
1	0.495	0.127	0.487	0.053
2	0.987	0.303	0.993	0.236
3	0.684	0.225	0.638	0.133
4	0.375	0.101	0.380	0.050
5	0.434	0.100	0.407	0.061
6	0.414	0.128	0.428	0.079
7	0.283	0.110	0.295	0.077
8	0.759	0.354	0.711	0.174

Table 3.2: Average and standard deviation of relative 18S-rRNA concentrations using the triplicate and the regression line readout methods

Gene	Correlation	Comment
lpl	0.940	1 EST (595/600bp)[a]
c-myc	0.896	3 ESTs, all with more than 95% of bases identical
incenp	0.892	2 ESTs, with more than 99% of bases identical
cyclinA 2	0.868	4 ESTs, all with more than 90% of bases identical
pparγ	0.838	1 EST (426/459bp)
mki67	0.830	1 EST (606/606bp)
decorin	0.828	4 ESTs, 2 with more than 95% of bases identical, other two have no detectable homology using the blast algorithm. All 4 ESTs have the same profile and were used for comparison.
nur77	0.733	1 EST (622/633bp) microarray profile with same trend but one outlying point. Without that point correlation would be 0.881.
klf5	0.781	No data for best match EST (411/413bp) available, other one used had no detectable homology using blast and was just once in 3 annotation software runs annotated as klf5 via unigene.
bteb 1	0.514	two ESTs annotated, but one has completely different profile. Two other ESTs had more then 99% identical bases and same profile as qPCR data but were not annotated at all. One EST had no blast homology but was annotated bteb1 and had similar profile.
nurr1		no datapoints from microarray available

[a]Numbers in brackets is similarity to EST (number of identical bases/EST bases).

Table 3.3: Correlation of qPCR results compared to microarray results

For 8 genes the microarray profile was confirmed using qPCR (lpl, c-myc, incenp, cyclin A2, pparg, mki67, decorin, nur77), for 2 genes no comparable

microarray data was available (klf5, nurr1). The profile of one gene (bteb1) did not match the profiles on the microarray. The correlation between qPCR and microarray data is shown in figure 3.1 (see A.2 for profile details).

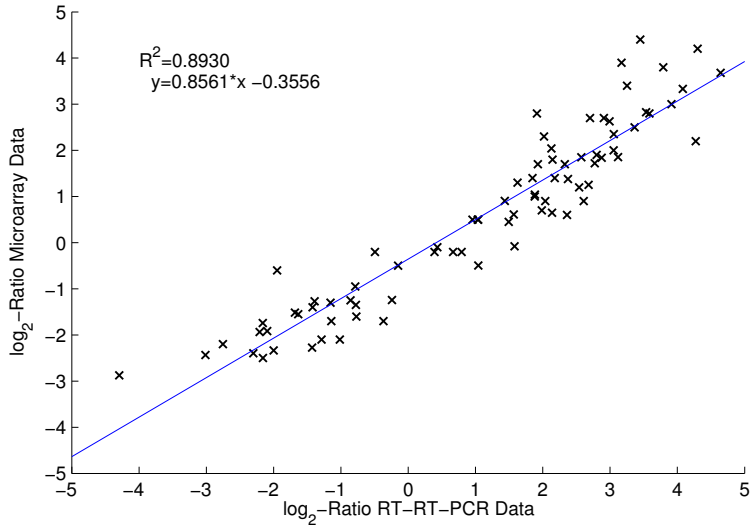


Figure 3.1: Correlation of expression data from qPCR and microarray

3.2 Transfections

3.2.1 Calcium-phosphate Coprecipitation

This method yielded rather low transfection efficiencies. The best results were obtained using transfection mixes containing the pcDNA4/HisMax©/lacZ-plasmid. The cells were incubated for 3h with the mix, afterwards a 4 minute long glycerol shock was applied. Transfection rates reached 3% this way. Due to the very fast handling steps and narrow timing intervals, in general the reproducibility of the method was poor.

The application of a glycerol shock increased the transfection rates, 6 comparable experiments showed the following:

- The shock never made the transfection perform significantly worse than without
- In two cases (of 6) no significant change was visible
- In the remaining cases transfection rate increased 2 to 10-fold, except for 2 cases where transfection events got only visible after glycerol treatment, therefore no ratio can be given.

3.2.2 Lipofectamine™ Reagent

Transfections using Lipofectamine™ Reagent reached transfection efficiencies (8%) comparable to Superfect® Transfection Reagent but at a much higher death rate of more than 90%. Another disadvantage was the very low transfection efficiency in the presence of serum or antibiotics (1/10th of transfection rate with antibiotics, about 1/100 with serum of serum/antibiotics-free transfection). Differentiated cells were also very hard to transfect with this method, highest transfection efficiencies reached using 3T3-L1 cells 2 days after induction were around 1%.

3.2.3 Lipofectamine™2000 Reagent

This method also proved to be not useful with 3T3-L1 cells. Although the death rate during transfection was much lower compared to Lipofectamine™ Reagent (around 60%), there was another phenomenon which may indicate negative impact on cell metabolism: After the transfection procedure the cells showed many small vesicles inside the cytoplasm that were stable for at least 48h, until the culture was fixated. This multitude of vesicles could adversely influence the lipid metabolism because the additional surfaces need lipids to form them.

3.2.4 Superfect® Transfection Reagent

The highest transfection rates were obtained using 20 μL Superfect® Transfection Reagent and 4 μg pcDNA4/HisMax©/lacZ-plasmid per well. This transfection method also appeared to be the most reproducible one. The average transfection rate under the conditions mentioned above and using preconfluent 3T3-L1 cells was 13.0% (standard deviation 2.2%). The death rate could be lowered below 30% during the experiments so that this method produced a higher number of transfected cells than the other methods.
The parameters for an optimal transfection rate vary with confluence, therefore imprecise confluence measurements shift the optimum. Figure 3.2 shows the differences in the number of transfected cells and transfection rate between an experiment conducted at higher (0.5) and at lower (0.2) confluence.

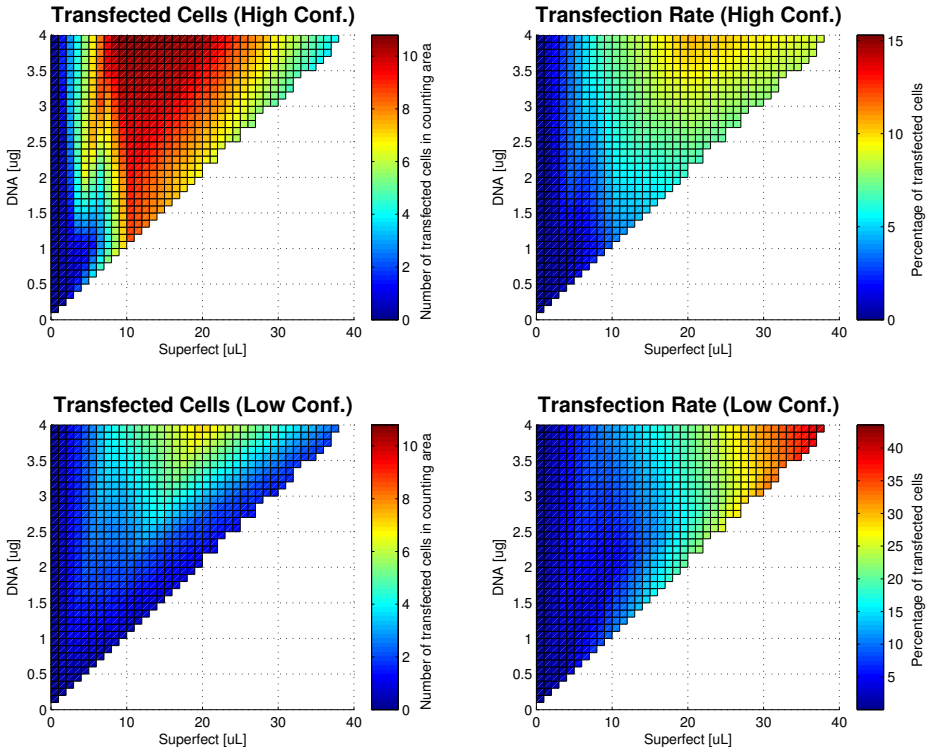


Figure 3.2: Impact of confluence on transfection experiment results.

Although the transfection rate is much higher at low confluence, the total number of transfected cells per area is lower. Transfections at lower cell density kill nearly all cells, but the few surviving cells are more likely to be transfected thus the transfection rates are very high.

The transfection method using Superfect® Transfection Reagent and the pEGFP-C1-plasmid yielded transfection rates of about 5% (only 1/3 compared to pcDNA4/HisMax©/lacZ). Transfection rates are known to vary when different plasmids are used but the change could also be linked to problems with the fluorescence microscopy equipment (see 4.2.6 for discussion).

3.2.5 Effectene® Transfection Reagent

The transfection method using Effectene® Transfection Reagent was able to produce high transfection rates (20%) but only at very high death rates. At death rates comparable to other methods this method did not perform better (12% transfection at 54% death rate). With differentiated cells the rate was as low as with other methods (around 1%).

3.3 Slide Transfections

Growth tests with 3T3-L1 cells on coated slides without spots showed that the cells adhere willingly to the poly-L-lysine and CMT-GAPS surfaces. Bacterial or fungal contaminations were no problem during 7 days of incubation in standard DMEM^{+}.

It was possible to reverse transfect cells on the slide using the pcDNA4/HisMax©/lacZ-plasmid and stain them using the X-Gal staining protocol. The blue stain was visible under the microscope but also with the GenePix Model 4000B Microarray Scanner(see figure 3.3).

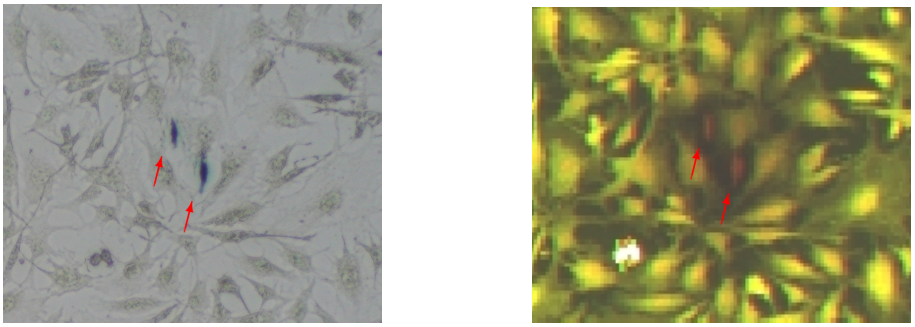


Figure 3.3: X-Gal positive cells (indicated by red arrows) under the microscope and in the scanner

In one experiment Superfect® Transfection Reagent and Effectene® Transfection Reagent activated DNA embedded in gelatin was spotted and cells were seeded onto these slides immediately after spotting. This method failed to produced a single transfected cell, the presence of gelatin hindered the reagent mediated transfection.

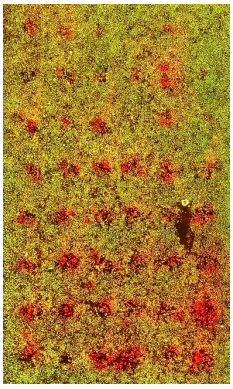
The method using spotted DNA-slides that were activated just before use proofed more successful. Two different transfectants were tested, Effectene® Transfection Reagent and TransMessenger™ Transfection Reagent. Both reagents were able mediate transfections but Effectene® Transfection Reagent produced less transfected cells and showed a bad spot-spot and inter-experiment reproducibility.

Because of these findings TransMessenger™ Transfection Reagent was used to do further optimisation. Two different concentrations were tested (slide activation with 7 μL and 16 μL TransMessenger™ Transfection Reagent, the higher one produced nearly 100 times more transfected cells.

The comparison of GAPS-slides and poly-L-lysine slides showed differences regarding the reproducibility. Spots with the same composition but at

distant positions on the spotting grid showed comparable numbers of transfected cells when poly-L-lysine slides were used. On GAPS-coated slides the DNA-activation or another reaction (e.g. adsorption of reagent to the activated surface) seems to be too fast, so that the DNA-spots where the transfection mix was added to the slide, were able to produce transfected cells. Spots that had no contact to the transfection mix until the coverslip was placed on the slide, which is usually 15 seconds after addition of the transfection mix, contained less transfected cells than spots with the same composition that were activated 15 seconds earlier.

A nonlinear dependency between spotted DNA concentration and number of transfected cells was found. Increasing the amount of DNA in the spotting solution up to 50 ng/μL increased the number of transfected cells rather linearly, above that concentration the increase got smaller, and concentrations of 250 ng/μL pcDNA4/HisMax©/lacZ could not be bound to the slide any more, thus resulting in transfected cells beside the spot. Best results were obtained using 50 to 65 ng/μL pcDNA4/HisMax©/lacZ and 0.2% gelatin, yielding 11 transfected cells per spot on average. The data readout for one slide is shown in figure 3.4.



6	7	1	0	8	3
0	6	7	9	3	0
10	8	14	2	15	3
9	12	9	14	14	8
10	9	13	11	8	25
12	12	15	11	11	9
16	8	13	10	7	28
7	5	13	20	9	19

DNA [ng/μL]	Cells/Spot
20	4.0
30	6.7
40	12.0
50	10.3
65	15.0
80	16.0
100	14.7

Figure 3.4: Reverse transfection experiment readout: 1) scanning of slide, 2) counting of transfected cells per spot, 3) calculation of the average of spots with same composition

Different concentrations of gelatin were tested, good results were found with concentrations ranging from 0.2 to 0.22%. The influence of the gelatin concentration, if kept in this range, on the number of transfected cells per spot was below statistical significance. DNA-gelatin spotting solutions with less than 0.2% gelatin had a negative effect on the transfection rate. Spots from a solution with 0.17% gelatin produced only 50% of transfected cells compared to spots containing 0.2% gelatin solution.

Slide activation was done with 100 μL activation mix, 50 μL were tested but yielded lower transfection rates together with high variability between spots of same composition. The mechanical handling of slide and coverslip with that low amount of mix in between was very difficult: it was impossible to move the cover slip from the position where it was placed on the slides, air bubbles stuck readily to the gel spots and could not be removed.

Spots with pEGFP-C1 plasmid DNA at 100 ng/μL could successfully transfect cells but the transfected cells also occurred beside the spots, the small pEGFP-C1 plasmid (4.7kb) seemed to be more mobile than larger pcDNA4/HisMax©/lacZ plasmid (8.3kb).

The slide fixation was critical to the outcome of the experiment. Although glutaraldehyde worked best, it produced a very high background fluorescence in the green channel thus making the discovery of GFP-positive cells impossible. Fixation with formalin 10% allowed readout of GFP-fluorescence but destroyed the β-galactosidase activity. Unfixated cells changed their morphology and tended to burst. UV crosslinking did not work either, the slides behaved like unfixated ones.

Chapter 4

Discussion

4.1 qPCR

4.1.1 General Aspects

Samples for qPCR standard curves are serial dilutions of DNA containing the target sequence to be amplified by the tested primer set. Usually some abundant DNA is used for the samples, the RT-PCR measurement afterwards yields precise information about the PCR efficiency, but not more. The target concentration in the calibrator cDNA sample is determined in different reactions (e.g. triplicate).

In the experiments discussed here, the calibrator cDNA was used for the standard curve samples. The idea proofed to be useful because it helped to improve the precision of the calibrator measurement: Standard setups use 3 replicates are to determine the calibrator concentration. By using calibrator cDNA for the standard curve samples, the standard curve data can be used to calculate the primer efficiency *and* the calibrator concentration. The 11 RT-PCR reactions per standard curve help also to improve the precision of the calibrator concentration measurement, what could be observed analysing multiple 18S-rRNA quantifications.

Although standard curves can increase precision, there are also two drawbacks:

- standard curve samples need large amounts of cDNA (about 10 times more than for a run with only a calibrator triplicate)
- the standard curve samples need space on the optical reaction plate

4.1.2 Properties of LUX™Primers

The quality of the data obtained using LUX™Primers regarding fluorescence intensity increase upon extension was below the values mentioned by the manufacturer. This is mainly because it was not possible to take full advantage of the LUX™Primers on the AbiPrism7000 machine. There were two main problems:

- Since LUX™Primers are quenched due to their secondary structure, they are highly sensitive to variations in the thermoblock temperature. A higher temperature will unquench the fluorophore even if the primer was not incorporated into an amplicon. Therefore small temperature variations, like they are more common in older machines, will cause changes in fluorescence intensity and reduce the precision of the analysis.

- The AbiPrism Sequence Detection Software in combination with the AbiPrism7000 does not allow to specify the exact time of the intensity measurement, it will always perform the measurement in the last of the repeated steps in the cycle, during elongation time. The high temperature during elongation time compared to the anneal step increases the fluorescence of free LUX™Primers, thus creating a high background fluorescence. The only way to overcome this drawback are firmware and software updates from the instrument manufacturer.

4.1.3 Data analysis

The use of Pearson's RSQ function to assess the correlation between the qPCR and the microarray datasets was problematic: The Pearson formula considers the distribution of the values and therefore may produce misleading results on non-gaussian distributed data. If e.g. the data contain one outlying datapoint then the RSQ function will report a very good correlation for the dataset but the value is mainly influenced by the outlier. A different approach is to use the RSQ function on logarithmic data, but this may pretend good correlation if the values show a common trend but reduce the effect of outliers to make the correlation results better comparable.

The comparison of microarray data to the qPCR data was done using RSQ-function on logarithmic data. The slope should be 1 (see figure 3.1), but is below. One reason is that spots of strongly down-regulated genes show a low fluorescence intensity near the background levels. Hence incomplete background subtraction causes underestimation of gene down-regulation, these points then reduce the slope of the regression line.

The regression line intercept of -0.36 instead of 0 shows that there is a systematic error in the analysis method. Two possible explanations exist:

- The microarray data was analysed using global normalisation. The method assumes, that the mRNA-level in average do not change (the average log ratio is 0). If this assumption is violated, the method may introduce a systematic bias into the data.
- The reference gene (18S rRNA) amount per cell changes between reference (confluent cells) and samples.

4.2 Transfections

4.2.1 General aspects of transfections

Reproducibility

From first experiments one could deduce that transfection methods generally have poor reproducibility. Subsequent experiments showed other interesting trends: The death rate decreased from one transfection experiment to the next and the parameters for optimal transfection efficiency stabilised. The late experiments showed the highest transfection rate always at the same parameters. These changes seem to be mainly a consequence of the increased speed of liquid handling, pipetting and other manual operations so that the negative effects onto the cells decreased.

These trends were visible with all transfection methods. It seems that cell culture experiments could be done in a reproducible manner but it is very hard to control the parameters that affect the outcome e.g. the experimenter's skills that cannot be quantified and which continuously increase during the work.

Media

Some transfection method protocols recommend the use of serum-free or antibiotics free transfection media formulations to increase efficiency. They also recommend to check the influences to decide which media suits best. The following list contains the tested media compositions used during the transfection experiment.

- Superfect® Transfection Reagent: Preparation of the transfection mix was done in serum- and antibiotics-free media as suggested by the manual.

- Effectene® Transfection Reagent: Transfection mix preparation was done in a buffer solution supplied by the manufacturer.
- Lipofectamine™ Reagent: Transfection complex formation was done in serum-free media, with and without antibiotics, respectively. The transfection rate without antibiotics was 8 times higher than in the other mix. Transfection process itself occurs in serum-free media.
- Lipofectamine™2000 Reagent: Transfection mix preparation was done in media without antibiotics, once including serum and once without. The two transfections yielded similar transfection efficiencies. The transfection itself was done in antibiotics-free media including serum.

Transfections in serum-free media i.e. with Lipofectamine™ Reagent can be problematic if the transfection procedure takes time and gene regulation is of interest because serum deprivation changes the regulation patterns since serum contains numerous growth hormones, some of them are still unknown [17].

Transfection Mechanism

A key component for transfection optimisation is the understanding of the underlying transfection mechanism.

- Kinetics: Transfections need complex interactions between the biological system (the cells) and chemical compounds (DNA, buffers, activators) but nevertheless it is possible to find correlations between the amount of substances and cells used and the results.

