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**SYNTHESIS AND PRELIMINARY EX VIVO TESTING OF
SIRNA TARGETING TCRB: A PROPOSED THERAPY FOR
THE TREATMENT OF AUTOIMMUNITY**

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Nutrition and Food Sciences

by
Nicholas Joseph Magazine
B.S., Louisiana State University, 2015
B.S., Louisiana State University, 2015
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List of Abbreviations

C – Constant region of the TCRb gene

cDNA – complimentary DNA

D – Diversity region of the TCRb gene

DNA – Deoxyribonucleic acid

dsRNA – Double-stranded RNA

G - Gravity

HPV – Human papilloma virus

J – Joining region of the TCRb gene

mRNA – messenger RNA

NIH – United States National Institutes of Health

PCR – Polymerase chain reaction

qPCR – Quantitative polymerase chain reaction

RISC – Ribosome induced silencing complex

RNA – Ribonucleic acid

RNAi – RNA interference

siRNA – Small interfering RNA

TCRb – T Cell receptor Beta chain gene

TdT – Terminal deoxynucleotidyl transferase

V – Variable region of the TCRb gene

Abstract

As of 2018, the United States National Institutes of Health estimate that over half a billion people worldwide are affected by autoimmune disorders. Though these conditions are prevalent, treatment options remain relatively poor, relying primarily on various forms of immunosuppression which carry potentially severe side effects and often lose effectiveness overtime. Given this, new forms of therapy are urgently needed. We propose small-interfering RNA (siRNA) for hypervariable regions of the T-cell receptor β -chain gene (*TCRb*) as a highly targeted, novel means of therapy for the treatment of autoimmune disorders.

In this experiment, both human Jurkat and human CD8+ anti-HPV T-cells were purchased and their mRNA isolated. A novel method for the production of siRNA was then employed to create an siRNA “cocktail” for particular hypervariable regions within *TCRb*. Additionally, a conventional method was employed to create siRNA for single computationally determined regions of *TCRb*. Synthesized siRNA was subsequently coated with lipofectamine to facilitate transfection. The coated siRNAs were then applied to respective patient cells at 10 pmol for 24h. Gene knockdown was quantified by qPCR, while cell viability was quantified by MTS assay and trypan blue count.

While all siRNA species demonstrated a significant level of knockdown on *TCRb*, *CD3*, and *CD137* ($P < 0.05$), the siRNA “cocktail” showed the highest level of *TCRb* knockdown ($P = 0.0406$). Moreover, siRNA developed in this manner had an extremely small effect on T-cells lacking affinity for different antigens (low off targeting). The effect of each treatment on cell viability was not statistically significant (For all comparisons, $P > 0.2$).

The results of this experiment demonstrate not only the high potency of the described siRNAs on gene knockdown, but the low levels of off-targeting they exhibit. Based on this, we believe that both the previously described siRNA cocktail as well as computationally created siRNAs could potentially be utilized as effective therapeutics in the treatment of autoimmunity.

Chapter 1. Introduction

As of 2018, the United States National Institutes of Health (NIH) estimate that over half a billion people worldwide are affected by various autoimmune conditions (NIH, 2018). These conditions range from cosmetic disorders (such as psoriasis), to potentially life-threatening diseases (such as lupus). In addition to presenting in a wide range of severity, the financial burden put onto patients with these conditions varies widely as well. In the case of a relatively mild condition such as psoriasis, the financial burden on a patient actively getting treatment for the condition can be as low as \$1757 annually (Evans, 2016). Though this is certainly not an insubstantial financial burden, it contrasts heavily with conditions such as multiple sclerosis, lupus, or Crohn's disease. The annual cost of conditions such as these easily exceed \$60,000 per year for many patients, with the majority of this cost being composed of direct medical expenses (Optum Health, 2019).

Over the past few decades, there have been many advances in the field of molecular biology, particularly in the area of its application to medicine. One of the most impactful of these technologies over these years has come from the advent of RNA interference (RNAi) technologies (Daneholt, 2006). RNA interference is a process by which specific genes are effectively silenced with short strands of inhibitory RNA molecules (Daneholt, 2006). One type of RNA molecule which has been shown to effectively accomplish this is small interfering RNA (siRNA) (Agrawal *et al*, 2003). The first step of siRNA creation involves the synthesis of a strand of double stranded RNA (dsRNA), and subsequently enabling its entrance into the cytoplasm of a mammalian cell (Agrawal *et al*, 2003). Upon entering a cell, this dsRNA is shortly thereafter bound by the protein DICER, which cleaves the dsRNA into siRNA (Agrawal *et al*, 2003). Key features of the resulting siRNA molecules are their typical length of 21-25 nucleotides, as well as a pair of overhanging

nucleotides on both 3' ends, hydroxylated 3' ends, and phosphorylated 5' ends (Agrawal *et al*, 2003). After the siRNA has been formed, RNA-induced silencing complex (RISC) loads a single strand (typically the antisense strand) into its complex (Agrawal *et al*, 2003). The loaded RISC then proceeds to bind messenger RNA (mRNA) strands complimentary to the loaded siRNA strand, cleaving and inactivating the mRNA in the process (Agrawal *et al*, 2003). This disruption in translation leads to silencing of the target gene as the proteins it codes for will ultimately not be created.

In 1999, when the effects of siRNA were first reported, its potential medical applications were immediately evident (Baulcombe, 1999). As the genes for DICER are relatively conserved among mammals, the gene silencing effects of siRNA potentially allow for treatment of diseases in which a human's own cells are responsible for all of, or part, of the pathology. This includes conditions ranging from cancer, autoimmunity, or even some viral infections. In fact, not surprisingly, in the past decade there have arisen clinical trials for drugs which utilize siRNA in the treatment of cancer, as well as, Ebola (Oha and Parkb, 2009). Unfortunately, the research for use of RNAi technologies in autoimmune diseases has been relatively sparse, likely owing primarily to the mechanism by which autoimmune diseases cause their pathology. Autoimmune disease pathology is caused by a small minority of immune cells acting in such a way that causes damage to the host (*Harrison's Principles of Internal Medicine*, 2012). Unfortunately, the differences between the cells causing autoimmune pathology versus those which are necessary for normal biological function and immunity are scarce. As a result, most RNAi therapies which seek to alleviate or eliminate a particular pathology will also end up causing damage to the healthy portion of the host's immune system. As a result, therapies for autoimmune diseases which seek to use RNAi technology will typically target genes which are significantly up-regulated as part of

the condition's pathology, such as pro-inflammatory factors in rheumatoid arthritis (Pauley and Cha, 2013). This being the case, the ideal target for treatment of an autoimmune disorder would be a gene expressed exclusively in the cell lines responsible for the pathology of the condition. Fortunately, there are a few genes that produce mRNA which differ between individual immune cell lines. These include the genes responsible for antibody production in B cells as well as antigen receptor chain production in T cells (*Janeways immunobiology*, 2012).

To deal with the vast array of pathogens which are able to infect humans, the body has evolved a remarkable system commonly referred to as adaptive immunity. In contrast with the innate immune system, the adaptive immune system has evolved to be able to deal with novel pathogens, allowing the body to rid itself of diseases not experienced by previous generations (*Janeways immunobiology*, 2012). This is achieved by the generation of variation in antigen recognition chains for T cells, and antibodies for B cells, a process which occurs in a series of steps which over the past century have become fairly well understood. Though the processes are extremely similar, for the sake of time, focus will be taken on antigen receptor chain in T cells (as they are more relevant to the scope of these writings).

To understand the gene arrangement coding for antigen receptor chain development, one must first understand the structure of the chains themselves. In 95% of cases, a T cell receptor is composed of both an *alpha*, and a *beta* chain (*Janeways immunobiology*, 2012). Each of these chains is formed by an amino terminal variable (V) region, which composes the antigen recognition portion of the receptor, and a constant (C) region, which composes the transmembrane portion of the receptor (*Janeways immunobiology*, 2012). Though the processes which govern the development of both of these chains are similar, the development of the β chain will be the focus of this chapter (the chain most pertinent in this experiment).

Development of the β chain is controlled by a gene segment designed to create the opportunity for a large degree of diversity for the antigen receptor it will ultimately encode. The β chain is composed of many of each of four separate segment types: variable (V) regions, joining (J) segments, diversity (D) segments, and constant (C) segments. These genes are arranged in an order as follows: 52 V regions, followed by a D region, followed by 6 J segments, followed by a C region, followed by an additional D region, followed by 7 additional J segments, followed by a final C region (*Janeways immunobiology*, 2012). The genes encoding the β chain receptor are located on chromosome 7 (Caccia *et al*, 1984). To produce a functional β chain, the variable, diversity, and joining regions undergo rearrangement and splicing to one another through a series of processes. This newly spliced gene is then linked to the constant gene regions to form the completed gene product which will ultimately produce the mRNA responsible for β chain production (*Janeways immunobiology*, 2012). Because of this series of gene recombinations, it is highly unlikely that the gene segment encoding for any two separate lines of T cells will be the same.

The proliferation of both B and T cells is dependent on how effective they are at recognizing antigen. With some exception, cells which successfully recognize antigen receive a variety of signals which stimulate their proliferation (*Janeways immunobiology*, 2012). Cells that do not receive these signals, however, tend not to reproduce and over time begin to die off (*Janeways immunobiology*, 2012). The lifespan of these cells ranges greatly, with the half-life of CD8 T cells being around 37 days (McDonagh and Bell, 1995) while their CD4 counterparts have been shown to survive for as long as 17 years in humans (Imamishi *et al*, 2014).

The gene rearrangements that cause the diversity seen in both T and B cells are an evolutionary necessity for the destruction of a variety of pathogens. These same rearrangements,

however, can potentially be problematic for the wellbeing of the same system they are trying to protect, as is the case in autoimmune disorders. Autoimmune disorders are caused when B or T cell receptors become specific for motifs on the host's own tissues (*Janeways immunobiology*, 2012). These tissue molecules which are targeted are termed autoantigens, and can vary greatly between individuals. The immune system has a number of mechanisms, as well as a vetting process, designed to prevent the occurrence of autoantigen (*Janeways immunobiology*, 2012). This being the case, however, given the rate at which new cells are produced, even a small slip in the system can lead to autoimmunity. An example of a disease in which this occurs is multiple sclerosis. In the case of multiple sclerosis, T cells gain specificity for protein present in the myelin sheath, the protective and insulative coating found surrounding neurons (Huseby *et al*, 2012). The recognition of myelin by a T cell causes inflammation as well as a disruption of the blood-brain barrier, leading to a variety of severely damaging neurological effects for the victim (Minager and Alexander, 2003).

Though significant advances in autoimmune disease treatment have been made over the past several decades, there is a large requirement for improvement. Most current therapies focus on down regulation of the immune system as a whole, as opposed to targeting the specific immune cell lines causing the pathology. As expected, this leads to a variety of problems and side effects, including global immune suppression. These side effects severely limit the dosing of these drugs, as high and long-term use of these therapies can ultimately lead to the patient's immune system becoming severely compromised (Williamson and Berger, 2015). It is therefore critical that treatments which are highly specific for the agents causing the specific pathology be the primary focus of the development of future therapies targeted at autoimmune disorders.

To develop therapies with high specificity, it is necessary to be able to identify and exploit the subtle differences between cells. In the case of autoimmune disorders, this means differentiating between specific lines of B or T cells. Given that there is genetic diversity among these lines in the form of the genes encoding the receptors found on their surfaces, these same genetic differences offer a viable target for the creation of a therapy with a high degree of specificity. Given that RNAi technology gives scientists the ability to target specific gene sequences with relatively high fidelity, RNAi can likely offer a highly effective means of treating a wide array of autoimmune disorders, provided the ability to elucidate the gene sequences required for B cell and T cell receptor production.

Therapy with a method as specific as RNAi in autoimmune conditions also has a number of other implications. If the cells lines affected by RNAi are unable to produce antigen receptors, they will, therefore, be unable to recognize antigen. A cell which is unable to recognize antigen will not receive proliferation signals, and slowly will begin to die off. This being the case, RNAi may potentially offer a means of therapy which can lead to remission of autoimmune diseases over time, as the cell lines causing the pathology may gradually begin to die off. This contrasts greatly with most current therapies, which show a high degree of relapse soon after treatment has been halted. This research aims to develop the methods required to synthesize siRNA for the T cell receptor beta chain (TCRb gene). Additionally, this research seeks to test the various siRNA species created on human immune cells ex-vivo to demonstrate their effectiveness.

Notes

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Chapter 2. The 51 Primer Method

2.1 Introduction

To Create siRNA Targeting the TCRb gene of a particular line of T cells, it is necessary to know the mRNA sequence of the TCRb gene within these cells. Though this may seem simple in principle, it presents quite a technical challenge due to the high levels of variation that occur within this gene. For a researcher to be able to sequence a gene, they must first amplify the gene to sufficient levels for analysis using Polymerase chain reaction (PCR). Unfortunately, one of the requirements to carry out a successful PCR reaction is designing primers targeting sequences bracketing the gene of interest. Unfortunately, due to the high levels of variation within the TCRb gene, this is not possible using conventional methods. As previously discussed, there are roughly 52 different variable regions which can be found on the 5' end of the mRNA for the TCRb gene. Additionally, there are two different variations (though highly similar) of the constant region found on the 3' end of the mRNA. Given this information, a conventional method used for the amplification of the TCRb gene has been to make two separate primer mixes, one containing primers for the 51 variable regions and the other containing primers for both of the constant region variants. One benefit of this method is the relative ease of amplification once the primer mixes have been created (given that a large number of aliquots can be made). Additionally, though many primers are required, these primers are extremely inexpensive (costing only approximately \$6 USD at the time of this writing) and come in quantities that can be used to perform hundreds of assays.

2.2 Methods

The methods used in this experiment were adapted from Robins *et al.* The primers used were taken from United States Patent Application: US 2016/0319340 A1. These Primers as well as primer pCB1 can be found in appendix A.

2.2.1 Creation of Primer Mix

Primers were ordered dry and desalted from Thermo Scientific and resuspended to a concentration of 200mM. One microliter of each of the 51 variable region primers were combined on ice and pipetted gently to mix. In a second microfuge tube, pCB1 primer (a primer which binds to both potential constant regions) was resuspended to 10mM.

2.2.2 mRNA Isolation

RNA was isolated from Both Human Jurkat and Human CD8+ anti-HPV cells. This was accomplished using an Absolutely RNA Nanoprep Kit (Agilent). Approximately 10,000 cells of each sample were combined with 100 μ l lysis buffer and 0.7 μ l Beta-Mercaptoethanol. These samples were then vortexed for approximately one minute until well homogenized. After homogenization, 100 μ l sulfolane was added to the lysate and the samples were again vortexed for approximately five seconds. It was noted that the samples became warm during this process. Samples were then transferred to individual RNA-binding spin cups and centrifuged at 12,000 x g for one minute. The filtrate was immediately discarded. Three hundred microliters of low-salt wash buffer was then added to the spin column and centrifuged at 12,000 x g for an additional minute with the filtrate again being discarded. The spin column was then centrifuged at 12,000 x g for two minutes in order to completely dry the spin column. After patting the edges of the column dry with a Kim-Wipe, 2.5 microliters RNase-Free DNase I was combined with 12.5 microliters DNase digestion buffer. The full 15 microliters of completed DNase solution was

then added to the spin column and allowed to incubate in a 37°C bead-bath for 15 minutes. After incubation, 300 microliters high-salt wash buffer was added to the column and centrifuged at 12,000 x g for one minute. The filtrate was again discarded and 300 microliters of low-salt wash buffer was added to the column and centrifuged at 12,000 x g for one minute. After discarding the filtrate, the column was spun for an additional three minutes to dry the column. After patting the column dry with a kim-wipe, the cup was placed into a fresh two milliliter collection tube. The provided elution buffer was warmed to 60°C, and 10 microliters of elution buffer was quickly added to each column and allowed to sit for two minutes at room temperature. The columns were then spun at 12,000 x g for five minutes and capped. A NanoDrop™ 2000 unit (Thermo Scientific) was used to quantify the concentration of RNA within the resulting solutions. NanoDrop™ analysis of the samples revealed a concentration of 10.3 nanograms/microliter for the human Jurkat cells and 5.1 nanograms/microliter for the human CD8+ Anti-HPV cells.

2.2.3 Reverse Transcription

For both the Human Jurkat and Human CD8+ Anti-HPV cells reverse transcription of the mRNA was performed. To do this, 20 nanograms of RNA (determined by nanodrop) was combined with one microliter oligo (dT)₂₀ (50 micromolar) and 1 microliter of a 10 mM dNTP mix (10mM dATP, dGTP, dCTP, dTTP). The samples were then brought to 13 microliters using PCR-grade water. These samples were then heated to 65°C for 5 minutes followed by immediate incubation on ice for 1 minute.

Using the reagents provided with a superscript RT kit, four microliters 5x first strand buffer, one microliter 0.1 M DTT, and one microliter Superscript III reverse transcriptase (200 units/microliter) were added to each sample. Additionally, 1 microliter RNaseIN™ was added to

each sample to maximize cDNA yield. The samples were then mixed by gentle pipetting and allowed to incubate at 50°C for one hour. Following this incubation, the reaction was inactivated by heating at 70°C for 15 minutes.

