Targeted miRNA suppression using sponges

Exploring the use of sponges for diagnostic purposes

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MSc Cellular and Molecular Biotechnology

Thesis Report

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Laboratory of Microbiology

Wageningen University & Research

January 13th, 2025

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ABSTRACT

The diagnosis of Multiple Sclerosis relies on time consuming clinical assessments, preventing time sensitive treatment options. In recent years, microRNA (miRNA) testing has become an upcoming field in diagnostics. Designing a clinical tool that detects dysregulated miRNA patterns will improve clinical assessments. However, methods based on concentration analysis and multiplexing for detecting multiple targets remain challenging.

The Wageningen University & Research 2024 iGEM team miRADAR aims to tackle these challenges. Toehold circuits are used on a paper-based testing platform to detect miRNA patterns. Toeholds are leaky, making the diagnosis less reliable. This study proposes a solution by creating a concentration threshold using sponges. Sponges are single stranded RNAs containing multiple binding sites, they can also form secondary structures, improving their stability. miRNA will anneal to sponges before they can bind to the toehold switch, containing a reporter gene, creating a binary output (ON/OFF).

An assembly method was developed for to expression of target miRNAs 16 and 203 and was confirmed by sequencing. The RNA production efficiency between different DNA templates was compared; on weight base the production of RNA was higher with the use of PCR templates. However, on molar base the production was higher with the use of plasmid templates. Subsequently, different sponge types were designed to evaluate miRNA suppression efficiency. However, the Golden Gate Assembly used to create expression constructs for these sponges was unsuccessful for realizing the planned designs. With a design for 8 sponge fragments with specific overhangs, only one insertion was realized. Whereas a strategy with side fragments annealing to the plasmid and 1, 2, or 4 internal sponges, only delivered insertion to the side fragments. Producing the desired sponge designs will require further optimization of the Golden Gate Assembly and sponge-oligo designs. Further testing of the obtained assemblies was postponed. Additionally, the development of the planned miRNA detection mechanism, using a toehold switch, failed. Therefore, it was necessary to seek for alternative, (semi-) quantitative miRNA detection methods. Base-stacking PCR for detecting miRNA in a one-pot reaction was tested. This direct miRNA detection approach failed to yield any results in a first attempt and will require further optimization.

Acknowledgements

I would like to thank Tim for his great support and valuable feedback. In addition, the whole Laboratory of Microbiology, especially Henrique for support during the summer and Agata for her support with iGEM. Special thanks to Nico and Rob for their advice and iGEM supervision. All the miRADAR team members, and especially Kette and Daisy, who (sometimes regrettably) lent me their ears for one of my several “gekwakels”. I would also like to thank the University for the opportunity of joining the iGEM team, this has been an unforgettable experience. Finally. I would like to thank my laptop for surviving the several blue screens of deaths, sometimes multiple per day, and allowing me to finish this report.

Contents

1.0: INTRODUCTION

Multiple Sclerosis (MS) is a chronic neurodegenerative autoimmune disease of the Central Nervous System (CNS) (1–4). affecting near 3 million people worldwide (4). People with the most risk are mostly affecting young adults (3–6) with a higher proportion of female patients (4). MS is clinically defined into several types, starting initially with 'mild' symptoms which worsen with disease progression. The most prevalent type is relapse-remitting MS; symptoms appear with acute attacks and are followed by complete or partial recovery. Following disease progression, attacks occur more often and with less relapses (Secondary Progressive MS). In the subsequent stage, Primary Progressive MS has no relapses or remissions (1,5,6). Currently no treatment is available, however symptoms can be suppressed with therapeutics (3). MS is characterized by inflammatory demyelination of myelin sheaths. Starting with inflammation reactions involving adaptive and innate immune cells who consider the myelin as foreign, followed by crossing the blood brain barrier, causing rapid demyelination. The lesions left behind can be repaired in initial stages of MS but are often incomplete. With disease progression neuronal loss becomes inevitable (1). It is therefore of utmost importance to diagnose MS in an early stage, allowing for timely treatment and prevention of disease progression.

Diagnosis of MS is based on ruling out other diseases, with clinical research following the in 2017 revised McDonald Criteria: relying on extensive patient history and neurological examination (3). MRI (Magnetic Resonance Imaging) is used to detect lesion dissemination in space (DIS) and time (DIT) of the CNS (1,3). In 2021 consensus recommendations were made on the standardization of MRI in patients with MS (3,7). Ruling out MS-mimics, diseases that show similar symptoms, and other diseases, is time consuming and costly. While it's not required for the McDonald Criteria, cerebrospinal fluid (CSF) analysis provides evidence of MS up to 90% of patients (3), which works great for ruling out mimic diseases, but is very invasive to the patient. Current methods are not suitable for early diagnosis of MS. Therefore, novel methods to identify and quantify disease state are highly desired (1).

One such method is by detecting the presence of a disease with the use of diagnostic biomarkers (1,8). An ideal biomarker has clinical relevance, is practical to sample, the detection method has a high sensitivity and specificity, and the test must be reliable, in addition, the simplicity of equipment required for detection is important for implementation (8,9). Ultimately, a biomarker indicates the development and presence of disease before any symptoms arise (10). In recent years, circulating microRNAs (miRNA) have been identified as promising biomarker for that reason (4,11).

Circulating miRNAs are post transcriptional gene regulators. They function by (partly-) annealing to complementary regions of DNA and mRNAs, resulting in DNA methylation, mRNA silencing and affecting mRNA translation (8). These short, single-stranded, non-coding RNAs influence over 30% of the human genome. A single miRNA can regulate over a 100 genes, underlining their vital role in cellular functions (8,12). As of now, 1917 miRNA sequences of Homo sapiens, were transcribed and catalogued in the MIRBase (www.mirbase.org; accessed on 4 December 2024). miRNAs present in blood-serum, are encapsulated in exosomes or bound to proteins (13,14), which make them resistant to severe physicochemical, which can be found in laboratory settings (11,14). Common methods for detection of miRNA are RT-qPCR, NGS (Next Gen Sequencing) and microarrays. While highly accurate, they often require skilled technicians, are labour intensive and expensive. Despite these current challenges, the advantages of miRNA biomarkers far outweigh their current limitations: Sampling of body fluids is minimally invasive for the patient, can be measured throughout disease progression and the miRNA sensitivity is so high that their levels can indicate the presence of a disease even before clinical symptoms arise (8,11).

This research was part of the 2024 Wageningen University & Research iGEM team 'miRADAR – miRNA analysis in Disease Assessment using Ribocomputation". Our team took on the challenge of designing a diagnostic tool for the detection of Multiple Sclerosis, targeting multiple miRNAs, with the objective for creating an easy to use, and cost-effective testing device. miRADAR makes use of toehold switches, specifically designed to detect miRNAs (figure 1). Toeholds are mRNA structures that contain a hairpin stem-loop at the 5' UTR region, obstructing the Ribosome Binding site (RBS) (figure 1,1). In the presence of a specific miRNA the structure will unfold by strand displacement (figure 1,2 and 1,3), exposing the RBS, which allows for translation of the repressed gene (figure 1,4) (15,16).

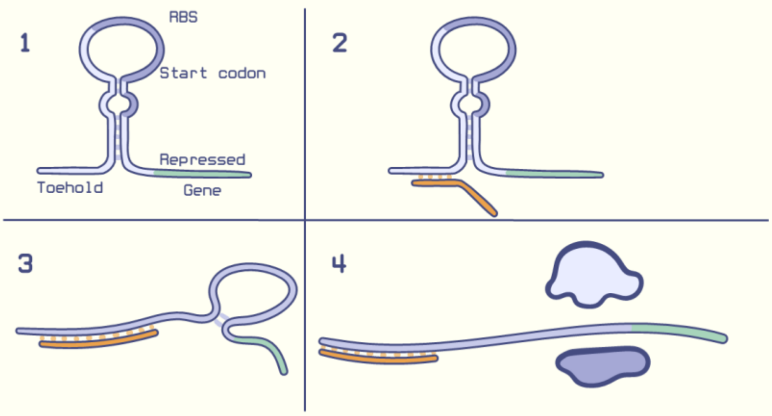

Figure 1: (1) Schematic structure of a RNA toehold switch with the following parts: Toehold, a hairpin-stem-loop containing the Ribosome Binding Site (RBS) and start codon of the downstream gene. (2) Trigger miRNA (yellow) binds toe the toehold region of the structure. (3) Trigger miRNA binds to the stem loop-binding region of the toehold switch by strand-displacement, linearizing the structure. (4) The toehold switch is fully linearized, exposing the RBS and start coding of the gene. The Ribosome can bind and translate the gene. Credits Illustration: Kette Troost

Toeholds produce an undesirable linear output in the presence of their trigger, whereas a binary output (ON/OFF) is preferred, creating a clear result for diagnostic purposes. To ensure the binary output, different strategies have been proposed. A promising strategy is the implementation of sponges: to catch the target miRNA up to a predetermined level, any leftover miRNA above the threshold would then give a positive signal with the toehold reaction (figure 3b).

Synthetic RNA constructs known as ‘sponges’, ‘miRNA inhibitors’ or “Decoys” contain one or multiple miRNA specific sequences, and function by supressing binding of mature miRNA onto their natural target (12,17). The concept of “natural target” and “pseudotarget” comes from competing endogenous RNA (ceRNA), which compete for the same pool of miRNA, influencing the expression of natural mRNAs (12). To understand the mechanisms behind artificial sponges we can investigate natural sponges, found in nature. The first ceRNA was discovered in plants: PHO2 (mRNA) avoids miRNA-guided cleavage by the transcription of ISP1 (ncRNA), which functions as decoy. The pseudotarget has an almost perfect complementary region to the miRNA, except for a 2-nt mismatch, which is crucial for avoiding miRNA-guided cleavage after annealing. This mismatched target sequence is conserved over different plant species (12,18,19). In humans, the first ceRNA discovered was a pseudogene. With advancements in sequencing techniques and bioinformatics, we rapidly learn more about the role of ceRNAs in humans. An abundant, new group of ceRNA was recently discovered, known as circular RNAs (circRNAs) (20,21). With an average size of 500 ribonucleotides (and with outliers of 1.5 kb) (12,20), circRNAs are more efficient at competing for miRNA than pseudogenes, In addition, circRNAs evades degradation by cellular mechanisms, due to their intramolecular structure (12,22). Several studies have shown the involvement of ceRNAs in tumorigenesis (12), and the loss of pseudogenes in patients with cancer (23). Suppression of mature miRNA by natural sponges seems to be a general occurrence and is of major importance to health.

Taking inspiration from natural occurring sponges, artificial sponges could be a potential tool in medicine and molecular biology by combatting viral infections or cancers (24). Sponges have been used to investigate loss-of-function phenotypes, as genetic knock-out of miRNA (families) can be challenging (25). For applications *in vivo* and *in vitro* sponges must be resistant to cellular degradation mechanisms, before and after annealing to target miRNA, for efficient miRNA suppression. In addition, it must contain multiple miRNA binding sites for a specific target (12). It was found that for linear (bulged) sponges, 6 to 12 target sites are most optimal (26), as longer RNAs are more likely to form secondary structures, hindering the miRNA binding. Therefore, designing sponges with a defined secondary structure is of utmost importance (12).

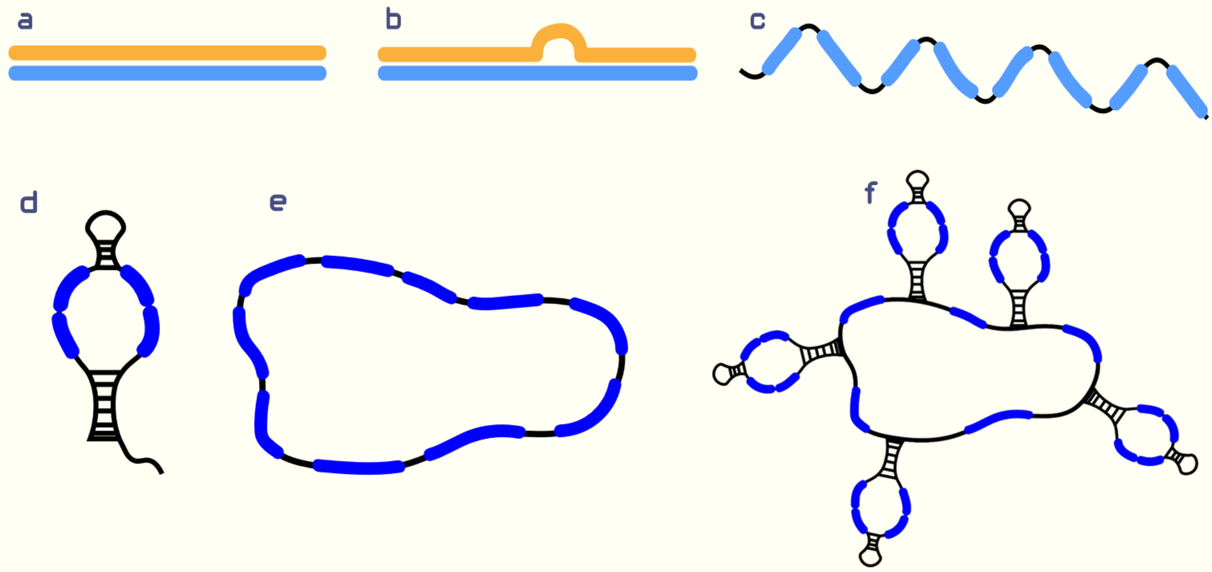

Figure 2: Schematic of sponge designs, inspired by Bak et al (17) (a): A perfect complementary sponge target site (blue) annealed to target miRNA (orange). (b): A bulged sponge (blue) is almost perfectly complementary to target miRNA (orange) except for 2 nt, creating a bulge. This improves stability against RNA degradation mechanisms of the cell and allows for targeting miRNA families. The sponge type in (a) and (b) can be used in all other sponge types, depending on its use. (c): The linear sponge, with 8 target sites (blue) and with small spacers in between each sponge fragment (black). (d): Tough-Decoy, here shown with 4 target sites. The secondary structure allows for perfect exposure of the target sites and increases the evasion of RNA degradation. (e): Circular sponge with 10 target sites (blue). (f) A hypothesized improvement of the circular sponge by including Tough-Decoys.

The linear sponges (figure 2c) have multiple miRNA target sites in tandem, they can be either perfect complementary or contain a 'bulge' in the seed region of each target site (figure 2 a and b). Most sponges have a small spacer sequence in-between target sites. The bulge allows for better stability against degradation mechanisms of the cell and to inhibit miRNAs from the same family (26–28). Tough Decoy sponges have a secondary structure that form a small hairpin between the two sides, increasing the overall stability and improving the accessibility of target sites (figure 2d). Several studies have shown that Tough Decoys are more efficient than linear sponges (29), which increases even more when placing Tough-Decoys in tandem (30). With recent improvements in making artificial circular sponges (figure 2e), it was found that they are also more efficient in suppressing miRNA than linear sponges (31). Bak et al have hypothesized that the efficiency of circular sponges could be further improved by the addition of Tough-Decoys to the template (figure 2f) (26). Designing such a template will be challenging.

Several objectives need to be achieved before we can investigate the efficiency of different RNA sponges using miRADARs test platform. First, the (bulged-) sponges and Tough-Decoys targeting miR16 and 203, as used by Bak *et al* (29), need to be synthesized. The (bulged-) sponge with 8 target sites and the Tough-Decoy with 2 target sites stood out from a side-by-side comparison of several miRNA-inhibitors *in vitro.* Next, it is essential to find the most suitable DNA template for RNA synthesis using cell-free components, for both the sponges and for the to be target miRNA. Then, finally, sponges can be applied to a paper-based disc and freeze-dried for storage (figure 3a). Once activated by sample material, the sponges will be able to target the specific miRNA and create the desired binary out-put (figure 3c), which can be measured by the colorimetric output, produced by the toehold switch, or the lack thereof (figure 3b). After experimentally testing the efficiency of sponges, the designs could be improved by changing the amount of target sites and the circular sponges in figure 2e and 2f could be considered as well.

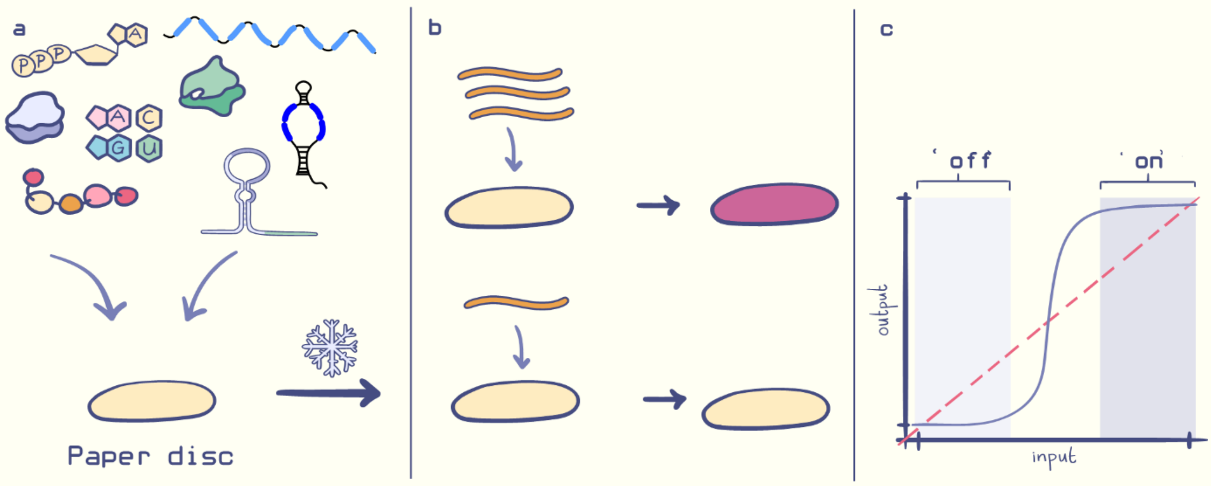

Figure 3: (a) Schematic overview of the cell-free components that are added onto the paper disc used by miRADAR, in addition to ATP, ribosomes, nucleotides, amino acids and T7 RNA polymerase, the toehold switch and the sponge types are also added to the paper disc containing CPRG substrate and RNase inhibitor. The disc is freeze dried for storage. (b) The test is reactivated with samples containing miRNA. In the top figure, more miRNA is present, and the threshold is reached, resulting in a colorimetric output generated by the activation of the toehold-switch. In the bottom figure, not enough miRNA is available to surpass the threshold and the system remains off. (c) Schematic of how the threshold-system works. The blue S-curve remains in the off-state until a certain concentration (input) is achieved, resulting in steep increase in generated output. The dashed red line shows how the toehold-switch would generate a linear output depending on the output. Credits: Kette Troost.