  With Superfect® Transfection Reagent for example, the transfection rate and death rate depends on the amount of complexes per cell and not on the complex concentration in the media. If cells are seeded with lower density but the complexes in the media have the same concentration then nearly all cells are killed while at recommended density only 30% would have been killed. This matches quite well the information from papers that suggest that most of the complexes are taken up via endocytosis, therefore cells at lower cell density have to take up more complexes per cell [22]. After incubation of the cells with the transfection media, the media is depleted of active complexes no matter how many cells were seeded.

  This shows that the reaction kinetics are a way to make assumptions on the underlying mechanism and possibly use them to optimise the transfections or reduce the transfection parameter space.

- Cell Cycle Synchronisation: All the transfection reagents used during this work mediated the endosomal uptake of DNA and delivered the DNA with great losses into the cytoplasm. One main problem with these methods seemed to be the transport of the DNA into the nucleus, mainly because of two reasons:
  - The cellular environment with a high content of macromolecules lets the cytoplasm act more like an agarose gel then a liquid media. So DNA-molecules above a certain mass are nearly immobile by diffusion and need much time to reach the nucleus [12].
  - The DNA has to get behind the nuclear membrane to get successfully transcribed.

  These two obstacles can be overcome during mitosis when the nuclear membrane is disintegrated. Shortly after transfection only paired cells express the plasmid-coded protein which corresponds to the theoretical background, that the entry of plasmid DNA through pores into the nucleus is a rare event and that it is more likely for plasmids to get into it when the nuclear membrane is reconstructed after mitosis.

Plasmid Influence

The plasmid used (pEGFP-C1 or pcDNA4/HisMax©/lacZ) to transfect the cells influenced the outcome of the experiment in many ways: the transfection rate with the same amount of plasmid (see transfection reagent discussion), the readout efficiency (see readout discussion) and the effect of the encoded protein on the cell was different.

Protein overexpression is an unnatural burden to the cell and does harm to the cell's metabolism if continued for many hours. This is mainly a problem when using the pEGFP-C1-plasmid, because high protein levels are needed to allow direct fluorescence detection.

3 hours after transfection the GFP-expressing cells occur only in pairs and there are no floating (dead) cells expressing the protein. The time for cells exposed to the protein was simply too short to damage the cells.

After 48h many of the pEGFP-C1-transfected cells were in groups of 3 or 4 cells because this time allowed 2 additional rounds in the cell cycle (see figure 4.1). The number was often below 4 because the continued overexpression of GFP promoted cell death. This was also the cause for the high number of GFP-positive cells floating in the medium (see [11]).

Figure 4.1: Dividing GFP-positive cells

4.2.2 Superfect® Transfection Reagent

The Superfect® Transfection Reagent consists of dendrimers with a rather fixed structure and it interacts with the DNA without the need of any complementary components. The chemically well-defined transfection mixture is perhaps the reason why these transfections were the most reproducible.

The toxic component in the transfections seems to be the Superfect® Transfection Reagent itself, transfections with equal amounts of Superfect® Transfection Reagent but different amounts of DNA resulted in similar death rates. Perhaps the effect is due to the buffering capacity of the dendrimere, thus changing the pH in endosomes on uptake, irrespective of the DNA bound.

Leaving the transfection mix on the cells increased the death rate significantly but only resulted in non-significant increase in transfection efficiency. It seems that the cells take up the Superfect® Transfection Reagent completely if left on the cells. This would also explain why the same amount of transfection mix with exactly the same composition is far more toxic for cells at lower confluence: the amount of complexes taken up by each cell is higher if the number of cells is smaller.

That also seems to be the cause why a 4 minute glycerol shock after treatment with Superfect® Transfection Reagent only increases the death rate but not transfection rate: the DNA-complexes are consumed or inactive so the transfection rate cannot be increased by glycerol-mediated uptake and so the toxic effects of this treatment dominate.

The transfections also worked also with the pEGFP-C1-plasmid. Although the pcDNA4/HisMax©/lacZ and the pEGFP-C1-plasmid showed different transfection rates, however the toxicity of the reagent was still comparable. This shows that the toxicity is mainly due to the Superfect® Trans-

fection Reagent.

The most interesting effect using Superfect® Transfection Reagent with pEGFP-C1 was that only 3 hours after transfection the first GFP-positive cells showed up. At this early stage the few positive cells occurred only in pairs, thus showing the all the transfected cells were strictly synchronised and had undergone mitosis since the start of the transfection procedure.

4.2.3 Lipofectamine™ Reagent

The Lipofectamine™ Reagent did not produce visible abnormalities in cell morphology but seemed to be very toxic. Even the optimisation of the lipofectamine-reagent/DNA ratio did not yield good transfection conditions and therefore the very high death-rate compared to the other methods was not acceptable.

4.2.4 Lipofectamine™2000 Reagent

The Lipofectamine™2000 Reagent-mediated transfection reagents promoted the formation of vesicles that looked like fat droplets, but the Oil-Red O staining did not stain them. The vesicles seem to gather around the nucleus but this effect may be due to the much greater cell thickness near the nucleus and so the number of vesicles per area in through light microscopy seems to be greater there (see fig. 4.2) and the number of vesicles showed no obvious correlation to the cell morphology: cells with and without vesicles had good contact to the surface, fibroblastic cell shape and a clearly visible, structured nucleus. Even the transfected cells had the same variation in the internal vesicle density.

The vesicles density in the cell at the time of the fixation (2 days after transfection) varied greatly. Some cells were nearly completely filled with vesicles, others showed no vesicles.

The vesicles cannot be fixation or staining artefacts because wells without Lipofectamine™2000 Reagent were fixated and stained in parallel and not a single cell was filled with vesicles. However in the Lipofectamine™2000 Reagent treated wells the vesicles were in some cells that numerous so that they filled a considerable amount of the cytoplasm and therefore may contain a nonneglectable amount of membrane lipids.

Since the transfection method should be suitable for experiments on fat cell differentiation, any interference with the fatty acid metabolism could be problematic. Due to this possible drawback within this experimental setup, this transfection reagent was discarded from the list of possible transfectants to use.

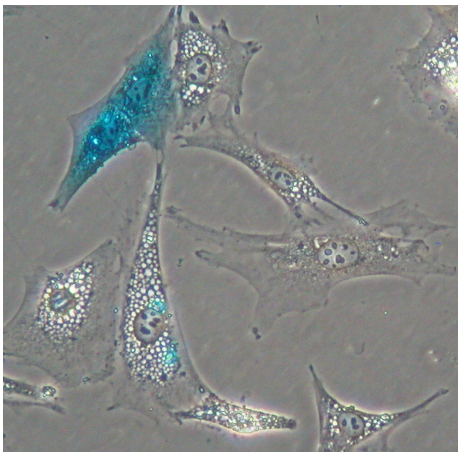


Figure 4.2: Vesicle formation with Lipofectamine™ 2000 Reagent

4.2.5 Calcium-phosphate Precipitation Method

With the 3T3-L1 cell line the glycerol shock was the only way to get detectable transfection efficiencies, therefore a statistically significant discussion of transfection efficiencies without glycerol is not possible.

The glycerol solution alone was not toxic to the cells if exposed to it for up to 20 minutes, the osmotic stress and the intracellular glycerol did not kill cells. The toxicity seems to be a synergistic effect together with other toxic reagents.

Together with the transfection mix, glycerol killed about 25% more cells compared to a well with the same transfection mix but without glycerol, but usually increased the transfection efficiency 10-fold. This leads to the conclusion that only glycerol solution together with transfection complexes significantly increase the uptake of complexes.

There seems to be a correlation between the duration of the glycerol shock and the transfection rate. While with 1 minute shock duration about 0.7% of the cells got transfected, a 4 minute shock produced 3.2% of transfected cells (a 4.4-fold increase). It was unexpected to find that the different glycerol shock durations did not yield significantly different death rates. Since the amount of transfection complex uptake changed, the toxicity has to be due to the first contact of glycerol solution and cells. It was shown in chondrocytes that most severe changes occur during the first 20 seconds after glycerol addition (see [21]). It seems that the complexes destabilise the membrane and the osmotic shock upon glycerol addition breaks it.

From the data it is not clear if 3h or 5h incubation of the cells with

transfection mix before glycerol addition is better, the influence (if any) is definitely below the regular experimental variation.

Finally the data also show the handling difficulties of the calcium phosphate precipitate transfection with glycerol shock: in 5 out of 6 comparable experiments, the addition of glycerol increased the transfection rate in a reproducible manner. In one experiment the addition of glycerol failed to produce the expected effects.

4.2.6 Readout

In general the $\beta -$ galactosidase detection was more sensitive than the GFP-detection. This was due to the indirect detection method using the enzymatic activity of $\beta -$ galactosidase to produce a coloured product.

One strange finding was that differentiated but non-transfected cells produced a much higher blue background after X-Gal staining compared to undifferentiated. The colour seems to originate from some other reaction then the one catalysed by the plasmid encoded β-galactosidase, but is not attached to the fat droplets (see figure 4.3):

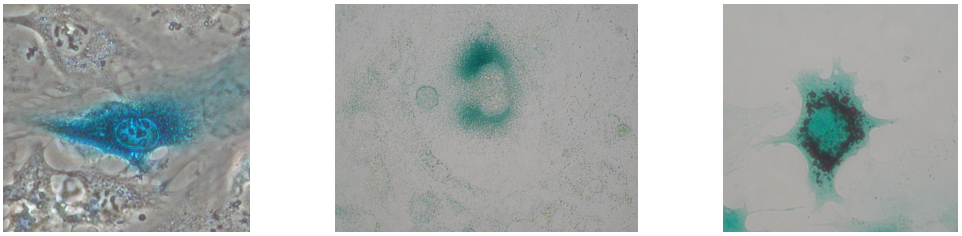


Figure 4.3: Stainings of 3T3-L1 cells: The left picture shows an undifferentiated cell, that was transfected with the lacZ-plasmid and successfully stained with X-Gal (true-positive), the picture in the middle displays a non-transfected adipocyte, that also shows a blue colour when stained with X-Gal (false-positive). The third picture shows an Oil-Red O stained adipocyte (the blue cell body originates from a light-green counter staining, not X-Gal).

The blue colour seems to be enclosed in small vesicles, probably lysosomes. Lysosomes are known to contain acidic β-galactosidase, but normally it should be active at pH 4, not pH 7.4 (the staining buffer pH and the optimum for the bacterial β-galactosidase).

The GFP-fluorescence readout is also problematic. The best time for readout would be 12 to 24 hours after transfection as the GFP-expression and fluorescence intensity is at its peak and cells intoxicated by GFP have

not lost contact to the surface yet [11]. The problem doing the readout after 12 hours is the higher degree of cell cycle synchronisation of the GFP-positive cells. This may lead to an overestimation of transfection efficiency because all positive cells have doubled, while many of the untransfected have not.

Beside that the ambient light in the fluorescence room made it very difficult to detect cells with low fluorescence intensities, therefore the true transfection rate will certainly be higher than the rate observed in these experiments.

4.3 Slide Transfections

The use of non cell culture treated plastic ware has proven to be advantageous over standard cell-culture dishes. The main problem with the cell culture treated dishes is that the cells adhere to the bottom surface after seeding. When the dish is manipulated afterwards, the slides move over the bottom and scrape off the cell layer. These cells may then seat onto the slide's surface and overgrow spots with lower cell densities, thus destroying the results for these spots.

It seems that the detection of X-Gal stained β-galactosidase positive cells with the GenePix scanner was only possible due to the model specific laser optics. The ArrayWoRx$^{©e}$ Biochip Reader set to a close excitation and emission wavelength was not able to detect a signal. The reason could be that the GenePix scanner uses excitation lasers and a photomultiplier detector while the ArrayWoRx$^{©e}$ Biochip Reader uses filtered white light and a CCD-camera. This could be especially problematic for people trying to reproduce the experiments since the exact origin of the signal is not yet known. Although it was possible to show that only cells that are blue under the microscope show a signal in the scanner, it is not clear if the scanner signal depends on the blue stain or the reduced colourless precursor substance. Experiments to show the real cause of the signal would need special photometric measurements to analyse the quenching effects of the colourless precursor or the blue product yielded by by breaking up the X-Gal substrate.

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Appendix A

Appendix

A.1 LUX™ Primer Sequences

Name	Ensemble ID	Amplicon Size	Primers Pos.	Sequence	Label
bteb 1	ENSMUST00000036884	82	499	catacagAAGGGCCGTTCACCTGTAtG	FAM
			464	TGGCTGTGGGAAAGTCTATGG	none
cyclin A2	ENSMUST00000029270	106	1273	gacctaGTGGCGCTTTGAGGTAGGtC	FAM
			1213	CAGAGCTGGCCTGAGTCATTG	none
cmyc 1 [a]	ENSMUST00000022971	97	1295	gactcgCGCTGCTGTCCTCCGAGtC	FAM
			1348	TCAGAGTCGCTGCTGGTGGT	none
cmyc 2	ENSMUST00000022971	102	1295	gactcgCGCTGCTGTCCTCCGAGtC	FAM
			1354	GCTCTTCTTCAGAGTCGCTGCT	none
decorin	ENSMUST00000020087	122	843	caccaaAGGGATCGCAGTTATGTTGGtG	FAM
			768	TCATAGAACTGGGCGGCAAC	none
incenp	ENSMUST00000025562	81	1784	cacgcAGGAGGTGAAGCTGAAGCGtG	FAM
			1818	TCCTTCATCTGTTCCACCCTCT	none
klf5	ENSMUST00000005279	108	1456	gactcatGGCTTCTCGCCCGTATGAGtC	FAM
			1396	AAGCGACGTATCCACTTCTGC	none
mki67	ENSMUST00000033310	82	1521	gacaatcGGGCCGTTCCTTGATGATTGtC	FAM
			1489	GAAACGCCAACCAAGAGGAAGT	none
nur77	ENSMUST00000023779	65	1030	gtacatctCTTGTTTGCCAGGCAGATGtAC	FAM
			1014	GGCTTCTTCAAGCGCACAGT	none
lpl	ENSMUST00000015712	152	330	caaccaAGGTCTTGCTGCTGTGGTtG	FAM
			223	AGCAGACGCGGGAAGAGATT	none
ppargγ	ENSMUST00000000450	132	1213	caccaTGCGGAAGCCCTTTGGtG	FAM
			1303	GGGCGGTCTCCACTGAGAAT	none

Table A.1: LUX™ Primers for qPCR

[a] The unlabelled primer has just one base near its 5´-end across the exon-exon border, so the unlabelled primer was redesigned manually

A.2 qPCR expression profiles

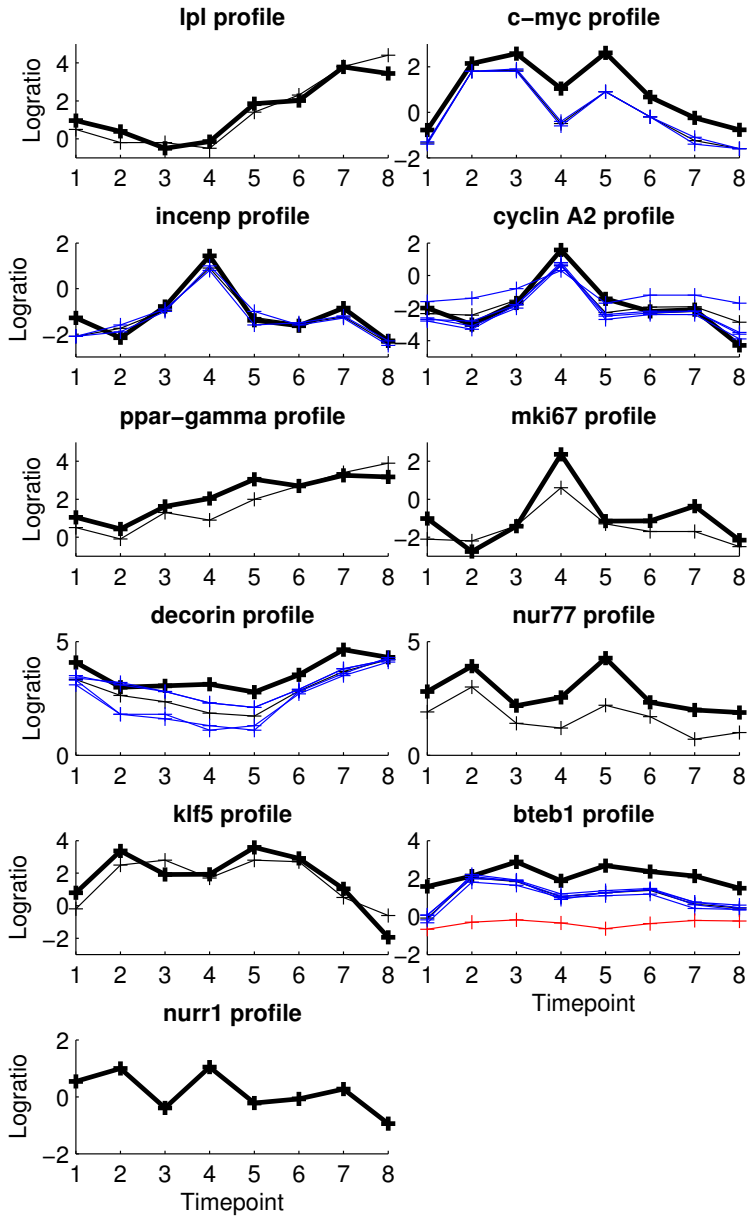


Figure A.1: Comparison of expression profiles from qPCR and microarray data: The bold black line shows the log-ratio from the qPCR data, the thin black line the microarray data log-ratio average of all spotted ESTs and the blue lines show the microarray profiles of different ESTs.

A.3 ExonCheck Software Usage

The ExonCheck software is intended to serve as a tool to find primers that span exon-exon boundaries. The programme does not design the primers itself, it combines data inputs from different sources and displays the results in a way to help the user to select suitable primers.

The first step in this process is to enter the target sequence, in most cases a completely processed mRNA containing exons. The sequence can be loaded online directly from the ENSEMBL database (using the transcript ID) or copied into the input area. Exon-exon boundaries are marked with an *x*.

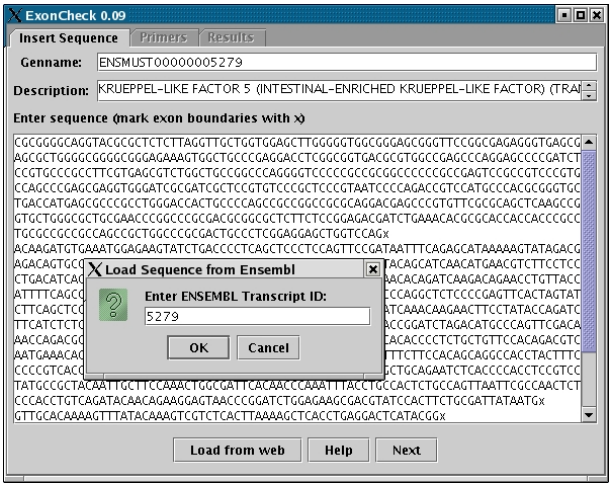


Figure A.2: Loading of a sequence from ENSEMBL

The next step is to enter the primer sequences to visualise. Normally these sequences originate from a primer design program that used the whole mRNA sequence to find suitable primers but did not consider the exon-exon boundaries. The software has special support to parse primer design results from the LUX™ Primer Design Software (Invitrogen) and to display the primers in a colour corresponding to the primer score (some calculated quality value) so that good primers are green and bad ones red.