To remove the mRNA strands from their cDNA counterparts, RNaseH (New England Biolabs) was used. This was accomplished by adding 2.1 microliters RNaseH buffer and 1 microliter RNase H to each sample. These samples were then incubated at 37°C for 20 minutes and immediately frozen and stored at -80°C.

2.2.4 PCR Amplification

In triplicate, both completed cDNA samples were combined in a PCR plate on ice with the following reagents: five microliters SYBR™ Green Master Mix (Thermo Scientific), 0.5 microliters variable region primer mix, 0.5 microliters pCB1 primer, 1 microliter of respective sample, and 3 microliters PCR grade water.

After the reagents were combined, the PCR reaction was carried out using the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
40 Cycles:		
Denaturation	94°C	15 seconds
Annealing and Extension	53°C	1 minute
Fluorescence read		

A positive result would be indicated by fluorescence in the respective qPCR reactions following amplification.

2.3 Results

None of the three samples for each of the two reactions showed any fluorescence during the qPCR reaction. It should be noted that this experiment was repeated an additional time with

similar results, however in the second run of the experiment one of the wells for human Jurkat cells began to show fluorescence after 19 cycles. Due to the fact that no other wells in the triplicate reaction showed any fluorescence, it is assumed that this false positive was the result of primer dimerization or contamination.

2.4 Discussion

It is uncertain why the 52-primer method was not successful. The theory behind the method was sound and the protocols outlined were relatively standard (though applied in a highly specialized manner). It is possible that the experiment failed due to human error (a primer not being added properly), though it is also possible that the sheer number of primers used in this experiment caused unanticipated interactions which prevented the reaction from working.

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Chapter 3. C Tailing Method

3.1 Introduction

Due to the failures experienced with the 51-primer method discussed in the previous chapter, it became necessary to explore alternative options for sequencing the TCRb gene. To accomplish this, a spin on the traditional “C-tailing” method was used. Though the methods used to achieve sequencing using C-tailing are relatively complex, the principle behind the method is fairly simple. In order to elucidate the sequence of a given DNA strand, the DNA strand must first be amplified using PCR. For a researcher to perform PCR, both a forward and reverse primer are required. Creating a forward and reverse primer requires the researcher to know the sequence of areas bracketing the DNA sequence of interest. In the case of mRNA for a gene like TCRb, this is problematic as the sequence of interest is highly randomized. Fortunately, because we are interested in the mRNA sequence of TCRb, we know that the 3’ end of the sequence of interest will be the constant region of TCRb, which is relatively conserved. Because of this, a primer for a sequence that is homologous for both constant region variants can be used. This means that we are only left needing a reverse primer to sequence TCRb. Unfortunately, the 5’ end of the mRNA (and therefore the 3’ end of its cDNA after reverse transcription) for TCRb begins with the hypervariable region of the gene. This means that under ordinary circumstances, a single primer would be unable to provide sufficient specificity to allow for amplification of the gene. This is remedied in the G-tailing method using an enzyme known as terminal deoxynucleotidyl transferase (TdT). TdT adds random nucleotides to the 3’ end of any given DNA sequence. To exploit this, we can allow TdT to perform its function on the cDNA of the TCRb gene except do so in a solution that is devoid of all nucleotides except for cytosine. By doing this, the cDNA has a poly-C repeat added to its 3’ end. This poly-C repeat allows us to

design a reverse primer in the form of a Poly-G primer. Desoxyinosine residues are interspersed within the sequence to lower the melting temperature of the primer and allow for successful PCR amplification. Verification of a successful assay can be performed by qPCR where amplification (indicated by fluorescence in the PCR reactions) is indicative that the Tailing reaction is successful.

3.2 Methods

3.2.1 mRNA Isolation

RNA was isolated from Both Human Jurkat and Human CD8+ anti-HPV cells. This was accomplished using an Absolutely RNA Nanoprep Kit (Agilent). Approximately 10,000 cells of each sample were combined with 100 µl lysis buffer and 0.7 µl Beta-Mercaptoethanol. These samples were then vortexed for approximately one minute until well homogenized. After homogenization, 100 µl sulfolane was added to the lysate and the samples were again vortexed for approximately five seconds. It was noted that the samples became warm during this process. Samples were then transferred to individual RNA-binding spin cups and centrifuged at 12,000 x g for one minute. The filtrate was immediately discarded. Three hundred microliters of low-salt wash buffer was then added to the spin column and centrifuged at 12,000 x g for an additional minute with the filtrate again being discarded. The spin column was then centrifuged at 12,000 x g for two minutes in order to completely dry the spin column. After patting the edges of the column dry with a Kim-Wipe, 2.5 microliters RNase-Free DNase I was combined with 12.5 microliters DNase digestion buffer. The full 15 microliters of completed DNase solution was then added to the spin column and allowed to incubate in a 37°C bead-bath for 15 minutes. After incubation, 300 microliters High-salt wash buffer was added to the column and centrifuged at 12,000 x g for one minute. The filtrate was again discarded and 300 microliters of low-salt wash

buffer was added to the column and centrifuged at 12,000 x g for one minute. After discarding the filtrate, the column was spun for an additional three minutes to dry the column. After patting the column dry with a kim-wipe, the cup was placed into a fresh two milliliter collection tube. After warming the provided elution buffer to 60°C, 10 microliters of elution buffer were quickly added to the column and allowed to sit for two minutes at room temperature. The column was then spun at 12,000 x g for five minutes and capped. A NanoDrop™ 2000 unit (Thermo Scientific) was used to quantify the concentration of RNA within the resulting solution. NanoDrop™ analysis of the samples revealed a concentration of 8.9 nanograms/microliter for the human Jurkat cells and 7.8 nanograms/microliter for the human CD8+ Anti-HPV cells.

3.2.2 Reverse Transcription

For both the Human Jurkat and Human CD8+ Anti-HPV cells reverse transcription of the mRNA was performed. To do this, 20 nanograms of RNA (determined by nanodrop) was combined with one microliter oligo (dT)20 (50 micromolar) and 1 microliter of a 10 mM dNTP mix (10mM dATP, dGTP, dCTP, dTTP). The samples were then brought to 13 microliters using PCR-grade water. These samples were then heated to 65°C for 5 minutes followed by immediate incubation on ice for 1 minute.

Using the reagents provided with a superscript RT kit, 4 microliters 5x first strand buffer, one microliter 0.1 M DTT, and 1 microliter Superscript III reverse transcriptase (200 units/microliter) were added to each sample. Additionally, 1 microliter RNaseIN™ was added to each sample to maximize cDNA yield. The samples were then mixed by gentle pipetting and allowed to incubate at 50°C for one hour. Following this incubation, the reaction was inactivated by heating at 70°C for 15 minutes.

To remove the mRNA strands from their cDNA counterparts, RNaseH (New England Biolabs) was used. This was accomplished by adding 2.1 microliters RNaseH buffer and 1 microliter RNase H to each sample. These samples were then incubated at 37°C for 20 minutes and immediately frozen and stored at -80°C.

3.2.3 DNA Cleanup

DNA cleanup was performed using a GeneJET PCR purification kit (Thermo Scientific). This step was necessary to remove any residual dNTPs which would be problematic for the future TdT enzyme reaction. To do this, a 1:1 volume of binding buffer (approximately 25 microliters) was added to the previously created samples and thoroughly mixed. It was noted that the samples were a bright yellow, indicating that the pH of the solution was correct for the assay. The 50 microliters of this solution were then added to the GeneJet purification column and centrifuged at 8,000 x g for 30 seconds, discarding the permeate. Seven hundred microliters wash buffer was then added to each sample and centrifuged for 30 seconds, discarding the permeate. The columns were then transferred to new microfuge tubes and centrifuged at 8,000 x g for one minute to ensure that the columns were dry. The columns were again transferred to fresh microfuge tubes and 20 microliters elution buffer was added to each column. After centrifuging at 8,000 x g for a final minute, the samples were quantified via NanoDrop™ and stored at -80°C for G-Tailing. NanoDrop™ assessment of the samples showed a concentration of 2.2 nanograms/microliter for the human Jurkat sample and 2.4 nanograms/microliter for the human CD8+ anti-HPV cells.

3.2.4 Poly-C Tailing

Poly-C tailing was carried out using a TdT enzyme reaction kit purchased from New England Biolabs. In clean microfuge tubes on ice, the following reagents were added in order: 5

microliters 5x TdT buffer, 18.5 microliters of the clean cDNA product from previous steps, 0.5 microliters 10mM dCTP, and 1.5 microliters TdT enzyme. This reaction mix was then pipetted gently to mix and gently spun to collect all reagents in the bottom of the microfuge tubes. The reaction mixes were then incubated at 37°C for 15 minutes for the tailing reaction to proceed. After the incubation, the samples were placed into a 70°C bead bath for 10 minutes to stop the reaction by inactivating the enzyme.

3.2.5 Verification of Product Formation

The formation of properly formed product was verified using qPCR. Five microliters SYBR™ Green PCR Master mix (Thermo Scientific) was combined on ice with 0.5 microliters 10mM pGI primer (primer which anneals to the poly-C sequence), 0.5 microliters 10mM pCB1 primer, two microliters of the tailed DNA product, and two microliters PCR grade water (The sequence of both primers used in this reaction can be found in Appendix A). This was done in triplicate for both samples. Thermocycling was then executed under the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
40 Cycles:		
Denaturation	94°C	15 seconds
Annealing and Extension	53°C	1 minute
Fluorescence read		

3.3 Results

All six samples that were run showed fluorescence by 30 cycles, indicating that the C-tailing reaction worked as intended. It should be noted that the product obtained from the PCR reaction would not be ideal for sequencing as the specificity of amplification is quite low as a result of effectively using only a single primer (due to the possibility of other unwanted genes being amplified as well). Future reactions that required sequencing of the PCR product used a

nested primer sequence in which two primers for the constant region were used to improve specificity.

3.4 Notes

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Chapter 4. Synthesis of SiRNA Using a Whole Strand “Shotgun” Method

4.1 Introduction

One major issue with developing siRNA for a particular gene is the high level of variability in siRNA effectiveness. Although a number of algorithms have been developed to predict the effectiveness of a given siRNA, these formulas are far from infallible and very often have no correlation with real-life applications.

The following methods were created as a highly reliable, low-cost technique to create large quantities of SiRNA for a given TCRb gene. The general idea behind the method is as follows:

- First, the cDNA for the mRNA of the TCRb gene is amplified using conventional PCR as well as the previously described C-Tailing method. A nested PCR reaction is used to increase specificity (needed as the pGI primer is not specific to the cDNA of interest).
- Next, a T7 promoter is hybridized to the cDNA samples using overhanging primers incorporated into a PCR reaction
- The dsDNA is melted and using the new T7 promoter attached to the cDNA templates, transcription to RNA is carried out using T7 RNA polymerase.
- The complimentary RNA strands created in the previous transcription reaction are allowed to slowly cool and anneal to form dsRNA strands.
- After cleaning the dsRNA using a column chromatography mini-prep, an RNase III enzyme (DICER) is used to cleave the dsRNA strands into a series of siRNA molecules.
- The siRNAs are then cleaned by glycogen/ethanol precipitation for use.

4.2 Methods

Note: all primer sequences can be found in appendix A.

4.2.2 Nested PCR Reactions

The PCR reactions in this portion of the experiment were carried out using Titanium™ Taq DNA polymerase (Clontech). In two separate microfuge tubes on ice, the 15 microliters of DNA created using the previously described C-tailing method for both human Jurkat and human CD8+ anti-HPV cells were combined with 0.5 microliters pGI primer, 0.5 microliters pCB1 primer, one microliter dNTP mix, five microliters 10x taq buffer, one microliter taq polymerase, and 27 microliters PCR grade water. After all reagents were added to their respective microfuge tubes, the samples were mixed by gentle pipetting. Samples were then split into three separate aliquots on a PCR plate (for a total of six total samples) and thermocycled under the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
20 Cycles:		
Denaturation	94°C	1 minute
Annealing	53°C	1 minute
Extension	68°C	30 seconds
Fluorescence read (only applicable to wells using SYBR™ Green)		

A parallel reaction using SYBR™ Green was carried out to verify that the PCR amplification was successful. In duplicate, one microliter of the respective DNA samples were combined with five microliters SYBR™ Green master mix as well as 0.5 microliters pGI primer and 0.5 microliters pCB1 primer and three microliters PCR grade water. Fluorescence in these samples using SYBR green after thermocycling could be used as a positive indication that the reaction using Titanium™ Taq polymerase was successful.

After this first PCR reaction had completed, a second nested PCR reaction using a second primer set was executed. Five microliters of DNA from each sample were placed into two separate

microfuge tubes on ice. To these tubes, the following reagents were added: Five microliters 10x taq buffer, 0.5 microliters pGI primer, 0.5 microliters pCB2 primer, one microliter dNTP mix, one microliter 50x Titanium™ taq polymerase, and 37 microliters PCR grade water. After all reagents were added to their respective microfuge tubes, the samples were mixed by gentle pipetting. Samples were then split into three separate aliquots on a PCR plate (for a total of six total samples) and thermocycled under the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
20 Cycles:		
Denaturation	94°C	1 minute
Annealing	53°C	1 minute
Extension	68°C	30 seconds
Fluorescence read (only applicable to wells using SYBR™ Green)		

Again, a parallel reaction using SYBR™ Green was carried out to verify that the PCR amplification was successful. In duplicate, one microliter of the respective DNA samples was combined with five microliters SYBR™ Green master mix as well as 0.5 microliters pGI primer and 0.5 microliters pCB2 primer and three microliters PCR grade water. Fluorescence in these samples using SYBR green after thermocycling could be used as a positive indication that the nested reaction using Titanium™ Taq polymerase was successful.

4.2.3 DNA Cleanup

DNA cleanup was performed using a GeneJET PCR purification kit (Thermo Scientific). This step was necessary to remove any residual dNTPs which would be problematic for the future TdT enzyme reaction. To do this, a 1:1 volume of binding buffer (approximately 25 microliters) was added to the previously created samples and thoroughly mixed. It was noted that the samples were a bright yellow, indicating that the pH of the solution was correct for the assay.

The 50 microliters of this solution were then added to the GeneJet purification column and centrifuged at 8,000 x g for 30 seconds, discarding the permeate. Seven hundred microliters wash buffer was then added to each sample and centrifuged for 30 seconds, discarding the permeate. The columns were then transferred to new microfuge tubes and centrifuged at 8,000 x g for one minute to ensure that the columns were dry. The columns were again transferred to fresh microfuge tubes and 20 microliters elution buffer was added to each column. After centrifuging at 8,000 x g for a final minute, the microfuge tubes were capped and stored at -80°C for later steps.

4.2.4 T7 Promoter Hybridization

In two separate microfuge tubes on ice, five microliters of clean DNA from each sample in the previous steps were combined with 0.5 microliters pGI+Promoter primer, 0.5 microliters pCB2+Promoter primer, one microliter dNTP mix, five microliters 10x taq buffer, one microliter taq polymerase, and 37 microliters PCR grade water. After all reagents were added to their respective microfuge tubes, the samples were mixed by gentle pipetting. Samples were then split into three separate aliquots on a PCR plate (for a total of six total samples) and thermocycled under the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
10 Cycles:		
Denaturation	94°C	1 minute
Annealing	53°C	1 minute
Extension	68°C	30 seconds
Fluorescence read (only applicable to wells using SYBR™ Green)		

A parallel reaction using SYBR™ Green was carried out to verify that the PCR amplification was successful. In duplicate, one microliter of the respective DNA samples were

combined with five microliters SYBR™ Green master mix as well as 0.5 microliters pGI+promoter primer and 0.5 microliters pCB2+promoter primer and three microliters PCR grade water. Fluorescence in these samples using SYBR green after thermocycling could be used as a positive indication that the reaction using Titanium™ Taq polymerase was successful.

After this first PCR reaction had completed, a second nested PCR reaction using a second primer set was executed. Five microliters of DNA from each sample were placed into two separate microfuge tubes on ice. To these tubes, the following reagents were added: Five microliters 10x taq buffer, one microliter T7 Promoter Primer, one microliter dNTP mix, one microliter 50x Titanium™ taq polymerase, and 37 microliters PCR grade water. After all reagents were added to their respective microfuge tubes, the samples were mixed by gentle pipetting. Samples were then split into three separate aliquots on a PCR plate (for a total of six total samples) and thermocycled under the following parameters:

Step	Temperature	Time
Denaturation	94°C	2 minutes
20 Cycles:		
Denaturation	94°C	1 minute
Annealing	53°C	1 minute
Extension	68°C	30 seconds
Fluorescence read (only applicable to wells using SYBR™ Green)		

Again, a parallel reaction using SYBR™ Green was carried out to verify that the PCR amplification was successful. In duplicate, one microliter of the respective DNA samples was combined with five microliters SYBR™ Green master mix as well as one microliter T7 Promoter Primer and three microliters PCR grade water. Fluorescence in these samples using SYBR green after thermocycling could be used as a positive indication that the nested reaction using Titanium™ Taq polymerase was successful.

