5. METHODS

5.1 RNA isolation

Modified RNEasy Mini Kit protocol.

1	Wash cells 2 times with PBS (20 000 to 100 000 cells were used per sample)
2	Solve cell pellet in 1 ml GTC (when working with small cell numbers use 600µl)
3	Dilute the cell-GTC solution 1 to 1 with Ethanol (70 %), Mix well with the pipette
4	Place 700 µl of the solution on the column. Centrifuge 15 sec at 15000g
5	Repeat step 4 until the whole volume is centrifuged
6	Add 350 µl of RW 1 buffer on the column. Centrifuge 15 sec at 15000g
7	In separate tube add 10 µl DNase to 70 µl RDD-Buffer. Mix well
8	Place 80 µl of DNA mix on the column. Incubate 15 min in room temperature.
9	Add 350 µl Buffer RW 1 on the column, centrifuge 15 sec at 15000g
10	Add 500 µl Buffer RPE on the column, centrifuge 15 sec at 15000g
11	Add 500 µl Buffer RPE on the column, centrifuge 2 minutes at 15000g
12	Replace column into the new tube
13	Add 50 µl RNase free water, centrifuge 1 minute at 15000g
14	RNA will be in the eluate
15	Freeze at –70°C or proceed with further protocol

5.2 DNA isolation

Modified PUREGENE DNA Purification Kit protocol.

	Cell Lysis
1	Add 1 ml whole blood to a 15 ml tube containing 3 ml RBC Lysis Solution. Invert to mix and incubate 5 minutes at room temperature. Invert again at least twice during the incubation.
2	Centrifuge at 2000g for 5 minutes. Remove supernatant leaving behind the white cell pellet and about 100 µl of the residual liquid.
3	Vortex the tube vigorously to resuspend the cells in the residual liquid. This speeds up the cell lysis in Step 4 below.
4	Add 1 ml Cell Lysis Solution to the resuspended cells and pipet up and down to lyse the cells, incubate at 37°C 30 minutes or until the solution is homogeneous.
	RNase Treatment
5	Add 5 µl RNase A Solution to the cell lysate.

6	Mix the sample by inverting the tube 25 times and incubate at 37°C for 15 minutes (the incubation can be prolonged up to 60 minutes without effect on DNA yield).
	Protein Precipitation
7	Cool sample to room temperature.
8	Add 333 µl Protein Precipitation Solution to the cell lysate.
9	Vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate, incubate on ice for 5 minutes.
10	Centrifuge at 2000g for 5 minutes. The precipitated proteins will form a tight dark brown pellet. If the protein pellet is not tight, repeat Step 9
	DNA Precipitation
11	Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 15 ml tube containing 1 ml 100% Isopropanol (2-propanol).
12	Mix the sample by inverting gently 50 times.
13	Centrifuge at 2000g for 3 minutes; the DNA will be visible as a small white pellet.
14	Pour off supernatant and drain tube briefly on clean absorbent paper. Add 1 ml 70% Ethanol and invert the tube several times to wash the DNA pellet.
15	Centrifuge at 2000g for 1 minute. Carefully pour off the ethanol. Pellet may be loose so pour slowly and watch pellet.
16	Invert and drain the tube on clean absorbent paper and allow to air dry 10 – 15 minutes.
	DNA Hydration
17	Add 100 µl DNA Hydration Solution (100 µl will give a concentration of 300 µg/ml if the total yield is 30 µg DNA).
18	Rehydrate DNA by incubating at 65°C for 1 hour and overnight at room temperature. If possible, tap tube periodically to aid in dispersing the DNA. Sample should be centrifuged briefly and then transferred to a 1.5 ml microfuge tube.
19	Quantify DNA and prepare dilution to 50µg/ml for conventional PCR analyses. Store DNA dilution for immediate use at 4°C. For long term storage, store stock at -20°C or -80°C.

