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„Gene expression analysis of DNA repair enzymes in women with type 2 diabetes mellitus“

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1 Introduction

Type 2 diabetes mellitus is not only a complicated disease in itself but also gives rise to major complications, with hyperglycaemia as the driving force behind them [American Diabetes Association, 2010]. One crucial consequence of hyperglycaemia is a higher formation rate of reactive oxygen species (ROS) leading to increased oxidative stress [Giacco and Brownlee, 2010]. High oxidative stress leads to increased DNA damage and can subsequently trigger the initiation of cancer via mutations in our DNA [Azqueta et al., 2009]. These mutations derive its origin from the incorporation of incorrect bases into the DNA due to non-canonical base pairing which is typically caused by DNA base lesions [Robertson et al., 2009]. These lesions can be the consequence of oxidative stress and are repaired by the base excision repair pathway (BER). Involving specific enzymes, a multitude of coordinated steps repairs damaged DNA bases and hereby restores genomic integrity [Parsons and Edmonds, 2016].

The Department of Nutritional Sciences of Vienna had already investigated the connections between diabetes and DNA damage [Müllner et al., 2013 a, b]. However, the influence of hyperglycaemia induced increased oxidative stress levels on DNA repair remained unclear. Therefore a new project was started in 2014 called MICRODIAB to investigate the influence of glycaemic control on DNA damage and repair levels in women with type 2 diabetes mellitus (T2DM). As a part of this project, the focus of this thesis was to analyse the gene expression levels of DNA repair enzymes taking part in short-patch BER. This was done by the implementation of a qPCR experiment to examine possible differences between female T2DM patients with good and poor glycaemic control (HbA1c <7.5>). Additionally possible correlations between short-patch BER enzymes were investigated.

2 Literature survey

2.1 Type 2 diabetes mellitus and its diagnosis

T2DM is described as a progressive defect in insulin secretion preceded by insulin resistance [American Diabetes Association, 2014]. The resulting chronic hyperglycaemia causes long-term damage, failure or dysfunction of many organs such as kidneys, nerves, eyes, blood vessels and the heart [American Diabetes Association, 2010]. With an estimated international amount of 415 million people having diabetes in 2015 and a predicted overall increase to 642 million until 2040, we can by now clearly speak of a growing worldwide health burden [International Diabetes Federation, 2015].

Diabetes is traditionally diagnosed by plasma glucose levels of ≥126 mg/dl in a fasting plasma glucose test or two hour plasma glucose levels of ≥200 mg/dl in an oral glucose tolerance test [American Diabetes Association, 2010]. Furthermore a threshold of ≥6.5% for glycated haemoglobin (HbA1c) was set as additional diagnostic criteria for diabetes [The International Expert Committee, 2009]. The underlying mechanism of HbA1c measurement is that circulating blood glucose binds irreversibly with the red blood cells' haemoglobin during their lifespan of 2-3 months [Munshi et al., 2015]. The amount of glycated haemoglobin which is generated is dependent on the red blood cells' exposure to glucose during their life [Motta et al., 2010]. Accordingly HbA1c reflects the average levels of blood glucose over a period of 2-3 months and is therefore also used as a marker for adequate glycaemic control [American Diabetes Association, 2010]. Furthermore it correlates with macro- and microvascular complications of T2DM [American Diabetes Association, 2010].

2.2 Mechanisms of damage by hyperglycaemia

Tissue damage by hyperglycaemia is induced by five basic mechanisms: increased polyol pathway percolation of sugars, elevated advanced glycation end products (AGEs) formation in the cell, higher expression of activating ligands and AGEs

receptors, activation of isoforms of protein kinase C and hexosamine pathway overactivity [Giacco and Brownlee, 2010]. This leads to various pathological events like a decrease in antioxidant defence mechanisms, an increase in inflammatory cytokines and a disruption of normal gene expression. However, this damage only occurs in cells that are not capable of down regulating glucose uptake during hyperglycaemic states such as the ones in the glomerulus, retina and the nerves. What distinguishes all of these cells, from the ones being able to control their glucose uptake, is an overproduction of ROS [Brownlee, 2004].

2.2.1 Reactive oxygen species (ROS)

ROS belong to the class of free radicals. They are described as unpaired electron bearing atoms [Devasagayam et al., 2004]. ROS are created by regular metabolic processes in our cells, when oxygen is involved [Azqueta et al., 2009] (Table 1). For example they play an important role in generating adenosine triphosphate (ATP), signalling mechanisms and immune defence [Devasagayam et al., 2004]. Under normal conditions ROS are sufficiently deactivated by antioxidants like superoxide dismutase (SOD) [Azqueta et al., 2009]. This is important because all cells in our body are in danger of being attacked and damaged by ROS [Devasagayam et al., 2004]. The healthy balance between free radical formation and deactivation can be shifted towards pro-oxidant formation in the diabetes disease causing oxidative stress [Devasagayam et al., 2004].

Table 1 Most important reactive oxygen species mod.[Devasagayam et al., 2004]

Reactive species	Symbol	Reactivity / Remarks
Superoxide	$O_2^{\bullet-}$	Generated in mitochondria, in cardiovascular system and others
Hydroxyl radical	$^{\bullet}OH$	Very highly reactive, generated during iron overload and such conditions in our body
Hydrogen peroxide	H_2O_2	Formed in our body by large number of reactions and yields potent species like $^{\bullet}OH$
Peroxyl radical	$ROO^{\bullet}$	Reactive and formed from lipids, proteins, DNA, sugars etc. during oxidative damage
Organic hydroperoxide	ROOH	Reacts with transient metal ions to yield reactive species
Singlet oxygen	$^1O_2$	Highly reactive, formed during photosensitization and chemical reactions
Ozone	O_3	Present as an atmospheric pollutant, can react with various molecules, yielding $^1O_2$

2.2.2 Oxidative stress and type 2 diabetes mellitus

Oxidative damage initiated by excess ROS formation is called oxidative stress [Sies, 1986]. Elevated levels of ROS can be found in diabetic patients and are caused by hyperglycaemia. During ATP synthesis in the cells' electron transport chain within the mitochondria, an increased flow of electron donors NADH and $FADH_2$ is generated in diabetic patients by pyruvate oxidation in the citric acid cycle derived from higher intracellular glucose levels than in healthy subjects. This drives the mitochondrial membrane voltage gradient over a certain threshold and subsequently blocks the electron transport. The electrons are backed up and donated to molecular oxygen by an enzymatic process. Superoxide is generated and therefore the ROS production is increased [Giacco and Brownlee, 2010]. ROS are not only damaging agents of our cells but are also endogenous factors for triggering DNA damage apart from the exogenous causes such as radiation, ultraviolet light, certain chemicals and toxins [Mills et at., 2003]. As the bases in our DNA are highly vulnerable and easily damaged [Lee and

Chan, 2015] oxidative DNA damage can remain unrepaired in times of oxidative stress [Azqueta et al., 2009]. The problem is that unrepaired DNA damage is the source of mutations [Nemec et al., 2010] which eventually lead to the formation of cancer [Cooke et al., 2003].

2.3 Oxidative DNA damage and carcinogenesis

Oxidative stress, generated by ROS, can lead to oxidised or methylated bases, apurinic/apyrimidinic sites (AP-sites) and single-strand breaks [Wilson III and Bohr, 2007]. One specific example for oxidative DNA damage is the 8-OHdG lesion, a very common biomarker for oxidative stress. Here the oxidised guanine has the inclination to base-pair with adenine. This non-canonical base pairing is highly mutagenic because it leads to a GC TA transversion [Cooke et al., 2003] (Figure 1). All mismatched bases that remain unrepaired, before the replication of DNA during cell division, can result in an unrecoverable mutation in the daughter cell [Tian et al., 2015].

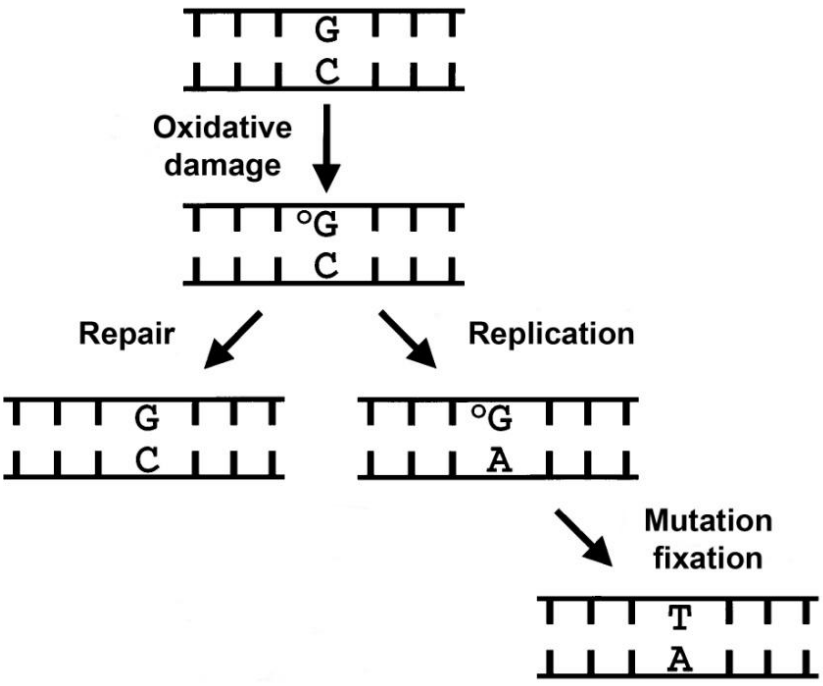

Figure 1 GC TA transversion mod.[Barnes and Lindahl, 2004]

Mutations play a very important role in carcinogenesis [Cooke et al., 2003] because they are the main factor for the initiation stage [Lee and Chan, 2015], which is the first step in the complex model of carcinogenesis [Cooke et al., 2003]. Initiation describes the stage in which proto-oncogenes, which are essential for normal cell division, differentiation and growth regulation, are transformed into oncogenes by mutations in the DNA. This is characterised by an over stimulated expression of their products leading to a constant signal for cell division. In addition, or on its own, a silencing of tumor-suppressor genes can happen due to mutations preventing the expression of growth repressor proteins or apoptosis. In the second stage, which is called promotion, these initiated cells are selectively affected by exogenous and endogenous tumor-promoters which further accelerate their proliferation rate. The final carcinogenesis step is called progression and is characterised by the formation of a malignant tumor [Mutschler et al., 2007]. Genomic integrity is therefore crucial for the prevention of cancer and can only be maintained if all DNA damages are repaired, which is done in the organism mainly by the base excision repair pathway [Robertson et al., 2009].

2.4 Base excision repair

BER can be split into two sub-pathways. The first option is short-patch BER, replacing only 1 single nucleotide and the second one is long-patch BER, replacing 2-13 nucleotides. Because short-patch BER is used in the majority of DNA repair events [Almeida and Sobol, 2007], this thesis will only focus on the mechanisms and DNA repair enzyme expression of short-patch BER.

Short-patch BER consists of five steps to repair a damaged base. First, a DNA glycosylase recognises a damaged base, removes it and leaves an AP-site. Second, an incision takes place at the AP-site, creating a single strand break, either by DNA endonuclease enzymatic activity or by DNA glycosylase lyase activity. Third, the various DNA break ends 3’ and 5’ are modified by various enzymes generating the needed 3’OH and 5’P for the insertion of a nucleotide. Fourth, the gap is filled with the correct

base by DNA polymerase. Fifth, the nick is sealed by DNA ligase [Robertson et al., 2009].

2.4.1 Enzymes involved in BER

In step 1 DNA glycosylases can be split into mono- and bifunctional branches. Monofunctional glycosylases, such as mutY homolog glycosylase (MUTYH), just remove the damaged base and produce an AP-site (Figure 2 (a) step 1), while bifunctional glycosylases, such as NTH endonuclease III-like (NTHL1), 8-Oxoguanine glycosylase (OGG1) and NEI endonuclease VIII-like (NEIL1) are also cleaving the DNA backbone to produce a single-strand break at the AP-site [Brenerman et al., 2014] (Figure 2 (b) step 1). Therefore an extra enzyme in step 2 is only necessary if monofunctional glycosylases have created the AP-site. In such a case apurimidinic endonuclease I (APEX1) produces the single strand break [Nemec et al., 2010] (Figure 2 (a) step 2). The action in step 3 depends on the initial glycosylases. If monofunctional glycosyles were active APEX1 has created the single-strand break which leaves behind 3'OH and 5'dRP ends (Figure 2 (a) step 3). At this point polymerase β (POLβ) has to change the 5'drP end to a 5'P end (Figure 3 (a)). If bifunctional glycosylases OGG1 or NTHL1 have created the gap by β-elimination, a 3'phospho-α,β-unsaturated aldehyde end is produced and changed by APEX1 to a 3'OH end [Brenerman et al., 2014] (Figure 2 (b) step 1-3 + Figure 3 (b)). If the bifunctional glycosylase NEIL1 has created the break by β,δ-elimination, a 3'P end is produced which has to be processed to a 3'OH end by polynucleotide kinase phosphatase (PNKP) [Wiederhold et al., 2004] (Figure 2 (b) step 1-3 + Figure 3 (c)). Both bifunctional glycosylase types will always create a 5'P end, so that in comparison to monofunctional glycosylases, no further action by POLβ is required at this part of the gap [Parsons and Edmonds, 2016] (Figure 2 compare steps 1-3 between (a) and (b)) + Figure 3 compare (b) and (c) to (a)). POLβ then fills the gap with the correct nucleotide in step 4 (Figure 2 (a) and (b) step 4). In the final step 5 the X-ray cross-complementing protein 1 (XRCC1) and DNA ligase IIIα (LIG3) complex is recruited by POLβ to seal the cleavage in the DNA backbone [Brenerman et al., 2014] (Figure 2 (a) and (b) step 5).

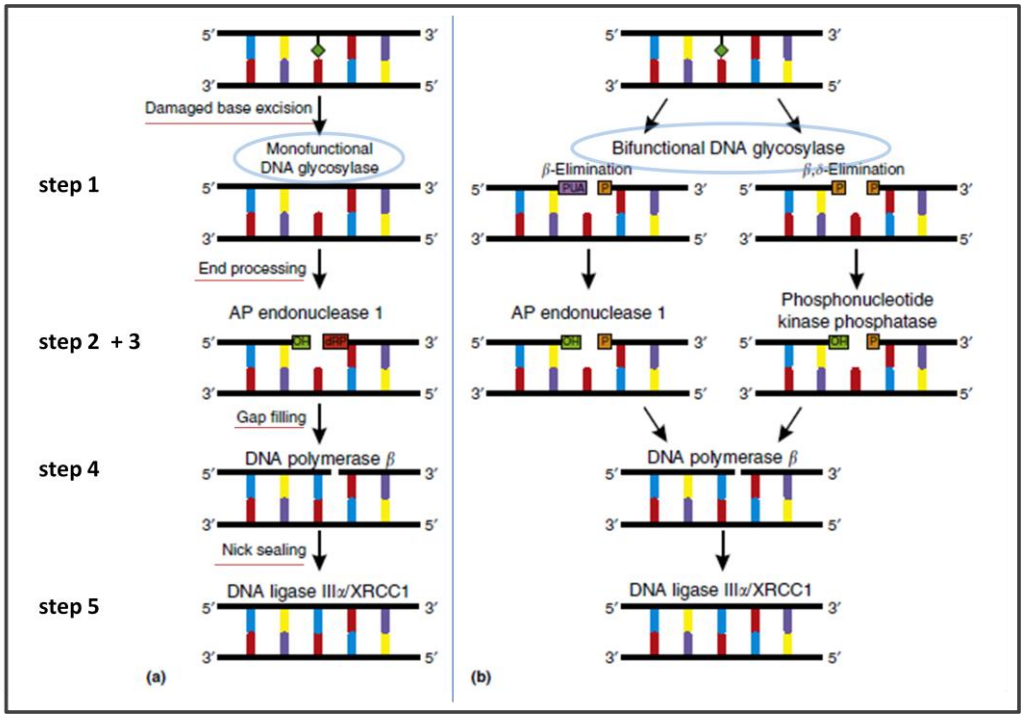

Figure 2 Short-patch base excision repair pathways mod.[Parsons and Edmonds, 2016]