4.2.5 DNA Cleanup

DNA cleanup was performed using a GeneJET PCR purification kit (Thermo Scientific). This step was necessary to remove any residual dNTPs which would be problematic for the future TdT enzyme reaction. To do this, a 1:1 volume of binding buffer (approximately 25 microliters) was added to the previously created samples and thoroughly mixed. It was noted that the samples were a bright yellow, indicating that the pH of the solution was correct for the assay. The 50 microliters of this solution were then added to the GeneJet purification column and centrifuged at 8,000 x g for 30 seconds, discarding the permeate. Seven hundred microliters wash buffer was then added to each sample and centrifuged for 30 seconds, discarding the permeate. The columns were then transferred to new microfuge tubes and centrifuged at 8,000 x g for one minute to ensure that the columns were dry. The columns were again transferred to fresh microfuge tubes and 20 microliters elution buffer was added to each column. After centrifuging at 8,000 x g for a final minute, the microfuge tubes were capped and stored at -80°C for later steps.

4.2.6 Transcription

For both the human Jurkat and human CD8+ anti-HPV cell DNA product, transcription was carried out using T7 RNA Polymerase (New England Biolabs). To transcribe the respective DNA samples, the following were combined in a PCR plate on ice: Two microliters of the respective DNA sample, four microliters PCR grade water, 10 microliters 2xNTP, two microliters T7 reaction buffer, 0.5 microliters RNASEin (thermo scientific), and two microliters T7 enzyme mix. It should be noted that there seemed to be a precipitate formed in the T7 reaction buffer that was re-dissolved by vortexing for several minutes. After mixing the reagents by gentle pipetting, the samples were incubated in a PCR thermocycler unit for 2 hours at 37°C.

4.2.7 Hybridization of ssRNA

After the incubation stage of the previous step, the PCR thermocycler was held at 75°C for 5 minutes followed by a steady reduction to 4°C over a one hour period.

4.2.8 RNA Cleanup

RNA samples were cleaned using an RNeasy™ miniprep kit (Qiagen). To do this, each sample had 20 microliters 70% EtOH added and mixed by pipetting. After this, the whole sample was transferred to an RNeasy™ spin column and centrifuged at 8,000 x g for 15 seconds, discarding the flow-through. Seven hundred microliters of reconstituted buffer RW1 was then added, centrifuged for 15 seconds at 8,000 x g, and the flow-through again discarded. Five hundred microliters buffer RPE was added to the spin columns and centrifuged at 8,000 x g for 15 seconds. A second 500 microliters buffer RPE was then added and centrifuged for two minutes to ensure that the column was completely dry. Finally, the Rneasy spin column was placed into a clean 1.5 microliter collection tube. Thirty microliters RNase-Free water warmed to 37°C (warmed to increase yield) was then added to the spin column membrane and spun at 8,000 x g for one minute. After this, siRNA synthesis was immediately proceeded to in order to maximize yield (RNA has poor stability).

4.2.9 SiRNA Synthesis by RNase III

To create siRNA, Shortcut® RNase III (New England Biolabs) was used. The following were combined on ice: The entire 30 microliters dsRNA from the previous step, 10 microliters 10x Shortcut Reaction Buffer, 10 microliters Shortcut RNase III, 10 microliters 10x MnCL₂, and 40 microliters PCR grade water. This solution was then mixed by gentle pipetting and incubated at 37°C for 20 minutes. The reaction was then halted by the addition of 10 microliters 10x EDTA solution.

4.2.10 siRNA Precipitation

To precipitate the siRNAs, one-tenth volume 3M NaOAc, 2 microliters Rnase-free Glycogen, and 360 microliters ice-cold 95% EtOH were added. This solution was then kept at -20°C for two hours. After incubation at -20°C, the samples were spun for 15 minutes at 12,000 x g. At this point, a small pellet had formed at the bottom of each tube. The supernatant was removed by gentle pipetting, taking care not to disturb the siRNA pellet. After wiping the top of the microfuge tubes with a kim-wipe, an additional 300 microliters of ice-cold 80% ethanol pipetted over the pellet and allowed to sit for 10 minutes at room temperature. The samples were again centrifuged at 12,000 x g for 15 minutes and the supernatant removed by gentle pipetting. The RNA was then resuspended in 30 microliters molecular biology grade water and stored at -80°C for future experimentation.

4.3 Results

All PCR samples ran successfully as indicated by fluorescence in the parallel qPCR reactions using SYBR green.

4.4 Notes

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Chapter 5. Synthesis of SiRNA Designed with a Computational Method

Note: This section contains code snippets written in the visual basic language. The full source code can be found in Appendix B.

5.1 Introduction

Although the previously described “shotgun” SiRNA synthesis method has many benefits, including low chance of failure in RNA interference, it presents a number of potential issues if intended for use as a therapeutic. Of these issues, the largest is likely the fact that with so many created siRNA species, some siRNA species will exhibit off-targeting effects. These effects can be avoided by creating specific siRNA species which have been blast searched against the human genome to avoid siRNA species with high levels of homology to genes that are unrelated to our target.

As previously mentioned, a major hurdle in creating siRNA for a particular gene is the high level of variability in siRNA effectiveness. Fortunately, many algorithms have been proposed based on meta data of successful siRNA species. Taken together, these algorithms can predict the effectiveness of a given siRNA species with a fair degree of accuracy. Of the prediction algorithms, one of the most popular and highly tested are the algorithms from Reynolds *et al* 2004. Roughly, the algorithms put forward by Reynolds *et al* state the following:

- An siRNA should have an overhang of two U residues on the 3' end of both strands.
- There should be an A residue in the 3 position
- There should be an A residue in the 19th position
- There should not be a G in position 13
- There should be a U in position 10
- There should not be a C or G in position 19

- There should not be a palindromic sequence of 4 or more bases
- The GC content of the siRNA should fall between 30% and 52%
- There should be at least one A or U residue in positions 15 through 19
- There should not be a run of four consecutive A residues
- There should not be a run of four consecutive U residues

After having established the rules for the design of siRNA in this experiment, it became necessary to consider the method of synthesis used to create the siRNA. As the siRNA is intended as a therapeutic, it would be ideal for whatever method is being used to be high throughput, have a low turnaround time, and require minimal sophisticated equipment. Based on these parameters, it was decided that a conventional oligo-based synthesis method be used (an example of an oligo-based siRNA design kit being a Silencer™ siRNA construction kit (Thermo scientific)). The general principle behind this type of method is that a DNA oligo for the complimentary sequence of the siRNA intended to be synthesized is purchased. To this DNA oligo, a T7 promoter is added. The compliment of this DNA sequence with a T7 promoter is also purchased. Both DNA strands are then transcribed into complimentary single stranded RNAs and allowed to anneal in such a way that they form completed siRNAs.

The methods required to both identify potential siRNA targeting sites as well as create templates to synthesize said RNAs are both complicated and time consuming. Due to the size and randomness of the TCRb gene, it is not viable to do most of these calculations by hand. To alleviate this problem, a computer program which automates the siRNA targeting site identification and subsequent DNA template design was created. This chapter outlines both the design philosophy and code used to meet this goal.

5.1.1 Creation of Scoring System and Implementation of Algorithms

For each discriminant put forward by Reynolds *et al*, a corresponding function was created. Each of these functions receives a variable “siRNA” (as text) and returns a true or false value as to whether or not the rules of the discriminant are met.

The following function checks position 3 of a given siRNA for the nucleotide “A”. If the nucleotide is present, the Boolean value “True” (1) is returned. Otherwise, “False” (0) is returned.

```
Function Discrim1(ByVal siRNA As String) As Boolean  
  
    Dim bool As Boolean = False  
  
    If GetChar(siRNA, 3) = "a" Or GetChar(siRNA, 3) = "A" Then  
  
        bool = True  
  
    End If  
  
    Return bool  
  
End Function
```

This function looks for an “A” nucleotide in position 19 and returns the Boolean value “True” if it is found or “False” if it is not.

```
Function Discrim2(ByVal siRNA As String) As Boolean  
  
    Dim bool As Boolean = False  
  
    If GetChar(siRNA, 19) = "a" Or GetChar(siRNA, 19) = "A" Then  
  
        bool = True  
  
    End If  
  
    Return bool  
  
End Function
```

In this function, “G” is checked for in position 13. If G is **not** found, “True” is returned (This is a negative discriminant).

```
Function Discrim3(ByVal siRNA As String) As Boolean

    Dim bool As Boolean = False

    If Not GetChar(siRNA, 13) = "g" And Not GetChar(siRNA, 13) = "G" Then

        bool = True

    End If

    Return bool

End Function
```

This function checks for a “U” residue in position 10. If the U residue is found, “True” is returned.

```
Function Discrim4(ByVal siRNA As String) As Boolean

    Dim bool As Boolean = False

    If GetChar(siRNA, 10) = "u" Or GetChar(siRNA, 10) = "U" Then

        bool = True

    End If

    Return bool

End Function
```

The below function checks position 19 for the absence of both “C” and “G” residues. If neither are present, “True” is returned (negative discriminant).

```
Function Discrim5(ByVal siRNA As String) As Boolean

    Dim bool As Boolean = False

    If Not GetChar(siRNA, 19) = "c" And Not GetChar(siRNA, 19) = "C" And Not
GetChar(siRNA, 19) = "g" And Not GetChar(siRNA, 19) = "G" Then

        bool = True

    End If

    Return bool

End Function
```

The following function checks for even numbered palindromes of length 4 or greater. If a Palindrome is found, “True” is returned.

```
Function Palindrome_check_4_units(ByVal siRNA As String) As Boolean

    Dim bool As Boolean = False

    Dim reverse_sequence As String = Strings.StrReverse(siRNA)

    Dim count As Integer = 0

    Dim forward As String = Nothing

    Dim reverse As String = Nothing

    Do While count < siRNA.Length - 4

        forward = siRNA.Remove(0, count)

        forward = siRNA.Substring(0, 4)

        Dim count2 As Integer = 0
```

```

Do While count2 < siRNA.Length - 4
    reverse = siRNA.Remove(0, count)
    reverse = siRNA.Substring(0, 4)
    If forward = reverse Then
        bool = True
    End If
    count2 = count2 + 1
Loop
count = count + 1
Loop
Return bool
End Function

```

This function checks for odd numbered palindromes of length 5 or greater. If a palindrome is found, “True” is returned.

```

Function Palindrome_check_5_units(ByVal siRNA As String) As Boolean
    Dim bool As Boolean = False
    Dim reverse_sequence As String = Strings.StrReverse(siRNA)
    Dim count As Integer = 0
    Dim forward As String = Nothing
    Dim reverse As String = Nothing
    Do While count < siRNA.Length - 5
        forward = siRNA.Remove(0, count)

```



```

forward = siRNA.Substring(0, 5)

Dim count2 As Integer = 0

Do While count2 < siRNA.Length - 5

    reverse = siRNA.Remove(0, count)

    reverse = siRNA.Substring(0, 5)

    If forward = reverse Then

        bool = True

    End If

    count2 = count2 + 1

Loop

count = count + 1

Loop

Return bool

End Function

```

This function Checks for the GC content of a given siRNA strand and returns a corresponding value between 0 and 1.0.

```

Function Get_GC(ByVal siRNA As String, siRNA_length As Integer) As Double

    Dim GC_content As Double = 0

    Dim count As Integer = 0

    Dim ATU_count As Integer = 0

    Do While count < siRNA.Length

```

```

    If GetChar(siRNA, count + 1) = "a" Or GetChar(siRNA, count + 1) = "A" Or
GetChar(siRNA, count + 1) = "t" Or GetChar(siRNA, count + 1) = "T" Or GetChar(siRNA,
count + 1) = "u" Or GetChar(siRNA, count + 1) = "U" Then

        ATU_count = ATU_count + 1

    End If

    count = count + 1

Loop

GC_content = 1 - (ATU_count / siRNA_length)

Return GC_content

End Function

```

The below function looks for A, U, or T residues between residues 15 and 19. The function returns “True” if a residue is found.

```

Function AUT_in_15_through_19(ByVal sirna As String, sirna_Length As Integer) As Boolean

    Dim bool As Boolean = False

    Dim char15 As Char = GetChar(sirna, 15)

    Dim char16 As Char = GetChar(sirna, 16)

    Dim char17 As Char = GetChar(sirna, 17)

    Dim char18 As Char = GetChar(sirna, 18)

    Dim char19 As Char = GetChar(sirna, 19)

    If char15 = "a" Or char15 = "A" Or char15 = "t" Or char15 = "T" Or char15 = "u" Or char15 =
"U" Then

        bool = True
    End If

End Function

```

```

End If

If char16 = "a" Or char16 = "A" Or char16 = "t" Or char16 = "T" Or char16 = "u" Or char16 =
"U" Then

    bool = True

End If

If char17 = "a" Or char17 = "A" Or char17 = "t" Or char17 = "T" Or char17 = "u" Or char17 =
"U" Then

    bool = True

End If

If char18 = "a" Or char18 = "A" Or char18 = "t" Or char18 = "T" Or char18 = "u" Or char18 =
"U" Then

    bool = True

End If

If char19 = "a" Or char19 = "A" Or char19 = "t" Or char19 = "T" Or char19 = "u" Or char19 =
"U" Then

    bool = True

End If

Return bool

End Function

```

This function checks for a leading “AA” sequence in the mRNA being targeted (The siRNA complement will be “UU”).

```
Function Check_for_AA(ByVal siRNA As String) As Boolean
```

```

Dim AA_Bool As Boolean = False

Dim Score As Integer = 0

If GetChar(siRNA, 1) = "A" Or GetChar(siRNA, 1) = "a" Then
    Score = Score + 1
End If

If GetChar(siRNA, 2) = "A" Or GetChar(siRNA, 2) = "a" Then
    Score = Score + 1
End If

If Score = 2 Then
    AA_Bool = True
End If

Return AA_Bool

End Function

```

In this function, the algorithm checks for a running repeat of either four “A” or four “T” residues. If this is found, “True” is returned.

```

Function Check_for_running_T_or_A(ByVal siRNA As String, sirna_length As Integer) As
Boolean

    Dim Poly_T_A As Boolean = False

    Dim count As Integer = 0

    Dim tempstring As String = Nothing

    Do While count < sirna_length - 4
        tempstring = siRNA

```

```

tempstring = tempstring.Remove(0, count) 'remove leading characters in temporary string
tempstring = tempstring.Substring(0, 4) ' trims temporary string to 4 characters

If tempstring = "aaaa" Or tempstring = "AAAA" Or tempstring = "TTTT" Or tempstring =
"tttt" Or tempstring = "UUUU" Or tempstring = "uuuu" Then

    Poly_T_A = True

    count = 9999999

    'breaks loop

End If

count = count + 1

Loop

Return Poly_T_A

End Function

```

5.1.2 SiRNA Scoring

Scoring of siRNA candidates is based on the level of statistical significance assigned to each individual discriminant by Reynolds *et al.* The respective “point value” of 7 of the 11 discriminants can be seen in the following list:

A in position 3	16.8
A in position 19	22.1
No G in position 13	14.1
U in position 10	30.6
No C or G in position 19	25.6
No palindromes of 4 or more	10.1
At least one A or U in 15 through 19	16.9

Four of the eleven discriminants were considered absolutely necessary for an siRNA to be successful (and were therefore not assigned a score). These four discriminants can be seen in the table below:

No four consecutive A residues
No four consecutive U residues
The GC content must fall between 30% and 52%
There should not be a palindrome of 4 or more bases

To apply the respective scores to each siRNA, the following code was used:

```
Do While count < codelength - SiRNA_Length

  Dim GC_content As Double = Get_GC(DataArray(count), SiRNA_Length)

  If Discrim1(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 16.8

  End If

  If Discrim2(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 22.1

  End If

  If Discrim3(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 14.1

  End If

  If Discrim4(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 30.6

  End If

  If Discrim5(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 25.6
```

```

End If

If not Palindrome_check_4_units(DataArray(count)) And not
Palindrome_check_5_units(DataArray(count)) Then

    SiRNA_Score = SiRNA_Score + 10.1

End If

If AUT_in_15_through_19(DataArray(count), DataArray(count).Length) Then

    SiRNA_Score = SiRNA_Score + 16.9

End If

If Check_for_running_T_or_A(DataArray(count), DataArray(count).Length) Then

    'Lowers score to a level where they will be ignored by future algorithms

    SiRNA_Score = SiRNA_Score - 20000

End If

If Not Check_for_AA(DataArray(count)) Then

    'Lowers score to a level where they will be ignored by future algorithms

    SiRNA_Score = SiRNA_Score - 20000

End If

If Get_GC(DataArray(count), DataArray(count).Length) < 0.3 Or
Get_GC(DataArray(count), DataArray(count).Length) > 0.52 Then

    'Lowers score to a level where they will be ignored by future algorithms

    SiRNA_Score = SiRNA_Score - 20000

End If

ScoreArray(count) = SiRNA_Score

SiRNA_Score = 0

```

```
count = count + 1
```

```
Loop
```

By assigning a score of “-20,000” to siRNAs that do not meet the necessary discriminants, these siRNA are never returned to the user.