5.3 siRNA design

Cautious design is required to achieve a high potency siRNAs. Sequences of the targeted genes WT1, PAX2, PAX8, GATA1 and PBGD were obtained from nucleotide online database PUBMED. As it is hard to predict the potency of a given siRNA, 4 to 5 target sequences 21bp long were chosen for a given gene spanning its entire coding sequence. As it was suggested that siRNAs against 3' UTR region might have high potency, one such target site per gene was also selected (McManus et al., 2002). The siRNA sequences were chosen according to present standards

(Tuschl, 2001). They were to lie further than 75bp from the start codon of the gene, to include a UU overhangs on both sides, to have GC content of around 50% for optimal activity and not to exceed 30bp of length to avoid activation of interferon-dependent gene suppression mechanisms (Elbashir et al., 2001). The BLAST search for short nearly exact matches excluded similarities to the other genes. The targeted sequences were chosen to be active across human and mouse species. As the in vitro transcribed siRNA seem to have higher potency than chemically synthesized, the method of siRNA construction with T7 polymerase was selected (Jarvis et al. 2002). This method of synthesis bases on ability of T7 polymerize to synthesize RNA on the ssDNA matrix (Milligan, 1987; Donze O. and Picard D., 2002). To synthesize siRNAs two separate reactions are needed in which sense and antisense strands are synthesized (the matrix ssDNA sequences for all synthesized siRNAs shown in section 4.11). After annealing the ssRNA overhangs are cut off with RNase. ssDNA matrices that are present in the reaction mix are digested with DNase. The product is further analyzed on the agarose gel to assure the purity and completeness of reaction. The general outline of the method is shown on figure 5.1. The siRNAs were synthesized and purified with Silencer siRNA Construction Kit. The siRNA was quantified on spectrophotometer and analyzed electrophoretically on low melting agarose gel. Typical yield of reaction was 100µl of 10µM solution of siRNA – which normally is a sufficient amount to perform at least 50 transfections. Figure 6.2 shows positions of siRNAs in targeted genes and positions of amplicon for qRT-PCR.

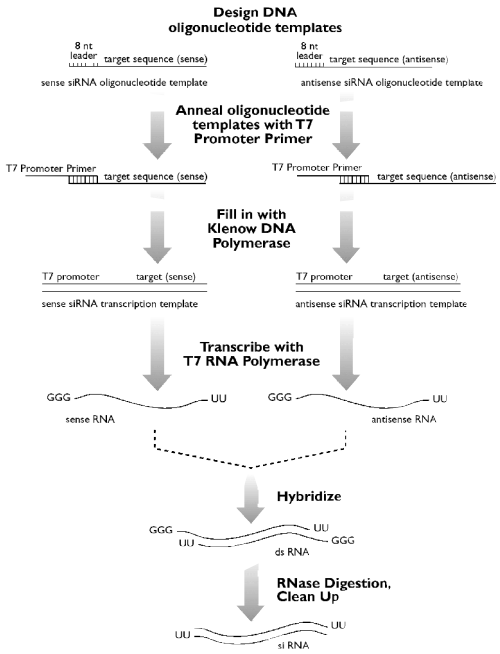

Figure 5.1: Synthesis of siRNA with Silencer siRNA Construction Kit (Printed with friendly agreement from Ambion, Cambridgeshire, UK).

