The finding of new targets of microRNA-21 in prostate cancer

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BPH	benign prostatic hypertrophy
PSA	Prostate-specific antigen
miRNA	microRNA
ssRNA	single-stranded RNA
nt	nucleotides
UTR	untranslated region
RISC	RNA-induced silencing complex
TPM1	Tropomyosin 1 (alpha)
PTEN	Phosphatase and tensin homolog
PDCD4	Programmed cell death 4
RECK	Reversion-inducing-cysteine-rich protein with kazal motifs
TIMP3	TIMP metallopeptidase inhibitor 3
BMPR2	Bone morphogenetic protein receptor type II
BTG2	BTG family member 2
MCS	multi-cloning site
LUC	luciferase binding site
-AAA...	poly-A-tail
rpm	rounds per minute
KpnI	An E. coli strain that carries the KpnI gene from Klebsiella pneumoniae OK8
BglII	A E. coli strain that carries the BglII gene from Bacillus globigii
MluI	An E.coli strain that carries the MluI gene from Micrococcus luteus

dNTPs	desoxyribonucleotide triphosphates
CIP	Calf Intestinal Alkaline Phosphatase (source: calf intestinal mucosa)

1. ABSTRACT

Prostate cancer is one of the leading cancers in men, but still all common treatment methods show a large number of disadvantages. A new approach is to find a treatment method, which does not harm the patient in such a great way as traditional methods such as radiation, hormone or chemotherapy. These new approaches look for treatment methods on the molecular level and in this specific case by using target specific microRNAs. It is believed that the regulation of microRNAs is possibly a new candidate to treat and regulate cancer as some microRNA levels have been found to be over-expressed in various cancer cells including breast, cervical, lung and colorectal cancer. The regulation or repression of microRNA-21 is believed to lead to apoptosis of the cells with an over- or higher expression of microRNA-21, which are mostly cancerous cells. Therefore, the overall goal is to find ways how to regulate microRNA-21 based on their role in mRNA regulation in different tissues.

2. ZUSAMMENFASSUNG

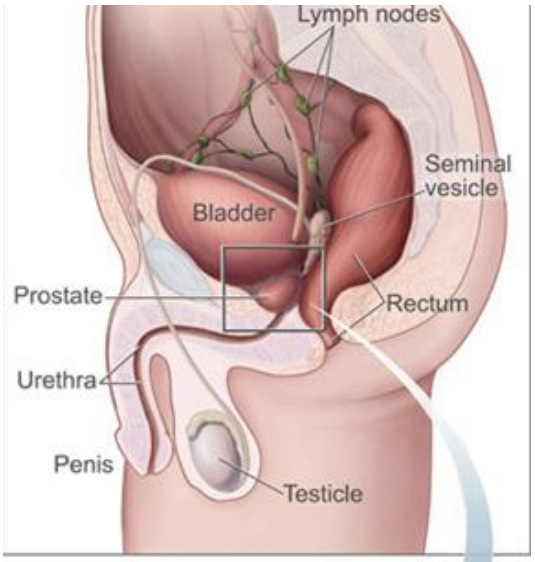
Prostatakrebs ist eine der führenden Krebsarten in Männern, doch immernoch zeigen die gebräuchlichen Behandlunsgmethoden eine Vielzahl an Nachteilen Ein neuer Ansatz ist es, Behandlungsmethoden zu finden, die dem Patienten nicht so sehr wie traditionelle Behandlungsmethoden unter die Bestrahlung, Hormon- & Chemotherapie, schaden. Diese neuen Ansätze suchen nach einer Behandlungsart auf dem molekularen Level und in diesem Fall durch die Verwendung von microRNAs. Es wird vermutet, dass die Regulation von microRNAs ein neuer, möglicher Kandidat ist, um Krebs zu behandeln und regulieren, da man in verschiedenen Krebsarten, wie Brust-, Gebärmutter, Lungen- & Darmkrebs, diverse microRNA Level als überexpressioniert gefunden hat. Es wird vermutet, dass die Regulation oder Unterdrückung von microRNA-21 zur Apoptose von Zellen mit hoher microRNA-21 Expression, welche meistens carzinogene Zellen sind, führen könnte. Darauf basierend ist es ein allgemeine Ziel neue Wege zu finden, um microRNA-21, basierend auf seiner Rolle in verschiedenen Geweben, zu regulieren.

3. INTRODUCTION

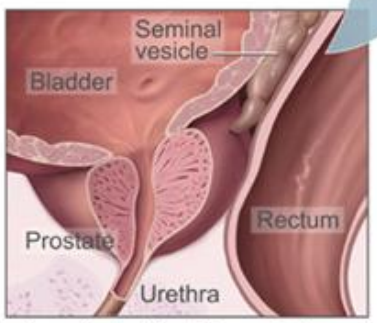
3.1. The prostate

The prostate is a compound tubuloalveolar exocrine gland of the male mammalian reproductive system [2]. To ensure proper work of the prostate, male hormones, also known as androgens, are needed; these hormones are also responsible for the male sex characteristics. Even though the main male hormone is testosterone, the prostate is regulated by the hormone dihydrotestosterone.

The size of a healthy human prostate is about the size of a walnut – in prostate cancer the size of the prostate is largely increased. The urethra, just below the urinary bladder, is surrounded by the prostate and called prostatic urethra within the prostate where it merges with the two ejaculatory ducts. The prostate itself is sheathed in the muscle of the pelvic floor (**Fehler! Verweisquelle konnte nicht gefunden werden.**).



This shows the prostate and nearby organs.



This shows the inside of the prostate, urethra, rectum, and bladder.

n

e

Figure 1. Prostate and its nearby organs in close caption

3.2. Prostate cancer

Prostate cancer is the most common malignancy among men in the United States, with an estimated 186 320 new cases and 28 660 prostate cancer–related deaths in 2008 [1]. The most common test for screening and detecting

prostate cancer is the PSA-test, which is based on the prostate-specific antigen levels in the blood. In case of increased levels of PSA – indicating the presence of a prostate cancer – mostly lead to a biopsy of the prostate to ensure the diagnosis.

The treatment for prostate cancer differs dependant on the stage and the spreading of the cancer, varying from the removal of the prostate, and possibly also the lymph nodes, to radiation as well as hormone therapy.

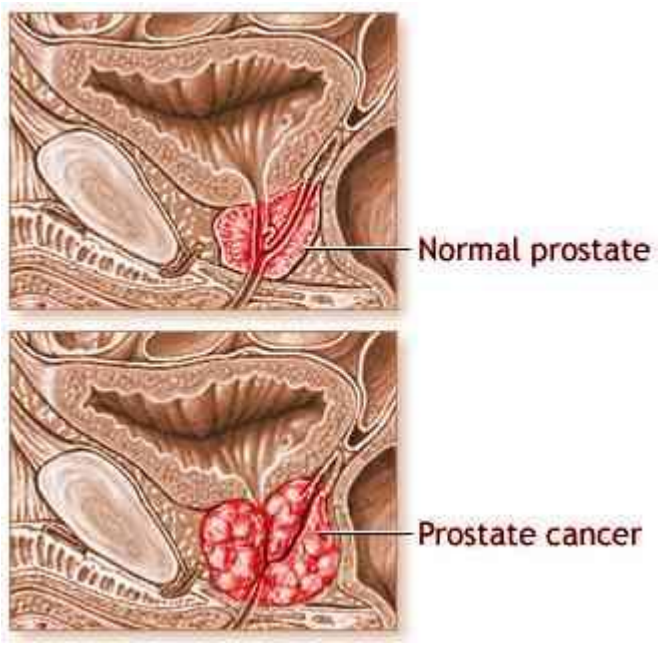


Figure 2. Comparison of a normal prostate to prostate cancer with increased cell growth

3.3. MicroRNAs

MicroRNAs are single-stranded RNAs of ~22 nt in length that are generated from endogenous hairpin-shaped transcripts [1]. miRNAs function as guide molecules in post-transcriptional gene regulation by base-pairing with the target mRNAs, usually in the 3' untranslated region. More than 500 species of miRNAs have now been identified in humans [5][6]and binding of a miRNA to the target mRNA typically leads to translational repression and exonucleolytic mRNA decay, although highly complementary targets can be cleaved endonucleolytically [7]. Over one third of human genes are predicted to be

directly targeted by miRNAs. Consequently, the unique combination of miRNAs in each cell type determines the use of thousands of mRNAs [7].

3.3.1. Biogenesis of miRNA

miRNAs are initially transcribed by RNA polymerase II as long primary transcripts (pri-miRNA). Pri-miRNAs are processed by Drosha and Pasha, producing 60- 70 nt stem-loop precursors miRNA (pre-miRNA). Pre-miRNAs are then exported to the cytoplasm by the nuclear export factor exportin 5 and cleaved further by Dicer, giving rise to transient ~22-nucleotide duplexes containing mature miRNA and their complementary nucleotides. Mature miRNA are then loaded onto the RNA-induced silencing complex, where they interact with target mRNA transcripts. When a miRNA and its cognate mRNA interact with perfect complementarity, RISC directly cleaves the target mRNA [8][9][10] (**Figure 3**).

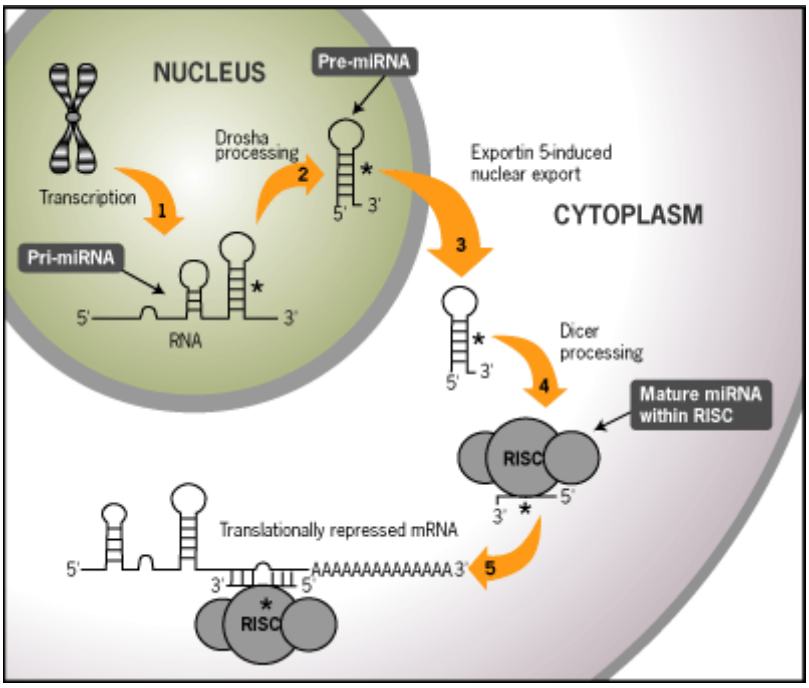


Figure 3. miRNA Processing Pathway

3.3.2. Regulatory roles of microRNAs

The most essential question arising from the discovery of hundreds of different miRNAs is what they are all doing. In some miRNAs, like lin-4 and let-7, crucial clues to their regulatory targets as well as their functions were identified even before they were known to be non-coding RNA genes.

The reported functions of these miRNAs is based on in vivo experiments and for some of these, the function has been determined by the phenotypic consequences of a mutated miRNA or an altered miRNA complementary site, either of which can disrupt miRNA regulation. In other cases, function was inferred from the effects of mutations or transgenic constructs that lead to ectopic expression of the miRNA [11].

For the vast majority of miRNAs, the phenotypic consequences of disrupted or altered miRNA regulation are not known. However, computational approaches are being developed to find the regulatory targets of the miRNAs, providing clues to miRNA function based on the known roles of these targets [12][13][14][15]. Different approaches are used to determine specific direct regulatory functions of miRNAs on different mRNAs.

3.4. microRNA-21

The microRNA-21 is located on the chromosome 17 at location 17q23.1. (

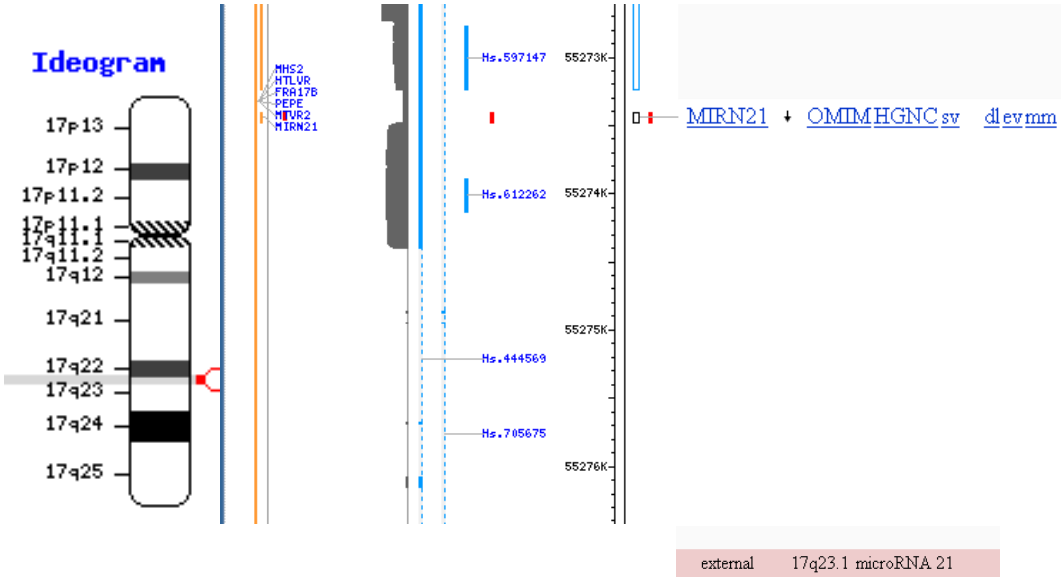


Figure 4. The chromosome 17 (a) & the specific location of miR-21 on the chromosome (b)

The microRNA-21 gene (miR-21) has been identified as the only miRNA commonly overexpressed in solid tumors of the lung, breast, stomach, prostate, colon, brain, head and neck, esophagus and pancreas [16][16]. Increased expression of miR-21 has been implicated in various processes involved in carcinogenesis, including inhibition of apoptosis [17], promotion of cell proliferation [18]and stimulation of tumor growth [19]. In addition, increased miR-21 has been associated with chemoresistance in human cholangiocarcinoma cells [20]. Interestingly, miR-21 seems to be involved in a number of positive and negative feedback loops, and therefore is a part of the complex regulatory network operating in both normal and diseased cells (

Figure 6).

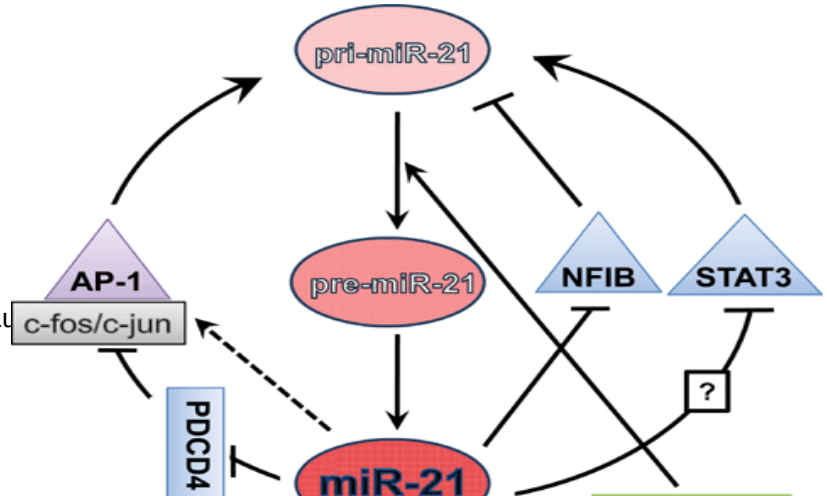


Figure 5. Model of miR-21 network and feedback regulation. Maturation of miR-21 from pri-miR-21 is shown in the center of the model. miR-21 direct target genes are depicted on blue background. Genes shown on green background are regulated (probably indirectly) by miR-21 and are involved in miR-21 processing from pri-miR-21 to pre-miR-21.

