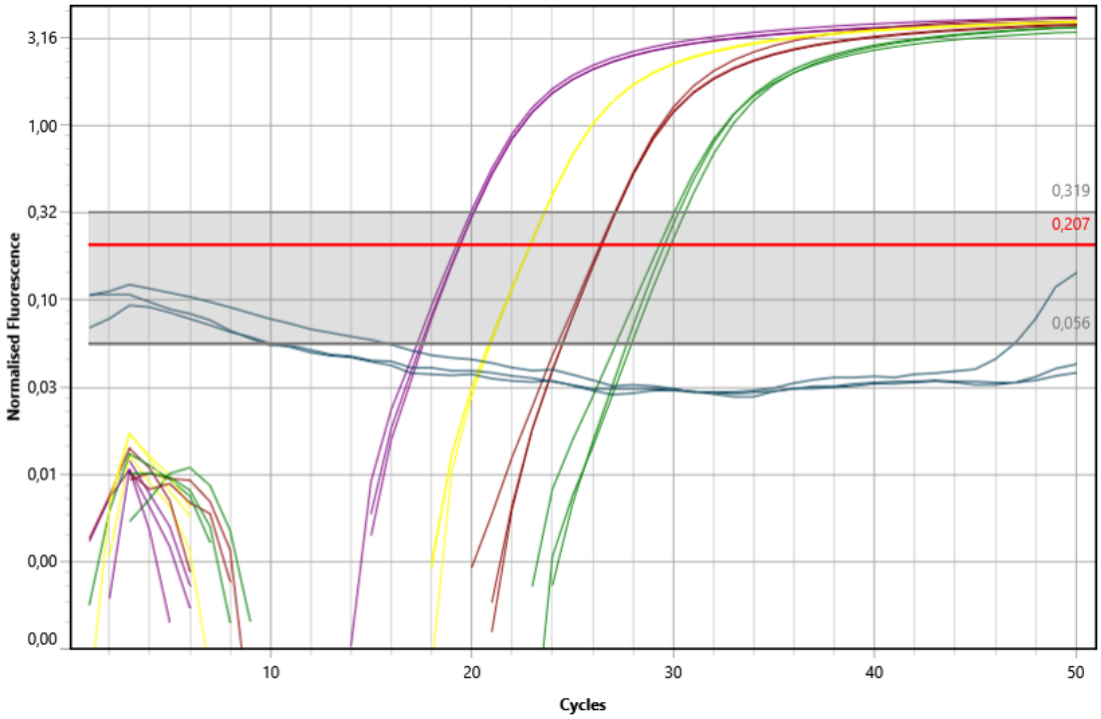

Development of analytical and sensitive methods to detect and quantify therapeutic oligonucleotides

Master's thesis in Biotechnology

Izabela Domagala

DEPARTMENT OF LIFE SCIENCES

CHALMERS UNIVERSITY OF TECHNOLOGY
Gothenburg, Sweden 2023
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Cover: Cycling graph generated by Mic qPCR cycler software, depicting results for elongated EBER1-5.

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Abstract

Therapeutic antisense oligonucleotides (ASOs) had a breakthrough in 1998 when fomivirsen, an inhibitor of human cytomegalovirus, was approved for medical use. Scientists discovered the medical potential of therapeutic oligonucleotides in treatments of various conditions and diseases, leading to the research field gaining more popularity. A challenge within this research field is the detection and evaluation of different oligonucleotide sequences, as no established quantitative and sensitive methodologies are present for these. The aim of this thesis is to develop analytical and sensitive methods for detection and quantification of therapeutic oligonucleotides. Particularly antisense oligonucleotides (ASOs), for application in *in vitro* experiments associated with fields such as cancer research. The ASOs included in this project are EBER1-5 and EBER1-6.

The two-tailed RT-qPCR method, used for detection of miRNA, was adapted and modified in this project. The method was changed so that instead of a reverse transciption, a PCR was performed to anneal and elongate the two-tailed primer with the ASO. The PCR was then followed by a qPCR analysis. Different deviations to the initial protocol as well as to the designs of the primers and oligonucleotides, were performed during the project. The results showed that the method is promising as detection was possible to approximately 10^5 molecules, which corresponds to a concentration of 83 fM. Still, improvements need to be done for the binding and the elongation in the two-tailed part of the method to be able to reliably detect lower amount of molecules.

Keywords: antisense, ASO, oligonucleotide, PCR, qPCR, therapeutic, two-tailed RT-qPCR.

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First of all I want to direct my deepest gratitude to my supervisor, Anders Ståhlberg, for giving me the opportunity to undertake this project in his research group. All guidance has been invaluable for my understanding and knowledge within this field. I am truly thankful for all the interesting discussions about this project, but also research overall. Your passion for research truly passes on.

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The thesis has taken a lot of time and energy, but I could not have wished for a better half a year. I am truly grateful for all the knowledge, new experience, and all the people I have met on the way. Lastly, but certainly not least, thank you to my family and close friends for all the support, motivation and kind words. It truly warms my heart.

Izabela Domagala, Gothenburg, December 2023

List of Acronyms

Below are the acronyms that have been used throughout the thesis. The list is alphabetically ordered.

2'-MOE	2'-O-methoxy-ethyl
2'-OME	2´-O-methyl
2Tp	two-tailed primer
A	Adenine
ASO	Antisense oligonucleotide
C	Cytosine
EBV	Epstein-Barr virus
Fwd	Forward
G	Guanine
LC-MS	Liquid chromatography-mass spectrometry
LNA	Locked nucleic acid
miRNA	Micro ribonucleic acid
mRNA	Messenger ribonucleic acid
NTC	Non template control
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
Rev	Reverse
RNAse H	Ribonuclease H
T	Thymine
TE buffer	Tris-EDTA buffer
U	Uracil

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1
Introduction

Zamecnik and Stephenson suggested, 1978, that oligonucleotides have the potential to be used therapeutically [1]. When issues with i.e. stability, delivery, and specificity were solved, medical use could be considered and then allowed. The first breakthrough was in 1998 when the first oligonucleotide, fomivirsen, was approved for medical use [2]. Fomivirsen is an antisense oligonucleotide that is inhibiting replication of human cytomegalovirus [3], which is a member of the herpesvirus family [4]. Since fomivirsen was approved, the field of therapeutic oligonucleotides has been growing and gaining popularity in the scientific world [5]. Therapeutics directly targeting the RNA are promising as applications can be found for various diseases. The latest new oligonucleotide-based drug is nusinersen [6], which was approved in 2016 by the US FDA. Nusinersen is used to treat spinal muscular atrophy, a diseases targeting the spinal cord, nerves, and the brainstem [7]. Other RNA targeting therapeutics that are currently under development include, for instance, those for Huntington's disease where brain nerve cells are progressively broken down, or for spinocerebellar ataxia that affects gait stability, eye movements, and speech [8]. As therapeutic oligonucleotides have big potential to be utilized in treatments of various diseases and conditions, it is of interest to study their modality. One challenge within this field is the design, detection, and evaluation of different oligonucleotide sequences, as there are no established quantitative and sensitive analytical methods for these short sequences.

1.1 Aim and clarification of task

The aim of the thesis is to develop analytical and sensitive methods for detection and quantification of therapeutic oligonucleotides, particularly antisense oligonucleotides (ASOs), for application in *in vitro* experiments associated with fields such as cancer research.

The main focus in this project is to modify and optimize the two-tailed RT-qPCR method, so that it is compatible for sensitive ASO quantification and detection. The ultimate goal is to be able to detect ASOs in biological samples such as liquid biopsies, but as the project is in an early stage, no clinical samples will be used. The detection and quantification of ASOs is essential to deepen the understanding of cellular uptake dynamics, pharmacokinetics, and the interaction of oligonucleotides with their targets.

2
Theory

To increase the understanding of the methodologies and results presented in this thesis, this section will explain concepts such as antisense oligonucleotides, antisense oligonucleotide detection, and polymerase chain reaction (PCR) methods.

2.1 Antisense oligonucleotides

Antisense oligonucleotides (ASOs) are short sequences of 12-30 nucleotides [9]. Those oligonucleotides act as antagonists of cellular RNA molecules, such as mRNA, pre-mRNA, viral RNA, miRNA, or telomerase-associated RNA present in cancer cells [1]. ASOs target their complementary mRNA sequences and inhibit the upcoming biosynthesis of protein [10]. The binding of the oligonucleotides to the RNA occurs through Watson-Crick base-pairing [1], [9], meaning that adenine (A) base pairs with thymine (T) by two hydrogen bonds and guanine (G) base pairs with cytosine (C) by three hydrogen bonds. Observe that thymine (T) is exchanged for uracil (U) when base pairing in RNA [11]. The two major gene silencing strategies applied by ASOs are Ribonuclease H (RNAse H) recruitment, and emergence of steric hindrance. The ASOs in this project are designed to function by the method of RNAse H recruitment. When ASOs bind the target mRNA, a DNA/RNA heteroduplex is formed, leading to RNAse H recruitment and mRNA degradation. In the case of steric hindrance, the ASO binds the target in a way that silences the gene. Silencing can occur due to various mechanisms, including obstruction of a ribosome from interaction, hindrance of the splicing machinery from interaction leading to new splicing patterns, or blockage of translational repressors leading to upregulation of the expression [12].

Oligonucleotides have short half-lives when unprotected in nuclease-rich cells or serum, as defence mechanisms try to degrade those. To improve the metabolic stability and binding affinity to target RNA, oligonucleotides are often modified chemically by for example incorporating sugar modifications. These modifications not only reduce adverse effects but also enhance uptake and mitigate potential phosphate modifications [1], [9]. Different modifications are associated with different generations of ASOs. There are first, second, and third generations of ASOs [13]. The initial DNA or RNA backbone is a phosphodiester. In first generation ASOs, the phosphodiester linkage between nucleotides is commonly exchanged by phosphorothioate (PS) linkage, where one non-bridging oxygen atom (O) is exchanged by a sulfur atom (S). Observe that, instead of sulfur, in some cases it can be a methyl

group giving rise to methyl phosphonates, or amine group giving rise to phosphoramidates. Figure 2.1 shows the different mentioned structures of first generation ASOs, as well as the initial phosphodiester oligonucleotide. The substitution from oxygen to sulfur enhances both nuclease resistance and cellular uptake, as negative charge is carried. In addition, the plasma half-life becomes longer. There is, however, a side effect with induced cellular toxicity as these modified molecules can interact and non-specifically bind serum proteins [10], [13].

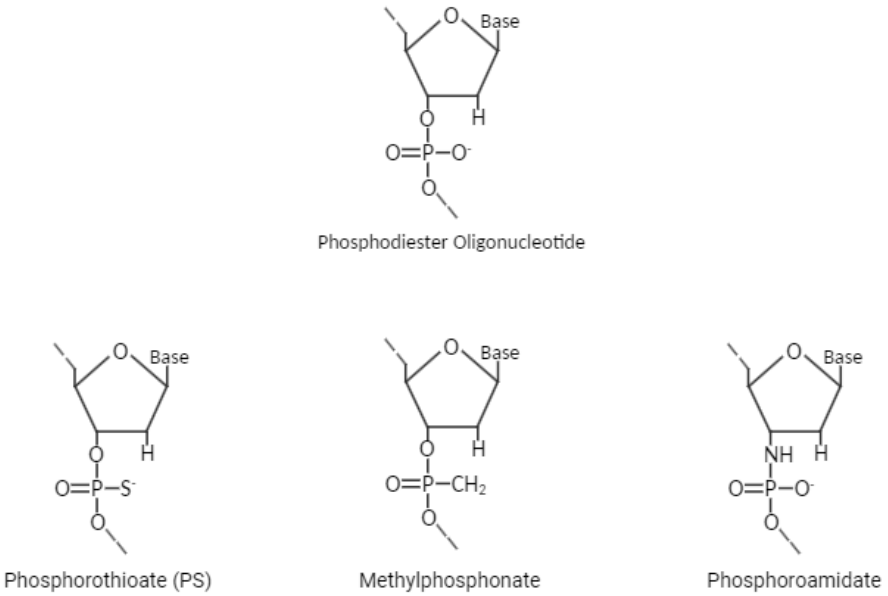

Figure 2.1: Phosphodiester oligonucleotide above the three different first generation antisense oligonucleotides. Phosphorothioate to the left, methylphosphonate in the middle, and phosphoroamidate to the right. Figure created in Biorender.com, based on "Advances in Antisense Oligonucleotide Development for Target Identification, Validation, and as Novel Therapeutics" by M. Mansoor and A. J. Melendez [14].