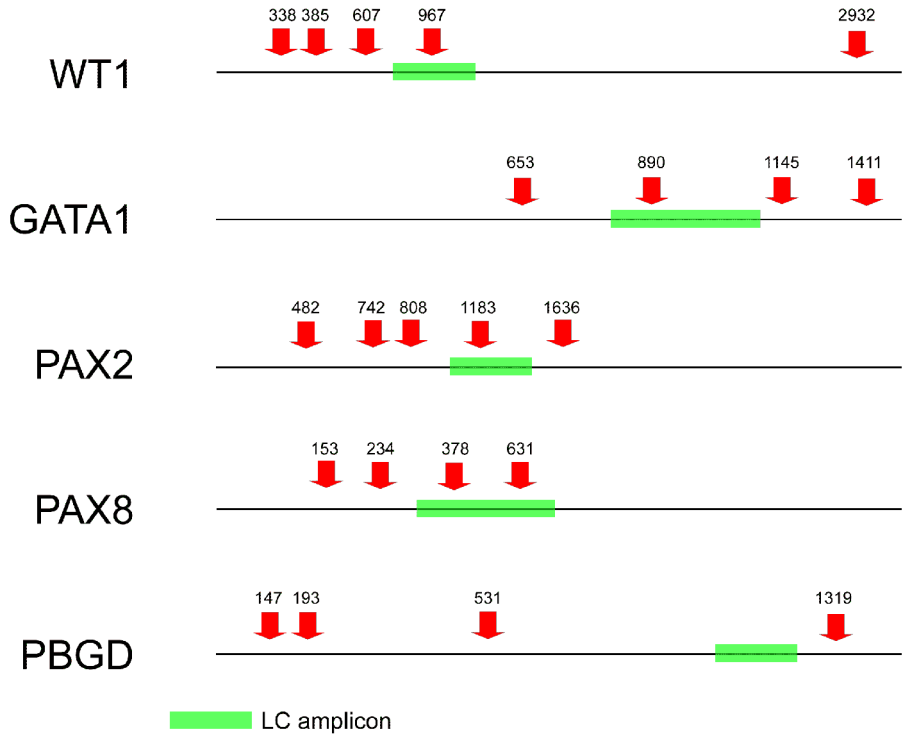

Figure 5.2: Schematic localization of target siRNA sequences on mRNA of studied genes. Green boxes are for LC amplicons. Arrows show positions of different siRNAs targets. Number states the first complementary nucleotide in mRNA

5.4 siRNA synthesis protocol

Modified manufacturer protocol for Silencer siRNA Construction Kit

All reactions should be performed in the conditions that minimize possibility of RNase contamination and assure sterility of siRNAs since they will be used in antibiotic free cell cultures.

	Transcription Template Preparation
1	Resuspend the Template oligonucleotides to 100 µM in nuclease-free water according to manufacturers instructions
2	**Hybridize each template oligonucleotide to the T7 Promoter Primer**. In separate tubes mix the following: 2 µl T7 Promoter Primer 6 µl DNA Hyb Buffer 2 µl of either sense or antisense template oligonucleotide Heat the mixture to 70°C for 5 min, then leave at room temp for 5 min.
3	Fill in with Klenow DNA Polymerase Add the following to the hybridized oligonucleotides: 2 µl 10X Klenow Reaction Buffer 2 µl 10X dNTP Mix 4 µl Nuclease-Free Water 2 µl Exo– Klenow Gently mix by pipetting or slow vortexing. Centrifuge briefly to collect the mixture at the bottom of the tube. Transfer to 37°C incubator and incubate for 30 min.
4	The siRNA templates can be used directly in a transcription reaction or stored at –20°C until they are needed for transcription.
	dsRNA Synthesis
5	Thaw the 2X NTP Mix and 10X T7 Reaction Buffer Keep the tube of T7 Enzyme Mix at –20°C and do not vortex it.