3.5. Databases predicting microRNA targets

In eukaryotes, the 3' UTR of the mRNA does not have to be perfectly complementary complementary to the seed sequence of the miRNA (binding site), whereas in prokaryotes, this binding is perfectly complementary (

Figure 6).

Due to this fact, there are many different ways how mRNAs and miRNAs can bind to each other and this is the reason why the prediction of miRNA targets is so difficult and why different algorithms for the prediction of miRNA targets exist.

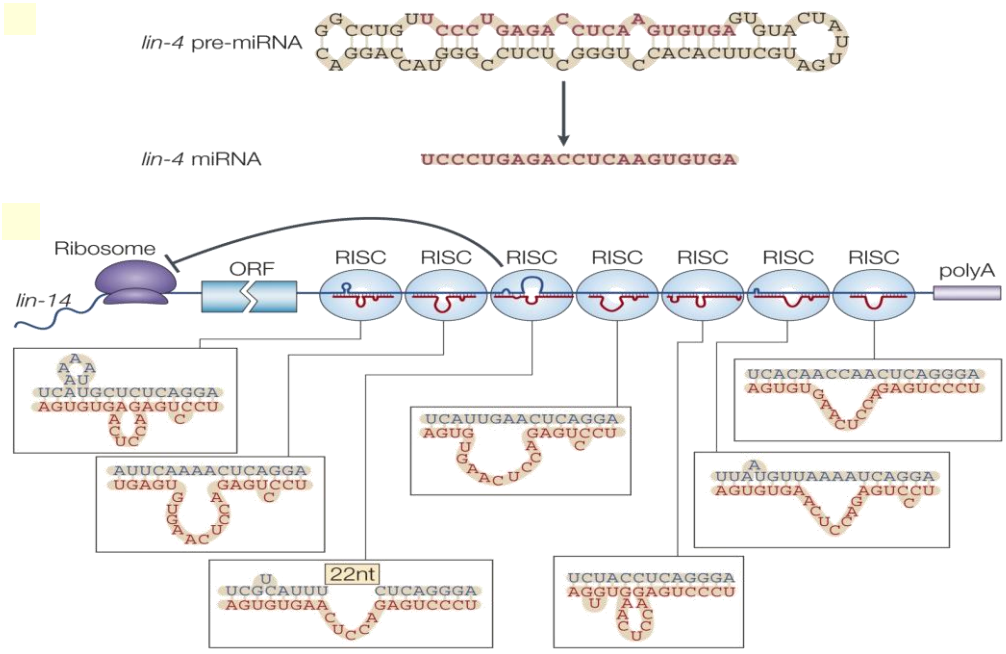


Figure 6. The different binding possibilities between mRNA and miRNA in eukaryotes with the example lin-4

3.5.1. The nomenclature of for binding possibilities

In the world of miRNA, there is a specific nomenclature used to describe the different binding possibilities between mRNAs and miRNAs (

Table 1[29]). This nomenclature is also used by the different algorithms to predict the targets and apportion them a ranking.

Table 1. The different binding possibilities between mRNA and miRNA and the nomenclature used

Position	Predicted Target Region (top) and has-let-7e (bottom)	Seed Match
Position 4-27 of HMGA2 3' UTR	5' ...CAAC-GUUCGAUUU-CUACCUCA... 3' UGAUAUGUUGGAGGAUGGAGU	8mer
Position 1089-1113 of HMGA2 3' UTR	5' ...GACCUGAAUA-CCACUUACCUCAA... 3' UGAUAUGUUGGAGGAUGGAGU	7mer-1A
Position 1239-1262 of HMGA2 3' UTR	5' ...CCACUACUCAAU--ACUACCUCU... 3' UGAUAUGUUGGAGGAUGGAGU	7mer-m8

The sites shown in the table are defined as following:

8mer site: an exact match to the positions 2-8 of the miRNA (= the seed + position 8) with the mRNA and a downstream 'A' across on position 1 of the miRNA.

7mer-1A: an exact match to the positions 2-7 of the miRNA (= the seed) with the mRNA and a downstream 'A' across on position 1 of the miRNA

7mer-m8: an exact match to the positions 2-8 of the miRNA (= the seed + position 8) with the mRNA, but no downstream 'A' across on position 1 of the miRNA

These binding possibilities are used in different algorithms to rank the target predictions as well as to show the actual binding positions between mRNA and miRNA.

3.5.2. The miRanda algorithm

The miRanda algorithm [[30][31] consists of 2 basic steps, supplemented by statistical and phylogenetical estimations to identify potential targets. miRanda reads the RNA sequences of one file (file 1), scans them against all sequences in another file (file 2) and reports all potential target sites. The target sites are identified based on an alignment score, which is based on sequence complementarity. There are non-specific penalties for mismatches,

gap openings and gap extensions. Important is that the miRNA positions 2-8 (seed region) are in perfect complementary match with the reference sequence. At a second stage, the Vienna package for RNA folding is employed [32] to estimate the thermodynamic properties of all predicted duplexes. The thermodynamic parameters related to an optimal folding of this artificial RNA allow scoring potential target sites by their folding energies [33].

3.5.3. The TargetScan algorithm

The program TargetScan – given a miRNA conserved in several organisms as wells as a set of orthologous 3' UTR sequences - searches the UTR for segments of perfect Watson-Crick complementarity to bases 2-8 of miRNA from its 5' end. Then, the program extends each seed match in every direction as far as possible – also allowing G:U pairs – but stops at mismatches. It then uses the RNA fold program of the Vienna package to complete the alignment. The scores are produced according to sites' binding energies and also searches for conserved regions in other species. The program gives the possibility to list the predicted targets either by 'total context score' or P_{CT} – the probability of conserved targeting as described in Friedman et al., 2008.

3.5.4. The PicTar algorithm

In the program PicTar, the approach heavily relies on cross-species comparisons through multiple sequence alignments of orthologous 3' UTRs, where potential miRNA targets are located. The PicTar algorithm implements a probabilistic model estimating the likelihood for a given sequence segment to function as binding site for a single miRNA or combination thereof and using general 3' UTR sequences as a background [33]. Firstly, conserved 3' UTR segments are checked for their minimal number of perfect and imperfect matches for a given miRNA set, which is specified by the user. Afterwards, it derives a Hidden Markov Model-based score for a given UTR to be targeted by the given miRNA.

A figurative explanation of the PicTar algorithm is given in Figure 7.

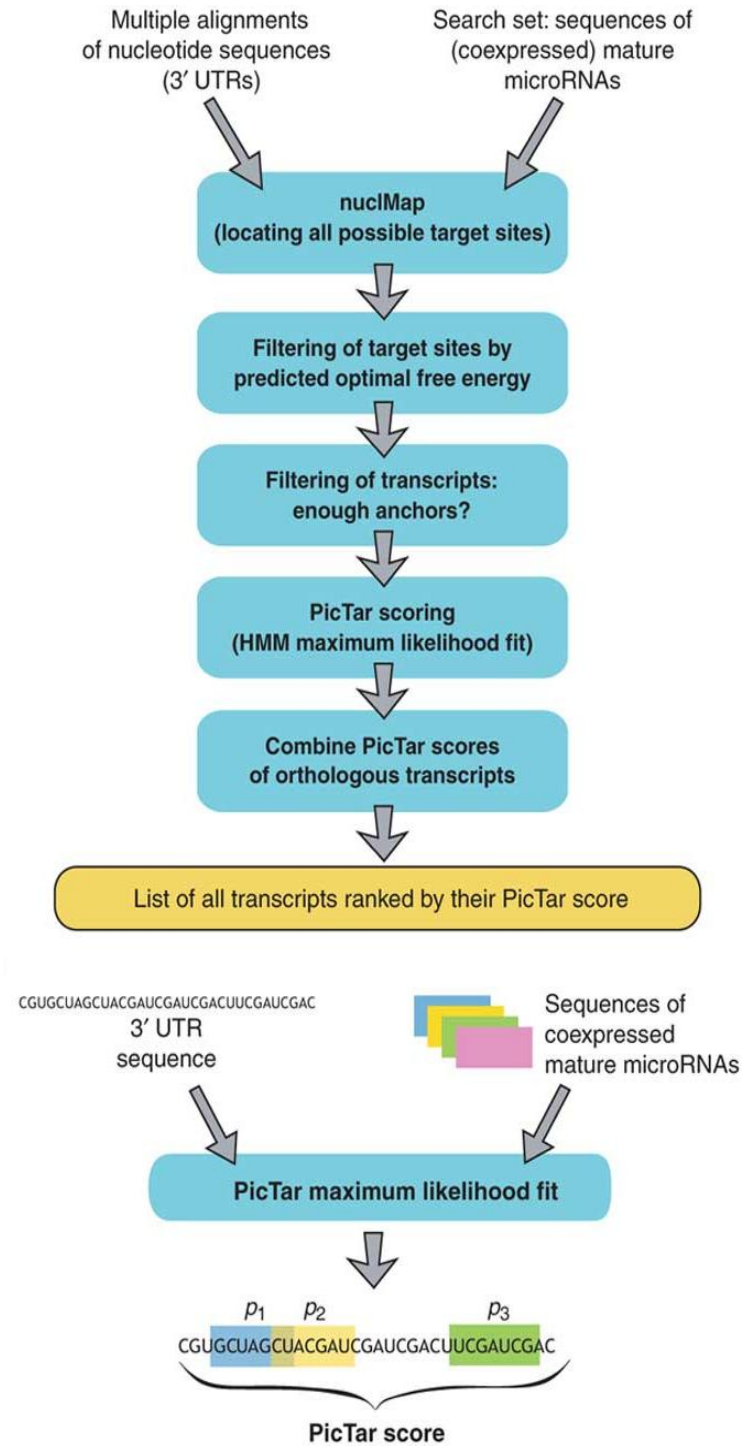


Figure 7. The PicTar

4. AIM

The overall aim of the experiments was to identify new targets of miR-21 which can be further used for in vivo studies and could give rise to new treatments method of prostate cancer on a molecular level – being not as invasive and dangerous for the patient.

In earlier experiments, a microarray was done, using LAPC-4 and LNCaP cells – both prostate cancer cells – to identify messenger RNAs up- and downregulated in these cell lines, which represented to be direct targets of miR-21.

The next step was to use a bioinformatical approach to narrow this list of potential targets down by predicting targets of human miR-21 with different algorithms and finding matches. The list of narrowed down targets was the starting point for further experimental plans.

The hypothesis was that by cloning the UTRs of predicted targets (messenger RNAs) into a vector construct, containing also a luciferase active site, amplifying the UTR using specifically designed primers and then transfect them with miR-21 a change should have been visible – if the mRNA was a direct target of miR-21.

Theoretically a direct regulation of miR-21 should be visible through either up- or downregulation of luciferase activity in the vector containing the UTR compared to a control vector.

Another aim was to construct a diverse set of vectors to confirm the findings of the luciferase reporter assay. The construction of the vectors had to be planned and carried out precisely, including different sets of analysis after each major step, in order to ensure that the vector construct used in the major experiments were correct.

5. GENERAL EXPERIMENTAL WORKFLOW

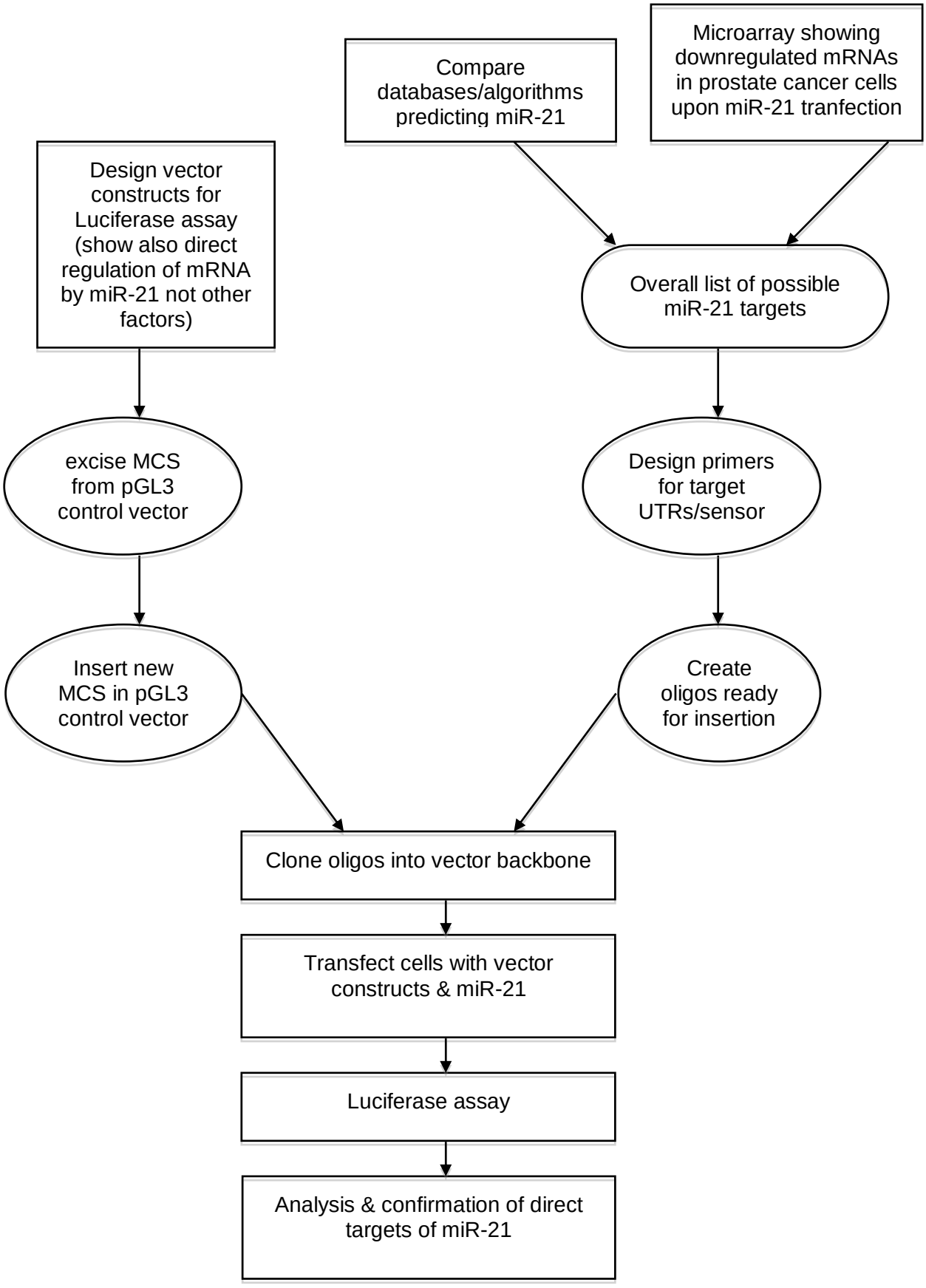


Figure 8. General workflow of experiments

6. METHODS

6.1. Prediction of mir-21 targets

6.1.1. Bioinformatical approach

For the prediction of miR-21 targets, three bioinformatical approaches were used, each characterized by a different algorithm. The three algorithms each represent a different online database predicting targets of microRNAs. Based on other findings usind prediction softwares, the three following databases were chosen: miRanda, TargetScan and PicTar. Each of them works with a different algorithm and has a specific sensitivity and specificity. An overview of their criteria for prediction and ranking mechanisms is given below:

Table 2. Comparison of the three databases used (m = mammalian/vertebrate, w = worms, f = fly, + = additional clades)

Tool	Clades	Criteria for prediction & ranking	Website URL	Recent teference
TargetScan	m	Stringent seed pairing, site number, site type, site context (which includes factors, that influence site accessibility); option of ranking by likelihood of preferential conservation rather than site context	http://targetscan.org	Friedman et al., 2008
PicTar	m, f, w	Stringent seed pairing for at least one of the sites for the miRNA, site number, overall predicted pairing stability	http://pictar.mdc-berlin.de	Lall et al., 2006
miRanda	m, f, w, +	Moderately stringent seed pairing, site number, pairing to most of the miRNA	http://www.microrna.org	Betel et al., 2008

The three databases gave different predictions as they use different algorithms – for the miRanda database, the sensitivity as well as selectivity settings were chosen to obtain more results. The outcomes of the databases were then transferred to an Excel spreadsheet and checked for any repetitions. The next step was to compare the three different lists to each other as well as comparing all three with each other.