However, early in the project, the designing, developing, and testing of toeholds was more challenging than initially thought, and could therefore not be used within the time of this project. This makes validating sponge-efficiency difficult. Conventional methods like RT-qPCR, are not able to discriminate between free-miRNA and miRNA bound to sponge, after sponge experiments, due to the heat-cycles. To measure free-miRNA, a way to separate the sponge from the free miRNA has to be investigated. However, due to time constraints this was not feasible. To check whether it made sense to separate the components, a method for selective detection of RNA was sought. Conventional methods like, RT-PCR, northern blotting, microarray, isothermal amplification were taken into consideration. However, they would require skilled personnel and would increase costs. To keep with the spirit of the iGEM project, an affordable, simple alternative was chosen. Base-stacking PCR (BS-PCR), developed by Cui-Yuan Yu, et. al. overcomes said problems (25). This method makes use of a single enzyme, DNA polymerase, which is more cost efficient and simplifies the reaction to a one-pot reaction. BS-PCR is based on hybridization between a weak forward primer (figure 4 step 1) and a specific miRNA that functions as an extension of the forward primer, stabilizing the weak forward primer (figure 4 step 2). BS-PCR will only succeed when the miRNA of interest is present, therefore, measuring the amount of dsDNA is directly proportional to the amount of miRNA. The system contains a single-stranded DNA template (guide-DNA), a perfect complementary reverse primer (RV) and a partial complementary forward primer (FW) annealing with 5nt. The forward primer is not stable enough to support the PCR reaction on its own. When target miRNA is present the miRNA stably anneals to guide-DNA, enhancing the stability of FW in the process – called base-stacking hybridization. Allowing polymerase to extend the reaction and synthesizing double stranded DNA (figure 4 step 3). In consequent PCR reactions, DNA is amplified as normal.

Figure 4: Schematic illustration of Base-stacking PCR proposed by Yu et al (32).
step 1: Guide DNA (dark blue) and fw primer (purple) cannot initiate elongation.
step 2: miRNA (pink) anneals to complementary region of guide DNA, hybridizing with the fw primer.
step 3: The hybridization between fw primer and miRNA is strong enough to initiate elongation by polymerase, as the first base pairs are formed, the guide RNA is kicked off and dsDNA is formed.
step 4: fw and reverse primer can anneal to DNA templates and support /// PCR reactions

Most studies describing *in vivo* or *in vitro* applications, focus on transgenic efficiency and on battling the degradation mechanisms of the cell. Which is not needed for our application, as the sponges will be protected from degradation by the addition of RNase inhibitors. To the best of my knowledge, the use of sponges in a cell-free environment for diagnostic purposes is an unexplored area and could therefore bring unforeseen challenges. Brauer and Rossbach did develop a procedure for the production and purification of Artificial Circular RNA using *In vitro* transcription, but did not contribute to cell-free applications (24).

Here it is hypothesized that the linear sponge would be the most suitable for miRADARs application. The amount of target sites can be directly compared to the amount of miRNA that is considered as the threshold, assuming that each miRNA binds to a single target site. To calculate how much sponge is needed for each target, the sponge target sites must be converted to moles, this can be done by using NEBioCaclculator. Next, the total miRNA suppressed, or the total number of sponges required for suppressing RNA, can be calculated using formula 1 and 2 below.

$$Total\ miRNA\ suppressed\ (mol) = Number\ of\ sponge\ molecules\ (mol) * total\ target\ sites$$

Equation 1: The total miRNA suppressed (mol) can be calculated by multiplying the Number of sponge molecules (mol) by the total target sites a single sponge possesses.

This formula can be converted to calculate the number of sponge molecules required to suppress a certain concentration of miRNA:

$$Number\ of\ sponge\ molecules\ (mol) = \frac{Total\ miRNA\ suppressed\ (mol)}{Total\ target\ sites}$$

Equation 2: The number of sponge molecules (mol) required for suppressing a certain concentration of Total miRNA (mol) can be calculated by dividing the Total miRNA suppressed (mol) by the total amount of target sites.

A sponge with 8 targets, each 22 nt in length, has a total length of 208 nt (spacer sequences of 4 nt included). With 1 ng of sponge you have 15,00 fmol RNA, this can suppress up to 120,00 fmol miRNA. Comparing this to a Tough-Decoy, with 2 target sites (with a total length of 128) the total amount of RNA that can be suppressed is 48,74 fmol. When 500 fmol needs to be suppressed for a binary output, 62,5 fmol of linear sponge (~ 4,17 ng) and 250 fmol of Tough-Decoy (~ 10,26 ng) would be required. Therefore, a linear sponge will be more effective for miRADARs application for creating the binary output.

This report addresses the challenges with constructing plasmids containing linear sponge fragments, either of predetermined lengths or that of varying sizes and for Tough-Decoys, using Golden Gate Assembly. Additionally, the effectiveness of various DNA templates for RNA synthesis using Cell-Free components was assessed. Finally, the replacement of the toehold-experiments, was evaluated, but showed that BS-PCR was not suitable for the intended application.

2.0: MATERIALS & METHODS

2.1 Bacterial strains & Plasmids:

Chemically competent *E. coli DH10beta* strains prepared in house were used for cloning experiments, except when stated otherwise. Competent cells were stored at -80 ºC in aliquots of 100 µl.

Table 1: Overview of successful bacterial transformation during this study

Name	Plasmid
E. coli DH10 beta pDHFR_miR16.5	pDHFR_miR16
E. coli DH10 beta pDHFR_miR203.8	pDHFR_miR203

2.2 Growth media, antibiotics, and growth conditions.

LB (Lysogeny Broth) medium: Dissolve 10 g/L Tryptone, 5 g/L Yeast extract, and 10 g/L NaCL, in 1L demineralized water, for LB agar, supplement with 15 g/L bacterial agarose. All media were sterilized with the autoclave.

Ampicillin: Stock 100 mg/ml stored in -20 ºC. The working concentration: 100 µg/ml, was prepared by diluting the antibiotic in media broth. The antibiotic was prepared by dissolving in MQ, filter sterilized and aliquoted.

Selection plates and medium were made with LB agar and LB medium, supplemented with ampicillin.

Growth conditions: Selection plates were inverted and incubated at 37 ºC. Overnight cultures were placed in the shaking incubator at 37 ºC at 180 RPM in selective medium. Glycerol 20% stocks were made from overnight cultures and stored at -80 ºC. All culture plates were stored at 4 ºC.

2.3 General protocols & techniques.

Oligo's were designed using SnapGene (Version 7.2.1), synthesized by Integrated DNA Technologies (IDT) and dissolved in sterile milliQ (mQ) to a stock concentration of 100 µM and a working stock of 10 µM. PCR reactions were performed as described by New England Biolabs (NEB) using Q5® High-Fidelity DNA Polymerase. Colony PCR was carried out with OneTaq® DNA Polymerase (NEB). All PCR programs can be found in Appendix 1.1: Settings of thermocycler, PCR DNA samples were analysed on 1% or 2% agarose gels (depending on expected size) and stained with SYBR™ Safe DNA Gel Stain (ThermoFisher). Gels ran at 100V for 30 minutes using (BioRAD) and imaged. PCR products were purified using the DNA

Clean & Concentrator kit (ZymoResearch) according to manufacturer's instructions. DNA cut from gel was purified using Zymoclean Gel DNA Recovery Kit, using the protocol provided. Plasmid isolation was carried out using qiaPREP Spin Miniprep Kit (QIAGEN) and provided instructions. For all before mentioned commercial kits the elution step was modified to use sterile mQ instead. Whole plasmid sequencing was done by PlasmidSaurus. Sanger Sequence was performed by MacroGen samples were sent as instructed by the companies. Primer JO_017 was used with Sanger Sequencing.

2.4 Sponges

2.4.1: Designing the sponges.

Sponges consist mostly of tandem repeats of a target sequence, making them challenging to synthesize commercially. To overcome this, Golden Gate Assembly was used for synthesising plasmids with sponge fragments. Golden Gate fragments were designed using the NEB Golden Gate Assembly tool (version 2.10.3: bsmBI-V2; 4BP, t4 DNA Ligase, 25C, 1h; Q5 High-fidelity 2X master mix; min length 18; max length 50). The oligo's for microRNA-16 and microRNA203, Sponge-16 and Tough-Decoy-16 (based on (29), each containing a unique 4 nucleotide sticky end for Golden Gate Assembly.

2.4.2: Restriction-Digest + ligation of miRNA expression vectors

Complementary oligo's were designed with an additional 4-nucleotide overhang, as mentioned above (2.4.1). Sponge fragments were made by combining oligo's to a final concentration of 5 µM in a reaction containing 10X NEBuffer 3.1 and mQ to a final volume of 20 µl. The reaction was performed with one of three protocols using a thermocycler*. (Appendix 1.2: Settings of thermocycler for oligo annealing)*

The high copy number pDHFR_control_template (Appendix B.6.1, for plasmid map and sequence) containing an ampicillin resistance gene, and reporter gene under control of a T7 promotor gene, was used for transformations and golden gate assembly. The plasmid was linearized to omit the current reporter gene and insert BsmBI restriction sites, this was achieved by PCR (primers: f_DHFR_BsmBI & r_DHFR_BsmBI), linearized plasmid available in appendix B.6.2. The PCR product was treated with DpnI (NEB), digesting methylated DNA, and purified using DNA Clean & Concentrator kit (ZymoResearch). Linearized pTarget was verified by gel, and the concentration was measured using a nanodrop.

The first Assembly was tried with miRNA fragments miR 16 and miR 203 (appendix: B.3, for fragment sequence), which were prepared in the same way as the sponge fragments (methods 2.4.2). In preparation of the assembly, the linearized pTarget was digested in a thermoblock at 55 ºC for 1 hour, using 1 µg of linearized pTarget in 10X NEBuffer 3.1, supplemented with of

BsmBI v2 (NEB) in a total volume of 20 µl, with mQ. Digested pTarget was isolated from gel and purified. The concentration was determined with the nanodrop. The assembly was performed using 0.02 pmol of digested pTarget and miRNA fragments were added to a 3:1 ratio, in a total volume of 20 µl containing 10X T4 DNA ligase buffer (NEB), mQ and T4 ligase buffer (NEB). The assembly incubated at 16 ºC for 1 hour and remained overnight in a heat block.

2.4.3: Golden Gate Assembly of Sponge fragments

To recreate the sponges from Bak et al., 8 sponge fragments targeting miR16 were made for Golden Gate assembly. The first sponge fragment was designed to anneal to the promotor region of linearized pTarget, the last sponge fragment to the terminator sequence of linearized pTarget containing BsmBI restriction sites. The Golden Gate assembly was performed using 0,02 pmol of digested pTarget and sponge fragments were added to a 3:1 ratio (sponge: pTarget) each, in a total volume of 20 µl containing 10X T4 DNA ligase buffer (NEB), mQ and T4 ligase (NEB). The mixture was ncubated at 16 ºC for 1 hour and remained overnight in a heat block. In attempts to improve the Golden Gate assembly, less sponge fragments were used in the assembly. To achieve this, existing sponge fragments were redesigned. The sponge-end annealing to pTarget remains the same, the other sponge-end changed to anneal to the “middle” sponge fragment. The middle sponge fragment was designed to anneal to itself and the other sponge fragments. The Golden Gate Assembly method remained the same. Further optimization of Golden Gate Assembly led to the redesign of sponge fragments, by introducing BsmBI restriction sites. This allowed for a one-pot reaction. The one-pot Golden Gate Assembly was prepared with a 5x master mix containing all reaction enzymes and buffer: mQ, T4 ligase buffer (NEB), T4 Ligase, (NEB), and BsmBI V2 (NEB). The assembly was mixed with 0.02 pmol linearized pTarget, the 3 new designed sponge fragments containing BsmBI restriction sites were added in a 20:1 or 60:1 ratio (sponge : pTarget) and samples were filled to a final volume of 8 µl with mQ. A thermocycler was used to perform the Golden Gate Assembly: 42 ºC for 5 minutes, 16 ºC and 42 ºC for 2 minutes with 35 cycles, 16 ºC for 5 minutes and 80 ºC for 10 minutes. Variations on this method were performed by omitting the pTarget or Sponge fragments in the first 10 cycles.

2.5. DNA transformations.

The protocol for transforming competent cells was adapted from Green and Rogers (32) *by Tim Althuis and further refined based on personal experience. The full protocol is in Appendix Protocols C.1: Heat shock transformation of competent BH10b.*

DNA transformations were performed following Golden Gate Assembly and assembly of miRNA expression vectors. Prior to transformation, chemically competent cells were thawed on ice. 1 ng DNA of interest was carefully added per sample, subsequently, the cells were incubated on ice for 30 minutes prior to heat-shock in a pre-warmed water bath at 42 ºC for 30 seconds, which was followed by immediately placing it on ice for a duration of 2 minutes. Cells were carefully transferred to 1 mL of prewarmed LB medium and incubated for recovery for 1 hour at 180 RPM at 37 ºC. Following recovery, 100 µl of the cell suspension was spread onto selection plates. The remaining cell suspension was pelleted, resuspended in 100 µl of supernatant and plated on selective plates. Plates were incubated overnight at 37 ºC. Single colonies were screened for successful transformation using colony PCR with primers (JO_017_control_f and JO_018_control_r), designed to flank the promotor and terminator regions. Agarose gel electrophoresis (2%) was performed to verify the correct product size. Colonies with the expected size were inoculated into 6 ml selection medium and incubated overnight at 37 ºC 180 RPM in Falcon tubes. A glycerol stock was prepared from each overnight culture for storage and the plasmid DNA was isolated with the commercial kit.

2.6. RNA synthesis

Plasmids were constructed with the assembly strategy. Successful integration was confirmed by complete plasmid sequencing. Linearized DNA templates were prepared by PCR using primers (JO_017 and JO_018). Oligo-dimers containing a T7 promotor and miRNA sequence were prepared as described in 2.4.2, all sequences are available in appendix X. All RNA synthesis reactions were carried out using 100 ng of DNA template and *in duplo*. Before starting, the workstation, pipettes, tip boxes and gloves were sterilized with RNAsezap. Reagents were thawed on ice, and working solutions were prepared at room temperature. A master mix containing reaction buffer and NTPs (A, G, U, C) was prepared for all reactions. Reaction mixes were made with a total volume of 20 µl in sterile PCR tubes with RNase inhibitor (1 Unit/µl), DTT (5 mM) and T7 RNA polymerase mix, as can be seen in appendix C.4. The components were mixed by vortexing, followed by a quick pulse-spin, used to collect the solution at the bottom of the PCR tube. The reaction was performed overnight in a thermocycler at 37 ºC Following the overnight reaction, DNAse I treatment was performed, to remove all template DNA.

Finally, RNA was purified using manufacturer's instructions, with a final elution in 50 µl RNAse-free water. The RNA concentration was quantified using the nanodrop.

2.7: Base-stacking PCR

Conditions for base stacking PCR were based on conditions described by (33).The guide DNA was specifically designed to target miR16. The complete protocol can be found in appendix C.5. All reagents were prepared using RNAse-free water and kept on ice to prevent RNA degradation. In a sterile 2 ml tube the following components were combined: RNase inhibitor (1 U/µl), RNase-free water, primers (600 nM), guide DNA (5 nM), RNA-16 (0,1 nM), and Q5 2x reaction mix (1x). After mixing, the reaction mixture was aliquoted into PCR tubes, 25 µl per reaction.

The BS-PCR was performed using a thermal cycler under the following conditions. The initial denaturation step was performed at 94 ºC for 30 seconds. This was followed by a denaturation step of 94 ºC for 5 seconds. The annealing and extension steps were combined into a single 30 second step and were performed according to the conditions outlined in the table below. This process was repeated for 30 cycles. Columns 3 to 10 were duplicates while reactions 1, 2, 11, and 12 were run as a single reaction.

Table 2: Columns indicating the thermocycler settings for Base-Stacking PCR

Column	1	2	3	4	5	6	7	8	9	10	11	12
ºC	54.0	55.1	56.6	58.4	60.6	62.8	65.2	67.4	69.6	71.4	72.9	74.0
Samples	1x	1x	2x	2x	2x	2x	2x	2x	2x	2x	1x	1x

Following amplification, 6x loading dye was added to each sample and loaded into a 2% agarose gel for electrophoresis and visualization of the PCR product.

3.0 Results & Discussion

3.1: Preparation of pTarget

In preparation for Golden Gate Assembly the pDHFR_control template had to be linearized, and restriction sites had to be inserted, as described under 2.5.4. The DpnI digested PCR product was evaluated by gel electrophoresis as shown in figure 5b. The linearized pTarget has an expected size of 2071 bp. Lane 2, with digested pTarget sample, clearly shows a band corresponding in size to the 2000 bp marker band in lane 1. The pDHFR_control_template (figure 5a) is 2727 bp and is not visible on the gel, indicating that linearization and amplification by PCR and the DpnI were successful. The linearized pTarget (figure 5c) was used for Golden Gate Assemblies.

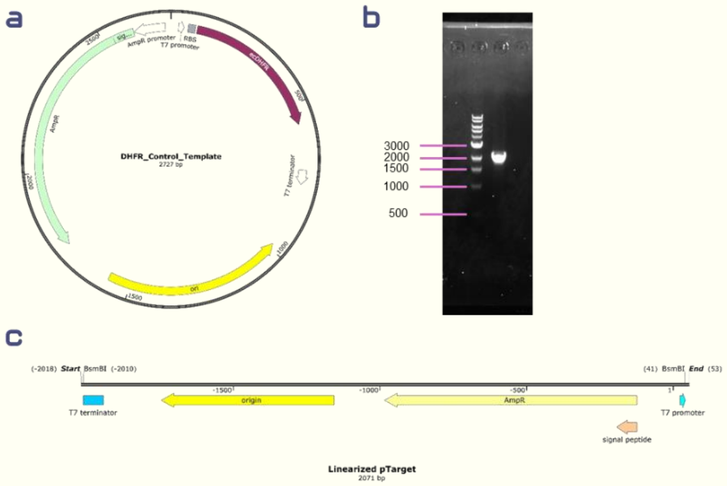

Figure 5: (a) Plasmid map of DHFR_control_template (2727 bp) is linearized with primers f_DHFR_BsmBI and r_DHFR_BsmBI, the linearized pTarget (2077 bp) with additional BsmBI restriction sites is shown at the bottom (c), note that the 480 bp ecDHFR control gene is not amplified and is removed from the template. (c) Image of 1% agarose gel with lane 1 – 1kb ladder (NO550S NEB) and lane 2 – a sample of linearized pTarget after DpnI treatment. The band in lane 2, at around the same position as the 2000 bp marker in lane 1, corresponds with the expected size of linear pTarget which is 2077 bp. The pDHFR_control_template is 2727 is not visible on the gel.

