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Modified oligonucleotides target the IRES in c–Myc to control gene expression

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Abstract

Regulation of gene expression is attracting more attention as a means to develop gene therapy for cancer, as it targets specifically cancer genes with no harm for healthy body cells. Deregulation of c–Myc protein is found to occur in more than half of human tumours and correlates to aggressive cancer stages, with its resistance to therapy, making it very difficult to be cured by traditional cancer therapy. Controlling of gene expression could be a promising approach to regulate this oncoprotein, in particular, stopping the translation of the c–Myc mRNA.

Translation from mRNA to protein requires initiation, elongation and termination of the peptide synthesis. Blocking the initiation factors, in particular binding of the ribosome to the start site of mRNA, could be a way worth trying to stop translation.

The possible secondary structure of the c–Myc mRNA gives a potential new method to control gene expression of mRNA. Apart from the common initiation sites, some mRNA contains an internal ribosomal entry site (IRES) element, which is being better understood in recent years. This could lead us to choose some more promising targets to control gene expression.

Oligonucleotides provide us a good resource to control the gene expression. Not only the standard DNA, but in particular some modified oligonucleotides, including locked nucleic acid (LNA) and phosphorothioate nucleic acid.

In this essay, we aimed to synthesise standard DNA and modified DNA, which target the IRES point of the c–Myc gene to inhibit the translation process.

Menu

Abbreviation

APS	Ammonium persulfate
CEP–CL	2–Cyanoethyl N,N–diisopropylchlorophosphoramidite
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle medium
DMSO	Dimethyl sulfoxide
DMT	4,4'–dimethoxytrityl group
DNA	Deoxyribonucleic acid
dsRNA	Double strand RNA
eIF	Eukaryotic initiate factor
FBS	Fetal bovine serum
GTP	Guanosine triphosphate
HPLC	High–performance liquid chromatography
HRP	Horseradish peroxidase
IRES	Internal ribosome entry site
kDa	Kilo dalton
LNA	Locked nucleic acid
Met–tRNAi	Methionine transfer ribonucleic acid
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
ON	Oligonucleotide
PS	Polystyrene
RIPA	Radio Immunoprecipitation Assay
RISC	RNA–Induced Silencing Complex
RNA	Ribonucleic acid
RNase	Ribonuclease

RT–PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
ssRNA	Single strand RNA
TLC	Thin layer chromatography
TMED	Tetramethylethylenediamine
tRNA	Transfer ribonucleic acid
UV–VIS	Ultraviolet–visible spectroscopy
WB	Western blot

Introduction

1.1 Gene expression

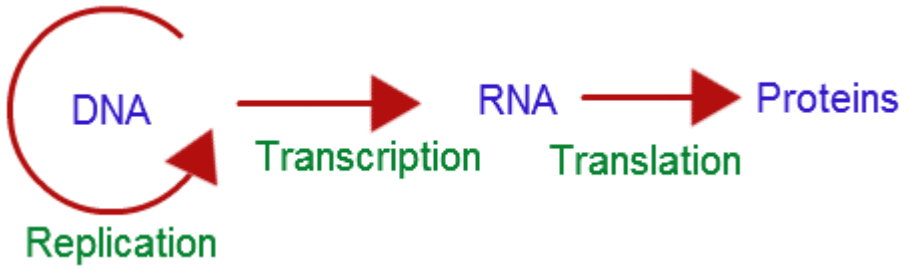


Figure 1.1–1 main process of the central dogma of biology.

Gene expression is a process to use information from genes to synthesis the gene products, which are often the proteins. The central dogma of biology is an explanation of the flow of genetic information from DNA to form a functional product of protein[1]. The general process that occurs in all the cells is: DNA is transcribed into mRNA, which in turn is translated into the protein. Naturally the DNA exists in form of a DNA–DNA duplex in all life forms except some viruses. According to the central dogma of biology, DNA undergoes conserved replication to increase the quantity during cell division with the aid of DNA helicase and polymerase. Transcription is also carried out using the DNA duplex. When DNA duplexes unwind, the DNA will bind the RNA nucleotide triphosphates through hydrogen bonding according to the complementary base pairing, and RNA polymerase will synthesise the complementary single strand of mRNA. The mRNA is not stable within the cell and is directly used for the next step in translation. So the single strand RNA will separate from the DNA, and then be bound by the ribosome to initiate protein synthesis. In this step, transfer RNA (tRNA) will be recruited following the triplet codon of the mRNA, and add the corresponding amino acid in steps catalysed by the ribosome. After the dehydration condensation, attaching of the amino acid will form the polypeptide. The protein consists of a numbers of amino acids to form a polypeptide.