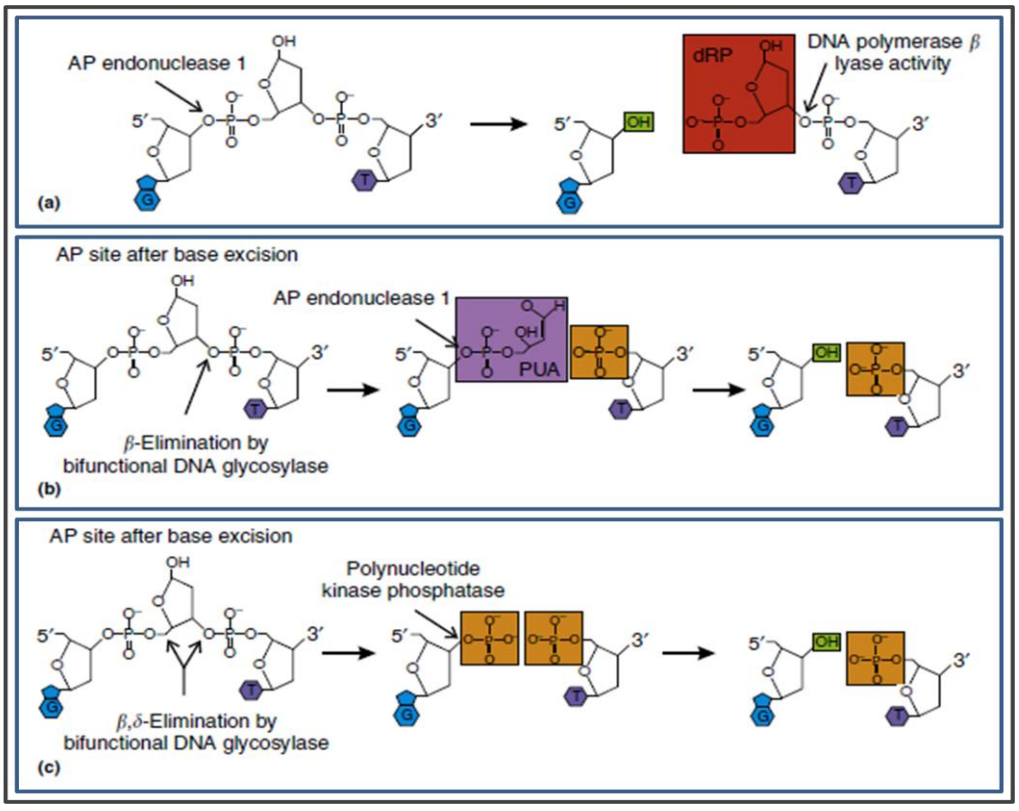

Figure 3 End modifications in short-patch BER [Parsons and Edmonds, 2016]

2.5 Real-time quantitative polymerase chain reaction

The basic steps of a classic polymerase chain reaction (PCR) cycle are denaturation of the genetic material by heat, annealing of the primers to each side of the target DNA sequence as well as DNA synthesis leading to a doubling of the initial amount of the desired DNA strand by polymerase. The amount of a specific DNA sequence will double from cycle to cycle creating millions of copies in less than an hour (Figure 4). The main goal is producing enough genetic material for further investigations from a very small and mostly insufficient starting amount [Powledge, 2004].

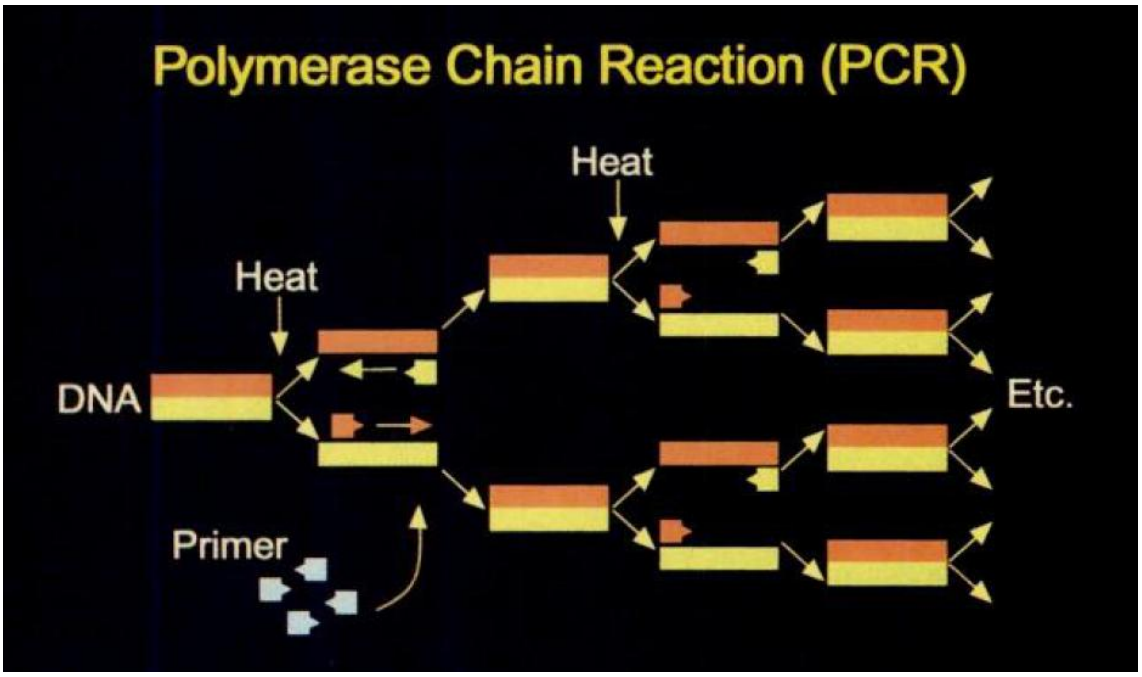

Figure 4 PCR amplification process [Wrobel, 1995]

In contrast, real-time quantitative PCR (qPCR) measures the DNA amplification progress in real time, to quantitate specific sequences and therefore give information about gene expression levels. The basic concept behind this relative quantification is that fluorescent probes that bind to the DNA, for example by dyes like SYBR green, emit a stronger signal with every cycle. This change in signal strength can be measured and expressed in graphic curves. Since the amplification is only efficient in the early so called exponential phase, until a plateau effect has developed, it is not possible to orderly calculate the DNA starting amount from the quantity of DNA at the end of the

PCR run. Due to the inefficiency of the amplification within the plateau phase every correlation between finishing and starting volume is lost. Thus the cycle of threshold value (Ct), the number of cycles at which the signal reaches a certain threshold, is determined instead (Figure 5). This Ct value lies within the exponential phase where DNA amplification is still efficient and therefore correlates with the starting volume of DNA. This way gene expression levels can be compared between samples because the quantity of a specific DNA sequence in a given sample is inversely proportional to its Ct value. Based on the premise of a perfectly efficient experiment, a difference in Ct values of one between samples means that the amount of a specific target was two times higher in the sample which had a lower Ct value compared to one with a higher Ct value [Valasek and Repa, 2005].

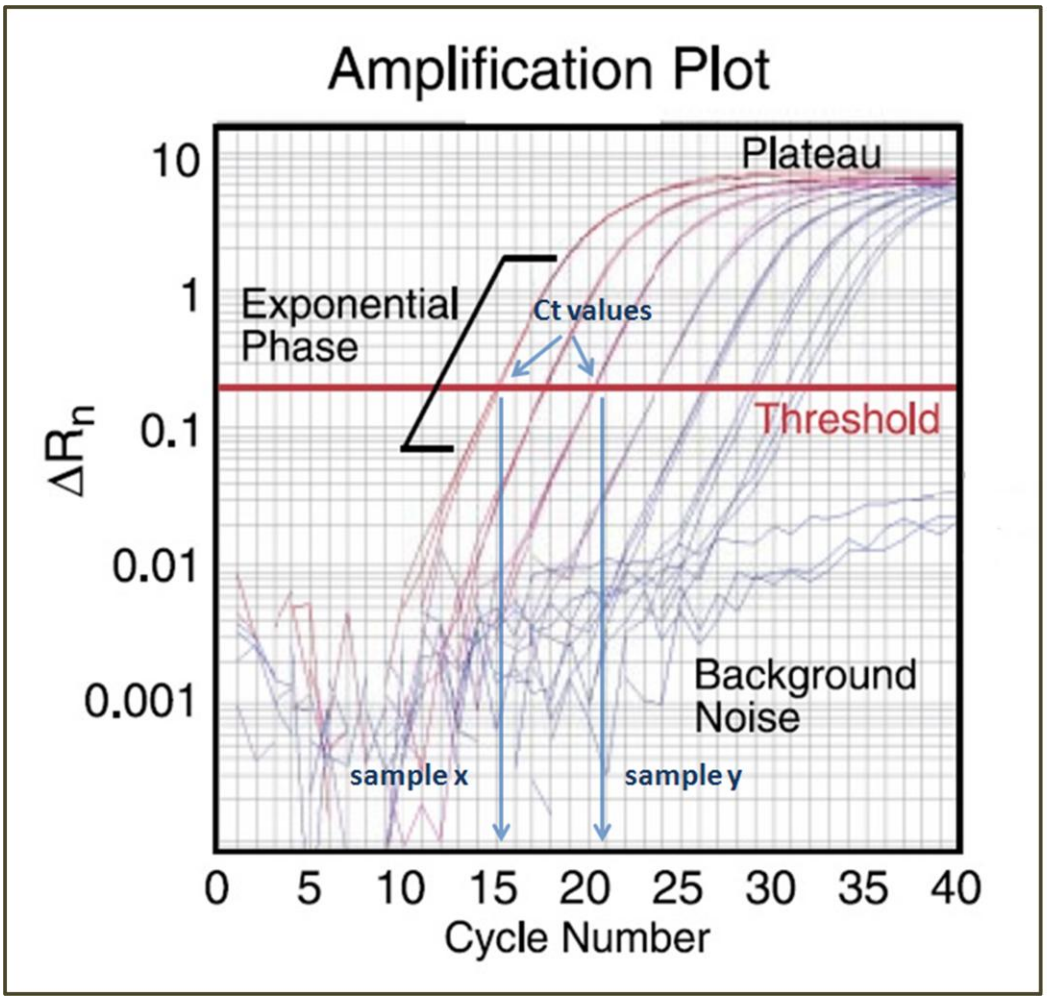

Figure 5 Real-time quantitative PCR amplification plot mod.[Valasek and Repa, 2005]

It is important to mention that messenger RNA (mRNA) levels are measured in a qPCR gene expression analysis. Because the DNA polymerase that is used in PCR does not function with RNA the mRNAs have to be converted to cDNA via reverse transcriptase [Valasek and Repa, 2005]. The popularity of qPCR has massively grown over the last decade as it has become the go-to technique to analyze varieties in gene expression levels [Taylor et al., 2010].

2.5.1 Real-time quantitative PCR and housekeeping genes

What has to be considered is that there are many variables in the multi-step preparation of a qPCR experiment, before we can finally analyse mRNA expression values via cDNA [Andersen et al., 2004]. These variable values include the amount of input material and enzymatic efficiencies in all preparation steps. The effects of this so called nonspecific variation must be eliminated by using a proper normalisation factor when calculating the results of the experiment. Without this we can not be sure if a discovered variation between samples really origins in gene-specific variation or is caused by sampling differences [Vandesompele et al., 2002]. What we consider as housekeeping genes (HKG) are genes that do maintenance work in our cells for basal cellular functions [Eisenberg and Levanov, 2013]. Accordingly, their expression levels should be stable regardless of cell type, tissue or experimental condition [Thellin et al., 1999] and should not vary between the investigated samples [Andersen et al., 2004]. Therefore housekeeping genes are used as internal reference to normalise the Ct values of the genes of interest for every sample in a qPCR experiment [Andersen et al., 2004].

For a longer time it was assumed that genes like ACTβ or GAPDH, once discovered as a housekeeping gene, are stably expressed genes in all future experiments and that taking one of them was enough to normalize the results of the investigation. In contrast to common practice, it is now clear that this is not the case and therefore an evaluation of housekeeping genes has to be done for every new experiment to find the most stably expressed ones for every experimental setup [Vandesompele et al., 2002].

3 Materials and Methods

3.1 Study design

Blood samples of 146 women over 30 years of age with diagnosed type 2 diabetes mellitus, treated with oral antidiabetica and/or insulin, were taken as part of the cross-sectional MIKRODIAB study in 2014, a collaboration between the diabetes clinic at the Health Centre South in Vienna and the Department of Nutritional Sciences at the University of Vienna. Sampling was done during a regular health assessment. Additional inclusion criteria to the ones stated above were a constant bodyweight for the last 4 weeks, constant nutritional and exercise behaviour and being a non-smoker for at least one year. Exclusion criteria were pregnancy or lactation, participating in a study 30 days prior to this one, new or changed medication concerning metabolic parameters within 4 weeks prior to the study, significant cardiovascular damage (NYHAY>III), liver disease (transaminase levels 3x higher than reference), dialysis, chronic kidney disease (serum creatinine>2mg/dl), carcinoma, organ transplantation, gastrointestinal malabsorption, steroid prescription, being HIV positive, chronic alcohol/substance abuse within the last 2 years and methadone use within the last 2 years. Details of the study can be found in the Master's Thesis of Bianca Guggenberger ("Assessment of DNA damage in females with type 2 diabetes mellitus"). The patients were divided into two groups regarding HbA1c, described as good (HbA1c < 7.5) or poor glycaemic control (HbA1c ≥ 7.5). Matching was done according to the following criteria: age, medication and smoking status (never smoked VS ex smoker longer than a year). For the final qPCR analysis 23 perfect matches (46 samples) were formed.

- ➔ 28 perfect matches were planned at the beginning of the preparations. Due to extraction inefficiencies we had to reduce them to 24. One perfectly matched couple (142(2)/211) had to be excluded from the final analysis due to a possible degradation in sample 142(2) which was discovered after looking at the PCR raw data. It produced inconsistent data within the triplicates and often even no Ct values at all.

Final 23 perfect matches in groups: 39(2)/19, 111/174, 86/55, 108/57, 128/115(2), 215(2)/45, 99/95, 6/36(2), 1/110, 89/50, 109(2)/18, 216(2)/206, 182/170, 80/60, 117/172, 94/11, 53/35, 136/147, 188/10, 123(2)/124, 100(2)/56(2), 101/24 and 127/159.

3.2 PBMC isolation

PBMCs were isolated from the fresh blood samples via density gradient centrifugation, treated with RNAlater to stabilize RNA content and frozen at -80°C. An aliquot of 5 million cells was used for RNA isolation.

➔ For the first 38 samples (#1-60) only 1 million cells were frozen and for 11 samples (#1-24) no RNA later was used. This explains the lower RNA extraction results for these samples. PBMCs consist of lymphocytes, monocytes and dendritic cells [Miyahira, 2012]. Changes in gene expression levels, induced by disease, are reflected in PBMCs. They are in continuous interaction with the whole body, have a fast turnover rate and express around 80% of all genes encoded by our genome [Liew et al., 2006]. PBMCs are therefore suggested as a model system for the studies of diabetic pathophysiology and complications [Balasubramanyam et al., 2002].

3.3 RNA extraction

Promega ReliaPrep^{TM} RNA Cell Miniprep System was used for all RNA extractions with slight modifications to the original protocol. At first 1-Thioglycerol and guanidine thiocyanate (GTC) inactivate all ribonucleases. GTC then disrupts the nucleoprotein comlplexes to release the RNA into the solution. Nucleic acids then bind to the membrane within the column by centrifugation because their adsorption is favoured when chaotropic salts disrupt the water molecules. All DNA is digested by adding DNase I and the remaining RNA is purified by various washing steps. RNA is eluted by adding nuclease free water to the membrane before a last centrifugation step [Promega, 2012].

➔ If the amount of cells frozen at the beginning of the PBMC isolation was less than five million, the amount of used nuclease free water (NFW) in the RNA elution step of the extraction protocol was reduced from 30 µl to 25 µl for those samples in an attempt to compensate the negative side effects of this error. If the extraction rate was too low in the initial samples RNA was also extracted from the backup samples in hope of better results, 20µl NFW was used here (extracted backup samples are marked with the corresponding number and the appendix "(2)" throughout this thesis). 25 µl were used for additional samples that were extracted to compensate for matching partners that were lost due to low extraction efficiencies in initial and backup samples. In total RNA was extracted from 95 samples (Table 2).