Second generation ASOs include modifications on the 2' position of the ribose. The changes on the 2' position are alkyl modifications and include 2´-O-methyl (2'-OME) and 2'-O-methoxy-ethyl (2'-MOE). See (A) in figure 2.2 for the two different structures. The alkyl modifications give rise to higher target specificity, higher binding affinity, higher nuclease resistance, and lastly higher hybridization stability with target mRNA. One downside is that 2´-OME and 2´-MOE are made incapable of activating Ribonuclease H (RNAse H). This as conformational changes appear due to the modifications, blocking the translational machinery. RNAse H is an important endonuclease that is recruited during activation of ASOs and if it does not get recruited and activated properly, its silencing efficiency decreases. To induce RNAse H activation, second generation ASOs form a DNA/RNA heteroduplex when RNA-like flanks are added to the DNA sequence, earning a gapmer configuration [13], see (B) in figure 2.2. The new configuration improves protection against nucleases and the molecules become more stable [15].

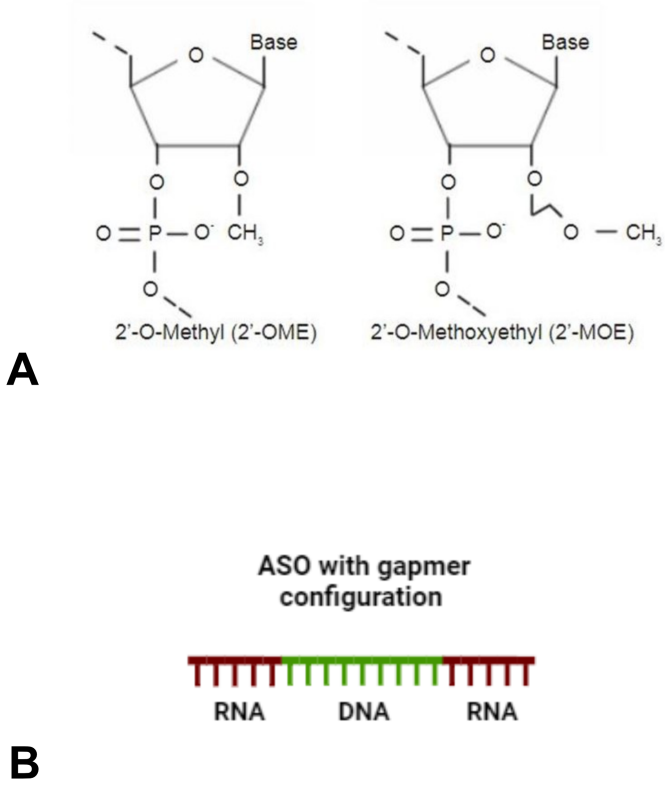

Figure 2.2: (A) The two second generation antisense oligonucleotides. 2'-OME to the left and 2'-MOE to the right. Figure is obtained from "Advances in Antisense Oligonucleotide Development for Target Identification, Validation, and as Novel Therapeutics" by M. Mansoor and A. J. Melendez [14]. (B) Antisense oligonucleotide with gapmer configuration. The DNA sequence is flanked by modified RNA bases, on each end. Figure created using Biorender.com.

The design of third generation ASOs builds upon the configuration of second generation ASOs. Hence, one type of third generation ASO includes gapmer configuration and a locked nucleic acid (LNA). LNA arises when 2'-oxygen is bridged to 4'-carbon of the ribose, by a methylene bridge [16]. See figure 2.3 for a third generation ASO with locked nucleic acid. This configuration protects the ASO from nucleases, increase the binding strength to the target, and enhances the potency [15]. One downside is that the enhanced potency allegedly can have a tendency to cause higher toxicity, such as nephrotoxicity or hepatotoxicity [17]. Changes on the 2' ribose can change the melting temperature (Tm) by 3-16 °C/modified base [18] and modifications where LNA gets incorporated can change the melting temperature by 3-8 °C/modified base [15]. ASOs that are included in this project are all gapmers flanked by LNA bases.

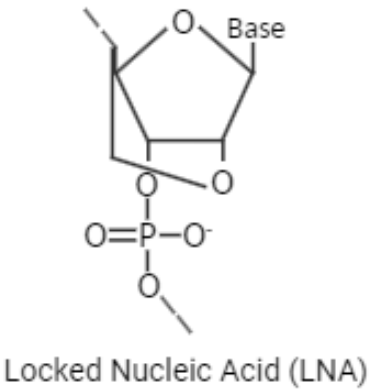

Figure 2.3: Third generation ASO with locked nucleic acid (LNA). Figure created in Biorender.com, based on "Advances in Antisense Oligonucleotide Development for Target Identification, Validation, and as Novel Therapeutics" by M. Mansoor and A. J. Melendez [14].

2.2 Antisense oligonucleotide detection

A major difficulty when working with ASOs is the detection and quantification. The methods available right now are expensive, labour intensive, and have a very limited sensitivity. The two most common methods are hybridization-based immunoassays and chromatography-based methods. Hybridization-based immunoassays have a lower limit of detection at 0.03 ng/mL and are more sensitive than chromatography-based methods. The most common chromatography-based method is liquid chromatography-mass spectrometry (LC-MS). This method is easier to develop and has the advantage of distinguishing intact ASOs from their metabolites [19].

Polymerase chain reaction (PCR) is an established detection method that is fast, specific, and simple. It is used in a wide variety of applications, but because ASOs are very short and have modified nucleotides, these are incompatible with the standard PCR methods [20]. In this project, one PCR approach, two-tailed RT-qPCR, is utilized and modified to assess the sensitivity and detection of ASOs.

2.3 qPCR

Quantitative polymerase chain reaction (qPCR) is a method used for detecting and quantifying amounts of a product (DNA), in real-time [21]. This method generates fluoresecence through three mechanisms: by a DNA-binding fluorochrome specifically binding to double-stranded DNA, a fluorophore being released when a probe is

digested, or by a fluorochrome binding a fluorescent probe upon binding the target. The fluorescence increases with each cycle when DNA amplification occurs and that increase is monitored. The obtained parameter is a quantification cycle or in other words a Cq value, which is the fractional number of cycles that the fluorescence needs to reach the quantification threshold. The threshold can either be predetermined by the instrument or chosen beforehand by the operator [22]. A sample with 1 molecule should obtain a Cq value in the range of 35-37, depending on the instrument and threshold chosen. Cq values above that range indicate background noise in form of other products being detected [23]. A schematic qPCR graph can be observed in figure 2.4.

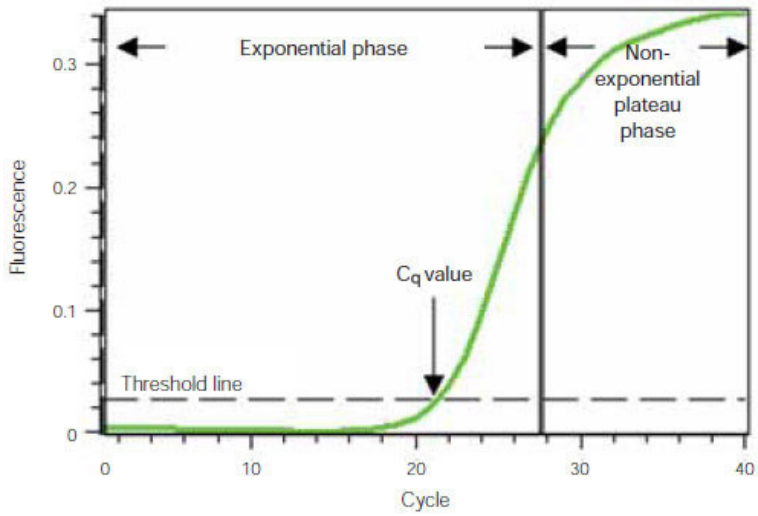

Figure 2.4: Schematic picture of a graph obtained after a qPCR analysis. The threshold line and Cq value are stated. Figure obtained from Bio-Rad Laboratories [24].

Cq values can be plotted against logarithmic amounts of DNA. The obtained slope can be used to calculate the qPCR efficiency, see equation 2.1. An efficiency between 90% to 110% is optimal, otherwise there are most probably errors present. The reason to why the efficiency can exceed 100% is because of the polymerase inhibition. The difference between Cq values is proportional to the difference between logarithmic amounts of DNA and the more target copies in the input, the fewer cycles of amplification, are needed to reach the quantification threshold. In other words, lower Cq values indicate higher amounts of target sequence in the initial sample. In some cases an addition of more template does not result in lower Cq values and with that the slope is lower. This results in a qPCR efficiency exceeding 100% [25], [26].

$$\text{qPCR efficiency } = 10^{\frac{-1}{\text{slope}}} - 1 \tag{2.1}$$

After a qPCR run, a melt curve analysis can be performed to observe if the correct target has been amplified and detected. Annealed products are melted at a constant

predetermined rate, such as for example 0.2 °C/s. The earlier mentioned fluorescence decreases when the products are melted and strands dissociate. This occurrence is monitored and a melting temperature (Tm) is established. Tm is the temperature at which half of the oligonucleotide molecules are double-stranded and half are single-stranded [27]. Tm often depends on the length of the sequence and the base composition within the sequence [28]. In some cases the Tm for the target is known beforehand and by that it is known which Tm should be obtained from the results. In other cases, the exact Tm is not known, and melting curve experiments get compared so that a feasible Tm can be obtained for the target in question [27]. Melt curve analysis can help detect primer-dimers, which are small by-products. These are formed when a primer binds itself or a different primer [29]. Owing it to the smaller size, primer-dimers, have lower Tm and can by that be easier distinguished from the target products.

2.4 Two-Tailed RT-qPCR

Two-tailed reverse transcription - quantitative polymerase chain reaction, in short two-tailed RT-qPCR, is a method developed by Androvic et al. by which miRNA can be detected. The main purpose of the two-tailed method is to elongate the molecules and transform RNA into cDNA [30].

The two conventional primers in standard PCR methods are longer or of the same length as the miRNA. To elude the issue of the miRNA having too short sequence, researchers have utilized a hairpin structure that elongates the miRNA. A down-side with this elongation and utilization of such structure, is compromised specificity and sensitivity. This is the case as one of the two primers senses the miRNA itself, while the other primer senses the extension [30].

In the two-tailed RT-qPCR method, illustrated in figure 2.5, a two-tailed RT primer is attached to a miRNA. The RT primer includes two hemiprobes, typically a slightly longer 5' hemiprobe and a shorter 3' hemiprobe. In addition, the RT primer contains a self-complementary structure, that enables the formation of a hairpin structure. The hairpin structure holds the two hemiprobes in good proximity to each other, facilitating stable binding of the target sequence. The target sequence, bound to the RT primer hemiprobes, is then used as a template. The 3' hemiprobe is elongated by a reverse transcriptase to obtain tailed cDNA. The 5' hemiprobe is not complementary to the target sequence and is not a part of the elongation process. The miRNA detaches from the 5' hemiprobe. It is important to note, that in spite of the fact that the 5' hemiprobe is not elongated, it is still an important part of the design. According to Androvic et al. the 5' hemiprobe greatly reduces the amounts of elongated RT primer. This improves the efficiency of the bindning between the target sequence and the RT primer and increases the probability of concurrent binding to the 3' hemiprobe and by that successful elongation. During qPCR, the 5' hemiprobe binds the forward primer and the elongated 3' hemiprobe binds the reverse primer [30].