A Venn-Diagramm was constructed to show the process of narrowing down the list of potential targets by only comparing different databases.

The usage of several different databases to predict targets of a specific microRNA was not described up to now in any paper or other experiments. A comparison to other approaches was therefore not possible.

6.1.2. Comparison with mRNA Microarray

A list of up- and downregulated mRNAs both in LNCaP and LAPC-4 cells was obtained from an earlier performed mRNA Microarray. This list precisely showed which of the mRNAs were affected in the two different types of prostate cancer cells.

The list of downregulated mRNAs (p-value < 0,02) was then compared to the predictions of the different databases, narrowing the number of potential targets down by either comparing it to one database, two databases or all three databases together. A Venn-Diagramm was constructed to show the process of narrowing down potential targets by comparing the different databases with the outcome of the Microarry as well as different Excel-Sheets showing the process of narrowing down targets.

The final list used for further experiments was obtained by comparing the downregulated mRNAs in LAPC-4 and LNCaP with only one database at a time to widen the result span.

6.2. Vector design

6.2.1. Basic flow of construct creation

The construction of five different vectors was designed for further experiments. The general vector forming the backbone for all five specifically designed vectors was the pGL3 control vector (**Fehler! Verweisquelle konnte nicht gefunden werden.**).

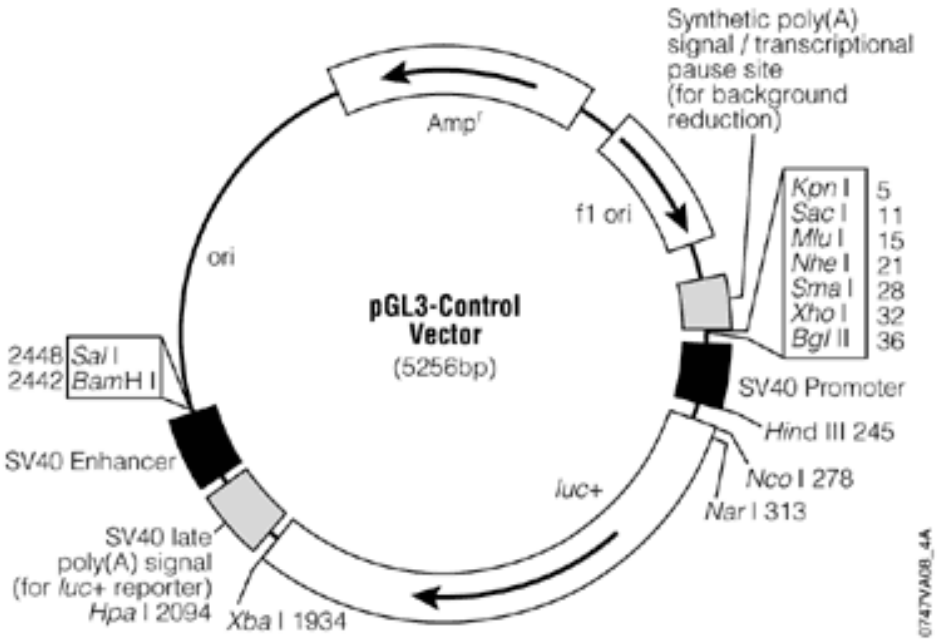


Figure 9. The pGL3 control vector in its basic form

For further vector design, it was planned to excise the original MCS from the pGL3 control vector by control vector by using doing a double digest with either MluI & BglII or KpnI & BglI – as shown in (as shown in (

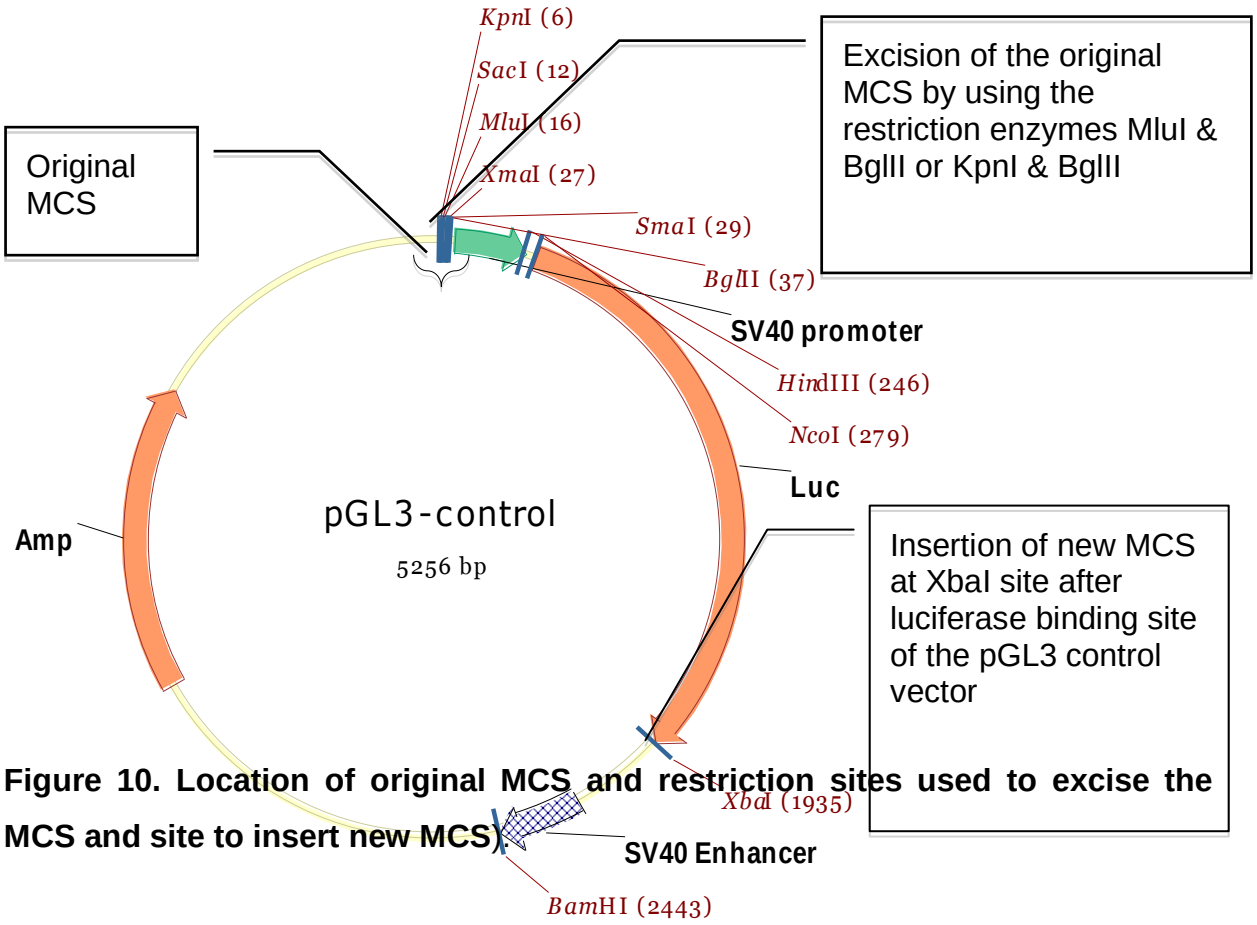


Figure 10. Location of original MCS and restriction sites used to excise the MCS and site to insert new MCS).

Figure 10. Location of original MCS and restriction sites used to excise the MCS and site to insert new MCS

To insert a new MCS after the luciferase binding site (Luc), the restriction enzymes XbaI was used and XbaI was used and after insertion of the new MCS at this site (

Figure 10. Location of original MCS and restriction sites used to excise the MCS and site to insert new MCS).

The newly inserted KpnI site (in the new MCS) was used to insert different UTRs and sequences for creating vectors with the following characteristics:

- ***Vector 1:*** a normal pGL3 control vector with a luciferase binding site

  The luciferase activity in the vector should be high as the promoter can bind to the luciferase site

- ***Vector 2:*** a pGL3 control vector with luciferase binding site and a 3' UTR with the putative miR-21 binding site (the seed sequence of miR-21 is in the UTR)

  The luciferase activity should be low as the miR-21 binds to the seed sequence in the UTR and blocks the luciferase

- ***Vector 3:*** a pGL3 control vector with luciferase binding site and a 3' UTR with a mutation in the putative miR-21 binding site

  The luciferase activity should be high as miR-21 cannot bind to the seed sequence due to the mutation and therefore doesn't block the luciferase

- ***Vector 4:*** a pGL3 control vector with luciferase binding site and a perfectly complementary sequence to miR-21 (only miR-21 can bind perfectly to the sequence)

  The luciferase activity should be low if miR-21 is the present in the sample as it binds to the complementary sequence and blocks the luciferase

- ***Vector 5:*** a pGL3 control vector with luciferase binding site and a mutation in the sequence perfectly complementary to miR-21

  The luciferase activity should be high again as miR-21 cannot bind as well as other microRNAs and therefore luciferase activity shouldn't be blocked anymore.

The site of insertion is for all vectors at the XbaI site of the pGL3 vector, after the luciferase binding site as shown in **Figure 10**.

In **Fehler! Verweisquelle konnte nicht gefunden werden.**, the basic design and major differences and characteristics of the five different vector constructs is shown to support the theoretical approach.

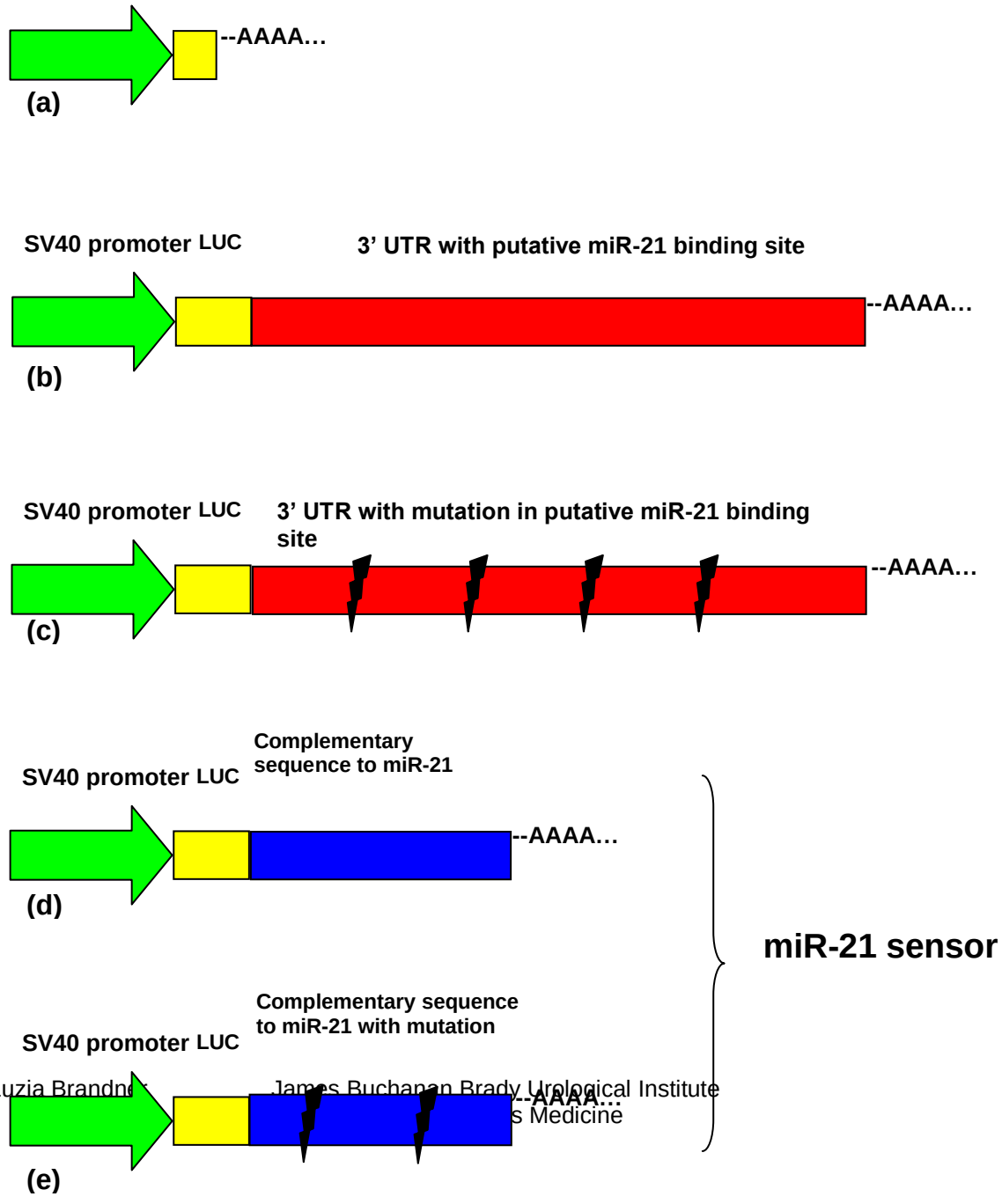


Figure 11. Basic scheme of the different vector constructs: (a) Vector 1 as described above, (b) Vector 2, (c) Vector 3, (d) Vector 4 and (e) Vector 5

6.2.2. Primer Design

The design of primers can be done with many different programs, all of them available online. There are also different programs available for the design of vectors and primers, but in most of the papers, it is not mentioned which programs were used to design primers or the steps of designing primers

For every predicted target, a specific set of primers was designed for later on inserting the targets mRNA into the different vector constructs. For each predicted mRNA targeted by miR-21, the UTR was obtained by entering the target as well as the miR-21 into the different databases. The sequence number used by Genome Browser was then generated and noted [36] (**Figure 12**).

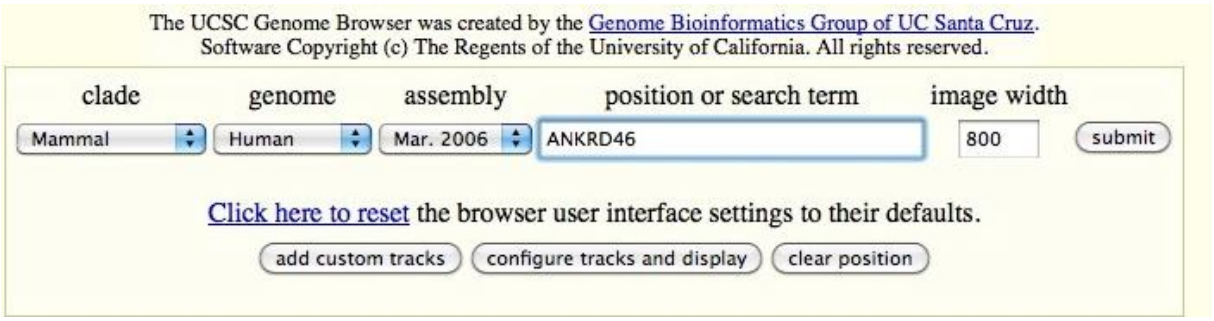


Figure 12. The Genome Browser search engine used to find mRNA sequences

From the Genome Browser website, the sequence of the specific UTR was obtained by entering the target into the “Genomes” search line. Then, the sequence with the specific sequence number – obtained from the databases – was opened (**Figure 13**).