5.1.3 Identification of V, D, J, and C Regions

For the user’s information, an algorithm was designed to identify the V, D, J, and C regions of a given RNA input. This was simply done by exploiting the fact that the V region is typically 340 residues long, the D region is 16 residues long, and the J region is 50 residues long. The following code was used to split the regions:

```
If Not CheckBox1.Checked Then
  Do While TextBox1.Text.Length > count
    If count <= 340 Then
      RichTextBox2.Text = RichTextBox2.Text + GetChar(TextBox1.Text, count + 1)
    End If
    If count > 340 And count <= 356 Then
      RichTextBox3.Text = RichTextBox3.Text + GetChar(TextBox1.Text, count + 1)
    End If
    If count > 356 And count <= 406 Then
      RichTextBox4.Text = RichTextBox4.Text + GetChar(TextBox1.Text, count + 1)
    End If
    If count > 406 Then
      RichTextBox5.Text = RichTextBox5.Text + GetChar(TextBox1.Text, count + 1)
```



```
End If
```

```
count = count + 1
```

```
Loop
```

```
End If
```

5.1.4 Addition of T7 Promoter

As the goal of this program is to advise the user on what DNA oligoes are required to create the siRNA of interest, an algorithm was used to systematically create complimentary primer pairs with T7 promoters designed to synthesize complimentary RNA strands which will create siRNA when hybridized. This is accomplished with the following code:

```
Do While count < codelength - SiRNA_Length
```

```
  If ScoreArray(count) > 0 Then
```

```
    'Displays siRNA candidate with respective score adjacent
```

```
    RichTextBox1.Text = RichTextBox1.Text + DataArray(count) + " " +
```

```
ScoreArray(count).ToString + vbCrLf
```

```
    'Displays siRNA candidate with the promoter sequence added
```

```
    RichTextBox6.Text = RichTextBox6.Text + DataArray(count) + "CCTGTCTC" + "
```

```
" + ScoreArray(count).ToString + vbCrLf
```

```
    'Removes leading AA to get compliment without overhang
```

```
    DataArray(count).Remove(0, 2)
```

```
    'Displays compliment and adds promoter sequence as well as AA overhang to the  
compliment
```

```
RichTextBox6.Text = RichTextBox6.Text + "AA" +  
Convert_to_Compliment(DataArray(count)) + "CCTGTCTC" + vbCrLf + vbCrLf  
  
End If  
  
count = count + 1  
  
Loop
```

5.1.5 User Interface

The user interface for this program was designed to both be all-inclusive and easily accessible. The two primary user inputs are the TCRb sequence as well as the desired siRNA length (with 23 as the optimal default). Additionally, the user may specify if their input only contains the CDR3 region, is the RNA compliment, or is the cDNA of the TCRb sequence. The user may also specify if they would like a number of residues trimmed from the front or back of the TCRb sequence (as interior sequences tend to be more favorable for inhibition by siRNA). The code for all of these user inputs (all of which are code-commented) can be found in appendix B. The user interface before having received input can be seen below:

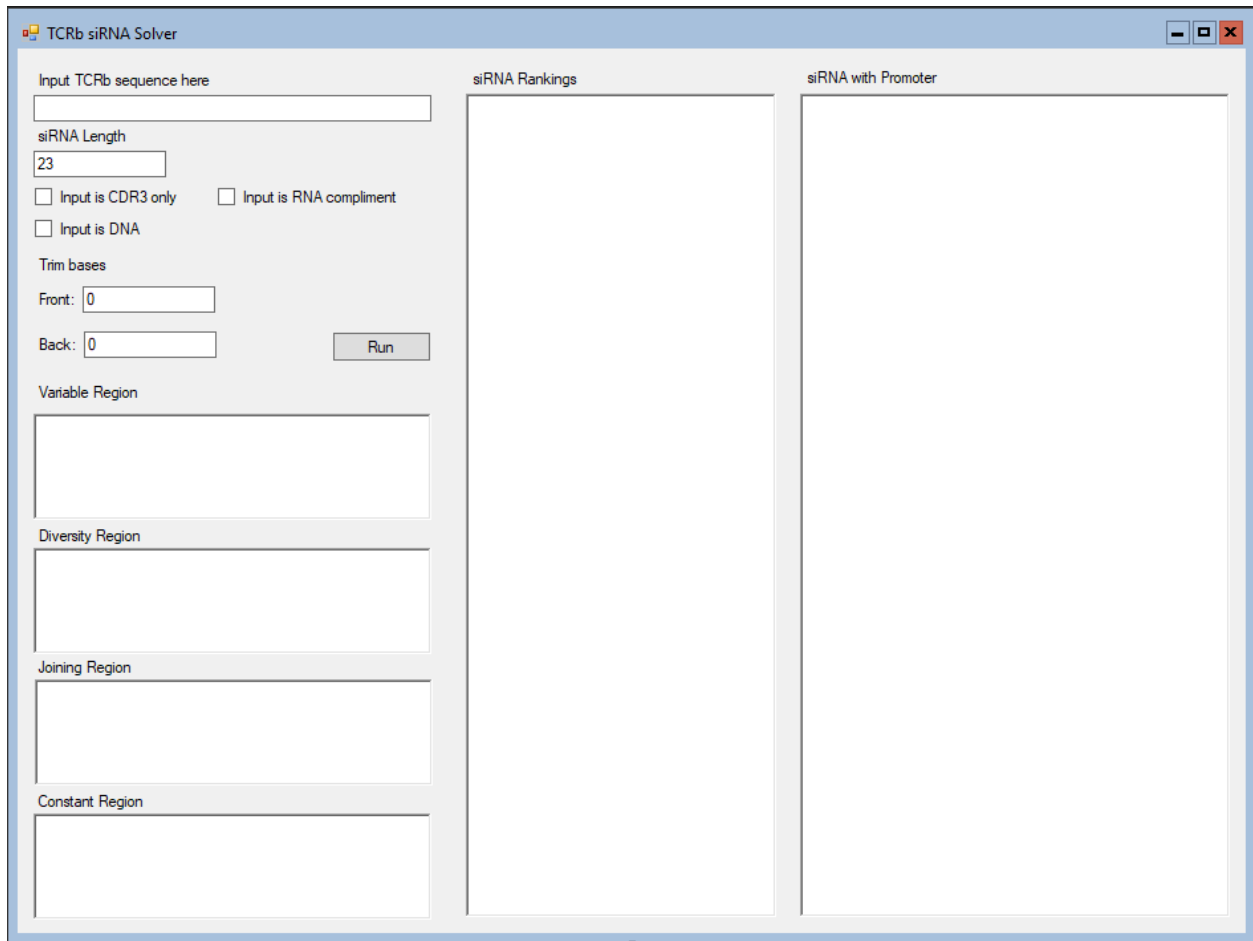


Figure 5.1. TCRb siRNA Solver user interface

The user interface after having received a TCRb sequence can be seen here:

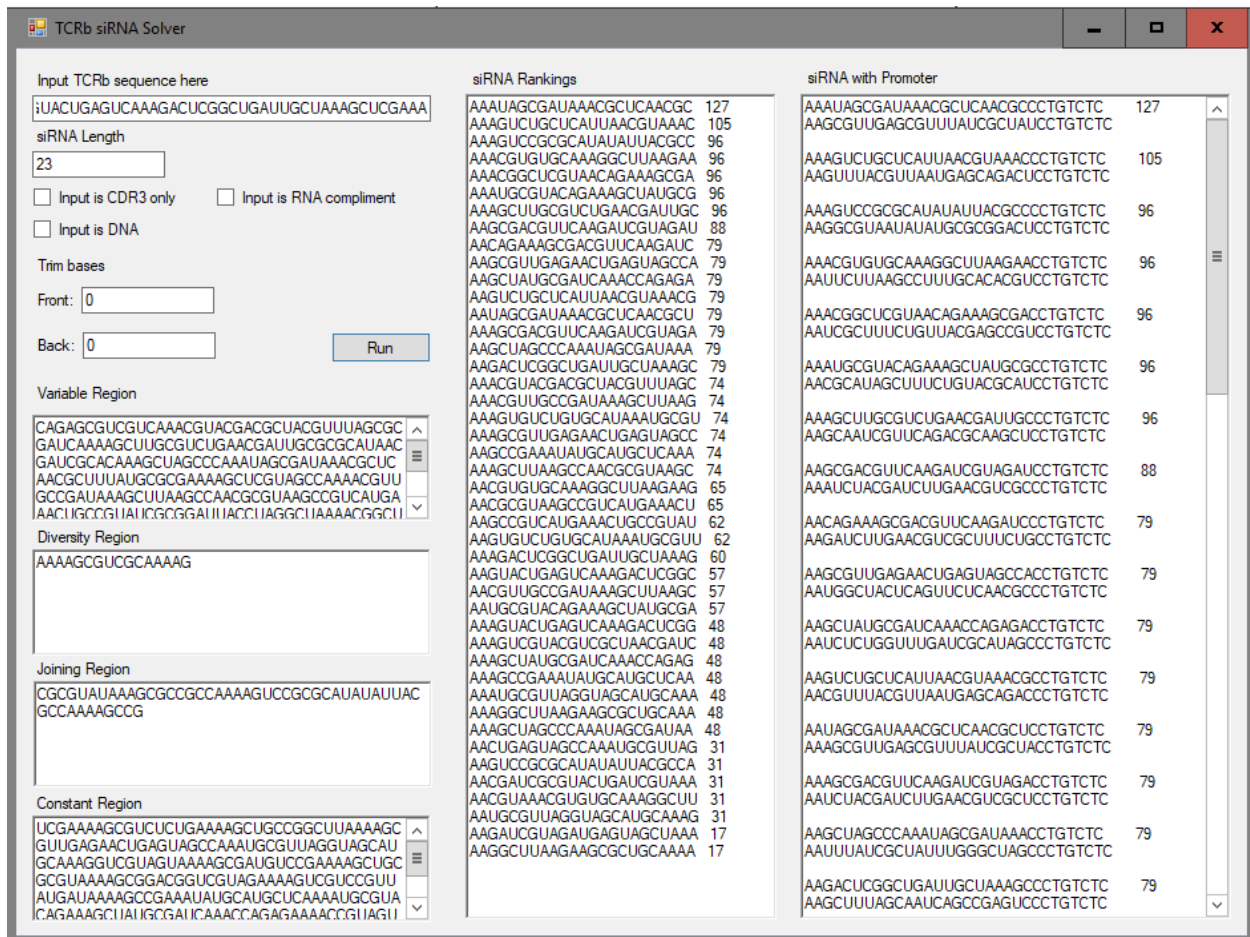


Figure 5.2. TCRb siRNA Solver user interface after having run data

5.2 Conclusion

The described program was tested for accuracy against hand calculations for the same TCRb sequences. After extensive debugging, it was found that the calculations were identical in all cases. Additionally, the calculations done by the program are not only far less time consuming but are also far less error prone (especially given the complexity required to make this type of calculation).

5.3 Notes

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Han, Y., He, F., Chen, Y., Liu, Y., & Yu, H. (2018). SiRNA silencing efficacy prediction based on a deep architecture. *BMC Genomics*, 19(S7). <https://doi.org/10.1186/s12864-018-5028-8>

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Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S., & Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nature Biotechnology*, 22(3), 326–330. <https://doi.org/10.1038/nbt936>

Chapter 6. Testing of siRNA on an *Ex Vivo* Immune Cell Model and Conclusions

6.1 Introduction

To test the efficacy of the various siRNA formulations previously described, qPCR was utilized. The qPCR experiment looked at the ability of each siRNA formulation to knock down three genes: TCRb, CD3, and CD137. In theory, TCRb should be directly knocked down as a result of the siRNA causing the TCRb mRNA to be degraded directly. CD3 is a co-receptor which conjugates to a completed T cell receptor upon its migration to the cell surface. A feedback loop decreases the level of CD3 in the absence of TCRb expression due to translated CD3 receptors being unable to migrate to the T cell surface. The final gene, CD137, is expressed by activated T cells. T cells which do not express their receptor complexes are unable to undergo activation and are therefore incapable of expressing CD137 at normal levels. β -actin was also measured to normalize the genes of interest.

In addition to testing for the expression of the aforementioned genes, cell viability was also tested. This is important as the intended mechanism of the siRNA is to prevent the cells from expressing their receptors while leaving their viability unaffected. Although T cells which do not express their receptors should be unable to proliferate at any significant level, the lack of receptors should not be directly toxic to the cells. Cell viability analysis was done both by MTS assay as well as direct count with trypan blue.

An important consideration for this type of therapy is that the siRNA synthesized for one particular T cell line should not be effective against other cell lines. This is important because the major benefit of using siRNA synthesized with the methods previously described is the isolation of the effect to only “pathogenic” T cell lines (as opposed to all cell lines being affected with a therapy such as corticosteroids). Additionally, negative controls were used to ensure any effects

witnessed were a result of the siRNA cocktails and not the vectors being used to deliver the therapies (lipofectamine™ (Thermo Scientific)).

6.2 Methods

6.2.1 SiRNA Synthesis Using a “Shotgun” Method

For both human Jurkat, and human CD8+ anti-HPV cells, siRNA was synthesized using the previously described “shotgun” method. These siRNAs were frozen and stored at -80°C for further experimentation.

6.2.2 SiRNA Synthesis Using a Computational Method

For both human Jurkat, and human CD8+ anti-HPV cells, the mRNA sequence of their respective TCRb genes were sequenced. After the sequence had been obtained for both samples, the three highest scoring primer pairs were determined using the previously created siRNA solver program. These primer pairs were purchased for use in the synthesis of siRNA for the respective TCRb genes. The six primer pairs used for siRNA synthesis and their corresponding scores can be found in the following table.

Table 6.1. List of Oligos and Corresponding Scores.

Sample	Oligo one	Oligo two	Score
Human Jurkat	AAGAAUCGAUUGUUGGGCAUACCCC TGTCTC	AAGGUAUGCCCAACAAUCGAUUCCC TGTCTC	110
	AAACCAGGACCCCAGAAGAGAAUCC TGTCTC	AAAUUCUCUUCUGGGGUCCUGGUCC TGTCTC	96
	AAAGGGUUAACAUGACCCGAAAUUCC TGTCTC	AAAAUUUCGGGUCAUGUAACCCUCC TGTCTC	82
Human CD8+ anti-HPV	AAGCUUAGCUCUAAGGUGUCAGGCC TGTCTC	AACCUGACACCUUAGAGCUAAGCCC TGTCTC	88
	AAGAUUAUCAUGUCCAGAAUUGCC TGTCTC	AACAAUUUCUGGACAUGAUAAUCCC TGTCTC	79
	AAGCCUGGAAACCAUGCUAAGCUCC TGTCTC	AAAGCUUAGCAUGGUUCCAGGCC TGTCTC	79

After the primers had been obtained, synthesis of siRNA was carried out using a Silencer™ siRNA construction kit (Thermo Scientific). To do this, template oligonucleotides

were suspended to a concentration of 100 micromolar in nuclease free water. The templates were then hybridized to the T7 promoter primer provided with the Silencer™ SiRNA construction kit by mixing two microliters of each one of the six oligonucleotides with six microliters hybridization buffer as well as two microliters T7 promoter primer. The mixture was then heated to 70 °C for 5 minutes and subsequently allowed to sit at room temperature for an additional 5 minutes. After hybridization with the T7 primer, the gap left behind was filled in with Klenow DNA polymerase. This was done by adding the following directly to each hybridization mixture: two microliters 10x Klenow reaction buffer, two microliters dNTP mix, four microliters nuclease-free water, and two microliters of Exo-Klenow enzyme. This mixture was mixed by slow pipetting and transferred to a 37°C bead-bath for 30 minutes.

To assemble the transcription reaction, two microliters of each of the previous samples combined in an ependorf tube on ice with the following: four microliters nuclease-free water, 10 microliters NTP mix, two microliters 10x T7 reaction buffer, and two microliters T7 enzyme mix. It should be noted that a considerable degree of precipitate had formed on top of the T7 reaction buffer which required vortexing to resuspend. All components were mixed by gentle pipetting and the reaction was incubated in a 37°C bead-bath for two hours. After the initial incubation, each of the primer pairs were combined (leaving six total samples) and allowed to continue incubation at 37°C overnight (12 hours).

After the samples had been incubated overnight, each ~40 microliter sample had the following added: six microliters digestion buffer, 2.5 microliters DNase, three microliters RNase, and 48.5 microliters nuclease-free water. This reaction was mixed by gentle pipetting and allowed to incubate for two hours at 37°C. After preparing both the siRNA binding buffer and siRNA wash buffer as described by the manufacturer, 400 microliters of siRNA binding buffer

was added to each sample and allowed to incubate for 5 minutes at room temperature. Nuclease-free water was then heated to 75°C for later use. Six siRNA filter cartridges were placed into 2mL collection tubes and 100 microliters siRNA wash buffer was added to each of these cartridges. Each siRNA sample with binding buffer was added to separate tubes and centrifuged at 10,000 x g for one minute. The flow-through of each sample was discarded and the collection tubes were replaced. Each cartridge was then washed twice with 500 microliters siRNA wash buffer by spinning the columns at 10,000 x G for one minute with the flow-through being discarded. Finally, siRNA was eluted from each cartridge by adding 100 microliters nuclease-free water heated to 75°C and centrifuging for 2 minutes at 12,000 x g. The siRNA created were stored at -80°C for later use.