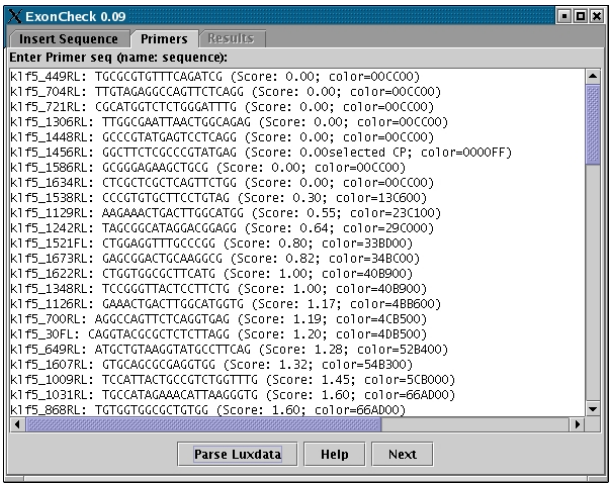


Figure A.3: Input of primer sequences

In the last step the results are generated and can be saved as an HTML report. The exons are coloured alternatingly in blue and black.

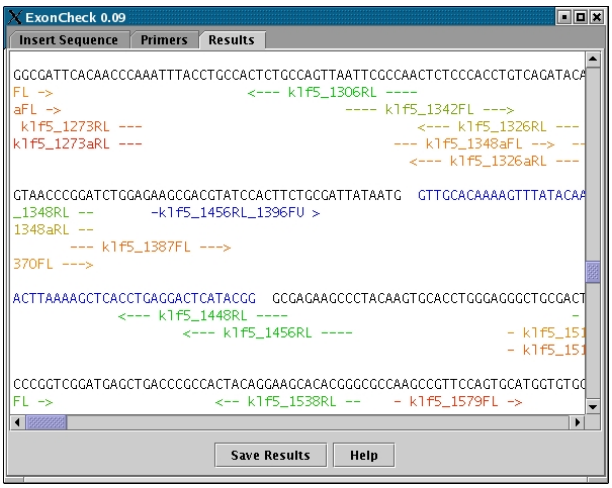


Figure A.4: Results showing primers across an exon-exon boundary

ExonCheck Source:

The source can be retrieved from the institute's CVS-repository with following command:

```
cvs -d :pserver:[username]@majestix:/home/CVS-Repository \\
checkout LuxPrimerExonCheck
```

```
package at.tugraz.genome.luxprimerexoncheck.util;

public class Sequence {

  public String         name;
  public String         description;

  private String        data;


  public Sequence(String name, String description, String data) {
    this.name=name;
    this.description=description;
    this.data=data;

    if(checkSequence(data)!=data.length()) {
      throw new IllegalArgumentException("Invalid char in sequence");
    }
  }


  public String getSequenceData() { return(data); }


  public Sequence getReversedSequence() {
    StringBuffer sbuf=new StringBuffer();

    for(int i=data.length()-1; i>=0; i--) {
      char c=data.charAt(i);
      switch(c) {
        case 'A': sbuf.append('T'); break;
        case 'T': sbuf.append('A'); break;
        case 'G': sbuf.append('C'); break;
        case 'C': sbuf.append('G'); break;
        default:
          throw new IllegalArgumentException("Wrong seq char '"+c+"' in \""+
                                             data+"\".");
      }
    }

    return(new Sequence(name, description, sbuf.toString()));
  }

  public static int checkSequence(String str) {

    for(int i=0; i<str.length(); i++) {
      char c=str.charAt(i);
      switch(c) {
        case 'A':
        case 'T':
        case 'G':
        case 'C':
          break;
        default:
          return(i);
      }
    }

    return(str.length());
  }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.util;

public class ExonSequence {

  public String         name;
  public String         description;

  private Sequence      exons[];

  public ExonSequence(String name, String description, Sequence exons[]) {
    this.name=name;
    this.description=description;
    this.exons=new Sequence[exons.length];
    System.arraycopy(exons, 0, this.exons, 0, exons.length);
  }


  public int getExonCount() { return(exons.length); }

  public Sequence getExon(int pos) {
    return(exons[pos]);
  }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.util;

import java.awt.Color;


/** Objects containing all necessary information about
 *  one unaligned primer
 */
public class Primer {
  public Sequence       sequence;
  public Sequence       revsequence;

  public String         comment;
  public Color          color;


  public Primer(Sequence seq) {
    sequence=seq;
    color=Color.black;
  }

  public Primer(String data) {
    String name=null;
    String sdata=null;

    int i=data.indexOf(':');
    if(i<0) name="";
    else {
      name=data.substring(0, i);
      data=data.substring(i+1).trim();
    }

    i=data.indexOf('(');
    if(i>=0) {
      sdata=data.substring(0, i).trim();
      data=data.substring(i).trim();
      //ARM ("#: braksplit to \""+sequence+"\" and \""+data+"\".");
    } else {
      sdata=data;
      data=null;
    }

    sequence=new Sequence(name, null, sdata);

    color=Color.black;

    if(data!=null) {
// parse the comment, has to be in brakets
      if((data.charAt(0)!='(')||(data.charAt(data.length()-1)!=')')) {
        throw new IllegalArgumentException("Missing () in comment");
      }

      data=data.substring(1, data.length()-1).trim();
      while(data.length()!=0) {
        i=data.indexOf(';');
        String test=data;
```

```
        if(i<0) data="";
        else {
          data=data.substring(i+1).trim();
          test=test.substring(0, i).trim();
        }

        if(test.startsWith("color=")) {
          test=test.substring(6).trim();
          color=new Color(Integer.parseInt(test, 16));
          continue;
        }

        if(comment==null) comment=test;
        else comment=comment+test;
      }
    }
  }



  public Sequence getSequence() {
    return(sequence);
  }


  public Sequence getReversedSequence() {
    if(revsequence==null) revsequence=sequence.getReversedSequence();
    return(revsequence);
  }



  public String format(int len, boolean reverse) {
    StringBuffer sbuf=new StringBuffer();

    int freespace=len-sequence.name.length();
    String nstr=sequence.name;

    if(freespace<=1) {
      nstr=sequence.name.substring(0, len-1);
      freespace=1;
    }

    int prelen=freespace>>1;
    int postlen=prelen;
    if(prelen+postlen!=freespace) {
      if(reverse) prelen++;
      else postlen++;
    }

    for(int i=0; i<prelen; i++) {
      if(i==0) {
        if(reverse) sbuf.append('<');
        else sbuf.append('-');
      } else if(i+1==prelen) sbuf.append(' ');
      else sbuf.append('-');
    }

    sbuf.append(nstr);

    for(int i=0; i<postlen; i++) {
      if(i+1==postlen) {
        if(reverse) sbuf.append('-');
        else sbuf.append('>');
      } else if(i==0) { sbuf.append(' ');
      } else {
        sbuf.append('-');
      }
    }

    return(sbuf.toString());
  }


  public String toString() {
    StringBuffer sbuf=new StringBuffer();
    sbuf.append(sequence.name).append(": ").append(sequence.getSequenceData());

    boolean commentstarted=false;
    if((comment!=null)&&(comment.length()!=0)) {
      if(!commentstarted) { sbuf.append(" ("); commentstarted=true; }
      else sbuf.append("; ");
      sbuf.append(comment);
    }
    if((color!=null)&&(!Color.black.equals(color))) {
      if(!commentstarted) { sbuf.append(" ("); commentstarted=true; }
      else sbuf.append("; ");
      sbuf.append("color=").
           append(Integer.toHexString(color.getRGB()|0xff000000).
                          substring(2).toUpperCase());
    }
    if(commentstarted) sbuf.append(')');
    return(sbuf.toString());
  }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.util;

/** This class holds one alignment (identity alignment, not
 *  basepaired.
 */
public class SequenceAlignment {

  private Sequence      aligned_sequence;
  private Sequence      base_sequence;

  private int           location;
  private int           length;

// private int          aligninfo[];


  public SequenceAlignment(Sequence aseq, Sequence bseq, int location) {
    aligned_sequence=aseq;
    base_sequence=bseq;

    this.location=location;
    this.length=aseq.getSequenceData().length();
  }

  public Sequence getASequence() { return(aligned_sequence); }

  public int getLocation() { return(location); }
  public int getLength() { return(length); }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.swing;

import java.awt.BorderLayout;
import java.awt.Color;
import java.awt.Dimension;
import java.awt.Font;
import java.awt.GridBagConstraints;
import java.awt.GridBagLayout;
import java.awt.event.ActionEvent;
import java.awt.event.ActionListener;
import java.awt.event.MouseEvent;
import java.awt.event.MouseListener;

import java.io.ByteArrayOutputStream;
import java.io.FileInputStream;
import java.io.FileOutputStream;
import java.io.InputStream;
import java.io.IOException;

import java.net.HttpURLConnection;
import java.net.URL;
import java.net.URLConnection;



import java.util.ArrayList;
import java.util.Hashtable;

import javax.swing.JButton;
import javax.swing.JEditorPane;
import javax.swing.JFileChooser;
import javax.swing.JFrame;
import javax.swing.JLabel;
import javax.swing.JMenuItem;
import javax.swing.JOptionPane;
import javax.swing.JPanel;
import javax.swing.JPopupMenu;
import javax.swing.JTextArea;
import javax.swing.JTextField;
import javax.swing.JTabbedPane;
import javax.swing.JScrollPane;
import javax.swing.text.BadLocationException;

import at.tugraz.genome.luxprimerexoncheck.util.*;

/** Disable following tabs, use next button
 *  First Pane: type data sequence or load it
 *i
 */
public class ExonCheckGUI implements ActionListener, MouseListener {

  private JFrame                frame;

  private JTabbedPane           tabpane;
  private JTextField            sequence_name;
  private JTextArea             sequence_description;
```

```
  private JTextArea             sequence_text;

  private JTextArea             primer_text;

  private JEditorPane           results_text;

  private ExonSequence          exondata;
  private Primer                primerlist[];

  private SequenceAlignment     alignments[];

  public static void main(String args[]) {
    new ExonCheckGUI();
  }

  public ExonCheckGUI() {
    frame = new JFrame("ExonCheck 0.09");
    frame.setDefaultCloseOperation(frame.EXIT_ON_CLOSE);

    tabpane=new JTabbedPane();
    tabpane.setOpaque(true);
    frame.setContentPane(tabpane);


// panel to enter the sequence

    JPanel sequence_panel=new JPanel();
    GridBagLayout gbl=new GridBagLayout();
    GridBagConstraints gbc=new GridBagConstraints();
    sequence_panel.setLayout(gbl);

    gbc.gridx=0;     gbc.gridy=0;


    gbc.gridwidth=1; gbc.gridheight=1;
    gbc.weightx=0.0;   gbc.weighty=0;
    gbc.fill=gbc.NONE; gbc.insets=new java.awt.Insets(4, 4, 4, 4);
    JLabel label = new JLabel("Genname:");
    sequence_panel.add(label, gbc);

    gbc.gridy++;
    label = new JLabel("Description:");
    sequence_panel.add(label, gbc);

    gbc.gridy--;
    gbc.gridx++;
    gbc.weightx=1.0;
    gbc.fill=gbc.HORIZONTAL;
    sequence_name=new JTextField("testgen");
    sequence_panel.add(sequence_name, gbc);

    gbc.gridy++;
    sequence_description=new JTextArea();
    sequence_description.setRows(2);
    JScrollPane scroll = new JScrollPane(sequence_description);
    scroll.setHorizontalScrollBarPolicy(scroll.HORIZONTAL_SCROLLBAR_NEVER);
    sequence_panel.add(scroll, gbc);

    gbc.gridwidth=2;
    gbc.gridx--;
    gbc.gridy++;
    label = new JLabel("Enter sequence (mark exon boundaries with x)");
//    gbl.setConstraints(label, gbc);
    sequence_panel.add(label, gbc);



    sequence_text=new JTextArea();
    sequence_text.setFont(new Font("monospaced", Font.PLAIN, 12));
    sequence_text.setToolTipText(
                   "Enter sequence (mark exon boundaries with x)");
    scroll = new JScrollPane(sequence_text);

    gbc.gridy++; gbc.weighty=1.0;
    gbc.fill=gbc.BOTH;
    sequence_panel.add(scroll, gbc);

    JPanel panel=new JPanel();
//    panel.add(makeButton("Load from file", "seq_load_file"));
    panel.add(makeButton("Load from web", "seq_load_web"));
    panel.add(makeButton("Help", "seq_show_help"));
    panel.add(makeButton("Next", "seq_nexttab"));

    gbc.gridy++;
    gbc.weighty=0.0;
    gbc.fill=gbc.NONE;
    sequence_panel.add(panel, gbc);
    tabpane.addTab("Insert Sequence", sequence_panel);



// panel to enter the primer and checksequences

    JPanel primer_panel=new JPanel();
    BorderLayout bdl=new BorderLayout();
    bdl.setHgap(3);
    bdl.setVgap(3);
    primer_panel.setLayout(bdl);

    label=new JLabel("Enter Primer seq (name: sequence):");
    primer_panel.add(label, BorderLayout.NORTH);

    primer_text=new JTextArea();
    primer_text.setFont(new Font("monospaced", Font.PLAIN, 12));
    primer_text.addMouseListener(this);
    primer_text.setToolTipText("Rightclick to add or edit primers");
    scroll = new JScrollPane(primer_text);
    primer_panel.add(scroll, BorderLayout.CENTER);

    panel=new JPanel();
    panel.add(makeButton("Parse Luxdata", "primer_parselux"));
    panel.add(makeButton("Help", "primer_show_help"));
    panel.add(makeButton("Next", "primer_nexttab"));
    primer_panel.add(panel, BorderLayout.SOUTH);
    tabpane.addTab("Primers", primer_panel);
    tabpane.setEnabledAt(1, false);


    JPanel result_panel=new JPanel();
    bdl=new BorderLayout();
    bdl.setHgap(6);
    bdl.setVgap(6);
    result_panel.setLayout(bdl);

    results_text=new JEditorPane("text/html", null);
    results_text.setEditable(false);
//    results_text.setFont(new Font("monospaced", Font.PLAIN, 12));
    scroll = new JScrollPane(results_text);
    result_panel.add(scroll, BorderLayout.CENTER);

    panel=new JPanel();
    panel.add(makeButton("Save Results", "result_save"));
    panel.add(makeButton("Help", "result_show_help"));
    result_panel.add(panel, BorderLayout.SOUTH);

    tabpane.addTab("Results", result_panel);
    tabpane.setEnabledAt(2, false);

    frame.setSize(new Dimension(800, 600));
//    frame.pack();
    frame.setVisible(true);
  }



  public JButton makeButton(String text, String cmd) {
    JButton button=new JButton(text);
    button.setActionCommand(cmd);
    button.addActionListener(this);
    return(button);
  }


  private final static String ENSEMBL_TRANSCRIPT_FIELD_START_STRING=
  "<th width=\"20%\">Ensembl Transcript ID</th>\n    \t<td width=\"80%\"><b>";

  private final static String ENSEMBL_DESCRIPTION_FIELD_START_STRING=
              "<th width=\"20%\">Description</th>\n    \t<td width=\"80%\">";


/** Try to parse the Sequence
 *  @returns a hint for name description and the exon parts in
 *  the string array
 */
  public ExonSequence parseExonSequence(String sequence, String format)
                                    throws IllegalArgumentException {
    ArrayList exonlist=new ArrayList();
    String name=null;
    String description=null;

    if("fips_format".equals(format)) {
      StringBuffer sbuf=new StringBuffer();
      for(int i=0; i<sequence.length(); i++) {
        char c=sequence.charAt(i);
        if(c<=0x20) continue;

        switch(c) {
          case 'x':
            if(sbuf.length()>0) exonlist.add(sbuf.toString());
            sbuf.setLength(0);
            break;
          case 'A':
          case 'T':
          case 'G':
          case 'C':
            sbuf.append(c);
            break;
          default:
            throw new IllegalArgumentException("Illegal character "+c+" in seq.");
        }
      }
      if(sbuf.length()>0) exonlist.add(sbuf.toString());
    } else if("exonview_format".equals(format)) {

      if(sequence.indexOf("<h3><h3>Ensembl Transcript Report</h3></h3>")<0) {
        throw new IllegalArgumentException(
                  "Page is not a valid Ensembl Transcript Report");
      }

// extract name from page, this are the fields
// Transcript  and  Ensembl Transcript ID

      int i=sequence.indexOf(
          "<H4>Transcript</H4></td>\n        <td class=\"background1\"><b>");
      if(i>=0) {
        int ne=sequence.indexOf("</b>", i+60);
        name=sequence.substring(i+60, ne);
      }

// name: copy the transcript ID again to have foolproof output
// with the ID of the page really fetched
      i=sequence.indexOf(ENSEMBL_TRANSCRIPT_FIELD_START_STRING);
      if(i>=0) {
        i+=ENSEMBL_TRANSCRIPT_FIELD_START_STRING.length();
        int ne=sequence.indexOf("</b>", i);
        if(name==null) name=sequence.substring(i, ne);
        else name=name+" "+sequence.substring(i, ne);
      }
      if(name!=null) name=name.replaceAll(" ", " ").trim();

// extract description:
```

```
      i=sequence.indexOf(ENSEMBL_DESCRIPTION_FIELD_START_STRING);
      if(i>=0) {
        i+=ENSEMBL_DESCRIPTION_FIELD_START_STRING.length();
        int de=sequence.indexOf('<', i);
        description=sequence.substring(i, de).replaceAll(" ", " ").trim();
      }
//      System.err.println("Description tag is "+description+", offs "+i);

      int seqstart=sequence.indexOf("<pre class=\"snpmarkup\">");
      int seqend=sequence.indexOf("</pre>");
      sequence=sequence.substring(seqstart+23, seqend);


      //TRACE ("#: sequence data is \""+sequence+"\"-END");

      StringBuffer currexon=new StringBuffer();
      String exoncolor="normal";
      String color=exoncolor;

      while(sequence.length()!=0) {
        for(i=0; i<sequence.length(); i++) {
          if(sequence.charAt(i)>0x20) break;
        }
        if(i>0) sequence=sequence.substring(i);

        if(sequence.length()==0) break;

        for(i=0; i<sequence.length(); i++) {
          char c=sequence.charAt(i);
          if((c!='A')&&(c!='T')&&(c!='G')&&(c!='C')) break;
        }
        if(i>0) { // bases
          if(!exoncolor.equals(color)) {
            exoncolor=color;
            if(currexon.length()!=0) exonlist.add(currexon.toString());
            currexon.setLength(0);
          }

          currexon.append(sequence.substring(0, i));
          sequence=sequence.substring(i);
          continue;
        }

        i=sequence.indexOf("<font");
        if(i==0) { // start of font
          color="abnormal";
          i=sequence.indexOf('>');
          sequence=sequence.substring(i+1);
          continue;
        }

        i=sequence.indexOf("</font>");
        if(i==0) { // start of font
          color="normal";
          sequence=sequence.substring(7);
          continue;
        }

        //TRACE ("#: Illegal string start \""+sequence+"\".");
        break;
      }
      if(currexon.length()!=0) exonlist.add(currexon.toString());


    } else {
// unknown format
      throw new IllegalArgumentException("Unknown format "+format);
    }

    if(exonlist.size()==0) return(null);

    Sequence esd[]=new Sequence[exonlist.size()];
    for(int i=0; i<esd.length; i++) {
      esd[i]=new Sequence("Exon "+(i+1), null, (String)exonlist.get(i));
    }

    ExonSequence es=new ExonSequence(name, description, esd);
    return(es);
  }



  public Primer[] parsePrimers(String data, String format)
                                throws IllegalArgumentException {
    ArrayList primerlist=new ArrayList();

    if("fips_format".equals(format)) {
// split into lines

      while(data.length()!=0) {
        int i=data.indexOf('\n');
        String line=(i<0)?data.trim():data.substring(0, i).trim();
        data=(i<0)?"":data.substring(i+1);

        if(line.length()==0) continue;
        primerlist.add(new Primer(line));
      }
    } else if("lux_format".equals(format)) {

// parsing will only use lines with Forward/Reverse and tab in the line
      int discardedlines=0;

      for(int spos=0; spos<data.length(); ) {
        int next=data.indexOf('\n', spos);
        if(next<0) next=data.length();

        String line=data.substring(spos, next).trim();
        spos=next+1;

        if((line.indexOf("Forward")<0)&&(line.indexOf("Reverse")<0)) {
          //ERROR ("#: line \""+line+"\" contains no Forward, Reverse keyword");
          discardedlines++;
          continue;
        }

// isolate the sequence

        String fields[]=new String[5];
        for(int fcnt=0; fcnt<fields.length; fcnt++) {
          int i=0;
          while((i<line.length())&&
                (!Character.isWhitespace(line.charAt(i)))) i++;

          if(i==line.length()) fields[fcnt]=line;
          else fields[fcnt]=line.substring(0, i);

          line=line.substring(i).trim();
          if(line.length()==0) break;
        }

        if((line.length()!=0)||(fields[4]==null)) {
          //TRACE ("#: invalid field count");
          discardedlines++;
          continue;
        }

        //ARM ("#: got 5 entries, doing valchecks");

        try {
          String seq=fields[1].toUpperCase();

// illegal chars ??
          if(!checkSequence(seq)) {
            //TRACE ("Illegal chars in sequence \""+seq+"\".");
            discardedlines++;
            continue;
          }

          seq=fields[1];
          int i=seq.lastIndexOf('t');
          if((i<0)||(i==seq.length()-1)||(i<seq.length()-4)) {
            //TRACE ("labeled thymine not in \""+seq+"\".");
            discardedlines++;
            continue;
          }
          seq=seq.substring(0, i);

// check for head
          i=0;
          while((i<seq.length())&&(Character.isLowerCase(seq.charAt(i)))) i++;
          seq=seq.substring(i);
          if(!checkSequence(seq)) {
            //TRACE ("invalid char in loopcleared seq \""+seq+"\".");
            discardedlines++;
            continue;
          }

          if((!fields[2].equals("Forward"))&&(!fields[2].equals("Reverse"))) {
            discardedlines++;
            continue;
          }

          i=Integer.parseInt(fields[3]);
          if(i<0) {
            discardedlines++;
            continue;
          }

          double score=Double.parseDouble(fields[4]);

          //TRACE ("#: adding primer "+primerlist.size());
          Primer p=new Primer(fields[0]+": "+seq);

// set primer color to green (score=0), orange (score 4), red (score 10)
          if(score<=4) {
// green 00CC00 to FF8800

            p.color=new Color((float)(score/4),
                              (float)(0.8-(score*0.3/4)),
                              (float)0.0,
                              (float)1.0);
          } else {
            p.color=new Color((float)(1.0),
                              (float)(0.5*(1-(score-4)/6)),
                              (float)0.0,
                              (float)1.0);
          }
//  p.color=new Color((float)0.0, (float)(1.0-score), (float)0.0, (float)1.0);

          p.comment="Score: "+fields[4];
          primerlist.add(p);
        } catch (Exception e) {
          e.printStackTrace();
          showErrorMessage(e);
          discardedlines++;
          continue;
        }

      }
      if(discardedlines>0) {
        //ERROR ("#: input contained "+discardedlines+" unreadable lines");
      }
    } else {
// unknown format
      return(null);
    }

    if(primerlist.size()==0) return(null);
    Primer ret[]=new Primer[primerlist.size()];
    primerlist.toArray(ret);
    return(ret);
  }
```

```
/** Align the primer sequences to the exonsequence
 */
  public SequenceAlignment[] alignPrimers() {
    ArrayList list=new ArrayList();

    StringBuffer sbuf=new StringBuffer();

    for(int ecnt=0; ecnt<exondata.getExonCount(); ecnt++) {
      sbuf.append(exondata.getExon(ecnt).getSequenceData());
    }

    String fullseq=sbuf.toString();
    Sequence bs=new Sequence(exondata.name, exondata.description,
                             fullseq);

    for(int pcnt=0; pcnt<(primerlist.length<<1); pcnt++) {
      Primer p=primerlist[pcnt>>1];

      Sequence ts=((pcnt&1)==0)?p.getSequence():p.getReversedSequence();

      for(int tpos=0; tpos<fullseq.length(); ) {
        tpos=fullseq.indexOf(ts.getSequenceData(), tpos);
        if(tpos<0) break;

        list.add(new SequenceAlignment(ts, bs, tpos));

        tpos++;
      }
    }


    if(list.size()==0) return(null);
    SequenceAlignment ret[]=new SequenceAlignment[list.size()];
    list.toArray(ret);
    return(ret);
  }



/** Check the sequencelist and the primer-list, build summary
 *  info
 *
 *
 */


  public String formatResults(boolean outputhtml) {
    StringBuffer sbuf=new StringBuffer();
    for(int i=0; i<exondata.getExonCount(); i++) {
      sbuf.append(exondata.getExon(i).getSequenceData());
    }

    String exonseparator="  ";
```

```
    String fullseq=sbuf.toString();

// List containing primer comments to sequence
    ArrayList commentstrings=new ArrayList();
    commentstrings.add("");

    int hitcnts[]=new int[primerlist.length<<1];
// list with start pos in exonstring (including exon separators)
// primer length, print row and primer ref
    int alignpos[]=new int[alignments.length<<2];

    int maxrows=-1;
    for(int acnt=0; acnt<alignments.length; acnt++) {
      int seqpos=0;
      alignpos[acnt<<2]=-1;
      alignpos[(acnt<<2)+1]=-1;
      alignpos[(acnt<<2)+2]=-1;
      alignpos[(acnt<<2)+3]=-1;

      for(int ecnt=0; ecnt<exondata.getExonCount(); ecnt++) {
        Sequence eseq=exondata.getExon(ecnt);

        seqpos+=eseq.getSequenceData().length();
        if(alignments[acnt].getLocation()<seqpos) {
          if(alignpos[acnt<<2]==-1) {
            alignpos[acnt<<2]=alignments[acnt].getLocation()+
                              exonseparator.length()*ecnt;
          }

          if(alignments[acnt].getLocation()+
             alignments[acnt].getLength()<=seqpos) {
            alignpos[(acnt<<2)+1]=alignments[acnt].getLocation()+
                                  alignments[acnt].getLength()+
                                 exonseparator.length()*ecnt;
            break;
          }
        }
      }


// now fix the printrow for the alignment
      int virtstart=alignpos[acnt<<2]-1;
      int virtend=alignpos[(acnt<<2)+1]+1;

      for(int row=0; ; row++) {
        int arcnt;
        for(arcnt=0; arcnt<acnt; arcnt++) {
          int cpos=arcnt<<2; // checkposition

          if(alignpos[cpos+2]!=row) continue;

          if((alignpos[cpos+1]>virtstart)&&(alignpos[cpos]<virtend)) break;
        }
        if(arcnt==acnt) {
          alignpos[(acnt<<2)+2]=row;
```

```
          if(row>maxrows) maxrows=row;
          break;
        }
      }

// find the primer for this alignment
      for(int i=0; i<primerlist.length; i++) {
        if((primerlist[i].getSequence()==alignments[acnt].getASequence())||
           (primerlist[i].getReversedSequence()==alignments[acnt].getASequence())) {
          alignpos[(acnt<<2)+3]=i;
          hitcnts[(i<<1)+
      (primerlist[i].getSequence()==alignments[acnt].getASequence()?0:1)]++;
          break;
        }
      }

      //TRACE ("#: got alignment "+acnt+" at location"+
      //TRACE  alignments[acnt].getLocation()+", outpt "+
      //TRACE  alignpos[(acnt<<2)]+", row "+alignpos[(acnt<<2)+2]+" for "+
      //TRACE  alignments[acnt].getASequence().name+", primer "+
      //TRACE  alignpos[(acnt<<2)+3]);
    }



// compile the row information and add it to output

    sbuf.setLength(0);
    maxrows++;

    if(outputhtml) {
      sbuf.append("<Html><Head><Title>Exoncheck Results: ").
           append(sequence_name.getText()).append("</Title></Head>\n").
           append("<Body bgcolor=\"#FFFFFF\"><Br>\n<B>Description:</B><Br><Pre>"
).
           append(sequence_description.getText()).append("</pre><Br><br>\n").
           append("<B>Testsequences:</B><Br>\n<Pre>\n");

      for(int i=0; i<primerlist.length; i++) {
        sbuf.append(primerlist[i].toString()).append('\n');
      }
      sbuf.append("</Pre>\n<Br><Br>\n<B>Mapping:</B>\n<Br><Br>\n<Pre>\n");
    }

    int alignseqs[][]=new int[maxrows][80];
    String lines[]=new String[maxrows];
    StringBuffer linebuf=new StringBuffer();

    for(int outptpos=0; outptpos<fullseq.length(); outptpos+=80) {
      int outptend=outptpos+80;
      if(outptend>fullseq.length()) outptend=fullseq.length();

      for(int i=0; i<maxrows; i++) alignseqs[i][0]=-1;

      for(int acnt=0; acnt<alignments.length; acnt++) {
// check if alignment acnt has something in common with outptpos - outptend
```

```
        if((alignpos[(acnt<<2)+1]<=outptpos)||
           (alignpos[(acnt<<2)]>=outptend)) continue;

// has something in common, add it to the alignseqlist
        int row=alignpos[(acnt<<2)+2];

        int inspos;
        for(inspos=0; inspos<alignseqs[row].length; inspos++) {
          if(alignseqs[row][inspos]==-1) break;
          if(alignpos[(acnt<<2)]<alignpos[alignseqs[row][inspos]<<2]) break;
        }

        if(alignseqs[row][inspos]==-1) {
          alignseqs[row][inspos++]=acnt;
          alignseqs[row][inspos]=-1;
        } else {
          System.arraycopy(alignseqs[row], inspos, alignseqs[row], inspos+1,
                           alignseqs[row].length-inspos-1);
          alignseqs[row][inspos]=acnt;
        }
      }


// now do the output

      int sppos=0;
      for(int exon=0; exon<exondata.getExonCount(); exon++) {
        if(sppos>=outptend) break;

        String exonstr=exondata.getExon(exon).getSequenceData();
        int nextpos=sppos+exonstr.length();

        if(outptpos<nextpos) {
          if(sppos<outptpos) {
            int shorten=outptpos-sppos;
            exonstr=exonstr.substring(shorten);
            sppos+=shorten;
          }

          if(sppos+exonstr.length()>outptend) {
            exonstr=exonstr.substring(0, outptend-sppos);
          }

          if((outputhtml)&&((exon&1)==0)) {
            sbuf.append("<Font color=\"#0000FF\">");
          }
          sbuf.append(exonstr);
          if((outputhtml)&&((exon&1)==0)) {
            sbuf.append("</Font>");
          }
        }


        sppos=nextpos;
        if(sppos+exonseparator.length()>outptpos) {
          int i=exonseparator.length();
```

```
          if(sppos<outptpos) i=sppos+exonseparator.length()-outptpos;
          else if(i+sppos>outptend) i=i+sppos-outptend;

          while(i>0) {
            sbuf.append(' ');
            i--;
          }
        }

        sppos+=exonseparator.length();
      }
      sbuf.append('\n');

/*
      String fspart=fullseq.substring(outptpos, outptend);
      sbuf.append(fspart).append('\n');
*/

      //ARM ("#: starting output for row "+outptpos+" to "+outptend);

      int lastboundarycrossrow=-1;
      for(int row=0; row<maxrows; row++) {
        lines[row]=null;
        linebuf.setLength(0);
        int lastend=outptpos;

        for(int refpos=0; refpos<alignseqs[row].length; refpos++) {
          if(alignseqs[row][refpos]==-1) break;

          int apos=alignseqs[row][refpos];
          int aoffs=apos<<2;


          if((alignpos[aoffs]<outptpos)||(alignpos[aoffs+1]>outptend)) {
            lastboundarycrossrow=row;
          }

          boolean revdir=
  primerlist[alignpos[aoffs+3]].getSequence()!=alignments[apos].getASequence();

          String pstring=primerlist[alignpos[aoffs+3]].
                         format(alignpos[aoffs+1]-alignpos[aoffs], revdir);

          //ARM ("#: output for alignment "+apos+" sequence \""+pstring+
          //ARM  "\", lastend "+lastend+", row "+row+
          //ARM  ", startpos "+alignpos[aoffs]+", endpos "+alignpos[aoffs+1]);

          if(alignpos[aoffs]<lastend) { // trim the primer seq
            pstring=pstring.substring(lastend-alignpos[aoffs]);
          }

          while(lastend<alignpos[aoffs]) {
            linebuf.append(' ');
            lastend++;
          }
```

```
          if(lastend+pstring.length()>outptend) {
            pstring=pstring.substring(0, outptend-lastend);
          }

          lastend=alignpos[aoffs+1];

          if(outputhtml) {
            pstring=pstring.replaceAll("<", "&lt;").replaceAll(">", "&gt;");

            linebuf.append("<Font color=\"#").
                    append(Integer.toHexString(primerlist[alignpos[aoffs+3]].
                            color.getRGB()|0xFF000000).substring(2, 8)).
                    append("\">").append(pstring).
                    append("</Font>");
          } else {
            linebuf.append(pstring);
          }
        }

        if(linebuf.length()!=0) lines[row]=linebuf.toString();
      }

      for(int row=0; row<maxrows; row++) {
        if(lines[row]==null) {
          if(row<=lastboundarycrossrow) sbuf.append('\n');
        } else {
          //TRACE ("#: adding line \""+lines[row]+"\".");
          sbuf.append(lines[row]).append('\n');
        }
      }
      sbuf.append('\n');  // one empty line to next row
    }

    sbuf.append("\n\n");

    for(int primernum=0; primernum<primerlist.length; primernum++) {
      Primer p=primerlist[primernum];
      sbuf.append(p.getSequence().name).append(": ").
           append(hitcnts[primernum<<1]).append(" matches forward, ").
           append(hitcnts[(primernum<<1)+1]).
           append(" matches backward\n");
    }

    if(outputhtml) {
      sbuf.append("</Pre><br><br>\n</Body></Html>\n");
    }

    return(sbuf.toString());
  }


/** Check if the sequence contains only ATCG characters.
 */
  private boolean checkSequence(String seq) {
    for(int i=0; i<seq.length(); i++) {
      switch(seq.charAt(i)) {
```

```
        case 'A':
        case 'T':
        case 'G':
        case 'C':
          break;
        default:
          return(false);
      }
    }
    return(true);
  }


//*************************************************************************//
//
//                      ActionListener impl
//
//*************************************************************************//


  public void actionPerformed(ActionEvent ae) {
    String command=ae.getActionCommand();

    if(command.endsWith("_show_help")) {
      String topic=command.substring(0, command.indexOf('_'));
      if(topic.equals("seq")) topic="sequence_tab_help.html";
      else if(topic.equals("primer")) topic="primer_tab_help.html";
      else if(topic.equals("result")) topic="results_tab_help.html";
      else topic="index.html";
      HelpWindow.show(frame, topic);
      return;
    }

    if("seq_nexttab".equals(command)) {
      try {
        ExonSequence es=parseExonSequence(sequence_text.getText(),
                                          "fips_format");
        if(es!=null) {
          exondata=es;
          exondata.name=sequence_name.getText();
          exondata.description=sequence_description.getText();
          tabpane.setEnabledAt(1, true);
          tabpane.setSelectedIndex(1);
          return;
        } else exondata=null;

      } catch (IllegalArgumentException iea) {
        iea.printStackTrace();
        showErrorMessage(iea);
      }

      tabpane.setEnabledAt(1, false);
      tabpane.setEnabledAt(2, false);
      return;
    }
    if("primer_nexttab".equals(command)) {
```

```
      try {
        primerlist=parsePrimers(primer_text.getText(), "fips_format");
        if(primerlist!=null) {
          alignments=alignPrimers();
          results_text.setText(formatResults(true));
          tabpane.setEnabledAt(2, true);
          tabpane.setSelectedIndex(2);
          return;
        }
      } catch (IllegalArgumentException iea) {
        iea.printStackTrace();
        showErrorMessage(iea);
      }

      tabpane.setEnabledAt(2, false);
      return;
    }


    if("primer_parselux".equals(command)) {
// display a popup window to enter data from lux primer design page

      JTextArea lux_text=new JTextArea(20, 80);
      lux_text.setFont(new Font("monospaced", Font.PLAIN, 12));
      JScrollPane scroll = new JScrollPane(lux_text);

      int state = JOptionPane.showOptionDialog(tabpane, scroll,
                    "Paste Lux-Primerdesign table into textfield below",
                    JOptionPane.OK_CANCEL_OPTION, JOptionPane.PLAIN_MESSAGE,
                    null, null, null);

      if(state!=JOptionPane.OK_OPTION)  return;

      String data=lux_text.getText();

      primerlist=parsePrimers(data, "lux_format");

      if(primerlist==null) {
        tabpane.setEnabledAt(2, false);
      } else {
        StringBuffer sbuf=new StringBuffer();

        for(int i=0; i<primerlist.length; i++) {
          sbuf.append(primerlist[i]).append('\n');
        }

        primer_text.setText(sbuf.toString());

        alignments=alignPrimers();
        results_text.setText(formatResults(true));
        tabpane.setEnabledAt(2, true);
        tabpane.setSelectedIndex(2);
      }
      return;
    }
```

```
    if((command.startsWith("primer_edit_line"))||
       (command.startsWith("primer_add_line"))) {
      int line=Integer.parseInt(command.substring(command.indexOf("line")+4));

      String linestr=null;
      int linestart=-1, lineend=-1;

      try {
        if(line>primer_text.getLineCount()) {
          linestart=lineend=
                  primer_text.getLineEndOffset(primer_text.getLineCount());
        } else if(command.indexOf("add")>=0) {
          linestart=lineend=primer_text.getLineStartOffset(line);
        } else {
          linestart=primer_text.getLineStartOffset(line);
          lineend=primer_text.getLineEndOffset(line);

          linestr=primer_text.getText(linestart, lineend-linestart).trim();

          if(linestr.length()==0) linestr=null;
        }
      } catch (BadLocationException ble) {
        System.err.println("Failed to get text for line "+line);
        ble.printStackTrace();
        linestr=null;
      }

      if((linestart<0)||(lineend<0)) return;

      Primer p=null;
      if(linestr!=null) {
        try {
          p=new Primer(linestr);
        } catch (IllegalArgumentException iea) {
          p=new Primer("x: ");
          p.getSequence().name=linestr;
        }
      } else p=new Primer("primer"+line+": ");

// show the dialog to modify the primer
      EditPrimerDialog epd=new EditPrimerDialog();
      p=epd.editPrimer(p, frame.getBounds());
      if(p==null) return;

      String allstr=primer_text.getText();
      primer_text.setText(allstr.substring(0, linestart)+
                          p+"\n"+allstr.substring(lineend, allstr.length()));
      return;
    }


    if("seq_load_web".equals(command)) {
      Object val=JOptionPane.showInputDialog(tabpane,
                "Enter ENSEMBL Transcript ID:", "Load Sequence from Ensembl",
                 JOptionPane.QUESTION_MESSAGE);
```

```
      String ensemblidstr=(String)val;
      if((ensemblidstr==null)||(ensemblidstr.trim().length()==0)) return;

      String urlstr=null;
      if(!ensemblidstr.startsWith("ENSMUST")) {
// check if remainings are only digits
        try {
          long l=Long.parseLong(ensemblidstr);
          ensemblidstr=Long.toString(l);
          while(ensemblidstr.length()<11) ensemblidstr="0"+ensemblidstr;

          urlstr=
    "http://www.ensembl.org/Mus_musculus/transview?transcript=ENSMUST"+
    ensemblidstr+"&db=core";
        } catch (NumberFormatException nfe) {}
      } else {
        urlstr="http://www.ensembl.org/Mus_musculus/transview?transcript="+
               ensemblidstr+"&db=core";
      }

      if(urlstr==null) {
        showErrorMessage("Error", "Invalid ENSEMBL Transcript ID",
                         ensemblidstr+
" is an invalid transcript ID, valid IDs would be ENSMUST00000022971 or just 22971");
        return;
      }

      String page=null;


      try {
        URL url =new URL(urlstr);
        URLConnection uc=url.openConnection();
        if(!(uc instanceof HttpURLConnection)) {
          throw new IllegalArgumentException("Illegal connection type "+
                                             uc.getClass());
        }

        HttpURLConnection connection = (HttpURLConnection)uc;

        InputStream in = connection.getInputStream();

        ByteArrayOutputStream bos=new ByteArrayOutputStream();
        int n;
        while((n=in.read())>=0) {
          bos.write(n);
        }

        in.close();
        connection.disconnect();

        page=new String(bos.toByteArray());

        //ARM ("#: read "+bos.size()+" bytes, page data is "+page);

      } catch (Exception e) {
```

```
        e.printStackTrace();
        showErrorMessage(e);
        return;
      }

      //TRACE ("#: make strict??");
//        if(urlstr.startsWith("http://www.ensembl.org/"))
      if(urlstr.indexOf("transview")>0) {
        //ARM ("#: parsing page "+page);
        ExonSequence es=null;
        try {
          es=parseExonSequence(page, "exonview_format");
        } catch (IllegalArgumentException iea) {
          iea.printStackTrace();
          showErrorMessage("Error Loading Transcript",
                           "Failed to load transcript:",
  "The page at "+urlstr+" didn't contain a valid ensembl transcript report."+
  " Try to open the page with IE or mozilla and check if your browser is "+
  "able to load it and if the page contains the header \"Ensembl Transcript "+
  "Report\". Other pages won't load.");
        } catch (Throwable t) {
          t.printStackTrace();
          return;
        }

        StringBuffer sbuf=new StringBuffer();

        if(es!=null) {
          if(es.name==null) es.name="testgen";

          sequence_name.setText(es.name);
          sequence_description.setText(es.description==null?"":es.description);
          exondata=es;

          for(int i=0; i<exondata.getExonCount(); i++) {
            String data=exondata.getExon(i).getSequenceData();

            for(int j=0; j<data.length(); j+=80) {
              if(j+80<=data.length()) {
                sbuf.append(data.substring(j, j+80)).append('\n');
              } else sbuf.append(data.substring(j));
            }

            if(i+1<data.length()) sbuf.append('x');
            sbuf.append('\n');
          }
        } else {
          //TRACE ("#: parsing returned null");
        }

        sequence_text.setText(sbuf.toString());
        primerlist=null;
        primer_text.setText("");
      } else {
        showErrorMessage("Invalid URL", "Invalid URL",
                         "Can't read message from url \""+urlstr+
```

```
                         "\", url doesn't contain exon data");
      }

      tabpane.setEnabledAt(1, false);
      tabpane.setEnabledAt(2, false);

      return;
    }


    if("result_save".equals(command)) {
// show file dialog and store html formated text

      JFileChooser fc;
      java.io.File file=null;
      fc=new JFileChooser(file);

 //     if(lastselecteddir!=null) fc.setCurrentDirectory(lastselecteddir);

      fc.addChoosableFileFilter( new javax.swing.filechooser.FileFilter() {
        public boolean accept(java.io.File file) {
          return (file.isDirectory() || file.getName().endsWith(".html"));
        }
        public String getDescription() {
          return "ExonCheck Report File *.html";
        }
      }
      );

      if(fc.showSaveDialog(null)==fc.APPROVE_OPTION) {
        file=fc.getSelectedFile();
      }
//      lastselecteddir=fc.getCurrentDirectory();

      if(file==null) return;

      try {
        String data=formatResults(true);
        FileOutputStream fout = new FileOutputStream(file, false);
        fout.write(data.getBytes());
        fout.close();
      } catch (IOException ioe) {
        ioe.printStackTrace();
        showErrorMessage(ioe);
      }

      return;
    }

    //TRACE ("#: unknown action "+command);
  }



//*********************************************************************//
//
```

```
//                      MouseListener impl
//


  public void mouseClicked(MouseEvent me) {
    if(me.getComponent()==primer_text) {
      //TRACE ("#: mouse clicked");

      if((me.getModifiers()&me.BUTTON3_MASK)==0) return;

      try {
        int clickoffset=primer_text.viewToModel(me.getPoint());
        int line=primer_text.getLineOfOffset(clickoffset);
        int linestart=primer_text.getLineStartOffset(line);
        int lineend=primer_text.getLineEndOffset(line);

        String linestr=primer_text.getText(linestart, lineend-linestart);

        //ARM ("#: selected line is \""+linestr+"\".");
        linestr=linestr.trim();

// show popup with delete, edit
        JPopupMenu  menu = new JPopupMenu();
        menu.add(createMenuItem("Add", "primer_add_line"+line, true));
        if(linestr.length()!=0) {
          menu.add(createMenuItem("Edit", "primer_edit_line"+line, true));
        }

        menu.pack();
        menu.show(me.getComponent(), me.getX(), me.getY());
      } catch (BadLocationException ble) {
      }
      return;
    }

    //WARNING ("Unknown event source "+me.getComponent());
  }

  public void mousePressed(MouseEvent me) {
  }

  public void mouseReleased(MouseEvent me) {
  }

  public void mouseEntered(MouseEvent me) {
  }

  public void mouseExited(MouseEvent me) {
  }


// ************************************************************************//
//
//                      GUI Glue                                          //
//
//**************************************************************************
```

```
  private JMenuItem createMenuItem(String text, String cmd, boolean enabled) {
    JMenuItem mi = new JMenuItem(text);
    mi.setActionCommand(cmd);
    mi.addActionListener(this);
    mi.setEnabled(enabled);
    return(mi);
  }


  private void showErrorMessage(Throwable t) {
    showErrorMessage("Program Error", "Caught Exception During Execution:",
                     t.toString());
  }


  private void showErrorMessage(String title, String header, String message) {
    JLabel label = new JLabel("header");
    JTextArea errortext=new JTextArea();
    errortext.setFont(new Font("monospaced", Font.PLAIN, 12));
    errortext.setText(message);
    errortext.setEditable(false);
    errortext.setPreferredSize(new Dimension(300,200));
    errortext.setLineWrap(true);
    JScrollPane scroll = new JScrollPane(errortext);
    scroll.setPreferredSize(new Dimension(300, 200));

    JPanel panel=new JPanel();
    panel.setLayout(new BorderLayout());
    panel.add(label, BorderLayout.NORTH);
    panel.add(scroll, BorderLayout.CENTER);

    JOptionPane.showMessageDialog(tabpane, panel, title,
                                  JOptionPane.ERROR_MESSAGE);
  }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.swing;

import java.awt.Color;
import java.awt.GridBagConstraints;
import java.awt.GridBagLayout;
import java.awt.Rectangle;
import java.awt.event.ActionListener;


import javax.swing.JButton;
import javax.swing.JColorChooser;
import javax.swing.JDialog;
import javax.swing.JLabel;
import javax.swing.JOptionPane;
import javax.swing.JPanel;

import at.tugraz.genome.luxprimerexoncheck.util.*;

import javax.swing.JTextField;


public class EditPrimerDialog implements ActionListener {

  JDialog                       dialog;

  Primer                        primer;

  JTextField                    name_textfield;
  JTextField                    sequence_textfield;
  JTextField                    comment_textfield;
  JTextField                    color_textfield;

  public EditPrimerDialog() {


    //TRACE ("Creating ColumnAdmin GUI");

    dialog=new JDialog();
    dialog.setTitle("Edit Primer");
    dialog.setModal(true);

    GridBagLayout gbl;
    GridBagConstraints gbc = new GridBagConstraints();
    dialog.getContentPane().setLayout(gbl=new GridBagLayout());

    gbc.gridwidth=1;
    gbc.gridx=0;
    gbc.gridy=0;
    gbc.weighty=0;
    gbc.weightx=0;
    gbc.fill=gbc.NONE;
    gbc.insets=new java.awt.Insets(3, 3, 3, 3);
    JLabel lab=new JLabel("Name:");
    gbl.setConstraints(lab, gbc);
    dialog.getContentPane().add(lab);
```

```
    gbc.gridy++;
    lab=new JLabel("Sequence:");
    gbl.setConstraints(lab, gbc);
    dialog.getContentPane().add(lab);

    gbc.gridy++;
    lab=new JLabel("Comment:");
    gbl.setConstraints(lab, gbc);
    dialog.getContentPane().add(lab);

    gbc.gridy++;
    lab=new JLabel("Color:");
    gbl.setConstraints(lab, gbc);
    dialog.getContentPane().add(lab);


    gbc.weightx=1.0;
    gbc.gridwidth=2;
    gbc.gridy=0;
    gbc.gridx=1;
    gbc.fill=gbc.HORIZONTAL;

    name_textfield=new JTextField();
    name_textfield.setColumns(20);

    gbl.setConstraints(name_textfield, gbc);
    dialog.getContentPane().add(name_textfield);

    gbc.gridy++;
    sequence_textfield=new JTextField();
    gbl.setConstraints(sequence_textfield, gbc);
    dialog.getContentPane().add(sequence_textfield);

    gbc.gridy++;
    comment_textfield=new JTextField();
    gbl.setConstraints(comment_textfield, gbc);
    dialog.getContentPane().add(comment_textfield);

    gbc.gridy++;
    gbc.gridwidth=1;
    color_textfield=new JTextField();
    color_textfield.setColumns(8);
    gbl.setConstraints(color_textfield, gbc);
    dialog.getContentPane().add(color_textfield);

    gbc.gridx++;
    gbc.weightx=0.0;
    JButton button=makeButton("Edit", "color_edit");
    gbl.setConstraints(button, gbc);
    dialog.getContentPane().add(button);


    gbc.gridy++;
    gbc.gridx=0;
    gbc.weightx=1.0;
    gbc.weighty=1.0;
```

```
    gbc.gridwidth=3;
    gbc.anchor=gbc.NORTH;
    gbc.fill=gbc.NONE;

    JPanel bpanel=new JPanel();
    bpanel.setLayout(new java.awt.FlowLayout());
    bpanel.add(makeButton("OK", "dialog_ok"));
    bpanel.add(makeButton("Cancel", "dialog_cancel"));

    gbl.setConstraints(bpanel, gbc);
    dialog.getContentPane().add(bpanel);

    dialog.pack();

  }


  public Primer editPrimer(Primer p, Rectangle center) {

    name_textfield.setText(p.getSequence().name);
    sequence_textfield.setText(p.getSequence().getSequenceData());
    comment_textfield.setText(p.comment);

    if(p.color!=null) {
      color_textfield.setText(Integer.toHexString(p.color.getRGB()|0xFF000000).
                              substring(2, 8));
    } else color_textfield.setText("000000");

    dialog.setLocation(center.x+((center.width-dialog.getWidth())>>1),
                       center.y+((center.height-dialog.getHeight())>>1));

    dialog.setVisible(true);

    return(primer);
  }


  private JButton makeButton(String name, String action) {
    JButton but=new JButton(name);
    but.setActionCommand(action);
    but.addActionListener(this);
    return(but);
  }



//************************************************************************//
//                                                                        //
//                      Event Handling                                    //
//                                                                        //
//************************************************************************//



  public void actionPerformed(java.awt.event.ActionEvent event) {
```

```
    String cmd=event.getActionCommand();

    //TRACE (".actionPerformed(cmd="+cmd+").");

    if("dialog_cancel".equals(cmd)) {
      primer=null;
      dialog.setVisible(false);
      return;
    }

    if("dialog_ok".equals(cmd)) {
      Sequence seq=null;

      try {
        seq=new Sequence(name_textfield.getText(), null,
                         sequence_textfield.getText());
      } catch (IllegalArgumentException iae) {
        return;
      }

      primer=new Primer(seq);
      primer.comment=comment_textfield.getText();
      try {
        int val=Integer.parseInt(color_textfield.getText(), 16);
        primer.color=new Color(val);
      } catch (IllegalArgumentException iae) {
        return;
      }

      dialog.setVisible(false);
      return;
    }

    if("color_edit".equals(cmd)) {

      Color color=null;
      try {
        int val=Integer.parseInt(color_textfield.getText(), 16);
        color=new Color(val);
      } catch (IllegalArgumentException iae) {
        return;
      }
      if(color==null) color=Color.black;

      color=JColorChooser.showDialog(dialog, "Edit Color", color);
      if(color!=null) {
        color_textfield.setText(Integer.toHexString(color.getRGB()|0xFF000000).
                                substring(2, 8));
      }
      return;
    }

    throw new InternalError("Unknown action command \""+cmd+"\"");
  }
}
```

```
package at.tugraz.genome.luxprimerexoncheck.swing;

import java.awt.BorderLayout;
import java.awt.Dimension;
import java.net.URL;
import java.util.ArrayList;

import javax.swing.JEditorPane;
import javax.swing.JFrame;
import javax.swing.JScrollPane;
import javax.swing.event.HyperlinkEvent;
import javax.swing.event.HyperlinkListener;
import javax.swing.text.html.HTMLDocument;
import javax.swing.text.html.HTMLFrameHyperlinkEvent;

/*
import java.awt.Color;
import java.awt.Font;
import java.awt.GridBagConstraints;
import java.awt.GridBagLayout;
import java.awt.event.ActionEvent;
import java.awt.event.ActionListener;
import java.awt.event.MouseEvent;
import java.awt.event.MouseListener;

import java.io.ByteArrayOutputStream;
import java.io.FileInputStream;
import java.io.FileOutputStream;
import java.io.InputStream;
import java.io.IOException;

import java.net.HttpURLConnection;
import java.net.URL;
import java.net.URLConnection;

import java.util.Hashtable;

import javax.swing.JButton;
import javax.swing.JFileChooser;
import javax.swing.JLabel;
import javax.swing.JMenuItem;
import javax.swing.JOptionPane;
import javax.swing.JPanel;
import javax.swing.JPopupMenu;
import javax.swing.JTextArea;
import javax.swing.JTextField;
import javax.swing.JTabbedPane;
import javax.swing.text.BadLocationException;

import at.tugraz.genome.luxprimerexoncheck.util.*;
*/

/** Disable following tabs, use next button
 *  First Pane: type data sequence or load it
 *i
 */
```

```
public class HelpWindow extends JFrame implements HyperlinkListener {

  private ArrayList     history;
  private JEditorPane   helppagepane;

  public HelpWindow(JFrame parent) {
    super("ExonCheck 0.09 Help");
    history=new ArrayList();

    helppagepane=new JEditorPane("text/html", null);
    helppagepane.setEditable(false);
    helppagepane.addHyperlinkListener(this);
//    results_text.setFont(new Font("monospaced", Font.PLAIN, 12));
    JScrollPane scroll = new JScrollPane(helppagepane);
    getContentPane().add(scroll, BorderLayout.CENTER);

    setSize(new Dimension(600, 600));
//    pack();
    setVisible(true);
  }


  public static void show(JFrame parent, String topic) {
    HelpWindow hw=new HelpWindow(parent);
    hw.show(hw.getClass().getResource("/resources/help/"+topic));
  }


  public void show(URL topicurl) {
    try {
      helppagepane.setPage(topicurl);
    } catch (java.io.IOException ioe) {
      ioe.printStackTrace();
    }
  }

  public void hyperlinkUpdate(HyperlinkEvent he) {
    if (he.getEventType() == HyperlinkEvent.EventType.ACTIVATED) {
      if (he instanceof HTMLFrameHyperlinkEvent) {
        HTMLFrameHyperlinkEvent  evt = (HTMLFrameHyperlinkEvent)he;
        HTMLDocument doc = (HTMLDocument)helppagepane.getDocument();
        doc.processHTMLFrameHyperlinkEvent(evt);
      } else {
        try {
          helppagepane.setPage(he.getURL());
        } catch (Throwable t) {
          t.printStackTrace();
        }
      }
    }
  }
}
```