Table 2 NFW used for every sample in the elution step of the RNA extraction

20µl	25(2), 31(2), 33(2), 35(2), 36(2), 39(2), 43(2), 44(2), 45(2), 47(2), 56(2), 94(2), 100(2), 108(2), 109(2), 114(2), 115(2), 117(2), 121(2), 123(2), 142(2), 182(2), 215(2), 216(2)
25µl	1, 6, 9, 10, 11, 13, 18, 19, 22, 23, 24, 25, 31, 33, 35, 36, 39, 43, 44, 45, 47, 50, 53, 55, 56, 57, 60, 77, 80, 81, 86, 93, 95, 99, 102, 107, 116, 124, 169, 186, 188
30µl	89, 94, 100, 101, 108, 109, 110, 111, 114, 115, 117, 121, 123, 127, 128, 136, 142, 147, 154, 159, 170, 172, 174, 182, 185, 206, 210, 211, 215, 216

3.4 Quantity and quality assessment of RNA

Thermo Scientific NanoDrop 2000c was used to quantitate nucleic acid concentrations and to control RNA quality. The absorbance at a RNA specific wavelength is measured and its concentration can be calculated via the Beer-Lambert equation. The absorbance ratio at the wavelengths of 260nm and 280nm (260/280 ratio) should be around 2 for pure RNA. Furthermore the absorbance ratio at the wavelengths of 260nm and 230nm (260/230 ratio), which tells you about nucleic acid purity, should be

between 1.8 and 2.2 to guarantee a sample to be free of contaminants [Thermo Scientific, 2009]. Furthermore it was calculated if there was enough RNA material available for cDNA conversion in the next step of qPCR preparation (Table 3).

Table 3 Example of the RNA extraction results

Samples which did not fit the required criteria are marked red

RNA extraction					**Material left**		**cDNA conv.: µl needed for 0.5µg RNA**	
sample	date	c ng/µl	260/280	260/230	µl	µg	RNA	H2O
39	16.01.2015	38.5	1.95	0.95	23	0.8855	12.987013	-0.98701299
39(2)	10.03.2015	88.8	2.09	2.08	18	1.5984	5.63063063	6.369369369
43	23.02.2015	7.2	2.06	0.35	23	0.1656	69.4444444	-57.4444444
43(2)	10.03.2015	24.1	2.12	1.76	18	0.4338	20.746888	-8.74688797
44	23.02.2015	28.7	2.01	1.91	23	0.6601	17.4216028	-5.42160279
44(2)	10.03.2015	38.4	2.13	2.07	18	0.6912	13.0208333	-1.02083333
45	23.02.2015	45.9	1.98	2.2	23	1.0557	10.8932462	1.106753813
45(2)	10.03.2015	51.3	2.1	2.25	18	0.9234	9.74658869	2.253411306
47	12.03.2015	86.7	1.94	1.81	23	1.9941	5.76701269	6.232987313
47(2)	13.03.2015	87.9	1.89	0.96	23	2.0217	5.68828214	6.311717861
50	23.02.2015	173.2	2.03	2.36	18	3.1176	2.88683603	9.113163972
53	15.01.2015	117.7	1.94	1.78	23	2.7071	4.24808836	7.75191164
55	24.02.2015	362.7	2.01	2.17	18	6.5286	1.37854977	10.62145023
56	16.01.2015	65.7	2,09	1.52	23	1.5111	7.61035008	4.389649924
56(2)	10.03.2015	105	2.01	2.2	18	1.89	4.76190476	7.238095238

RNA quality was also assessed by gel-electrophoresis. Within the gel an electric field is created where negatively charged molecules migrate towards the anode. Larger molecules are kept in the porous gel causing smaller molecules to move faster than them. Therefore this test tells you if the DNase in the RNA extraction kit really wiped out all existing DNA because it would have bulged up at the start if present. The lighter RNA molecules should have travelled further in the gel than DNA [Rogers, 2015] and formed 28s and 18s bands [Taylor et al., 2010] (Figure 6).

➔ RNA was extracted from around 8 PBMC samples per day. From every extraction day the sample with the highest concentration was taken for the gel-electrophoresis as a random test. Those were samples nr: 24, 50, 55, 81, 93, 147, 170, 206, 210, 123(2). A 1.2% agarose gel was used with 80 volts (constant) for 120min.

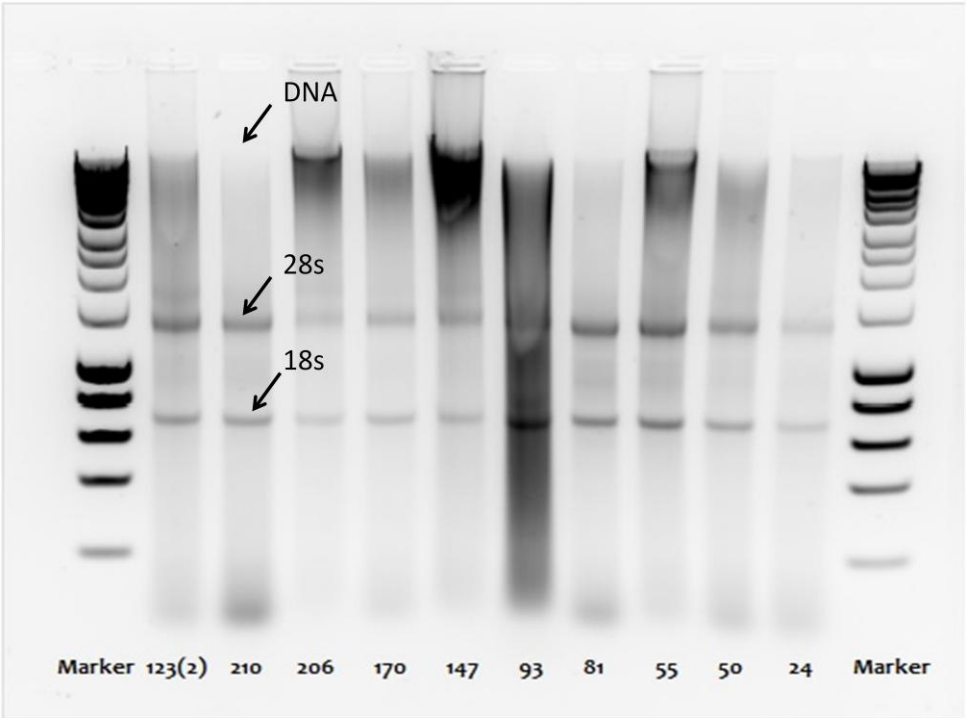

Figure 6 Quality assessment of the RNA extraction via gel-electrophoresis

There are no visible DNA bands and clearly visible 28s and 18s RNA bands. What is also visible is a possible degradation of the sample 93. The dark area at the top of sample 147 is not caused by DNA but by a contamination from spillage with sample 93. Receiving this picture as a random test tells you that RNA extraction should have happened successfully in general.

3.5 Reverse transcription

For the reverse transcription of the sufficiently extracted RNA samples into cDNA the Qiagen QuantiTect® Reverse Transcription Kit in combination with a peqlab Primus 25 advanced thermo cycler was used. After the elimination of genomic DNA (gDNA) with a gDNA wipeout buffer the mRNA templates are converted into single-stranded cDNA by reverse transcription and degradation of the mRNAs [Qiagen, 2009]. Incubation for gDNA elimination was done for 2 minutes at 42°C. Incubation for reverse transcription was done for 15 minutes at 42°C and for 3 minutes at 95°C to inactivate the transcriptase.

➔ The desired input of every RNA sample was 0.5µg. For –RT, needed in the actual qPCR runs as control, a pool of RNA samples was made. To reach a total input of 0.5µg RNA the needed volume of four templates was calculated to reach an amount of 0,125µg each. The samples 111 (c=211ng/µl -> 0.6µl), 128 (c=122.7 -> 1µl), 136 (c=121.8ng/µl -> 1µl) and 159 (c=113.5 -> 1.1µl) were taken from different extraction dates and age groups to mirror the whole study population.

3.6 Quantity and quality assessment of cDNA

NanoDrop was used to measure the cDNA concentration but more importantly the 260/280 and 260/230 absorbance ratios. The 260/280 ratio should change from the results of 2.0 for RNA to around 1.8 for cDNA to proof its purity and successful conversion [Thermo Scientific, 2009] (Table 4).

➔ Based on a volume of 18µl cDNA eluate every sample was diluted with 192µl NFW to reach a total volume of 200µl. A concentration of around 162ng/µl was reached, based on a rough estimation of an average of 1800ng/µl in the undiluted samples. Exact cDNA concentration measurements are not necessary for the final qPCR experiment because the cDNA output is proportional to the RNA input [Valasek and Repa, 2005]. The input of RNA

was precisely calculated to be 0.5µg for every sample at the cDNA conversion step.

Table 4 Example of the reverse transcription results

Reverse transcription		**NanoDrop**		
sample	date	c ng/µl	260/280	260/230
39(2)	26.03.2015	1906	1.82	2.19
19	26.03.2015	2142.3	1.8	2.2
142(2)	26.03.2015	1905.6	1.8	2.23
211	26.03.2015	2129.7	1.81	2.15
86	26.03.2015	1893.3	1.82	2.2
55	26.03.2015	1895.4	1.82	2.13
108	26.03.2015	1847.9	1.82	2.21
57	26.03.2015	2062.8	1.8	2.21
128	27.03.2015	1868.4	1.8	2.21
115(2)	27.03.2015	1656.4	1.81	2.19
215(2)	27.03.2015	2019.7	1.8	2.13
45	27.03.2015	1688	1.81	2.22
99	27.03.2015	1684	1.81	2.22
95	27.03.2015	1816.5	1.81	2.23

3.7 Primer research

Literature research was made with the search engines PubMed, ScienceDirect and ISI Web of Knowledge. The goal was to identify the most important short-patch BER enzymes and to find models for primer versions of these enzymes in gene expression studies. In addition we searched for utilised housekeeping genes and their primer versions in these types of experiments.

We decided to focus on the following genes of interest taking part in short-patch BER: APEX1, LIG3, MUTYH, NEIL1, NTHL1, OGG1, POLB and XRCC1. It is controversially discussed if polyADP-ribose polymerase-I (PARP1) plays a role in BER during single-strand break repair or not [Caldecott, 2014]. Nevertheless, the PARP1 enzyme expression was also investigated in this study. The involvement of PNKP was neglected during the planning of this study and was therefore not included into the investigation. In addition we chose to add mammalian heme oxygenase 1 (HMOX1) to our investigated enzymes because it is considered as a key marker for cellular oxidative stress [Poss and Tonegawa, 1997]. For the use as internal control we decided to evaluate the housekeeping genes ACTB, B2M, GAPDH and HPRT1. No version of the GAPDH primer gave specific results in primer testing and so it was decided to work only with the other three housekeeping genes in the study, because three are enough for the calculation of an accurate normalization factor [Vandesompele et al., 2002].

3.8 Primer design

Primer design was done with Primer-BLAST (Ye et al., 2012) partially based on reference material (Table 5+6). In primer design a balance has to be found between primer specificity and efficiency which is dependent on many variables. For example, adding bases to a primer increases its specificity but decreases its annealing speed and therefore its efficiency. The goal is to avoid unspecific amplicons and reach optimal amplification rates at the same time. The general recommendation is to use primers with a length of 18 to 24 bases, a melting temperature (Tm) between 54°C and 62°C and a G+C content of around 50% [Dieffenbach et al., 1993]. The product length should be between 75 and 150 basepairs [Taylor et al., 2010]. In addition the selfcomplementarity rate should be kept low, especially at the 3' end, to avoid primer-dimer formation [Dieffenbach et al., 1993]. The term "primer-dimer" describes interactions between the primers themselves which can lead to amplified products, when high primer concentrations are present. Subsequently the true results can be falsified [Brownie et al., 1997]. Primer pairs with the ability to additionally amplify unintended templates should not be used also [Ye et al., 2012] (Figure 7). Another

topic that is important in qPCR gene expression measurements is the not wanted amplification of genomic DNA (gDNA) when samples get eventually contaminated [Powledge, 2004]. Designing primers with exon-exon junctions should prevent them from amplifying gDNA [Laurell et al., 2012].

POLβ Primer Pair 3	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GGGCTGAAATATTTTGGGGACT	Plus	22	585	606	58.63	45.45	8.00	1.00
Reverse primer	ACTGGACTCTGCACCTCTTC	Minus	20	734	715	59.03	55.00	4.00	0.00
Product length	150								
Exon junction	592/593 (forward primer), 720/721 (reverse primer) on template NM_002690.2								

Products on intended target:
>NM_002690.2 Homo sapiens polymerase (DNA directed), beta (POLB), mRNA
>XM_005273539.2 PREDICTED: Homo sapiens polymerase (DNA directed), beta (POLB), transcript variant X6, mRNA
Products on potentially unintended templates:
>XM_011529951.1 PREDICTED: Homo sapiens meiosis inhibitor 1 (MEI1), transcript variant X22, mRNA
>XM_011516823.1 PREDICTED: Homo sapiens rhophilin, Rho GTPase binding protein 1 (RHPN1), transcript variant X10, mRNA

Figure 7 Example of a rejected primer pair for POLβ

This could have been a primer version worthy of testing with its basic characteristics but had to be rejected because of unspecificity regarding the amplification of unintended targets (only an excerpt is shown).

3.9 Primer pre-testing via PCR and gel-electrophoresis

Different primer versions for each gene of interest and housekeeping gene were ordered and tested. This was done via the Thermo Scientific DreamTaq PCR Master Mix (2X) and the peqlab Primus 25 advanced thermo cycler according to the manual [Thermo Scientific, 2013] followed by gel-electrophoresis (1.5% agarose gel with 100 volts (constant) for 60min). By this test we could see if our ordered primer versions were able to produce amplicons from a cDNA pool in a traditional PCR by the presence

or absence of bands after gel-electropohresis [D'haene et al., 2010]. To test if the primers were capable of unwanted genomic DNA amplifying gDNA was used as another template [Laurell et al., 2012]. Furthermore NFW as a no template control (NTC) was used to check for primer-dimers [Taylor et al., 2010].

- ➔ The cDNA pool consisted of the samples (perfect matches in groups) 123(2)/124, 100(2)/56(2), 101/24, 127/159, 182/170, 117/172, 39(2)/19, 111/174, 142(2)/211 and 86/55. They were taken from different extraction dates and age groups to reflect the whole study population. The gDNA sample was human DNA extracted from PBMCs. The input of cDNA pool in the master mix was 324ng and the gDNA input was 25ng. Unfortunately a miscalculation was made for all primer concentrations because µM was treated as an indicator of quantity instead of concentration. Consequently primers with 2µM were used instead of a maximum of 1µM according to the protocol (Figure 8). The main issue was that it was unclear if positive results occurred because of a real specific reaction or because of primer-dimer formation [Taylor et al., 2010]. Therefore everything was repeated in a second round. Primer versions that did not work at all in the first round were excluded. The input of cDNA pool in the master mix was 243ng and the gDNA input was again 25ng. Regrettably a calculation error occurred for the primers of APEX1, MUTYH, NEIL1 (Version2), OGG1, PARP1 and POLβ. This time the dilution by the total volume of reagents was not included into the calculation. Here the concentration was 0.02µM whereas the minimum concentration according to the protocol was 0.1 µM (Figure 9). For ACTB, B2M, GAPDH, HMOX1, HPRT1, LIG3, NTHL1 and XRCC1 (Version2) this error was detected beforehand and their concentration was correctly calculated to be 0.15µM (Figure 10). Due to the mistakes the results from the first gel-electrophoresis are not impeccable because of the miscalculated primer concentrations. Unfortunately, the reassurances of the findings from the first round were also hampered due to some mistakes in the second round. What can be said is that the overall results were sufficient enough to select the

better working primer version when more than one was ordered for later testing with qPCR. Gels are anyway prone to contamination from one well to the other and data interpretation is highly subjective so that results from these tests should always be reassured by another method [Taylor et al., 2010], which we did by qPCR melting curve analysis.