6.2.3 SiRNA Lipofection

The eight separate siRNA samples created from previous steps were lipofected using Lipofectamine™ RNAiMax lipofection reagent (Thermo Scientific). For each sample, fifty microliters Opti-MEM™ (Thermo Scientific) medium was combined with three microliters Lipofectamine™ RNAiMAX reagent. For each sample, in a separate tube, 50 microliters Opti-MEM™ medium was combined with 10 picomoles siRNA (concentration determined by nanodrop). The diluted lipofectamine and diluted siRNA for each respective sample were then combined and allowed to incubate at room temperature for 5 minutes.

6.2.4 Treatment of Cell Lines with SiRNA

After allowing both the human Jurkat and human CD8+ anti-HPV cells to grow for 48 hours, Cells were seeded at 200,000 cells per 190 microliters of media in a 96 well plate in triplicate. To each of these wells, ten microliters of the respective lipofected siRNA mix was

added (for an addition of one picomol siRNA per well). Samples were then allowed to incubate for 24 hours at 37°C and analyzed after incubation.

6.2.5 qPCR

One hundred microliters of each sample (approximately 100,000 cells) were removed from each well and processed through an RNeasy mini kit (Qiagen). Samples were spun down and their supernatant removed by gentle pipetting. The cell pellets that formed were resuspended in 350 microliters buffer RLT. These cells were then vortexed to homogenize, and added to RNeasy spin columns placed in collection tubes. These samples were then spun at 8,000 x g for 15 seconds and their flow-through discarded. After this, 700 microliters buffer RW1 was added to each sample, centrifuged at 8,000 x g for 15 seconds, and the flow-through again discarded. Two separate 500 microliter aliquots of buffer RPE were then added to each sample, centrifuged at 8,000 x g for 15 seconds, and the flow through discarded. After having placed each sample in a fresh collection tube, the RNA was eluted by placing 30 microliters of RNase-free water to each column and centrifuging at 8,000 x g for a final minute.

After each samples RNA had been isolated, qPCR was performed using a SuperScript™ IV One-Step RT-PCR system (Invitrogen). The three genes being tested for each sample were TCRb, CD3, CD137, and β -actin (normalization). These samples used primers TCRb-f, TCRb-r, CD3-f, CD3-r, CD137-f, CD137-r, β -actin-f, and β -actin-r respectively. The sequences for these primers can be found in appendix a. To execute qPCR, 25 microliters 2x Platinum™ RT-PCR Master Mix was combined with two microliters of the previously created RNA samples, 2.5 microliters of each sample's respective primers at 10 micromolar (5 microliters primer per sample), 0.5 microliters superscript IV enzyme mix, and 17.5 microliters nuclease-free water.

These samples were then mixed by gentle pipetting and run under the following thermo-cycler parameters:

Step	Temperature	Time
Reverse Transcription	55C	1 minutes
Denaturation	98C	2 minutes
Amplification (40 cycles)	98C	10 seconds
	58C	10 seconds
	72C	1 minute
Final Extension	72C	5 minutes

After thermocycling, samples had their CT values normalized by their respective β -actin expression and were compared by the $\Delta\Delta$ CT method. All qPCR was performed in triplicate for each of the three replicates for each sample (for a total of 9 replicates for each treatment group).

6.2.6 Cell Viability by Trypan Blue Count

To quantify cell viability by direct count, 10 microliters of each sample were removed from each well and placed into separate microfuge tubes. To each tube, an equal volume of trypan blue was added. These cells were then counted under a microscope and their values compared to one another. This assay was performed for each of the triplicate for all samples.

6.2.7 Cell Viability by MTS

To quantify cell viability, MTS reagent (Abcam) was obtained. The remaining 90 microliters microliters (~90,000 cells) of each well was combined with 10 microliters MTS reagent and incubated for 4 hours at 37°C. After brief agitation, the plate was analyzed spectrophotometrically at an absorbance value of 490 nm.

6.3 Results

6.3.1 qPCR Results

Gene expression for all samples were quantified relative to the negative control. The results for each of the three genes for both human Jurkat and human CD8+ anti-HPV cells can be seen in the graphs below:

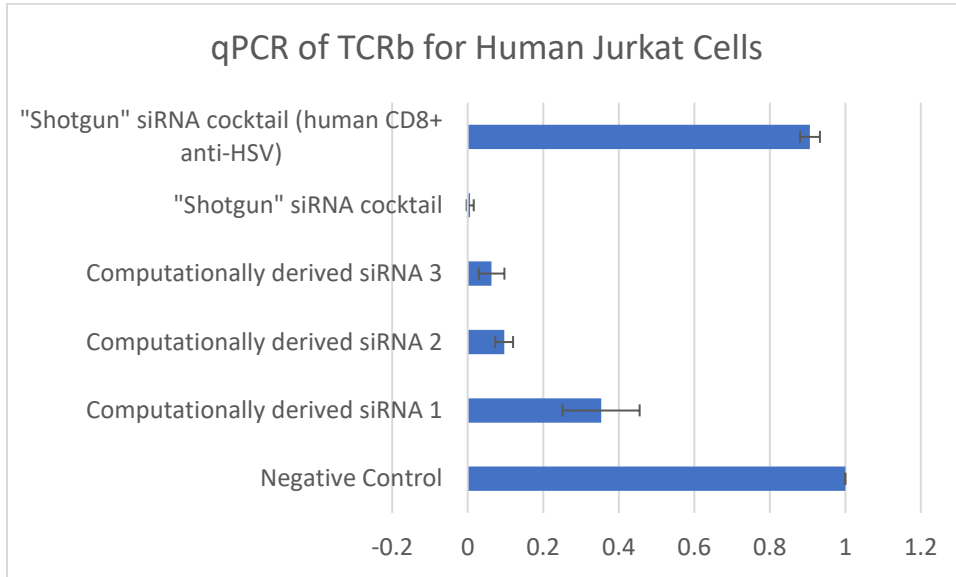


Figure 6.1. qPCR results for TCRb on human Jurkat cells.

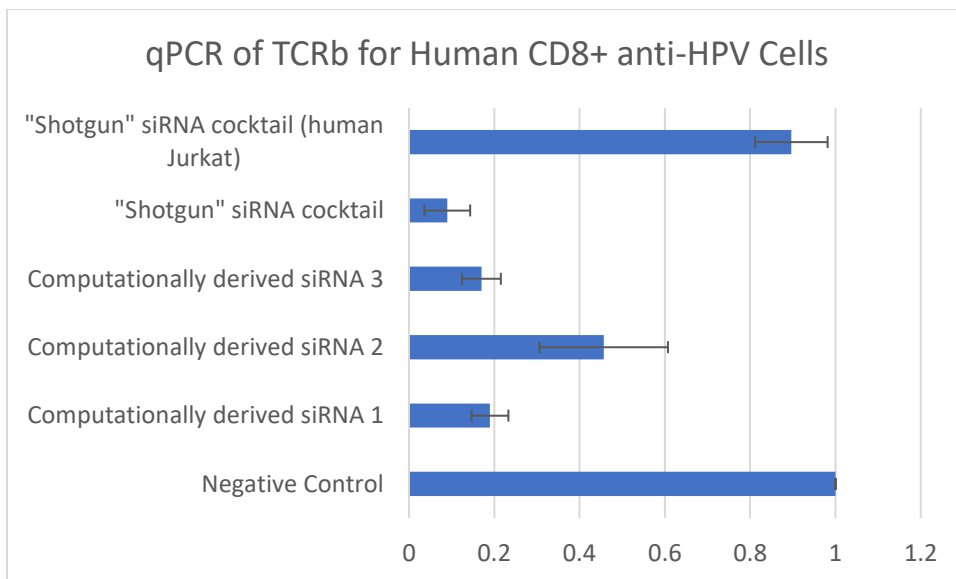


Figure 6.2. qPCR of TCRb for human CD8+ anti-HPV cells

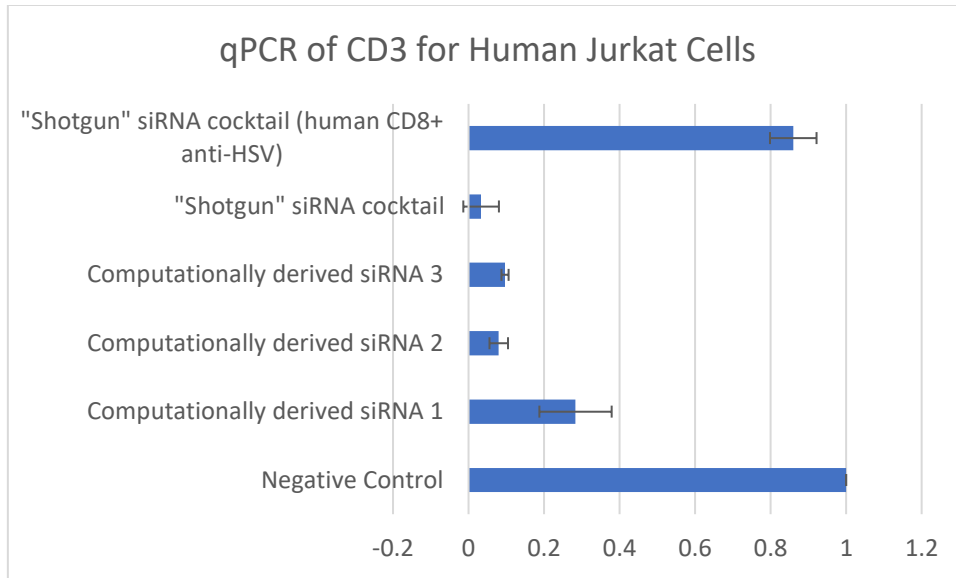


Figure 6.3. qPCR results for CD3 on human Jurkat cells.

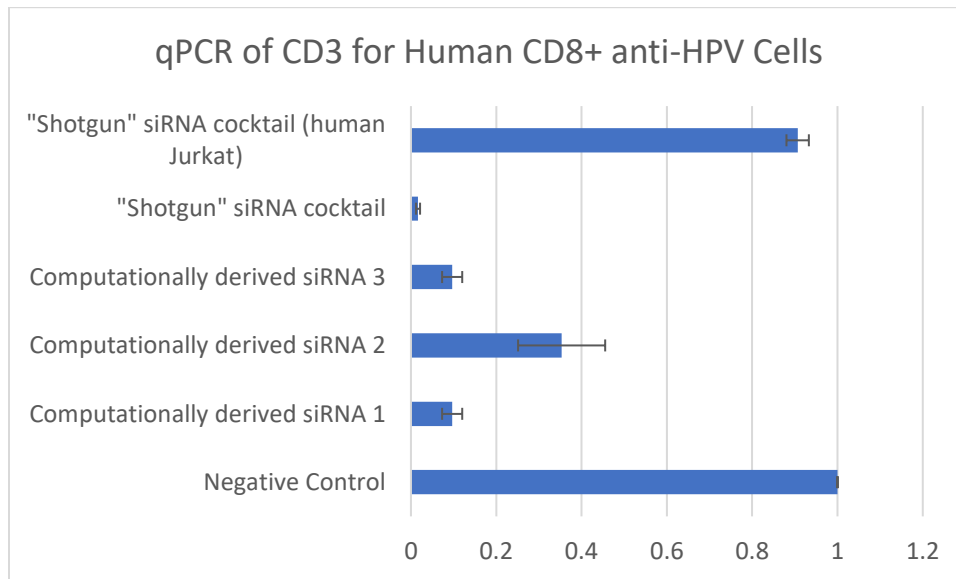


Figure 6.4. qPCR of CD3 for human CD8+ anti-HPV cells

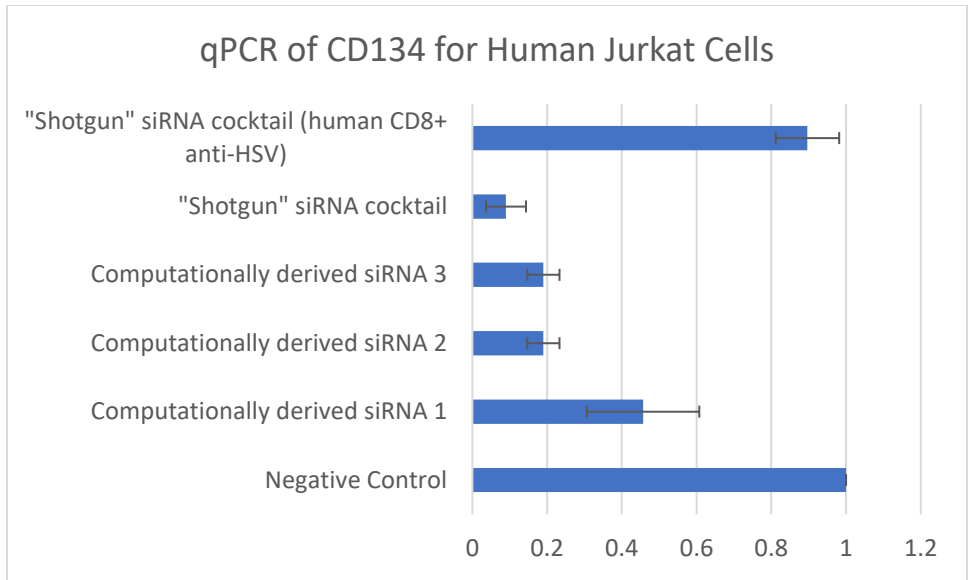


Figure 6.5. qPCR results for CD137 on human Jurkat cells.

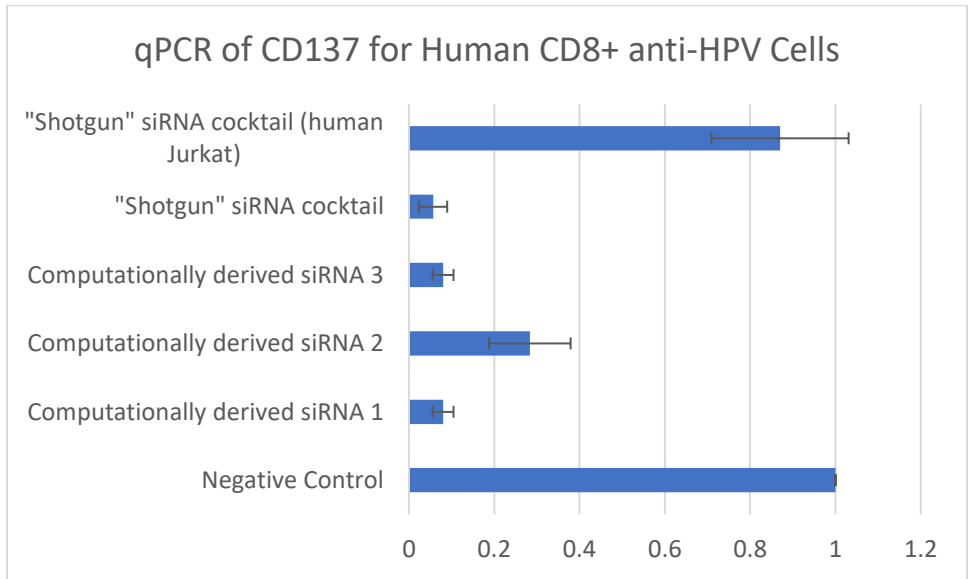


Figure 6.6. qPCR of CD137 for human CD8+ anti-HPV cells

6.3.2 Cell Viability by MTS

Cell viability via MTS was calculated relative to the negative control. The results for the human Jurkat cells can be seen in the graph below:

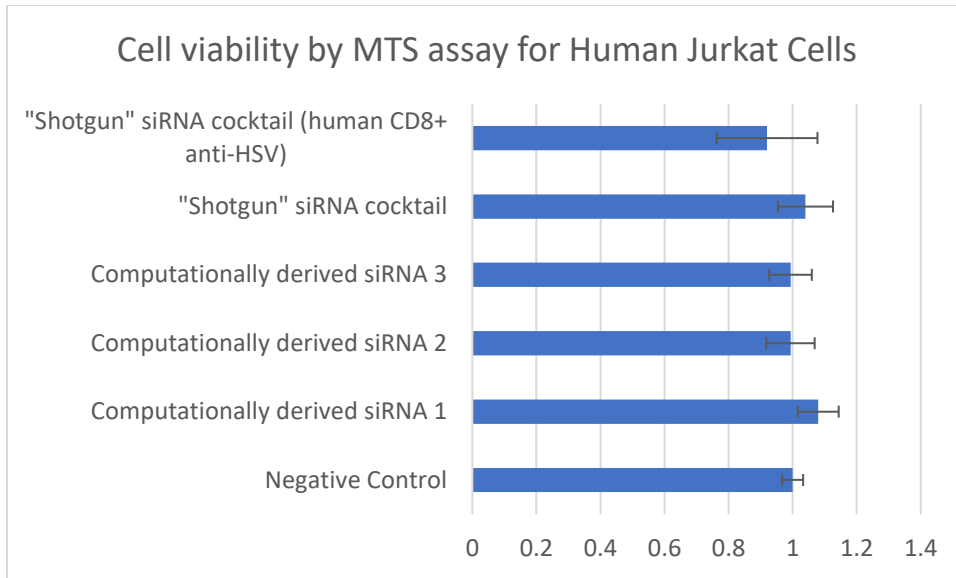


Figure 6.7. Cell viability count results utilizing MTS on human Jurkat cells

All samples demonstrated no significant difference from the control with a P value > 0.05 for all comparisons.