Institute of Genomics and Bioinformatics

Graz University of Technology
Erzherzog-Johann-University

Standard Operating Procedure

TITLE: **Realtime-RT-PCR Run on AbiPrism7000 with Luxprimers**		
SOP-NO: METxxx_xx	REPLACES: none	PAGE: 1 of 25
REWIEV PERIOD: biannual	VALID FROM: xx/xx/2004	

originated: R. Fiedler the date

approved by: Dipl.-Ing. Dr.techn., Univ.- Prof. Z. Trajanoski the date

Contents

I.Purpose

This SOP contains a detailed description of all steps to perform a Realtime - RT-PCR run on the AbiPrism 7000:

- Material/Considerations: Decisions about the experiment, materials needed for Realtime - RT-PCR
- Sample Preparation: Preparation of cDNA from RNA with SuperScriptIII
- Functional Setup: Short description how to define your plate setup with the AbiPrism Software
- Plate Preparation / Run: Guideline how to prepare the plate
- Data Analysis: How to get the template concentration per well from the amplification plot
- Calculations: Compare the template concentrations to get information about regulation

This SOP contains also background information about the steps. The information is given for a better understanding of the process. You should mark the parts containing the manual work steps before taking it to the lab.

II.Scope

This procedural format is utilized within the Institute of Genomics and Bioinformatics.

III. Procedure

III-1. Material / Considerations

The principle of the RT-PCR is that RNA-samples are reverse transcribed (e.g. via SuperScriptIII) and the generated cDNA is used for the absolute quantitation in the AbiPrism7000. Depending on your experiment you have to decide about following points:

- Calibration curve: If you use primers or calibration samples for the first time you have to do one calibration curve per primer on the plate, for each successive run you can omit the calibration curve and copy it but that may reduce the precision of your results. There are two possibilities:
  - Firstly absolute calibration curve: it uses calibration samples with known template concentration.
  - Secondly a relative calibration curve you can use your custom sample RNA for that but consider that you will need rather a lot of sample (30x times more cDNA than for one normal sample well).

- Sample selection: Decide how many different RNA samples you want to quantify using realtime RT-PCR. Some sample numbers give better plate utilization (lower resource consumption). You will need a large amount of cDNA if you want to do calibration curves in every RT-PCR run (recommended). Therefore the cDNA for that purpose has to be available in great excess (30x times more) compared to sample cDNA. Hence the cDNA for that purpose should be easily accessible in great amounts in your experiment (e.g. use RNA from preconfluent cells or pool of time points). You may want to use RNA from your calibrator sample for that purpose because that increases the precision of the analysis.

Materials:

- Check that pipette tip trays with 96x 10uL-tips are available. Use only cone-shaped tips (like B), not the ones with the cylindric body (like A), this increases precision. It will help if you draw a 2x2 grid onto the top of the plastic tray holding the tips. It is easier to find e.g. the tip position C5 in the box when the grid is visible. With the pipetting hints from III-4 this will significantly reduce pipetting errors.

Fig. 1: Sampler tips

- Reconstitute all the necessary primers if you use them for the first time. Therefore refer to the primer manufacturer's protocol and dilute the reconstituated primer to 10uM. It's recommended to aliquot the 10uM dilution to 50uL aliquots and store them dark at -20°C.
- You will need RNAse-free water for the reverse transcription step.

AbiPrism 7000 Maintenance: It is recommended to perform background runs every week to look for thermoblock contamination (this happens rather frequently). Ask the person in charge for the AbiPrism7000 to do that.

III-2. Sample Preparation

The actual concentration of the sample is not important if all samples are treated the same way. Only the No-RT sample needs some special handling according to the treatment of the other samples. See the No-RT sample dilution point (b) in the SuperScriptIII Reverse Transcription protocol (i).

i. Reverse Transcription with SuperScript III First-Strand Synthesis System for RT-PCR Cat No. 18080-051:

Check the SuperScriptIII protocol for the reverse transcriptase reaction basics or if you want to make modifications to the protocol. The SuperscriptIII reaction accepts up to 5ug of RNA per reaction, 2.5ug is an acceptable value. If higher precision is wanted the RNA should be diluted to 312.5ng/uL, this dilution can be used directly for the reaction (no additional water needed), that's the case in the following protocol.

The cDNA from 2.5ug RNA (one reaction) is suitable to fill 210 sample wells (with 5uL sample per well), so one reaction will satisfy sample cDNA needs. **Since large amounts of cDNA are needed for the calibration curves**, a 5x or 10x bigger reaction should satisfy your needs. You will have to prepare at least a 5x reaction to use the pipetting scheme for the calibration curve given below (e).