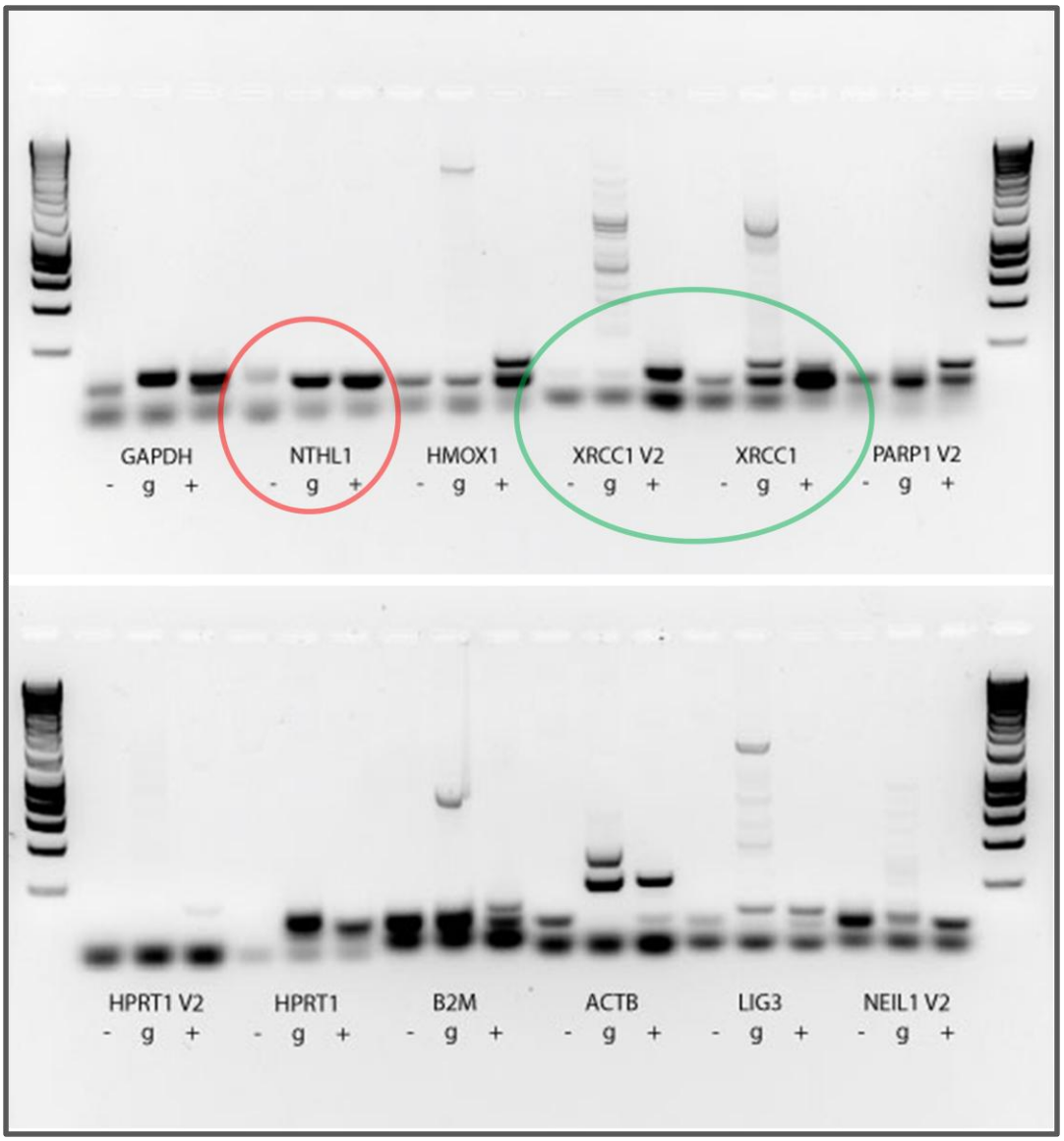

Figure 8 Primer pre-testing via PCR and gel-electrophoresis. 1st round, c too high (2µM instead of max. 1µM). NTC = -, gDNA = g, cDNA = +

When looking at the NTHL1 results as an exmple it is unclear if the spots for cDNA are caused by template amplification or primer-dimer formation because oft he present spot fort the NTC. The same is true for gDNA. Though the decision for the second version oft he XRCC1 primer was possible for example.

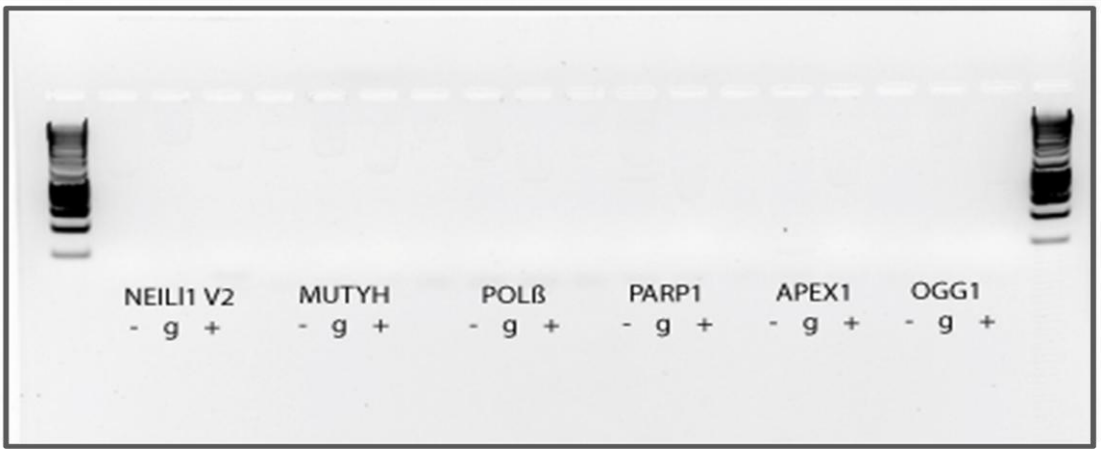

Figure 9 Primer pre-testing via PCR and gel-electrophoresis. 2nd round, c too low (0.02µM instead of min. 0.1µM). NTC = -, gDNA = g, cDNA = +

This gel just proofed that PCR obviously does not work if the used primer concentration is below the required minimum.

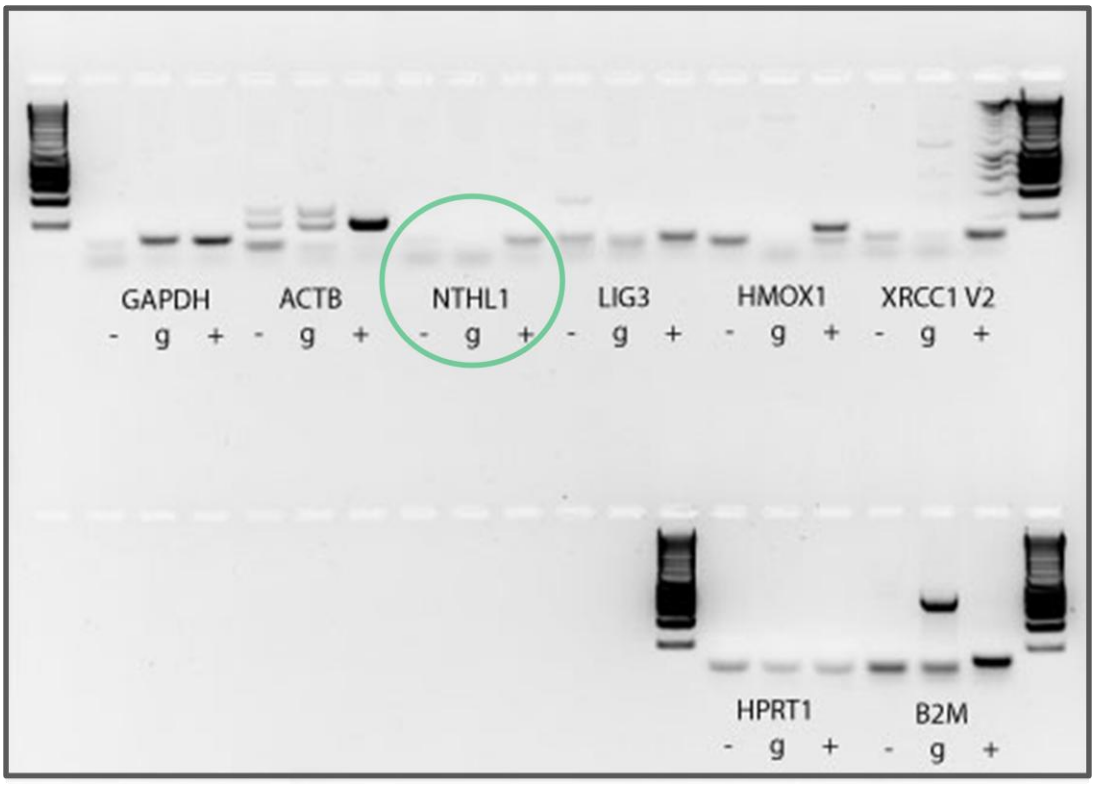

Figure 10 Primer pre-testing via PCR and gel-electrophoresis. 2nd round, c okay (0.15µM). NTC = -, gDNA = g, cDNA = +

Spots for cDNA of the NTHL1 primer that had appeared in figure 8 did also appear here. This time without NTC or gDNA spots, making this version a candidate for qPCR testing.

3.10 Primer testing via qPCR

The pre-tested primer versions were tested with qPCR with melting curve analysis to secure veritable results in the real qPCR runs at the end of the study. This again was a test for the specificity of the primers [D'haene et al., 2010] and therefore also verified the decisions made because of the gel-electrophoresis testing. The used materials were the Applied Biosystems 7300 Real Time PCR System (7300 Real-Time PCR System Sequence Detection Software Version 1.3.1) with 96 well plates (20µl per well) and the Applied biosystems by life technologies™ SYBR® Select Master Mix. Primers were tested in 4 concentrations: 100nM, 200nM, 300nM and 400nM. Each concentration was tested with the cDNA pool, gDNA and NFW as NTC in duplicates.

The thermal profile of the qPCR machine was:

- Stage 1: 50°C 2min
- Stage 2: 95°C 2min
- Stage 3: 40 repetitions of 95°C 15sec + 60°C 1min
- Dissociation stage: 95°C 15 sec, 60°C 1min, 95°C 15sec, 60°C 15sec

Specifically and therefore correctly working primers should show one single peak in the melting curve analysis [D'haene et al., 2010] (Figure 11 (b)). More than one peak would suggest that there was no specific amplification for one exclusive DNA target because two or more sequences were amplified and obtained [Valasek and Repa, 2005] (Figure 14 (b) and (c)). The usage of NTCs tests for possible primer-dimer formation and utilising samples without added reverse transcriptase (-RT) tests for genomic DNA contamination [Laurell et al., 2012]. As stated before, gDNA templates were run to check if primers are capable of amplifying genomic DNA. This is important because if your cDNA samples or solutions get contaminated with genomic DNA you do not know if your signal is generated by cDNA, gDNA or a combination of both in the final experiment, which might lead to wrong conclusions [Powledge, 2004] (Figure 12). If Ct values for gDNA appear in testing nonetheless, a minimum difference of five quantification cycles frames them as an irrelevant influence for accurate gene

expression measurement [Laurell et al., 2012] (Figure 13 (a)). It has to be said that using DNase I during RNA extraction, gDNA wipeout buffer during cDNA transcription and running NTC and –RT controls in the final qPCR analysis should have given us enough confidence to clearly pronounce veritable results. Checking the primers for their reaction to gDNA was just another precaution.

- ➔ The amounts of input were 81ng for cDNA and 5ng for gDNA. The 96 well plates allowed a testing of 4 different primers at the same time with this setup. After the first test run we saw that a concentration of 100nM often produced later Ct and also lower melting curve values than the other three concentrations whereas the other three gave similar results compared to each other. We therefore concluded that 200nM and 300nM primer concentrations worked efficiently enough and that the inclusion of a 400nM concentration was not necessary. All further testing was done with 200nM and 300nM primer concentrations. OGG1, APEX1 and MUTYH (Figure 12) primers produced problematic gDNA template results in the first qPCR test and new primers were designed, ordered and tested directly with qPCR on the 96 well plate machine without gel-testing beforehand. Unfortunately NEIL1 (Figure 14) from the first shipping and OGG1 From the second proofed to be not working correctly in the real runs because of specificity issues. The testing of those versions was done on a different and much older PCR machine than the actual runs. Maybe that is one reason why the unspecificity of the primers was discovered only at that stage. New versions of OGG1 primers were designed and ordered for the third and for NEIL1 for the second time. Correctly working versions were detected by qPCR with melting-curve analysis with the same new 384 well plate PCR machine as the real runs were performed with later on. During a run of working primers a test for newer versions was done on the empty wells of the plate. We tested each at 200nM primer concentration with cDNA pool, gDNA and NTC in triplicates.

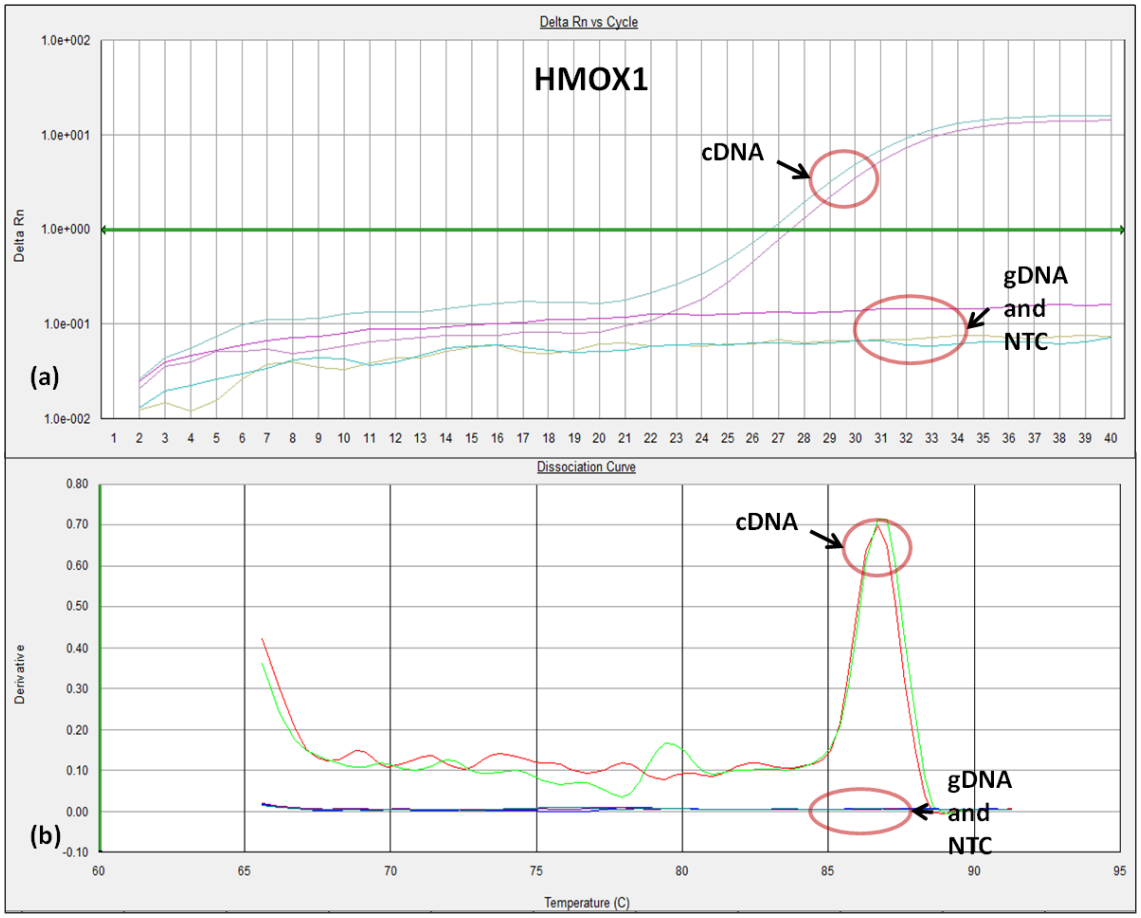

Figure 11 Primer testing via qPCR, HMOX1: (a) Ct values, (b) melting curve

An example for a perfect result can be presented by the HMOX1 primer. Ct values are only present from the cDNA pool and there are no values for gDNA or NTC (a). The melting curve analysis shows one single peak for the cDNA pool and again no peaks for gGNA or NTC (b).