The results for the human CD8+ anti-HPV cells can be seen in the below graph:

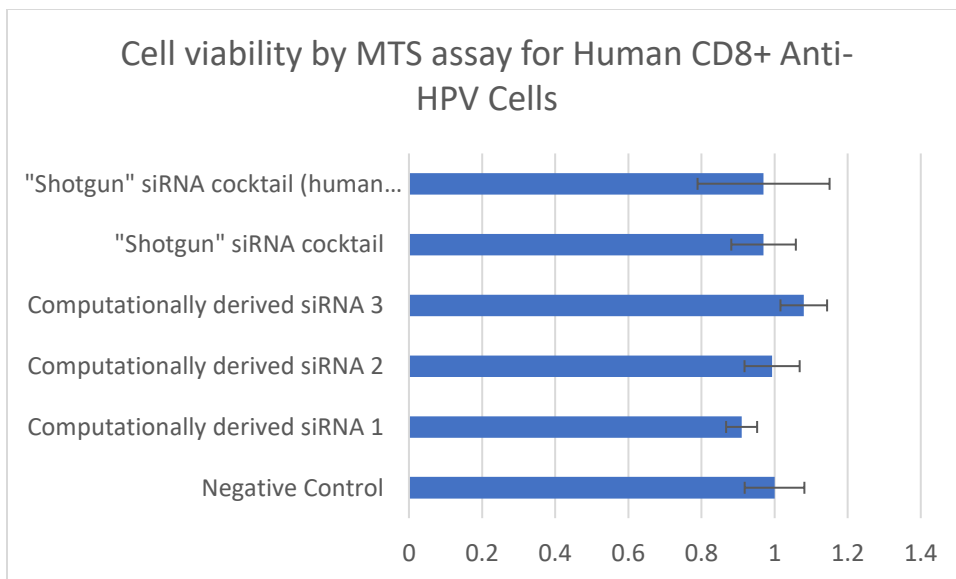


Figure 6.8. Cell viability results utilizing MTS on human CD8+ anti-HPV cells

All samples demonstrated no significant difference from the control with a P value > 0.05 for all comparisons.

6.3.3 Cell Viability by Trypan Blue

Cell viability via Trypan blue was quantified relative to the negative control. The results for human Jurkat cells can be seen below:

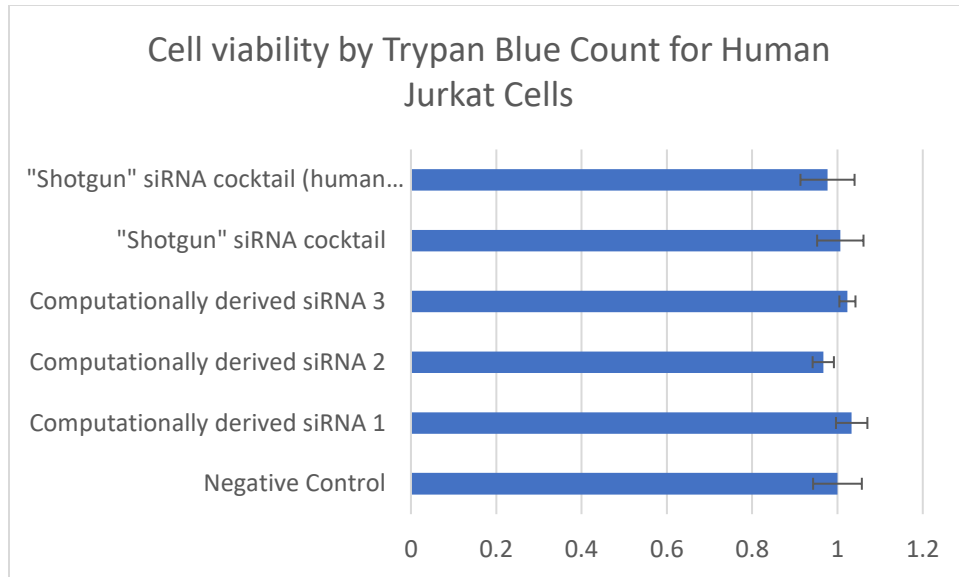


Figure 6.9. Cell viability count results utilizing trypan blue on human Jurkat cells

All samples demonstrated no significant difference from the control with a P value > 0.05 for all comparisons.

Results for the human CD8+ anti-HPV cells can be seen here:

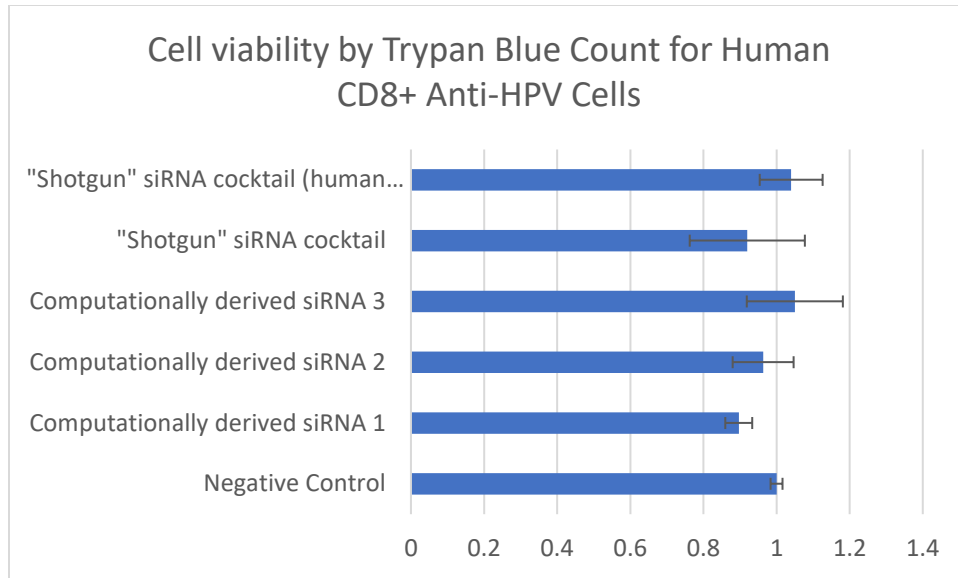


Figure 6.10. Cell viability count results utilizing trypan blue on human CD8+ anti-HPV cells

All samples with the exception of the computationally derived siRNA #1 did not vary significantly from the control with a P value > 0.05.

6.4 Discussion

These results demonstrated a high level of effectiveness for all siRNA species targeting TCRb. Additionally, cocktails for human Jurkat used to treat human CD8+ anti-HPV cells had a minimal effect on gene expression. The reverse observation was the same, with siRNA synthesized for human CD8+ anti-HPV cells having minimal effect on human Jurkat cells. These observations taken together not only demonstrate the potency of siRNA synthesized with the methods outlined, but the high level of targeting specificity was demonstrated as well. It should be noted that only a single siRNA species seemed to have an impact on cell viability, meaning that the siRNAs elicit their effect on cells by inhibiting gene expression and not by a directly toxic mechanism. Though all siRNA were effective, siRNA synthesized with the previously described “shotgun” method demonstrated the highest level of effectiveness at gene suppression

for all genes tested. This is likely because of the large array of siRNA species created when making siRNA in this manner.

T cells that are incapable of expressing TCRb are physiologically unable to elicit an inflammatory response. If a T cell cannot elicit an inflammatory response, it is unable to proliferate. Given the high level of effectiveness for siRNAs which have been prudently synthesized to knock down the expression of TCRb, these siRNA species are potentially a potent, novel means of therapy for the treatment of autoimmunity.

6.5 Notes

1. Abcam, "MTS Cell Proliferation Assay Kit (Colorimetric)." MTS Cell Proliferation Assay Protocol, Abcam, 2019, [www.abcam.com/ps/products/197/ab197010/documents/ab197010%20MTS%20Cell%20Proliferation%20Colorimetric%20Assay%20Kit%20Protocol%20v3b%20\(website\).pdf](http://www.abcam.com/ps/products/197/ab197010/documents/ab197010%20MTS%20Cell%20Proliferation%20Colorimetric%20Assay%20Kit%20Protocol%20v3b%20(website).pdf).
2. Biolabs, New England. "ShortCut RNase III Digestion Protocol (M0245)." NEB, New England Biolabs, 2019, www.neb.com/protocols/0001/01/01/shortcut-rnase-iii-digestion-protocol-m0245.
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4. Oha, Y., Parkbm T. 2009. "siRNA delivery systems for cancer treatment". Advanced Drug Delivery Reviews. 61(10): 850-862.
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6. "Universal SYBR Green Quantitative PCR Protocol." Millipore Sigma, Sigma Aldrich, 2019, www.sigmaaldrich.com/technical-documents/protocols/biology/sybr-green-qpcr.html.

Appendix A. Primer Sequences

Oligo Name	Oligo Sequence
TRB2V10-1	AACAAAGGAGAAGTCTCAGATGGCTACAG
TRB2V10-2	GATAAAGGAGAAGTCCCCGATGGCTATGT
TRB2V10-3	GACAAAGGAGAAGTCTCAGATGGCTATAG
TRB2V6-2/3	GCCAAAGGAGAGGTCCCTGATGGCTACAA
TRB2V6-8	CTCTAGATTAAACACAGAGGATTTCCAC
TRB2V6-9	AAGGAGAAGTCCCCGATGGCTACAATGTA
TRB2V6-5	AAGGAGAAGTCCCCAATGGCTACAATGTC
TRB2V6-6	GACAAAGGAGAAGTCCCGAATGGCTACAAC
TRB2V6-7	GTTCCCAATGGCTACAATGTCTCCAGATC
TRB2V6-1	GTCCCAATGGCTACAATGTCTCCAGATT
TRB2V6-4	GTCCCTGATGGTTATAGTGTCTCCAGAGC
TRB2V24-1	ATCTCTGATGGATACAGTGTCTCTCGACA
TRB2V25-1	TTTCTCTGAGTCAACAGTCTCCAGAATA
TRB2V27	TCCTGAAGGGTACAAAGTCTCTCGAAAAG
TRB2V26	CTCTGAGAGGTATCATGTTTCTTGAAATA
TRB2V28	TCCTGAGGGGTACAGTGTCTCTAGAGAGA
TRB2V19	TATAGCTGAAGGGTACAGCGTCTCTCGGG
TRB2V4-1	CTGAATGCCCAACAGCTCTCTCTTAAAC
TRB2V4-2/3	CTGAATGCCCAACAGCTCTCACTTATTC
TRB2V2P	CCTGAATGCCCTGACAGCTCTCGCTTATA
TRB2V3-1	CCTAAATCTCCAGACAAAGCTCACTTAAA
TRB2V3-2	CTCACCTGACTCTCCAGACAAAGCTCAT
TRB2V16	TTCAGCTAAGTGCCTCCCAAATTCACCCT
TRB2V23-1	GATTCTCATCTCAATGCCCAAGAACGC
TRB2V18	ATTTTCTGCTGAATTTCCCAAAGAGGGCC
TRB2V17	ATTCACAGCTGAAAGACCTAACGGAACGT
TRB2V14	TCTTAGCTGAAAGGACTGGAGGGACGTAT
TRB2V2	TTCGATGATCAATTCTCAGTTGAAAGGCC
TRB2V12-1	TTGATTCTCAGCACAGATGCCTGATGT
TRB2V12-2	GCGATTCTCAGCTGAGAGGCCTGATGG
TRB2V12-3/4	TCGATICICAGCTAAGATGCCIAATGC
TRB2V12-5	TTCTCAGCAGAGATGCCTGATGCAACTTA
TRB2V7-9	GGTTCTCTGCAGAGAGGCCCTAAGGGATCT
TRB2V7-8	GCTGCCAGTGATCGCTTCTTTGCAGAAA
TRB2V7-4	GGCGGCCAGTGGTCGGTTCTCTGCAGAG
TRB2V7-6/7	ATGATCGGTTCTCTGCAGAGAGGCCTGAGG
TRB2V7-2	AGTGATCGCTTCTCTGCAGAGAGGACTGG
TRB2V7-3	GGCTGCCCAACGATCGGTTCTTTGCAGT

TRB2V7-1	TCCCCGTGATCGGTTCTCTGCACAGAGGT
TRB2VII- 123	CTAAGGATCGATTTTCTGCAGAGAGGCTC
TRB2V13	CTGATCGATTCTCAGCTCAACAGTTCAGT
TRB2V5-1	TGGTCGATTCTCAGGGCGCCAGTTCTCTA
TRB2V5-3	TAATCGATTCTCAGGGCGCCAGTTCCATG
TRB2V5-4	TCCTAGATTCTCAGGTCTCCAGTTCCTA
TRB2V5-8	GGAAACTTCCCTCCTAGATTTTCAGGTCTG
TRB2V5-5	AAGAGGAAACTTCCCTGATCGATTCTCAGC
TRB2V5-6	GGCAACTTCCCTGATCGATTCTCAGGTCA
TRB2V9	GTTCCCTGACTTGC ACTCTGAACTAAAC
TRB2V15	GCCGAACACTTCTTTCTGCTTTCTTGAC
TRB2V30	GACCCAGGACCGGCAGTTCATCCTGAGT
TRB2V20-1	ATGCAAGCCTGACCTTGTCCTACTCTGACA
TRB2V29-1	CATCAGCCGCCCAAACCTAACATTCTCAA
pCb1	TTCACCCACCAGCTCAGCTCC
pCb2	ATACGCGTAGATCTCTGCTTCTGATGGC
pGI	CACCGGGIIGGGIIGGGII
Human Jurkat 1-1	AAGAAUCGAUUGUUGGGCAUACCCCTGTCTC
Human Jurkat 1-2	AAGGUAUGCCCAACAAUCGAUUCCTGTCTC
Human Jurkat 2-1	AAACCAGGACCCCAGAAGAGAAUCCTGTCTC
Human Jurkat 2-2	AAAUUCUCUUCUGGGGUCCUGGUCCTGTCTC
Human Jurkat 3-1	AAAGGGUUAUCAUGACCCGAAAUUCCTGTCTC
Human Jurkat 3-2	AAA AUUCGGGUCAUGUAACCCUCCTGTCTC
Human CD8+ Anti-HPV 1-1	AAGCUUAGCUCUAAGGUGUCAGGCCTGTCTC
Human CD8+ Anti-HPV 1-2	AACCUGACACCUUAGAGCUAAGCCCTGTCTC
Human CD8+ Anti-HPV 2-1	AAGAUUAUCAUGUCCAGAAAUUGCCTGTCTC
Human CD8+ Anti-HPV 2-2	AACAAUUCUGGACAUGAUAAUCCCTGTCTC
Human CD8+ Anti-HPV 3-1	AAGCCUGGAAACCAUGCUAAGCUCCTGTCTC
Human CD8+ Anti-HPV 3-2	AAAGCUUAGCAUGGUUCCAGGCCCTGTCTC

Appendix B. TCRb siRNA Solver Source Code

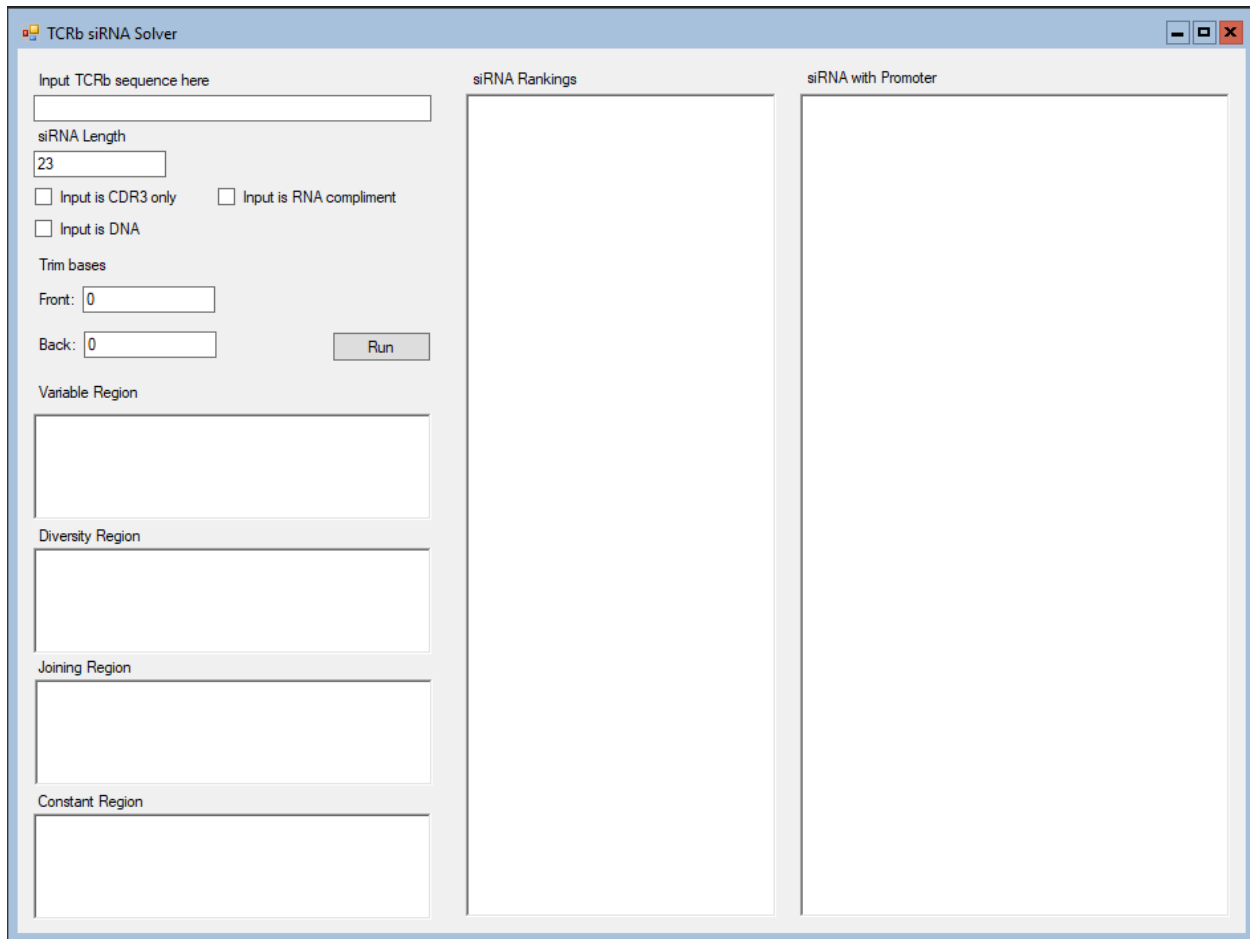


Figure 8.1. User Interface

8.1 User Interface Source Code

```
_ Partial Class Form1 Inherits System.Windows.Forms.Form

'Form overrides dispose to clean up the component list.
<System.Diagnostics.DebuggerNonUserCode()> _
Protected Overrides Sub Dispose(ByVal disposing As Boolean)
    Try
        If disposing AndAlso components IsNot Nothing Then
            components.Dispose()
        End If
    Finally
        MyBase.Dispose(disposing)
    End Try
End Sub
```

'Required by the Windows Form Designer

Private components As System.ComponentModel.IContainer

'NOTE: The following procedure is required by the Windows Form Designer

'It can be modified using the Windows Form Designer.