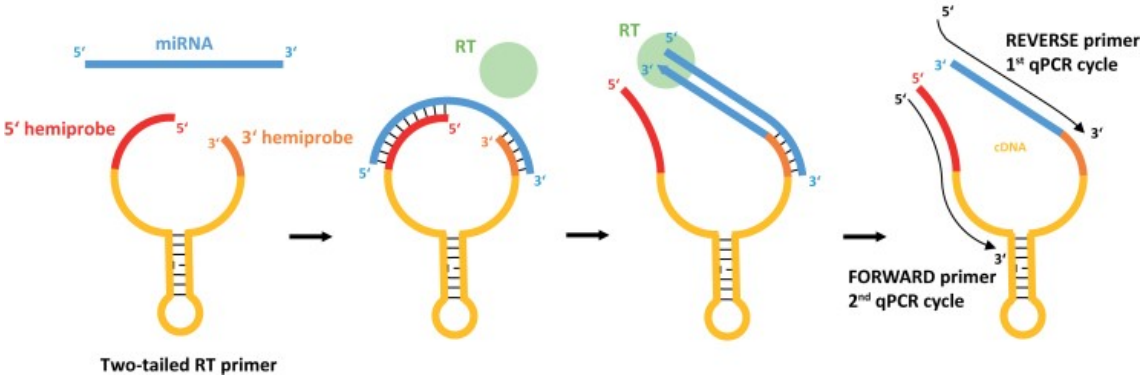

Figure 2.5: Schematic depiction presenting the two-tailed RT-qPCR method developed by Androvic et al. and the different steps involved. Figure is obtained from "Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification" by Androvic et al. [30].

In this project, the two-tailed RT-qPCR method is adapted for ASO detection, see figure 2.6. The RT primer is exchanged with a single stranded DNA molecule, called two-tailed primer (2Tp). The method has been adapted so that no reverse transcription occurs, instead a PCR is performed so that the 2Tp attaches to the ASO. The reason to why a PCR can be performed instead of a reverse transcription is the fact that the ASO is a DNA sequence and not a miRNA sequence that needs to be reversibly transcribed. The reverse transcriptase is by that exchanged with a DNA polymerase to obtain the full length of the target sequence. The modified two-tailed RT-qPCR method will be refereed to as two-tailed PCR-qPCR.

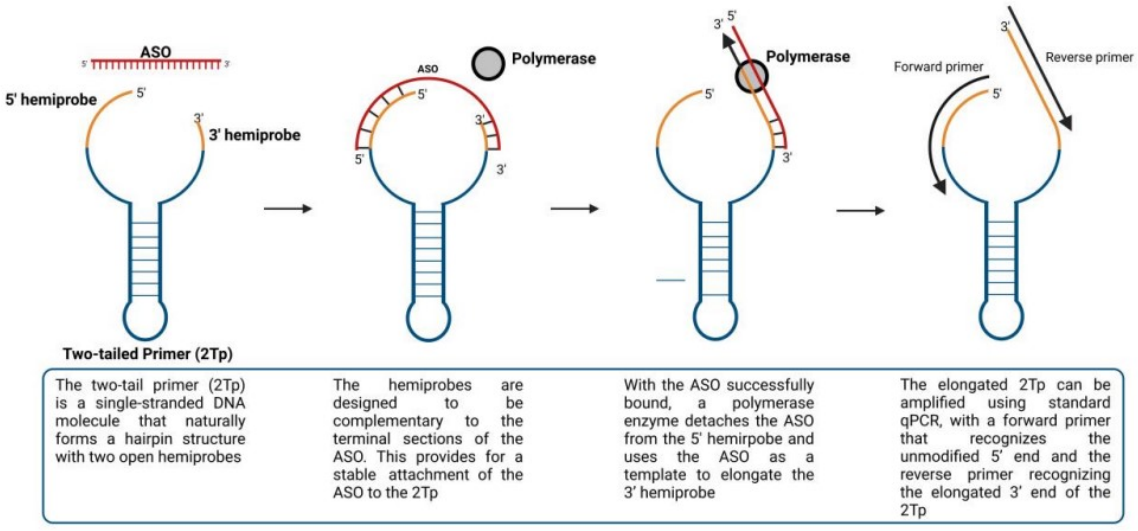

Figure 2.6: Schematic depiction presenting the modified two-tailed RT-qPCR method and the different steps involved. Figure is obtained from the unpublished paper "Development of assays for the detection of therapeutic oligonucleotides" by A. Mofers [31].

3
Methods

The following section will describe the necessary preparations, material, methodologies, and data analysis in this project. Oligonucleotide and primer designs will be explained, as well as the two-tailed PCR-qPCR method. As the project progressed, modifications were made to the initial designs and protocol. These will be presented in the results section.

3.1 Oligonucleotide and primer designs

In this project, both modified (marked "ASO") and non-modified (marked "DNA") ASOs were included. The ASOs used were EBER1-5 and EBER1-6 which are ASOs coupled to the Epstein-Barr virus, see sequences in appendix 1, table A.1. Epstein-Barr virus (EBV) causes multiple types of malignancies in epithelia and lymphoids. Epstein-Barr virus encoded small RNAs (EBERs) are non-coding RNAs that are abundantly expressed in dormant EBV-infected cells [32]. The non-modified ASOs were the primary ones to be used due to the less expensive price and easier purchase accessibility from Integrated DNA Technologies, Coralville, IA, USA. The modified ASOs were kindly gifted by Ka-Wei Tang's research group that work with EBV, and were intended for use if significant results were obtained when initially using the non-modified ASOs. The ASOs were received as lyophilized pellets and were resuspended in Tris-EDTA buffer, also called TE buffer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The ASOs were stored at -80 °C for long-term storage and at -20 °C if used consistently. The ASOs were diluted to concentrations appropriate for the experiments, and to minimize the impact of too many freeze-thaw cycles, aliquots were prepared. For the dilutions, a combination of TE buffer and bovine serum albumin (Thermo Fisher Scientific), in a 1:20 ratio, was used.

Parts of the designed sequences did not get involved in any complimentary binding or secondary structures. These parts were designed to be as non-reactive as possible, so the bases A and T were preferred over G and C. For the two-tailed primers (2Tp) a set hairpin structure was used, that previously has been developed in Anders Ståhlberg's research group. See the 2Tp sequences in appendix 1, table A.2. The 2Tp designed for EBER1-5 (16 bases) had both hemiprobes consisiting of 6 bases each and the 2Tp designed for EBER1-6 (20 bases) had both hemiprobes consisting of 7 bases each. See table A.2 in appendix 1, for the hemiprobes. The forward and reverse qPCR primers used for amplification of 2Tps depended on the ASO binding region and were designed to have suitable melting temperatures. The forward

primer for EBER1-5 was 17 bases long, while the reverse primer was 15 bases long. For EBER1-6, the forward primer was 18 bases long and the reverse primer was 17 bases long. All primers were ordered from Integrated DNA Technologies and the sequences are presented in appendix 1, table A.3.

The designed oligonucleotides could possibly form homodimers as well as heterodimers. If homodimers occur, it could interfere with the binding to the target sequences and if heterodimers occur it could interfere with the amplification of the desired product. To assess homodimer and heterodimer occurence in the oligonucleotide designs, IDTDNA Online OlogiAnalyzer™ Tool was used. If ΔG was below the empirical value of -6 kcal/mol for the binding, the non-predetermined regions were altered to heighten the binding energy.

3.2 Two-tailed PCR-qPCR

For the two-tailed PCR-qPCR reaction, the annealing and elongation mixture consisted of 2 µl of ASO at a chosen concentration, 5 µl iQ SYBR® Green Supermix at 1x final concentration (Bio-Rad Laboratories, Hercules, CA, USA), 2Tp at final concentration of 50 nM and nuclease-free water (Invitrogen, Thermo Fisher Scientific) to achieve a final mixture volume of 10 µl. Two controls were used for this experiment. One control was called "No ASO" and it had all the reagents just mentioned, except the ASO. It was volumetrically replaced by nuclease-free water instead. The second control was called "No 2Tp" and it included all reagents except the 2Tp. Instead it was volumetrically replaced by nuclease-free water. Note that the ASO concentration in the second control was the highest among all concentrations used in the entire experiment setup. All mixtures were loaded into a transparent 96 well, PCR plate (Sarstedt, Nümbrecht, Germany), where every well contained 10 µl. The prepared plate was then put into an T100 thermocycler (Bio-Rad) to perform enzyme activation, annealing and elongation. See table 3.1 for the empirically determined time and temperature profiles for the thermocycler.

Table 3.1: Time and temperature profile used in thermocycler for the annealing and elongation. Observe that the first step was cycled only once, whereas the second and third steps were together cycled 10 times in total.

Temperature	Time
95 °C	2 min
40 °C	3 min
70 °C	20 s

A qPCR analysis was performed after the PCR was finished. The mixture for the analysis consisted of 5 µl iQ SYBR® Green Supermix at 1x final concentration, pooled forward and reverse primers to obtain a final concentration of 0.4 µM (Integrated DNA Technologies) and nuclease-free water to reach the volume of 8 µl in total. This mixture was loaded into Mic strip tubes (Bio Molecular Systems, Upper

Coomera, QLD, Australia) and 2 µl of the thermocycled mixture was added in, to reach a total volume of 10 µl. A non template control (NTC) was included using 8 µl of the qPCR mixture and 2 µl of nuclease-free water. The Mic tubes were covered with included caps and placed into a Mic qPCR cycler (Bio Molecular Systems), where the qPCR temperature and cycling profile, outlined in table 3.2, was executed. Note that a melt curve analysis was performed in the same run, where the temperature changed from 60 °C to 95 °C by 0.2 °C/s.

Table 3.2: The qPCR temperature and time profile used in Mic qPCR cycler. Observe that first step was cycled once, all subsequent steps were together cycled 50 times in total.

Temperature	**Time**
95 °C	2 min
95 °C	5 s
60 °C	20 s
70 °C	20 s

3.3 Data analysis

Raw data was generated through Mic qPCR cycler software. A cycling graph with corresponding Cq values and a melting curve graph with corresponding Tm values was obtained. After being assessed, the data was exported to excel for further analysis. Mean values and standard deviations were calculated from all the replicates of the same sample. The mean Cq values were plotted against the corresponding amounts of ASO for all samples. A plot with Cq mean values relative $\log_{10}$(Amount of ASO molecules) was also conducted. The slope that was obtained from the curve was used to calculate the qPCR efficiency using equation 2.1.

4

Results

In this part, results from experiments with EBER1-5 (DNA) and EBER1-6 (DNA) will be presented. Firstly, results from initial designs and protocol will be presented. This will be done through cycling graphs, a table with Cq and Tm values, a plot of Cq values relative amount of ASO molecules, a plot of Cq values relative $\log_{10}$(Amount of molecules) including trendlines and corresponding equations, and lastly a melt curve graph. Subsequently, results from setups containing the different design and protocol deviations will follow. There, the results will be presented only through tables and a plot of Cq values relative amount of ASO molecules. The plots of Cq values relative $\log_{10}$(Amount of molecules) including trendlines and corresponding equations can be found in appendix 2, if relevant for the experimental setup. See figure 4.1 for an overview of the different design and protocol deviations included in this project.

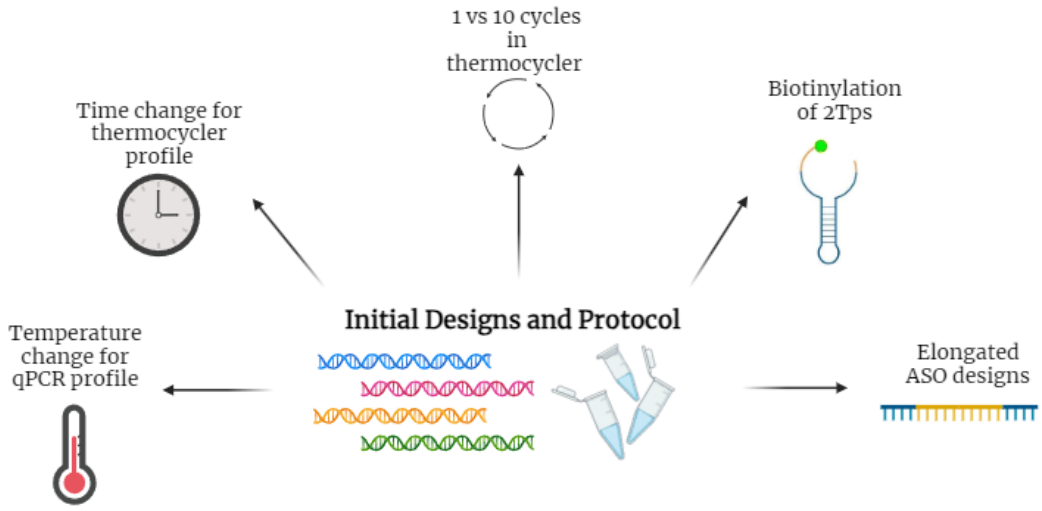

Figure 4.1: Overview of the different modifications made to the initial designs and protocol. Figure created using Biorender.com.