Use the tetrade to perform the reaction, it's much simpler than the waterbath method and more reproducible. If you use less than 5 eppendorfer tubes then put additional 5 to 10 empty eppis into the tetrade because the pressure on only 5 eppis at 85°C will cause severe deformation or even break them. Per reaction you should get 21uL of cDNA product (after RNAse H digest).

a. Dilution of the samples: Make an RNA-Dilution of 312.5ng/uL with water to add it to the SSIII reaction. Per reaction you would need 8uL but make at least 10uL so that something remains in the tube (if the SSIII reaction fails you can use the remainder to check the RNA quality on the BioAnalyzer). Prepare a bigger dilution volume if you want to make more than one SSIII reaction. The remaining RNA dilution can be stored at -80°C.
Example: Dilution of RNA with 2.11ug/uL to a final volume of 10uL. Calculate the volume of RNA needed:

$$v_{RNA} = {v_{final} \cdot c_{final}} \Big/ {c_{RNA}} = {10uL \cdot 0.3125ug \cdot uL^{-1}} \Big/ {2.11ug \cdot uL^{-1}} = 1.481uL$$

Prepare the RNA-Dilution:

Substance	Volume [uL]
DEPC H_2O	8.519
RNA	1.481
Sum	**10**

b. Dilution of the No-RT control: The data evaluation will be simpler if the No-RT sample contains the same volume from the RNA sample compared to the samples that were reverse transcribed (so your No-RT sample and the normal samples will contain the same amount of genomic DNA). Use the 312.5ng/uL RNA-dilution and dilute it 1:131.25 in ddH_2O. You can use any sample RNA for that but most likely you want to use the RNA used for the calibration curve because of its good availability. The factor depends on the 8uL RNA sample used for the SSIII reaction, the 21uL SSIII product made from the 8uL and the 1:50 cDNA dilution: f=1:50x21/8.

Substance	Volume [uL]
DEPC H_2O	130.25
RNA (312.5ng/uL)	1.000
Sum	**131.25**

c. SuperScriptIII Reaction: Prepare the SSIII RNA mixes with the diluted RNAs from above:

Substance	Volume [uL]
RNA (312.5ng/uL)	8
Random Hexamer Primers (50ng/uL)	1
dNTP-Mix (10mM)	1
Sum	**10**

Heat the SSIII RNA mixes to 65°C for 5min and put on ice for at least 1 min afterwards (You may want to use the tetrade (Program STDPRO > SS3PRE). Make sure to enable the lid heating to a temperature greater than 90°C (110°C is default, that's ok for our reaction; value can be changed via SETUP). The lid heating is finally enabled when you start the thermocycler run. The tetrade won't beep after the cooling the sample to 4°C, just terminate the program after more than 5 min and put the sample on ice. Put

additional empty 0.2uL eppis into the thermoblock if you have less then 10 eppis and shut the lid only slightly (**The eppis could get deformed otherwise!).**

Prepare the cDNA Synthesis mix during the 5min incubation time, mix all substances in the order given below. **Don't forget to multiply the volumes with the number of reactions you want to run.**

Substance	Volume [uL]
10x RT Buffer	2
MgCl2 (25mM)	4
DTT (0.1M)	2
RNaseOut (40U/uL)	1
SuperScriptIII RT (200U/uL)	1
Sum	**10**

Add 10uL of cDNA Synthesis Mix to each RNA sample mix. Heat for 10min to 25°C and then 50min to 50°C. Terminate the reaction by heating to 85°C for 5min. This is all performed by the tetrade programm STDPRO > SS3MAINH.

Do a RNAse H digest of the remaining RNA: add 1uL of RNAse H solution to each sample and put the samples to 37°C for 20min. You can use the tetrade program STDPRO > FIXT37 and terminate it after 20min.

The undiluted SSIII product should be stable when stored at -20°C.

d. Dilution of the cDNA samples: A sample dilution of 1:50 is a rather good trade-off between sample demand and quality of the results. The 18S reference will show up very soon in the amplification plot but the plot data analysis is still possible. Lowcopy mRNAs will show up 5-10 cycles before the NTC (No template control). Dilute 2uL of SSIII cDNA product with 98uL of ddH_2O which is suitable for 20 wells a 5uL (or bigger amount if needed).

Substance	Volume [uL]
ddH_2O	98
SSIII Product	2
Sum	**10**

e. Dilution of the cDNA used for relative calibration: Prepare dilutions of 1:5, 3.15:50, 1:50, 3.15:500 and 1:500 of the cDNA product from the SuperScriptIII reaction. From here the 1:5 cDNA solution for the calibration curve will be referred as *Std 10x*, the 3.15:50 as *Std 3.15x*, 1:50 as *Std 1x*, 3.15:500 as *Std 0.315x* and 1:500 as *Std 0.1x*.
The concentrations look a little bit queer but form a good logarithmic ladder ranging from 10x to 1:10x compared to the samples (the samples are 1:50 dilutions of the SSIII product, so the *Std 1x* dilution will contain the same SSIII product amount as the samples.).
Per calibration curve you will need 15uL of the *Std 1x* dilution and 10uL of the remaining dilutions.

Prepare the 1:5 dilution (*Std 10x*) at first:

Substance	Volume [uL]
ddH_2O	400
SSIII Product	100
Sum	**500**

Prepare the remaining calibration curve point solutions using the 1:5 dilution (*Std 10x*) from above:

Dilution	Vol ddH_2O [uL]	Vol 1:5 Dilution [uL]	Final Vol [uL]
Std 10x		316.5	316.5
Std 3.15x	217.5	100	300
Std 1x	630	70	700
Std 0.315x	307.5	10	317.5
Std 0.1x	346.5	3.5	350
Sum		**500**	

If you don't want to continue with the protocol on the same day, then the put all samples into the fridge to -20°C after mixing. If you continue with the protocol then just put the eppis into an ice box.

III-3. Functional Setup

Enter all the data for the platesetup into the computer using the Abi SDS software.

a. Start the software via the windows start menu – programs – AbiPrism 7000 – AbiPrism 7000 SDS Software. The AbiPrism machine doesn't need to be switched on when doing this.

b. Choose File – New from the menu. A popup appears asking you about the type of analysis you want to perform. Select *Absolute Quantitation* in the *Assay*-field. Our AbiPrism7000 machine only accepts 96 well plates so select *96-Well Clear* in the *Container*-field.
You should create and use a template if you have to do many similar runs. See the AbiPrism user manual for more information. Select the template you want to use via the *Browse…*-button if you have already created one.
If you press OK and the PCR machine is switched off you will get an error message (Cannot detect instrument). It's save to say Cancel.

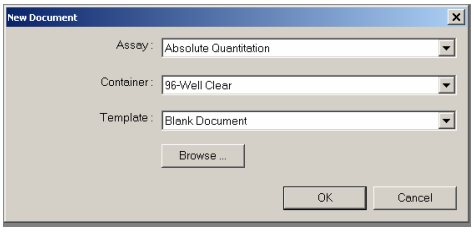


Fig. 2: Create Well Setup for new Quantitation

c. Start with the *Detector Manager* (Menubar – Tools – Detector Manager) to add the necessary detectors. A detector is roughly a primer set with one fluorophore labelled primer and one unlabelled. You have to create at least one detector for each primer pair because all readout settings (like baseline, threshold, calibration curve) are applied to one detector.

Add the missing detectors via the Button *File* – *New* in the bottom left corner of the detector manager. Don't set the *Quencher Dye* field when creating new detectors for *LuxPrimers.*

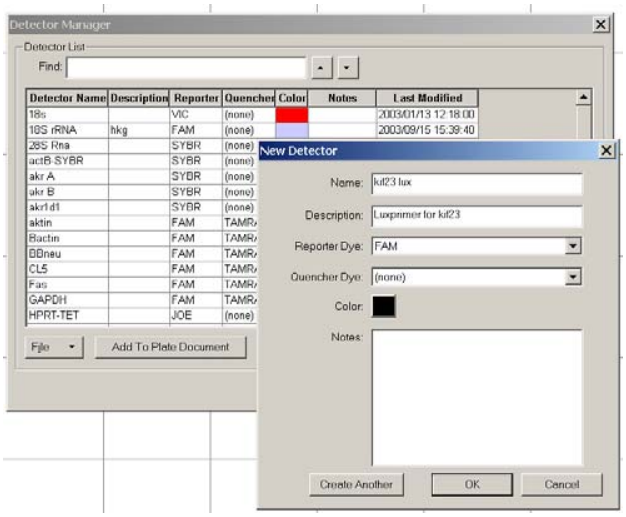


Fig. 3: Detector manager

d. After that define the detectors you want to use for this run: select them in the list and press the *"Add To Plate Document"*- button (you won't notice any change when pressing the button but the software got everything right).

e. Enter the well-specific data with the *Well Inspector* (via Menubar-View-Well Inspector or the icon at the top):

- Select one or more wells in the plate document and set the detector type and the task (what's the well used for: Unknown if filled with sample (or No-RT control), NTC if used as no template control and Standard if it defines a point in standard curve) of the well.

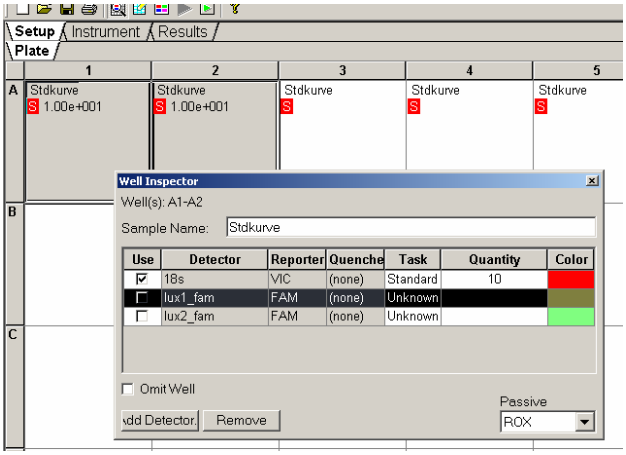


Fig. 4: Use *Well Inspector* to define properties of wells

- Make sure the the *Passive*-field at the right bottom is set to correct passive dye. Rox reference dye is recommended together with FAM-labelled lux primers. The reference dye's signal is used by the AbiPrism software to normalize the fluorescence signals from the primers. The signal from the Rox dye is eliminated in the normalization process, you can see it only in the *Raw Spectra* tab during datareadout.

- You need to define the template quantity in a well if you set the well's task to *Standard*. With an absolute calibration curve insert the concentration without the unit (e.g. in pg). With a relative calibration curve just insert values that reflect the correct concentration ratios between the calibration liquids (i.e. 10, 3.15, 1, 0.315 and 1 if you use the calibration curve mentioned in III-2).

- It is wise to create plate setups that reduce pipetting errors. This can be achieved if e.g. the mastermixes containing different primerpairs (detectors) are adjusted as plate columns and the samples to rows. Here is an example setup containing the wells for calibration in the top three rows and the samples below:

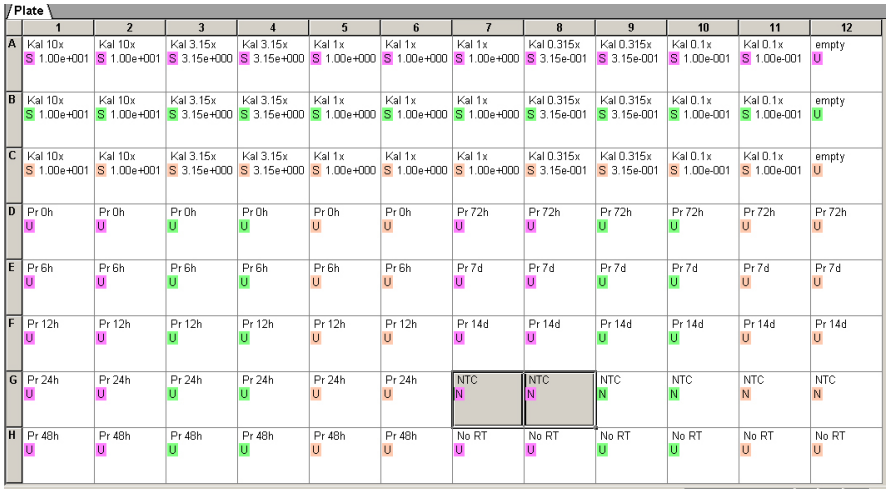


Plate	1	2	3	4	5	6	7	8	9	10	11	12
A	Kal 10x S 1.00e+001	Kal 10x S 1.00e+001	Kal 3.15x S 3.15e+000	Kal 3.15x S 3.15e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 0.315x S 3.15e-001	Kal 0.315x S 3.15e-001	Kal 0.1x S 1.00e-001	Kal 0.1x S 1.00e-001	empty U
B	Kal 10x S 1.00e+001	Kal 10x S 1.00e+001	Kal 3.15x S 3.15e+000	Kal 3.15x S 3.15e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 0.315x S 3.15e-001	Kal 0.315x S 3.15e-001	Kal 0.1x S 1.00e-001	Kal 0.1x S 1.00e-001	empty U
C	Kal 10x S 1.00e+001	Kal 10x S 1.00e+001	Kal 3.15x S 3.15e+000	Kal 3.15x S 3.15e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 1x S 1.00e+000	Kal 0.315x S 3.15e-001	Kal 0.315x S 3.15e-001	Kal 0.1x S 1.00e-001	Kal 0.1x S 1.00e-001	empty U
D	Pr 0h U	Pr 0h U	Pr 0h U	Pr 0h U	Pr 0h U	Pr 0h U	Pr 72h U	Pr 72h U	Pr 72h U	Pr 72h U	Pr 72h U	Pr 72h U
E	Pr 6h U	Pr 6h U	Pr 6h U	Pr 6h U	Pr 6h U	Pr 6h U	Pr 7d U	Pr 7d U	Pr 7d U	Pr 7d U	Pr 7d U	Pr 7d U
F	Pr 12h U	Pr 12h U	Pr 12h U	Pr 12h U	Pr 12h U	Pr 12h U	Pr 14d U	Pr 14d U	Pr 14d U	Pr 14d U	Pr 14d U	Pr 14d U
G	Pr 24h U	Pr 24h U	Pr 24h U	Pr 24h U	Pr 24h U	Pr 24h U	NTC N	NTC N	NTC N	NTC N	NTC N	NTC N
H	Pr 48h U	Pr 48h U	Pr 48h U	Pr 48h U	Pr 48h U	Pr 48h U	No RT U	No RT U	No RT U	No RT U	No RT U	No RT U

Fig. 5: Wellsetup for 3 Genes (Calibration Curve, 8 Samples, No-RT and NTC per Gene)

f. It's good to make the *Thermocycler Setup* now, so that you can start immediately after plate preparation. The setup depends on the supermix and the primers you are going to use.

- <u>Thermocycle Setup for Platinum Quantitative PCR SuperMix UDG (Invitrogen 11730) with Luxprimers:</u>

The standard profile from the datasheet works fine:

50°C for 2min
95°C for 2min
45 cycles of:
95°C, 15sec
55°C, 30sec (anneal)
72°C, 30sec (elongate)

Select the "Dissociation Protocol" checkbox to enable meltingpoint analysis (product characterization).

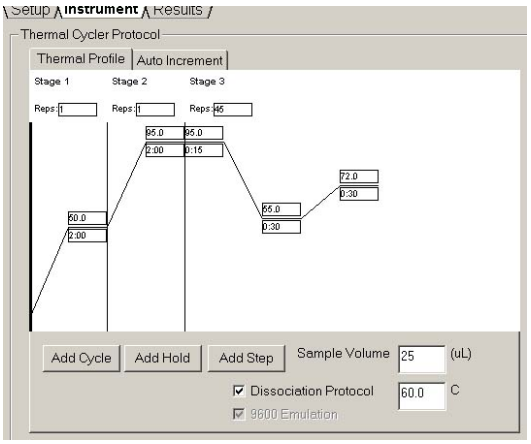


Fig 6: Thermocycle Setup

You may want to raise the annealing temperature with some primers to increase specificity.

Don't change the sequence of the steps in the cycle, this will have influence on the time when the AbiPrism instrument will make its fluorescence level measurement because (at least the SDS 1.0 version) performed the measurement in the second step of the cycle. The measurement won't work if not done during annealing.

g. Store the plate setup to the local hard drive. **Don't store data on network disks,** the computer should be disconnected from the net during the run.

III-4. Plate Preparation / AbiPrism Run

i: Thawing of reagents and samples:

- Place the primer, sample and PCR-mix eppis in an eppirack and put aluminia foil around it to shield off light (The labelled primers are sensitive to light). Put the rack into the 4°C fridge and let thaw. You can use a prechilled eppirack to make sure that the temperature doesn´t rise above 4°C. (for small amounts below 200uL 10min are sufficient, but especially for the supermix longer times would be needed. Transfer all thawed samples onto ice after 10min and let the supermix stay at 4°C longer or thaw in hands).
- Mix well after thawing: Samples can be vortexed, but the supermix should only be mixed by inflicting the eppi several times because the mix may take up air in stable small bubbles while vortexing. Spin down immediately after mixing.
- Make aliquots from the PCR-Mix if the liquid is still in the 10mL bottle from the factory to avoid frequent freezing and thawing: A good choice are 98x reactions aliquots in 2mL Eppendorf tubes or if you want to be sure that you don't run out of mastermix use 100x.
- Put all reagents onto ice immediately after thawing. Use an icebox can be closed light-tight. The luxprimers and the Rox reference dye are sensitive to light.

ii: Pipetting Scheme: The next step after creation of the complete plate setup in III-2 is to calculate the values for the pipetting scheme. Calculate the values depending on your reaction size (50uL/Well or 25uL/Well) and the PCR-mix. Here is an example:

ii-a: Pipetting Scheme for a 96-Well Plate with 3 Genes and 32 Reactions a 25uL per Gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen 11730-017) and Lux Primers:

Assume your genes are named A, B and C and you have primer pairs for each gene. Label one 2mL eppi with S (Supermix Tube) and 1.5mL eppis A, B, C (Mastermixes with primerpair for genes A, B, C).

a. Prepare the supermix for 98 Reactions in the 2mL eppi labelled S:

Substance	Volume [uL]
Platinum UDG Supermix	1225
ddH_2O	588
Rox Reference Dye	49
Sum	**1862**

Mix well by slowly inverting the tube 5 times (do not use the vortex, this will create many small bubbles). Spin down.

b. Transfer 618uL supermix from tube S to eachof the two labelled eppis (A, B). Spin down eppi S and transfer 618uL to C (8uL should remain in S).

c. Now add 16.26uL of each primer of pair A to eppi A. The name of the labelled lux primer ends with FL (forward labelled) or RL (reverse labelled), the name of the unlabelled primer ends with FU or RU (forward/reverse unlabelled). Example: nur77-1030RL (labelled) and nur77-1030RL-1014FU (unlabelled).

Substance	Volume [uL]
Supermix	618
Labelled Primer	16.26
Unlabelled Primer	16.26
Sum	**650.52**

Mix by inflicting the tube, spin down and put the eppi on ice. Make sure that the icebox is light-tight so that the labelled primers won't bleach.
Also add the primer pair B to eppi B and pair C to eppi C.

d. Put one coolpack (cooled to -20°C) onto a styropore icebox top cover. Take out fresh optical reaction plate (e.g. AbiPrism 96-Well optical reaction plate No. 4306737) and put it into a 96-wellplate holder and that onto the coolpack (this avoids direct contact between the optical plate and the coolpack).

Use the *Eppendorf Multipette* to transfer the mastermixes to the wells. Make sure that the multipette is configured to your volume (with the 500uL tips set the volume control wheel to 2 to get 20uL/well. See the *multipette manual* for more information.). The handling of the multipette is a little bit tricky, so if you do this for the first time, get yourself an old plate and try to fill it with water to get used to the tool. Don't push the trigger to fast, this may sputter liquid out of the well. Although the pipette tip can hold 500uL (Eppendorf combitip No. 0030 069.226) you shouldn't use the whole volume because the last push will be imprecise.

Fig 7: Multipette Tip

After the practising take a fresh tip and push up the handle to suck up some air (move the plunger from the mark A up to B). After that suck up as much mastermix as available. The small amount of air above the liquid doesn't cause imprecision but allows to empty the tip completely. **Push the trigger two times to eject some liquid back into the**

mastermix eppi because the first one or two pushes won't dispense the 20uL volume precisely.

Fill the wells but don't empty the tip completely. The last wells have to be filled with a normal sampler: transfer the remaining liquid from the multipette tip back to the mastermix eppi and spin down.