6	Assemble the transcription reactions and mix gently For each siRNA, assemble 2 transcription reactions (separate reactions for each strand) at room temperature to synthesize the sense and antisense RNA strands of the siRNA. For each transcription reaction, mix the following components in the order shown: 2 µl sense or antisense siRNA template (from step 4) 4 µl Nuclease-free Water 10 µl 2X NTP Mix 2 µl 10X T7 Reaction Buffer 2 µl T7 Enzyme Mix Gently mix contents thoroughly by flicking or brief vortexing and then microfuge briefly to collect the reaction mixture at the bottom of the tube
7	Incubate transcription reactions for 2 hr at 37°C, preferably in a cabinet incubator. (This will prevent condensation, which may occur if the tube is incubated in a heat block.). When cabinet incubator is not accessible use heat block and cover samples with insulation e.g. polyester block.
8	Combine in one tube the sense and antisense transcription reactions and incubate at 37°C overnight. Try (as in step 7) to prevent condensation.
	siRNA Preparation/Purification
9	Digest the siRNA with RNase and DNase Thaw the Digestion Buffer at room temperature and vortex the tube to mix the contents thoroughly. To the tube of dsRNA from step 8, add the following reagents in the indicated order: 6 µl Digestion Buffer 48,5 µl Nuclease-free Water 3 µl RNase 2,5 µl DNase Mix gently, and incubate for 2 hr at 37°C.
10	Add 400 µl siRNA Binding Buffer and incubate 2–5 min at room temperature
11	Meanwhile preheat Nuclease-free Water. It will be used to elute the siRNA from the Filter Cartridge in step 14.
12	For each siRNA preparation, place a Filter Cartridge in a 2 ml Tube (provided with the kit). Apply 100 µl of siRNA Wash Buffer to the filter of the Filter Cartridge to prewet the filter. Add the siRNA in the siRNA Binding Buffer from step 10 to a prewet Filter Cartridge and spin at 5000g for 1 min. Avoid higher speeds due to high siRNA loss Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 ml Tube (same tube, do not use new tube).

13	Prewet a Filter Cartridge with 100 µl siRNA Wash Buffer and bind the siRNA For each siRNA preparation, place a Filter Cartridge in a 2 ml Tube (provided with the kit). Apply 100 µl of siRNA Wash Buffer to the filter of the Filter Cartridge. Add the siRNA in the siRNA Binding Buffer from step 3 to a prewet Filter Cartridge and spin at5000g for 1 min. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge in the 2 ml Tube (new tube).
14	Add 100 µl of the preheated Nuclease-free Water to the filter of the Filter Cartridge and incubate at room temperature for 2 min (to obtain better yield a short incubation 1 min at 75°C may be of use). Spin the Filter Cartridge at 8500g for 2 min. The purified siRNA will be in the eluate (in the2 ml Tube).
15	Quantify siRNA. Store at –20°C or –80°C.

5.5 siRNA labeling protocol

Silencer siRNA Labeling Kit manufacturer protocol.

1	Add 100 µl Reconstitution Solution to Labeling Reagent and mix well
2	Assemble the labeling reaction and mix well 18,3 µl Nuclease-free Water 5,0 µl 10x Labeling Buffer 19,2 µl 21 mer duplex siRNA at 20 µM (~5 µg – the amount of siRNA should remain at this level if lower or higher concentrations are used the volume should be adjusted with water) 7,5 µl Cy3 or FAM Labeling Reagent
3	Incubate at 37°C for 1 hr in the dark, avoid condensation using cabinet incubator
4	Store in the dark at –20°C or –80°C until the use.

5.6 Nucleic acid quantification

1	Measure the A_{260} of a 1:25 dilution of DNA, RNA or siRNA sample
2	For absorption A_{260} = 1 the sample contains: 50µg/ml dsDNA, 40µg/ml ssDNA/ssRNA/siRNA, 30µg/ml oligoDNA
	The ratio A_{260}/A_{280} value between 1,8 and 2,0 proves optimal quality of sample

5.7 Gel electrophoresis analysis of nucleic acids

5.7.1 PCR and qRT-PCR products

1	Mix: 1,5% Agarose TBE buffer Warm in microwave till agarose dissolves in buffer. Add Ethidium bromide to 0,005% Wait till the mix cools down. Cast in the chamber. Wait till gel forms. Pour buffer to the chamber
2	Mix 5µl of DNA solution with 5µl of loading buffer and pipet down to gel pocket
3	For small gel chambers (8 wells) use 50V voltage
4	Let the gel run for around 30 minutes
5	Make a Polaroid photo of gel