Observe that the NTC controls are not indicated in the presented graphs, but that these were examined for each experiment. If signal was detected in these controls, it meant some contamination or primer dimer formation must have occured as no target should be detected there. The "No ASO" and "No 2Tp" controls are included in the graphs as signals were detected repeatedly. If a signal is detected, it might indicate that unwanted binding has occured leading to formation of PCR products.

As it theoretically should not be any signal due to the fact that either the ASO or the 2Tp is missing, it is of interest to study the controls in comparison to the samples to identify limitations in the protocol or designs that could be modified to improve the method.

4.1 Initial designs and protocol

For the two-tailed PCR-qPCR method to work, the 2Tp needs to efficiently anneal to the ASO. Initial experiments were conducted using EBER1-5 (DNA) and EBER1-6 (DNA) using the initial method and oligonucletide designs presented in the method section. This was done to observe if the ASO concentrations could be differentiated both from each other and the control samples. Concentrations ranging from 830 zM to 830 pM were included in a 10 time dilution series, corresponding to molecule numbers between 1 and 10^9.

Cycling graphs from the experiment are presented in figure 4.2. For EBER1-5 it is possible to observe a clear and more uniform signal from the replicates that are 10^9 to 10^6 molecules. For EBER1-6 it is a little less clear. All the replicates that have amounts below 10^6 molecules have a more spread out signal within the same sample. This is the case for both ASOs. Additionally, some replicates have a signal detected later than 37 cycles, indicating that no molecules are detected but instead background noise in form of other products. The replicates, shown as horizontal lines, do not have any signal at all. For EBER1-5 there was no signal in the No 2Tp control for all replicates in comparison to EBER1-6 where there was a signal in all the replicates for that control. No ASO control provided signals for both ASOs.

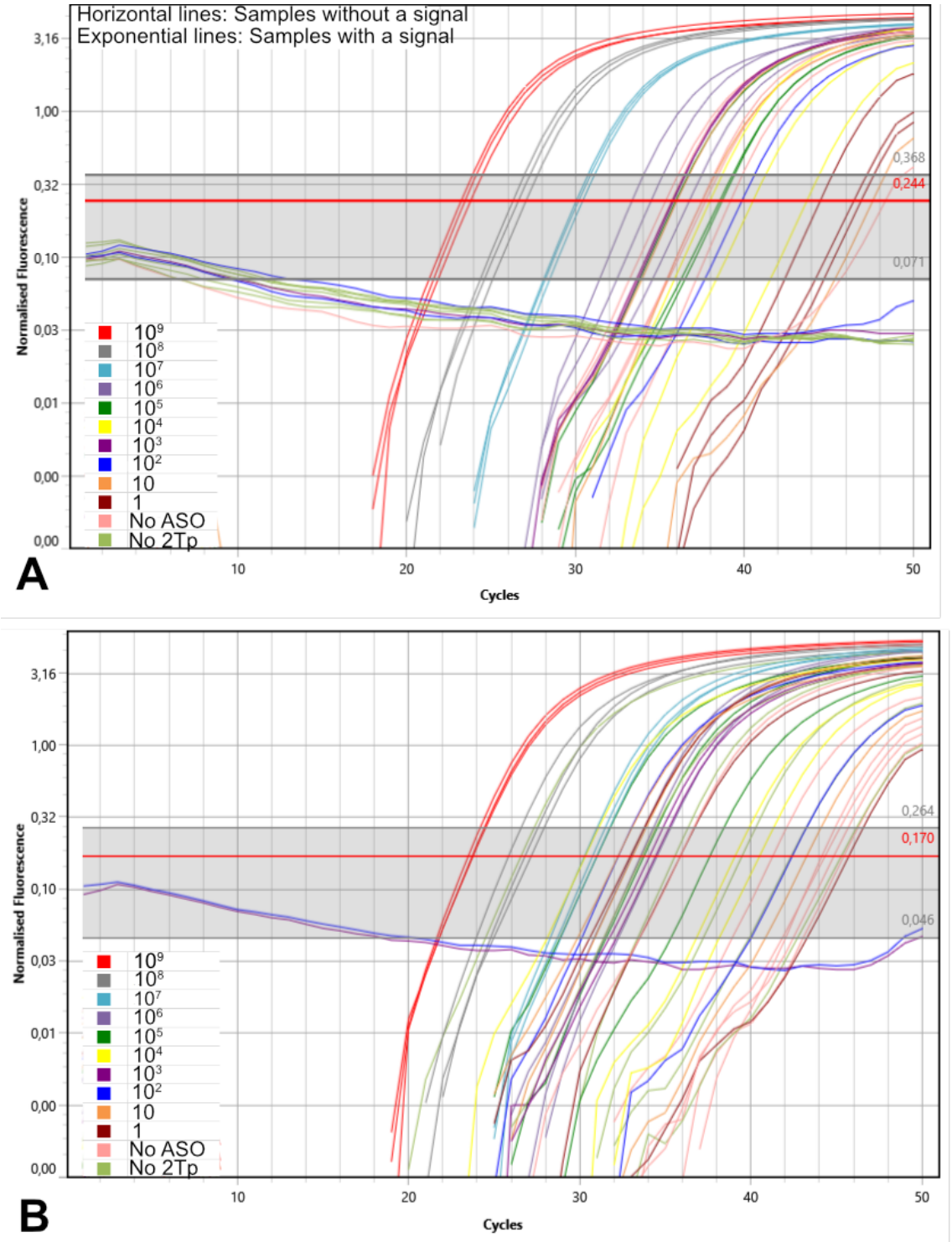

Figure 4.2: Cycling graph for (A) EBER1-5 (DNA) and (B) EBER1-6 (DNA). All samples were made in three replicates, beside the control samples that were done in 6 replicates.

Table 4.1 provides a summary of all Cq values with the associated standard deviations and Tm values with the associated standard deviations for both EBER1-5 and EBER1-6 samples. The values for each sample were obtained by calculating the mean of all the replicates for that specific sample. DNA can double, at most, for

each cycle. As there is a factor 10 between the amount of ASO molecules between each adjacent sample, the Cq value difference should be $\log_2(10) = 3.32$ between these samples. For EBER1-5 samples containing 10^9 to 10^4 molecules, that seems to approximately be the case. For lower amounts of molecules the Cq values diverge from the pattern. Observe that numerous Cq values exceed the range of 35-37. For EBER1-6 samples containing 10^9 to 10^6 molecules, the difference between adjacent samples is approximately 3.32. However, for lower molecule amounts, the Cq values exhibit variability similarly to the ones for EBER1-5. Tm values for EBER1-5 are very similar and the standard deviations are low. For EBER1-6 the Tm values have a larger variability for the lower molecule amounts and the standard deviation are higher there. Overall the melt curve graph depicts a higher variability in Tm values for EBER1-6, see figure 4.3.

Table 4.1: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for EBER1-5 (DNA) and EBER1-6 (DNA). "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^9	23.44	0.28	80.94	0.02
EBER1-5 10^8	26.61	0.41	80.94	0.01
EBER1-5 10^7	30.18	0.17	80.92	0.01
EBER1-5 10^6	34.79	1.73	80.94	0.07
EBER1-5 10^5	37.67	1.53	80.67	0.18
EBER1-5 10^4	40.68	2.85	80.73	0.22
EBER1-5 10^3	35.72**	0.05	80.83**	0.35
EBER1-5 10^2	39.82*	0	80.60*	0
EBER1-5 10	40.33	6.28	80.69	0.33
EBER1-5 1	45.91	1.31	80.72	0.13
EBER1-5 No ASO	37.02	1.58	80.84	0.13
EBER1-5 No 2Tp	-	-	-	-
EBER1-6 10^9	23.59	0.16	80.65	0.02
EBER1-6 10^8	26.56	0.69	80.63	0.06
EBER1-6 10^7	30.63	0.39	80.63	0.01
EBER1-6 10^6	33.65	0.73	80.64	0.03
EBER1-6 10^5	34.08	3.28	80.82	1.11
EBER1-6 10^4	36.59	5.52	78.98	1.68
EBER1-6 10^3	34.21**	0.21	74.95**	0.34
EBER1-6 10^2	37.27**	7.17	79.35*	0
EBER1-6 10	36.15	6.12	77.21	3.84
EBER1-6 1	38.15	6.66	82.18	4.87
EBER1-6 No ASO	42.60	3.61	77.15	3.15
EBER1-6 No 2Tp	37.08	6.72	76.94	2.84

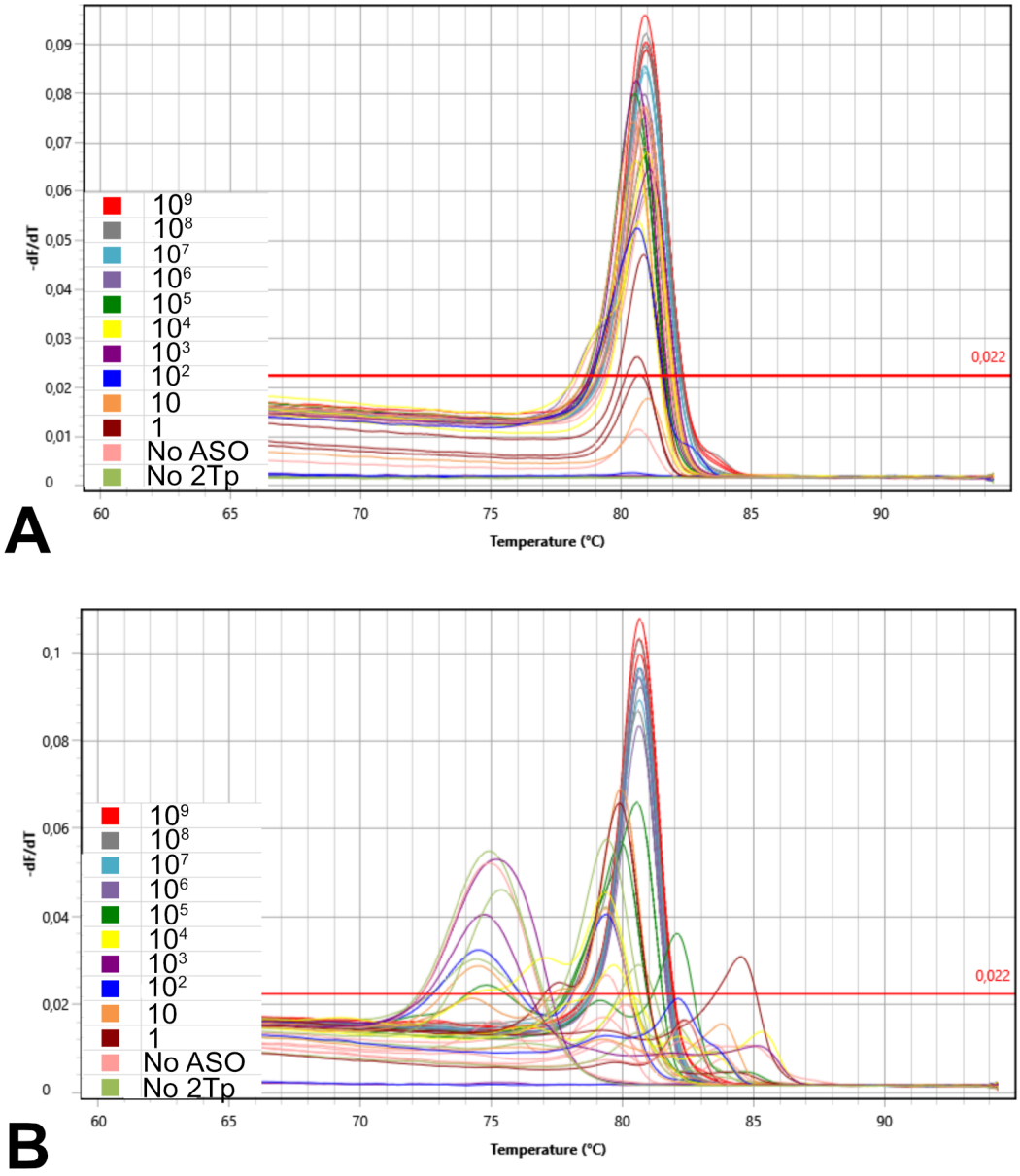

Figure 4.3: Melt curve graph for (A) EBER1-5 (DNA) and (B) EBER1-6 (DNA) for all the amounts of molecules stated in table 4.1. All samples were made in three replicates, except for the control samples that were done in 6 replicates.