This is the basic process for the cells to proliferate. However destroying the regular cancer

cell–cycle progression and division is the key role to control cancer [2]. So the project is designed to break this cell circle and eliminate the cancer cell proliferation.

1.2 Translation

Translation is the process to add one amino acid each time to the end of the polypeptides. Then polypeptides will fold in to protein and function in the cells.

The whole translation process is in three phases: initiation, elongation and termination.[3] Initiation: ribosomes will bind to the target mRNA. The first tRNA will attach to the starting codon. Elongation: after tRNA transfer the amino acid to the codon of next tRNA, ribosomes translocate to the next tRNA to continue the whole process. Termination: when the stop codon is reached, the process stops and the polypeptide will fold into its final structure with the function of the ribosome.

1.2.1 5–cap dependent initiation

Cap–dependent translation is the accepted mechanism for the vast majority of mRNAs [4]. The synthesis of protein from mRNA is a multistep process, involving initiation, elongation and termination. With the help of eIF1A (eIF1 as well) and eIF6 bind with 40S and 60S respectively, the ribosomes are disassociated. The ribosomal 40S subunit, bind with the eIF2–GTP/Met–tRNA complex, eIF3, eIF1 and eIF1A, forms the complex to recruit tRNA [5]. And the 'scaffold' protein eIF4G bind eIF4E, Mnk (eIF4E kinase), eIF4A and PABP. As the 5' end of the mRNA is capped with m^7G, eIF4E interact with m^7G and contribute to the ribosome binding. After that the 48S pre–initiation complex is formed, which is the scaffold protein eIF4G bind with the 48S complex through eIF3 as the bridge. Once the ribosome connect to m^7G and mRNA is unwind, the ribosome 40S plus subunit will start to scan mRNA form 5' to 3' until the first start codon (AUG) is recognized [6]. After that, the large subunit 60S will combine with 40S subunit to form a functional ribosome. EIF5 and eIF5B will promote hydrolysis of the eIF2–bound GTP, which will make all the eIFs be substituted. Also the 60S can join 40S and the protein starts to be synthesised.

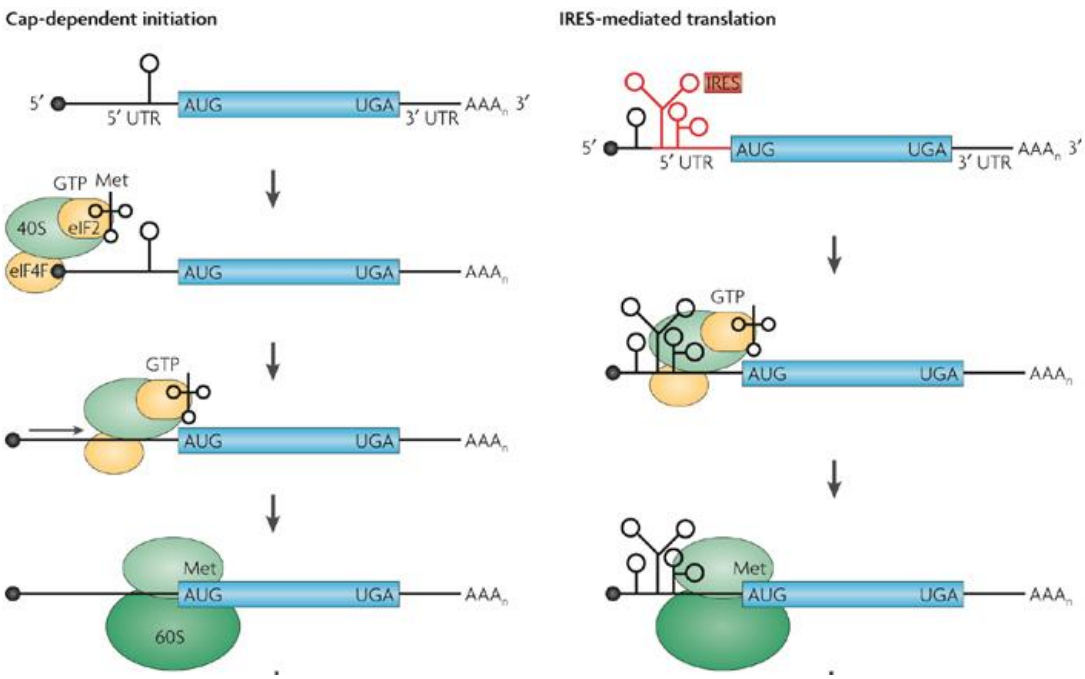


Figure1. 2–1 Cap–dependent initiation and IRES–mediated translation initiation. Adopted from Nature Reviews Genetic 8, 711–723(2007) [7]