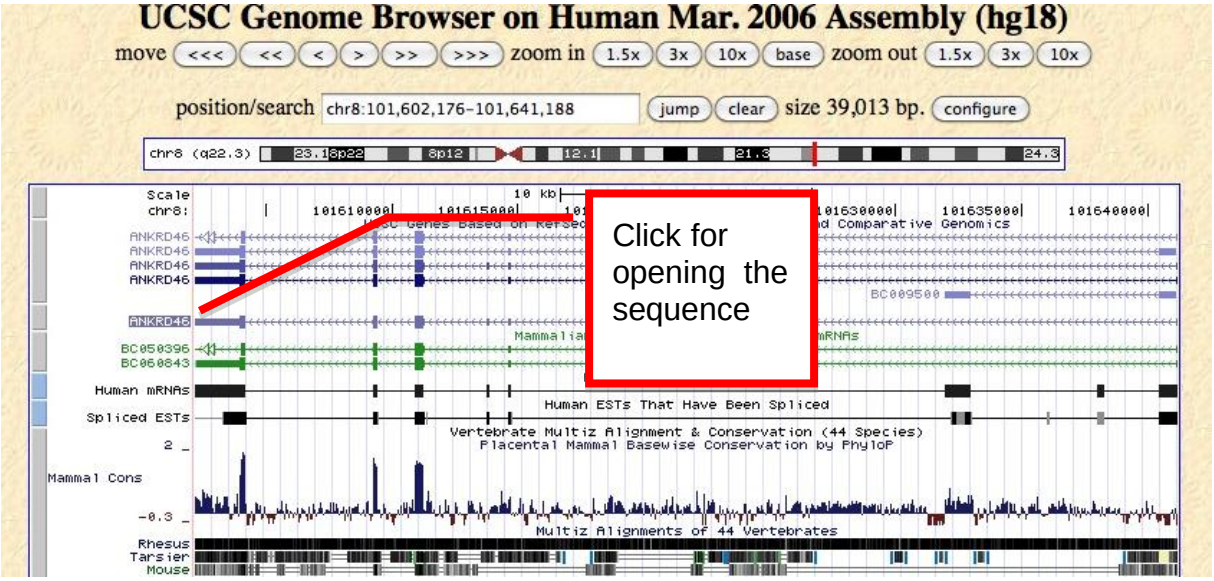
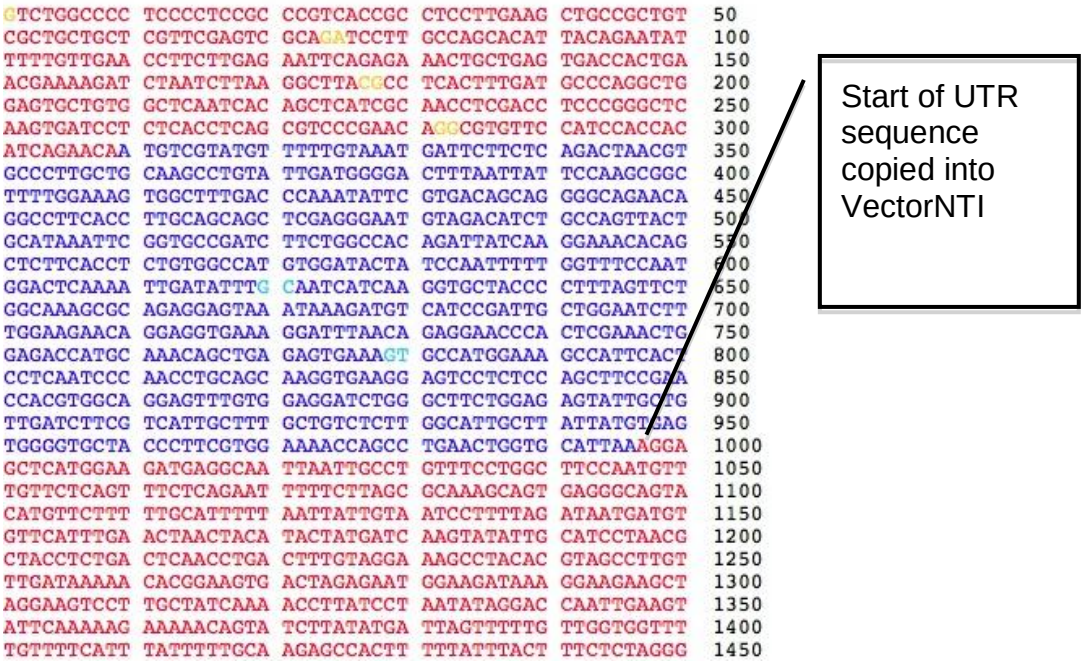


Figure 13. Selection of the right mRNA in order to obtain the sequence of the UTR

From the sequence, the UTR was selected – starting with the first A of the UTR (**Figure 14**) – and copied into the program VectorNTI.

cDNA NM_198401



Start of UTR sequence copied into VectorNTI

Figure 14. Selection of UTR - starting with A - from the sequence, copied into VectorNTI

In VectorNTI, the sequence was then checked for the presence of the miR-21 seed sequence (obtained sequence (obtained from the different databases). Afterwards, the online tool "Primer3" [37] was used to "Primer3" [37] was used to design forward and backward primers. A part of the UTR, which had the miR-21 seed sequence somewhere in the middle, was copied into the field for the sequence (Figure 15. Field for entering the sequence used to design primers in Primer3

) and the product size range was set between 1300 and 1800 bp.

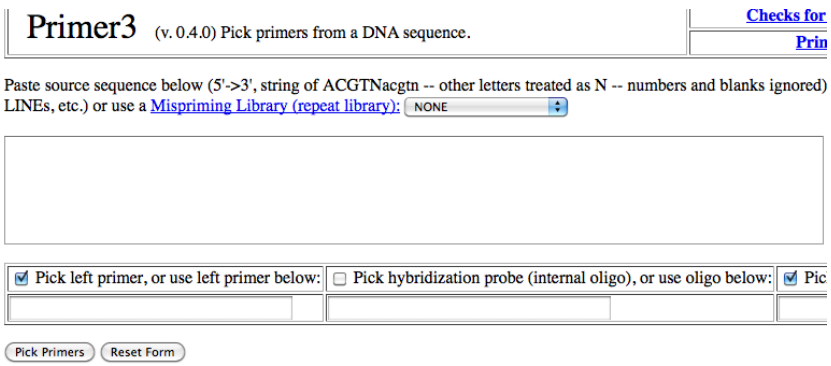


Figure 15. Field for entering the sequence used to design primers in Primer3

The primers obtained from Primer3 were then copied into a Word-Document and additionally, the sites for the restriction enzymes BglII and XhoI were added to the

primers, as those sites were then used to insert the UTR into the vector. The following sequences were added to the primers:

Forward primers: G G G A G A T C (BglII)

Reverse primers: G G G C T C G A G (XhoI)

The primers were then analyzed in VectorNTI – first alone and then as primer pairs aligned to each other. The focus was set, that the 3' ends were without any binding at the first position. If a primer pair fulfilled these requirements it was further analyzed and the theoretical PCR product was obtained. The rating for the primer pairs was secondary in this case.

Afterwards, Genome Browser PCR [38] was used to check whether the primers would amplify only one product in order to exclude the possibility of multiple, non-wanted products from the PCR. If the outcome was that only one chromosome was amplified by the primers, the designs of this primer pair were saved and ordered from Sigma-Aldrich.

6.3. Vector Backbone construction

6.3.1. DNA extraction using Miniprep

The approach of extracting DNA by using a Miniprep Kit is followed by most laboratories as it can be easily done and a complete Kit with all solutions necessary including a proven protocol can be obtained from different manufacturers.

Firstly, shaking cultures with 5 mL LB Medium with 5 µL Ampicillin (final concentration: 50 µg/mL) and a colony, picked with an inoculation loop from a LB Agar plate with pGL3 control colonies, were set up. The cultures were incubated overnight at 37° C and 225 rpm in a shaking incubator. For the DNA extraction, the QIAGEN QIAprep Miniprep Kit© [42] was used, following the bench protocol using a vacuum manifold.

From the overnight culture, 3 mL were used and distributed equally between two 1,5 mL microcentrifugation tubes, each containing 1,5 mL culture. The samples were then spun down for one minute at 13 300 rpm and the supernatant removed from the tubes. The pelleted bacterial cells were then resuspended in a total of 250 µL resuspension buffer (P1) and pooled in one microcentrifugation tube. After the addition of 250 µL of buffer P2, the tube was inverted 4-6 times to mix the solution. Due to the addition of LyseBlue © reagent to the buffer P2, the solution turned blue. Then, 350 µL buffer N3 were added and it was mixed immediately upon addition by inverting the tube 4-6 times. Due to the LyseBlue reagent system, the solution turned milky white to colorless. The sample was then centrifuged for 15 minutes at 13300 rpm in a table-top microcentrifuge.

In the meantime, the vacuum manifold as well as the QIAprep spin columns were prepared as described in the handbook. After centrifugation, the clear supernatant was applied to the spin column by pipetting. The vacuum source was switched on and the solution drawn through the spin columns. As soon as the supernatant passed through the column, the vacuum source was switched off again. The spin column was then washed by applying 500 µL of buffer PB and switching on the vacuum source again, drawing the buffer through the column. Afterwards, the vacuum source was switched off again and 750 µL of buffer PE were applied to the column. The vacuum source was switched on again and as soon the buffer had passed through, it was switched off and the column was transferred to a microcentrifugation tube. It was then centrifuged for one minute at 13000 rpm to remove residual wash buffer. The column was then placed in a new microcentrifugation tube and 50 µL of elution buffer were added to the center of the spin column. After 5 minutes, not one minute as recommended in the handbook, the tube was centrifuged again for 1 minute at 13000 rpm. Afterwards, the spin column was removed from the microcentrifugation tube and the tube was placed on ice to avoid DNA degradation. The DNA concentration of the sample was measured using the DNA concentration feature on the Nanodrop.

6.3.2. Restriction digest to excise the MCS

The usage of restriction enzymes to excise parts from a plasmid or creating an open site to insert sequences into a plasmid is widely spread and there are different manufacturers offering restriction enzymes with the according buffers. The set up of restriction digests can be varied, depending on the amount of sample as well as the amount of digested plasmid needed.

For the excision of the original MCS of pGL3, a restriction double digest was set up with the following enzyme pairs: KpnI & BglII and MluI & BglII. The enzymes have to following cutting patterns:

BglII
A | G A T C T
T C T A G | A

KpnI
G G T A C | C
C | C A T G G

MluI
A | C G C G T
T G C G C | A

The amount of enzyme used was determined by the amount of DNA in the sample. The enzymes as well as the buffers used were obtained from New England Biolabs and the buffers used for the double digest were based on the recommendation of the manufacturer's handbook. The unit definition of the manufacturer is that unit is defined as the amount of enzyme required to digest 1 µg of DNA in 1 hour at 37° C in a total reaction volume of 50 µL. The digestion was set up using 20 µL of the sample obtained from the Miniprep in a total reaction volume of 100 µL. Another consideration was not to exceed 10% of the total reaction volume with the enzymes used to avoid oversaturation. A restriction digest was then set up as following:

Table 3. Set up of the restriction digests

	Sample volume	Buffer # & volume	Enzyme 1 Name & volume	Enzyme 2 Name & volume	DEPC-treated water volume	BSA volume
Digest 1	20 µL	B2, 9 µL	KpnI, 5 µL	BglII, 5 µL	60 µL	1 µL
Digest 2	20 µL	B3, 10 µL	MluI, 5 µL	BglII, 5 µL	60 µL	-

The restriction digest was then carried out overnight by placing the samples in a waterbath with 37° C warmth to activate the enzymes.

To confirm the excision of the MCS using restriction enzymes, 2 µL of the sample were taken after overnight digestion, mixed with 1 µL 5X loading buffer and run in a 2% Argarose gel with a 1kb DNA ladder size marker to analyze the bands obtained. The gel was run for 30 minutes in 1X TAE buffer by applying 100V.

6.3.3. DNA purification

After a positive confirmation of the MCS excision, the sample was purified from the enzymes by performing an Ethanol precipitation. The protocol for the Ethanol precipitation was created in this laboratory and is only used in this laboratory.

The set up was as following, the numbers of 96% Ethanol and 3M Natrium acetate based on the sample volume (X):

Table 4. Set up and sample amount used for Ethanol precipitation

	Sample volume	96% Ethanol	3M Natrium acetate	Glycoblue©
Sample	X µL	3*X µL	0,1*X µL	1-3 µL

The amount of Glycoblue used was based on the final reaction volume – for volumes larger than 200 µL, 3 µL of Glycoblue were used.

The mixture with the different components, according to **Fehler! Verweisquelle konnte nicht gefunden werden.**, was placed at -80° C for 30 minutes. Afterwards, it was centrifuged for 15 minutes at 13300 rpm and a temperature of 4° C. The supernatant was pipetted off and replaced with 1 mL 75% Ethanol. The sample was then centrifuged again for 10 minutes at 13300 rpm. The supernatant was once more removed carefully, not destroying the pellet and the pellet was dried for 5 minutes at room temperature. The remaining dried pellet was then resuspended in 20 µL of DEPC-treated water. Once more the DNA concentration was measured using the DNA concentration feature on the Nanodrop.

6.3.4. Digestion of 3’ and 5’ overhangs

In order to ensure a proper ligation, the 3' and 5' overhangs, created by the different restriction enzymes used before, had to be digested. For this step, three different nucleases were used: Mung Bean, DNA Polymerase I Large (Klenow) fragment and Klenow fragment (3'→ 5' exo). The nucleases as well as the buffers were obtained from New England Biolabs and the buffers used were as recommended by the manufacturer. Also, the digests needed an addition of dNTPs which were also obtained from New England Biolabs.

For the sample digested with MluI and BglII, the Klenow fragment (3' → 5' exo) was used and for the sample digested with KpnI and BglII, one part was digested with DNA Polymerase I Large (Klenow) and the other part with first Klenow fragment (3' → 5' exo) and then Mung Bean.

The set up for the digestions looked as following, using a 1:10 diluted buffer, 1:40 diluted dNTPs and 1:10 diluted nucleases:

Table 5. Set up of the three reactions using different nucleases

Sample	Sample volume	DEPC-treated water volume	Buffer # & volume	dNTPs volume	Nuclease & volume	Nuclease & volume
MluI&BglII	8,4 µL	6,6 µL	B2, 2 µL	2 µL	Klenow 3'→5', 1 µL	-
KpnI&BglII	3,7 µL	11,3 µL	B2, 2 µL	2 µL	Klenow 3'→5', 1 µL	Mung Bean, 1 µL
KpnI&BglII	3,7 µL	11,3 µL	B2, 2 µL	2 µL	Klenow large, 1 µL	-

After addition of the first nuclease, the samples were placed at 25° C for 15 minutes. For the KpnI & BglII sample digested with the Klenow fragment (3' → 5' exo), 1 µL of diluted Mung Bean was added and the sample was placed at 30° C for 1 hour. The other two samples were removed from the 25° C and 80 µL of DEPC-treated water were added. The sample having the Mung Bean digest was removed from the 30° C after an hour and also 80 µL of DEPC-treated water were added to a final volume of 100 µL in all three samples.

6.3.5. Nuclease inactivation by Phenol Chloroform preciptiation

The protocol for the Phenol Chloroform precipitation was created in this laboratory, like the protocol for the Ethanol precipiation, it is therefore possible that other laboratories have different ways to inactivate enzymes.

In order to inactivate the nucleases in the sample, a Phenol cholorform precipitation was performed. Each 100 µL sample were mixed with 100 µL of Phenol chloroform (45%/58% v/v) and the samples were centrifuged for 15 minutes at 13300 rpm at 4° C. Afterwards, the organic phase of the supernatant was taken up and transferred into a new microcentrifugation tube.

After the phenol chloroform precipitation, the sample (taken up supernatant) was purified again by performing another Ethanol precipitation as described before.

6.3.6. Plasmid ligation

After the 3' and 5' overhangs in samples were removed, the plasmid was ligated using T4 DNA Ligase, obtained from New England Biolabs. The set up of the experiment was as following:

Table 6. Set up and volumes used for plasmid ligation

	Sample volume	T4 buffer volume	T4 ligase volume	DEPC-treated water volume
Sample	10 µL	2 µL	1 µL	7 µL

Each of the three samples, obtained after Ethanol precipitation, was used for the ligation, the remains of the samples (each 10 µL) were stored at -20° C.

After adding the buffer as well as the ligase and the DEPC-treated water, the samples were kept at room temperature for 1 hour. Afterwards, they were placed on ice for several hours and later on placed at 16° C overnight.

The ligation protocol was created in this laboratory based on a number of earlier experiments. It is individual for this laboratory and will most likely differ from other ligation protocols.