Figure 4.4 portrays a plot of Cq values relative ASO molecule amounts from table 4.1. As previously mentioned, it is possible to observe that the Cq values for the samples with higher molecule amounts follow a pattern whilst the samples with lower molecule amounts have more variability. In the same figure it is possible to observe a graph representing Cq values relative $\log_{10}$(Amount of ASO molecules). From that graph and the equations a qPCR efficiency of 84% was calculated for EBER1-5 and 96% for EBER1-6. Observe that only samples containing 10^6 to 10^9 molecules were included in that graph and the efficiency calculations, as the other samples had large variability. The graph representing Cq values relative $\log_{10}$(Amount of ASO

molecules), for all molecule amounts included in the experiment, can be found in figure B.1, in appendix 2.

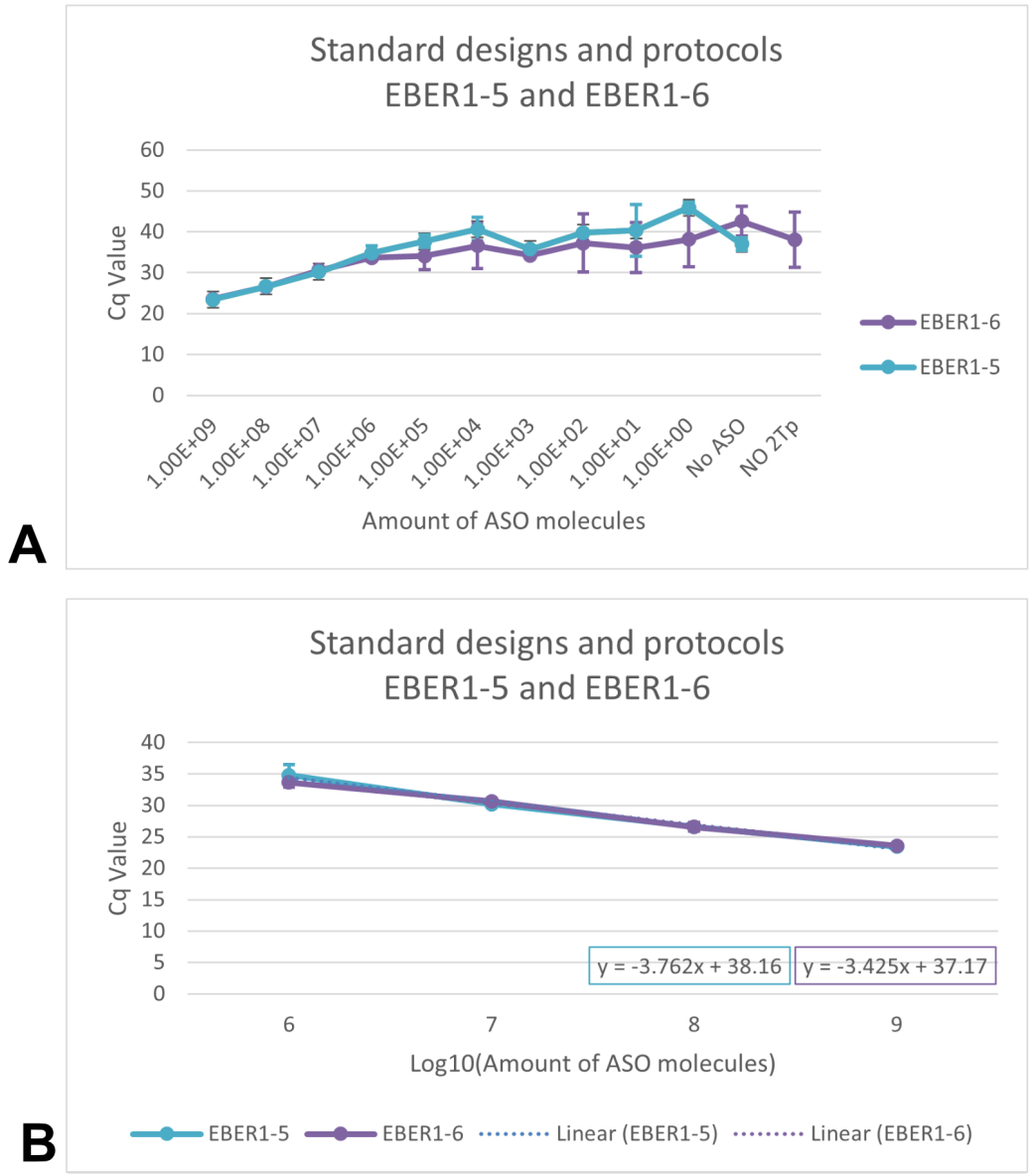

Figure 4.4: (A) Graph representing Cq values relative amount of ASO molecules for EBER1-5 (DNA) and EBER1-6 (DNA). (B) Graph representing Cq values for samples containing 10^6 to 10^9 molecules relative $\log_{10}$(Amount of ASO molecules) for EBER1-5 (DNA) and EBER1-6 (DNA). Trendlines and corresponding equations are included. Error bars represent standard deviations of the mean for 3 independent replicate experiments, this for both graphs.

4.2 Biotinylated 2Tps

The first deviation from the initial methodology was made for the designs. A biotinylated version of the 2Tp was designed, both for EBER1-5 (DNA) and EBER1-6

(DNA), see appendix 1, table A.2. A biotin molecule was covalently attached to the 5' end of the 2Tps [33] to reduce unwanted bindings that could result in unwanted products such as primer-dimers. The biotin itself does not impact the 2Tp properties [34].

Cq values and Tm values are presented with the corresponding standard deviations in tables 4.2 and 4.3. This includes results both for when initially designed 2Tps and biotinylated 2Tps were used. For EBER1-5 (DNA), the Cq values for samples with biotinylated 2Tp are slightly higher than the ones for the equivalent samples with initial 2Tp, except for the 10^5 sample. The Tm is quite uniform for all samples with low standard deviation. For EBER1-6 (DNA), the Cq values for samples with biotinylated 2Tp are slightly lower than the ones for the equivalent samples with initial 2Tp. The Tm is quite uniform for all samples with low standard deviation. All controls for both ASOs presented comparable behaviours to the initial designs and protocol.

In figure 4.5 it is visible that the difference in Cq values is small both between the two ASOs and between initial 2Tp and biotinylated 2Tp. Differences occur in the two controls. The qPCR efficiency was 84% for EBER1-5, 95% for biotinylated EBER1-5, 96% for EBER1-6, and 100% for biotinylated EBER1-6. The graph utilized for efficiency calculations, representing Cq values relative log10(Amount of ASO molecules), can be observed in figure B.2, in appendix 2.

Table 4.2: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for EBER1-5 (DNA). Biotinylated samples are marked as "Biotin". "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^9	20.14	0.36	80.74	0.02
EBER1-5 10^9 Biotin	21.12	0.40	80.67	0.09
EBER1-5 10^8	23.77	0.40	80.80	0.01
EBER1-5 10^8 Biotin	24.84	0.47	80.69	0.10
EBER1-5 10^7	27.32	0.13	80.75	0.01
EBER1-5 10^7 Biotin	27.90	0.13	80.74	0.01
EBER1-5 10^6	30.78	0.10	80.74	0.02
EBER1-5 10^6 Biotin	32.17	0.80	80.57	0.21
EBER1-5 10^5	35.52	0.33	80.69	0.06
EBER1-5 10^5 Biotin	34.75	1.93	80.71	0.06
EBER1-5 No ASO	37.18**	2.61	80.38**	0.36
EBER1-5 No ASO Biotin	42.26*	0	80.95*	0
EBER1-5 No 2Tp	-	-	-	-
EBER1-5 No 2Tp Biotin	-	-	-	-

Table 4.3: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for EBER1-6 (DNA). Biotinylated samples are marked as "Biotin". "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-6 10^9	21.01	0.17	80.74	0.04
EBER1-6 10^9 Biotin	20.22	0.14	80.73	0.06
EBER1-6 10^8	24.29	0.21	80.80	0.02
EBER1-6 10^8 Biotin	23.76	0.17	80.76	0.03
EBER1-6 10^7	27.40	0.60	80.78	0.01
EBER1-6 10^7 Biotin	27.02	0.12	80.76	0.05
EBER1-6 10^6	31.39	0.57	80.79	0.03
EBER1-6 10^6 Biotin	30.50	0.22	80.76	0.05
EBER1-6 10^5	34.60	1.32	80.74	0.06
EBER1-6 10^5 Biotin	33.42	1.25	80.71	0.02
EBER1-6 No ASO	40.98*	0	75.39*	0
EBER1-6 No ASO Biotin	43.39*	0	80.36*	0
EBER1-6 No 2Tp	39.30**	0.51	81.52**	1.10
EBER1-6 No 2Tp Biotin	35.27**	0.80	78.89**	0.83

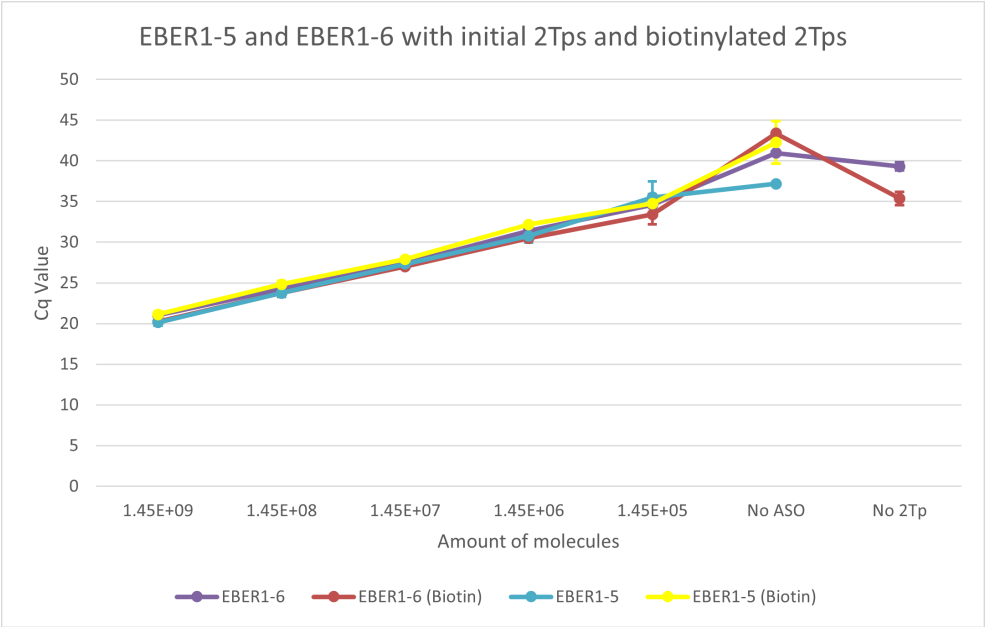

Figure 4.5: Cq values relative the amount of molecules for setup with initial 2Tps and biotinylated 2Tps for both EBER1-5 (DNA) and EBER1-6 (DNA). Error bars represent standard deviations of the mean for 3 independent replicate experiments.