3.2: Assembly of miRNA 16 and 203 constructs.

Introducing miRNA 16 and miRNA 203 into the linearized pTarget was a test case for fragment annealing, and a test for the functionality and selectivity of the plasmid. The procedure is described under 2.4.2. Colony PCR on 4 colonies of pDHR_miR16 and 4 of pDHFR_miR203 indicated the correct insertion of one fragment and were inoculated for cloning in selective media (a representative agarose gel can be found in appendix D.1). Following plasmid isolation, samples were checked on the nanodrop. Based on the concentration and 260/280 value, one sample of each was sent to PlasmidSaurus for sequencing (139,464 ng/µl, 1,72 260/280 for pDHFR_miR16 and 115,316 ng/µl, 1,76 260/280 for pDHFR_miR203). The sequence data showed correct insertion of the fragments with no deletions, additions, duplications, or other mutations. The BsmBI restriction sites were also deleted in the cloning process as planned, which can be seen by the partial plasmid map with the AB1 file in figure 6.

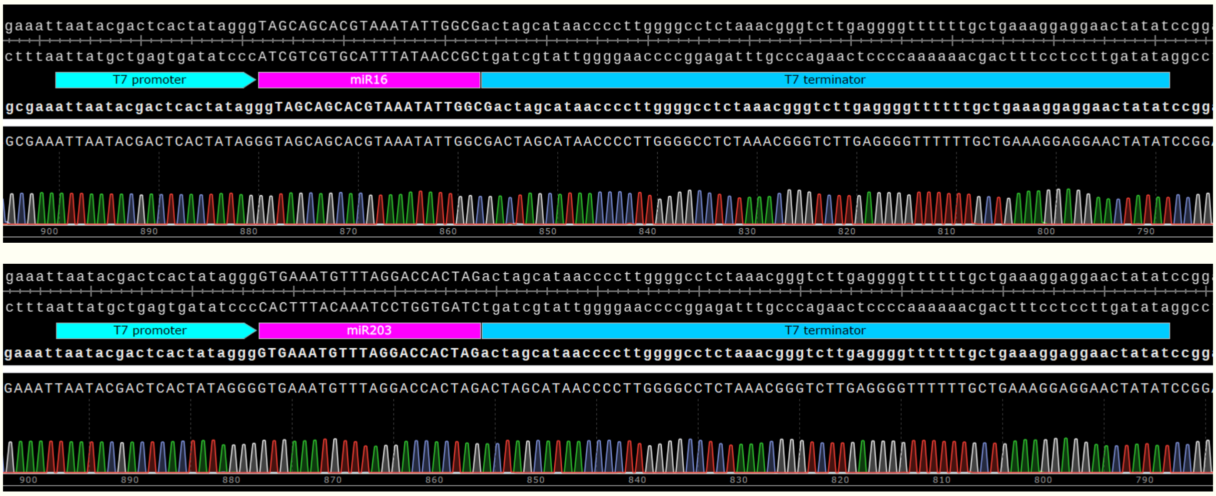

Figure 6: 135 bp segment of pDHFR_miR16 and pDHFR_miR203, showing successful integration of the miRNA 16 and the miRNA 203 fragments using linearized pDHFR_control_template. Whole plasmid sequencing by PlasmidSaurus shows correct insertion of the fragments with no deletions, additions, duplications or mutations.

Based on the results obtained, it can be concluded that the assembly of miRNA 16 and 203 plasmid constructs was successful (sequences can be found in appendix D.2), and the (BsmBI digested) linearized pTarget has proven effective for fragment insertion and selective growth of transformed *E. coli*. Constructs made here were used for RNA synthesis protocol.

3.3: Assembly of sponge constructs - 8 fragment insertion using overhangs.

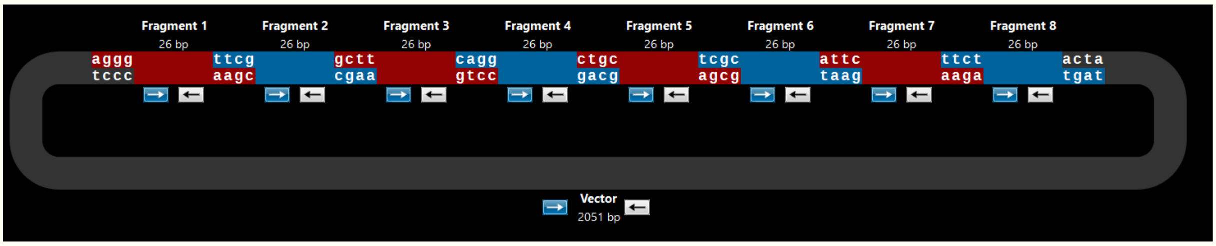

Figure 7: Planned Golden Gate Assembly using 8 sponge-fragments (blue and red) with specific overhangs using digested pTarget (grey).

In the first Golden Gate Assembly, a design was created with pre-digested pTarget, combined with 8 sponge fragments, containing 4 nucleotide overhangs. The overhangs were designed in such a way that a pre-determined sequence of the fragments would be formed upon annealing (figure 7).

Following transformation of competent cells, colonies grew on selective plates, albeit in limited numbers. Colony PCR showed only a single insert (D.5.A). In attempts of improving this Golden Gate Assembly, sponge fragments were made again, using different annealing strategies (Appendix A.2). Still, Sanger sequencing of three plasmids, isolated from individual colonies, showed that only one fragment was inserted and that no additional fragments were introduced (table 3 and further appendix D4). It is rather unexpected that the 3'-overhang of fragment 1 "AAGC" would anneal to the 5'-end of plasmid "ACTA" (both marked yellow in table 3). One explanation could be that during the making of sponge fragments, parts were mixed up, however this is unlikely, as this mistake would have been repeated up to 3 separate times. Another explanation could be that the first fragment was successfully inserted, and plasmid remained linearized. Upon transformation, selective pressure resulted in closing the plasmid via alternative end-joining (34). However, random sequences should've been inserted and visible with Sanger sequencing. Alternatively, if fragment 1 and 8 were both successfully inserted, alternative end-joining could overlap both fragments. Such an event would result in insertion of a single sponge fragment with the correct 3'- linker sequence of fragment 1 and the correct 5'-linker sequence of fragment 8. Tabel 3 compares one of the plasmids that was sequenced with the expected plasmid. The promotor and terminator region are a perfect match. The sponge part is also a perfect match, except for the overhangs, which seems to be part of both sponge fragment 1 and 8.

Table 3: Part of the planned Golden Gate Assembly using 8 sponge-fragments, compared to sequenced plasmid after cloning. Only the region in between promotor and terminator (both included), capital letters of expected sponge sequences are sponge sites, lowercase letters are plasmid regions and spacer sites. The blue marked DNA was the expected spacer of sponge fragment 1, instead the yellow marked DNA was found after aligning sequencing data to the expected sponge sequence.

Sample	Sequence 5'->
Expected sponge sequences	taatacgactcactataggg CGCCAATATTTACGTGCTGCTAttcgCGCCAATATTTACGTGCTGCTAgcttCGCCAATATTTACGTGCTGCTAcaggCGCCAATATTTACGTGCTGCTActgcCGCCAATATTTACGTGCTGCTAtcgcCGCCAATATTTACGTGCTGCTAattcCGCCAATATTTACGTGCTGCTAttctCGCCAATATTTACGTGCTGCTAactagcataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatat
Sequence pDHFR_S16.1	TAATACGACTCACTATAGGGCGCCAATATTTACGTGCTGCTAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

Based on the hypothesis that the large number of fragments was the bottleneck of this Golden Gate Assembly design, a new strategy using only three sponge-fragments (named 16A, 16B and 16C) was tested, as described in, 3.4, Assembly of sponge constructs – simplifying the reaction.

3.4: Assembly of sponges constructs - Simplifying the reaction.

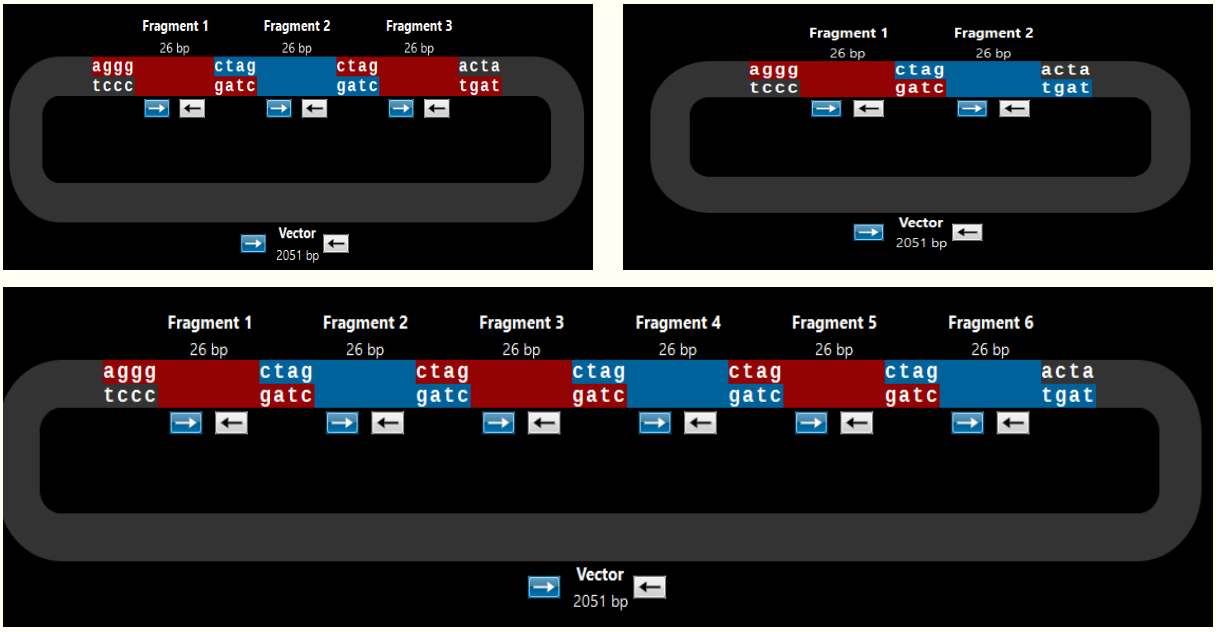

Figure 8: Golden Gate Assembly attempt 2 makes use of 3 sponge fragments. The side fragments anneal to the digested pTarget, can anneal to each other and to the middle fragment. There are three possible results. (a) The side fragments (blue) anneal to pTarget, and one additional sponge is inserted (red). (b) Only the two side fragments have annealed, closing the sponge before other sponge fragments could be inserted. (c) Several sponge fragments have been inserted, creatin a sponge with 6 target sites.

Following the initial trial, a new strategy was designed in which Sponge-16A and Sponge-16C (the side fragments in figure 8) anneal to the digested pTarget with specific overhangs. Sponge-16B anneals to both sponge fragments and itself, ensuring that at least 2 sponge fragments would be inserted in the plasmid (figure 8b), with each inserted sponge-B16 as bonus, one B16 fragment has been inserted in figure 8a and four additional B16 fragments have been inserted in figure 8c. Despite this, colony PCR repeatedly showed two inserts or less (D.5.b). One explanation could be that the plasmid closes after annealing with 16A and 16C, before sponge 16B could anneal, as shown in figure 8a. Sponge-16A and Sponge-16C overhangs are perfectly complementary, as they have the same overhangs as Sponge-16B. Upon further investigation, it was discovered that oligo's ordered from IDT were not phosphorylated, which was contrary to my first assumptions. The phosphate is required for ligation, as this provides the energy for ligation. This could also explain the previous failed attempt, with the 8-fragment insertion using overhangs.

To overcome this, sponge-fragments were redesigned to contain BsmBI restriction sites (now called 16X, 16Y, 16Z). Additionally, a short conversation with people from BacGen resulted into the insight, that with small fragments a high insert-to-plasmid ratio (20:1 or 60:1) improves assembly efficiency compared to the 3:1 ratio that was used till now. With this new and improved Golden Gate Assembly, a one-pot reaction could be performed, as described in a one pot Golden Gate Assembly.

3.5: Assembly of sponges – One pot Golden Gate Assembly.

In the new approach, the sponge sequences were aligned with BsmBI overhangs (appendix B.5). After restriction, sticky ends of both sponges and pTarget allows for annealing of sponge X and sponge Y as side sponges, and annealing to each other and with fragment Y as intermediate sponge.

The new sponge fragments were used at a 20/50/20:1 (X/Y/Z: PTarget) and 20:1 ratio. Additionally, pTarget was omitted from the first 10 cycli, allowing the sponge-fragments to anneal first, and prevent the plasmid from closing too early. However, evaluation of initial results, shows that only two fragments were inserted with this strategy. Clearly, the procedure favours annealing of sponge X and sponge Z to the plasmid and each other, leaving no room for fragment Y. Based on the initial results with this strategy, the experiment was repeated using adjusted conditions: now with 20:1, 20/60/20:1, and 60:1 ratio, and sponge-Z was omitted instead of linearized pTarget, to favour introduction of sponge Y. Transformations were carried out using DH10alpha cells (NEB), kindly provided by Tim Althuis, DH10beta was used for a transformation with linearized pTarget as control. After two days colonies started to appear on selective plates, except on the 20:1 plate, which stayed empty. The 20/60/20:1 plate had one large colony and three smaller ones; plate 60/1 had two small colonies and the control plate was completely full. Colonies were streaked on a new selection plate and used for colony PCR. The PCR yielded several unexpected results. First, the negative control (figure 9 con2) has a bright band at the same level as positive control (figure 9 con1), which suggests that either DpnI treatment was not successful or samples were contaminated with positive control. Secondly, almost all samples have 2 or more bands, which are at the same level as the positive control. Further indicating that DpnI treatment was not efficient in removing original plasmid or further proving a contamination of the colony PCR.

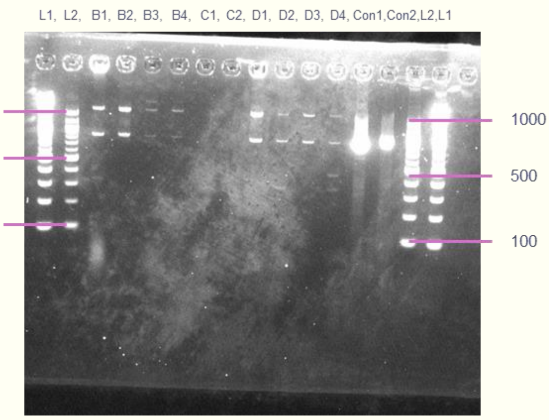

Figure 9: Image of 2% agarose gel, in a slightly crooked positioned. Lanes L1 – 1kb plus ladder (NEB N0550S), Lanes L2 – 100 bp ladder (NEB N3231S). The Lanes L1 were not used, as the bands are not clearly separated. For lanes L2, the bands indicating 100 bp, 500 bp and 1000 bp have been marked with pink. Lane B1 – B4 are colony PCR samples of Golden Gate Assembly 20:1 ratio. Lanes C1 and C2 are colony PCR samples of Golden Gate Assembly 20/60/20:1 ratio. Lanes D1 – D4 are colony PCR samples of 60:1 ratio. Lane con1 – is the positive control of the colony PCR with control_template_pDHFR. Lane con2 – is the negative control for the colony PCR, which is DpnI treated pTarget. No bands are visible for lanes C1 and C2. Lanes B1, B2, D1, D2, D3 all appear to have two bands at the same position. The highest band of the two, is around the same position as the 1000 bp marker of the 100 bp ladder in L2. The lower bands are around the 700 bp marker. Lane B3 has an additional band, corresponding to the 1200 bp marker. Lane D4 appears to have two additional bands, corresponding to 500 bp marker and the 400 bp marker. Lane Con1 appears to have 2 bright bands, and 2 small bands. The two bright bands are near the 1000 bp marker and the 700 bp marker, the 2 small bands near the 1500 marker and the last band is near the 400 bp marker. Unexpectedly, DpnI treated pTarget contains a bright band on C2, while it does not contain any PCR sites. The band corresponds to the 1000 bp marker.

This does leave some questions: If pDHFR_control_template was still present, why didn't the DH10alpha plates overgrow with colonies containing the original plasmid, alpha cells are more efficient than the beta cells; why did it take two days before colonies grew? Another interesting observation, plate (20/60/20:1) did not have any bands after PCR. However, they did grow on the new selection plates, albeit pink / light red in colour. This was observed once before in a previous transformation attempt. It was hypothesized that a colony picked up 'floating plasmid' containing a colorimetric reporter gene, like ruby, which is used by the department. Sequencing of said plasmid would give more clarity, but was not pursued due to time constraints.

3.6: Assembly of sponges – Tough Decoy Design.

Figure 10: Planned Golden Gate Assembly using 3 fragments (left) or 2 larger fragments (right)) with specific overhangs using digested pTarget (grey).

In parallel with the 8-fragment Golden Gate Assembly (3.2), several designs with pre-digested pTarget, to create Tough Decoy constructs, using 4 nt overhangs were made (figure 10). The first design used 3 small fragments, colony PCR indicated no successful insertion of all fragments. Improving the fragment constructs let to the design of 2 larger fragments. This was possible by ordering PAGE-purified oligo's, which allowed for the design of larger constructs. All the constructs used for the Tough Design can be found in appendix B.9. Sanger sequence of four plasmids (figure 11) showed a similar result found earlier in section 3.3, alternative end-joining likely overlapped two sponge-fragments into one.