6.3.7. Transformation with DH5α

After overnight ligation, the samples were placed on ice again. Each sample was transformed in an individual tube. In 3 PCR tubes 30 µL of DH5α cells were placed and then 5 µL were added. The mixture was kept on ice for 5 to 30 minutes. Afterwards, they were heat shocked for 30 seconds at 42° C and immediately afterwards placed on ice again. To each tube, 250 µL of room temperature warmed S.O.C. media (Invitrogen©) were added and the tubes were placed horizontally in a shaking incubator at 37° C and 225 rpm for an hour.

After incubation, 250 µL of sample were spreaded on LB Agar plates with Ampicillin and the plates were placed in an incubator at 37° C overnight.

The next day, the plates were checked for colonies and put at 4° C.

The DH5α transformation is based on the user manual from the TOPO TA Cloning® Kit for Sequencing from Invitrogen. The steps for transformation can differ other to the transformation protocols used by other laboratories.

6.3.8. DNA extraction from transformed cells

The plates showing cultures were used to set up overnight shaking cultures. Depending on the number of usable colonies on the plates – not counting satellite colonies – a number of shaking cultures was set up, following the same procedure as before. For each culture, one inoculation loop with a bacterial colony was mixed into 5 mL of LB medium containing 5µL Ampicillin (final concentration: 50 µg/mL).

The cultures were placed in a shaker and incubated overnight at 37° C and 225 rpm.

From the shaking cultures, 3 mL were used to next day to perform a Miniprep following the standard protocol, except for the last step where the elution buffer (EB) was left on the spin column for 5 minutes instead of 1 minute to increase the yield.

6.3.9. Confirmation of ligation & further digestion

To confirm the excision of the MCS as well as the ligation and transformation, a small amount of the samples obtained from the Miniprep were used for further restriction digests. Three different digestions were set up for each Miniprep culture. The set up was as described below:

Table 7. Set up of the three different restriction digests

	Sample volume	Enzyme 1 volume	Enzyme 2 volume	Buffer volume	BSA volume	DEPC-treated water volume
Digest 1	1 µL	MluI, 1 µL	BglII, 1µL	Buffer 3, 14 µL	6 µL	14 µL
Digest 2	1 µL	XbaI, 1 µL	BglII, 1µL	Buffer 2, 14 µL	6 µL	14 µL
Digest 3	1 µL	KpnI, 1 µL	XbaI, 1 µL	Buffer 2, 14 µL	6 µL	14 µL

The enzymes have the following cutting patterns:

KpnI
G G T A C | C
C | C A T G G

MluI
A | C G C G T
T G C G C | A

XbaI
T | C T A G A
A G A T C | T

The buffer and BSA conditions were based on the manufacturer's recommendations for each double digest. The amount of enzymes used was chosen not to exceed 10%

of the total reaction volume to avoid oversaturation. The mixtures were incubated in a 37° C waterbath overnight.

The samples were analyzed the next day by running them on a 1% Argarose gel. The total of 20 μL sample were mixed with 10 μL 10X loading buffer and from this 5 μl were applied on the gel. Additionally, 3 μL of a1kB ladder size marker were loaded as well as 2 μL of original pGL3 vector (undigested) mixed with 1 μL of 10X loading buffer. The gel was run for 30 minutes in 1X TAE buffer by applying 100 V. It was then stained with Ethidium bromide and analyzed under UV-light. For the samples showing the expected results and therefore confirming the positive excision of the MCS, ligation and transformation, another restriction digest was set up.

The aim of this digest was to open up the plasmid again at the XbaI site, positioned after the luciferase binding site, to further be able to insert the new MCS.

The set up for the restriction digest was as following:

Table 8. Set up of the restriction digest with XbaI

	Sample volume	Enzyme volume	BSA volume	Buffer volume	DEPC-treated water volume
Digest 1	15 μL	XbaI, 8 μL	7 μL	25 μL	25 μL

The amount of enzyme added was not to exceed 10% of the total reaction volume. The buffer and BSA conditions were based on the manufacturer's recommendations. The samples were placed in a waterbath at 37° C overnight.

To confirm the digest, the samples were analyzed the following digest by running them on a 1% Argarose gel. From each sample, 1 μL was taken and mixed with 1 μL 10X loading buffer and then applied to the gel. Additionally, 3 μL of a 1kB ladder size DNA marker were applied to the gel. It was run for 30 minutes in 1X TAE buffer by applying 100 V and afterwards stained with Ethidium bromide and analyzed under UV-light. For positive samples – where the restriction digest could be confirmed – the remaining amount of sample from the digest (79 μL) were mixed with 21 μL DEPC-treated water, adding up to a total volume of 100 μL.

6.3.10. Enzyme inactivation & DNA purification

In order to inactivate the enzyme present in the sample, a Phenol chloroform precipitation was performed. To the 100 µL of sample (diluted with water, as described before) an equal amount of Phenol chloroform (45%/58% v/v) was added. They were mixed by inverting the microcentrifugation tube several times until the mixture appeared milky. The sample was then centrifuged for 15 minutes at 13300 rpm at 4° C. Afterwards, the organic phase of the supernatant (approximately 100 µL) was taken up with a pipette and transferred into a new microcentrifugation tube.

The amount of organic phase was the basis for the set up of the following Ethanol precipitation, as shown in **Fehler! Verweisquelle konnte nicht gefunden werden.**. The same protocol was followed as in earlier Ethanol precipitations, but resuspending the DNA pellet in 11 µL of DEPC-treated water instead of 20 µL. The amount of DNA in the samples was then determined, using the DNA concentration feature on the Nanodrop.

6.3.11. CIP treatment

In order to remove the 5' and 3' phosphoryl groups at both ends of the vector due to the cut with XbaI, a CIP treatment was performed in order to avoid religation between the open ends of the vector.

The DNA obtained after Ethanol precipitation was resuspended with 1X Buffer 3 (New England Biolabs©) to a final concentration of 0,5 µg/10 µL. Then 0,5 units of CIP per µg of vector DNA were added and the mixture was incubated in a waterbath at 37 °C for one hour.

Afterwards, the samples were filled up to a total volume of 100 µL with DEPC-treated water and a Phenol chloroform precipitation was performed, followed by an Ethanol precipitation. The resuspended DNA was then stored at -20° C until further usage.

6.3.12. Construction of new MCS using self-annealing oligo linkers

In order to insert the new MCS for the vector, two different oligo linkers were obtained from the company Sigma-Aldrich, based on the following sequences:

Table 9. Sequences of sense & antisense oligos

MCS Sense Sequence 5' – 3'	3CTAGAGGTACCGAGCTCTTACGCGTGCTAGCCCGGGCTCGAGCCATGGAGATCTA
MCS Antisense Sequence 5' – 3'	3CTAGTAGATCTCCATGGCTCGAGCCCGGGCTAGCACGCGTAAGAGCTCGGTACCT

The sequences were designed using the program VectorNTI and the primers are based on the sequence to be inserted. The new MCS was designed to insert new restriction sites into the plasmid, which differed from the original restriction sites in the original MCS.

From each oligo, 220 pmol were placed in 50 μL 1X TE buffer. The sample was then annealed following the temperature set-up:

1) 95 °C for 3 minutes
2) 95 °C for 1 minute
   - 1° C per cycle (0,1° C/sec.)

} 50 cycles

After ligation, the sample was kept at 4° C until further processing.

The ligation protocol was created by Shawn Lupol and is therefore individual for this laboratory.

6.3.13. Ligation of vector with new MCS

For the ligation of the vector with the new MCS, two different conditions were set up which differed in the amount of oligos (the new MCS) added. The vector was

removed from the -20° C and placed on ice. After complete thawing, the two conditions were set up as shown in **Table 10**.

Table 10. Set up of the ligation of the vector with the new MCS

	Sample volume	T4 buffer volume	T4 DNA ligase volume	DEPC-treated water volume	Oligo volume
Condition 1	5 µL	2 µL	1 µL	11 µL	1 µL
Condition 2	5 µL	2 µL	1 µL	11 µL	1 µL*

The asterix in the **Fehler! Verweisquelle konnte nicht gefunden werden.** indicates that the oligo in this condition was diluted 1:10 beforehand, so still 1 µL of oligo was added, but 10-times less concentrated.

The samples were incubated at room temperature for one hour and then placed on ice. After 2 hours, the samples were incubated at 16° C overnight.

6.3.14. Transformation with DH5α cells

After overnight incubation at 16° C, 5 µL of the sample were mixed with 40 µL of DH5α cells and the mixture was placed on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42° C and immediately afterwards placed on ice again for approximately 5 minutes. They were then mixed with 250 µL of room temperature warm S.O.C. medium (Invitrogen©) and placed horizontally in the shaking incubater. The cells were then incubated for 1 hour at 37° C and 225 rpm.

In the meantime, for each sample a LB Agar plate with Ampicillin (final concentration: 50 µg/mL) was placed at 37° C.

After the incubation time, the entire amount of sample & S.O.C. media (250 µL) was pipetted on an agar plate and spreaded. The plates were then incubated overnight at 37° C, removed the next day and placed at 4° C.

6.3.15. DNA extraction from transformed cells

From the plates with the transformed DH5α cells, colonies were picked and a shaking culture was set up over night. The colonies were picked up with an inoculation loop and each colony was mixed into 3 mL of LB Medium with 3 µL Ampicillin (final concentration: 50 µg/mL). The cultures were then incubated overnight at 37° C at 225 rpm in a shaking incubator. The next day, the cultures were transferred into bigger tubes and 2 mL of LB medium with 2 µL Ampicillin (final concentration: 50 µg/mL) were added. After 4 hours incubation at 37° C at 225 rpm in the shaking incubator, the DNA with extracted using the QIAgen Miniprep Kit following the general protocol, but increasing the DNA yield by leaving the elution buffer (EB) on the spin column for 5 minutes instead of 1 minute. The Minipreps were then kept on ice until further usage. The DNA concentration in the samples were measured by using the DNA concentration feature on the Nanodrop.

To the remaining shaking cultures, which were not used for the Minipreps, 2 mL of LB with 2 µL of Ampicillin (final concentration: 50 µg/mL) were added and the cultures were incubated once more overnight at 37°C at 225 rpm.

6.3.16. Upscaling of shaking cultures

The 5 mL shaking cultures were transferred into 1000 mL flasks the next day and 150 mL of LB medium with 150 µL Ampicillin (final concentration: 50 µg/mL) were added. The cultures were then incubated once again over night at 37° C and 225 rpm.

6.3.17. DNA extraction from large overnight cultures

To extract the DNA from the 150 mL shaking cultures, the Genelute HP Plasmid Maxiprep Kit© from Sigma-Aldrich was used. This Kit is used by many laboratories, as it provides the solutions and the protocol needed for the extraction of DNA from large cultures, in one Kit.

The standard protocol was followed; first, the cultures were transferred into 150 mL tubes and then centrifuged for 10 minutes at 5000 g. The supernatant was then discarded and the pellet was resuspended in 12 mL of cold resuspension solution by vortexing. Then 12 mL of Lysis solution were added, the tube was inverted 6 to 8 times to mix and then 3 to 5 minutes were waited to allow the solution to clear. After the solution had cleared, 12 mL of neutralization solution were added and the tube was gently inverted for 6 to 8 times to mix. Afterwards, 9 mL of binding solution were added, the solution was mixed by inverting 1 to 2 times and then the solution was added to the barrel of the filter syringe. The solution was then let sit for 5 minutes. In the meantime, the binding columns were placed into the vacuum manifold. To the columns, 12 mL of column preperation solution were added and dranw through the column by applying vacuum. After letting the sample sit for 5 minutes, syringe was held over the column and the solution was applied to the column by inserting the plunger into the syringe and therefore expelling the cleared lysate. After the lysate had passed through the column, 12 mL wash solution A were added to the column and allowed to pass through. Then 12 mL of wash solution B were added to the column and passed through. The vacuum was left on for 10 more minutes after passage of the wash solution B to dry the column. After 10 minutes, the column was transferred into a new collection tube (50 mL tube provided with the kit) and 3 mL of elution solution were applied to the column. The tube was then centrifuged for 5 minutes at 3000 g.

The solution obtained was transferred into a 1,5 mL microcentrifugation tube and the DNA concentration was determined using the DNA concentration feature on the Nanodrop.

6.3.18. Confirmation of new MCS at XbaI Site

The next step was to confirm whether the insertion of the new MCS at the XbaI site after the luciferase site worked. The samples obtained from the Miniprep were used for three different restriction digests. The three digests were done to confirm not only that the new MCS was inserted, but also that the old MCS was excised.

Besides the samples from the Miniprep, an undigested, original pGL3 control was used as a control. The three digests were set up as following:

Table 11. Set up of the three different restriction digests

	Sample volume	Enzyme 1 volume	Enzyme 2 volume	Buffer volume	BSA volume	DEPC-treated water volume
Digest 1	3 μL	XbaI, 1 μL	BglII, 1μL	Buffer 2, 6 μL	3 μL	6 μL
Digest 2	3 μL	XmaI, 1 μL	XbaI, 1 μL	Buffer 4, 6 μL	3 μL	6 μL
Digest 3	3 μL	NcoI, 2 μL	-	Buffer 3, 6 μL	3 μL	6 μL

The samples were incubated at 37° C for 2 hours. Then, 10 μL of the samples were run on a 1% Argarose gel to check the progress of the digestion. The 10 μL sample were mixed with 5 μL loading buffer and 10 μL of the mixture were loaded onto the gel. Additionally, 3 μL of a 1kB size DNA ladder were loaded. The gel was run for 30 minutes at 100 V in 1X TAE buffer. It was then stained for 10 minutes with Ethidium bromide and analyzed under UV-light.

6.4. Creation of sequences & ligation with backbone vector

6.4.1. Creation of miR-21 sensor inserts

To create the inserts for the miR-21 sensor vectors – with the complementary sequence to miR-21 as well as the complementary site with mutations – a set of primers was designed, a sense and an antisense primer.

The primers were diluted with DEPC-treated water to a final concentration of 100 μM and then 2,2 μL of each primer were mixed with 1X TAE buffer to a final volume of 50 μL. The annealing was done wit the following temperature set up:

1) 95 °C for 3 minutes
2) 95 °C for 1 minute
   - 1° C per cycle (0,1° C/sec.)

} 50 cycles

After the 50 cycles, the samples were kept at 4° C until further usage.

6.4.2. Creation of UTR inserts

6.4.2.1. Primer Design

The primers used for creating the UTRs with the miR-21 seed sequence by using PCR were designed as described before. The primers were obtained from Sigma-Aldrich and the annealing temperature was based on Primer3 as well as VectorNTI. It was made sure that the secondary structures of the primers were either non-existent or weak. The extension time chosen for each primer pair was based on the fragment to be amplified. As a rule of thumb it was assumed that the DNA polymerase polymerized 1000 bases per minute. The final list of primers used for each target was as shown below:

Figure 16. The list of forward and reverse primers used for the PCR to create the inserts with the UTRs of the targets, containing the miR-21 seed sequence, later to be inserted into the backbone vector

Name of mRNA	Forward primer	Reverse primer
ANKRD46	TCTGATGGCAAGAATGGATG	CTCTGCAAGCTGATCCTGAA
C1orf96	GGGAGAGAATGGTGGTGTGT	GCTTTGCTCCTCCTCCTCTT
EIF1AX	TCCTGGTAGGAATAGCACAGC	GGTCTCTTTAGGGGCCAGAT
KIAA0256	TCCTTTGCTCGATGTCAGTG	TCCTCCCCCAGCTTATTTAGA
KLF5	GACACCTCAGCTTCCTCCAG	TGGCTTTTCACCAGTGTGAGT
MATN2	TGAGGTCGTGAAGCAGTTTG	TGCGATGTTCAGGGCATAC
NAP1L5	CAGCGGCAGAGGAGGTAAT	TACTTCTTGGCGTCATCAGG
NIPBL	GGGAGATCCACCATGCCACTTTGTGAAC	GGGCTCGAGAACCAGTGCTGTAACCATCTGA
PPP13B	CAGCAAGAATGAAGCCAGTG	TCTGTTGCTGTCCCAGTACG
RAB37	AGTCCGAGCTACGACCTCAC	TTCACATGAAGGAGCAGCAG
S100A10	TGGAACACGCCATGGAA	GCCCGCAATTAGGGAAA

SKI	GGGAGATCTTTGGAACCCACT GCAAAAT	GGGCTCGAGGAAGCACGCCAACA CACTTA
ZBTB47	AGTGCCCACGAGTTTTCAA	TGTGTGGGTCTTCATGTGCT

6.4.2.2. PCRs to amplify UTR fragments

The annealing temperatures as well as extension times were based on informations given by Primer3 as well as VectorNTI (for the annealing temperature) and the fragment size to be amplified.