4.3 Time change for thermocycler program

The second deviation was to the temperature and time protocol for the thermocycler, see table 3.1. The 40 °C step was performed for 3 min, as in the initial protocol, as well as 60 min. This was done to asses if the time impacts the two-tailed reaction part that seems to be problematic to an extent. The sample with 10^7 molecules was chosen as it had previously shown detectable results in earlier experiments. This selection ensured that any impact resulting from changes made would most likely be detectable. Cq values and Tm values obtained from the experiment are stated in table 4.4.

No major changes occurred for the 10^7 molecules sample when changing the time from 3 min to 60 min. There is a small difference in the Cq value for EBER1-5 (DNA) as the value is higher for 60 min and a bigger difference for EBER1-6 (DNA) as the value is lower for 60 min. Important to note is that the standard deviations are larger for EBER1-6. All controls for both ASOs presented comparable behaviours to the initial designs and protocol. Tm values for EBER1-5 are alike and deviations are minimal. The Tm values obtained from the controls for EBER1-6 exhibit slightly more variation, a pattern consistent with observations from previous experiments presented in table 4.3 and 4.1.

In figure 4.6 it is clear that EBER1-6 (60 min) is the sample that differed the most from the rest. It is also the only sample that had a signal in the No 2Tp control. The No ASO control had an overall slightly higher value for EBER1-5 than EBER1-6.

Table 4.4: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for EBER1-5 (DNA) and EBER1-6 (DNA). Both 3 min and 60 min samples are presented. "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq Mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^7 (3 min)	28.59	0.32	80.74	0.03
EBER1-5 10^7 (60 min)	29.23	1.10	80.72	0.07
EBER1-5 No ASO (3 min)	38.33	3.52	80.60	0.15
EBER1-5 No ASO (60 min)	36.55	0.96	80.62	0.21
EBER1-5 No 2Tp (3 min)	-	-	-	-
EBER1-5 No 2Tp (60 min)	-	-	-	-
EBER1-6 10^7 (3 min)	29.72	1.42	80.68	0.04
EBER1-6 10^7 (60 min)	25.63	1.92	80.65	0.15
EBER1-6 No ASO (3 min)	35.28*	0	79.47*	0
EBER1-6 No ASO (60 min)	34.99**	0.11	77.37**	3.11
EBER1-6 No 2Tp (3 min)	-	-	-	-
EBER1-6 No 2Tp (60 min)	33.93*	0	80.72*	0

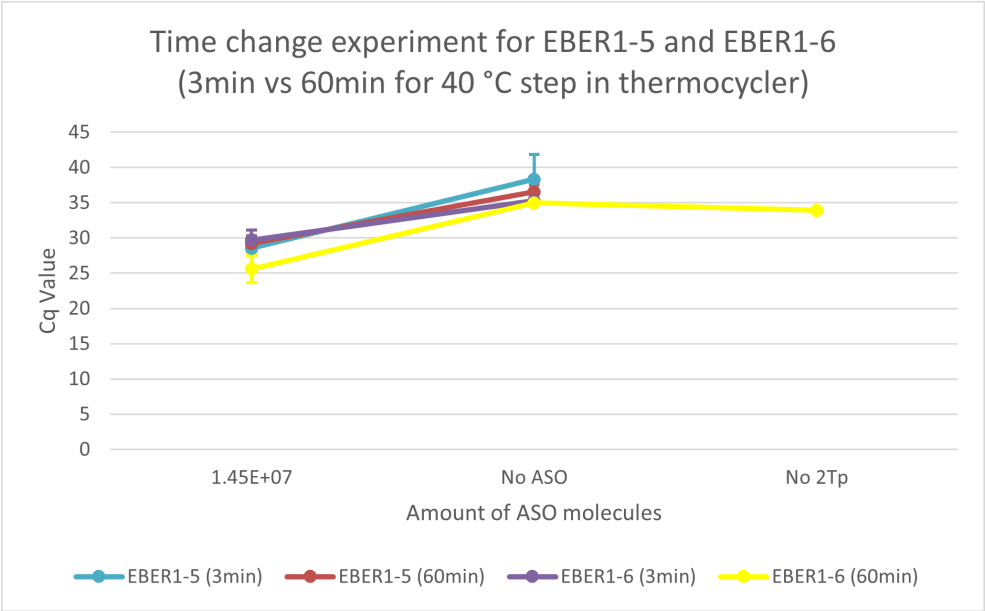

Figure 4.6: Cq values relative the amount of molecules for EBER1-5 (DNA) and EBER1-6 (DNA). This for 3 min compared to 60 min for 40 °C step in thermocycler temperature and time profile. Error bars represent standard deviations of the mean for 3 independent replicate experiments.

4.4 Cycle changes in thermocycler program

The third deviation was performed to assess whether the number of cycles for step two and step three in the thermocycler temperature and time profile, had an impact. An experiment comparing 1 cycle and the typically performed 10 cycles, was conducted.

For EBER1-5 (DNA) there is a slight difference between the different numbers of cycles for the sample with 10^7 molecules. The difference is approximately three times larger for the same samples for EBER1-6 (DNA). EBER1-5 No 2Tp control has previously not given any signal and the case was the same in this experiment for the 10 cycles. For the sample that was cycled once there was a signal in two out of three replicates. Tm values for 10^7 molecules sample for both ASOs does not differ from previous experiments and has low standard deviation.

From data presented in figure 4.7, it is possible to distinguish that EBER1-6 (1x) for the 10^7 molecules sample, exhibited a slightly more noticeable Cq value in comparison to the other 10^7 molecules sample. The difference, however, is not that significant in the broader context. The biggest difference in Cq value is between the No 2Tp controls for all samples.

Table 4.5: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for EBER1-5 (DNA) and EBER1-6 (DNA). Both one cycle and ten cycle samples are presented. "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq Mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^7 (1x)	27.58	0.23	80.74	0.01
EBER1-5 10^7 (10x)	28.87	0.38	80.72	0.01
EBER1-5 No ASO (1x)	39.28*	0	80.39*	0
EBER1-5 No ASO (10x)	36.21*	0	81.3*	0
EBER1-5 No 2Tp (1x)	26.71**	0.29	80.73**	0.25
EBER1-5 No 2Tp (10x)	-	-	-	-
EBER1-6 10^7 (1x)	31.07	0.18	80.67	0.03
EBER1-6 10^7 (10x)	28.09	0.07	80.71	0.02
EBER1-6 No ASO (1x)	40.26*	0	82.19*	0
EBER1-6 No ASO (10x)	42.03*	1.42	79.53**	3.80
EBER1-6 No 2Tp (1x)	44.76*	0	80.69*	0
EBER1-6 No 2Tp (10x)	40.22*	0	84.09*	0

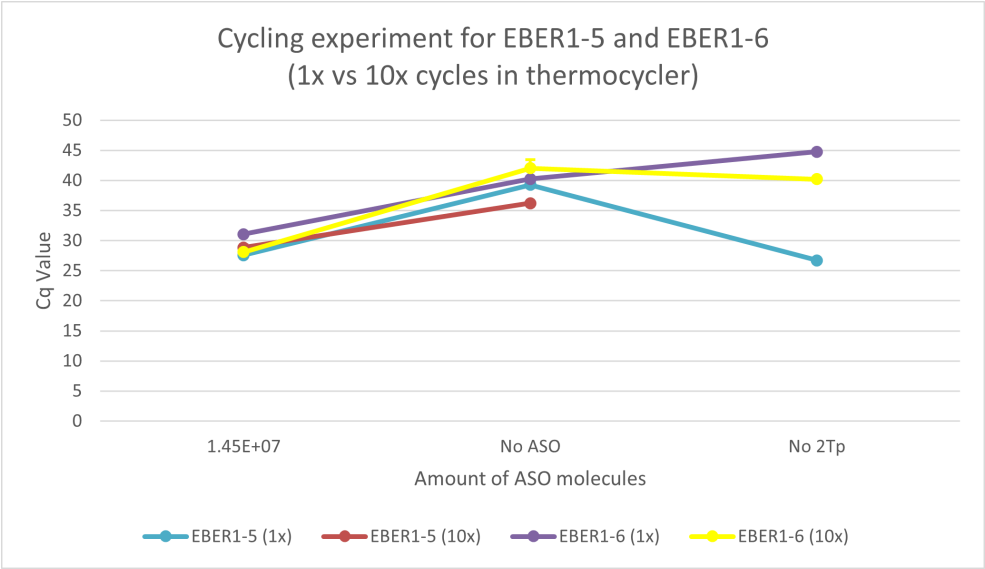

Figure 4.7: Cq values relative the amount of molecules for setup with 1 or 10 cycles for EBER1-5 (DNA) and EBER1-6 (DNA). Error bars represent standard deviations of the mean for 3 independent replicate experiments. Observe that the values for some error bars in the following graph are too low to be visualized.

4.5 Elongated ASOs

The fourth deviation was made to the designs, where the ASO sequences got elongated by the addition of sequences to each end of the sequence, matching a qPCR primer pair previously tested and used in the research group. Specifically, the MBNL1 forward primer sequence was added to the 5' end and the MBNL1 reverse primer sequence to the 3' end, see appendix 1, table A.1. The standard two-tailed PCR-qPCR protocol, as well as, the standard qPCR protocol without the two-tailed part were performed. Forward and reverse MBNL1 primers were used in the experiment involving just the qPCR and it was those that were quantified (even when attached to the ASO). Data from the two experiments could be compared and by that it was possible to observe if it was the two-tailed part that was the ineffective one.

The data from the two-tailed PCR-qPCR experiment involving MBNL1 elongated ASOs can be observed in table 4.6. Results do not follow the pattern where approximately 3.32 cycles can be observed between adjacent samples. Additionally, the Cq standard deviations are large for both ASOs. All controls for both ASOs presented comparable behaviours to the initial designs and protocol. The Tm values vary among all samples for EBER1-6, whereas there is almost no variation for EBER1-5.

Table 4.6: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for the extended EBER1-5 (DNA) and EBER1-6 (DNA). "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^5 (MBNL1)	34.82	0.93	80.87	0.01
EBER1-5 10^4 (MBNL1)	39.00	7.13	80.92	0.01
EBER1-5 10^3 (MBNL1)	37.12	3.41	80.90	0.29
EBER1-5 10^2 (MBNL1)	36.70	2.36	80.95	0.28
EBER1-5 10 (MBNL1)	36.14	2.88	80.63	0.04
EBER1-5 1 (MBNL1)	37.73	1.28	80.69	0.18
EBER1-5 No ASO (MBNL1)	37.67	1.28	80.77	0.12
EBER1-5 No 2Tp (MBNL1)	-	-	-	-
EBER1-6 10^5 (MBNL1)	37.19**	6.63	77.05**	3.60
EBER1-6 10^4 (MBNL1)	36.43*	0	81.5*	0
EBER1-6 10^3 (MBNL1)	-	-	-	-
EBER1-6 10^2 (MBNL1)	33.94	1.52	79.83	0.33
EBER1-6 10 (MBNL1)	44.85*	0	80.23*	0
EBER1-6 1 (MBNL1)	34.03**	2.50	77.31**	3.27
EBER1-6 No ASO (MBNL1)	32.38**	6.73	80.05**	0.63
EBER1-6 No 2Tp (MBNL1)	34.24**	1.61	79.59**	0.01

The results from just the qPCR are presented below in table 4.7. It is clear that the difference in Cq values between adjacent samples is approximately 3.32 for all samples, except between the sample containing 10 molecules and the one containing 1 molecule. Tm values do not differ much between samples and between the two ASO, except for sample with 1 molecule.

In figure 4.8 there is a visible pattern where it is approximately 3.32 difference between adjacent Cq values. The sample with one molecule deviated and was by that excluded from the qPCR efficiency calculations, see figure B.3 in appendix 2. Without that sample included, the efficiency was 95% for EBER1-5 (MBNL1) and 98% for EBER1-6 (MBNL1).

When comparing samples from table 4.6 to the corresponding samples from table 4.7 it is clear that the Cq values are much lower when performing the experiment excluding the two-tailed part. The samples with 1 molecule are an exception.