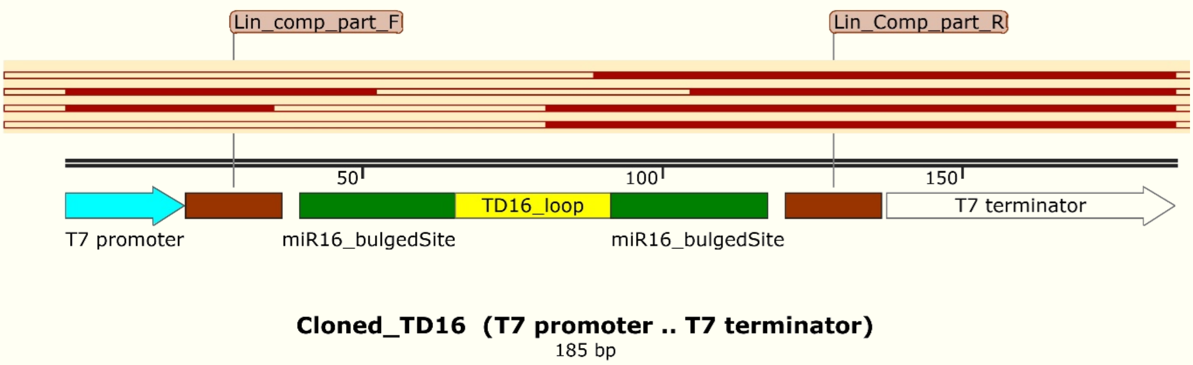

Figure 11 Expected plasmid fragment after Golden Gate assembly with Tough-Decoy fragments, aligned against 4 plasmid that were sent for sanger sequencing. The first and last sequences seem to miss the first half of the Tough-Decoy fragment (promotor region), indicated by the transparent beam. The remaining 2 sequences seem miss the middle section of the Tough Decoy.

With the challenges that were already discovered during the assembly of linear sponges, it was decided to not continue with the Tough-Decoy design, and focus more on the linear sponge design.

With the results obtained thus far, it is clear that building a sponge expression vector with a variable number of sponge fragments is not a straight forward task, and confirmation of the final products is an absolute requirement.

Golden Gate assembly was chosen for its efficient assembly of multiple fragments into a plasmid, it has been reported that up to 35 fragments have been inserted into a plasmid using this technique (35,36). Kluiver and colleagues used a method similar to the (final-) one used in this thesis, using SanDI (type-II restriction enzyme) for directional cloning. Their approach allowed them to efficiently generate sponges with over 10 sites (25). The key difference is that in Kluiver's method, the annealed sponges create a new SanDI site, which enables the insertion of additional sponges. Other methods were also considered, Gibson assembly requires 20 to 40 nucleotide overlapping sequences to efficiently work (37,38), the sponge fragments are 26 nucleotides. The repetitive nature of sponge fragments is incompatible with Gibson assembly. Using BioBricks, a standardized method for making genetic circuits using only a few restriction sites (39), is more laborious than the other methods, but with recent advancements it is a promising solution. Yamazaki and colleagues developed Quick Gene Assembly method, based on BioBricks, using magnetic beads and PCR amplification, the need for *in vivo* cloning screening process was rendered unnecessary. Improving the time needed to generate sponges substantially, and lowering the costs as well (37). A different approach altogether, could be by using sponge constructs that contain multiple different target sites, which can be ordered as a single DNA template. However, this would complicate measuring the concentration needed to create the binary output for the threshold in miRADARs system.

3.6: In vitro transcription reactions for miRNA

According to the manufacturer instructions of high scribe T7 synthesis kit (NEB), RNA synthesis works best with linear DNA fragments lacking a terminator sequence. The T7 RNA polymerase will "run off" after synthesizing RNA. To investigate whether this is true for short RNA templates (22 bp), three DNA templates (plasmid, PCR, and an oligo-dimer) were expressed *in duplo*. All constructs contain either the miR16 or miR203 miRNA sequence under the control of the T7 promotor and terminator except for the oligo-dimer, which lacked the terminator. Template sequences and the expected RNA sequence can be found in appendix D.7.1 and D.7.2. The RNA has been produced using 100 ng of template, this does not reflect biosynthesis on the molar level. For a fair comparison, the data must be normalized. The RNA can be normalized by calculating the total mole of total RNA yield, by using the NEBioCaclculator for ssRNA mass to moles convertor (version 1.15.8) and dividing this value with mole of DNA template (moles of RNA / moles of DNA). The data was evaluated using the F-test on equality of variance and statistically evaluated with the corresponding T-test.

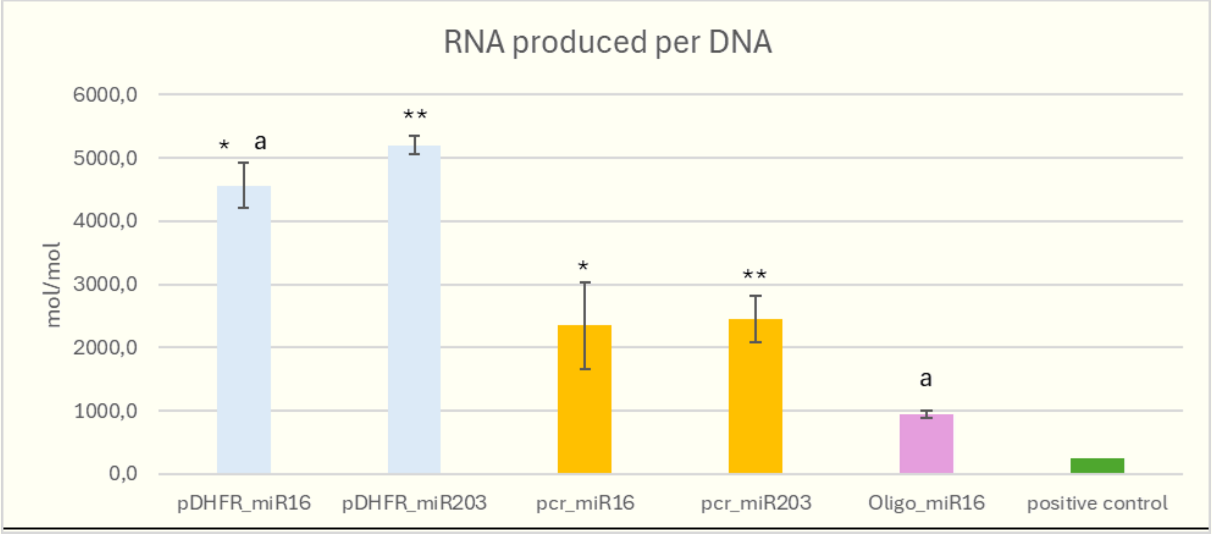

Figure 12: Average mole of RNA produced per mol of DNA template was calculated for each RNA producing DNA template: Plasmids (pDHFR_miR16 and pDHFR_miR203 – light blue), PCR fragments (pcr_miR16 and pcr_miR203 – orange), oligo-dimer (Oligo_miR16 – dark purple) and positive control (green). Significance was calculated between the same template (e.g. pcr_miR16 and pcr_miR203) and template encoding for the same RNA (e.g. pDHFR_miR16 and pcr_miR16). A significant higher value was found for both plasmid templates compared to the corresponding PCR templates, shown by "" and "**." In addition, the miR16 producing plasmid was also significantly higher than the oligo-dimer template, shown by the "a". The variation in RNA production with both RNA templates is high, this hampers the statistical evaluation.*

According to the results in Figure 12, plasmid is a significantly better template for the synthesis of miRNA than PCR and oligo-dimer, which contradicts the prediction from NEB. The control gene (1,8 kb), provided by NEB, has not been expressed as expected. According to NEB, 100 ng of control template should yield more than 40 µg of RNA (40). In our experiment, this protocol only yielded 568,48 ng (appendix D.2). The results of the positive control suggest that the reaction conditions were sub-optimal, at least for large fragments. Longer fragments require more template to achieve RNA yields comparable to that of shorter transcripts (41). In addition, the miRNA synthesis used the reaction mixture optimal for fragments smaller than 300 bp.

To find the best performing DNA template, experiments must be repeated with more replicates, and most important, templates need to be present at the same molar concentration. To investigate if the presence of terminator sequence influences the concentration and quality of RNA, templates without terminator should be included. The length and quality of RNA can be evaluated by running a denaturing polysaccharide gel (PAGE) (42,43). The PAGE is needed for small RNA constructs; loading the control (1.8 kb) onto the same gel as the small fragments (~26 bp) might not be favorable, as the small fragments will have the risk of running of the gel.

3.7: Base-stacking PCR for quantifying miRNA.

Base-Stacking PCR (BS-PCR) was performed as a replacement of the toehold switch, for detecting and quantifying miRNA. With BS-PCR the weak forward primer is combined with a specific miRNA to assist PCR based amplification. The guide-DNA from Yu et al (33), was adapted to anneal miR16. Optimal conditions as described by Yu were used for finding the most optimal temperature of the reaction. The expected size of the PCR fragment was ~100 bp. After PCR, gel electrophorese (figure 13) showed that no amplification had occurred.

Figure 13: Image of Agarose gel 2% with in lane 1 and 22 – 100 bp ladder (NEB) lane 2 to 21 are samples of the tested annealing temperatures (ºC): 54.0 (2) 55.1 (3), 56.6 (4,5), 58.4 (6,7), 60.6 (8,9), 62.8 (10,11), 65.2 (12,13), 67.4 (14,15), 69.6 (16,17), 71.4 (18,19), 72.9 (20), 74.0 (21). Samples are not visible on the gel. The 100 bp band of NEB has a mass of 48 ng and is quite difficult to see on the gel, no positive control was performed.

The 100 bp lane of the ladder is difficult to see, weak amplified samples would be very difficult to see also. The picture of the gel is quite 'dirty' making it more difficult to see weak bands. Changing the intensity of the gel picture does not improve the visibility by much (inverted colours, appendix D.6).

The original paper used non-denaturing polyacrylamide gel electrophoresis (PAGE) to visualize the DNA. PAGE makes the DNA collect in tight bands, which makes DNA more visible with lower concentrations. However, it was expected that PCR-reactions of 100 bp should be visible on agarose gels, when starting with 1 nM template. No positive control was included in the BS-PCR reaction. Introducing a strong fW promotor would allow for amplification of the guide-DNA without the need of miRNA.

RNA is prone to degradation; therefor preventive measures were taken. Before preparing all solutions, a RNAse free work environment was created using RNAZAP. The solutions were then created with RNAse free water and added to sterile PCR tubes. The PCR reaction contained 1 U/µl RNAse inhibitor. Making it unlikely that RNA degraded before the reaction had taken place. Comparing the reagents between Yu and those used in this experiment was not possible, as the components of Q5 High-Fidelity 2X Master Mix are a trade secret. However, the original paper made use of *TransStart*® Taq DNA Polymerase, while for this experiment *Q5 High-Fidelity DNA Polymerase* was used. In Protocol for Q5® High-Fidelity 2X Master Mix, point 4*: Q5 High-Fidelity "DNA Polymerase cannot incorporate dUTP and is not recommended for use with uracil-containing primers or templates."* While the RNA-primer is used for stabilizing the fW primer and for allowing the polymerase to anneal to the template (figure 4), it is likely the cause for unsuccessful BS-PCR. With the addition of a positive control, explained above, it can be evaluated if this is true or not. When Q5-polymerase is not optimal, the experiment should be repeated with the exact reagents used in the original experiment. Alternatively, the use of Taq polymerase, used in colony PCR, can also be tried.

The method behind Base-Stacking PCR looks similar with that of RACE PCR (Rapid Amplification of cDNA Ends), used for amplification of mRNA (44). Commercial kits are available for RACE-PCR, since the methods are similar, inspiration can be taken from troubleshooting guidelines. e.g. from ThermoFisher (45): The concentration of amplified product may be too low for detection with the staining dye, a Southern Blot analysis could be done instead. In addition, the guide-DNA could form secondary structures, interfering with the annealing of the polymerase. The secondary structures of the guide-DNA used in this study and that of by Yu *et. al.* (appendix x) were compared in figure 14. The secondary structures were made using the basic setting of the tool: Secondary structure tool for ssDNA, in SnapGene (8.0.1) with the following temperatures: 54 ºC, 64 ºC and 70 ºC.

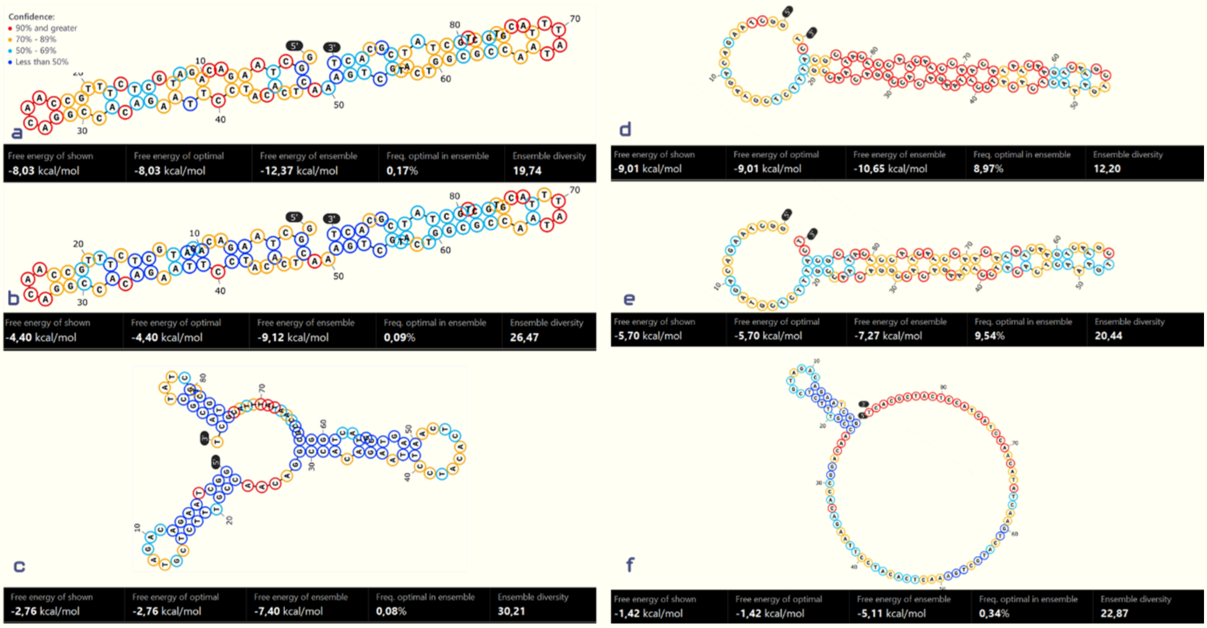

Figure 14: Predicted secondary structures of guide-DNA used in this study (on the left a-c) and the guide-DNA used by Yu et al (on the right side d-f) at three different temperatures. From top to bottom 70 ºC, 64 ºC and 54 ºC. The coloured circles indicate the confidence of the model that the templates will form said secondary structure: Red: 90% or more, orange: 70 to 89 %, light blue: 50 to 69% and dark blue: 50% or less. Below every secondary structures the following information is provided, from left to right: Free energy of shown, free energy of optimal, free energy of ensemble, Frequence optimal in ensemble, and ensemble diversity. The free energy is given in kcal/mol and indicates whether the structure is thermodynamically stable or not, the more negative this value is, the more energetically favourable the secondary structure is. Frequence optimal ensemble (foe) indicates how often the secondary will form, a low percentage means that this structure is relatively rare, a high percentage that is more common. The ensemble diversity means how many predicted structures the template can form. (a) The predicted secondary structure formed at 70 ºC has the highest confident score of the guide-DNA used in this study, the negative free-energy indicated that this structure is thermodynamically stable, although, according to the foe it is not likely to form. (b) Formed at 64 ºC the secondary structure is thermodynamically stable, but less likely to form indicated by a low foe. (c) At 54 ºC the secondary structure has the lowest confidence score, and is the least thermodynamically stable, and also the least likely to form. (d) The guide-DNA from Yu et al is thermodynamically stable at 70 ºC, with a foe of almost 9% it is likely that this structure will form. (e) At 64 ºC the DNA is less energy stable but still likely to form. (f) The secondary structure is not thermodynamically favourable to sustain the secondary structure, in addition it is not likely to form with a low foe score.

Yu concluded that for their purpose the optimal temperature for BS-PCR was 60 ºC. This suggest that the secondary structure of the guide-DNA at 60 ºC, is more dynamic and less stable than the secondary at 64 ºC (Figure 14e, the red and yellow circles will likely change to a lower level at 60 ºC). The secondary structure at 54 ºC (figure 14f) looks more favorable for annealing of primers and polymerase binding, however, the lower temperature might interfere with the PCR efficiency. The optimal temperature of 60 ºC provides a compromise between the stability of the secondary structure and BS-PCR conditions. These findings help to understand that the balance at 64 ºC (figure 14e) is too obstructive and those at 54 ºC (figure 14f) are suboptimal for PCR reactions. This knowledge can be directly applied to the guide-DNA for BS-PCR used in this study. The secondary structure at 64 ºC (figure 14b) looks promising. Indicated by the somewhat favorable free energy and the low confidence regions (the blue circles), showing more flexibility. The confidence of the secondary structure at 70 ºC (figure 14a) is similar to that of 64 ºC in figure 14e, which was too high for the BS-PCR conditions of Yu. it is likely that conditions for BS-PCR at 70 ºC will be too high for successful amplification. Similar to the secondary structure of Yu (14f), our guide-DNA also looks suboptimal for BS-PCR at 54 ºC (14c). When designing new experiments to determine the optimal reaction temperature, testing additional samples at incremental temperatures between 60 ºC and 70 ºC is recommended. This range helps to find the balance between the interference of the secondary structure and BS-PCR conditions.

4.0: Conclusion

In this thesis it was attempted to create an RNA based threshold system using sponges in cell-free systems as diagnostic toll, for the iGEM project miRADAR.

As a first contribution to miRADAR, an assembly method was created for introduction of microRNA sequence into a pre-digested pTarget.

The pTarget was used to clone the miRNAs miR16 and miR203. The miRNAs were successfully introduced, as confirmed by whole plasmid sequencing. The constructs were positively tested on for miRNA production. Here, the plasmid performed better than PCR or oligo-dimer constructs, when considering production of miRNA on a molar base. This was a contradiction compared to the suppliers' advises, which suggests that a linearized production system would perform better. Based on miRNA yields compared to the suppliers' result and control reactions, it is suggested that the reactions were still suboptimal, and miRNA yields can be further improved by optimizing reaction mixes and template concentrations.