1.2.2 IRES (internal ribosome entry site) mediated initiation

Some mRNAs, however, are translated by a cap–independent mechanism, mediated by ribosome binding to internal ribosome entry site (IRES) elements located in the 5′—untranslated region. IRESs are the elements or structures of the RNA of eukaryotes or viruses. Similar to other gene involving in regulation of cell growth, c–Myc mRNA has long and potentially highly structured 5'_untranslation region (5'UTR). The 5'UTR is the region located upstream of the start codon in the mRNA. Non–coding or untranslated regions mediate for the majority of the gene expression regulation[8] and evidence shows that some 5'UTRs contains IRES [9, 10].

IRES elements are found in the mRNAs of several survival factors, oncogenes and proteins crucially involved in the control of apoptosis. It was first discovered in the picornaviral RNAs as cis–acting RNA [11].These mRNAs are translated under a variety of stress conditions, including hypoxia, serum deprivation, irradiation and apoptosis. Thus, IRES–mediated

translational control might have evolved to regulate cellular responses in acute but transient stress conditions that would otherwise lead to cell death[12]. Also as the bridge complex between the cap–binding protein eIF4E, the adaptor protein eIF4G and 3'poly(A) tail can mediate the circulation of mRNA [13]. There are multiple mechanism can contribute to enhance translation. In recent studies, there are more and more experiments show that the initiation translation is via IRES. For example, the apoptotic protease activating factor (Apaf–1), which plays a vital role in cell apoptosis. Through the study of this protein in Hela, HepG2, MCF7, HK293, COS7 and MRC5 cells, the Apaf–1 IRES can recruit the translation process even the cap–dependent translation is not available [14]. And for c–Myc, there is also a lot of evidence show that the IRES exists in 5'UTR. There is study using a dicistronic reporter vector to insert between the two cistrons in c–Myc 5'UTR transfected into both HepG2 and HeLa cells, the downstream ciscron was increased by 50 times [15]. This demonstrating that the translation of c–Myc is mediated via cap–independent mechanism and suggest that c–Myc 5'UTR contains IRES segments. With more and more study proves that the IRES exists in the 5'UTR in the oncogene, the c–Myc IRES structure was extracted and optimized in the past decades [16].

Different from the mechanism of siRNA, binding the IRES can also activate the translation of RNA fragment. The study shows that targeting the c–Myc IRES RNA in the myeloma cells during endoplasmic reticulum stress increased the activity of c–Myc IRES [17].