Table 4.7: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for quantification of the extended EBER1-5 (DNA) and EBER1-6 (DNA). "**" states that not all replicates had a signal in that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-5 10^5 (MBNL1)	19.29	0.08	78.31	0.02
EBER1-5 10^4 (MBNL1)	22.87	0.03	78.29	0.04
EBER1-5 10^3 (MBNL1)	26.35	0.04	78.26	0.02
EBER1-5 10^2 (MBNL1)	29.52	0.29	78.26	0.03
EBER1-5 10 (MBNL1)	33.22	1.11	78.22	0.04
EBER1-5 1 (MBNL1)	42.10**	0.54	79.87**	0.04
EBER1-6 10^5 (MBNL1)	19.18	0.08	78.43	0.02
EBER1-6 10^4 (MBNL1)	22.62	0.04	78.41	0.01
EBER1-6 10^3 (MBNL1)	26.15	0.06	78.38	0.02
EBER1-6 10^2 (MBNL1)	29.26	0.22	78.39	0.02
EBER1-6 10 (MBNL1)	32.77	1.31	78.35	0.01
EBER1-6 1 (MBNL1)	37.47**	4.55	77.11**	1.73

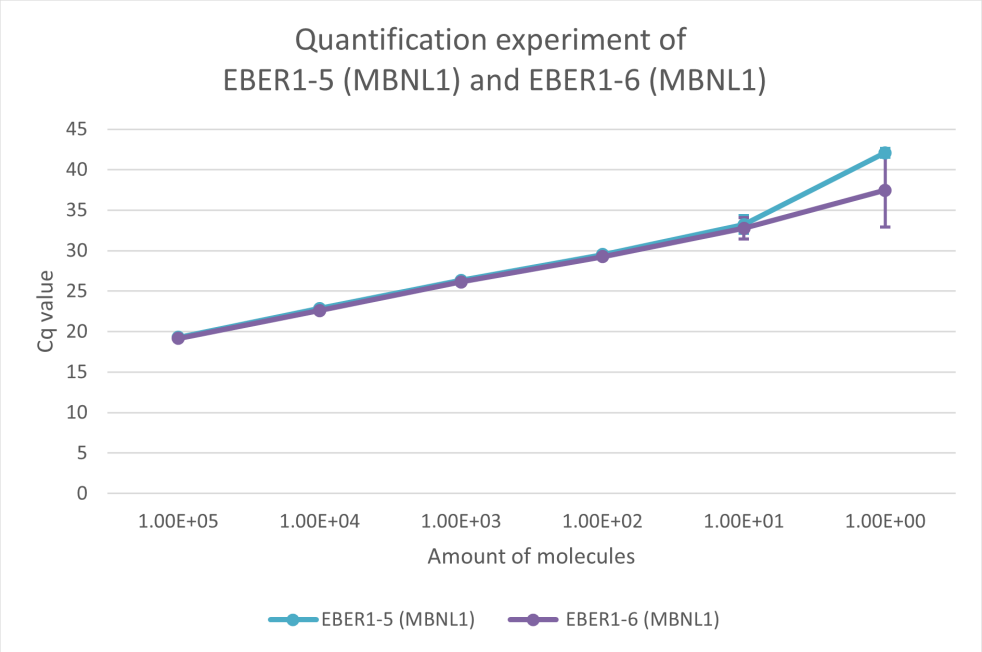

Figure 4.8: Cq values relative the amount of molecules for the extended EBER1-5 (DNA) and EBER1-6 (DNA). Error bars represent standard deviations of the mean for 3 independent replicate experiments.

4.6 Temperature and cycle changes in Mic qPCR cycler for the extended ASOs

The reverse qPCR primers for both ASOs have an overhang consisting of three bases. As it was found that this overhang impacts the melt temperature to an extent and that the temperatures in the qPCR program might have been incorrectly chosen, a fifth and last deviation was made. A changed protocol was used for the qPCR part of the two-tailed PCR-qPCR. The profile in table 3.2 was changed so that the first 5 cycles of the 50 total cycles, were performed with a temperature of 50 °C for the third step. The rest 45 cycles were performed according to the profile without changes.

Results from the experiment are summarized in table 4.8. No major differences are noted from results presented in table 4.6. Samples with bigger differences in Cq value have larger standard deviation or just one replicate that emits a signal, which makes it hard to draw reliable conclusions. As the results for EBER1-6 did not give any improvements in the results, no experiment was performed for EBER1-5. Figure 4.9 shows the inconsistent results described in table 4.8. No pattern can be distinguished and there is no signal at all for sample with 10^3 molecules.

Table 4.8: Cq mean values, Cq standard deviations, Tm mean values, and Tm standard deviations for the extended EBER1-5 (DNA) and EBER1-6 (DNA). "-" states that there was no signal at all detected, "**" states that not all replicates had a signal in that sample and "*" states that it was only one replicate that had a signal for that sample.

Sample	Cq mean	Cq standard deviation	Tm mean [°C]	Tm standard deviation
EBER1-6 10^6	26.99	1.50	80.63	0.09
EBER1-6 10^6 (MBNL1)	27.81	0.65	80.65	0.02
EBER1-6 10^5	31.63*	0	79.99*	0
EBER1-6 10^5 (MBNL1)	31.44	2.54	79.89	1.37
EBER1-6 10^4	33.15**	0.17	80.67**	0.93
EBER1-6 10^4 (MBNL1)	30.99**	1.69	78.71**	2.74
EBER1-6 10^3	-	-	-	-
EBER1-6 10^3 (MBNL1)	27.17*	0	79.44*	0
EBER1-6 10^2	26.63*	0	79.48*	0
EBER1-6 10^2 (MBNL1)	34.09	4.25	78.8	1.04
EBER1-6 No ASO	34.81**	2.65	79.35**	1.02
EBER1-6 No ASO (MBNL1)	-	-	-	-
EBER1-6 No 2Tp	36.90*	0	80.72*	0
EBER1-6 No 2Tp (MBNL1)	33.55*	0	82.39*	0

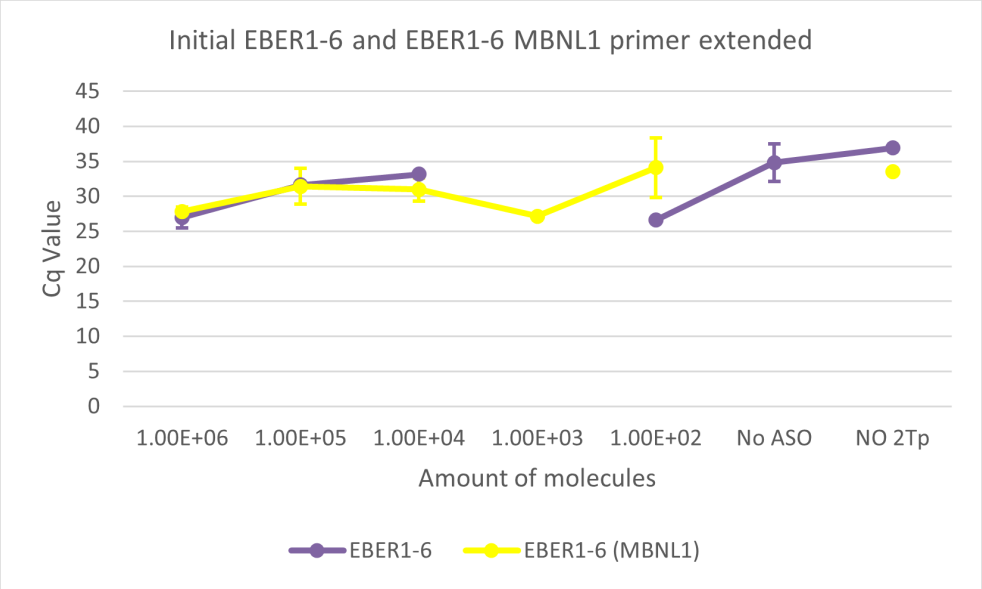

Figure 4.9: Cq values relative the amount of molecules with initial EBER1-6 (DNA) and the extended EBER1-6 (DNA). Error bars represent standard deviations of the mean for 3 independent replicate experiments.

5
Discussion

In this project the aim was to develop analytical and sensitive methods for detection and quantification of ASOs. This was achieved by utilizing and modifying the two-tailed RT-qPCR method. The experiments deducted with initial designs and initial protocol clarified that the detection of samples containing fewer than 10^5 molecules is less successful and effective for both EBER1-5 (DNA) and EBER1-6 (DNA). This is evidenced by the absence of the pattern displaying 3.32 cycles between adjacent samples as well as the fact that the Cq values exceed the 35-37 value range. Additionally, deviations between replicates within the same sample are apparent already from 10^6 molecules for EBER1-5 and 10^5 molecules for EBER1-6 and increases with decreasing number of molecules, visualized in figure 4.2. Tm values for the lower amounts of molecules for EBER1-6 have a larger variation and differ from the approximate 80 °C that the ASOs normally portray, see figure 4.3. This could mean that the binding in the two-tailed reaction is problematic or that other bindings occur and by that other products form than the ones expected. The control samples should theoretically not portray any signal, as either ASO or 2Tp is missing. Consequently, the two-tailed reaction cannot be performed as intended and target product should not be present. No signal is detected in the No 2Tp control for EBER1-5, but signal is detected for EBER1-6. This has been an occurrence in all performed experiments and is of interest to investigate. It could be that the qPCR primers form primer dimers, that the ASOs bind each other, or that the qPCR primers in some way bind the ASO itself and that is where the signal is detected from. The No ASO control portrays signal for both ASOs, meaning some entity is detected even when two-tailed reaction is not finalized. In this case it could also be qPCR primer dimer formation that is detected, 2Tps binding each other, or 2Tps bound to the qPCR primers. From the experiments it is possible to observe that the No ASO controls have Cq values that are close to the Cq values for the samples with lower amounts of molecules. This indicates once again that after reaching approximately 10^5 molecules it is mostly background noise in form of other product formed, that is detected and not the actual molecules in question. This occurrence can be seen in all experiments throughout this project. The efficiency for both ASOs resides approximately within the desired frame of 90%-110%, for the samples containing higher amounts of molecules. This is not the case if samples with fewer molecules are included suggesting that some errors must have occurred. This is logical as the samples with lower molecule amounts deviate from the earlier mentioned pattern.

Biotinylation of the 2Tps was intended to reduce other unwanted bindings that seem to be detected in the experiments. What was expected was that the Cq values

would be lower for the biotinylated 2Tps as less background would be detected. For EBER1-6 samples it is the case, but the difference is very subtle. For EBER1-5 the Cq values are instead slightly higher for the biotinylated samples. The results support the suspicion that the problem is not in the qPCR but in the PCR where the two-tailed reaction takes place. The Tm values are approximately 80 °C for both ASOs, with the exceptions for the EBER1-6 controls. The biotinylation resulted in minimal to no improvements.

The time change for the 40 °C step in the thermocycler temperature and time profile shows on some differences in the Cq values. However, the differences are small and the deviations within the samples are large. Therefore, it is concluded that the change had minimal to no impact on the results.

The cycling experiment, where step two and step three were together cycled different number of times in the thermocycler, did not reveal any substantial changes for EBER1-5. For EBER1-6 there was a slightly larger difference for samples cycled 10 times, as the Cq values were lower than for the samples that were cycled only once. It could be of interest to investigate this further with the other amounts of molecules. Although, it is important to decide if the difference is sufficient enough to be explored. It could be interesting to try cycling samples five, ten and fifteen times to see if the results vary much.

As doubts arouse if the two-tailed part of the two-tailed PCR-qPCR was efficient, extended ASOs were designed. The two-tailed PCR-qPCR and just a qPCR was performed with the new ASOs. The results show that there is a big possibility that the two-tailed part of the method is the problematic part. The Cq values for the qPCR follow the pattern of having approximately 3.32 cycles between adjacent samples, except for the one molecule sample, see figure 4.8. It is important to note that one molecule is very hard to detect so the deviating results for that sample are not very surprising. The Cq value for one molecule should approximately be in the range of 35-37, when having that in mind as well as the fact that the Cq values for adjacent samples should differ by 3.32, it can be concluded that the samples obtain Cq values within the expected ranges. This meaning the qPCR itself is efficient. When comparing the Cq values from experiment including the two-tailed part to the experiment without, it can be noted that the Cq values are overall much lower for just the qPCR, see table 4.7. The Cq values for the two-tailed PCR-qPCR present the problem of deviating and irregular Cq values, meaning that there might be issues with binding in the two-tailed part or formation of other products than the desired one. This means that it could be essential to revise the 2Tp designs, the protocol or look into other possible approaches than the two-tailed PCR-qPCR approach.