The development of Golden Gate Assembly to anneal different types of sponges proved to be quite challenging. With different approaches, it was attempted to introduce multiple sponges. When applying an overhang strategy with unique overhang sequences to introduce eight sponges by directed design, only the first was introduced. When applying an adapted overhang strategy, using a middle sponge, an start and an end sponge, only the start and end sponges were introduced. And when attempting a strategy with restriction sites, mainly the start and end were introduced, again leaving out the middle sponges.

With the results so far, it is clear that building a sponge expression vector with a variable number of sponge fragments is not a straightforward task. The introduction of sponge fragments could be further optimized, by optimizing ratios of plasmid to sponge fragments, by adapting temperature protocols for assembly or by using other restriction sites. An alternative system for multi-fragment cloning could be the bio-brick system.

Whatever system to be used for future work, confirmation of the number of sponges introduces is an absolute requirement for further use in a threshold system for miRNA assessment.

Awaiting the development of the toehold-switch, used for detection and quantification of miRNAs, an alternative was tested, Base-Stacking PCR. So far, this approach did not yield positive results yet in a first attempt. The approach can be further optimized by testing different DNA polymerases that are more adaptable to the miRNA, that acts as a stabilizing primer, and further optimization of temperatures for the reaction steps.

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Appendix A: Thermocycler settings

A.1: Settings of thermocycler for PCR

Table 4: PCR settings for linearization of pTarget with Q5

Step	Temperature ºC	Time
Initial denaturation	98	30 seconds
35 Cycles	98 69 72	10 seconds 30 seconds 60 seconds
Final extension	72	2 minutes
Storage	12	Storage

Table 5: PCR settings for colony PCR with OneTaq

Step	Temperature ºC	Time
Initial denaturation	94	5 minutes
35 cycles	94	30 seconds
	xx	xxx seconds
	68	60 seconds
Final Extension	68	5 minutes
Storage	12	Storage

A.2: Settings of thermocycler for oligo annealing.

Three different programs to anneal sponge/miRNA oligo's together. The difference lies in ramp speed, a slower ramp could help with better annealing. Table 7 differs from table 6 with a slower ramp speed. Table 8 differs from the other programmes by staying on the melting point of oligo's for 1 minute.

Table 6: Heat-cycle program 1 to anneal oligo's of sponge fragments and miRNA.

Initial strategy	Temperature ºC	Time (min)	Ramp (ºC/s)
Step 1	95	5	-
Step 2	12	-	0.1

Table 7: Heat-cycle program 2 to anneal oligo's of sponge fragments and miRNA.

Advanced strategy 1	Temperature ºC	Time (min)	Ramp (ºC/s)
Step 1	95	2	-
Step 2	25	-	0.028
Step 3	12	store	

Table 8: Heat-cycle program 3 to anneal oligo's of sponge fragments and miRNA.

Advanced strategy 2	Temperature ºC	Time (min)	Ramp (ºC/s)
Step 1	95	1	-
Step 2	82	1	0.0162
Step 3	12	Store	0.0162

Appendix B: Sequences

B.1: All oligo's

First strategy for Golden Gate assembly, without restriction and digestion of DNA-oligo's. Sponge 16 was used as proof-of-concept for the Golden-Gate assembly protocol. JO_001_f16s1 = forward sponge 16 site 1; JO_gg_f16s2 = forward sponge 16 site 2; JO_009_r16s1 = reverse sponge 16 site 1; etc.

Capital letters show sponge-fragment, the lowercase letters show the specific overhang.

The forward and reverse sponge are annealed together with the thermocycler, protocol:

Table 9: Sponge and miRNA fragments

Fragment	Sequence 5' > 3'
f_gg_mir16	agggTAGCAGCACGTAAATATTGGCG
r_gg_mir16	tagtCGCCAATATTTACGTGCTGCTA
f_gg_miR203	agggGTGAAATGTTTAGGACCACTAG
r_gg_mirR203	tagtCTAGTGGTCCTAAACATTTCAC
JO_001_f16s1	agggCGCCAATATTTACGTGCTGCTA
JO_002_f16s2	ttcgCGCCAATATTTACGTGCTGCTA
JO_003_f16s3	gcttCGCCAATATTTACGTGCTGCTA
JO_004_f16s4	caggCGCCAATATTTACGTGCTGCTA
JO_005_f16s5	ctgcCGCCAATATTTACGTGCTGCTA
JO_006_f16s6	tcgcCGCCAATATTTACGTGCTGCTA
JO_007_f16s7	attcCGCCAATATTTACGTGCTGCTA
JO_008_f16s8	ttctCGCCAATATTTACGTGCTGCTA
JO_009_r16s1	cgaaTAGCAGCACGTAAATATTGGCG
JO_010_r16s2	aagcTAGCAGCACGTAAATATTGGCG
JO_011_r16s3	cctgTAGCAGCACGTAAATATTGGCG
JO_012_r16s4	gcagTAGCAGCACGTAAATATTGGCG

JO_013_r16s5	gcgaTAGCAGCACGTAAATATTGGCG
JO_014_r16s6	gaatTAGCAGCACGTAAATATTGGCG
JO_015_r16s7	agaaTAGCAGCACGTAAATATTGGCG
JO_016_r16s8	tagtTAGCAGCACGTAAATATTGGCG

B.2: The sponge fragments for 8-part “Golden Gate Assembly”

Sponge 1 anneals to T7p and to sponge 2. Sponge 2 anneals to sponge 1 and sponge 3, etc.

Sponge 8 anneals to T7t. closing the sponge.

Table 10: Sponge fragments when they are annealed together.

Sponge Fragment	Sequence
Sponge16_1	agggCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATaagc
Sponge16_2	ttcgCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATcgaa
Sponge16_3	gcttCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATgtcc
Sponge16_4	caggCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATgacg
Sponge16_5	ctgcCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATagcg
Sponge16_6	tcgcCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATtaag
Sponge16_7	attcCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATaaga
Sponge16_8	ttctCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATtgat

B.3: miRNA fragments for Golden Gate assembly.

Table 11: all miRNA fragments in the final form

miRNA Fragment	Sequence
miR16	agggTAGCAGCACGTAAATATTGGCG ATCGTCGTGCATTTATAACCGCtgat
miR203	agggGTGAAATGTTTAGGACCACTAG CACTTTACAAATCCTGGTGATCtgat
miR16 with T7 promotor	cgccaatatttacgtgctgctaccctatagtgagtcgtattaatt gcggttataaatgcacgacgatgggatatcactcagcataattaa

B.4: The sponge fragment for 3-part "Golden Gate Assembly

The sponge fragment 16a: anneals to 3' of T7p and can anneal to 5' 16B and 5' 16C.

The sponge fragment 16b: anneals to 3' prime of 16a and 5' of 16C and itself.

The sponge fragment 16c: anneals to 5' of T7t and can anneal to 3' of 16A and 16B.

Table 12: Sponge fragments used in the 3-part Golden Gate assembly.

Sponge Fragment	Sequence
Sponge_16A	agggCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATgatc
Sponge_16B	ctagCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATgatc
Sponge_16C	ctagCGCCAATATTTACGTGCTGCTA GCGGTTATAAATGCACGACGATtgat

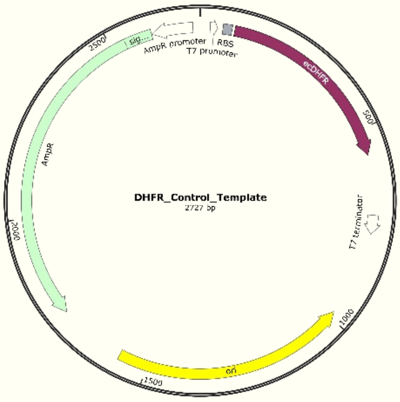
B.5: Sponges used in the one-pot reaction

The sponge fragments containing a BsmBI restriction sites. The lowercase letters will be the phosphorylated overhang. For sponge a this will be "aggg" and "agcg", this will ensure that sponge_a anneals to the T7p of pTarget and can anneal to sponge_b and sponge_c.

Table 13: Sponge fragments with BsmBI restriction sites, made for golden gate assembly.

Sponge Fragment	Sequence
BsmBI_S16A	ACGTCTCAagggCGCCAATATTTACGTGCTGCTAtcgcTGAGACGT TGCAGAGTtcccGCGGTTATAAATGCACGACGATagcgACTCTGCA
BsmBI_S16B	ACGTCTCAtcgcCGCCAATATTTACGTGCTGCTAtcgcTGAGACGT TGCAGAGTagcgGCGGTTATAAATGCACGACGATagcgACTCTGCA
BsmBI_S16C	ACGTCTCAtcgcCGCCAATATTTACGTGCTGCTAactaTGAGACGT TGCAGAGTagcgGCGGTTATAAATGCACGACGATtgatACTCTGCA

B.6.1: Plasmid map and sequence of DHFR_control_template



Sequence of DHFR_control_Template: 5'-prime

GCTAGTGGTGCTAGCCCCGCGAAATTAATACGACTCACTATAGGGTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATACATATGATCAGTCTGATTGCGGCGTTAGC
GGTAGATCGCGTTATCGGCATGGAAAACGCCATGCCGTGGAACCTGCCTGCCGATCTCGCCTGGTTTAAACGCAACACCTTAAATAAACCCGTGATTATGGGCCGCCATA
CCTGGGAATCAATCGGTCGTCCGTTGCCAGGACGCAAAAATATTATCCTCAGCAGTCAACCGGGTACGGACGATCGCGTAACGTGGGTGAAGTCGGTGGATGAAGCCA
TCGCGGCGTGTGGTGACGTACCAGAAATCATGGTGATTGGCGGCGGTCGCGTTTATGAACAGTTCTTGCCAAAAGCGCAAAAACTGTATCTGACGCATATCGACGCAGA
AGTGGAAGGCGACACCCATTTCCCGGATTACGAGCCGGATGACTGGGAATCGGTATTCAGCGAATTCCACGATGCTGATGCGCAGAACTCTCACAGCTATTGCTTTGAG
ATTCTGGAGCGGCGGTAATGAGGATCCCGGGAATTCTCGAGTAAGGTTAACCTGCAGGAGGCCTTTAATTAAGGTGGTGCGGCCGCGCTAGCGGTCCCGGGGGATCGA
TCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTT
GCTGAAAGGAGGAACTATATCCGGAAGCTTGGCACTGGCCGACCGGGGTCGAGCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACT
CAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGG
CGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTG
GAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTA
TCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCC
GCTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGG
CTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGT
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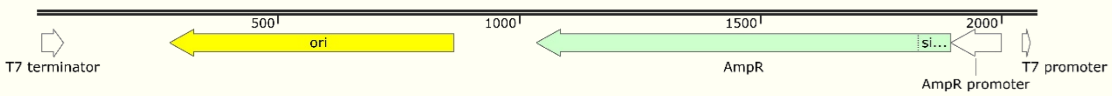
CCGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTT
ACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCAT
CTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCA
ACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTG
TCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCC
GATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGA
GTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCA
TCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTT
TCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATT
GAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGT

B.6.2: Linearized pTarget and sequence



Linearized_pDHFR_BsmBI overhangs
2071 bp

Sequence of linearized pTarget containting BsmBI restriction sites: 5'-prime

acgtctcaactagcataaccccttgggGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAAGCTTGGCACTGGCCGACCGGGGTCGAGCACTGAC
TCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCA
AAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAG
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GCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGCTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG
GTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAA
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ATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAG
TTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACA
TGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAA
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CAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT
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GGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAA
CAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCTAGTGGTGCTAGCCCCGCGAAATtaatacgactcactatagggtgagacgt

B.7.1: DNA Templates used in miRNA synthesis.

Template	Sequence 5'>
pDHFR_miR16	Full sequence – see D.1
pDHFR_miR203	Full sequence – see D.1
PCR_miR16	caaaaaacccctcaagacccgtttagaggccccaaggggttatgctagtcgccaatatttacgtgctgctaccctatagtgagtcgtattaatttcgcggggctagcaccactagcacttttcgggg gaaatgtgcg cggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttc cgtgtcgcccttattcccttttt tgcggcattttgcc
PCR_miR203	caaaaaacccctcaagacccgtttagaggccccaaggggttatgctagtctagtggtcctaaacatttcacccctatagtgagtcgtattaatttcgcggggctagcaccactagcacttttcggg ggaaatgtgcg cggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacattt ccgtgtcgcccttattcccttttt tgcggcattttgcc
Oligo_dimer16	aattaatacgactcactatagggtagcagcacgtaaatattggcg
Pos_control	CCTTTAATTAAGGTGGTGCGGCCGCGCTAGCGGTCCCGGGGGATCGATCCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTG CCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCGGAAGCT TGGCACTGGCCGACCGGGGTCGAGCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAAT ACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTG CTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAG ATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGG GAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCC GTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGCTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGG TAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGG TATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTT TGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA

	CTCACAGATCCGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTAT ATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACT CCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAG ATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCC GGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTA TGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGA TCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTT TCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACC GCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTT CGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCG CAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCTAGTGGTGCTAGCCCCGCGAAAT TAATACGACTCACTATAGGGTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATAACCATGAAAATCGAAGAAGGTAAAGGTCACCATCACC ATCACCACGGATCCATGGAAGACGCCAAAAACATAAAGAAAGGCCCGGCGCCATTCTATCCTCTAGAGGATGGAACCGCTGGAGAGCAACTG CATAAGGCTATGAAGAGATACGCCCTGGTTCCTGGAACAATTGCTTTTACAGATGCACATATCGAGGTGAACATCACGTACGCGGAATACTTCG AAATGTCCGTTCGGTTGGCAGAAGCTATGAAACGATATGGGCTGAATACAAATCACAGAATCGTCGTATGCAGTGAAAACTCTCTTCAATTCTTT ATGCCGGTGTTGGGCGCGTTATTTATCGGAGTTGCAGTTGCGCCCGCGAACGACATTTATAATGAACGTGAATTGCTCAACAGTATGAACATTT CGCAGCCTACCGTAGTGTTTGTTTCCAAAAAGGGGTTGCAAAAAATTTTGAACGTGCAAAAAAAATTACCAATAATCCAGAAAATTATTATCATGG ATTCTAAAACGGATTACCAGGGATTTCAGTCGATGTACACGTTCGTCACATCTCATCTACCTCCCGGTTTTAATGAATACGATTTTGTACCAGAGT CCTTTGATCGTGACAAAACAATTGCACTGATAATGAATTCCTCTGGATCTACTGGGTTACCTAAGGGTGTGGCCCTTCCGCATAGAACTGCCTG CGTCAGATTCTCGCATGCCAGAGATCCTATTTTTGGCAATCAAATCATTCCGGATACTGCGATTTTAAGTGTTGTTCCATTCCATCACGGTTTTG GAATGTTTACTACACTCGGATATTTGATATGTGGATTTCGAGTCGTCTTAATGTATAGATTTGAAGAAGAGCTGTTTTTACGATCCCTTCAGGATTA CAAAATTCAAAGTGCGTTGCTAGTACCAACCCTATTTTCATTCTTCGCCAAAAGCACTCTGATTGACAAATACGATTTATCTAATTTACACGAAATT GCTTCTGGGGGCGCACCTCTTTCGAAAGAAGTCGGGGAAGCGGTTGCAAAACGCTTCCATCTTCCAGGGATACGACAAGGATATGGGCTCAC TGAGACTACATCAGCTATTCTGATTACACCCGAGGGGGATGATAAACCGGGCGCGGTCGGTAAAGTTGTTCCATTTTTTGAAGCGAAGGTTGT GGATCTGGATACCGGGAAAACGCTGGGCGTTAATCAGAGAGGCGAATTATGTGTCAGAGGACCTATGATTATGTCCGGTTATGTAAACAATCCG GAAGCGACCAACGCCTTGATTGACAAGGATGGATGGCTACATTCTGGAGACATAGCTTACTGGGACGAAGACGAACACTTCTTCATAGTTGAC CGCTTGAAGTCTTTAATTAAATACAAAGGATATCAGGTGGCCCCCGCTGAATTGGAATCGATATTGTTACAACACCCCAACATCTTCGACGCGG GCGTGGCAGGTCTTCCCGACGATGACGCCGGTGAACTTCCCGCCGCCGTTGTTGTTTTGGAGCACGGAAAGACGATGACGGAAAAAGAGAT CGTGGATTACGTCGCCAGTCAAGTAACAACCGCGAAAAAGTTGCGCGGAGGAGTTGTGTTTGTGGACGAAGTACCGAAAGGTCTTACCGGAA AACTCGACGCAAGAAAAATCAGAGAGATCCTCATAAAGGCCAAGAAGGGCGGAAAGTCCAAACTCGAGTAAGGTTAACCTGCAGGAGG

B.7.2: Expected miRNA constructs.