The individual annealing temperatures and extension times were as following:

mRNA name	Annealing temperature	Extension time
ANKRD46		
C1orf96	56° C	2 min. 30 sec.
EIF1AX	54° C	50 sec.
KIAA0256	55° C	1 min.
KLF5	55° C	1 min. 30 sec.
NAP1L5	55° C	50 sec.
NIPBL	55° C	1 min. 30 sec.
PPP1R3B	55° C	50 sec.
RAB37	55° C	55 sec.
S100A10	54° C	30 sec.
ZBTB47	55° C	55 sec.

The PCRs were run according to the following temperature protocol:

Step 1	95° C	5 min.
Step 2	95° C	30 sec.
Step 3	Individual annealing temp.	30 sec.
Step 4	72° C	Individual extension time
Step 5	72° C	10 min.
Step 6	4° C	∞

Steps 2 through 4 were repeated for 40 cycles in total.

For the PCRs, the SYBR® Green JumpStart™ Taq ReadyMix™ was used in order to simplify and speed up the process. The set up of each reaction was as following:

Table 12. Set up per tube for PCR

	cDNA templated volume	Taq Ready Mix	Sense primer	Antisense primer	DEPC-treated H_2O
Reaction	2 μL	12,5 μL	1 μL	1 μL	8,5 μL

If the PCR products were not satisfying – checked through analysis in a 1% Argarose gel – the PCR was set up again with a temperature gradient.

6.4.2.3. PCR with temperature gradient

For the creation of the UTRs, later to be cloned into the vector backbone, a PCR was set up with a temperature gradient to determine a good annealing temperature for the primers. For the PCR, the GoTaq™ DNA Polymerase Kit by Promega was used. The primers amplified fragments of approximately 500 bp and MCF-7 cDNA was used as template for the PCR. For each well, a 25 μL reaction was set up with the following requirements:

Table 13. Set up per well for PCR

	Final sample concentration	Taq Go Polymerase (2X) volume	Final primer concentration	DEPC-treated water volume
Reaction	10 ng	10 μL	500 nM	Fill up to 25 μL

The amount of primer used for the PCR was not as recommended by the manufacturer, but they were rather used in excess. The temperature set up for the PCR was as following:

95° C for 5 minutes
95 °C for 30 seconds
50-65° C for 30 seconds } 40 cycles
72° C for x minutes
72° C for 10 minutes

The extension time (72° C for x minutes) was chosen based on the fragments to be amplified. The last step with 72° C for 10 minutes were added to the normal PCR cycle to amplify anything that was not completed after the 40 cycles.

6.4.2.4. Restriction digest & gel purification

The PCR products obtained were digested with BglII and XhoI at 37° C overnight to excise the fragments needed from the MCF-7 cDNA. The samples were then applied to a 1% Argarose Gel, additionally a λ DNA/ HindIII marker (concentration: 83,3 ng/μL) was applied and the gel was then run at 130 V for 30 minutes.

The gel was stained with SYBR Gold Nucleic Acid stain (10000X concentrated) for 20 minutes and then analyzed under UV-light. The bands of the right size were then cutted from the gel and a gel extraction was done using the QIAQuick Gel Extraction Kit from QIAGEN. The protocol for the extraction included with the Kit was followed exactly – only instead of using the EB buffer in the last step, the same amount of DEPC-treated water was used instead. The samples were then stored at -20° C until further usage.

6.4.3. Cloning of UTR segments into vector

6.4.3.1. Restriction digest to open insertion site

After the UTR segments were created, they were cloned into the pGL3 vectors with the new MCS. First, the vectors were digested with BglII and XhoI to open the site of insertion at the two sites. The digestion with BglII and XhoI was done to make sure that the UTR sequences were cloned in directionally. The restriction digest was set up was following:

Table 14. Set up of restriction digest to open insertion site

	Sample	Enzyme	Enzyme	BSA	Buffer	DEPC-

	volume	1 volume	2 volume	volume	volume	treated water volume
Digest 1	15 µL	BglII, 4 µL	XhoI, 4 µL	7 µL	25 µL	25 µL

The amount of enzyme, BSA and buffer used were based on the amount of sample used. The digest was done overnight by incubating the cells in a waterbath at 37° C and therefore activating the enzyme. After incubation, the DNA concentration was measured using the DNA concentration feature on the Nanodrop.

6.4.3.2. CIP digestion of vector

After digestion with XbaI, the vectors were digested with CIP to remove the phosphates at the open ends, created by the XbaI digest, to avoid religation of the vector. Before setting up the digestion, the CIP was diluted with DEPC-treated water to a final concentration of 10 µg/µL and the buffer used was diluted 1:10 to obtain a 1X buffer.

For the CIP digestion, 0,5 µg of DNA were used and mixed with 1X Buffer to a final volume of 10 µL. Also, 1 µL of diluted CIP were added and then the sample was incubated for 1 hour at 37° C. After incubation, DEPC-treated was added to the sample to a final volume of 100 µL. To inactivate the CIP, a Phenol chloroform preciptiation was performed which was followed by an Ethanol precipitation.

6.4.3.3. Ligation of UTR or sensor segment with vector

After the Ethanol precipiation, the vector was ligated with the UTR segment created by annealing of the primers. For each different vector type, the primers were different as different sequences were cloned into the vector construct. For the ligation, the following set up was chosen:

Table 15. Set up for ligation of UTR segment into vector

	Sample volume	T4 buffer volume	T4 DNA ligase volume	DEPC-treated water volume	UTR volume
Ligation 1	5 µL	2 µL	1 µL	11 µL	1 µL

The sample was incubated at room temperature for 1 hour and then incubated over night at 16° C.

6.4.3.4. Confirmation of insertion of UTRs/miR-21 sensor

To confirm the ligation of the backbone vector with either the UTR sequences or the miR-21 sensor sequences, two methods were chosen, a diagnostic double digest and a PCR.

To confirm the insertion of the UTRs at the new MCS, a diagnostic digest with BglII and XmaI was done using 1 μL sample in a total reaction volume of 20 μL using the buffers recommended by the manufacturer.

For confirming the insertion of the miR-21 sensor, a PCR was done as the primers confirmed the right orientation of the insert only if a PCR product was obtained.

The samples with the cloned in sequences were then kept at -20° C until further usage.

6.5. Direct miR-21 target confirmation

6.5.1. Transfection of different cell lines

For the Luciferase assay, two different cell lines were chosen – LAPC-4 and 4.2 cells as both cell lines are easy to handle and transfection efficiency is high. The cells were plated in 100 μL of complete media without antibiotics in 96 well plates and incubated at 37° C until confluency had reached 40 – 80%.

For the tranfection, Lipofectamin 2000 was used based on earlier experiments, which confirmed it was the most efficient tranfection agent for these cell lines. Additionally, Renilla was added as a control to check if tranfection was succesfull or not.

When the confluency was in range, two solutions were prepared for the tranfection, solution A and B.

Table 16. Set up of solution A per well

	Optimem	DNA	Renilla	miR-21
Set up/well	24,4 μL	180 ng	18 ng	15 nmol

The solution was mixed as described above and after vortexing incubated for 5 minutes at room temperature. In the meantime, solution B was prepared as following:

Table 17. Set up of solution B per well

	Optimem	Lipofectamin 2000
Set up/well	32,5 μL	180 ng

After solution A was incubated for 5 minutes, solution A and B were mixed together, vortexed and incubated at room temperature for 20 minutes. After the incubation time, 100 μL of the transfection mix were added to each well. For each condition, 24 wells were tranfected. The plates were then incubated at 37° C and the Luciferase activity was read after 24, 48 and 72 hours.

6.5.2. Luciferase assay

The Luciferase activity was analyzed and it was checked whether the mRNAs of which the UTRs were cloned into the vector, were directly targeted by miR-21 or not. Due to the set up of five different vectors of which two were sensors specifically for miR-21, the influence of miR-21 on the mRNAs could be shown precisely.

7. RESULTS

For analyzing the fragments in a 1% Argarose gel, a 1 kB Plus DNA ladder was applied every time. The band sizes are as following:

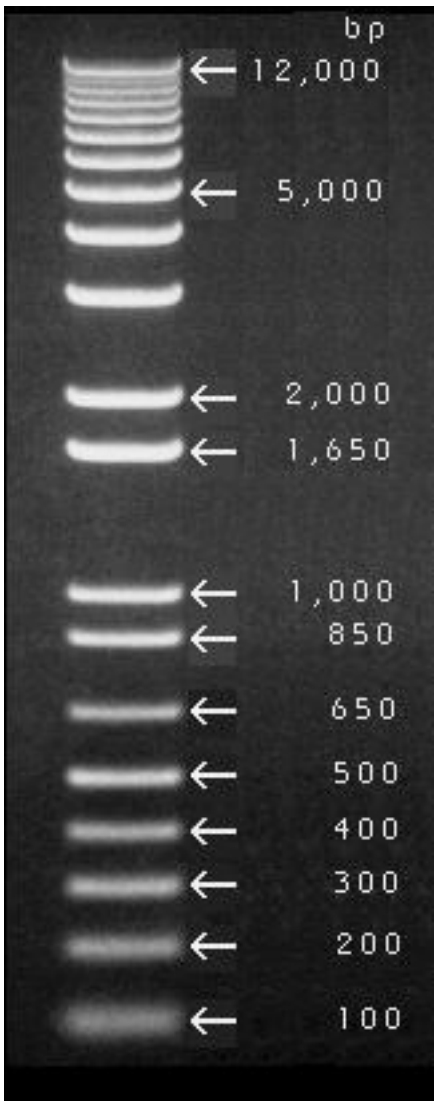


Figure 17. 1 kB Plus DNA ladder. Band sizes are according to manufacturer's information (Invitrogen)

7.1. Confirmation of excision of old MCS

The excision of the old MCS of the pGL3 control vector was checked through digestion with AvaI of the samples overnight. The samples were afterwards run in a 1% Argarose gel with a 1kB Plus ladder.

As a negative control the undigested pGL3 control vector was also applied to the gel. If the excision worked only one band should be visible, whereas if the excision did not work two bands should be visible – as in the negative control - as it can be seen in the figure below.

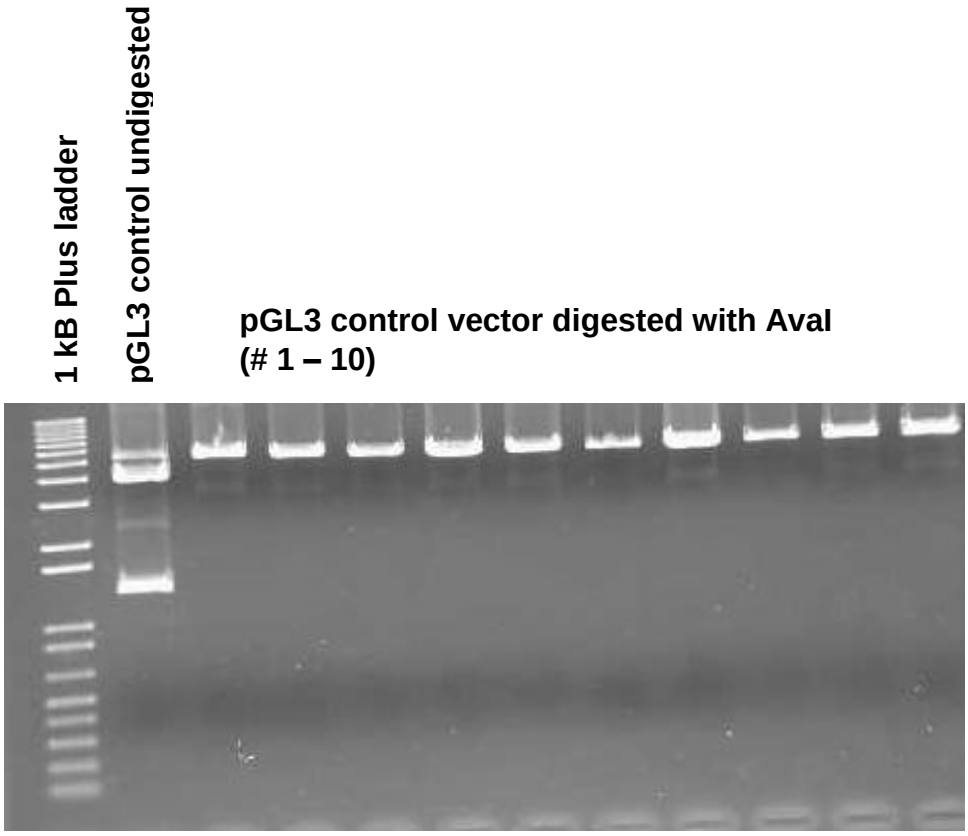


Figure 18. 1% Argarose Gel under UV-light. 1 kB Plus ladder in first slot, pGL3 control undigested vector as negative control, pGL3 control samples were digested with AvaI,

In earlier attempts to excise the old MCS, two distinctive bands could be seen when when applying the samples on the gel (

Figure 19). After troubleshooting and excluding several possibilities, the cause was found in a parasitic contamination of the original sample used for the excision. The bands are indicated in the figure by the two red arrows. For further experiments a new sample of pGL3 control vector was used in order to avoid further errors due to this contamination.

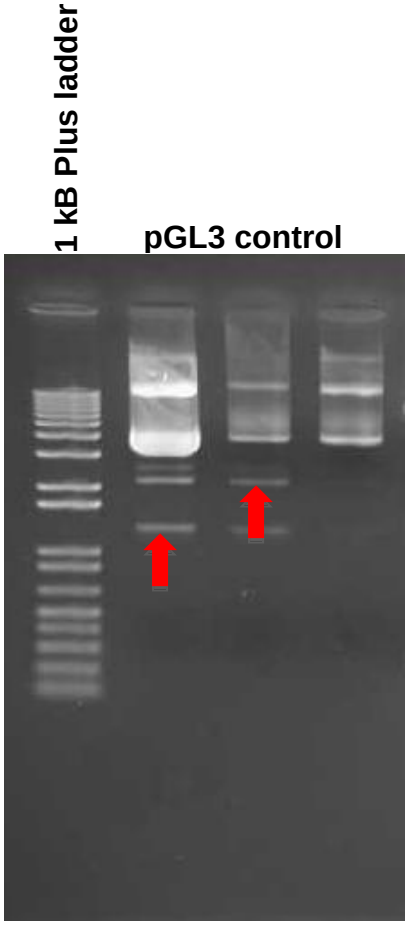


Figure 19. 1% Argarose Gel showing 1 kB Plus ladder and pGL3 control with parasitic bands (indicated by the two red arrows)

7.2. Confirmation of insertion of new MCS

To confirm the excision of the old MCS as well as the insertion of the new MCS, the samples were digested with AvaI or BamHI & XbaI overnight. They were applied to a 1% Argarose Gel and checked under UV-light for bands. If the old MCS was excised and the new MCS inserted a digest with AvaI should lead to the following band sizes: 3,9 kB, 1,3 kB and 6 bp. A successful excision and insertion would show the following bands upon digestion with

BamHI and XbaI: 4,7 kB and 500 bp. Samples which showed successful excision and insertion in both digests were used for further steps.