During this project it was observed that the temperature profile might have been chosen incorrectly for the qPCR part. To investigate that, an experiment with an adjusted profile, was performed. As the adjusted protocol did not give any improvements in the results, it could be concluded that it still was most probably an issue with the two-tailed part of the method.

6

Conclusions and Future Perspectives

There is still a long way to go and plenty of room for improvement as for finding sensitive methods to detect and quantify therapeutic oligonucleotides. The two-tailed PCR-qPCR method provides overall promising results as detection is possible for samples containing higher amounts of molecules. In this project the threshold for detection with that method was approximately 10^5 molecules, which is a concentration of 83 fM. It has been observed from the results that the two-tailed part of the method is most likely inefficient and that is particularly visible in samples with lower molecule amount. More specifically the problem most likely occurs with the 2Tp binding the ASO or during the elongation of the molecules. Most probably just a limited amount of ASOs that initially are added to the samples bind and elongate correctly, resulting in limited detection when few molecules are present from the beginning. It is therefore of interest to examine the designs of the 2Tps and qPCR primers, as well as the protocol, to see if there could be changes made that improve the results. It could also be of interest to analyze the qPCR products with capillary electrophoresis to distinguish what is formed in the samples and the controls, particularly the No 2Tp control for EBER1-6. This way it would be easier to distinguish the target products from other off-target products.

Further approaches could be to try a completely different method than the two-tailed one. An approach that was discussed during this project is using the so called padlock probes. Adapting these to work for ASO detection could have a potential to work.

Finally, if the methods prove to be efficient, it is crucial to apply them to other ASOs, as they are intended to be applicable to various ASO designs. Including different ASOs from those tested could yield interesting insights in the method efficiency. Additionally, it is crucial to try these methods with biological samples, as these present more complexity than the optimized buffer conditions used in this project.

7

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A

Appendix 1

In this appendix it is possible to observe sequences of the ASOs and primers used in this project.

Table A.1: Sequences for the modified (ASO), non-modified (DNA), and MBNL1 elongated ASOs used in this project. The green marked part of the sequence is the MBNL1 forward primer and the orange marked part of the sequence is the MBNL1 reverse primer.

ASO	Sequence
EBER1-5 (DNA)	CACGTCTCCTCCCTAG
EBER1-5 (ASO)	LNA-CAC, DNA-G, RNA-U (methylated), DNA-CTCCTCCC, LNA-TAG
EBER1-5_MBNL1 (DNA)	AAAATGGCTAACACTGGAAGTATGTCACGTCT CCTCCCTAGTCTTCATCCACCCCCACATTT
EBER1-6 (DNA)	CAGAGTCTGGGAAGACAACC
EBER1-6 (ASO)	LNA-CAGA, DNA-G, RNA-U (methylated), DNA-CTGGGAAGAC, LNA-AACC
EBER1-6_MBNL1 (DNA)	AAAATGGCTAACACTGGAAGTATGTCAGAGTC TGGGAAGACAACCTCTTCATCCACCCCCACATTT

Table A.2: Sequences of the two-tailed primers (2Tps) used in this project. The red marked bases are the 5' hemiprobe and the blue marked bases are the 3' hemiprobe.

2Tp	Sequence
EBER1-5 2Tp	GACGTGCCATCCTCTCCAGGTACAGTTGGTA CCTGTCTGCACTTCTAGGG
EBER1-6 2Tp	GACTCTGCCATCCTCTCCAGGTACAGTTGGTAC CTGTCTGCACTTGGTTGTC
EBER1-5 2Tp (Biotinylated)	Biotin-GACGTGCCATCCTCTCCAGGTACAGTTGGTA CCTGTCTGCACTTCTAGGG
EBER1-6 2Tp (Biotinylated)	Biotin-GACTCTGCCATCCTCTCCAGGTACAGTTGGTAC CTGTCTGCACTTGGTTGTC

Table A.3: Sequences of the forward (Fwd) and reverse (Rev) primers used in this project.

Primer	Sequence
EBER1-5 Fwd	GACGTGCCATCCTCTCC
EBER1-5 Rev	CCGCACGTCTCCTCC
EBER1-6 Fwd	GACTCTGCCATCCTCTCC
EBER1-6 Rev	CCGCAGAGTCTGGGAAG
MBNL1 Fwd	AAAATGGCTAACACTGGAAGTATGT
MBNL1 Rev	AAATGTGGGGGTGGATGAAGA

B

Appendix 2

In this appendix graphs presenting Cq values relative the $\log_{10}$(Amount of molecules), are collected.

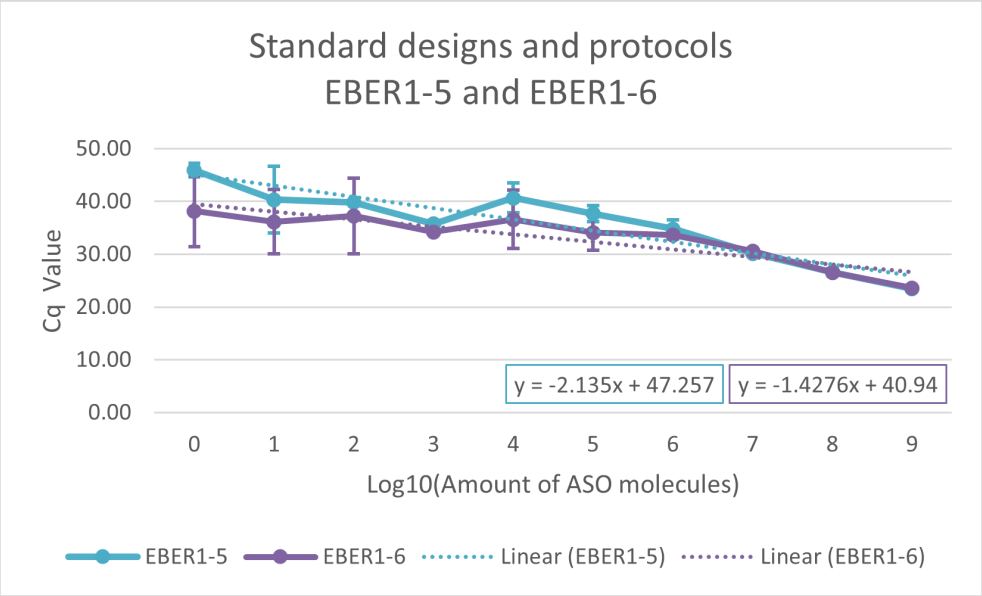

Figure B.1: Cq values relative the $\log_{10}$(Amount of molecules) for all molecule amounts, for EBER1-5 (DNA) and EBER1-6 (DNA). Error bars represent standard deviations of the mean for 3 independent replicate experiments.

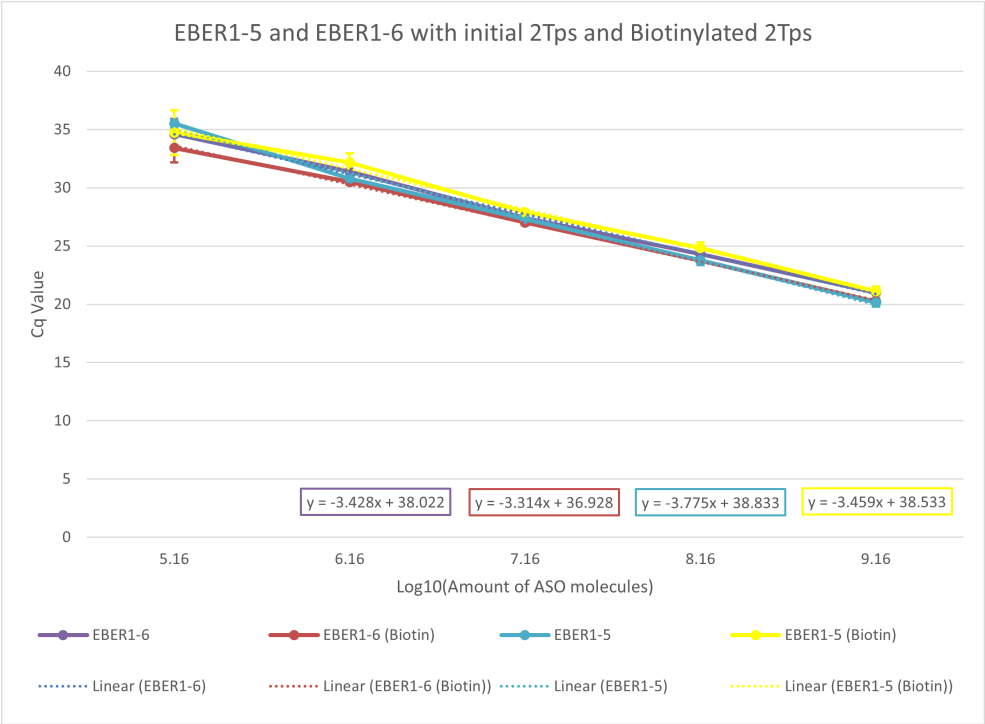

Figure B.2: Cq values relative the $\log_{10}$(Amount of molecules) for setup with initial 2Tps and biotinylated 2Tps for both ASOs. Error bars represent standard deviations of the mean for 3 independent replicate experiments.

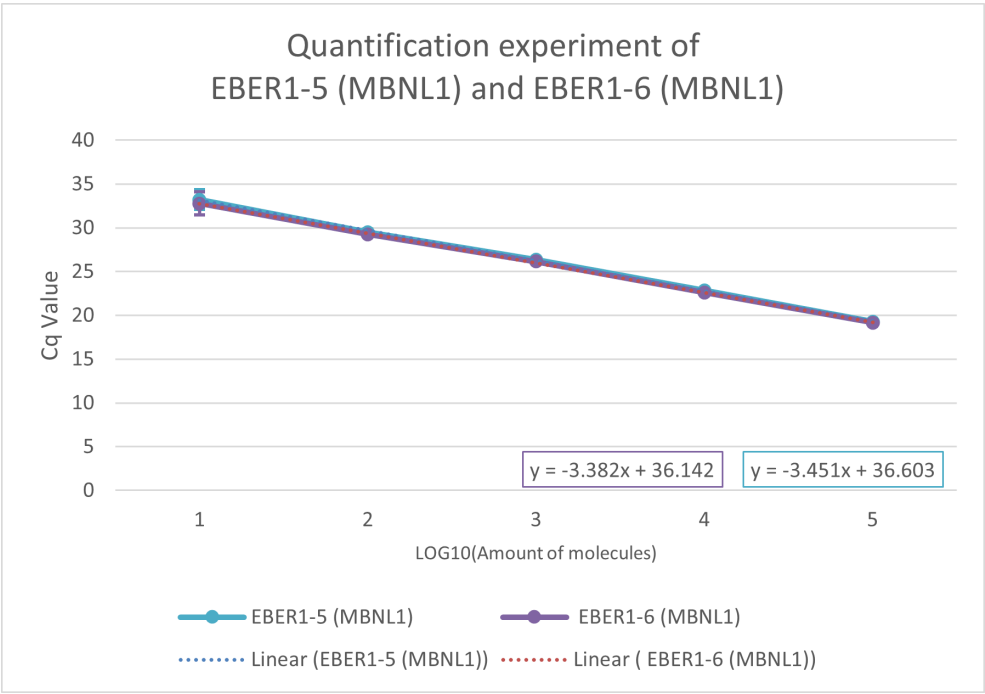

Figure B.3: Cq values relative the $\log_{10}$(Amount of molecules) for quantification of MBNL1 primer extended EBER1-5 and EBER1-6. Error bars represent standard deviations of the mean for 3 independent replicate experiments.

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