Template	Sequence 5'>
pDHFR_miR16	guagcagcacguaaauauuggcgacuagcauaaccccuuggggccucuaaacgggucuugagggguuuuuug
pDHFR_miR203	ggugaaauguuuaggaccacuagacuagcauaaccccuuggggccucuaaacgggucuugagggguuuuuug
PCR_miR16	guagcagcacguaaauauuggcgacuagcauaaccccuuggggccucuaaacgggucuugagggguuuuuug
PCR_miR203	ggugaaauguuuaggaccacuagacuagcauaaccccuuggggccucuaaacgggucuugagggguuuuuug
Oligo_dimer16	guagcagcacguaaauauuggcg
Pos_control	UCUAGAAAUAAUUUUGUUUAACUUUAAGAAGGAGAUAUAACCAUGAAAAUCGAAGAAGGUAAAGGUCACCAUCACCAUCACCACGGAUCC AUGGAAGACGCCAAAAACAUAAAGAAAGGCCCGGCGCCAUUCUAUCCUCUAGAGGAUGGAACCGCUGGAGAGCAACUGCAUAAGGCUAU GAAGAGAUACGCCCUGGUUCCUGGAACAAUUGCUUUUACAGAUGCACAUAUCGAGGUGAACAUCACGUACGCGGAAUACUUCGAAAUGU CCGUUCGGUUGGCAGAAGCUAUGAAACGAUAUGGGCUGAAUACAAAUCACAGAAUCGUCGUAUGCAGUGAAAACUCUCUUCAAUUCUUU AUGCCGGUGUUGGGCGCGUUAUUUAUCGGAGUUGCAGUUGCGCCCGCGAACGACAUUUAUAAUGAACGUGAAUUGCUCAACAGUAUGA ACAUUUCGCAGCCUACCGUAGUGUUUGUUUCCAAAAAGGGGUUGCAAAAAAUUUUGAACGUGCAAAAAAAAUUACCAAUAAUCCAGAAAA UUAUUAUCAUGGAUUCUAAAACGGAUUACCAGGGAUUUCAGUCGAUGUACACGUUCGUCACAUCUCAUCUACCUCCCGGUUUUAAUGAA UACGAUUUUGUACCAGAGUCCUUUGAUCGUGACAAAACAAUUGCACUGAUAAUGAAUUCCUCUGGAUCUACUGGGUUACCUAAGGGUGU GGCCCUUCCGCAUAGAACUGCCUGCGUCAGAUUCUCGCAUGCCAGAGAUCCUAUUUUUGGCAAUCAAAUCAUUCCGGAUACUGCGAUUU UAAGUGUUGUUCCAUUCCAUCACGGUUUUGGAAUGUUUACUACACUCGGAUAUUUGAUAUGUGGAUUUCGAGUCGUCUUAAUGUAUAGA UUUGAAGAAGAGCUGUUUUUACGAUCCCUUCAGGAUUACAAAAUUCAAAGUGCGUUGCUAGUACCAACCCUAUUUUCAUUCUUCGCCAA AAGCACUCUGAUUGACAAAUACGAUUUAUCUAAUUUACACGAAAUUGCUUCUGGGGGCGCACCUCUUUCGAAAGAAGUCGGGGAAGCGG UUGCAAAACGCUUCCAUCUUCCAGGGAUACGACAAGGAUAUGGGCUCACUGAGACUACAUCAGCUAUUCUGAUUACACCCGAGGGGGAU GAUAAACCGGGCGCGGUCGGUAAAGUUGUUCCAUUUUUUGAAGCGAAGGUUGUGGAUCUGGAUACCGGGAAAACGCUGGGCGUUAAUC

	AGAGAGGCGAAUUAUGUGUCAGAGGACCUAUGAUUAUGUCCGGUUAUGUAAACAAUCCGGAAGCGACCAACGCCUUGAUUGACAAGGAU GGAUGGCUACAUUCUGGAGACAUAGCUUACUGGGACGAAGACGAACACUUCUUCAUAGUUGACCGCUUGAAGUCUUUAAUUAAAUACAA AGGAUAUCAGGUGGCCCCCGCUGAAUUGGAAUCGAUAUUGUUACAACACCCCAACAUCUUCGACGCGGGCGUGGCAGGUCUUCCCGAC GAUGACGCCGGUGAACUUCCCGCCGCCGUUGUUGUUUUGGAGCACGGAAAGACGAUGACGGAAAAAGAGAUCGUGGAUUACGUCGCCA GUCAAGUAACAACCGCGAAAAAGUUGCGCGGAGGAGUUGUGUUUGUGGACGAAGUACCGAAAGGUCUUACCGGAAAACUCGACGCAAGA AAAAUCAGAGAGAUCCUCAUAAAGGCCAAGAAGGGCGGAAAGUCCAAACUCGAGUAAGGUUAACCUGCA

B.8: Templates used in Base-Stacking PCR.

Construct	Sequence 5'>
JO_033_guide_miR16	GGCTAAGACAGATGCTCTTTGCCAACAGGCCACAGAATTCCTACACTCAAA GTCGTACTGgCGCCAATATTTACGTGCTGCTATCGCACT
JO_034_guide_RV	GGCTAAGACAGATGCTC
JO_035_guide_FW	GATGCGACGATGCGA
JO_RNA_16	UAGCAGCACGUAAAUAUUGGCG
Yu et al template (33):	GGCTAAGACAGATGCTCTTTGCCAACAGGCCACAGAATTCCTACACTCAAA GTCGTACTGAACTATACAACCTACTACCTCATCGCACT

B.9: Constructs used for Tough-Decoy Assembly.

TD16_1	agggGACGGCGCTAGGATCATCAACCGCCAATATTTAGATCCGTGCTGCTACAAGT CTGCCGCGATCCTAGTAGTTGGCGGTTATAAATCTAGGCACGACGATGTTCAtaag
TD16_2	attcTGGTCACAGAATACAACCGCCAATATTTA ACCAGTGTCTTATGTTGGCGGTTATAAATctag
TD16_3	gatcCGTGCTGCTACAAGATGATCCTAGCGCCGTC GCACGACGATGTTCTACTAGGATCGCGGCAGtgat
ToughDecoy16_1	agggGACGGCGCTAGGATCATCAACCGCCAATATTTAGATCCGTGCTGCTACAAGTATTCTGGT CTGCCGCGATCCTAGTAGTTGGCGGTTATAAATCTAGGCACGACGATGTTCATAAGACCAgtgt
ToughDecoy16_2	cacaGAATACAACCGCCAATATTTAGATCCGTGCTGCTACAAGATGATCCTAGCGCCGTC CTTATGTTGGCGGTTATAAATCTAGGCACGACGATGTTCTACTAGGATCGCGGCAGtgat

Appendix C: General protocols & techniques used in the present study.

C.1: Heat shock transformation of competent BH10b

The protocol for transforming competent cells was adapted from Green and Rogers (32) *by Tim Althuis and further refined based on personal experience.*

Transformation of competent cells is used to select successful golden gate assembly and for cloning plasmids.

C.1.1: Materials, Reagents & Templates

- PCR tubes with chemically competent DH10b cells
- Plasmid of interest
- Transformation construct of interest
- Eppendorf Tubes
- LB medium
- Selection plates
- Water bath
- Ice

C.1.2: Protocol

1. Equilibrate a water bath to 42 ºC.
2. Thaw chemically competent cells on ice, one for each transformation.
3. Prepare Eppendorf tubes with 1 ml of LB medium and place at 37 ºC.
4. Prepare the selection plates using protocol xxx.
5. Add plasmid.
    a. Add 1 or 5 µl ligation mix to the thawed cells, do not pipette up and down.
    b. Add 1 ng of plasmid to the thawed cells, do not pipette up and down.
6. Incubate on ice for 30 minutes.
7. Heat shock cells for 30 seconds in the water bath.
8. Place back on ice for 2 minutes.
9. Carefully pipette the cells to 1 ml of pre-warmed LB medium.

10. Incubate at 37 ºC 180 RPM for 1 hour. Use tape to hold down on the mat.
11. Plate 100 µl and "rest" (pellet, resuspended with 100 µl supernatant) and place upside down in the incubator.
12. Count colonies and follow with colony PCR (X.2.2)

C.2: Colony PCR

Colony PCR is used to analyse the correct number of inserts during Golden Gate Assembly

C.2.1: Materials, reagents, and templates:

- PCR tubes
- milliQ
- Thingy to pick the colonies (1 µl) loop.
- OneTaq 2x
- Primers
  - JO_017 (10 µM)
  - JO_018 (10 µM)

JO_017_DHFRc_f Control PCR ggcaaaatgccgcaaaaaggg

JO_018_DHFRc_r Control PCR caaaaaacccctcaagacccgt

C.2.2: Protocol

Method 1:

1. Prepare the following master mix for colony PCR, adapt the amounts to your needs.

Table 14: Master mix colony PCR.

Components	1x	5x	xx
milliQ	4,25	21,25	xx
JO_017	0.25	1,25	xx
JO_018	0,25	1,25	xx
Buffer OneTaq 2x	6,25	31,25	xx

2. Prepare 2 sets of PCR tubes.

   a. 5 or 10 µl milliQ
   b. 11.5 µl of master mix.
3. Pick a colony and place it in the ‘a’ tube, twist, go to the ‘b’ tube and twist again. Or twist in the ‘a’ tube and pipette up and down and add 1 µl in the ‘b’ tube.
4. Place the PCR tubes of ‘a’ at 4 ºC for storage and later use.
1. Set the thermocycler as follows (Appendix 1.1: colony PCR)

Method 2:

2. Follow step 1 of method 1.
3. Prepare PCR tubes with master mix.
4. Pick a colony and make streak on a new selection plate. Dip and twist in the master mix.
5. Set the thermocycler a follows (Appendix 1.1: colony PCR)

C.3 RNA synthesis using different DNA templates

RNA synthesis protocol adapted from NEB with templates smaller than 0.3 kb.

C.3.1 Materials, Reagents & Templates

- Gloves (black)
- Nuclease-free tubes
- RNAsezap
- RNAse-free water
- DNase I buffer (10x)
- DNAse I (NEB)
- DTT (0.1 M)
- RNase inhibitor
- Ice

Templates (see B)

- pDFHR_miR16
- pDHFR_miR203
- PCR_miR16
- PCR_miR203
- Oligo_miR16
- Oligo_miR203
- Positive control ()

C.3.2: Protocol

1. Wear gloves and clean everything with the RNAsezap, including gloves, pipette boxes pipettes, reagents and the RNAsezap itself – so clean everything!
2. Thaw everything on ice, mix and pulse-spin, keep on ice.
3. Prepare the following master mix, pipette up and down before use.

Table 15: master mix RNA synthesis for 1 reaction.

Reagent	Volume (µl)	Final concentration
10X reaction buffer	1,5	0.75 x
ATP (100 nM)	1.5	7.5 nM
GTP (100 nM)	1.5	7.5 nM
UTP (100 nM)	1.5	7.5 nM
CTP (100 nM)	1.5	7.5 nM

4. Assemble the following reaction at room temperature in the following order.

Table 16: reagents for RNA synthesis at room temperature

Reagent	**Volume (µL)**	**Final concentration**
RNase inhibitor (40 U. µl)	0,5	1 unit / µl
Nuclease free water	To a total of 20	
Master mix	7,5	1x
Template DNA (100 ng) *	1	5 ng
DTT (0.1M)	1	5 mM
T7 RNA polymerase mix	2	U.µl
Total volume	**20**	

*Adding 1 µg of DNA template if preferred for the highest yield of RNA, however, I never managed to get this high of DNA template. So, I added 100 ng template to the reactions.

5. Vortex and pulse-spin. Incubate at 37 ºC overnight in a thermocycler (roof at 96 ºC).
6. Add 70 µl of RNAse-free water, 10 µl of 10X DNAse I Buffer, and 2 µl of DNAse I. Mix and incubate for 15 minutes at 37 ºC.

7. Purify the RNA with RNA purification kit ZymoResearch, elute with 50 µl RNAse free water instead.
8. Use the nanodrop for quantification, write down everything.

A Nanodrop spectrophotometer can read RNA concentrations from 10 ng/µl to 3000 ng/µl directly. For single stranded RNA, 1 A260 is equivalent to RNA concentration of 40 µg/ml. The RNA concentration can be calculated as follows: A260 x dilution factor x 40 = xxx µg/ml RNA.

9. Store the RNA in -20 °C.

C.4: Proof of concept Base-Stacking PCR

C.4.1 Material, Methods & Templates

Table 17: Constructs used for BS-PCR.

Construct	Sequence 5’>
JO_033_guide_miR16	GGCTAAGACAGATGCTCTTTGCCAACAGGCCACAGAATTCCTACACTCAAA GTCGTACTGgCGCCAATATTTACGTGCTGCTATCGCACT
JO_034_guide_RV	GGCTAAGACAGATGCTC
JO_035_guide_FW	GATGCGACGATGCGA
JO_RNA_16	UAGCAGCACGUAAAUAUUGGCG

Table 18: Concentrations needed for table 18.

Sample	Concentration		Concentration 2	
Stock	100	µM	1,00E-04	M
Working stock	10	µM	1,00E-05	M
Prep Stock	100	nM	1,00E-07	M
A	10	nM	1,00E-08	M
B	5	nM	5,00E-09	M
C	1	nM	1,00E-09	M
D	500	pM	5,00E-10	M
E	100	pM	1,00E-10	M
F	50	pM	5,00E-11	M
G	5	pM	5,00E-12	M

Table 19: RNA Dilutions for BS-PCR

Sample	CONC		Sample:sample	Dilution	takeSample	miliQ	D
Stock	1,00E-04	M	S:S	1	0	100	
Working stock	1,00E-05	M	S:W	10	10	90	10
Prep Stock	1,00E-07	M	W:P	100	10	990	100
A	1,00E-08	M	P:A	10	10	90	10
B	5,00E-09	M	P:B	20	5	95	20
C	1,00E-09	M	A:C	10	10	90	10
D	5,00E-10	M	B:D	10	10	90	10
E	1,00E-10	M	C:E	10	10	90	10
F	5,00E-11	M	D:F	10	10	90	10
G	5,00E-12	M	F:G	10	10	90	10

C.4.2: Protocol Base stacking PCR

1: Prepare all reaction components with RNAse free water. Work on ice.

2: Master mix for all planned reactions according to table Z.

Table 20: Reaction scheme master mix for BS-PCR

Component	**1 reaction**	**Final concentration**	**20 reactions**
Q5 2x	12.5	1x	250
10 µM FW	1,5	600 nM	30
10 µM RV	1,5	600 nM	30
Template 50 nM	2,5	5 nM	50
RNA-16 (1 nM = B) / mQ	2,5	100 pMol	50
RNase inhibitor???		1 U/µL	
mQ	4.5		90
Total	25		500

3: Aliquot the mix in PCR tubes.

4: Make sure to place the tubes horizontally in the PCR machine.

5: Use the following program.

94 ºC – 30 seconds

94 ºC – 5 seconds + 60 ºC 30 seconds x 30 cycli.

Select Gradient: with ~2.0 temperature difference per column.

5: Load the PCR reaction with 6x loading dye and load on a 2% gel 100v 30 minutes. The expected size is 98 nt.

Repeat the protocol with the best performing temperature. Make a series of different concentrations in duplo.

C.5: Golden Gate assembly

C.5.1: Introduction

Method A, a 2-pot reaction for an 8-part Golden Gate Assembly. Inserts were designed to fit in the correct orientation, with specific overhangs. Inserts are made from oligo's pair that are perfectly complementary with a 4-nucleotide overhang on each site. The pTarget is made from a linearized plasmid that is digested beforehand. The Golden Gate assembly makes use of the T4-ligase and is run overnight at 16 ºC.

C.5.2: Materials

T4 DNA ligase buffer (10x)

T4 ligase

Sterile milliQ – prepared in house.

Q5 High-fidelity 2x master mix

Agarose gel (2%)

10X NEBuffer r3.1

BsmBI v2

ZymoResearch gel clean up kit.

Primers:

Primers used for the linearization of template DNA.

f_DHFR_BsmBI:	5'	-	acgtctcaactagcataaccccttggg
r_DHFR_BsmBI:	5'	-	acgtctcaccctatagtgagtcgtatta

Primers used for colony PCR.

JO_017_DHFR_c 5' - ggcaaaatgccgcaaaaaggg

JO_018_DHFR_c 5' - caaaaaacccctcaagacccgt

Template: DHFR_control_template - appendix x

Inserts: See appendix:

C.5.3: Protocol Annealing/combining/making/ of sponge fragments.

Oligo's are diluted with milliQ to create 100 μM stocks.

Assemble the annealing reaction into PCR tubes according to the following table.

Table 21: reaction mixture containing everything for annealing RNA or sponge fragments.

Component	Stock Concentration	Final concentration	Volume μL
Forward oligo	100 μM	5 μM	1
Reverse oligo	100 μM	5 μM	1
10X NEBuffer r3.1	10X	1X	2
milliQ	-	-	16

Place the PCR tubes into a thermocycler and set to one of the three programs (appendix xxx)

Preparation of pTarget by PCR

1. Add the following components together for the linearization of pTarget, work on ice.

Table 22: Q5 PCR reaction mixture for linearization of pTarget

Component	Volume μL
milliQ	19
f_DHFR_BsmBI (10 μM)	2,5
r_DHFR_BsmBI (10 μM)	2,5
pDHFR_C (1 ng/μl)	1

Q5 High-Fidelity 2X Master Mix	25

2. Set up the thermocycler with the following protocol.

Table 23: Thermocycler setting for linearization of pTarget.

Step	Temperature ºC	Time
Initial denaturation	98	30 seconds
35 Cycles	98 69 72	10 seconds 30 seconds 60 seconds
Final extension	72	2 minutes
Storage	12	Storage

3. Prepare a 1% agarose gel in the meantime.
4. Load 5 µl PCR product with 1 µl of 6x purple loading dye*.
5. Add to the gel: 1kb ladder and the loaded PCR products.
6. Run the gel at 100v for 30 minutes.
7. Take a picture of the gel with
8. Check if the expected size of the band is 2071 bp.
9. Purify PCR product with ZymoResearch DNA clean & concentrator kit according to manufacturer instructions, use 11 µl milliQ for the dilution step instead of the provided elution buffer.
10. Measure the DNA concentration with the nanodrop.

* You can do 1 µl with 0.2 µl, but this is at your own risk of having to do it again with more product anyway.

Restriction and digestion of linearized plasmid with BsmBI v2.

11. Assemble the following reaction in a 2,0 ml Eppendorf tube.

Table 24: Assembly mixture for digestion of linearized pTarget with BsmBI

Component		Volume (µL)
Linearized pTarget	1 µg	
10X NEBuffer 3.1	1X	2,0
MilliQ	To 20 µL	1x
BsmBI V2	5 – 10 units per µg DNA	0,4

12. Pipette up and down and incubate the reaction at 55 ºC in the thermoblock for 1 hour.
13. Prepare a 1% agarose gel in the meantime.
14. Add 6x loading dye to the mixture and load on the gel, run for 30 minutes at 100v.
15. Take a picture of the gel.
16. Cut the linearized plasmid from the gel with a scalpel and store in a 2 mL Eppendorf tube.
17. Purify the gel with Zymoclean Gel purification kit according to manufactures instructions, elute in 11 µL mQ instead.
18. Quantify the DNA concentration by nanodrop.
19. Store at -20 ºC

C.5.4: Golden Gate Assembly – Method A

1. Before you prepare the following ligation reaction, calculate how many moles need to be added with the help of: https://nebiocalculator.neb.com/#!/ligation (version 1.15.8)
2. Assemble the following reaction in a 2,0 mL Eppendorf tube.

Table 25: Reaction mixture for Golden Gate Assembly Method A

Component	Amount	Volume (µl)
pTarget (25ng/µl)	0,02 pmol	1
Annealed oligo's (2,5 µM)		2 each (8*2 = 16)
T4 DNA ligase buffer (10x)		2
MilliQ	To 20 µl	0
T4 ligase (x U/ ml)	xxx U / µl	1

3. Vortex and spin down shortly
4. Incubate for 1 hour at 16 ºC in a heat-block.
5. Remove 5 µl of the ligation reaction and use this for transformation protocol ()
    a. Heat inactivated at 60 ºC for 10 minutes.
6. Place the remaining solution back in the heat-block overnight.