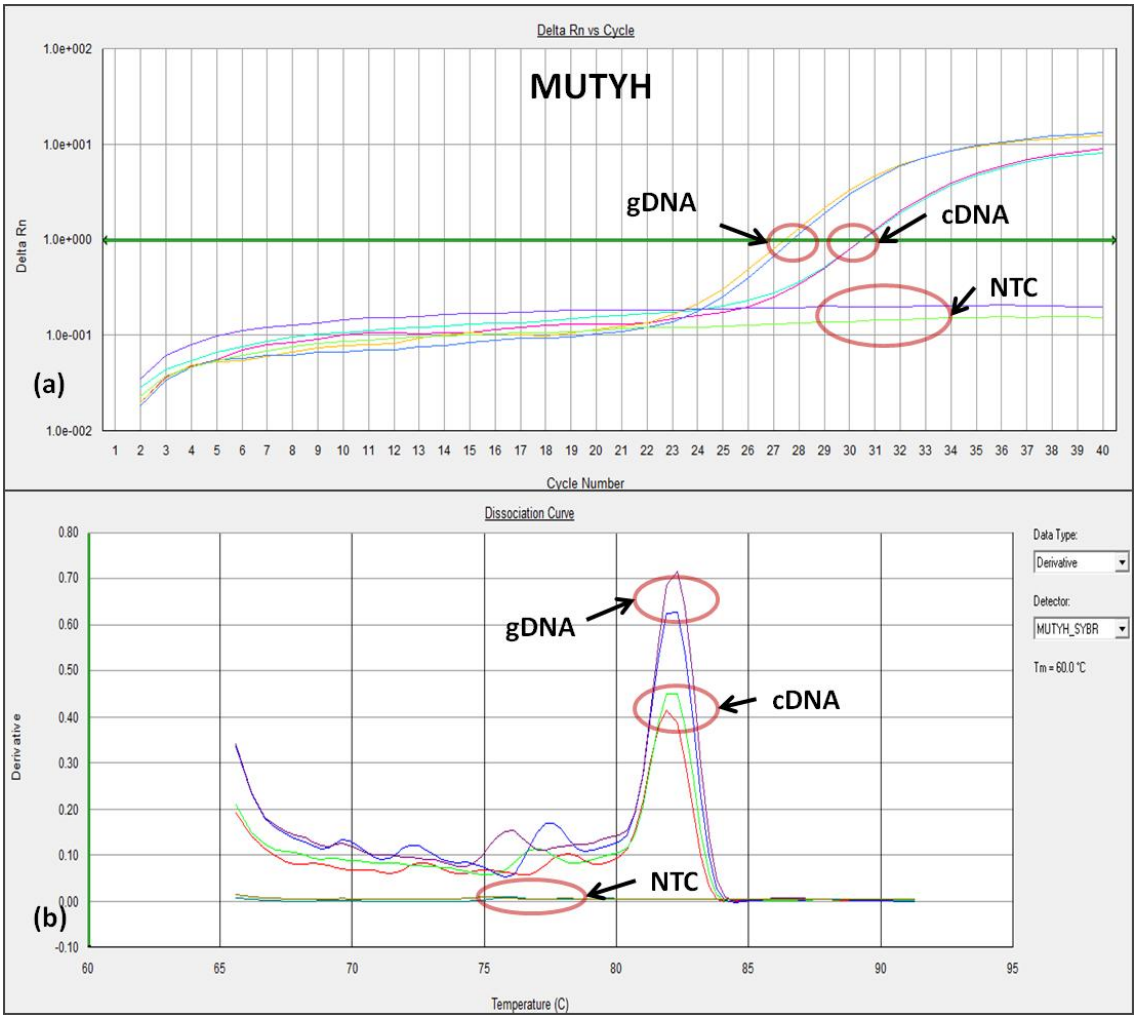

Figure 12 Primer testing via qPCR, MUTYH: (a) Ct values, (b) melting curve

An example of a worst case scenario is presented with the initial MUTYH primer version. There are no NTC Ct values but values for gDNA appear earlier in comparison to the cDNA pool which is higly unwanted (a). The melting curve analysis shows a specific reaction of the primer for our gene of interest but inappropriately also pronounced specific curves for genomic DNA (b). In case of an undetected contamination of a tested sample the signal from gDNA would add to the signal from cDNA and therefore corrupt our results. Therefore no veritable results can be guaranteed.

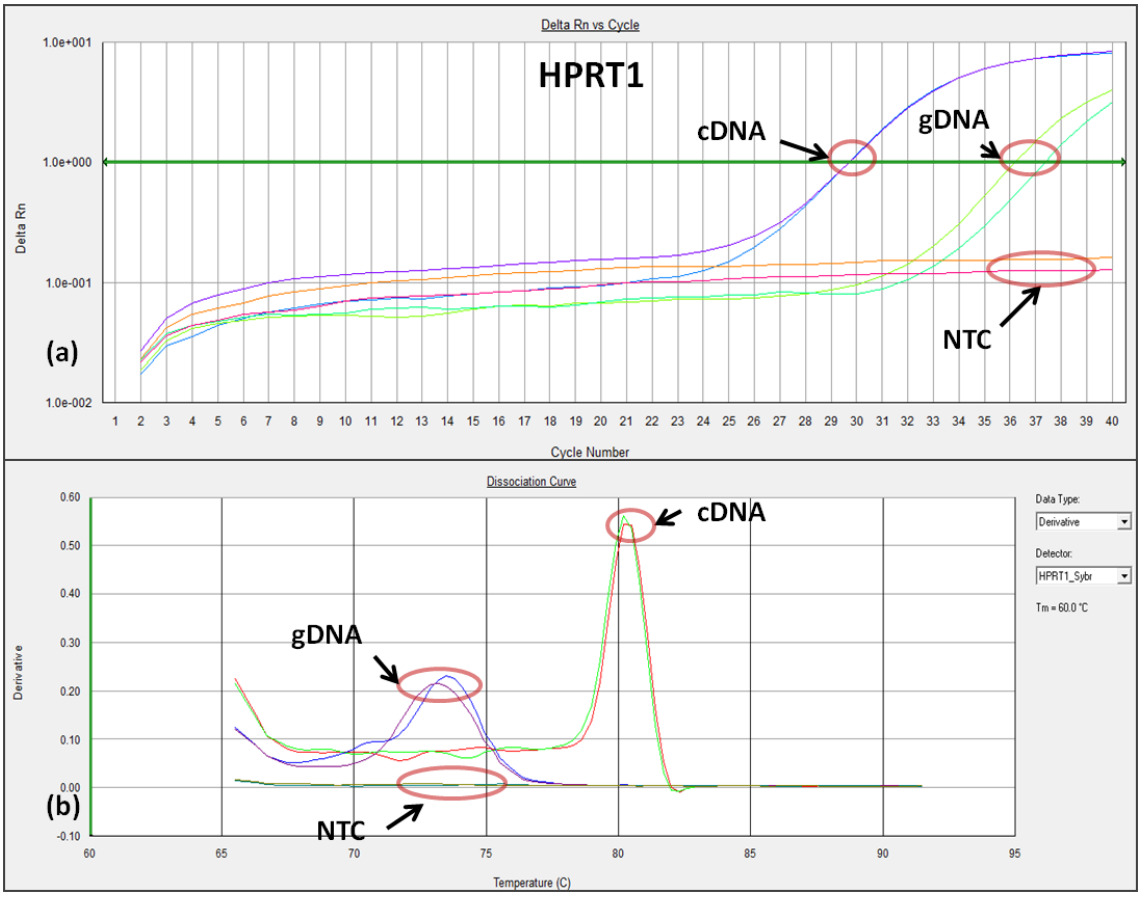

Figure 13 Primer testing via qPCR, HPRT1: (a) Ct values, (b) melting curve

At first sight the results for the HPRT1 primer version do look problematic but are revealed as acceptable in the end. Ct values from gDNA are visible but so late that they can be claimed as irrelevant. No existing NTC values can be seen (a). The melting curve analysis reveals the gDNA values as a weak and distinguishable product. Again no NTC values were obtained (b). Therefore veritable results can still be guaranteed because a possible undetected contamination would not add relevance to the cDNA signal.

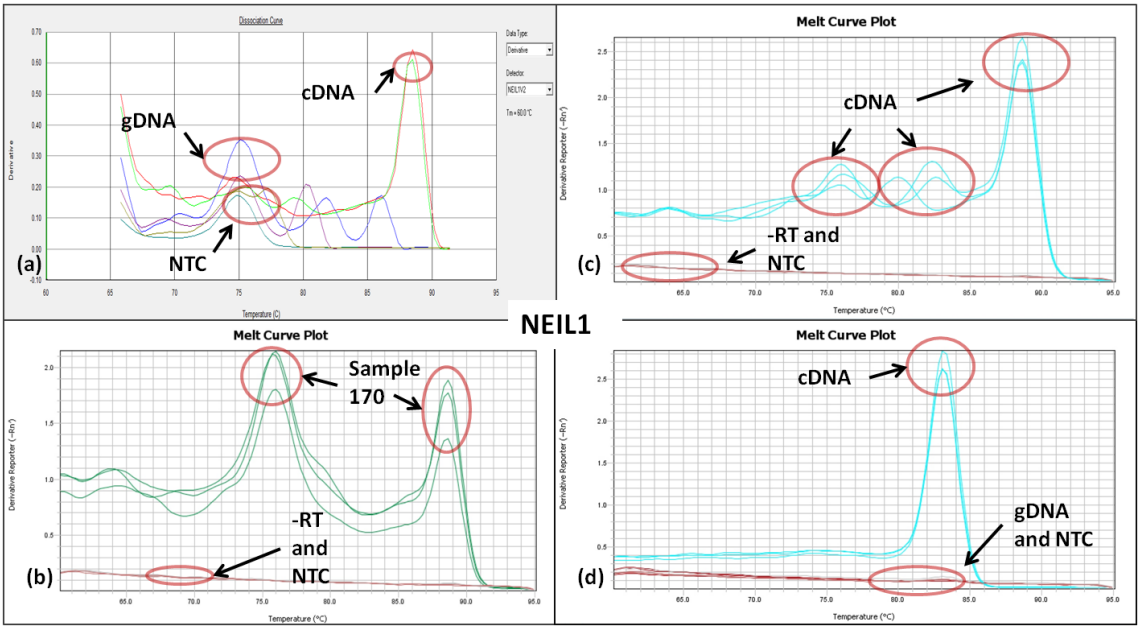

Figure 14 Primer testing via qPCR, NEIL1: melting curves (a) initial test, (b) qPCR run sample 170, (c) qPCR run cDNA pool, (d) qPCR test final version

An example for surprises in the actual runs was the NEIL1 primer. The tested version that was judged as critical but acceptable in testing showed specificity issues in the actual runs. In the initial test the primers produced weak, unspecific gDNA and even lower NTC curves (a). Because the Ct values proofed to be irrelevant (data not shown) we decided to try it with this version for the qPCR analysis of the samples. Nonetheless melting curve analysis in those qPCR runs revealed unspecific product formation in many samples like the presented sample with the number 170 (b). NTC and –RT were negative. This was also revealed when looking at the cDNA pool that was run in the qPCR analysis as reference for this kind of errors (c). These specificity issues forced us to test new primer versions and to repeat the experiment for NEIL1 with correctly functioning primers. The final primer version proofed to be working specifically without curves for gDNA or NTC in testing (d) and qPCR analysis (data not shown).

The following primer versions were tested as working correctly and therefore used in the final qPCR runs (Table 5+6). If not stated otherwise primers were self-designed with the help of Primer-BLAST (Ye et al., 2012):

Table 5 Final primer versions GOI

Gene symbol	Primer sequence	Length	Tm (°C)	GC content (%)	Product length (bp)	Accession number
APEX1	Forward: CGGACAAGGAAGGGTACAGT Reverse: CTCCTCATCGCCTATGCCGTA	F 20 R 21	F 62.9 R 68.2	F 55 R 57.1	83	NM_001641.3
HMOX1	Forward: CTGCGTTCCTGCTCAACATC Reverse: GCAGAATCTTGCACTTTGTTGC	F 20 R 22	F 66 R 65.8	F 55 R 45.4	130	NM_002133.2
LIG3	Forward: AGAGCGAGTCCAGGTGCATA Reverse: GTGGGCCACCTTGTGAGGAA	F 20 R 20	F 64.9 R 69.6	F 55 R 60	88	NM_013975.3
MUTYH	Forward: TCCACCGCCATGAAAAAGGT Reverse: TGGGACCTTTTGGAACCCATA	F 20 R 21	F 68.6 R 67.1	F 50 R 47.6	77	NM_001293190.1
NEIL1	Forward: GACAGAGTGGAGGACGCTTT Reverse: GCTGGGTTGCAGTCCTCTTA	F 20 R 20	F 63.4 R 64.3	F 55 R 55	91	NM_001256552.1
NTHL1	Forward: CAGACAGATGATGCCACGCT Reverse: TGTATTTCACCTTGCTCCTCCA	F 20 R 22	F 66.6 R 65.6	F 55 R 45.4	70	NM_002528.5
OGG1	Forward: CCGAGCCATCCTGGAAGAAC Reverse: CAGATGCAGTCAGCCACCTTG	F 20 R 21	F 68.1 R 68.2	F 60 R 57.1	129	NM_016821.2
PARP1*	Forward: CCACACACAATGCGTATGACT Reverse: CCACAGCAATCTTCGGTTATGA	F 21 R 22	F 63.5 R 65.9	F 47.6 R 45.4	113	NM_001618.3
POLβ*	Forward: AAAAGTGGATTCTGAATACATT GCTA Reverse: GGCTGTTTGGTTGATTCTGAAG	F 26 R 22	F 62.6 R 64.6	F 30.7 R 45.4	123	NM_002690.2
XRCC1	Forward: AAGAAGACCCCCAGCAAACC Reverse: CGAGTTGGAGCTGGCAATTT	F 20 R 20	F 66.3 R 66.3	F 55 R 50	77	NM_006297.2

* taken from [Slyskova et al., 2012]

Table 6 Final primer versions HKG

Gene symbol	Primer sequence	Length	Tm (°C)	GC content (%)	Product length (bp)	Accession number
ACTB*	Forward: TGGCACCCAGCACAATGAA Reverse: AGTCATAGTCCGCCTAGAAGCA	F 19 R 22	F 69 R 64	F 52.6 R 50	183	NM_001101.3
B2M**	Forward: CACCCCCACTGAAAAAGATGAG Reverse: CCTCCATGATGCTGCTTACATG	F 22 R 22	F 66.6 R 66.5	F 50 R 50	106	NM_004048.2
HPRT1	Forward: TGCTTTCCTTGGTCAGGCAG Reverse: TTCAAATCCAACAAAGTCTGGC	F 20 R 22	F 67.6 R 65	F 55 R 40.9	110	NM_000194.2

*taken from [Acs et al., 2014] ** taken from [Valceckiene et al., 2010]

3.11 Real-time quantitative PCR of the samples

To measure the amount of DNA repair enzyme gene expression, qPCR was used for analysing the samples in the course of this thesis. This was done on a Applied biosystems by life technologies™ Quant Studio 6 Flex machine (Quant Studio Real Time PCR Software v1.1) with 384 well plates (10µl per well) and the Applied biosystems by life technologies™ SYBR® Select Master Mix. As experiment type comparative Ct (ΔΔCt) was selected. The run method included:

- Hold stage: 50°C 2min, 95°C 2min
- PCR stage: 40 cycles of 95°C 15sec + 60°C 1min
- Melt curve stage: 95°C 15 sec, 60°C 1min, 95°C 15sec, 60°C 15sec

➔ The primary design was to run two primers with all perfect matches each on one plate including NTC and -RT as control on every plate. As additional control all three HKGs with cDNA pool as template and one cDNA pool template for every primer were run on every plate (Figure 15). Every sample

was done in triplicates. The primer concentration was 200nM. The cDNA sample and cDNA pool input was 81ng. The cDNA pool was of the same origin as in the primer tests.

	HPRT1												PARP1											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	39(2)			19			94			111			39(2)			19			94			11		
B	11			174			53			35			111			174			53			35		
C	142(2)			211			136			147			142(2)			211			136			147		
D	86			55			188			10			86			55			188			10		
E	108			57			123(2)			124			108			57			123(2)			124		
F	128			115(2)			100(2)			56(2)			128			115(2)			100(2)			56(2)		
G	215(2)			45			101			24			215(2)			45			101			24		
H	99			95			127			159			99			95			127			159		
I	6			36(2)			NTC			minusRT			6			36(2)			NTC			minusRT		
J	1			110			cDNApool						1			110			cDNApool					
K	89			50									89			50								
L	109(2)			18									109(2)			18								
M	216(2)			206									216(2)			206								
N	182			170									182			170						cDNA ACTB		
O	80			60									80			60						cDNA B2M		
P	117			172									117			172						cDNA HPRT1		

Figure 15 Example of a typical qPCR plate setup

3.12 Housekeeping gene evaluation and analysis of real-time quantitative PCR

The evaluation of the housekeeping genes, as internal control, was done with the Thermo Fisher Cloud qPCR analysis software which uses the geNorm algorithm [Vandesompele et al., 2002]. These results were controlled by self-calculation of M-scores ("The average pair wise variation of a particular gene with all other control genes" [Vandesompele et al., 2002]) according to the literature [Hellemans et al., 2007]. The general recommendation from the geNorm authors is to exclude the least stable HKG from the pool of candidates until no improvement in M-scores can be seen. This way a combination from at least two but optimally three HKGs can be identified from a pool of candidates. ACTB, B2M and HPRT1 were all shown to be very stable by

having M-scores way lower (average of 0.043) than required (below 0.5). An exclusion of the least stable one did not bring any improvement (data not shown). Analysis of the final qPCR data was done using the Thermo Fisher Cloud qPCR analysis software. To calculate our results the $2^{-\Delta\Delta Ct}$ method was used [Livak and Schmittgen, 2001]. As a slight adjustment a normalization factor as internal control was calculated based on the results from the HKG evaluation instead of using a single reference gene as recommended [Vandesompele et al., 2002]. The geometric mean of the ACTβ, B2M and HPRT1 Ct value results were taken for the calculation of the normalisation factor [Vandesompele et al., 2002]. Within the perfectly matched couples patients with an HbA1c <7.5 were taken as a calibrator. Software of choice for all calculations was Microsoft Excel.