Use a 20uL sampler to fill the remaining (in most cases 2 or 3) wells. After that check that you haven't left out a well, all wells have equal volume of mastermix and that no liquid was sputtered across the plate when working with the multipette.

e. Use a 10uL sampler to add 5uL from your cDNA-dilutions to each of the wells. You can use the tip box prepared in III-1. Use the tip from position A1 to transfer sample to well A1, then the tip box will reflect the position of the wells without sample. Make sure that the tip is dipped into the master mix when adding the sample to the well. After dispensing, rinse the tip 2x with the mastermix volume to leave no residue in the tip but don't produce bubbles in the mastermix. Pull out the tip a little bit above the liquid and empty the tip completely.
Add 5uL of ddH2O to the NTC wells and 5uL of the No-RT sample to the No-RT wells.

iii: Finish plate: Close the plate with an optical adhesive cover sheet (e.g. AbiPrism 4313663): Remove the large white middle part from the sheet to uncover the sticky surface and put it with the sticky side down onto the plate. Use a soft rubber tool to press the sheet onto the plate and remove the two side parts of the sheet afterwards (you should be able to tear them off but sometimes they won't go off. If this is the case, press the sheet down with the rubber tool right beside the outmost column of wells and use a stanly knife or scalpel to cut the ends).

Spin down the liquids in the 96-well plate by centrifugation with 400rcf for 2min. (that is 1500rpm with the 158mm swingtrays in the Jouan CR422 centrifuge or recall program 2).

The time during centrifugation is good to switch on the AbiPrism machine and to start the software (The centrifugation time plus the 4min denaturation steps at the start of the thermocycler program should be satisfactory for lamp warm-up – 10min warm-up are recommended): The most failsafe way to do this is to disconnect laptop's network cable, switch the laptop on and wait for it to boot. Use the username "lab1", password "xxxxx" to log on as local user (no Genome network logon). Then switch on the AbiPrism machine and wait for 20 seconds (don't forget to write a logbook entry). Now start the SDS software and load your plate setup.

After centrifugation put the grey-brown rubber-foam mate with the grey side down onto the plate and put the plate into the AbiPrism 7000 machine. If there were problems with the software startup, put the plate onto the coolpack again and that into the 4°C fridge until problems are fixed.

Start the run by clicking onto the start button located in the instrument setup tab.

iv: After run steps: Remove the plate from the thermoblock and verify that the thermoblock wasn't contaminated during the run by checking the background fluorescence.

iv-a Background fluorescence check: Use therefore the *Instrument-Calibrate*-item from the SDS software menu bar. The calibration window will allow to see a fluorescence image of the thermoblock. Therefore select the *Open Shutter* checkbox, set the camera exposure time to 1024ms, select the filter A and click the *Snapshot* button. If you can hardly see the shape of the wells on the snapshot picture, then the block seems to be clean. Try the filters

B, C and D and look at these snapshot pictures too. If only one of them shows gray or white spots inside a well. Then you need to clean the thermoblock.

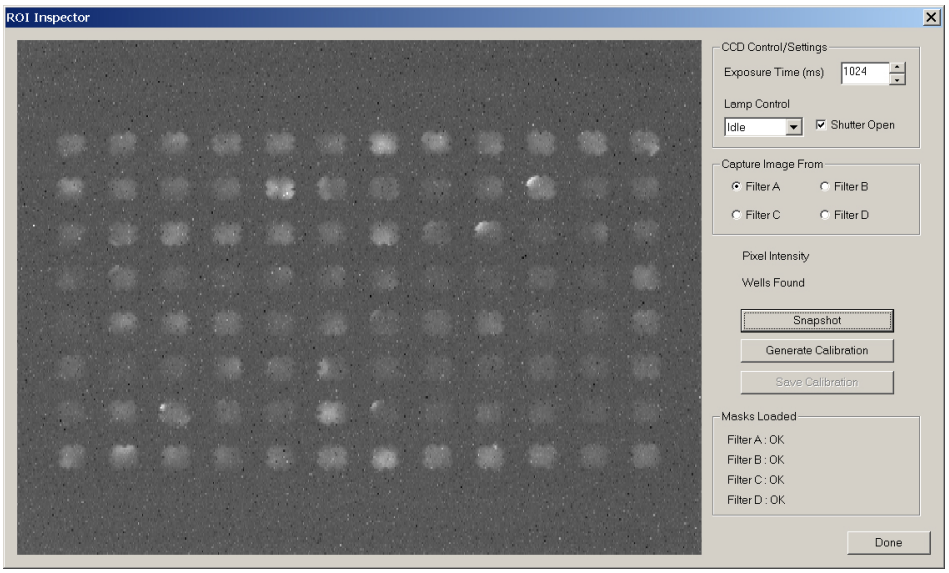


Fig. 8: Fluorescence image of the thermoblock. The wells A7, B5, B10, C9, G6 and H7 show minor contamination, cleaning in this case is not necessary but recommended.

iv-b Cleaning of the thermoblock: Be careful when performing the cleaning steps. Don't scratch any surface with hard tools, if you have to apply soft force try to wrap e.g. a forceps with a kimwipe and use this. Good cleaning results could be archived with this procedure:

- Take a small piece of kimwipe, roll it to a cigar-like shape and make it a little bit wet (use ddH2O) Don't make it that wet so that the water gets pressed out when pushing it into the contaminated well.
- Push the piece into the contaminated well and move it around a little bit in the well and then leave it in the well for a minute. (Don't move the wipe too much, it will loose fibers and that may contaminate other wells)
- Take out the wet kimwipe and use a piece of dry one to dry the well
- Use the air-duster to blow away fibers from the thermoblock. Do this with all wells because it's highly likely that you contaminated other wells with fibers during the cleaning operation.

If the well couldn't be cleaned with this procedure then you may use ethanol to clean it. Take care with the ethanol, it shouldn't touch any other surface than the metal thermoblock. **Don't use acidic or oxidizing agents in any case!** After your cleaning efforts, make snapshot pictures again to see if the efforts were successful.

iv-c Background run: Perform a background run if the thermoblock seems clean. Therefore create a new plate and select the *Background Run*-option. Save the plate to the maintenance/background_runs directory and start the run. After the run look at the fluorescence intensities measured in each empty well. If the block wasn't

contaminated or the cleaning was successful, then the maximal intensity in each well should be below 400 units. If the intensity is higher, start again at point iv-a.

III-5 Data Analysis:

After the RT-PCR run was completed, switch off the AbiPrism machine because you don't need it to be online during readout (it's obligatory for all performers to add a logbook entry).

i. Baseline Settings:

Switch to the Results-tab in the SDS-Software and select the *Amplification Plot* subtab. Set the baseline for each detector (Don't use the autobaseline option, it doesn't work very good with luxprimers) using a linear view of *Delta Rn vs Cycle*. Select some or all wells using one detector. It's easier to look at the graph if it doesn't contain all lines at once, but if you use only some wells for baseline setting, make sure that the well with the highest and the lowest concentration is included (No-RT and NTC wells shouldn't be included when defining the baseline).
The best choice are the wells for the calibration curve, if available, because the calibration concentrations normally cover a broader range than the samples. At first set the baseline start and end cycle both to 1 and press the "Analyze" button. This will disable the baseline correction and you can see the fluorescence signal directly. The SDS software sometimes fails to scale the y-axis properly, to solve the problem, click the y-axis and a popup for manual configuration will show.

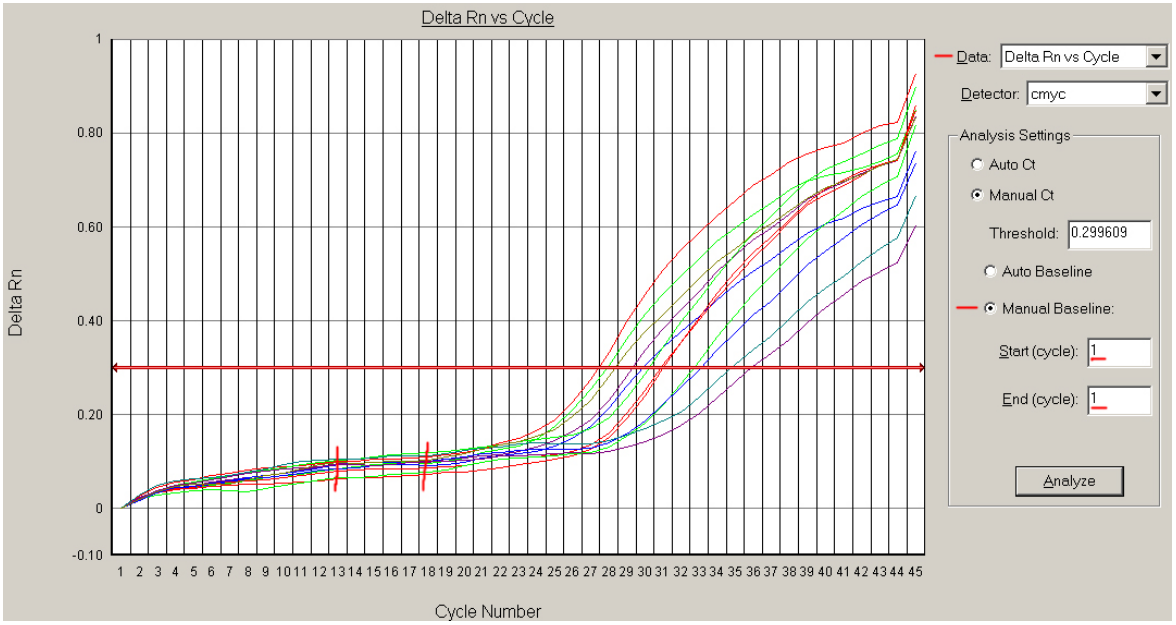


Fig 9: *Amplification plot* of calibration curve wells

Set the baseline end to a cycle where **no** line has started to rise yet (e.g. 17) and the baseline start to the cycle where the baseline already got stable (e.g. 12, where the slope of the baseline is rather constant). Then press "Analzye".

The next step is to set an appropriate threshold value for each detector using the semilogarithmic *Delta Rn vs Cycle* view. The possible range of the threshold value is limited: The reaction shifts from exponential kinetics to linear kinetics due to chemical limitations in the PCR. Only the exponential reaction is suitable for quantitative PCR (The *Amplification Plot* will show a straight line in the semilogarithmic plot) and thus limits the maximal threshold value.
The minimal threshold value is limited by the instrument's noise interfering with the fluorescence signal from the plate. The noise is relatively smaller at high fluorescence values.

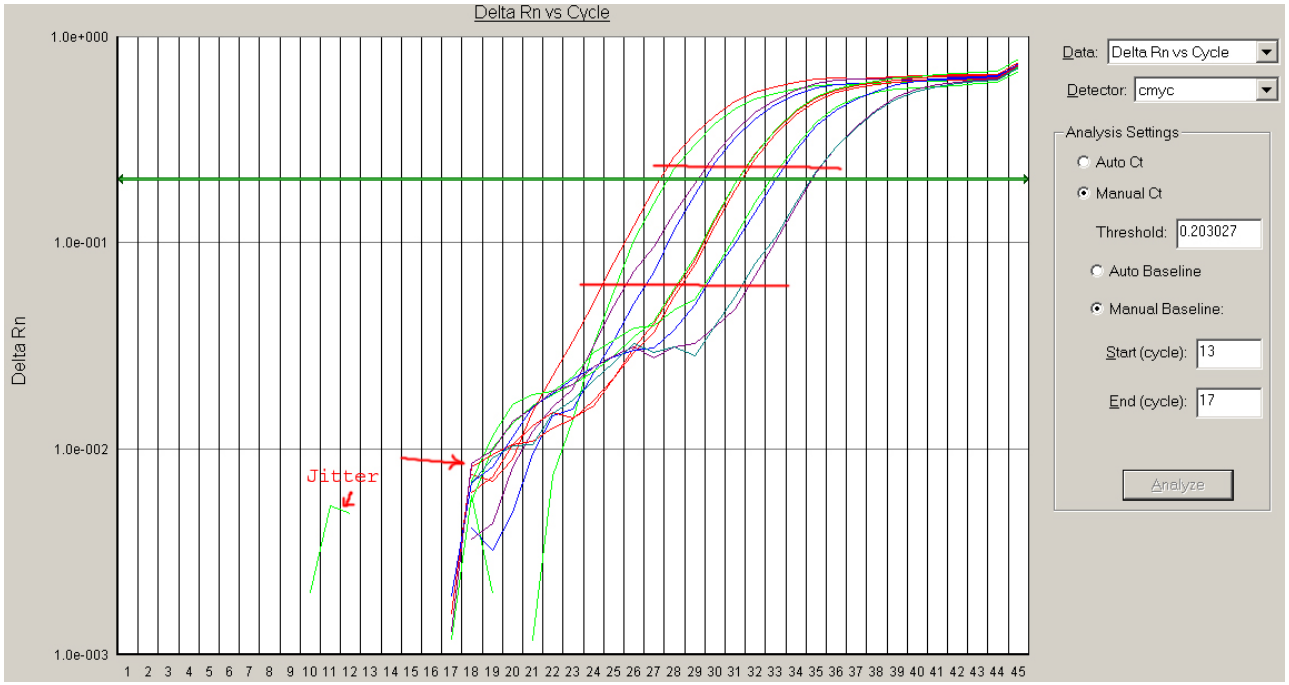


Fig 10: Setting an Appropriate Threshold Value

Now set the threshold to a value above the noise but still in the linear part of the graph and press "Analyze".

Common problems:

The setting of the baseline can be rather tricky (especially with luxprimers). A good way to find your way through is to look at the standard curve (next tab after "Amplification Plot" tab). The samples for the calibration curve are at least duplicates (or even triplicates). If the results for one concentration lie more than one half cycle apart then a baseline problem could be the reason: Adjust the end cycle by adding or subtracting one and press "Analyze" to

see if the standard curve changes (look at the distribution of the points, not the R-square value. When optimizing the R-square value and calibration sample dilutions are imprecise you could correct that with wrong baseline settings). Try to change the threshold a little bit to see if the divergent duplicate points were caused by jitter.

In many cases the calibration curve has a U-like shape. In some cases different baseline settings (moving the baseline end cycle away from the signal to lower values) may ease the problem. A good solution is to look at the Ct values of your samples and then use only these calibration curve points with Ct values near the sample Ct values (Set the task of the wells you don't want to include in the calibration curve from "standard" to "unknown"). By shortening the calibration curve you can get a better linear fit to the calibration curve points. But be careful: by reducing the point number it gets more likely that pipetting errors in the calibration curve affect the quality of your data adversely.

ii. Quality Control:

Each primer pair should amplify one welldefined sequence only. Look at the dissociation graphs in the tab labelled *Dissociation*. All graphs for one detector (primer pair) should look much the same. A curve separated from the others may indicate a failure of the PCR in this well. Don't include this well(s) in your data analysis if it is a sample well. Remove the point from the calibration curve if it is part of it (use the well manager to set the task from *Standard* to *Unknown*).

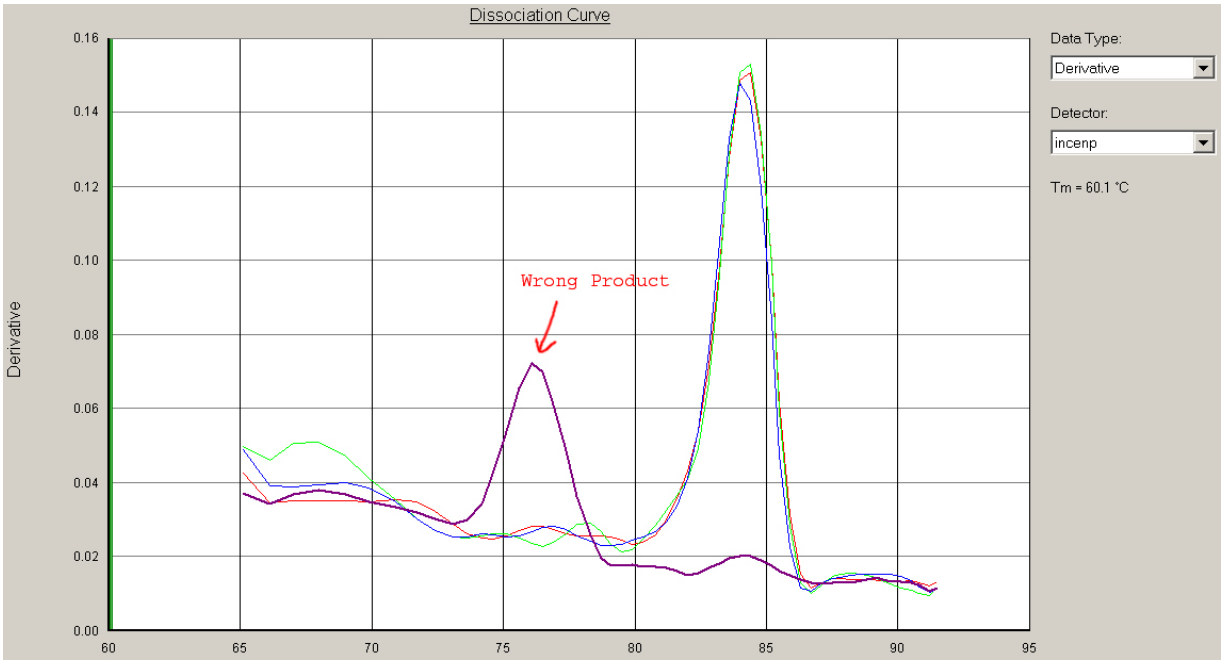


Fig 11: Melting curves of different products

No Template Control and No-Reverse Transcription Control Handling:

- NTC Wells: In most cases the NTC wells will produce inhomogeneous product without peaks or very broad peaks in the dissociation plot. The peaks should be much lower (if here the peaks don't reach even 1/3 of the height of a real product peak) than the normal product peaks and may show at different temperatures for NTC or No-RT duplicates. The peaks are mainly caused by primer dimerisation.
  One exception are wells with 18S lux primers. The primers fit on human, mouse and rat genomic DNA templates and will produce a reproducible sharp peak at a welldefined temperature. Even a very small contamination will be amplified to a detectable product. As long as the Ct value of the NTC lies significantly behind the one for the No-RT, the peak can be ignored.

- No-RT wells: Also the dissociation curves for the No-RT wells shouldn't show any peak if primers spanning exon-exon boundaries were used (so that genomic DNA can't act as template). This is not the case for the Lux 18S rRNA primers because they amplify a sequence that is used in the ribosomes without splicing, so the genomic DNA may act as template and will produce the same product compared to reverse transcribed rRNA. In all other cases a detectable product may indicate problems with the primer design.

Data Export:

Select the "Report" subtab in the "Results" tab. Select all the wells in the well-list at the bottom of the page (by clicking the small square above A-row label and left from the 1-column label). Trigger the export via the menubar "File" – "Export" – "Results". In most cases it's simpler to select all the wells and remove additional data in excel instead of selecting only some wells for the result report. If you decided to use an absolute calibration curve then the data in the result file contains already the absolute concentrations for all unknown samples.

III-6 Calculations

If the plate doesn't contain an absolute calibration curve or if information about the change of regulation (change of ratio of two genes) is wanted, then additional calculations are needed. The two possible methods are ddCt and REST.

ddCt: This method doesn't consider the PCR efficiency, it assumes an efficiency of E=1 for all primers which means that the template concentration doubles in every PCR cycle. It doesn't use all the information from the calibration curve and wastes data. Therefore this method shouldn't be used if calibration data is available and higher precision is wanted. If you still want to use it, perform the calculations with the formulas from the *REST*-Method section but set E_x=1 for all genes.

REST:

This method also includes information about the PCR efficiency. More information about the basics can be found in papers about that topic (look at the AbiPrism Absolute Quantitation User Bulletin stored in TWiki > Laboratory > DeviceAbiPrism7000Data). The formulas are more complicated that's why it's recommended to create one spreadsheet that contains the result data table from the SDS software and calculates the REST results from that data. If one and the same plate layout is used more than once then only the result data from the SDS software has to be exchanged (and perhaps the result columns have to be renamed).