'Do not modify it using the code editor.

<System.Diagnostics.DebuggerStepThrough(> _

Private Sub InitializeComponent()

```
Me.Button1 = New System.Windows.Forms.Button()
Me.TextBox1 = New System.Windows.Forms.TextBox()
Me.TextBox2 = New System.Windows.Forms.TextBox()
Me.RichTextBox1 = New System.Windows.Forms.RichTextBox()
Me.CheckBox1 = New System.Windows.Forms.CheckBox()
Me.CheckBox2 = New System.Windows.Forms.CheckBox()
Me.RichTextBox2 = New System.Windows.Forms.RichTextBox()
Me.CheckBox3 = New System.Windows.Forms.CheckBox()
Me.TextBox3 = New System.Windows.Forms.TextBox()
Me.Label1 = New System.Windows.Forms.Label()
Me.Label2 = New System.Windows.Forms.Label()
Me.Label3 = New System.Windows.Forms.Label()
Me.TextBox4 = New System.Windows.Forms.TextBox()
Me.RichTextBox3 = New System.Windows.Forms.RichTextBox()
Me.RichTextBox4 = New System.Windows.Forms.RichTextBox()
Me.RichTextBox5 = New System.Windows.Forms.RichTextBox()
Me.Label4 = New System.Windows.Forms.Label()
Me.Label5 = New System.Windows.Forms.Label()
Me.Label6 = New System.Windows.Forms.Label()
Me.RichTextBox6 = New System.Windows.Forms.RichTextBox()
Me.Label8 = New System.Windows.Forms.Label()
Me.Label9 = New System.Windows.Forms.Label()
Me.Label10 = New System.Windows.Forms.Label()
Me.Label7 = New System.Windows.Forms.Label()
Me.Label11 = New System.Windows.Forms.Label()
Me.SuspendLayout()
```

```
'
'Button1
'
```

```
Me.Button1.Location = New System.Drawing.Point(237, 213)
Me.Button1.Name = "Button1"
Me.Button1.Size = New System.Drawing.Size(75, 23)
Me.Button1.TabIndex = 0
Me.Button1.Text = "Run"
Me.Button1.UseVisualStyleBackColor = True
```

```
'
'TextBox1
'
```

```

Me.TextBox1.Location = New System.Drawing.Point(12, 35)
Me.TextBox1.Name = "TextBox1"
Me.TextBox1.Size = New System.Drawing.Size(300, 20)
Me.TextBox1.TabIndex = 2
'
'TextBox2
'
Me.TextBox2.Location = New System.Drawing.Point(12, 77)
Me.TextBox2.Name = "TextBox2"
Me.TextBox2.Size = New System.Drawing.Size(100, 20)
Me.TextBox2.TabIndex = 4
Me.TextBox2.Text = "23"
'
'RichTextBox1
'
Me.RichTextBox1.Location = New System.Drawing.Point(338, 34)
Me.RichTextBox1.Name = "RichTextBox1"
Me.RichTextBox1.Size = New System.Drawing.Size(234, 620)
Me.RichTextBox1.TabIndex = 5
Me.RichTextBox1.Text = ""
'
'CheckBox1
'
Me.CheckBox1.AutoSize = True
Me.CheckBox1.Location = New System.Drawing.Point(13, 104)
Me.CheckBox1.Name = "CheckBox1"
Me.CheckBox1.Size = New System.Drawing.Size(114, 17)
Me.CheckBox1.TabIndex = 6
Me.CheckBox1.Text = "Input is CDR3 only"
Me.CheckBox1.UseVisualStyleBackColor = True
'
'CheckBox2
'
Me.CheckBox2.AutoSize = True
Me.CheckBox2.Location = New System.Drawing.Point(13, 128)
Me.CheckBox2.Name = "CheckBox2"
Me.CheckBox2.Size = New System.Drawing.Size(86, 17)
Me.CheckBox2.TabIndex = 7
Me.CheckBox2.Text = "Input is DNA"
Me.CheckBox2.UseVisualStyleBackColor = True
'
'RichTextBox2
'
Me.RichTextBox2.Location = New System.Drawing.Point(12, 275)
Me.RichTextBox2.Name = "RichTextBox2"
Me.RichTextBox2.Size = New System.Drawing.Size(300, 80)

```

```

Me.RichTextBox2.TabIndex = 8
Me.RichTextBox2.Text = ""
'
'CheckBox3
'
Me.CheckBox3.AutoSize = True
Me.CheckBox3.Location = New System.Drawing.Point(151, 104)
Me.CheckBox3.Name = "CheckBox3"
Me.CheckBox3.Size = New System.Drawing.Size(142, 17)
Me.CheckBox3.TabIndex = 9
Me.CheckBox3.Text = "Input is RNA compliment"
Me.CheckBox3.UseVisualStyleBackColor = True
'
'TextBox3
'
Me.TextBox3.Location = New System.Drawing.Point(49, 179)
Me.TextBox3.Name = "TextBox3"
Me.TextBox3.Size = New System.Drawing.Size(100, 20)
Me.TextBox3.TabIndex = 10
Me.TextBox3.Text = "0"
'
'Label1
'
Me.Label1.AutoSize = True
Me.Label1.Location = New System.Drawing.Point(13, 156)
Me.Label1.Name = "Label1"
Me.Label1.Size = New System.Drawing.Size(58, 13)
Me.Label1.TabIndex = 12
Me.Label1.Text = "Trim bases"
'
'Label2
'
Me.Label2.AutoSize = True
Me.Label2.Location = New System.Drawing.Point(13, 182)
Me.Label2.Name = "Label2"
Me.Label2.Size = New System.Drawing.Size(34, 13)
Me.Label2.TabIndex = 13
Me.Label2.Text = "Front:"
'
'Label3
'
Me.Label3.AutoSize = True
Me.Label3.Location = New System.Drawing.Point(13, 216)
Me.Label3.Name = "Label3"
Me.Label3.Size = New System.Drawing.Size(35, 13)
Me.Label3.TabIndex = 14

```



```

Me.Label3.Text = "Back:"
'
'TextBox4
'
Me.TextBox4.Location = New System.Drawing.Point(50, 213)
Me.TextBox4.Name = "TextBox4"
Me.TextBox4.Size = New System.Drawing.Size(100, 20)
Me.TextBox4.TabIndex = 15
Me.TextBox4.Text = "0"
'
'RichTextBox3
'
Me.RichTextBox3.Location = New System.Drawing.Point(12, 376)
Me.RichTextBox3.Name = "RichTextBox3"
Me.RichTextBox3.Size = New System.Drawing.Size(300, 80)
Me.RichTextBox3.TabIndex = 16
Me.RichTextBox3.Text = ""
'
'RichTextBox4
'
Me.RichTextBox4.Location = New System.Drawing.Point(13, 475)
Me.RichTextBox4.Name = "RichTextBox4"
Me.RichTextBox4.Size = New System.Drawing.Size(300, 80)
Me.RichTextBox4.TabIndex = 17
Me.RichTextBox4.Text = ""
'
'RichTextBox5
'
Me.RichTextBox5.Location = New System.Drawing.Point(12, 576)
Me.RichTextBox5.Name = "RichTextBox5"
Me.RichTextBox5.Size = New System.Drawing.Size(300, 80)
Me.RichTextBox5.TabIndex = 18
Me.RichTextBox5.Text = ""
'
'Label4
'
Me.Label4.AutoSize = True
Me.Label4.Location = New System.Drawing.Point(13, 252)
Me.Label4.Name = "Label4"
Me.Label4.Size = New System.Drawing.Size(82, 13)
Me.Label4.TabIndex = 19
Me.Label4.Text = "Variable Region"
'
'Label5
'
Me.Label5.AutoSize = True

```

```

Me.Label5.Location = New System.Drawing.Point(13, 360)
Me.Label5.Name = "Label5"
Me.Label5.Size = New System.Drawing.Size(84, 13)
Me.Label5.TabIndex = 20
Me.Label5.Text = "Diversity Region"
'
'Label6
'
Me.Label6.AutoSize = True
Me.Label6.Location = New System.Drawing.Point(13, 459)
Me.Label6.Name = "Label6"
Me.Label6.Size = New System.Drawing.Size(77, 13)
Me.Label6.TabIndex = 21
Me.Label6.Text = "Joining Region"
'
'RichTextBox6
'
Me.RichTextBox6.Location = New System.Drawing.Point(590, 34)
Me.RichTextBox6.Name = "RichTextBox6"
Me.RichTextBox6.Size = New System.Drawing.Size(324, 620)
Me.RichTextBox6.TabIndex = 22
Me.RichTextBox6.Text = ""
'
'Label8
'
Me.Label8.AutoSize = True
Me.Label8.Location = New System.Drawing.Point(341, 16)
Me.Label8.Name = "Label8"
Me.Label8.Size = New System.Drawing.Size(85, 13)
Me.Label8.TabIndex = 24
Me.Label8.Text = "siRNA Rankings"
'
'Label9
'
Me.Label9.AutoSize = True
Me.Label9.Location = New System.Drawing.Point(593, 15)
Me.Label9.Name = "Label9"
Me.Label9.Size = New System.Drawing.Size(104, 13)
Me.Label9.TabIndex = 25
Me.Label9.Text = "siRNA with Promoter"
'
'Label10
'
Me.Label10.AutoSize = True
Me.Label10.Location = New System.Drawing.Point(13, 560)
Me.Label10.Name = "Label10"

```

```

Me.Label10.Size = New System.Drawing.Size(86, 13)
Me.Label10.TabIndex = 26
Me.Label10.Text = "Constant Region"
'
'Label7
'
Me.Label7.AutoSize = True
Me.Label7.Location = New System.Drawing.Point(13, 17)
Me.Label7.Name = "Label7"
Me.Label7.Size = New System.Drawing.Size(136, 13)
Me.Label7.TabIndex = 27
Me.Label7.Text = "Input TCRb sequence here"
'
'Label11
'
Me.Label11.AutoSize = True
Me.Label11.Location = New System.Drawing.Point(13, 59)
Me.Label11.Name = "Label11"
Me.Label11.Size = New System.Drawing.Size(73, 13)
Me.Label11.TabIndex = 28
Me.Label11.Text = "siRNA Length"
'
'Form1
'
Me.AutoScaleDimensions = New System.Drawing.SizeF(6.0!, 13.0!)
Me.AutoScaleMode = System.Windows.Forms.AutoScaleMode.Font
Me.ClientSize = New System.Drawing.Size(926, 666)
Me.Controls.Add(Me.Label11)
Me.Controls.Add(Me.Label7)
Me.Controls.Add(Me.Label10)
Me.Controls.Add(Me.Label9)
Me.Controls.Add(Me.Label8)
Me.Controls.Add(Me.RichTextBox6)
Me.Controls.Add(Me.Label6)
Me.Controls.Add(Me.Label5)
Me.Controls.Add(Me.Label4)
Me.Controls.Add(Me.RichTextBox5)
Me.Controls.Add(Me.RichTextBox4)
Me.Controls.Add(Me.RichTextBox3)
Me.Controls.Add(Me.TextBox4)
Me.Controls.Add(Me.Label3)
Me.Controls.Add(Me.Label2)
Me.Controls.Add(Me.Label1)
Me.Controls.Add(Me.TextBox3)
Me.Controls.Add(Me.CheckBox3)
Me.Controls.Add(Me.RichTextBox2)

```

```

Me.Controls.Add(Me.CheckBox2)
Me.Controls.Add(Me.CheckBox1)
Me.Controls.Add(Me.RichTextBox1)
Me.Controls.Add(Me.TextBox2)
Me.Controls.Add(Me.TextBox1)
Me.Controls.Add(Me.Button1)
Me.Name = "Form1"
Me.Text = "TCRb siRNA Solver"
Me.ResumeLayout(False)
Me.PerformLayout()

```

End Sub

```

Friend WithEvents Button1 As Button
Friend WithEvents TextBox1 As TextBox
Friend WithEvents TextBox2 As TextBox
Friend WithEvents RichTextBox1 As RichTextBox
Friend WithEvents CheckBox1 As CheckBox
Friend WithEvents CheckBox2 As CheckBox
Friend WithEvents RichTextBox2 As RichTextBox
Friend WithEvents CheckBox3 As CheckBox
Friend WithEvents TextBox3 As TextBox
Friend WithEvents Label1 As Label
Friend WithEvents Label2 As Label
Friend WithEvents Label3 As Label
Friend WithEvents TextBox4 As TextBox
Friend WithEvents RichTextBox3 As RichTextBox
Friend WithEvents RichTextBox4 As RichTextBox
Friend WithEvents RichTextBox5 As RichTextBox
Friend WithEvents Label4 As Label
Friend WithEvents Label5 As Label
Friend WithEvents Label6 As Label
Friend WithEvents RichTextBox6 As RichTextBox
Friend WithEvents Label8 As Label
Friend WithEvents Label9 As Label
Friend WithEvents Label10 As Label
Friend WithEvents Label7 As Label
Friend WithEvents Label11 As Label
End Class

```

8.2 Non-interface source code

```

Public Class Form 1
'Halts current procedure and throws error to the user
Sub Throw_error()

```

```
MsgBox("An error has occurred. Please check your inputs and try again.")
```

```
Stop
```

```
End Sub
```

```
'Scans respective siRNA regions for whether or not they meet The first Descrip as defined by  
Reynalds et al
```

```
'SiRNA has a base of A in position 3
```

```
'Returns a boolean value
```

```
Function Discrim1(ByVal siRNA As String) As Boolean
```

```
Dim bool As Boolean = False
```

```
'Checks specified position for specified character
```

```
If GetChar(siRNA, 3) = "a" Or GetChar(siRNA, 3) = "A" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

```
End Function
```

```
'Scans respective siRNA regions for whether or not they meet The second Descrip as defined by  
Reynalds et al
```

```
'SiRNA has a base of A in position 19
```

```
'Returns a boolean value
```

```
Function Discrim2(ByVal siRNA As String) As Boolean
```

```
Dim bool As Boolean = False
```

```
'Checks specified position for specified character
```

```
If GetChar(siRNA, 19) = "a" Or GetChar(siRNA, 19) = "A" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

```
End Function
```

```
'Scans respective siRNA regions for whether or not they meet the third Descrip as defined by  
Reynalds et al
```

```
'SiRNA does not have a base of G in position 13
```

```
'Returns a boolean value
```

```
Function Discrim3(ByVal siRNA As String) As Boolean
```

```
Dim bool As Boolean = False
```

```
'Checks specified position for specified character
```

```
If Not GetChar(siRNA, 13) = "g" And Not GetChar(siRNA, 13) = "G" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

```
End Function
```

```
'Scans respective siRNA regions for whether or not they meet the fourth Discrim as defined by  
Reynalds et al
```

```
'SiRNA has a base of U in position 10
```

```
'Returns a boolean value
```

```
Function Discrim4(ByVal siRNA As String) As Boolean
```

```
Dim bool As Boolean = False
```

```
'Checks specified position for specified character
```

```
If GetChar(siRNA, 10) = "u" Or GetChar(siRNA, 10) = "U" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

```
End Function
```

```
'Scans respective siRNA regions for whether or not they meet the fifth Discrim as defined by  
Reynalds et al
```

```
'SiRNA does not have a base of C or G in position 19
```

```
'Returns a boolean value
```

```
Function Discrim5(ByVal siRNA As String) As Boolean
```

```
Dim bool As Boolean = False
```

```
'Checks specified position for specified character
```

```
If Not GetChar(siRNA, 19) = "c" And Not GetChar(siRNA, 19) = "C" And Not  
GetChar(siRNA, 19) = "g" And Not GetChar(siRNA, 19) = "G" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