5.7.2 siRNA

1	Mix: 4% Low melting agarose XI TBE buffer The best way to make such high concentration gel is to add agarose to buffer. Agarose should be added slowly with caution to assure proper dispersion in buffer. The mixture can be supplied with distilled water (a detailed mass of added water should be noted). The agarose should be left in buffer for few minutes before warming up to avoid clumping. Warm in microwave. Low voltages should be used at the beginning. Allow boiling till the added mass of water evaporates. Add Ethidium bromide to 0,005% Cast in the chamber. Wait till gel forms. Pour buffer to the chamber
2	Mix 5µl of siRNA solution with 5µl of loading buffer and pipet down to gel pocket.
3	For small gel chambers (8 wells) use 25V voltage
4	Let the gel run for around 120 minutes
5	Make a Polaroid photo of gel

5.8 cDNA synthesis

Modified Omniscript Kit protocol.

1	Prepare mRNA solution of concentration not higher than 133µg/ml
2	Put 15µl of the mRNA solution into two tubes
3	Incubate at 65°C for 5 minutes to relax RNA secondary structure
4	Cool down on ice for 5 minutes
5	Mix (total reaction volume 7,5µl): 2 µl Omniscript buffer 2 µl dNDTP (5 mM) 2 µl Oligo-dT-Primer 0,5 µl RNase Inhibitor (40U/µl) 1 µl Omniscript Reverse Transcriptase Mix well Second mix without Reverse Transcriptase should be made (Replace RT with DEPC water)

6	Add to RNA (one tube with RT positive mix, other with RT negative mix), Prepare one tube where RT positive mix will be added to DEPC water as a control, centrifuge down and incubate 1h at 37°C
7	Inactivate enzyme with 5 minute incubation at 95°C
8	Reaction stop – 5 minutes on ice
9	Store cDNA at –20°C

5.9 siRNA transfection protocol

Modified protocol siPORT Lipid and Oligofectamine.

First step should be dilution of transfection agent into the medium. During the 20 minutes of incubation of transfection agent in the medium the cell preparations should be undertaken. After that the siRNA is added to the mix and incubated for 15 minutes. This leaves enough time to incubate cells on ice (if needed) and divide them to wells. The addition of siRNA mix to the cells is the last step of protocol.

	Cell preparation
1	Allow cells prepared to transfection to grow in the media without antibiotics for at least 48 hours prior to transfection. Change medium one day prior to transfection (medium without antibiotics should be used). Harvest cells at exponential growth phase.
2	Centrifuge cells and remove the medium at the begging of transfection. Replace cells medium with Opti-MEM I. Dilute cells to final concentration (The recommended amount per well: 50 000 cells for K562, 20 000 cells for HEL and 50 000 cells for HL60, 70% confluence of HBL100). Divide not adherent cells suspension 200µl per well in 24 well dish. Replace medium in wells with adherent cells with Opti-MEM I. Transfection media must not contain FBS or antibiotics to assure proper transfection rate and activity of siRNA. The best time to perform this procedures is during the incubation of transfection agent mix with medium – step 5 of this procedure
3	Incubate cells on ice for 10 min (if needed) – during step 7 of this procedure
	Transfection mix preparation The volumes presented here are adjacent for one well of the 24 well dish, the volumes should be multiplied for used number of wells. All reactions should be made in double as internal controls for reproducibility.
4	Dilute 3,3 µl of transfection agent in Opti-MEM I to 8,25 µl
5	Vortex well and incubate at room temperature for 30 minutes (step 2 procedures of cell preparation to be performed at that time)
6	In different tube: dilute siRNA in Optimem to 46,75 µl (use final siRNA concentrations of 10-100nM)

7	Add siRNA solution to transfection agent solution, mix gently and incubate at room temperature for 15 minutes (during this time incubate cells on ice if needed and divide cells to wells (steps 2 and 3 of this protocol))
8	Add transfection mix to cells in well, pipet up and down 15 times to mix well.
9	Incubate 2-4 hours at normal cell culture conditions. Shake plate gently for 1 min each 30 minutes.
10	Add 1ml fresh normal growth medium to well (e.g. RPMI 1640, 20% FBS).
11	Assay for target gene activity 8 – 72 h post transfection.