- ➔ There are of course other options and ways than GeNorm for the evaluation of housekeeping genes like NormFinder [Andersen et al., 2004] and BestKeeper [Pfaffl et al., 2004]. However, comparisons did show mostly minor differences in results between them [Piehler et al., 2010; Ren et al., 2010; Hildyard and Wells, 2014].

3.13 Statistics

Tests for normal distribution were done by a Kolmogorov-Smirnov test. A t-test was used to examine differences between the good and poor glycaemic control group. A one-sample t-test against 1 was used to examine differences in gene expression fold-changes. The significance level was set to 0.05. Spearman correlation was used to analyse possible correlations between the DNA repair enzymes. Statistical analysis was done with SPSS version 22.

A more detailed list of all working steps and materials of this investigation can be found in the appendix.

4 Results

4.1 Study population

The goal was to create study groups that significantly differ in HbA1c which was achieved (p=0.000) for the whole MICRODIAB study population (Table 7) as well as for the reduced gene expression analysis study population (Table 8).

Table 7 MICRODIAB study population

		Age (years)	Duration of T2DM (years)	HbA1c	BMI
Good glycaemic control (HbA1c <7.5)	Mean	68.3	13.5	6.8*	34.0
	Std. Deviation	9.8	9.1	0.5	7.4
	Minimum	40.0	0.0	5.9	21.2
	Maximum	86.0	54.0	7.4	58.6
	N = 69				
Poor glycaemic control (HbA1c ≥7.5)	Mean	66.8	15.2	8.6*	35.9
	Std. Deviation	10.1	7.0	1.3	7.7
	Minimum	45.0	0.0	7.5	19.0
	Maximum	84.0	33.0	16.3	61.2
	N = 77				
Total	Mean	67.5	14.4	7.8	35.0
	Std. Deviation	10.0	8.0	1.3	7.6
	Minimum	40.0	.0	5.9	19.0
	Maximum	86.0	54.0	16.3	61.2
	N = 146				

*significant difference (α=5%)

Table 8 Gene expression analysis study population

		Age (years)	Duration of T2DM (years)	HbA1c	BMI
Good glycaemic control (HbA1c <7.5)	Mean	67.3	15.3	7.0*	34.4
	Std. Deviation	9.2	9.7	0.3	5.2
	Minimum	40.0	3.0	6.1	25.4
	Maximum	79.0	46.0	7.4	45.5
	N = 23				
Poor glycaemic control (HbA1c ≥7.5)	Mean	68.0	16.7	9.1*	34.9
	Std. Deviation	8.0	8.3	1.9	5.6
	Minimum	45.0	.0	7.5	25.2
	Maximum	79.0	32.0	16.3	47.1
	N = 23				
Total	Mean	67.7	16.0	8.0	34.6
	Std. Deviation	8.5	9.0	1.8	5.4
	Minimum	40.0	.0	6.1	25.2
	Maximum	79.0	46.0	16.3	47.1
	N = 46				

*significant difference (α=5%)

4.2 Real-time quantitative PCR analysis

Trends were shown in terms of fold changes for APEX1, LIG3, NEIL1, NTHL1, PARP1 and XRCC1 (Figure 16) but only APEX1 (30%, p=0.018, SD 0.57), LIG3 (31%, p=0.016, SD 0.58) and XRCC1 (28%, p=0.020, SD 0.53) showed significant up regulation in the poor glycaemic control group in comparison to good control.

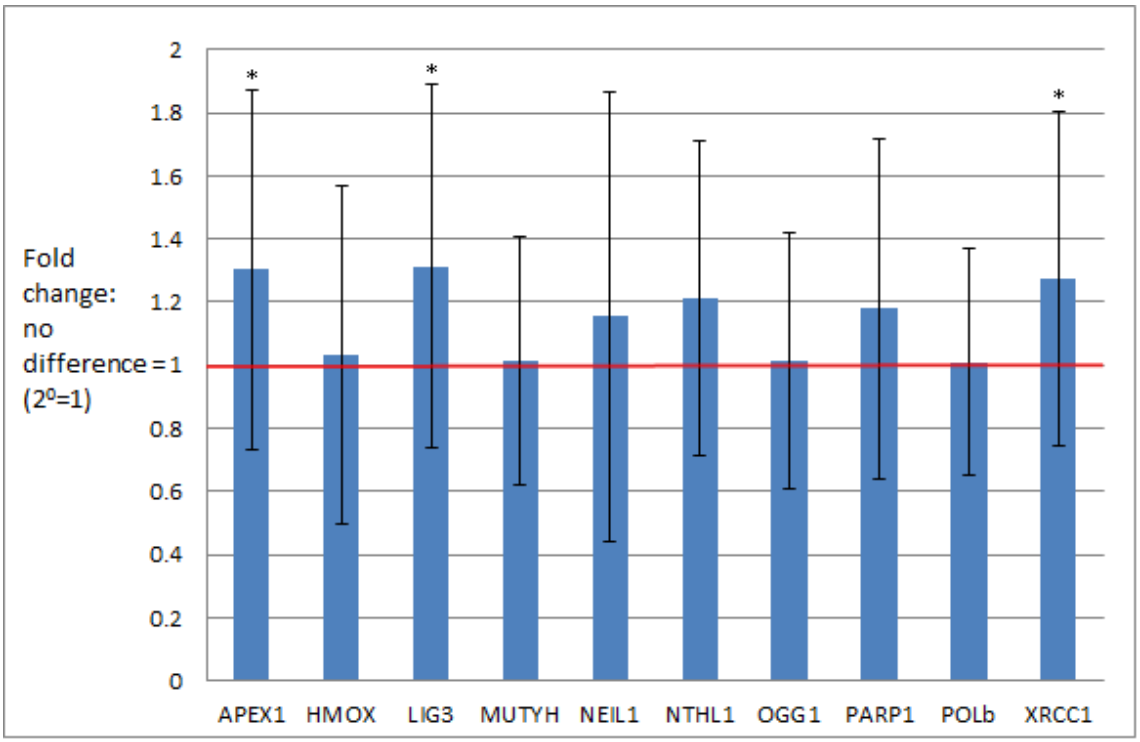

Figure 16 Fold changes good VS poor glycaemic control *sign. (α=5%)

Because NEIL1, OGG1 and POLβ did prove to be not normally distributed, they were again tested in a Wilcoxon test against 1, but no significant results were obtained either (data not shown).

4.3 Correlations

In addition we looked at possible correlations between the repair enzymes (Figure 17) and found strong and highly significant (α=1%) positive correlations for APEX1 with XRCC1: 0.675 (p=0.000), LIG3 with XRCC1: 0.654 (p=0.001) and NTHL1 with OGG1: 0.647 (p=0.001). No correlation was found between any DNA repair enzyme expression level and HbA1c levels (data not shown).

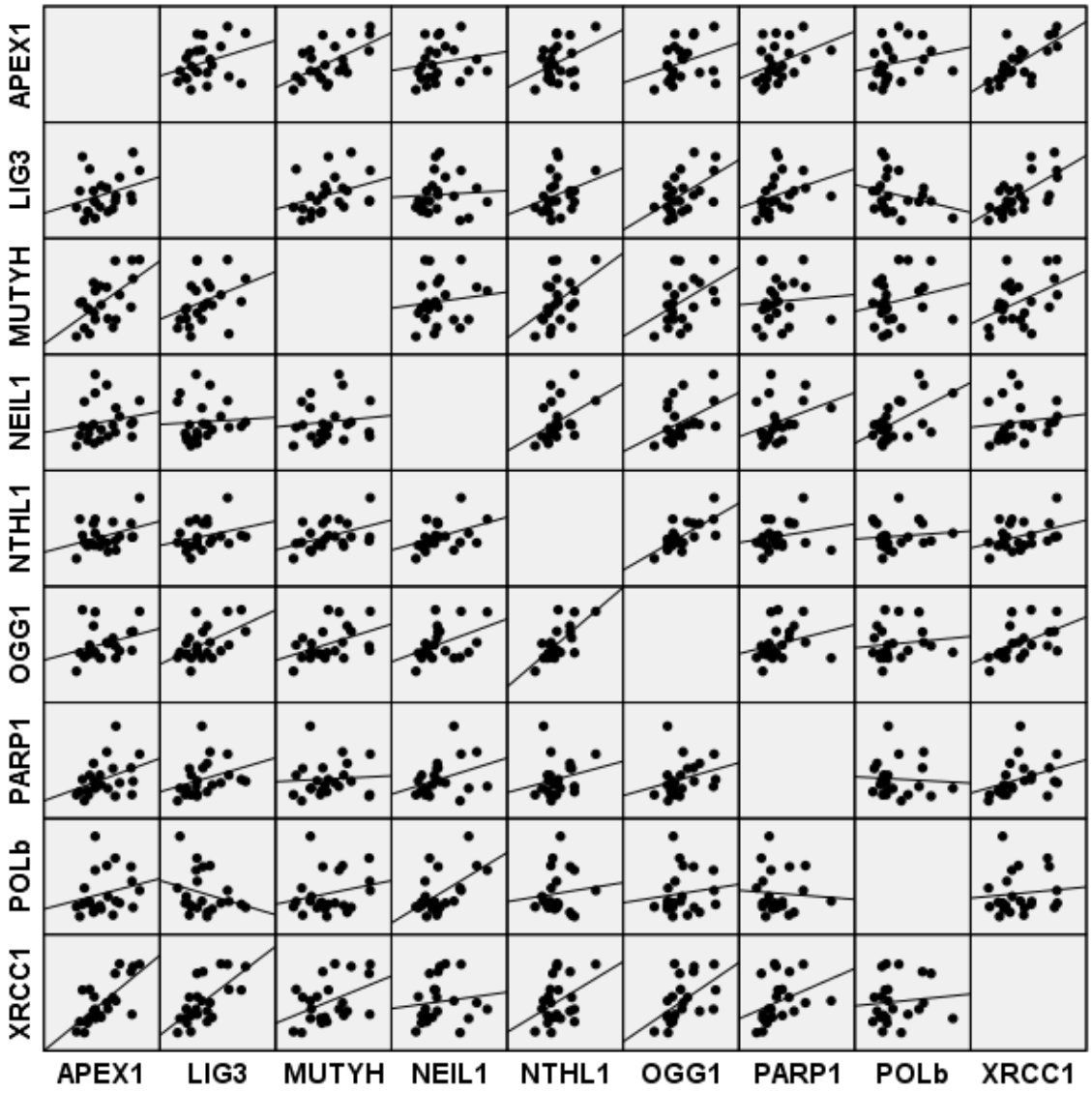

Figure 17 DNA repair enzyme correlation: matrix scatter plot

5 Discussion

Not only pathophysiological knowledge suggests that oxidative DNA damage should be increased in diabetes type 2, which has already been outlined within this thesis, but also various studies came to the same conclusion within their experiments. Lodovici et al. showed significantly higher levels of DNA damage, via the comet assay, for patients with type 2 diabetes mellitus, in comparison to healthy controls [Lodovici at el., 2008]. Arif et al. came to comparable results but additionally found a significant correlation between DNA damage and levels of fasting blood glucose [Arif et al., 2010]. Dincer et al. observed significantly higher levels of DNA damage, again via the comet assay, for T2DM patients with poor glycaemic control (HbA1c > 6.2) in comparison to those with good glycaemic control and significantly higher levels for T2DM patients with good glycaemic control in comparison to healthy control subjects. In their study HbA1c levels correlated with DNA damage in the diabetic as well as in the control group [Dincer et al., 2002]. Another study by Choi et al. showed a direct and significant correlation between DNA damage and HbA1c levels [Choi et al., 2005]. A more recent study by Xavier et al. investigated DNA damage via the comet essay and DNA repair mRNA expression profiles via microarray technology. Interestingly the diabetes type 2 patients were split into hyperglycaemic and non-hyperglycaemic patients with a cut-off point of 7 for HbA1c. This study design was even more similar to our own investigation than the one from Dincer et al. In addition they added a control group of healthy subjects. In this study DNA damage was significantly higher for the hyperglycaemic group compared to the others and among the differentially expressed gene sets GO: 0006282 (“regulation of gene repair”) was induced in the hyperglycaemic group [Xavier et al., 2015].

For all these reasons we could have expected a higher expression of DNA repair enzymes in T2DM patients with poor glycaemic control (≥7.5 HbA1c) in our experiment. Such expectations were not only rejected in order to avoid bias but also because there were other studies that gave quite contrary results in comparison to the ones above.

Ibarra-Costilla et al. found only slightly elevated levels of DNA damage in patients with type 2 diabetes mellitus which were not significant and concluded therefore to not have any differences in comparison to the healthy control group [Ibarra-Costilla et al., 2010]. Jones et al. even found lower levels for DNA damage in T2DM patients [Jones et al., 2014].

In our investigation we saw significantly higher DNA repair enzyme mRNA expression levels for APEX1, LIG3 and XRCC1 and therefore speculated on having higher DNA repair rates in T2DM patients with poor glycaemic control in comparison to T2DM patients with good glycaemic control, supposedly driven by higher DNA damage. XRCC1 strongly correlated with LIG3, since they build a work-complex in short-patch BER. It was interesting that XRCC1 strongly correlated with APEX1 while POLß, which is supposed to play a role in an own BER step between them [Brenerman et al., 2014], did not. Whether the slightly weaker correlation between NTHL1 and OGG1 was beyond the fact that they both use β-elimination in contrast to NEIL1 (β,δ-elimination) [Parsons and Edmonds, 2016] was not revealed in spite of thorough literature research. Unfortunately no new knowledge was gained regarding PARP1 by our data. In total the enzymes with the strongest correlation were also the ones that were significantly up-regulated. Nevertheless the results showed only a mild increase in expression of about 1.3 fold (30%) for only 3 of the 9 investigated enzymes. This increase may be significant but its biological relevance can be questioned as a 2-fold difference (100%) is mostly considered as the cut-off point for relevant biological results. Although DNA repair was higher in the poor glycaemic control group there was no detected correlation between HbA1c and DNA repair enzyme expression. As all studies listed above were not able to present completely sufficient explanations for the results in our experiment we had to consult the literature again.

One possible explanation was found in a lower repair efficiency of DNA in T2DM patients, regardless of higher damage, in comparison to healthy subjects in two polish studies by using the comet assay [Merecz et al., 2015; Blasiak et al., 2004]. In addition a gene expression analysis via microarray hybridization by Manoel-Caetano et al.

showed a significant down regulation of genes associated with DNA repair for T2DM patients in comparison to healthy controls [Manoel-Caetano et al., 2012]. So it was very tempting to say that we could not see a strong increase in DNA repair enzyme expression in our experiment, because DNA repair is generally down-regulated in diabetes mellitus type 2, regardless of potentially higher DNA damage. That this would have been a wrong direction was revealed when looking at our data again. As we did not only measure gene expression levels of DNA repair enzymes but also for HMOX1 we were also able to speculate about DNA damage rates, as HMOX1 is a well accepted indicator for oxidative stress. We could clearly notice that HMOX1 was not differentially expressed in the poor glycaemic control group compared with the good glycaemic control group, we could not even observe a trend. We could therefore only conclude to not have higher oxidative stress levels in the poor glycaemic control group compared to the ones with good glycaemic control and therefore supposedly no difference in DNA damage. When there was no difference in DNA damage the slight increase in DNA repair can no longer be explained by decreased repair efficiencies. To solve this puzzle one important point has to be considered. Just because we did not find differences between the T2DM groups it does not mean that there was no oxidative stress present in the diabetic patients, it was just not greater when measured. In our case it almost seemed as if oxidative stress and its consequential DNA damage had somehow levelled off regardless of the existing hyperglycaemia, which should induce it according to the majority of the literature, leading to only minor differences in DNA repair gene expression between both study groups that theoretically could have been much larger.