The method uses the slope (k) and intercept (d) values of the regression line. The easiest way to acquire them is to read them out from the "Calibration Curve" tab in the SDS software. The SDS software used the log10 to calculate the logarithmic concentration for the regression line. If you calculate the values by yourself make sure to use the log10. Calculate the PCR efficiency with the use of the k-value from the regression line:

k_x	Slope of regression line for calibration data with primers x
E_x	PCR efficiency for primers x

$$E_x = 10^{-k_x} - 1$$

Calculate the average threshold for all duplicated samples. Don't include points that are obviously wrong (strange line in amplification plot or wrong dissociation data).

The main formula uses the assumption that all the cells contain the same amount of housekeeping gene RNA. Differences in the housekeeping RNA result from errors during RNA extraction, reverse transcription and diluting but all errors had the same effects on the housekeeping RNA and the test gene RNA. Therefore the ratio of the concentration of housekeeping gene RNA in the samples ($c_{Ref,rel}$) quantifies the difference between the dilution factors of both samples. The dilution factors in ratio of relative concentrations of sequence x in a sample and the calibrator can be corrected by division by $c_{Ref,rel}$.

E_{Ref}, E_x	PCR efficiency with primers of reference and gene x
$Ct_{Ref,Kal}$, $Ct_{Ref,Sample}$	Threshold cycles for the reference cDNA in the calibrator and in a sample
$Ct_{x,Kal}$, $Ct_{x,Sample}$	Threshold cycles for the gene x cDNA in the calibrator and in a sample
$c_{Ref,rel}$, $c_{x,rel}$	Concentrations of reference and gene x cDNA in one sample relative to the calibrator.
R	Ratio of gene x in sample to calibrator.

$$R = \frac{c_{x,rel}}{c_{\mathrm{Re}f,rel}} = \frac{(E_x - 1)^{(Ct_{x,Kal} - Ct_{x,Sample})}}{(E_{\mathrm{Re}f} - 1)^{(Ct_{\mathrm{Re}f,Kal} - Ct_{\mathrm{Re}f,Sample})}}$$

The $Ct_{x,Kal}$ value is exactly the d-value from the calibration data regression line. This value should reflect the real $Ct_{x,Kal}$ value more precisely than the average of the measured $Ct_{x,Kal}$ values, because the d-value contains more data than the single measurements.

IV. Short Protocol

IV-1. Preparations

- 96x 10uL-tips (in grided box)
- The reconstituted primers (10uM)
- RNAse-free water
- AbiPrism7000 ready (maintenance done)?

IV-2. Sample Preparation

i. Reverse Transcription with SuperScript III First-Strand Synthesis System for RT-PCR Cat No. 18080-051:

a. Dilution of the samples: Make an RNA-Dilution of 312.5ng/uL with water.

Example: Dilution of RNA with 2.11ug/uL to a final volume of 10uL. Calculate the volume of RNA needed

$$v_{RNA} = {v_{final} \cdot c_{final}} \Big/ {c_{RNA}} = {10uL \cdot 0.3125ug \cdot uL^{-1}} \Big/ {2.11ug \cdot uL^{-1}} = 1.481uL$$

Prepare the RNA-Dilution:

Substance	Volume [uL]
DEPC H_2O	8.519
RNA	1.481
Sum	**10**

b. Dilution of the No-RT control: Use the 312.5ng/uL RNA-dilution and dilute it 1:131.25 in ddH_2O

Substance	Volume [uL]
DEPC H_2O	130.25
RNA (312.5ng/uL)	1.000
Sum	**131.25**

c. SuperScriptIII Reaction: Prepare the SSIII RNA mixes with the diluted RNAs from above:

Substance	Volume [uL]
RNA (312.5ng/uL)	8
Random Hexamer Primers (50ng/uL)	1
dNTP-Mix (10mM)	1
Sum	**10**

Heat the SSIII RNA mixes to 65°C for 5min and put on ice for at least 1 min afterwards. Use the thermoblock or the tetrade (Program STDPRO > SS3PRE, lid heating 90°C or above).

Prepare the cDNA Synthesis mix during the 5min 65°C incubation time (multiply volume as needed)

Substance	Volume [uL]
10x RT Buffer	2
MgCl2 (25mM)	4
DTT (0.1M)	2
RNaseOut (40U/uL)	1
SuperScriptIII RT (200U/uL)	1
Sum	**10**

Add 10uL of cDNA Synthesis Mix to each RNA sample mix. Heat for 10min to 25°C and then 50min to 50°C. Terminate the reaction by heating to 85°C for 5min (tetrade program STDPRO > SS3MAINH).

RNAse H digest: add 1uL of RNAse H solution to each sample, put the samples to 37°C for 20min (Thermoblock or tetrade program STDPRO > FIXT37).

d. 1:50 Dilution of the cDNA samples:

Substance	Volume [uL]
ddH_2O	98
SSIII Product	2
Sum	**10**

e. Dilution of the cDNA used for relative calibration: Prepare dilutions of 1:5, 3.15:50, 1:50, 3.15:500 and 1:500 of the cDNA product from the SuperScriptIII reaction.

Prepare the 1:5 dilution (*Std 10x*) at first:

Substance	Volume [uL]
ddH_2O	400
SSIII Product	100
Sum	**500**

Prepare the remaining calibration curve point solutions using the 1:5 dilution (*Std 10x*) from above:

Dilution	Vol ddH_2O [uL]	Vol 1:5 Dilution [uL]	Final Vol [uL]
Std 10x		316.5	316.5
Std 3.15x	217.5	100	300
Std 1x	630	70	700
Std 0.315x	307.5	10	317.5
Std 0.1x	346.5	3.5	350
Sum		**500**	

IV-3. Functional Setup

- Enter the plate setup into the SDS software
- Enter the correct thermocycle configuration
    - Thermocycle Setup for Platinum Quantitative PCR SuperMix UDG (Invitrogen 11730) with Luxprimers:

      The standard profile from the datasheet works fine:

      50°C for 2min
      95°C for 2min
      45 cycles of:
          95°C, 15sec
          55°C, 30sec (anneal)
          72°C, 30sec (elongate)

      Select the "Dissociation Protocol" checkbox to enable meltingpoint analysis (product characterization).
- Store the plate setup to the local hard drive.

IV-4. Plate Preparation

a. Thawing of reagents and samples:

- Thaw the reagents in the 4°C fridge.
- Mix samples (but don't vortex the supermix) and spin down immediately afterwards
- Make aliquots from the PCR-Supermix if the liquid is still in the 10mL bottle (98x or 100x reactions)
- Put reagents into light-tight icebox

b. Prepare the plate according to the pipetting scheme

b-I Pipetting Scheme for a 96-Well Plate with 3 Genes and 32 Reactions a 25uL per Gene using Platinum Quantitative PCR SuperMix-UDG (Invitrogen 11730-017) and Lux Primers:

Assume your genes are named A, B and C and you have primer pairs for each gene. Label one 2mL eppi with S (Supermix Tube) and 1.5mL eppis A, B, C (Mastermixes with primerpair for genes A, B, C).

i. Prepare the supermix for 98 Reactions in the 2mL eppi labelled S:

Substance	Volume [uL]
Platinum UDG Supermix	1225
ddH_2O	588
Rox Reference Dye	49
Sum	**1862**

Mix well by slowly inverting the tube 5 times, spin down.

ii. Transfer 618uL supermix from tube S to each of the two labelled eppis (A, B). Spin down eppi S and transfer 618uL to C (8uL should remain in S).
iii. Add 16.26uL of each primer of pair A to eppi A, primers B to eppi B, ….

Substance	Volume [uL]
Supermix	618
Labelled Primer	16.26
Unlabelled Primer	16.26
Sum	**650.52**

iv. Put an optical reaction plate (e.g. AbiPrism 96-Well optical reaction plate No. 4306737) onto a 96-wellplate holder and that onto a coolpack (cooled to -20°C) lying in a styropore icebox top cover.
v. Transfer 20uL of mastermix to each well using an *Eppendorf Multipette* with an *Eppendorf combitip* No. 0030 069.226. Use a 20uL sampler for the last few wells.
vi. Transfer 5uL from your cDNA-dilutions to each of the wells. Add 5uL of ddH2O to the NTC wells and 5uL of the No-RT sample to the No-RT wells.

c. Close the plate with an optical adhesive cover sheet (e.g. AbiPrism 4313663).

d. Spin down the liquids in the 96-well plate by centrifugation with 400rcf for 2min. (1500rpm with the 158mm swingtrays in the Jouan CR422 centrifuge recall program 2).

e. Switch on the AbiPrism machine and to start the software during the centrifugation time

f. After centrifugation put the grey-brown rubber-foam mate with the grey side down onto the plate and put the plate into the AbiPrism 7000 machine.

g. Start the run by clicking onto the start button located in the instrument setup tab.

V.Definitions, Abbreviations

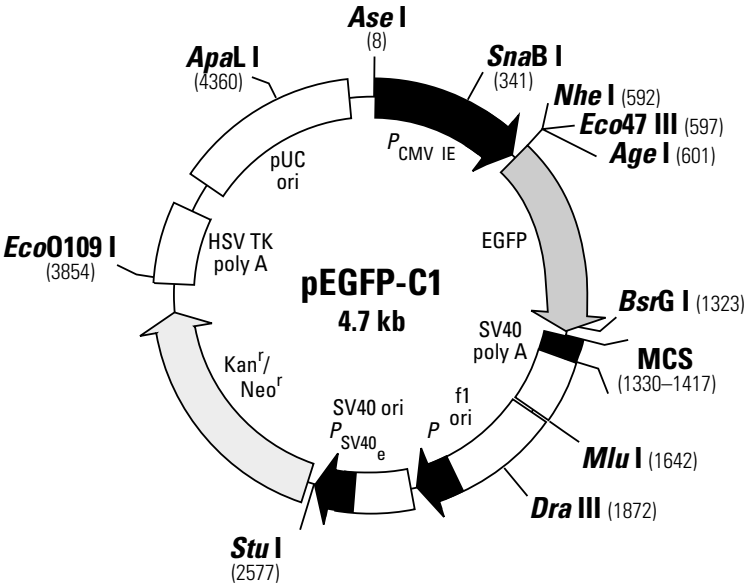
- *Calibration Curve*: Measure the reaction kinetics with a defined sample composition. This has basically nothing to do with the *Calibrator*
- *Absolute Calibration Curve*: Absolute concentration in calibration samples is known (e.g. in fg/uL)
- *Calibrator*: The calibrator sample is used in relative quantitation assays. The ratio of gene of interests' concentration and the reference gene's (e.g. 18S) is set to 1 in this sample. The calibrator is the reference that defines a gene's unregulated state.
- *Mastermix: Supermix* plus primers, only the template DNA is missing
- *No-RT:* No reverse transcription control, a well where RNA was added instead of cDNA sample.
- *NTC:* No template control, a well where water was added instead of sample
- *SDS*: Sequence Detection System (The control software for the AbiPrism7000 machine, also used for data evaluation)
- *Supermix*: Mix including polymerase, buffer and dNTPs but without primers or template.

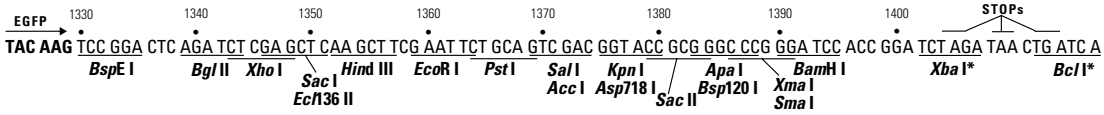
VI.ENCLOSURE

No enclosure given.

pEGFP-C1 Vector Information PT3028-5

GenBank Accession #: U55763 Catalog #6084-1





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C1. All restriction sites shown are unique. The *Xba* I and *Bcl* I sites (*) are methylated in the DNA provided by BD Biosciences Clontech. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam*– host and make fresh DNA.

Description

pEGFP-C1 encodes a red-shifted variant of wild-type GFP (1–3) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-C1 encodes the GFPmut1 variant (4) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences (5). Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site (6) to further increase the translation efficiency in eukaryotic cells. The MCS in pEGFP-C1 is between the EGFP coding sequences and the SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. SV40 polyadenylation signals downstream of the EGFP gene direct proper processing of the 3' end of the EGFP mRNA. The vector backbone also contains an SV40 origin for replication in mammalian cells expressing the SV40 T-antigen. A neomycin resistance cassette (Neor), consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. A bacterial promoter upstream of this cassette expresses kanamycin resistance in *E. coli.* The pEGFP-C1 backbone also provides a pUC origin of replication for propagation in *E. coli* and an f1 origin for single-stranded DNA production.

(PR29971; published 03 October 2002)

Use

Fusions to the C terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo*. The target gene should be cloned into pEGFP-C1 so that it is in frame with the EGFP coding sequences, with no intervening in-frame stop codons. The recombinant EGFP vector can be transfected into mammalian cells using any standard transfection method. If required, stable transformants can be selected using G418 (7). pEGFP-C1 can also be used simply to express EGFP in a cell line of interest (e.g., as a transfection marker).

Location of features

- Human cytomegalovirus (CMV) immediate early promoter: 1–589
  - Enhancer region: 59–465; TATA box: 554–560
  - Transcription start point: 583
  - C→G mutation to remove *Sac* I site: 569
- Enhanced green fluorescent protein gene
  - Kozak consensus translation initiation site: 606–616
  - Start codon (ATG): 613–615; Stop codon: 1408–1410
  - Insertion of Val at position 2: 616–618
  - GFPmut1 chromophore mutations (Phe-64 to Leu; Ser-65 to Thr): 805–810
  - His-231 to Leu mutation (A→T): 1307
  - Last amino acid in wild-type GFP: 1327–1329
- MCS: 1330–1417
- SV40 early mRNA polyadenylation signal
  - Polyadenylation signals: 1550–1555 & 1579–1584; mRNA 3' ends: 1588 & 1600
- f1 single-strand DNA origin: 1647–2102 (Packages the noncoding strand of EGFP.)
- Bacterial promoter for expression of Kan^r gene
  - –35 region: 2164–2169; –10 region: 2187–2192
  - Transcription start point: 2199
- SV40 origin of replication: 2443–2578
- SV40 early promoter
  - Enhancer (72-bp tandem repeats): 2276–2347 & 2348–2419
  - 21-bp repeats: 2423–2443, 2444–2464, & 2466–2486
  - Early promoter element: 2499–2505
  - Major transcription start points: 2495, 2533, 2539 & 2544
- Kanamycin/neomycin resistance gene
  - Neomycin phosphotransferase coding sequences:
  - Start codon (ATG): 2627–2629; stop codon: 3419–3421
  - G→A mutation to remove *Pst* I site: 2809
  - C→A (Arg to Ser) mutation to remove *Bss*H II site: 3155
- Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal
  - Polyadenylation signals: 3657–3662 & 3670–3675
- pUC plasmid replication origin: 4006–4649

Primer Locations

- EGFP-N Sequencing Primer (#6479-1): 679–658
- EGFP-C Sequencing Primer (#6478-1): 1266–1287

Propagation in *E. coli*

- Suitable host strains: DH5α, HB101, and other general purpose strains. Single-stranded DNA production requires a host containing an F plasmid such as JM109 or XL1-Blue.
- Selectable marker: plasmid confers resistance to kanamycin (30 μg/ml) to *E. coli* hosts.
- *E. coli* replication origin: pUC
- Copy number: ≈500
- Plasmid incompatibility group: pMB1/ColE1

References

1. Prasher, D. C., *et al.* (1992) *Gene* **111**:229–233.
2. Chalfie, M., *et al.* (1994) *Science* **263**:802–805.
3. Inouye, S. & Tsuji, F. I. (1994) *FEBS Letters* **341**:277–280.
4. Cormack, B., *et al.* (1996) *Gene* **173**:33–38.
5. Haas, J., *et al.* (1996) *Curr. Biol.* **6**:315–324.
6. Kozak, M. (1987) *Nucleic Acids Res.* **15**:8125–8148.
7. Gorman, C. (1985) In *DNA Cloning: A Practical Approach, Vol. II,* Ed. Glover, D. M. (IRL Press, Oxford, UK) pp. 143–190.

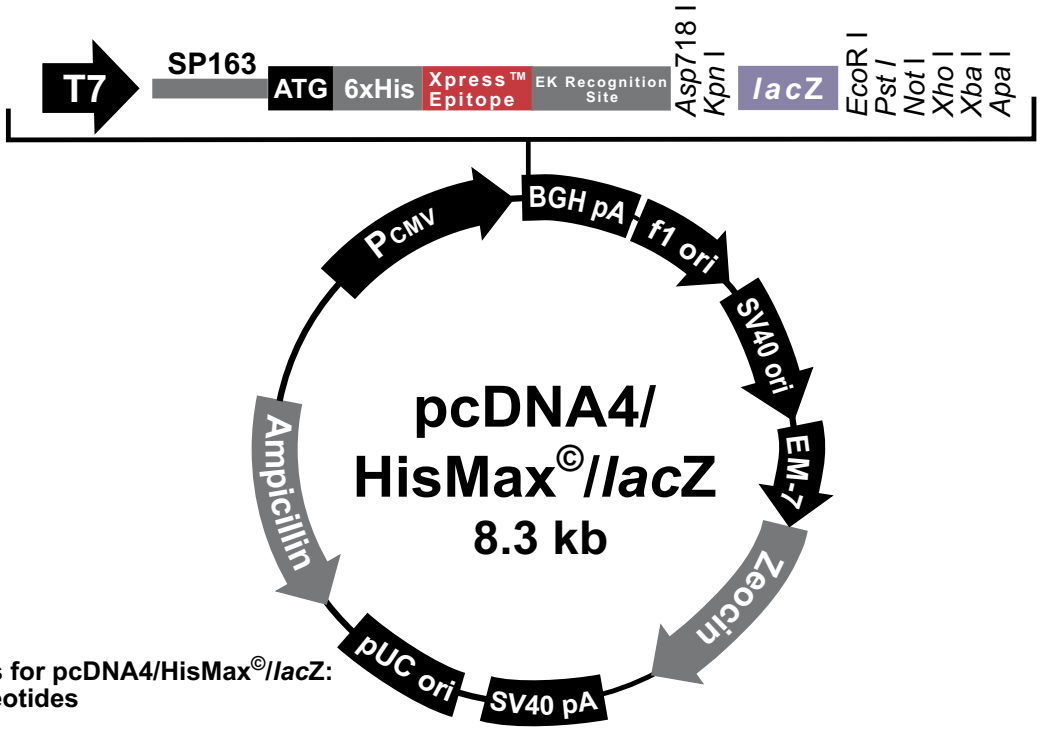
pcDNA4/HisMax©/*lacZ*

Description

pcDNA4/HisMax©/*lacZ* is a 8321 bp control vector containing the gene for β-galactosidase. This vector was constructed by ligating a 3.1 kb *Kpn* I-*Eco*R I fragment containing the *lacZ* gene into the *Kpn* I-*Eco*R I site of pcDNA4/HisMax©.

Map of Control Vector

The figure below summarizes the features of the pcDNA4/HisMax©/*lacZ* vector. **The complete nucleotide sequence for pcDNA4/HisMax©/*lacZ* is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (see page 19).**



Comments for pcDNA4/HisMax©/*lacZ*: 8321 nucleotides

CMV promoter: bases 232-819
T7 promoter/priming site: bases 863-882
QBI SP163 translational enhancer: bases 917-1079
ATG initiation codon: bases 1080-1082
Polyhistidine tag: bases 1092-1109
Xpress™ epitope: bases 1149-1172
Enterokinase recognition site: bases 1158-1172
LacZ ORF: bases 1197-4247
BGH reverse priming site: bases 4328-4345
BGH polyadenylation sequence: bases 4331-4558
f1 origin: bases 4604-5032
SV40 promoter and origin: bases 5059-5368
EM-7 promoter: bases 5416-5471
Zeocin™ resistance gene: bases 5490-5864
SV40 polyadenylation sequence: bases 5994-6124
pUC origin: bases 6507-7180
Ampicillin resistance gene: bases 7325-8185

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