C.5.5: Golden Gate assembly – Method B

This method uses a different combination of Sponge parts, and a different reaction scheme. This method does use the digested pTarget from 5.3.1.

pTarget: 1275626.04 g/mol

pInsert: 18515.08 g/mol

Component	3:1	20:1	60:1
pTarget	25 ng	25 ng	25 ng
Insert	1,1 ng	7,25 ng	21,75 ng

pTarget, 25 ng in 8 µl = 2.450 nM

inset: 1,1 ng in 8 µl = 7.426 nM

Inset, 7,25 ng in 8 µl = 48.95 nM

Inset, 21,75 ng in 8 µl = 146.8 nM

1. Prepare the following master mix, work on ice.

Table 26: META mix (5 reactions) for Golden Gate Assembly Method B.

milliQ	5 µl
T4 ligase buffer	3 µl
T4 ligase	2 µl

2. Prepare the following assembly mix, work on ice, make the Assembly mix.

Table 27: Reaction mixture for Golden Gate Assembly Method B

Component	Amount / Volume
META mix	2 µl
pTarget	25 ng
Insert 1	See ratio-table
Inset 2	See ratio-table

Insert 3	See ratio-table
milliQ	Fill to 8

3. Follow one of the protocols for the annealing reaction.

Table 28: Golden Gate assembly for one pot reaction, either by 1 or 2 steps

One-step-one-pot reaction	Two-step-one-pot reaction
42 ºC for 5 minutes [16 ºC for 2 minutes + 42 ºC for 2 minutes] – 35 cycli 16 ºC for 5 minutes 80 ºC for 10 minutes	42 ºC for 5 minutes 16 ºC for 2 minutes + 42 ºC for 2 minutes – 10 cycli. Add pTarget to the mixture. 42 ºC for 5 minutes 16 ºC for 2 minutes + 42 ºC for 2 minutes – 30 cycli 16 ºC for 5 minutes 80 ºC for 10 minutes

C.5.6: Golden Gate Assembly – Method C one-pot

This Golden Gate Assembly method makes use of the new sponge parts that include BsmBI restriction sites, all the components can be added in one-pot. Digestion of pTarget prior is not needed.

1. Anneal the oligo's following protocol *Annealing/combining/making/ of sponge fragments.*

2. Prepare the META mix on ice.

Table 29: META mixture (5 reaction) for Golden Gate Assembly Method C.

MQ	3 µl
T4 Ligase buffer	3 µl
T4 Ligase	2 µl
BsmBI V2	2 µl

3. Prepare the following assembly mix on ice.

Table 30: Golden Gate Assembly mix for one pot reaction

	20:1	60:1	20:60:20: 1
META mix	2 µl	2 µl	
pTarget	25 ng	25 ng	25 ng
16A	7,25 ng	21,75 ng	7,25 ng
16B	7,25 ng	21,75 ng	21,75 ng
16C	7,25 ng	21,75 ng	7,25 ng
mQ	Fill to 8	Fill to 8	Fil to 8

4. Follow one of the protocols for the Golden Gate Assembly, the left one is preferred.

Table 31: Golden Gate assembly for one pot reaction, either by 1 or 2 steps.

One-step-one-pot reaction	Two-step-one-pot reaction
42 ºC for 5 minutes [16 ºC for 2 minutes + 42 ºC for 2 minutes] – 35 cycli 16 ºC for 5 minutes 80 ºC for 10 minutes	42 ºC for 5 minutes 16 ºC for 2 minutes + 42 ºC for 2 minutes – 10 cycli. Add pTarget to the mixture. 42 ºC for 5 minutes 16 ºC for 2 minutes + 42 ºC for 2 minutes – 30 cycli 16 ºC for 5 minutes 80 ºC for 10 minutes

5. Transform DHF10b with 8 µl of the assembly mix, follow protocols.
6. The following inserts are expected after colonyPCR.

Expected sizes:

1 insert – 293; 2 insert – 319; 3 insert – 345 ; 4 insert – 371 ; 5 insert - 397 ; 6 insert – 423 ; 7 insert – 449 ; 8 insert – 475 ; 9 insert – 501 ; 10 insert – 527 ; 20 insert – 787.

7. Select colonies with 8 inserts (preferably).
8. Selected colonies, continue with C3.

C.5.6: Golden Gate Assembly – Method D Two-step-one-pot

This method is a two-step-one-pot reaction. The sponge fragments A and B are first annealed together with pTarget, then sponge C is added*

pTarget could be added from the start or together with fragment C.

1. Anneal the oligo's following protocol *Annealing/combining/making/ of sponge fragments.*
2. Prepare the META mix on ice.

Table 32: META mixture (5 reaction) for Golden Gate Assembly Method D.

MQ	3 µl
T4 Ligase buffer	3 µl
T4 Ligase	2 µl
BsmBI V2	2 µl

3. Prepare the following assembly mix on ice.

	20:1	60:1	20:60:20: 1
META mix	2 µl	2 µl	
pTarget	25 ng	25 ng	25 ng
16A	7,25 ng	21,75 ng	7,25 ng
16B	7,25 ng	21,75 ng	21,75 ng
16C	7,25 ng	21,75 ng	7,25 ng
mQ	Fill to 8	Fill to 8	Fil to 8

*Add 16C later – see step 4.

4. Use the following settings for the thermocycler.

   42 ºC for 5 minutes

   16 ºC for 2 minutes + 42 ºC for 2 minutes – 10 cycli.

   Add fragment C to the reaction (and add pTarget if not done yet)

   42 ºC for 5 minutes

   16 ºC for 2 minutes + 42 ºC for 2 minutes – 30 cycli.

16 ºC for 5 minutes

80 ºC for 10 minutes

5. Transform DHF10b with 8 µl of the assembly mix, follow protocols*

*This is too much plasmid, I usually add 1 ng

6. The following inserts are expected after colonyPCR.

Expected sizes:

1 insert – 293 ; 2 insert – 319 ; 3 insert – 345 ; 4 insert – 371 ; 5 insert - 397 ; 6 insert – 423 ; 7 insert – 449 ; 8 insert – 475 ; 9 insert – 501 ; 10 insert – 527 ; 20 insert – 787.

7. Select colonies with 8 inserts (preferably).

Appendix D: Results

D.1: Annealing DNA transcribed miRNA fragments into pTarget gel.

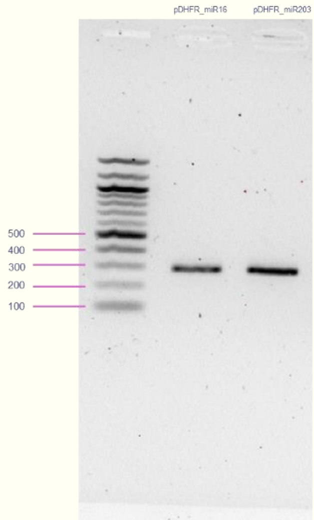


Figure 15: Agarose gel 2% the expected sizes of colony PCR products are for both samples 294 bp (pDHFR_miR16 and pDHFR_miR203), the bands of both samples appear to be on the same height as the 300 bp marker of the 100 bp DNA ladder (NEB).

D.2: Annealing DNA transcribed miRNA fragments into pTarget full sequence.

Table 33: pDHFR_miR16.5 PlasmidSaurus and pDHFR_miR203.8. annotated by PlasmidSaurus.

pDHFR_miR16	ttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttt tccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccg cctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttagcgggttggactcaagacgatagttaccggat aaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtga gctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgaggg agcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggag cctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggata accgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgctcgaccccggtcggccagtgcc aagcttccggatatagttcctcctttcagcaaaaaacccctcaagacccgtttagaggccccaaggggttatgctagtcgccaatatttacgtgctgct accctatagtgagtcgtattaatttcgcggggctagcaccactagcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattc aaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttatt cccttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacat cgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggt attatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcat cttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggagga ccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacga cgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaat agactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgt gggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaa cgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttc

	atttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatcccggatctgtgagttttcgttccactgagcgtcagaccccgta gaaaagatcaaaggatcttc
pDHFR_miR203	ttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaaaaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttt tccgaaggtaactggcttcagcagagcgcagataccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccg cctacatacctcgctctgctaatcctgttaccagtggctgctgccagtggcgataagtcgtgtcttagcgggttggactcaagacgatagttaccggat aaggcgcagcggtcgggctgaacggggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctacagcgtga gctatgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacgaggg agcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgacttgagcgtcgatttttgtgatgctcgtcaggggggcggag cctatggaaaaacgccagcaacgcggcctttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttatcccctgattctgtggata accgtattaccgcctttgagtgagctgataccgctcgccgcagccgaacgaccgagcgcagcgagtcagtgctcgaccccggtcggccagtgcc aagcttccggatatagttcctcctttcagcaaaaaacccctcaagacccgtttagaggccccaaggggttatgctagtctagtggtcctaaacatttca cccctatagtgagtcgtattaatttcgcggggctagcaccactagcacttttcggggaaatgtgcgcggaacccctatttgtttatttttctaaatacattc aaatatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaaggaagagtatgagtattcaacatttccgtgtcgcccttatt ccctttttttgcggcattttgccttcctgtttttgctcacccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggttacat cgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatgatgagcacttttaaagttctgctatgtggcgcggt attatcccgtattgacgccgggcaagagcaactcggtcgccgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcat cttacggatggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataacactgcggccaacttacttctgacaacgatcggagga ccgaaggagctaaccgcttttttgcacaacatgggggatcatgtaactcgccttgatcgttgggaaccggagctgaatgaagccataccaaacga cgagcgtgacaccacgatgcctgtagcaatggcaacaacgttgcgcaaactattaactggcgaactacttactctagcttcccggcaacaattaat agactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggtttattgctgataaatctggagccggtgagcgt gggtctcgcggtatcattgcagcactggggccagatggtaagccctcccgtatcgtagttatctacacgacggggagtcaggcaactatggatgaa cgaaatagacagatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatactttagattgatttaaaacttc atttttaatttaaaaggatctaggtgaagatcctttttgataatctcatgaccaaaatcccggatctgtgagttttcgttccactgagcgtcagaccccgta gaaaagatcaaaggatcttc

D.2: RNA synthesis using several templates

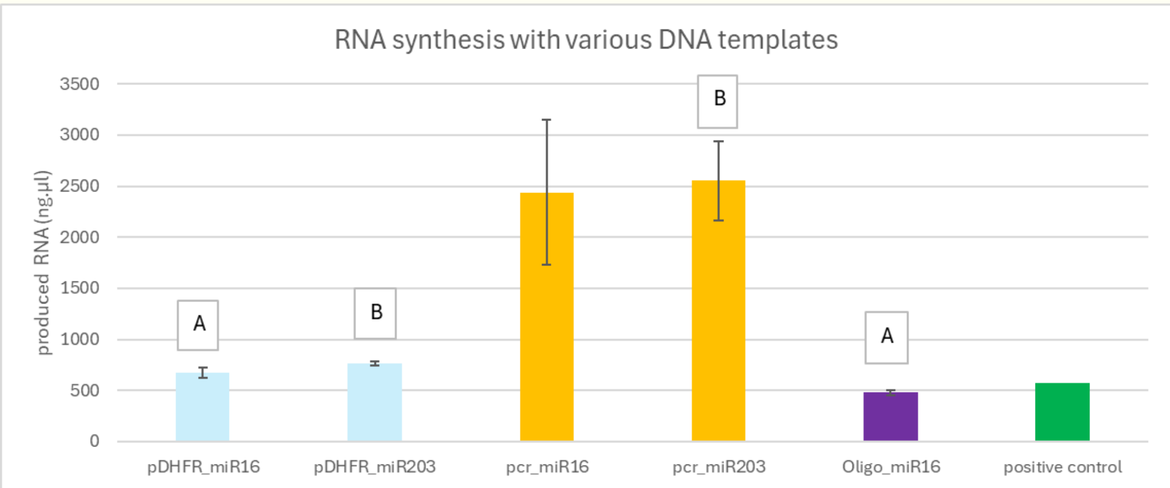


Figure 16: : Average RNA yield (ng/µl) with 100 ng of DNA template measured with a nanodrop using the following templates: Plasmids (pDHFR_miR16 and pDHFR_miR203 – light blue), PCR fragments (pcr_miR16 and pcr_miR203 – orange), oligo-dimer (Oligo_miR16 – dark purple) and positive control (green). RNA was synthesized in duplo with T7 RNA polymerase. Significance was calculated between the same template (e.g. pcr_miR16 and pcr_miR203) and template encoding for the same RNA (e.g. pDHFR_miR16 and pcr_miR16). A significant difference was found between pDHFR_miR16 and oligo_miR16 (A), and between pDHFR_miR203 and pcr_miR203 (B).

Raw data and calculations: supplemented file 1.

D.3: Golden Gate Assembly Tough Decoy 16

Table 34: Tough-Decoy: Sequence shown form Promotor to Terminator sequenced by MacroGen.

Expected sequence	taatacgactcactatagggGACGGCGCTAGGATCAAACCGCCAATATTTAGATCCGTGCTGCTACA AGTATTCTGGTCACAGAATACAACCGCCAATATTTAGATCCGTGCTGCTACAATGATCCTAG CGCCGTCactagcataaccccttggggcctctaaacgggtcttgaggggtttttg
Sequence TD16_1.1	TAATACGACTCACTATAGGGGACGGCGCTAGGATCAAACCGCCAATATTTAGATCCGTGCT GCTACAATGATCCTAGCGCCGTCACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGA GGGGTTTTTTG
Sequence TD16_1.2	TAATACGACTCACTATAGGGGACGGCGCTAGGATCAAACCGCCAATATTTAGATCCGTGCT GCTACAATGATCCTAGCGCCGTCACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGA GGGGTTTTTTG
Sequence TD16_2.1	TAATACGACTCACTATAGGGGACGGCGCTAGGATCCAGAATACAACCGCCAATATTTAGATC CGTGCTGCTACAATGATCCTAGCGCCGTCACTAGCATAACCCCTTGGGGCCTCTAAACGG GTCTTGAGGGGTTTTTTG
Sequence TD16_2.4	TAATACGACTCACTATAGGGCAGAATACAACCGCCAATATTTAGATCCGTGCTGCTACAATG ATCCTAGCGCCGTCACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTT TG

D.4: Golden Gate Assembly Sponge 16 – 8 fragment method

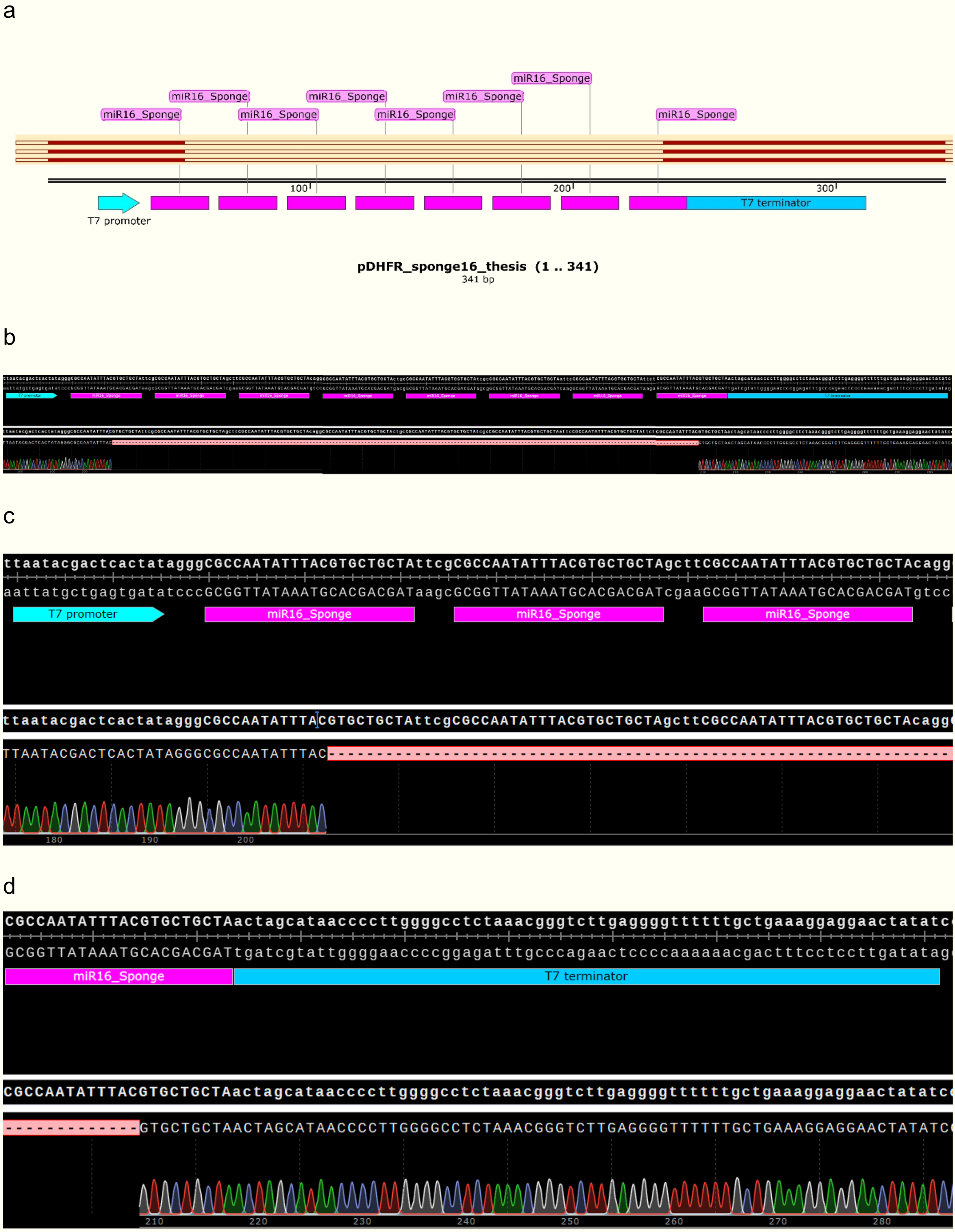


Figure 17: (a) Expected plasmid fragment after Golden Gate assembly with 8 Sponge fragments, aligned against the 3 plasmids that were sent for sanger sequencing. All of them seem to contain the first half of the first sponge fragment and the last half of the 8th sponge fragment. (c) Sequence alignment with one of the sequenced plasmids,(c) enlarged region of promotor region plasmid with sequence pDHFR_S16.1 (table 34). (d) enlarged region of terminator region of plasmid. NOTE: figure a has been pieced together, as the map was not able to be copied in a single go, nothing else has been edited from this figure.