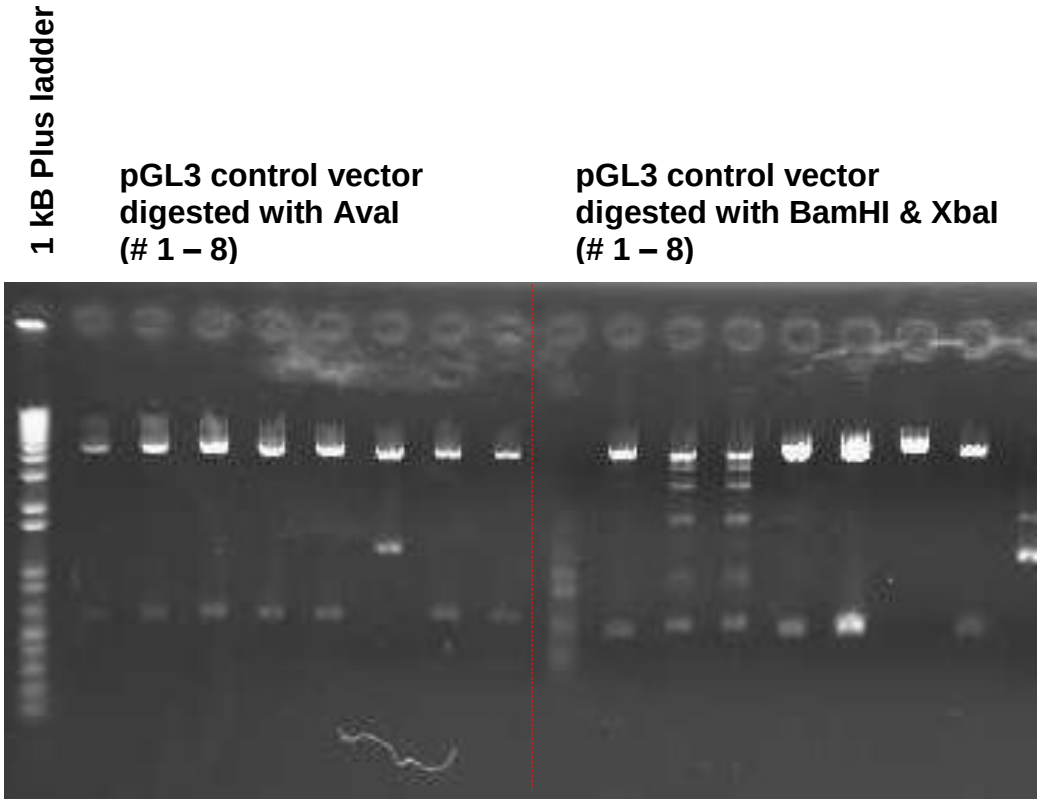


Figure 20. 1% Argarose Gel under UV-light. 1 kB Plus ladder, pGL3 control without old MCS & new MCS - digested with AvaI or digested with BamHI & XbaI

The sample with the number 5 showed a success in both excision as well as insertion and was used for further steps. The samples number 2 as well as 8 showed success and were stored until further usage.

7.3. Confirmation of primers for target UTRs

The primers designed as described in the Methods section were checked for their efficiency by using them for a PCR with a cDNA template (MCF-7, C4.2 or LNCaP). The outcome was then checked on a 1% Argarose gel with a 1kB

Plus ladder. If the gel was showing the expected bands, the primers were used for creating the target UTR.

The amplified fragments of each target UTR containing the miR-21 seed sequence had the following sizes:

Table 18. mRNAs and their according fragment sizes amplified through PCR & analyzed in a 1% Argarose Gel

mRNA	UTR fragment size
ANKRD46	
C1orf96	2375 bp
EIF1AX	633 bp
KIAA0256	975 bp
KLF5	909 bp
NAP1L5	487 bp
NIPBL	1317 bp
PPP1R3B	556 bp
RAB37	496 bp
S100A10	270 bp
ZBTB47	563 bp

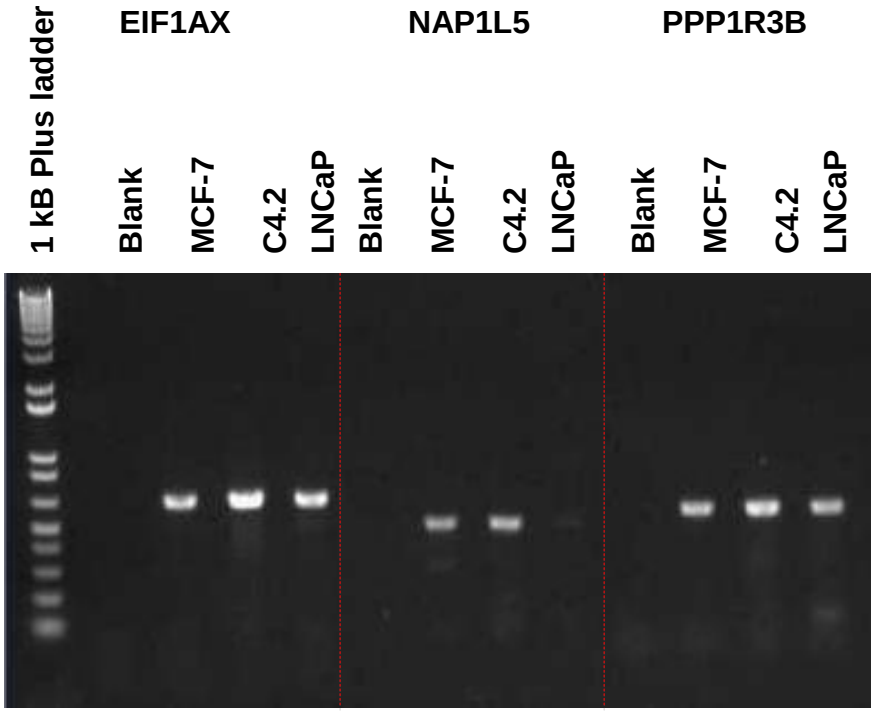


Figure 21. 1% Argarose Gel with 1kB Plus ladder. PCRs for EIF1AX, NAP1L5 & PPP1R3B. Blank as well as all 3 cDNA templates used were applied

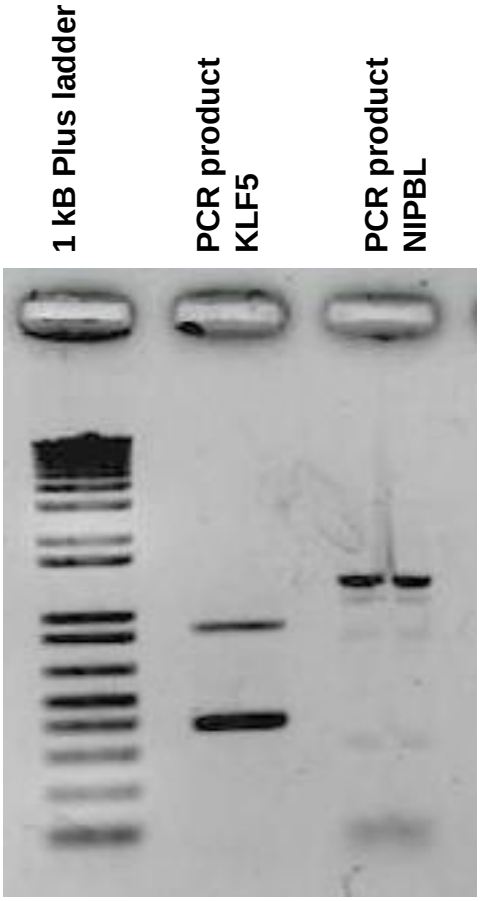


Figure 22. 1% Argarose Gel with 1kB Plus ladder. PCRs for KLF5 & NIPBLMCF-7 cDNA sample used was applied

The pGL3 control vector with new MCS was then ligated with the PCR products (= UTRs), transformed with DH5α, plated and shaking cultures were set up. The cultures were checked for a success in ligation with a BglII and XhoI digest. kl

8. DISCUSSION

The finding of new targets of miR-21 in prostate cancer is an area which is not investigated up to now. The experimental approach is therefore complicated as standard protocols do not exist yet.

The experimental workflow was followed step by step, but several errors occured as well as other factors slowing down the process which could not be foreseen.

The parasitic bands in the pGL3 control vector obtained were not visible in the beginning and could be removed upon a very digestion, therefore it was thought the bands occured due to supercoiled DNA in the plasmid, but not a contamination. After several experiments and troubleshooting sessions the realization of a contamination occured and the backbone vector had to be obtained once more.

Also the efficiency of the restriction enzymes used seemed to be impaired which is usually not the case. Several diagnostic digests as well as sequential digests had to be done in order to check for the efficiency of the different enzymes.

Another errors occured which showed, after sequencing the pGL3 control vector with the new MCS, that the MCS was inserted in the wrong direction. This danger had to be taken when ligating the backbone vector (without MCS) with the new MCS. Therefore the original primer designs had to be changed in order to create UTRs that could be inserted into a inverted MCS.

9. OUTLOOK

The finding of new targets of miR-21 especially in prostate cancer is a broad field which needs advancement on every level. The bioinformatical approach is investigated more and more and new programs and techniques are created to refine the search of new targets.

The molecular biological approach needs the set up of more protocols dealing with microRNAs and the factors influencing them as well as what microRNAs themselves influence.

The work in this field still shows many unsolved issues as well as that the function for several targets of miR-21 is not known yet. Also the finding of new targets is time-consuming and publication of new targets takes very long.

The broad range of downregulated mRNAs in prostate cancer makes it also difficult to chose a set of targets to be analyzed and checked for miR-21 influence.

There is a need for finding the influences of miR-21 in prostate cancer to develop new cancer treatment which is not as invasive as traditional methods up to now. If the influence of miR-21 can be found and also the mRNAs it is regulating another mystery in cancer could be solved and used for treatment.

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[44] QIAGEN, Bench Protocol: QIAquick Gel Extraction Kit Protocol using a vacuum manifold

A) APPENDIX

1. Microarray outcome compared to different algorithm predictions

Targetscan Predictions				
ZYG11B	SKI	NR2C2	FAM63B	ASPN
ZNF704	SFRS3	N-PAC	FAM46A	ASF1A
ZNF367	SCRN1	NFIB	FAM13A1	ARHGEF12
ZNF217	SCML2	NFAT5	FAM107B	ARHGAP24
ZFP36L2	SC5DL	NBEA	EPHA4	AP3M1
ZCCHC3	SATB1	MTMR12	ELF2	ALX1
ZBTB47	RPS6KA3	MSX1	EIF4EBP2	AIM1L
ZADH2	RP2	MSL-1	EIF2C4	ACVR2A
YOD1	RMND5A	MSH2	EHD1	ACVR1C
YAP1	RHOB	MRPL49	EGR3	ABCD2
XKR8	RGS7BP	MPRIP	DUSP8	
WWP1	RFFL	MICALL1	DNAJC16	
WNK3	RECK	MEIS1	DNAJA2	
VCL	RBPJ	MBNL1	DCUN1D3	
UQCRB	RBMS3	MATN2	CREBL2	
UBE2D3	RASGRP1	MAP3K1	CPEB3	
TRMT5	RAB6A	MAP2K3	COL4A1	
TRIM33	RAB22A	LRRC57	CNTFR	
TNS1	RAB11A	LRP6	CHL1	
TNRC6B	PURB	LOC153364	CHIC1	
TNPO1	PTCH1	LMBR1	CHD7	
TNKS	PRPF4B	LIFR	CDK6	
TIMP3	PRKCE	LEMD3	CDC25A	
TIAM1	PPP3CA	LANCL1	CD69	
THRB	PPP1R3B	KRIT1	CCR7	
THBD	PPARA	KLHDC5	CCL22	
TGFBR2	POM121C	KLF5	CCL20	
TGFBI	POM121	KLF3	CCL1	
TET1	PLEKHA1	KLF12	CCDC34	
tcag7.1228	PLAG1	KIAA1012	CBX4	
TBX2	PITX2	KIAA0256	CASKIN1	
TAGAP	PIP5K3	KCNA1	C4orf16	
STK40	PIK3R1	KBTBD6	C2orf18	
STK38L	PI15	JPH1	C17orf39	
STAG2	PHF14	JHDM1D	C14orf101	
SRL	PELI1	JAG1	C10orf12	
SPRY2	PDZD2	ITGB8	BTK	
SPRY1	PDCD4	IL12A	BTG2	
SPG20	PCSK6	HNRNPU	BRWD1	
SOX7	PCBP2	GTPBP1	BOLL	
SOX5	PCBP1	GRAMD3	BNC2	
SOX2	PBRM1	GPR64	BMPR2	
SOCS6	PANK3	GLT8D3	BMP3	
SNX29	PAN3	GLIS2	BEST3	
SNTB2	PAIP2B	GATAD2B	BCL7A	
SMARCD1	PAG1	GABRB2	BCL2	
SMAD7	OSR1	FGF1	BCL11B	
SLC8A3	OLR1	FCHO2	BCL11A	
SLC30A10	ODZ4	FBXO11	BAHD1	
SLC10A7	NTF3	FASLG	ATPAF1	

Miranda Predictions						
ZYG11B	WSB1	TSC1	TFRC	SPDYA	SKI	RNASE4
ZSWIM6	WNK3	TRPM7	TFDP1	SPATS2	SIX4	RMND5A
ZRANB2	WDR68	TRPC5	TEX261	SPATA18	SIX2	RIT1
ZRANB1	WDR26	TRO	TEX12	SPANXN5	SH3GLB1	RICTOR
ZNF767	WDR23	TRIO	TET1	SPAG11B	SH2D1A	RHOB
ZNF704	WDR22	TRIM4	TESK2	SPA17	SGIP1	RGS7BP
ZNF662	WDFY3	TRIM33	tcag7.1228	SP8	SGCB	RGNEF
ZNF652	WDFY1	TRIM2	tcag7.1015	SOX6	SFRS5	RGMB
ZNF609	WAPAL	TRAK2	TBX2	SOX5	SFRS3	RG9MTD2
ZNF592	WAPAL	TRAK1	TBL1XR1	SOX2	SFRS2IP	RFFL
ZNF589	VWA3B	TPRG1	TBC1D22B	SOX11	SFRS2B	REEP1
ZNF587	VTA1	TPR	TBC1D17	SOSTDC1	SFRS18	RECK
ZNF576	VSNL1	TPK1	TAT	SORL1	SETX	RDX
ZNF507	VLDLR	TP63	TAOK2	SOCS6	SET	RCN1
ZNF449	VIM	TP53INP1	TANC2	SOCS5	SESTD1	RCBTB1
ZNF367	VGLL2	TOPORS	TANC1	SNX19	SERTAD2	RBPJ
ZNF365	VGLL1	TOMM40	TAL1	SNX13	SERP1	RBM8A
ZNF326	VEGFA	TOMM22	TAF8	SNTB1	SEPT3 _	RBM44
ZNF295	VDAC1	TNRC6B	TAF15	SNORA4	SEL1L	RBM33
ZNF294	VCL	TNKS	SYT14L	SNCAIP	SEC63	RBM27
ZNF280D	VAPA	TNFRSF11B	SYPL1	SMS	SEC24C	RBM24
ZNF236	VAMP7	TMTC4	SUZ12	SMCR8	SEC22A	RBM22
ZNF207	UTX	TMLHE	SUMF1	SMC1A	SDHC	RASSF8
ZNF189	USP47	TMEM74	SUCLG2	SMARCE1	SCML2	RASSF6
ZNF10	USP34	TMEM68	STXBP5	SMARCD1	SCLT1	RASGEF1B
ZMYM6	USP31	TMEM55A	STX16	SMAP2	SCFD1	RASA1
ZMYM5	USP24	TMEM45A	STT3A	SMAD7	SCARB2	RAPGEF2
ZMYM4	UPP2	TMEM39A	STK36	SMAD5	SC5DL	RANBP3L
ZMYM2	UNQ1887	TMEM34	STK33	SLMAP	SATB2	RAD51L3
ZMAT2	UHMK1	TMEM27	STK3	SLC9A4	SATB1	RAD23B
ZFYVE16	UGCGL1	TMEM208	STCH	SLC9A11	SASH1	RAD21
ZFPM2	UFC1	TMEM16F	STAT3	SLC8A3	SAMD9L	RAB6B
ZFP62	UBR3	TMEM163	STAG2	SLC6A15	SAMD12	RAB37
ZFP14	UBR2	TMEM14A	ST8SIA4	SLC5A7	SACM1L	RAB2B
ZEB2	UBE2NL	TMEM137	ST8SIA2	SLC5A3	S100A7A	RAB27B
ZCCHC3	UBE2D3	TMEM106B	ST6GAL1	SLC39A14	S100A10	RAB22A
ZBTB47	UBE2D2	TMED10	SSR2	SLC39A11	RTN4	RAB21
ZBTB44	UBE2D1	TSC1	SSPN	SLC35F1	RTKN2	RAB11A
ZBTB41	UBASH3B	TRPM7	SSFA2	SLC34A2	RSRC2	PURB
ZBTB39	TXNRD1	TRPC5	SS18	SLC30A10	RPS6KA3	PTX3
ZBTB38	TUG1	TRO	SRI	SLC2A4RG	RPA2	PTPRU
ZBTB34	TUBG2	TRIO	SR140	SLC2A13	RP2	PTPRG
ZBTB2	TTC33	TRIM4	SPTY2D1	SLC26A3	RNF6	PTPN9
YOD1	TTC30A	TRIM33	SPRY4	SLC26A2	RNF38	PTPN20B
YAP1	TSPYL4	TRIM2	SPRY2	SLC25A26	RNF2	PTPN20A
XRN1	TSPAN9	TRAK2	SPPL2A	SLC1A1	RNF180	PTGFR
XKR6	TSPAN2	TRAK1	SPOCK1	SLC16A12	RNF144A	PTGER3
XAGE2	TSN	TPRG1	SPIN4	SLC14A1	RNF138	PTGDR
WWP1	TSHZ3	TPR	SPHKAP	SLC13A3	RNF111	PSRC1
WWC1	TSC22D2	TPK1	SPG3A	SLC12A6	RNF103	PSMA2
PSG8	PFKM	NPPB	MID1IP1	LOC649553	KLHDC5	
PSG1	PFKFB2	NPAT	MIB1	LOC619208	KLF9	
PSD3	PER3	NPAL2	MGC21881	LOC441204	KLF8	
PRTFDC1	PER2	NOVA1	MFAP5	LOC440416	KLF12	
PRRG1	PELI1	NOL14	METTL10	LOC440338	KIF3B	
PRR13	PEAR1	NME7	MEIS1	LOC440295	KIF3A	
PRR13	PDGFD	NIPBL	MEGF9	LOC401577	KIDINS220	
PRPF39	PDE4B	NIN	MEGF11	LOC401498	KIAA1804	