5.10 Light Cycler reaction conditions

Primers	Product size	Cycle number	Denaturation		Annealing		Elongation		$MgCl_2$ conc.
			temperature	time	temperature	Time	temperature	time	
WT1	218bp	55	95°C	1''	65°C	12''	72°C	10''	2mM
GATA1	259bp	55	95°C	1''	50°C	12''	72°C	10''	4mM
PAX2	266bp	55	95°C	1''	57°C	12''	72°C	10''	3mM
PAX8	343bp	55	95°C	1''	57°C	12''	72°C	10''	4mM
PBGD	187bp	45	95°C	1''	65°C	12''	72°C	10''	4mM
GAPDH	257bp	45	95°C	0"(peak)	58°C	12''	72°C	10''	2mM
WT1 enhancer*	426bp	40	95°C	5''	61°C	10''	72°C	20''	6mM

*with LightCycler-DNA Master SYBR Green I

Gene expression levels measured with LightCycler were either shown as a ratio to housekeeping gene or as an absolute value per sample. On most of the figures the values are shown as a percent value of appropriate control.

5.11 Conventional PCR reaction conditions

Primers	Product size	Cycle number	Denaturation		Annealing	
			temperature	time	temperature	Time
WT1 promoter proximal	391bp	36	95°C	60''	60°C	60''
WT1 promoter distal	265bp	36	95°C	60''	60°C	60''
WT1 enhancer	426bp	30	95°C	60''	53°C	45''
PAX8 promoter	396bp	40	95°C	60''	66°C	60''
PAX2 promoter	462bp	40	95°C	60''	66°C	60''
GATA1 promoter	418bp	40	95°C	60''	66°C	60''

5.12 Restriction Protocols

All reactions can be scaled up to obtain higher yield of restricted DNA.

	Hga I Restriction Protocol
1	Dilute DNA sample to 5µg/ml
2	In clean tube place 25µł of DNA solution, avoid contamination with restrictase
3	Add: 5 µl NEBuffer 1 10x 1 µl of Hga I (2 U) 19 µl of H_2O
4	Incubate at least 1 hour at 37°C (incubation can be prolonged up to 16 hours without star activity)
5	Heat inactivate at 65°C for 25min
6	Analyze by quantitative PCR

	Hha II Restriction Protocol
1	Dilute DNA sample to 5µg/ml
2	In clean tube place 25µł of DNA solution, avoid contamination with restrictase
3	Add: 5 µl NEBuffer 4 10x BSA to 100µg/ml 1 µl of Hha II (20 U) 19 µl of H_2O
4	Incubate at least 1 hour at 37°C (incubation can be prolonged up to 16 hours without star activity)
5	Heat inactivate at 65°C for 25min
6	Analyze by quantitative PCR

	Hpa II Restriction Protocol
1	Dilute DNA sample to 5µg/ml
2	In clean tube place 25µł of DNA solution, avoid contamination with restrictase
3	Add: 5 µl NEBuffer 1 10x 1 µl of Hpa II (25 U) 19 µl of H_2O
4	Incubate at least 1 hour at 37°C (incubation can be prolonged up to 16 hours without star activity)
5	Heat inactivate at 65°C for 20min
6	Analyze by quantitative PCR

5.13 Methylation protocol

All reactions can be scaled up to obtain higher yield of modified DNA.

	DNA methylase M.Sss I protocol
1	Prepare DNA dilution of less than 50µg/ml
2	In clean tube place 13µl of DNA solution
3	Add: 2 µl 10x NEBuffer 2 1 µl 20x SAM solution 0,5 µl of M.Sss I (2U) 3,5 µl H_2O Mix well.
4	Incubate at least 1 hour at 37°C
5	Heat inactivate at 65°C for 20min
	Reaction control
6	Samples of methylated and unmethylated DNA should be digested with Hpa II.
7	Digested DNA should be analyzed on agarose gel

5.14 Statistical analysis

The statistical analyses were performed with Statistica, Excel, LightCycler Data Analysis and DakoCytomation Summit Software