```
End Function
```

'Checks a substring of each siRNA for a palindromic sequence encompassing of 4 bases
'Returns a boolean value

Function Palindrome_check_4_units(ByVal siRNA As String) As Boolean

Dim bool As Boolean = False

Dim reverse_sequence As String = Strings.StrReverse(siRNA)

Dim count As Integer = 0

Dim forward As String = Nothing

Dim reverse As String = Nothing

'Splits sequence into substrings and compares them against their compliment

Do While count < siRNA.Length - 4

forward = siRNA.Remove(0, count)

forward = siRNA.Substring(0, 4)

Dim count2 As Integer = 0

Do While count2 < siRNA.Length - 4

reverse = siRNA.Remove(0, count)

reverse = siRNA.Substring(0, 4)

If forward = reverse **Then**

bool = True

End If

count2 = count2 + 1

Loop

count = count + 1

Loop

Return bool

End Function

'Checks a substring of each siRNA for a palindromic sequence encompassing of 5 bases
'Returns a boolean value

Function Palindrome_check_5_units(ByVal siRNA As String) As Boolean

Dim bool As Boolean = False

Dim reverse_sequence As String = Strings.StrReverse(siRNA)

Dim count As Integer = 0

Dim forward As String = Nothing

Dim reverse As String = Nothing

'Splits sequence into substrings and compares them against their compliment

```
Do While count < siRNA.Length - 5
    forward = siRNA.Remove(0, count)
    forward = siRNA.Substring(0, 5)
    Dim count2 As Integer = 0
    Do While count2 < siRNA.Length - 5
        reverse = siRNA.Remove(0, count)
        reverse = siRNA.Substring(0, 5)
        If forward = reverse Then
            bool = True
        End If
        count2 = count2 + 1
    Loop

    count = count + 1
```

Loop

Return bool

End Function

'Checks for the GC content of an siRNA input

'Returns a value between 0 and 1

Function Get_GC(ByVal siRNA As String, siRNA_length As Integer) As Double

```
Dim GC_content As Double = 0
```

```
Dim count As Integer = 0
```

```
Dim ATU_count As Integer = 0
```

```
Do While count < siRNA.Length
```

```
    If GetChar(siRNA, count + 1) = "a" Or GetChar(siRNA, count + 1) = "A" Or
    GetChar(siRNA, count + 1) = "t" Or GetChar(siRNA, count + 1) = "T" Or GetChar(siRNA,
    count + 1) = "u" Or GetChar(siRNA, count + 1) = "U" Then
```

```
        ATU_count = ATU_count + 1
```

```
    End If
```

```
    count = count + 1
```

```
Loop
```



```
GC_content = 1 - (ATU_count / siRNA_length)
```

```
Return GC_content
```

End Function

'Checks whether or not an A or U is present in positions 15-19 as specified in Reynolds et al

'Returns boolean value

```
Function AUT_in_15_through_19(ByVal sirna As String, sirna_ength As Integer) As Boolean
```

```
Dim bool As Boolean = False
```

'Load characters from siRNA into variables

```
Dim char15 As Char = GetChar(sirna, 15)
```

```
Dim char16 As Char = GetChar(sirna, 16)
```

```
Dim char17 As Char = GetChar(sirna, 17)
```

```
Dim char18 As Char = GetChar(sirna, 18)
```

```
Dim char19 As Char = GetChar(sirna, 19)
```

'Check above variables for presence of A, U, or T

```
If char15 = "a" Or char15 = "A" Or char15 = "t" Or char15 = "T" Or char15 = "u" Or char15 = "U" Then
```

```
    bool = True
```

```
End If
```

```
If char16 = "a" Or char16 = "A" Or char16 = "t" Or char16 = "T" Or char16 = "u" Or char16 = "U" Then
```

```
    bool = True
```

```
End If
```

```
If char17 = "a" Or char17 = "A" Or char17 = "t" Or char17 = "T" Or char17 = "u" Or char17 = "U" Then
```

```
    bool = True
```

```
End If
```

```
If char18 = "a" Or char18 = "A" Or char18 = "t" Or char18 = "T" Or char18 = "u" Or char18 = "U" Then
```

```
    bool = True
```

```
End If
```

```
If char19 = "a" Or char19 = "A" Or char19 = "t" Or char19 = "T" Or char19 = "u" Or char19 = "U" Then
```

```
    bool = True
```

```
End If
```

```
Return bool
```

End Function

'Checks for a leading sequence of AA at the start of respective siRNA
'returns boolean value

Function Check_for_AA(ByVal siRNA As String) As Boolean

Dim AA_Bool As Boolean = False

Dim Score As Integer = 0

'For each A in the first two residues, the variable "score" is iterated by 1

If GetChar(siRNA, 1) = "A" Or GetChar(siRNA, 1) = "a" Then

Score = Score + 1

End If

If GetChar(siRNA, 2) = "A" Or GetChar(siRNA, 2) = "a" Then

Score = Score + 1

End If

'Score being equal to 2 indicated that both of the leading residues Is A

If Score = 2 Then

AA_Bool = True

End If

Return AA_Bool

End Function

'Checks for running sequences of A or T (various literature indicates that this can cause
dissociation of siRNA from RISC)

'returns boolean value

Function Check_for_running_T_or_A(ByVal siRNA As String, sirna_length As Integer) As
Boolean

Dim Poly_T_A As Boolean = False

Dim count As Integer = 0

Dim tempstring As String = Nothing

Do While count < sirna_length - 4

tempstring = siRNA

tempstring = tempstring.Remove(0, count) 'remove leading characters in temporary string

tempstring = tempstring.Substring(0, 4) ' trims temporary string to 4 characters

'Checks for a polly A or poly T sequence

```

    If tempstring = "aaaa" Or tempstring = "AAAA" Or tempstring = "TTTT" Or tempstring =
    "tttt" Or tempstring = "UUUU" Or tempstring = "uuuu" Then
        Poly_T_A = True
        count = 9999999 'breaks loop
    End If

```

```

    count = count + 1

```

```

Loop

```

```

Return Poly_T_A

```

```

End Function

```

'Converts an input of a DNA sequence into its reverse complimentary RNA counterpart

'Returns string a value

```

Function Convert_to_RNA(Input As String)

```

```

    Dim Sequence(Input.Length) As String
    Dim Working_String(Input.Length) As String
    Dim count As Integer = 0

```

'Loads input into an array for conversion

```

Do While count < Input.Length
    Sequence(count) = Input(count)

```

```

    count = count + 1

```

```

Loop

```

'Reverses the DNA input (this is important because we are interested in the reverse compliment sequence)

```

Array.Reverse(Sequence)

```

```

count = 1

```

```

Dim Return_string As String = Nothing

```

'Converts first array sequence into complimentary values for second array

```

Do While count < Working_String.Length - 1

```

```

    Select Case Sequence(count)

```

```

        Case "A"

```

```

            Working_String(count) = "U"

```

```

        Case "a"

```

```

            Working_String(count) = "U"

```

```

Case "T"
    Working_String(count) = "A"

Case "t"
    Working_String(count) = "A"

Case "C"
    Working_String(count) = "G"

Case "c"
    Working_String(count) = "G"

Case "G"
    Working_String(count) = "C"

Case "g"
    Working_String(count) = "C"

Case Else
    Throw_error()

End Select

count = count + 1

Loop

count = 0

'Converts array of characters into a normal string for the function to return
Do While count < Working_String.Length

    Return_string = Return_string + Working_String(count)

    count = count + 1

Loop

Return Return_string

End Function

'Converts an input of a RNA sequence into its reverse complimentary RNA counterpart
'Returns string a value
Function Convert_to_Compliment(Input As String)

```

```
Dim Sequence(Input.Length) As String
Dim Working_String(Input.Length) As String
Dim count As Integer = 0
```

'Loads input into an array for conversion

```
Do While count < Input.Length
    Sequence(count) = Input(count)
```

```
    count = count + 1
```

Loop

'Reverses the DNA input (this is important because we are interested in the reverse compliment sequence)

```
Array.Reverse(Sequence)
```

```
count = 1
```

```
Dim Return_string As String = Nothing
```

'Converts first array sequence into complimentary values for second array

```
Do While count < Working_String.Length - 2
```

```
    Select Case Sequence(count)
```

```
        Case "A"
```

```
            Working_String(count) = "U"
```

```
        Case "a"
```

```
            Working_String(count) = "U"
```

```
        Case "U"
```

```
            Working_String(count) = "A"
```

```
        Case "u"
```

```
            Working_String(count) = "A"
```

```
        Case "t"
```

```
            Working_String(count) = "A"
```

```
        Case "T"
```

```
            Working_String(count) = "A"
```

```
        Case "C"
```

```
            Working_String(count) = "G"
```

```
        Case "c"
```

```
            Working_String(count) = "G"
```

```

    Case "G"
        Working_String(count) = "C"

    Case "g"
        Working_String(count) = "C"

    Case Else
        Throw_error()

End Select

count = count + 1

Loop

count = 0

'Converts array of characters into a normal string for the function to return
Do While count < Working_String.Length

    Return_string = Return_string + Working_String(count)

    count = count + 1

Loop

Return Return_string

End Function

'Checks that values within text boxes are of the data type needed
'Calls Throw_error() sub-procedure if any of the tests are positive
Sub Error_Checks()

    TextBox1.Text.ToUpper()

    Dim Check_TB2 As Integer

    If Not Integer.TryParse(TextBox2.Text, Check_TB2) Then
        Throw_error()
    End If

    Dim Check_TB3 As Integer

    If Not Integer.TryParse(TextBox3.Text, Check_TB3) Then

```

```

    Throw_error()
End If

Dim Check_TB4 As Integer

If Not Integer.TryParse(TextBox4.Text, Check_TB4) Then
    Throw_error()
End If

End Sub

```

'Sequence of events called when User hits the "Run" button
Private Sub Button1_Click(sender As Object, e As EventArgs) **Handles** Button1.Click

```

'Resets output
RichTextBox1.Text = Nothing

```

Region "Trim nucleic acid sequence"

```

'Trims front of siRNA sequence if specified by user to do so
If Not TextBox2.Text = Nothing Then
    Dim FrontTrim As Integer = CInt(TextBox3.Text)
    TextBox1.Text = TextBox1.Text.Remove(0, FrontTrim)
End If

```

```

'Trims back of siRNA sequence if specified by user to do so
If Not TextBox3.Text = Nothing Then
    Dim BackTrim As Integer = CInt(TextBox4.Text)
    Dim TempVar As Integer = TextBox1.Text.Length
    TextBox1.Text = TextBox1.Text.Substring(0, TempVar - BackTrim)
End If

```

End Region

'Calls subprocedure to convert input from a DNA sequence to a reverse complimentary RNA sequence if specified by user

```

If CheckBox2.Checked Then
    TextBox1.Text = Convert_to_RNA(TextBox1.Text)
End If

```

'Calls subprocedure to convert input from a RNA sequence to a reverse complimentary RNA sequence if specified by user

```

If CheckBox3.Checked Then
    TextBox1.Text = Convert_to_Compliment(TextBox1.Text)
End If

```

```

Dim SiRNA_Score As Integer = 0

```

'Initializes arrays which store siRNA candidates and their corresponding scores

```
Dim dataArray(50000) As String  
Dim scoreArray(50000) As Double
```

```
Dim codeLength As Integer = 0  
codeLength = TextBox1.Text.Length  
Dim siRNA_Length = Convert.ToInt32(TextBox2.Text)  
Dim count As Integer = 0
```

'Loads siRNA candidates into an array of length specified by user

Region "Load siRNA Array"

```
Do While count < codeLength - siRNA_Length 'load siRNAs into array
```

```
    dataArray(count) = TextBox1.Text  
    dataArray(count) = dataArray(count).Remove(0, count) 'remove leading characters in  
string  
    dataArray(count) = dataArray(count).Substring(0, siRNA_Length) 'remove following  
characters after siRNA
```

```
    count = count + 1
```

```
Loop
```

End Region

```
count = 0
```

'Separates V, D, J, and C regions

Region "separate regions"

```
If Not CheckBox1.Checked Then
```

```
    Do While TextBox1.Text.Length > count
```

```
        If count <= 340 Then
```

```
            richTextBox2.Text = richTextBox2.Text + GetChar(TextBox1.Text, count + 1)
```

```
        End If
```

```
        If count > 340 And count <= 356 Then
```

```
            richTextBox3.Text = richTextBox3.Text + GetChar(TextBox1.Text, count + 1)
```

```
        End If
```



```

If count > 356 And count <= 406 Then

    RichTextBox4.Text = RichTextBox4.Text + GetChar(TextBox1.Text, count + 1)

End If

If count > 406 Then

    RichTextBox5.Text = RichTextBox5.Text + GetChar(TextBox1.Text, count + 1)

End If

count = count + 1

Loop

End If

```

End Region

```
count = 0
```

'Adds scores

'Values added to each siRNA score are largely determined by their level of significance-

'-RNAi as determined by Reynolds et al

Region "Tally Scores"

```
Do While count < codelength - SiRNA_Length
```

'calls function to determine GC content of siRNA

```
Dim GC_content As Double = Get_GC(DataArray(count), SiRNA_Length)
```

```
If Discrim1(DataArray(count)) Then
```

```
    SiRNA_Score = SiRNA_Score + 16.8
```

```
End If
```

```
If Discrim2(DataArray(count)) Then
```

```
    SiRNA_Score = SiRNA_Score + 22.1
```

```
End If
```

```
If Discrim3(DataArray(count)) Then
```

```
    SiRNA_Score = SiRNA_Score + 14.1
```

```
End If
```

```
If Discrim4(DataArray(count)) Then
```

```
    SiRNA_Score = SiRNA_Score + 30.6
```

```
End If
```

```
If Discrim5(DataArray(count)) Then
    SiRNA_Score = SiRNA_Score + 25.6
End If
```

```
If not Palindrome_check_4_units(DataArray(count)) And not
Palindrome_check_5_units(DataArray(count)) Then
    SiRNA_Score = SiRNA_Score + 10.1
End If
```

```
If AUT_in_15_through_19(DataArray(count), DataArray(count).Length) Then
    SiRNA_Score = SiRNA_Score + 16.9
End If
```

```
'SiRNA with running A or T regions are not candidates for successful inhibition
If Check_for_running_T_or_A(DataArray(count), DataArray(count).Length) Then
    'Lowers score to a level where they will be ignored by future algorithms
    SiRNA_Score = SiRNA_Score - 20000
End If
```

```
'SiRNA with running A or T regions are not candidates for successful inhibition
If Not Check_for_AA(DataArray(count)) Then
    'Lowers score to a level where they will be ignored by future algorithms
    SiRNA_Score = SiRNA_Score - 20000
End If
```

```
'SiRNA with running A or T regions are not candidates for successful inhibition
If Get_GC(DataArray(count), DataArray(count).Length) < 0.3 Or
Get_GC(DataArray(count), DataArray(count).Length) > 0.52 Then
    'Lowers score to a level where they will be ignored by future algorithms
    SiRNA_Score = SiRNA_Score - 20000
End If
```

```
ScoreArray(count) = SiRNA_Score
```

```
SiRNA_Score = 0
```

```
count = count + 1
```

```
Loop
```

```
End Region
```

```
'Sorts array in ascending order based on the assigned score
Array.Sort(ScoreArray, DataArray)
```

```
'Reverses array so it will be sequenced in descending order for the user
Array.Reverse(ScoreArray)
Array.Reverse(DataArray)
```

```

count = 0

'Displays resulting siRNA candidates and their respective scores to the user
Do While count < codelength - SiRNA_Length

    If ScoreArray(count) > 0 Then

        'Displays siRNA candidate with respective score adjacent
        RichTextBox1.Text = RichTextBox1.Text + DataArray(count) + " " +
ScoreArray(count).ToString + vbCrLf

        'Displays siRNA candidate with the promoter sequence added
        RichTextBox6.Text = RichTextBox6.Text + DataArray(count) + "CCTGTCTC" + "
" + ScoreArray(count).ToString + vbCrLf

        'Removes leading AA to get compliment without overhang
        DataArray(count).Remove(0, 2)

        'Displays compliment and adds promoter sequence as well as AA overhang to the
compliment
        RichTextBox6.Text = RichTextBox6.Text + "AA" +
Convert_to_Compliment(DataArray(count)) + "CCTGTCTC" + vbCrLf + vbCrLf

    End If

    count = count + 1

Loop

End Sub
End Class

```

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Vita

Nicholas Magazine, born in Baton Rouge, Louisiana, worked as a programmer and pharmacy technician in the years leading up to the reception of his bachelor's degrees from Louisiana State University. During graduate school, Nicholas explored his interest in the sciences by working as a biochemist at the University laboratories. Upon completion of his doctorate, Nicholas intends to seek commercialization of his intellectual property relating to biochemistry.