ProSAPiP1	PDE3A	NFXL1	MEF2C	LOC401351	KIAA1804
PROCR	PDCD4	NFIB	MED27	LOC400950	KIAA1586
PRKAB2	PCTK2	NFAT5	MED21	LOC400680	KIAA1333
PRICKLE2	PCSK6	NEU1	MBTD1	LOC348120	KIAA1310
PPP6C	PCNXL2	NETO2	MBNL3	LOC339977	KIAA1211
PPP3CA	PCMTD2	NELL2	MAT2A	LOC339862	KIAA1128
PPP1R3A	PCLO	NEK1	MAST2	LOC338579	KIAA1012
PPP1R16B	PCGF5	NAV1	MARVELD2	LOC286059	KIAA0907
PPP1CC	PCDH19	NAT8L	MARS2	LOC285965	KIAA0644
PPP1CB	PCBP2	NAT13	MARCH7_	LOC285194	KIAA0460
PPM1L	PCBP1	NAT12	MARCH5_	LOC285045	KIAA0408
PPIE	PBRM1	NARG1L	MAPK10	LOC284600	KIAA0355
POLS	PAX5	NAP1L5	MAP4K3	LOC284561	KIAA0256
POLE3	PART1	NAP1L2	MAP3K8	LOC255025	KIAA0240
PNRC2	PAQR3	N4BP2L2	MAP3K7IP3	LOC221442	KIAA0146
PMEPA1	PAPOLG	MYO6	MAP3K2	LOC202459	KIAA0114
PLP1	PANK3	MYO3A	MAP3K1	LOC154092	KHDC1
PLK4	PAN3	MYCL1	MAP2K6	LOC150577	KCTD5
PLEKHH1	PALM2-AKAP2	MX2	MAP2	LOC149134	KCTD18
PLEKHA1	PALM2	MUTED	MAN1A2	LOC126536	KCTD16
PLCB1	PAG1	MTRF1	MAMDC2	LOC100133207	KCTD12
PLAG1	PABPC5	MTPN	MACROD2	LOC100132618	KCNQ5
PJA2	P15RS	MTMR12	LZTFL1	LOC100132169	KCNMB2
PITX2	OTUD4	MTCP1	LYST	LOC100132167	KCNMA1
PITPNB	OSTM1	MTAP	LYCAT	LOC100129792	KCNJ10
PITPNA	OSR1	MSTN	LUM	LIN28B	KCNC2
PIP5K3	OSBPL8	MSR1	LTBP2	LIMCH1	KCNA3
PIK3R1	OR8G1	MSN	LTBP1	LIFR	KCNA1
PI15	OR13A1	MRPS10	LRRTM2	LHX8	KBTBD6
PHOX2B	ONECUT2	MRPL9	LRRFIP1	LHFPL3	JPH1
PHLDB2	ODZ4	MRPL42	LRRC57	LEPR	JHDM1D
PHLDB1	ODZ1	MPHOSPH10	LRRC55	LEMD3	JAG1
PHF6	NXF5	MORC4	LRRC44	LCOR	ITGB8
PHF20L1	NXF4	MON2	LRRC2	LATS2	ITGB3
PHF20	NUP153	MOBP	LRP12	LATS1	ITGA1
PHF2	NTRK2	MOBKL3	LRAT	LARP2	IRAK3
PHF17	NTF3	MMP7	LPP	KTELC1	IRAK1BP1
PHF16	NSL1	MMP16	LPIN2	KRIT1	IQCH
PHF14	NRSN1	MLSTD2	LPGAT1	KPNA1	IPO5
PGM1	NRP1	MKX	LOC729603	KLHL6	INSIG2
PFTK1	NRK	MKRN1	LOC729051	KLHL15	ING3
PFN2	NRIP1	MIER3	LOC728142	KLHL14	ILF3
IL1RAP	GNG2	FES	EDNRB	CRIM1	CCDC85A
IL1B	GNG12	FCHSD2	DZIP1	CREBL2	CCDC4
IL17RD	GLT8D3	FCHO2	DYRK2	CREB5	CCDC25
IL12A	GLIS2	FBXO4	DYNLT3	CPEB3	CCDC22
IGF2BP1	GLDC	FBXO11	DUXAP10	COPS7A	CCDC150
IGF1	GLCCI1	FBXL2	DUSP5P	COL5A2	CCDC141
IFT57	GK5	FBXL11	DUSP5	COL4A4	CCDC117
HTRA2	GJB6	FAT3	DTL	COL4A1	CCDC109A
HSPH1	GJB1	FAM92A3	DSG1	COL19A1	CC2D2B
HSP90B3P	GIMAP2	FAM73A	DSC2	COL12A1	CBX4
HSDL1	GEM	FAM70A	DPY19L4	COBL	CBFB
HSD17B4	GCET2	FAM66C	DPY19L2P1	CNTN3	CBFA2T2

HSD17B13	GBAS	FAM63B	DPY19L2	CNTFR	CAST
HS2ST1	GATAD2B	FAM46A	DPP10	CNOT8	CASR
HPGD	GATA3	FAM45B	DOCK8	CNOT6	CASC2
HNRNPU	GARNL1	FAM33A	DNM1L	CNKSR2	CAPN3
HNRNPK	GAPVD1	FAM19A2	DNAJB14	CLOCK	CAMTA1
HNRNPA3	GALNT12	FAM156A	DNAJA2	CLLU1	CAMSAP1L1
HNRNPA1	GABRG2	FAM152A	DMXL1	CLK4	CALD1
HMGCR	GABRB2	FAM149B1	DMN	CLIC5	CACNA1E
HMGCLL1	G6PC	FAM13C1	DLX3	CLIC2	CA5B
HMGA2	G3BP1	FAM136A	DLG2	CLEC4A	C9orf82
HIC2	FZD3	FAM135A	DLAT	CLDN1	C9orf144
HEY2	FUNDC1	FAM131A	DKK2	CLCN5	C9orf122
HEXA	FUBP3	FAM127C	DKFZp686D0853	CKAP2	C9orf100
HERPUD2	FUBP1	FAM126B	DIO2	CHUK	C8orf33
HERPUD1	FTHL2	FAM123B	DIAPH2	CHST2	C7orf41
HERC1	FRS2	FAM110B	DEPDC7	CHST1	C7
HECTD2	FRS2	FAM102B	DDEF2	CHN2	C6orf32
HCG27	FRMD3	EVI5	DDB2	CHL1	C6orf170
HAX1	FOXP1	ETS1	DDAH1	CHIC1	C6orf10
HAP1	FOXI1	ETNK1	DCX	CHD7	C5orf41
GTPBP1	FOXG1	ESCO1	DCUN1D1	CHCHD4	C5orf30
GRM5	FNDC3A	ERMN	DCTN4	CFL2	C5orf3
GRIPAP1	FMR1	ERG	DCTN3	CECR7	C5orf29
GRIN3A	FMO2	EPM2AIP1	DAZL	CEACAM7	C5orf24
GRIA4	FLNB	EPM2A	DAG1	CDK6	C4orf34
GRAP2	FLJ45055	EPDR1	CYBRD1	CDK5RAP1	C4orf16
GRAMD3	FLJ41047	EP400NL	CYB5B	CDH6	C3orf57
GPRASP1	FLJ37543	ENC1	CXorf20	CDGAP	C2orf14
GPR85	FLJ30838	ENAH	CXCL14	CDC42SE1	C21orf91
GPR68	FLJ26245	EMR2	CUL3	CDC25A	C20orf114
GPR64	FLJ23834	ELF2	CTSC	CD69	C1orf96
GPR180	FLJ21438	EIF4EBP2	CTNND1	CD48	C1orf9
GPR158	FLJ16124	EIF2C4	CTNNAL1	CD47	C1orf21
GPD2	FLJ12993	EIF2C2	CTCFL	CD160	C1orf181
GPATCH2	FKBP5	EIF1AX	CTCF	CCT6AP1	C1orf128
GPAM	FGF7	EGLN1	CSNK1G1	CCL22	C1orf101
GP5	FGF5	EFNA1	CSGALNACT1	CCL20	C18orf62
GNL3L	FGF1	EFHA2	CRKL	CCL1	C17orf39
C16orf75	ATF7	ACBD5			
C16orf70	ATAD2B	ABR			
C15orf2	ASPA	ABI2			
C14orf4	ARNT	ABCD3			
C14orf147	ARMCX1	ABAT			
C14orf118	ARMC8	A2ML1			

C16orf75	ATF7	ACBD5
C16orf70	ATAD2B	ABR
C15orf2	ASPA	ABI2
C14orf4	ARNT	ABCD3
C14orf147	ARMCX1	ABAT
C14orf118	ARMC8	A2ML1
C14orf101	ARL6	
C13orf33	ARL4D	
C12orf40	ARL14	
C12orf23	ARID4A	
C11orf59	ARHGEF3	
C10orf81	ARHGEF12	
C10orf128	ARHGAP5	
C10orf12	ARHGAP24	
BZRAP1	ARGLU1	
BVES	APPL1	
BTK	APPBP2	
BTBD3	APOC1	
BTBD11	API5	
BTAF1	AP1G1	
BRMS1L	AOF1	
BRD2	ANXA4	
BRD1	ANLN	
BPESC1	ANKS1B	
BOLL	ANKRD55	
BOC	ANKRD50	
BNIP3L	ANKRD46	
BNC2	ANKRD37	
BMPR2	ANKRD34A	
BMPER	ANKFY1	
BIVM	ALX1	
BCL7A	ALS2CR4	
BCL2L15	ALKBH8	
BCL11A	ALG9	
BCAP29	ALDH1A1	
BBS12	AKAP7	
BAZ1A	AKAP2	
BAT2	AIM1L	
BACH1	AHCYL1	
B3GNT1	AFG3L1	
B3GALNT1	AFF1	
AZI2	ADNP	
ATXN10	ADCY2	
ATRNL1	ADARB1	
ATR	ADAM9	
ATPAF1	ADAM7	
ATP2B1	ACYP1	
ATP13A4	ACVR2B	
ATP10D	ACTR2	
ATMIN	ACTA1	

PicTar Predictions		
ZNF367	PPARA	ELF2
ZDHHC17	PLEKHA1	EIF2C2
ZCCHC3	PLAG1	EIF1AX
ZADH2	PITX2	EHD1
YAP1	PIP3AP	E2F3
WWP1	PELI1	DLX2
TRPM7	PDZK3	DDA3
TRIM9	PDCD4	CRIM1
TRIM2	PCSK6	CREBL2
TNFSF6	PCDH17	CPEB3
TIMP3	PCBP1	CNTFR
TGFBI	PB1	CHD7
TESK2	PAN3	CDC25A
TAGAP	P66beta	CCM1
TAF5	NTF3	CCL1
STAT3	N-PAC	CASKIN1
STAG2	NFIB	C8orf21
SSFA2	NBEA	C4orf16
SPRY2	MRPL9	C17orf39
SPRY1	M-RIP	BTG2
SPIN	MGC4796	BTBD3
SPG20	MGC26598	BRD2
SOX7	MBNL1	BRD1
SOX5	MATR3	BNC2
SOX2	MATN2	BCL2
SMARCD1	MAPRE1	BAHD1
SMAD7	MAMDC1	ATXN10
SLC9A6	MAK3	ASPN
SLC7A6	LOC51136	ASF1A
SKI	LOC153222	ARMC8
SFRS8	LEMD3	ARID1A
SFRS3	KIAA2024	ARHGEF7
SET8	KIAA1468	ARHGAP24
SCRN1	KIAA1194	ADNP
SCN8A	KCNA3	ACVR2
SATB1	KBTBD6	ACBD5
RSBN1	JAG1	ABCD2
RP2	IGSF4D	
RNF111	HNRPK	
RNF103	HIP2	
RHOB	HBP1	
RECK	H63	
RASGRP1	GANC	
RASA1	FLJ36812	
RAB6C	FLJ35409	
RAB6A	FLJ13910	
RAB11A	FLJ10154	
PURB	FBXO11	
PPP3CA	FBXL17	
PPP1R3A	EPHA4	

2. Downregulated mRNAs in both LAPC-4 and LNCaP cells according to Microarray outcome

Downregulated mRNAs in both cell lines (LAPC-4 & LNCap) and alphabetically ordered		
ZBTB47	MAGI2	BX413319
WWP2	LTBP4	BX409884
UBE2DNL	LOC442332	BX344068
TPM4	LOC441616	BI771091
TMEM179	LOC441073	BE072767
THRAP3	LOC440917	BC054888
THC2714497	LOC388969	BC003075
THC2580884	LOC286044	B3GALT4
THC2576044	LDB3	AU184995
THC2567636	KLF5	AQP10
THC2507877	KLC4	ANKRD46
TCL1A	KIAA1922	AL359650
TCF12	KIAA0319L	AK3L2
TBXA2R	KIAA0256	AK130038
TAPBP	ITSN1	AK093987
SOCS5	IGKV1D-8	AK021467
SLC24A1	HLA-DPB2	AI133203
SKI	hCG_26523	AF159295
SCARF2	GPR101	AF086329
S100A10	GIPR	ACPP
RYBP	FLJ36848	AA887462
RPL26	FAM79A	AA581414
RP11-262H14.4	FAM46C	AA563626
RHO	ERN1	A_24_P631625
RAB37	ERGIC1	BX413319
PPP1R3B	EP300	BX409884
PP8961	ENST00000283964	BX344068
PLEKHA2	EIF1AX	BI771091
OTUD7A	DIP2A	BE072767
OPTN	CYP4F2	BC054888
ONECUT1	CPA1	BC003075
OAZ1	COL20A1	B3GALT4
NTNG1	CECR4	AU184995
NOTCH2NL	CEBPD	AQP10
NIPBL	CDKN2B	ANKRD46
NEXN	CCDC123	AL359650
NCK2	C9orf18	AK3L2
NBN	C5orf21	AK130038
NAP1L5	C1orf96	AK093987
MPZ	C18orf10	AK021467
MLLT1	C12orf42	AI133203
MATN2	C11orf2	AF159295