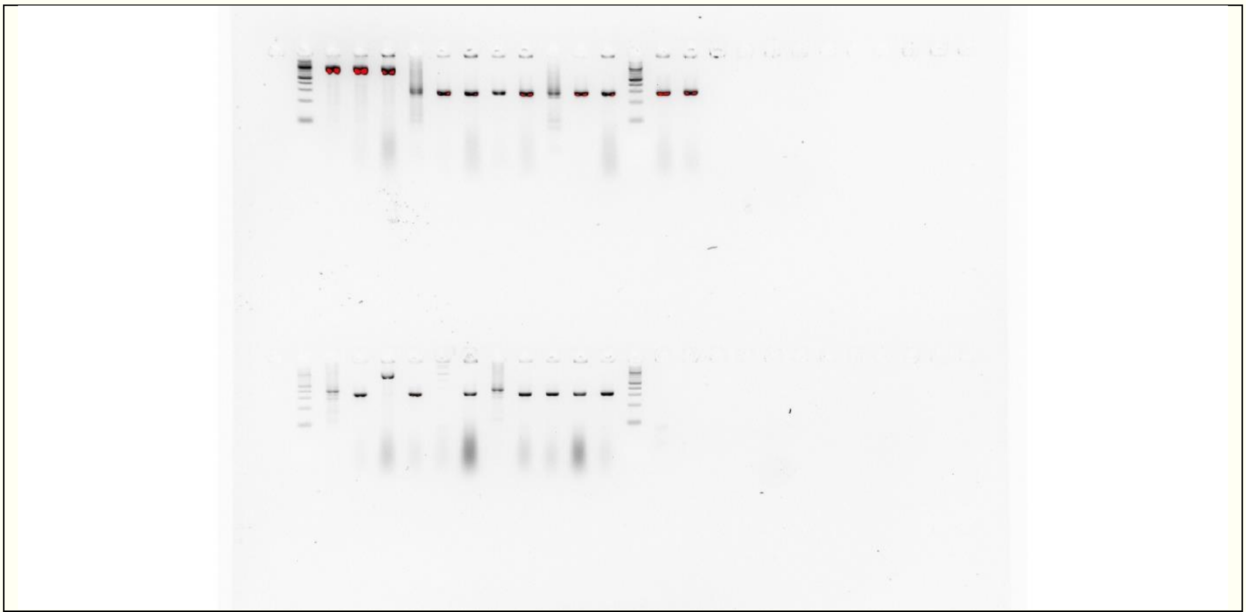
Table 35: Sponge sequence shown form Promotor to Terminator region, sequenced by MacroGen.

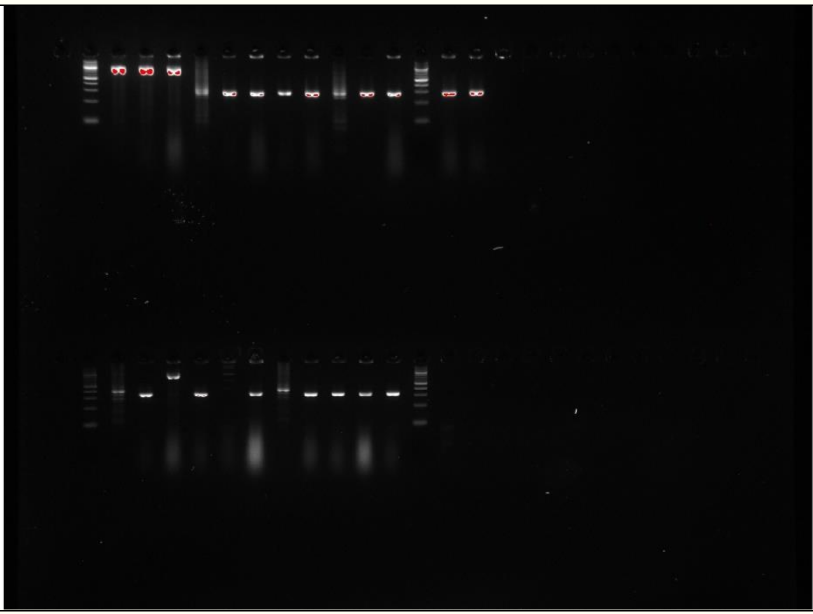
Expected sequence 8 sponges	taatacgactcactatagggCGCCAATATTTACGTGCTGCTAttcgCGCCAATATTTACGTGCTGCTAgcttCGCCAATATTTACGTGCTGCTAcaggCGCCAATATTTACGTGCTGCTActgcCGCCAATATTTACGTGCTGCTAtcgcCGCCAATATTTACGTGCTGCTAattcCGCCAATATTTACGTGCTGCTAttctCGCCAATATTTACGTGCTGCTAactagcataaccccttggggcctctaaacgggtcttgaggggttttttgctgaaaggaggaactatat
Sequence pDHFR_S16.1	TAATACGACTCACTATAGGGCGCCAATATTTACGTGCTGCTAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
Sequence pDHFR_S16.2	TAATACGACTCACTATAGGGCGCCAATATTTACGTGCTGCTAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG
Sequence pDHFR_S16.3	TAATACGACTCACTATAGGGCGCCAATATTTACGTGCTGCTAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG

D.5: Golden gate assemblies agarose gels

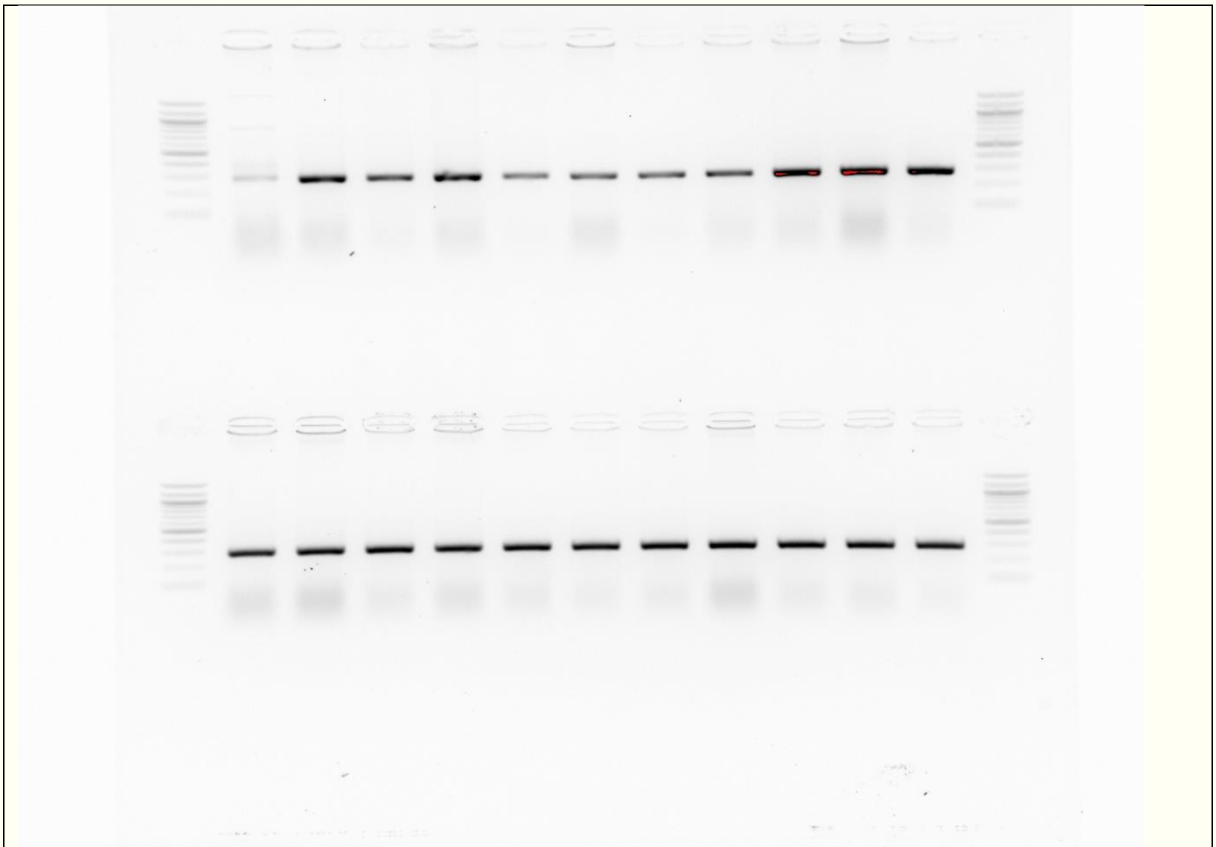
Representative agarose gels of Golden Gate Assemblies

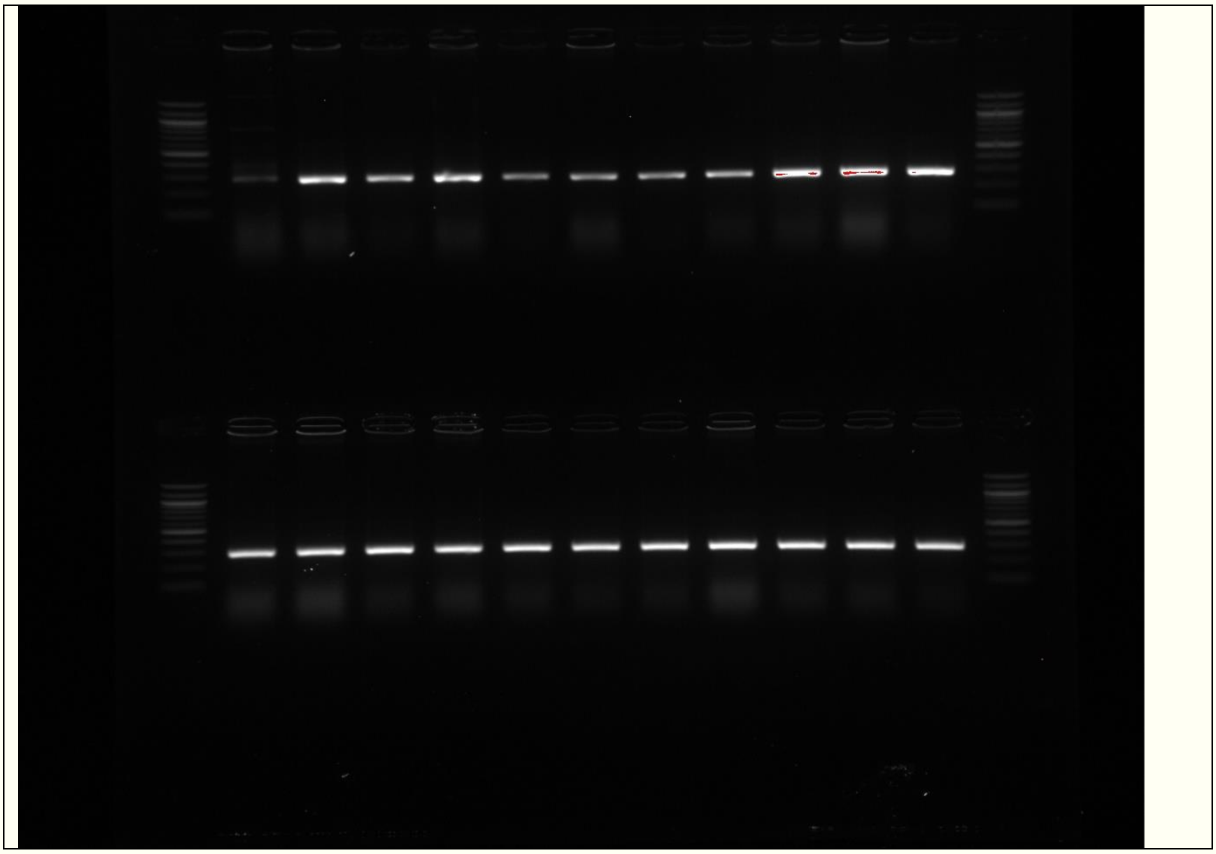
D.5.A: The 8-sponge fragment golden gate gel





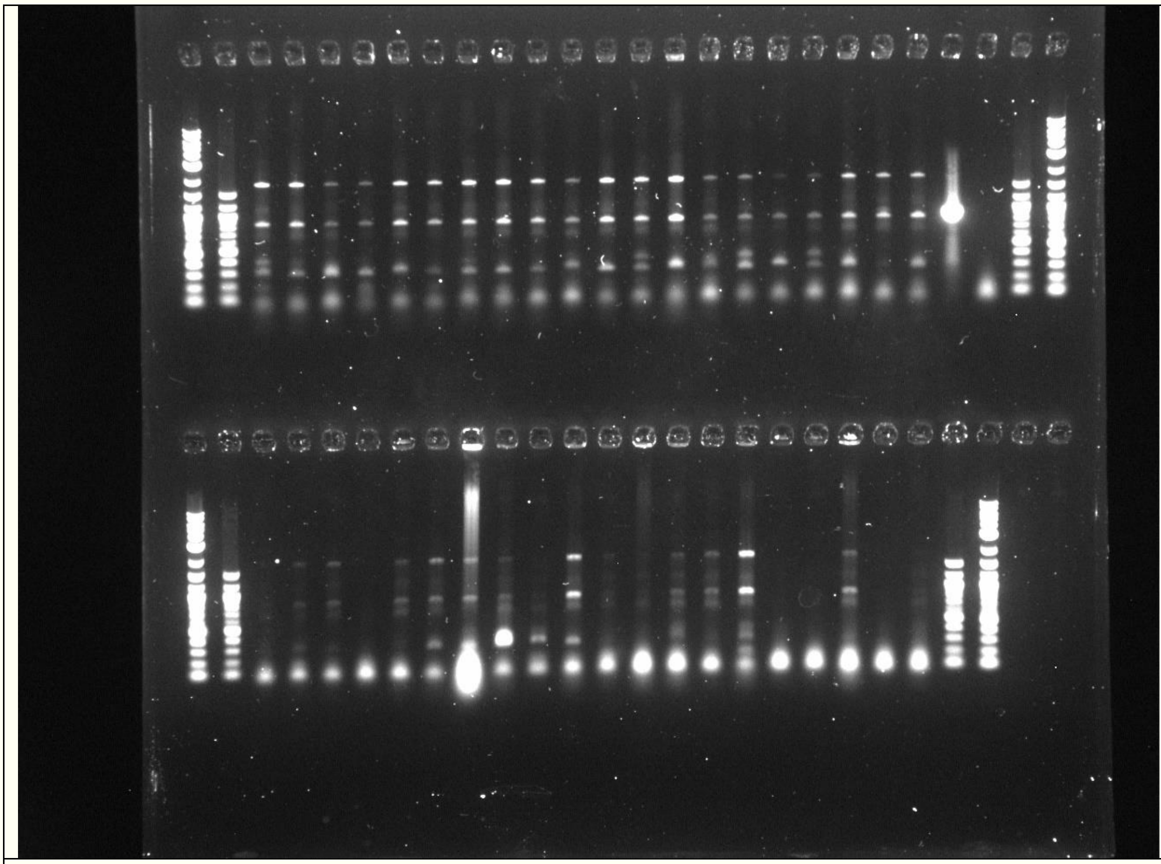
D.5.B: The 3-sponge fragment golden gate gel (ABC)





Ladder used 100bp. PCR sponge of colony PCR (both the 100 and rest plate) all showing near the 300 bp marker = 1 or 2 inserts.

D.5.C: The 3-sponge fragment golden gate gel (xyz) the first time



Ladder (1000 bp +) and Ladder (100 bp). Top part gel, first 10 samples: colony PCR 20:1 golden gate. Next to the ladder on the right side is positive control and negative control, both successful.
Bottom part gel, 20 samples from colony PCR 20/50/20:1 golden gate assembly

D.6: Gel of BS-PCR.

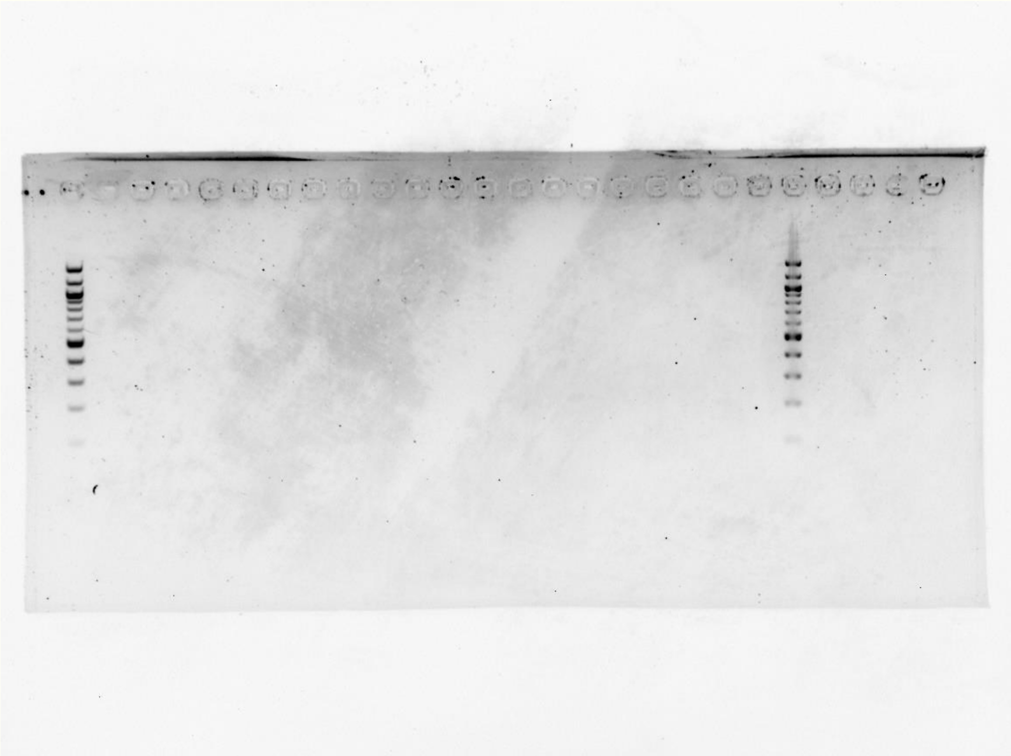


Figure 18: Picture of agarose gel (2%): Base-stacking PCR. Inverting the colour does not help much with greater visibility