A plausible explanation for this effect is the antidiabetic medication that all of the included matched couples received. Metformin as an example can reduce oxidative stress and DNA damage [Algire et al., 2012], as well as gliclazide that also reduced oxidative DNA damage in patients with type 2 diabetes mellitus [Sliwinska et al., 2008]. Also Xavier et al. draw this conclusion for the explanation of their results. After a one week intervention period with a diabetic diet, significantly decreased DNA damage levels could be found in all subjects, whereas the damage levels of the diabetic

patients were still significantly higher than those of the healthy controls. Interestingly gene expression of genes that are related to antioxidant defence and oxidative stress via qPCR did not show significant changes in expression profiles between the study groups [Xavier et al., 2014]. Furthermore only women were included in the present study as intended focus. Interestingly other studies have reported significantly lower levels of DNA damage in diabetic women compared to men [Dincer et al., 2002][Choi et al., 2005]. Another fact that has to be considered is diabetes duration and homeostasis. Both Ibarra-Costilla et al. and Anderson et al. claim that DNA damage levels decrease when oxidative stress becomes a chronic state in long-term T2DM, due to adaptive mechanisms within the stressed cells, leading to no statistical difference in DNA damage between diabetic patients and healthy control subjects [Anderson et al., 1998; Ibarra-Costilla et al., 2010].

Further, HbA1c levels of the grouped study population are narrow, which could also explain the small differences in levels of repair enzyme expression. When taking this direction two major points have to be discussed: the reliability and relevance of qPCR results in general. The reliability of qPCR results is strongly influenced by various sources of variability. Therefore a thorough documentation of the method and the fulfilment of certain criteria are necessary to avoid misleading results [Bustin, 2010]. This is why the “Minimum Information for Publication of Quantitative Real-Time PCR Experiments” (MIQE) guidelines were written in 2009, in order to make this type of experiment more comparable. The impact of technical variability that can be controlled for, starts at the sample acquisition stage where mRNA profiles can be perturbed quite easily by collection, processing and storage issues. Quantity and quality assessment of the extracted RNA as well as DNase treatment is also very important to ensure sound and comparable results [Bustin et al., 2009]. Another cause of variability is the reverse transcription step due to possible errors and various options of reagents and protocols [Ståhlberg et al., 2004]. Also the final qPCR has many factors that can introduce variability, such as the specificity of the primers that has to be tested and NTCs that have to be run to check for primer-dimer formation to name only a few [Bustin et al., 2009]. Furthermore the experimental setup can also influence

your results due to “run to run” variations [Derveaux et al., 2010]. Also the final data analysis can give rise to variability when normalisation is done just with one, probably also not evaluated, reference gene or due to variations in PCR amplification efficiencies (E) that were not taken into account [Bustin, 2010].

When looking at this thesis we can say that mostly all major MIQE requirements were fulfilled. PBMCs were extracted from fresh blood, RNA and cDNA were both tested with NanoDrop for quantity and quality and the RNA was also tested on a gel for purity and possible degradation. The reverse transcription step was performed with a kit that included a blend of oligo-dT and random primers, because both were described as the advantageous method throughout the literature [Bustin et al., 2005; Ginzinger, 2002; Kubista et al., 2006; Nolan et al., 2006]. Primers were carefully selected and thoroughly tested employing gel-electrophoresis and melting curve analysis. As basic plate setup the sample maximisation strategy was used as recommended [Derveaux et al., 2010] and also NTCs and –RT control samples were run in the final qPCR experiment. Normalisation when calculating results was done with carefully evaluated multiple reference genes, a required criterion that can not be emphasised enough because using just one single reference gene with a supposedly unstable gene expression just introduces more variability into the experiment [Vandesompele et al., 2002]. What normalisation is supposed to do is to subtract the effects of technical variation from the results, revealing the genuine biological changes [Derveaux et al., 2010], which are again obscured if normalisation is done improperly [Bustin, 2010]. The only major issue we had has its roots in the $2^{-\Delta\Delta Ct}$ method which assumes 100% PCR amplification efficiency (E=1) at all times, leading to a doubling of specific amplicons with each cycle [Schefe et al., 2006]. Consequently the PCR amplification efficiencies are expected to be equal between target and reference, which is necessary for a valid calculation [Livak and Schmittgen, 2001]. While this assumption holds true when using TaqMan probes [Life Technologies, 2012] this can not be said for SYBR green [Goni et al., 2009], which is what we used in this study. Therefore primer efficiencies should have been measured for all target genes [Giulietti et al., 2001], which was not included in our experimental design. Ignoring PCR efficiency differences that were relatively low (0.15)

have been shown to result in a measured gene expression difference of 16 fold for templates with identical initial template amounts in a review of qPCR analysis concepts [Schefe et al., 2006]. An estimation of the PCR efficiencies of the genes of interest compared with the housekeeping genes can be calculated from the slope of a standard curve that is drawn from the plotted ΔCt values of a serial dilution of a pool of samples versus their logarithmised concentrations [Livak and Schmittgen, 2001]. This could have been easily implemented into the workflow when doing qPCR runs. The calculated efficiencies could have then been taken into the equation with a more advanced formula by Pfaffl or by Hellemans et al. [Pfaffl, 2001; Hellemans et al., 2007]. Interestingly we are in good company when looking at the results of a review that investigated the validity of qPCR experiments in cancer research. Here 91% of the 179 reviewed papers did use analysis methods that are efficiency dependent, but PCR efficiencies were only reported by 18% [Dijkstra et al., 2014]. By fair means the use of an external standard curve for the measurement of PCR efficiencies has also been criticised for its strong susceptibility to errors originating from pipetting and the ignorance of possible inter-well E variations. If these measurements are wrong and taken into the equations mentioned earlier another vector than can confound data analysis is produced [Schefe et al., 2006]. As one brief comment it may be appropriate to ask if the variability from not taking different efficiencies into the equation or taking wrong measured efficiencies into the equation does result in the same distortion of results. Of course all this is no excuse for the fact that the importance of PCR efficiencies was overseen during the implementation of the present qPCR experiment.

In summary, it is not possible to say for sure if our discovered small differences in gene expression are rooted in real biological differences or technical variability because of the noted limitations. As wrongly assumed PCR efficiencies mostly result in falsely significant differences in mRNA expression [Schefe et al., 2006] an error corrected investigation would have supposedly resulted in not finding differences in gene expression levels between the groups of good and poor glycaemic control. However, this would not have had any influence on the final conclusion, because when discussing qPCR results also the question of biological relevance always has to be

answered [Bustin, 2010]. When looking at mRNA expression we are on transcriptome level which is not a perfect predicator for the proteome level [Chassy, 2010], because many genes control their expression not via the level of transcription but by posttranscriptional regulation [Tian et al., 2004]. This is mirrored in mammalian studies [Schwannhäusser et al., 2013; Tian et al., 2004; Xu et al., 2012] and human cancer cell studies [Chen et al., 2002; Shankavaram et al., 2007] revealing that only averagely 46% of the protein variability can be explained by mRNA levels. This is important because the execution of biological functions in our bodies is not handled by mRNAs but their corresponding proteins [Tian et al., 2004]. Additionally if the biological relevance of a fold change of 2 is already questionable [Bustin, 2010] the findings of averagely 1.3 in fold-change are probably of no biological relevance.

In conclusion, the expression of the DNA repair enzymes APEX, LIG3 and XRCC1 in our study was significantly different between the good and poor glycaemic control group in women with type 2 diabetes mellitus. However, this relatively low fold change of averagely 1.3 only affected three of the nine investigated enzymes participating in short-patch BER. The other six showed the same expression values between the groups. Furthermore HMOX1, as a marker for oxidative stress, was not differentially expressed between poor and good glycaemic control study groups. Therefore, well aware of the above mentioned limitations, whether it originates from medication, gender, adaptive mechanisms or even study design, based on the results of this thesis Austrian female T2DM patients with poor glycaemic control seemed to have the same oxidative stress and DNA repair levels as their counterparts with good glycaemic control regardless of their higher potential for DNA damage as a negative consequence of chronic hyperglycaemia. If it is to conclude that they are also of lower risk to suffer from negative health outcomes of T2DM, which correlate with DNA damage such as cancer, lies beyond the scope of this experiment and has to be investigated by future studies with a different study design and even greater accordance with the MIQE guidelines.

6 Summary

BACKGROUND: Increased levels of oxidative stress induced by hyperglycaemia are one negative side effect of type 2 diabetes mellitus. Apart from the damage to the micro- and macrovascular system also elevated levels of DNA damage can be observed in diabetic patients. Unrepaired DNA damage is the source of mutations which are the main factor in the initiation stage of carcinogenesis. To maintain genomic integrity and therefore prevent the formation of mutations our bodies use the base excision repair pathway to repair oxidatively damaged DNA. The aim of this thesis was to investigate differences in gene expression levels of short-patch base excision repair enzymes and one marker for oxidative stress between female T2DM patients with either better (HbA1c < 7.5) or poorer glycaemic control (HbA1c ≥ 7.5).

METHODS: For this master-thesis a gene expression analysis by qPCR was implemented and performed for 43 patients and ten genes of interest.

RESULTS: A significant up regulation was found for the enzymes APEX1 (30%, p=0.018), LIG3 (31%, p=0.016) and XRCC1 28%, p=0.020). No differences were found for the oxidative stress marker HMOX1. Strong correlations were discovered for XRCC1 with APEX1 (0.675, p=0.000), XRCC1 with LIG3 (0.654, p=0.001) and NTHL1 with OGG1 (0.647, p=0.001).

CONCLUSION: We only noticed a low difference in fold change between the good and poor glycaemic control groups for only three of the nine investigated enzymes and no difference in the marker for oxidative stress. Therefore the poor glycaemic control group seemed to have the same oxidative stress and DNA repair rates regardless of higher HbA1c levels in comparison to the better glycaemic control group. This might be explained by the antioxidative effects of the received antidiabetic medication, generally lower DNA damage levels in women, adaptive mechanisms in long-term T2DM and a narrow HbA1c distribution between both study groups.

7 Zusammenfassung

HINTERGRUND: Eine negative Auswirkung von Diabetes mellitus Typ 2 besteht in erhöhtem oxidativen Stress verursacht durch die chronische Hyperglykämie. Neben den Schäden die dadurch am micro- und macrovaskulären System verursacht werden ist auch die Schädigungsrate der DNS erhöht. Werden diese nicht repariert können Mutationen entstehen, welche als Hauptfaktor der Initiationsphase bei der Krebsentstehung angesehen werden. Um seine genomische Integrität zu erhalten bedient sich der Körper hierbei der Basenexzisionsreparatur. Das Ziel dieser Masterarbeit war es, die Unterschiede hinsichtlich der Genexpression verschiedener Enzyme dieses Reparaturmechanismus, sowie zusätzlich eines Markers für oxidativen Stress, zwischen Typ 2 Diabetikerinnen mit besserer (HbA1c < 7,5) und schlechterer glykämischen Kontrolle (HbA1c ≥ 7,5), zu untersuchen.

METHODEN: Für die vorliegende Masterarbeit wurde eine Genexpressionsanalyse mittels qPCR implementiert und an 43 Patientinnen für zehn selektierte Gene durchgeführt.

ERGEBNISSE: Es konnten signifikante Steigerungen der Genexpression für die Enzyme APEX1 (30%, p=0,018), LIG3 (31%, p=0,016) und XRCC1 (28%, p=0,020) gemessen werden. Für den Marker HMOX1 ergaben sich allerdings keine signifikanten Unterschiede. Darüber hinaus zeigten sich starke Korrelationen zwischen den Enzymen XRCC1 und APEX1 (0,675, p=0,000), XRCC1 und LIG3 (0,654, p=0,001) sowie NTHL1 und OGG1 (0,647, p=0,001).

FAZIT: Die Auswertung der Daten zeigte eine nur leicht gesteigerte Genexpression bei drei von neun untersuchten Reparaturenzymen und keine Unterschiede hinsichtlich des Markers für oxidativen Stress. Daraus ergab sich die Annahme, dass die Höhe an oxidativem Stress und der DNS Reparaturraten, in der Gruppe mit schlechter glykämischer Kontrolle, unabhängig von höheren HbA1c Werten, auf gleichem Niveau mit der Gruppe mit besserer glykämischen Kontrolle lagen. Erklären lässt sich dies möglicherweise anhand der antioxidativen Wirkung der eingesetzten Antidiabetika,

einer generell niedrigeren Höhe an DNS Schäden bei Frauen, adaptiven Mechanismen bei Langzeit Typ 2 Diabetikern und einer relativen engen Verteilung der HbA1c Werte zwischen den beiden Studiengruppen.

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9 Appendix

Materials

RNAlater:

Sigma-Aldrich RNAlater®

RNA extraction:

Promega ReliaPrep™ RNA Cell Miniprep System

Sigma-Aldrich RNaseZAP™

Reverse transcription:

Qiagen QuantiTect® Reverse Transcription Kit

Thermo cycler: peqlab Primus 25 advanced

UV-Vis Spectrophotometer:

Thermo Scientific NanoDrop 2000c (with according software)

Gel-electrophoresis:

Gel: Lonza SeaKem® LE Agarose

Gel stain: Biotium GelRed™ Nucleic Acid Gel Stain, 10.000X

Sample stain: New England BioLabs®Inc. Gel loading Dye, Blue (6X)

Marker: Biozym Quantitas 200bp-10kb

Buffer: TAE Buffer 10X

Power supply: BIO RAD PowerPac™ HC

Chamber: Biozym

Imaging: BIO RAD ChemiDoc™ XRS+ System (with Image Lab™ 3.0 software)

TAE Buffer

10X TAE Electrophoresis Buffer was self-made according to the following protocol:

Materials:

- 48,4g of Tris base (tris[hydroxymethyl]aminomethane)
- 11,4mL of glacial acetic acid (17.4 M)
- 3,7g of EDTA disodium salt
- deionised water

Preparation:

- Dissolve the Tris base, glacial acetic acid and EDTA in 800 ml of deionised water
- Dilute the buffer to 1 litre
- You do not need to sterilize the solution

Primer testing:

Gel:

Thermo cycler: peqlab Primus 25 advanced

Thermo Scientific DreamTaq PCR Master Mix (2X)

PCR:

Applied Biosystems 7300 Real Time PCR System (7300 Real-Time PCR System Sequence Detection Software Version 1.3.1)

Master Mix: Applied biosystems by life technologies™ SYBR® Select Master Mix

96 well plates (20µl per well)

qPCR:

PCR: Applied biosystems by life technologies™ Quant Studio 6 Flex (Quant Studio Real Time PCR Software v1.1)

Master Mix: Applied biosystems by life technologies™ SYBR® Select Master Mix

384 well plates (10µl per well)

UV-hood:
LTV Labortechnik GmbH & Co KG DNA/RNA UV- Cleaner Box

Protocol

PBMCs

A minimum of 5 million cells should be frozen in at -80°C to guarantee 1 million cells to survive for RNA extraction. Furthermore the cells should be treated with RNAlater before freezing.

RNA

RNA is highly unstable for a sufficient extraction it is therefore necessary to create an RNase free, as sterile as possible environment. All extractions are done under a previously thoroughly cleaned hood. Wearing gloves at every step and cleaning your hands with RNaseZAP every time you go under the hood is mandatory. All required reagents, cups, pipettes, tip boxes etc. must also be cleaned with RNaseZAP before placing it under the hood.

- Prepare all solutions according to the protocol
    - The amount of prepared DNase I should match your calculated need, do not prepare all of it
    - The rehydrated DNase I should be dispensed into working aliquots. 25-26µl aliquots are recommended if you plan to do 8 samples per extraction for example.
    - In this kit 1-Thioglycerol has to be added to the BG buffer to create a working solution for the RNA extraction
    - This BL +TG Buffer solution can only be stored for a maximum of 30 days at 2-10°C.
    - If it is not planned to use the whole kit up within this time period, which is highly unlikely, only small quantities of BL+TG Buffer should be prepared according to your extraction plans

- Extraction:
    - Gloves!
    - Prepare a sufficient mix of BL+TG Buffer according to the protocol, e.g. 32.5µl 1-Thioglycerol + 3.25ml BL Buffer if you plan to extract 10 samples or 65µl TG + 6.5ml BL Buffer for 20 samples etc.
    - Put all required solutions under the hood (remember to clean your hands and everything you take with you with RNaseZAP)
    - On ice get your frozen PBMC samples (8 samples per extraction can be handled at ease, doing too much at once may interferes with your results because auf prolonged standing times between the first and the last sample)
    - Follow the Promega Quick protocol, with minor modifications for optimal performance:
        - 14) Stick to the protocol but do not discard the collection tube.
        - 15) A. Add 300µl of RNA wash solution and centrifuge for 30sec.
        - 15) B. Place the column into a new collection tube and centrifuge at 14 000 x g for 2 min.
        - 16) Stick to the protocol but put the tubes on ice after centrifugation and transfer the eluate from the elution tube back to the membrane in the column and centrifuge again for 1 min.
            - You can reduce the amount of used nuclease free water (NFW) from 30µl to 25µl or even 20µl if poor results occur (e.g. due to a lower input than 1 million cells)
        - All centrifugation steps are performed with 14 000 x g and 24°C.
        - Except for the last step, working on ice is not necessary
    - Measure RNA quantity and quality via NanoDrop, transport on ice.

- NanoDrop:
    - Gloves!
    - Samples on ice!
    - Start computer and measuring software
    - Raise the sampling arm, wipe the upper and lower pedestal with a laboratory wipe splashed with distilled water, wipe it with a dry laboratory wipe
    - Pipette 2µl of distilled water onto the lower pedestal
    - Lower the arm and start the initial spectral measurement that is demanded by the software
    - Raise the arm, wipe the pedestals
    - Pipette 2µl of NFW onto the lower pedestal, lower the arm
    - Click “Blank” for measuring and storing the spectrum of your blanking buffer
    - Raise the arm, wipe the pedestals
    - Make sure “RNA” is selected in the software as measuring target
    - Pipette 2µl of your sample onto the lower pedestal, lower the arm
    - Click “Measure” to measure the concentration of nucleic acid in ng/µl, 260/280 and 260/230 ratios
    - Raise the arm, wipe the pedestals FIRST!
    - THEN note your measurements (doing so prevents your sample from drying out and corrupting the machine)
    - Go on like this for all samples
    - After you finish, wipe the pedestals with a slightly wet laboratory wipe and then wipe it dry
    - Close the arm and software
    - Shut down the computer
    - Freeze your measured RNA samples at -80°C

- Gel-electrophoresis:

  To confirm your results from the NanoDrop measurements gel-electrophoresis is performed, but it is not necessary to test all samples. The RNA sample with the highest concentration of each extraction day is taken for the gel-electrophoresis test (e.g. 8 extractions = 8 test samples).

  - Gloves!
  - Prepare a 1X TAE Buffer by mixing 100ml of 10X TAE Buffer with 900ml of distilled water
  - For a 1.2% gel mix 1.44g of agarose powder with 120ml TAE(1X) Buffer in an Erlenmeyer flask (250ml)
  - Microwave the mixture but first put a little piece from a paper towel into the opening of the flask to prevent the gel from spilling out, make sure that steam can still come out to prevent it from exploding
  - Microwave at “medium high” setting for around 4-5mintues.
  - The powder should have completely dispersed but do not overcook it to keep the desired gel concentration
  - You can take it out after 2 min., swirl it a little and put it back in for another two for example
  - BE CAREFUL the flask is extremely hot!
  - After the gel has cooled down a little bit, so that you at least dare to touch it for a longer time, but do not let it get too cold, pipette 12µl of GelRed Stain into the gel and mix it by swirling the flask
  - Pour the still hot gel into the prepared gel form (rubber borders attached)
  - With a tip move all bubbles to the side of the form
  - Put in the comb

- While the gel is cooling down and hardening for around 40min. you can start to prepare the RNA samples
    - Thaw on ice
    - Spin down the samples
    - Samples: 5µl RNA sample, 3µl NFW, 2µl loading dye (in new cups of course)
    - Marker: 8µl marker, 2µl loading dye
    - Freeze away original RNA samples
- After the gel is ready remove the comb and rubber borders, put the form into the chamber, in the right running direction: from negative (= black) to positive (= red)
- Fill up the electrophoresis chamber with TAE buffer
- Pipette 10µl of each sample and the marker into the wells
- Put the marker at first and last position of your gel setup
- Close the chamber
- Attach the cables to the power supply (left socket = negative =black, right socket = positive = red)
- Switch on the power supply
- 80 volts (static), 120min.
- Start
- After the run has finished start the computer
- Turn on the imaging device and the camera
- Open the software
- Carefully take out the gel by taking out the form
- Let all liquid run off
- Open the drawer of the imaging device and let the gel carefully slide from the form onto the plate of the drawer
- Close the drawer, start imaging with the software
- Click “New Protocol”
- Under “Application” click on “Select”, “Nucleic Acid Gels”, “GelRed”

- Under “Imaging Area” – “Select Gel Type” choose “Bio-Rad Ready Gel”
- Click “Position Gel” and fit the gel into the red frame
- Make sure that camera filter 1 is selected
- Click “Run Protocol”
- Adjust image after clicking on the icon “Image Transform”
- Save your image by clicking on “Snapshot”, “To file”(jpeg)
- After you have saved your image the gel can be thrown away in the black bin
- Clean the imaging surface with distilled water and then with ethanol
- Empty the electrophoresis chamber and clean every part with distilled water
- Switch everything off

<u>cDNA</u>

- Conversion:
  - Gloves!
  - Work on ice!
  - Program the thermo cycler before starting
  - Mix by flicking the cups and not by vortexing
  - Spin them down before opening for the first time, before you put them into the thermo cycler and after the thermo cycler has finished
  - No changes were made to the Qiagen protocol
    - Preparing a master mix of gDNA wipeout buffer and RNase-free water is not necessary

        - Notes Step 1:
            - Volume of RNA used depends on planned input and RNA sample concentration
            - RNase-free water is used to reach the total reaction volume of 14µl and therefore depends on RNA volume
                - E.g.: your RNA samples has a concentration of 88.8 ng/µl and you plan to use 0.5µg of RNA:
                    - gDNA Wipeout Buffer 2µl
                    - RNA 5.6µl
                    - RNase-free water 6.4
        - Notes Step 2:
            - Preparing a volume of master mix 10% greater than required has proven to be unnecessary
    - Remember to not only convert your RNA samples but to produce –RT and NTC as well
        - For –RT a pool of RNA samples was made:
            - To reach a total input of 0.5µg RNA the needed volume of four templates was calculated to reach an amount of 0.25µg each
            - Be careful not to exceed total reaction volume

- NanoDrop
    - Perform similar to RNA samples
    - Switch target to DNA
    - Freeze cDNA at -20°C

- cDNA dilution

  Dilute the finished cDNA samples with NFW
    - Gloves
    - On ice

- Thaw samples
- Spin them down
- Based on a volume of 18µl cDNA (20µl eluate, 2µl used for NanoDrop) dilute with 192µl NFW to reach a total volume of 200µl cDNA

Primer testing

Primer versions are tested by PCR combined with gel-electrophoresis for a quick overview over primer efficiencies. The best versions are then tested with qPCR with melting-curve analysis for confirmation of these results.

- DreamTaq PCR + Gel-electrophoresis
  - Each primer is tested with three different templates. One time with a cDNA pool, one time with a gDNA template and one time with NFW
  - For easier labeling use symbols for the different templates. E.g. + for cDNA, g for gDNA, - for NFW
  - Gloves!
  - Work on ice
  - No changes were made to the Thermo Scientific protocol
  - Set the required program for the thermo cycler before starting!
    - Individual setup where required:
      - Temperature:
        - at the annealing step the temperature has to be set as melting temperature of the primer minus 5°C (Tm-5)
        - It is not possible to do this for every sample on its own
        - Groups have to be formed
        - The maximum difference from the desired temperature should not get too high and should be equal between the groups

- When forming groups keep maximum capacity of thermo cycler and gel in account
    - 20 wells per gel, 40 if working with two combs
    - The thermo cycler has space for 25 samples
        - Day 1: 6 primers (tested with 3 templates each => 18 slots needed), Tm-5 = 56°C
        - Day 2: 8 primers (tested with 3 templates each => 24 slots needed), Tm-5 = 59°C

- Time:
    - Initial denaturation: 4min
    - Final extension: 10min
- Number of cycles:
    - Denat.Anneal.Ext.: 35 cycles

- Follow the Thermo Scientific protocol
    - Notes Step 2:
        - Prepare master mixes (always one time more, if you have 6 samples prepare it for 7) for cDNA, gDNA and NFW templates
        - Forward and reverse primer concentrations have to be equal
            - E.g.: the correct amount can be reached via taking 7.5µl of a 1µM primer (diluted beforehand: 5µl of 100µM Primer mixed with 495µl NFW) so that the final concentration in the final reaction volume of 50µl is 0.15µM

- DNA templates:
    - 25ng gDNA sample
        - human DNA extracted from PBMCs (person unknown)
        - CTRL A, 23.04.15, 2.5ng/µl
        - 10µl = 25ng
    - 243ng cDNA sample
        - cDNA pool of 10 perfect matches (= 20 samples)
        - 10µl of each sample mixed together (=200µl in total) with a concentration of 162ng/µl
        - 1:2 dilution with NFW => final concentration of 81ng/µl in 400µl
        - 3µl = 243ng
- NFW
    - Volume depends on all other components of the master mix
    - Used to reach a total reaction volume of 50µl

- Gel-electrophoresis of the amplified cDNA:
    - Same working procedure as with RNA
    - Exceptions:
        - 1.5% Gel:
            - 1,8g agarose powder
            - 120ml 1xTAE buffer
        - 80 volts (constant), 45 min.
            - This makes working with 2 combs in one gel possible
            - Therefore more than 6 triplicate samples (18 in total) can be done at once (2x20 wells!)

        - Marker at first and last position of every section => 36 samples per gel possible

    - 8µl cDNA sample + 2µl loading dye

- qPCR
    - Before starting design your plate and make your calculations!!
    - Master mixes and finished plate should never be exposed to direct light!!
    - Prepare eventual dilutions before starting the first round
    - Gloves!
    - On ice
    - Thaw primer and cDNA, spin down before opening the first time
    - Dilute primers to 4µM (20µl stock primer (100µM) + 480µl NFW)
    - Freeze away stock primers (-20°C)
    - 1:2 dilution of existing cDNA pool (used for DreamTaq PCR + Gel-electrophoresis)
        - Check how much is left via pipette
        - Mix it with the same amount of NFW (e.g. 370µl cDNA +370µl NFW)
        - c = 40.5ng/µl
    - primers are tested in 4 concentrations:
        - 100nM, 200nM, 300nM, 400nM
    - Each concentration is tested with cDNA, gDNA and NFW in duplicates
    - 96 well plate allows testing of 4 different primers at the same time with this setup
    - Prepare master mixes under the UV-hood (no ice necessary for mixing part)
        - Switch on switch 2 (UV- Recirculator/UV- light)
        - Start UV-light for 15 minutes (set timer in UV- light time controller)

- When timer has finished mix master mixes according to this scheme:
    - E.g.: 100nM:
        - 7µl NFW
        - 0.5µl forward primer
        - 0.5µl reverse primer
        - 10µl SYBR Select Master Mix
        - 20µl total reaction volume (2µl cDNA/gDNA/NFW are added later)
    - Be careful! In this setup we have 6 samples (3 templates in duplicates) for every single primer concentration
    - Master mixes are therefore prepared for 8 samples due to possible losses!
    - So the real pipeting scheme looks like this:
        - E.g.: 100nM:
            - 56µl NFW
            - 4µl forward primer
            - 4µl reverse primer
            - 80µl SYBR Select Master Mix
            - Total volume of 144µl
    - At the end you should have prepared 4 master mixes for everyone of the 4 primers like this (16 in total)
- Store finished master mixes on ice
- Freeze away primer dilutions
- Divide the 96 well plate into 4 areas (one for every primer) with a marker
- Divide each area into 4 zones (one for every concentration)
- Mark where cDNA, gDNA or NFW is added in a later step
- Pipet master mixes according to your plate design (18µl per well)

- Leave UV-hood and work under normal hood
- Add templates (2µl per well)
- Seal the plate with a sealing film (tightly!!)
- Make sure there are not too many bubbles inside the wells (softly knock them to the work bench)
- Wrap it in aluminum foil for transport or short waiting times, place it on ice
- Freeze away template material
- Spin down the plate with a centrifuge (3000g, 2min)
- Start computer, start PCR machine, start software
- Setup run according to your plate design with the software
    - Setup qPCR:
        - Click “create new document”
            - Assay: standard curve (absolute quantitation)
            - Container: 96-well clear
            - Template: blank document
            - Run mode: standard 7300
        - Click “weiter”
        - Click “new detector”
            - Here you can create your primers
            - Reporter = SYBR
            - Quencher = ROX
        - Click “add” to add your primers to the experiment
        - Click “weiter”
        - Select wells with the mouse and assign them to a primer (according to your plate design)
        - Click “fertig stellen”

- Setup:
    - Click on specific well
    - Right click on it, click “well inspector”
        - Name well (according to your sample names, e.g. 215)
- Instrument:
    - Sample volume 20µl
    - Thermal profile:
        - Stage 1: 50°C 2min
        - Stage 2: 95°C 2min
        - Stage 3: 40 reps of 95°C 15sec + 60°C 1min
        - Click “add dissociation stage” (=melting curve): 95°C 15 sec, 60°C 1min, 95°C 15sec, 60°C 15sec
- Start qPCR
- When finished analyze, save and export results
- Throw away the plate (black bin)
- After everything is finished clean UV-hood surfaces with alcohol, then with water
- Set a timer for 15 minutes, let it run, switch off hood afterwards

<u>qPCR</u>

- Basics:
    - Primer dilution 4µM
    - Primer concentration 200nM
    - cDNA sample dilution 40.5 ng/µl (50µl from original cDNA sample (162ng/µl) + 150µl NFW into new cups)
        - by doing so the original samples are not used up and can be used for repetitions or even other studies
    - cDNA sample input 81ng

    - cDNA pool same as in primer test with qPCR, c=40.5ng/µl
    - cDNA pool input 81ng
    - 384 well plate
    - 5µl SYBR Select Master Mix
    - 10µl total reaction volume per well
    - Primary design: 2 primers tested with all perfect matches each on one plate
    - NTC, -RT, cDNA pool as template for every primer as control on every plate
    - All three HKGs with cDNA pool as template as control on every plate
    - This time all templates in triplicates of course
    - Master mixes and finished plate should never be exposed to direct light!!
- Gloves!
- On ice
- Thaw cDNA and primers
- Flick cups
- Spin down
- Mark your plate according to your setup and personal preferences
- UV hood:
    - Mix master mixes for every primer
    - Pipet master mixes into wells (8µl)
- Freeze away primers
- Under normal hood:
    - Pipet templates (2µl)
    - Seal plate with sealing foil (tightly!)
    - Make sure there are not too many bubbles inside the wells (softly knock them to the work bench)

    - Wrap it in aluminium foil for transport or short waiting times, place it on ice

- Freeze away template material
- Spin down plate (3000g, 2min)
- Start computer
- Start machine
- Start software
- Setup run:
    - Create new experiment
    - Setup:
        - Experimental properties:
            - Instrument type: Quant Studio 6 Flex System
            - Block: 384 well
            - Type of experiment: comparative Ct (ΔΔCt)
            - Reagents: SYBR® green reagent
            - Properties: standard
        - Define:
            - Here you can create and define your primers (targets)
            - Targets:
                - Target name: name of primer
                - Reporter: SYBR
                - Quencher: none
                - Color: that is up to you
            - Passive reference: ROX
        - Samples
            - Here you can create and define your samples
            - Click “new” and write in name (e.g. 215)

        - Assign:
            - Assign each well to a sample
                - Click on desired well
                - In “samples” area select specific sample
            - Assign wells to a primer
                - Mark all desired wells with mouse
                - In “target” area select specific primer
        - Run method:
            - Reaction volume per well: 10µl
            - Hold stage: 50°C 2min, 95°C 2min
            - PCR stage: 40 cycles of 95°C 15sec + 60°C 1min
            - Melt curve stage: 95°C 15 sec, 60°C 1min, 95°C 15sec, 60°C 15sec
    - To start from here you have to save this setup as an experiment
    - You can also save your primary setup as a template by “clicking save as template”
        - Next time you can start by clicking “create new experiment from template” and just change the names of the primers
- Insert plate
- Start run
- Analyze, save, export run
- Throw away the plate