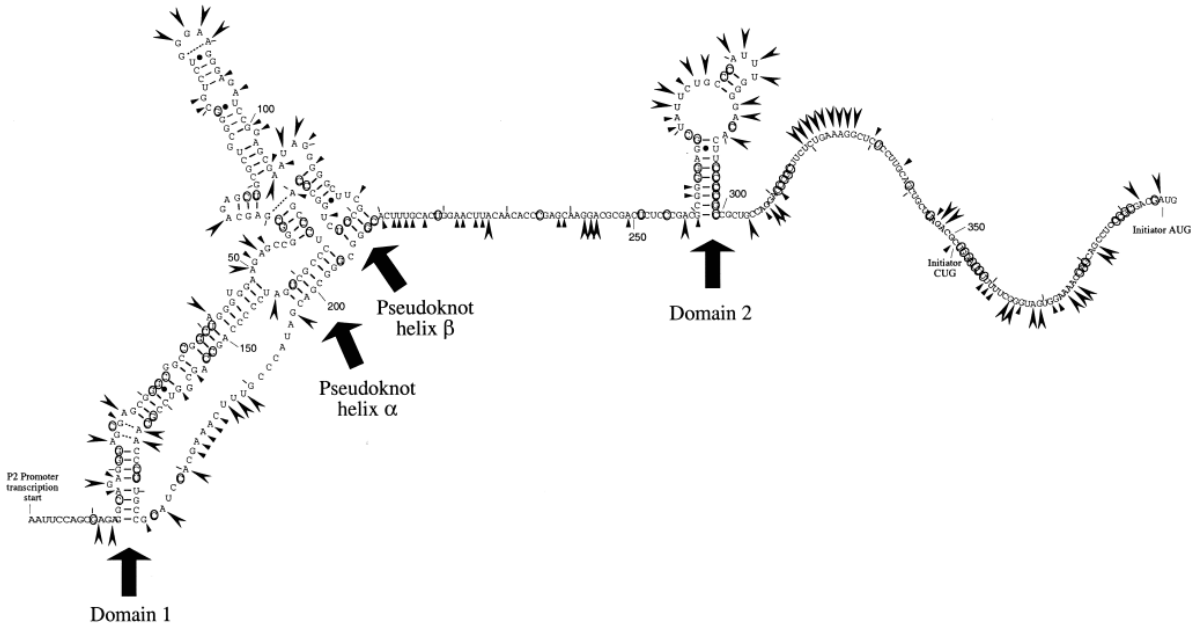


Figure 1.2–2 The possible secondary structure of IRES in c–Myc [16]

1.3 C–Myc deregulation in cancer

1.3.1 General cancer

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. Exposure to particular substances (carcinogens) could increase the risk of specific types of cancer. The carcinogens can be from different factors including lifestyle (nutrition, tobacco use, etc.), naturally occurring exposure (ultraviolent light, radon gas, infectious agents, etc.), medical treatment, pollution and others[18]. The metabolism will change when the normal cells transform to cancer cells, which is activated by amplification or translocation, or as a downstream effect of proto–oncogene. As the cancer cells grow rapidly and have unlimited proliferation, other cells in the body will be affected negatively as the cancer cells will capture most nutrition. This leads to the organ failure and endanger human's lives.

Remove surgery is the most effective way for the undifferentiated cancer. However, if the cancer cells have separated, remove surgery becomes impossible. The traditional cancer therapy is mainly focused on radiation and chemicals. While they will bring very serious

side–effect, which will also limit their function [19]. From the protein aspect, cancer is the result from deregulation of a gene, which means the overexpression and limited expression are both related to the cancer. Therefore, regulating the expression of specific cancer related proteins could be an effective way for cancer therapy.

1.3.2 C–Myc

C–Myc is the first protein found in the Myc family. The Myc proto–oncogene has been implicated to relate to most type of human tumours. Besides, it is a typical molecular feature of initiation and maintenance of tumorigenesis [19, 20]. As a transcription factor, Myc not only affects the process of transcription and co–transcription, but also has impact on the RNA metabolism, including constitutive and alternative splicing, mRNA stability and translation efficiency[21]. The abnormal activation of the c–Myc oncogene can be attributed to either transcriptional overexpression and/or protein stabilization. The c–Myc protein is a 64 kDa protein that is encoded by the c–Myc gene on human chromosome 8[22], which controls many aspects of both processes, and is also a proto–oncogene. Deregulation of c–Myc occurs in more than half of human tumours occur in colorectal, gastric, gallbladder, hepatic, mammary, ovarian, endometrial, head and neck, pulmonary, prostatic, thyroidal, oral, ocular, nasopharyngeal, endocrine, as well as hematopoietic neoplasms.

Also its aberrant expression correlates with many aggressive stages that show resistance to the traditional therapy. This makes it urgent and significant to find an efficient way to control the expression of c–Myc protein. In previous studies using mouse models[23], genetic tools were used to eliminate the expression of the c–Myc protein, which showed a possibility to develop a potential therapy.

1.4 Antisense strategy

Antisense strategy is a method to inhibit or downregulate the expression of target protein by using the antisense oligonucleotides (ONs), which can be DNA, RNA and chemically

modified ONs. The process could be triggering RNAse H [24], blocking translation[25] or alerting the correct splicing [26].

The basic idea is to use a complementary DNA to the target mRNA, and RNase H is recruited to degrade the mRNA. In humans, RNase plays the vital important role to eliminate the viral oligonucleotide. The hybridisation of the mRNA and the synthesized oligonucleotide could activate the RNase to trigger the degradation of the duplex as a DNA–RNA hybrid is not naturally occurring in cells but indicates invasion of foreign RNA [27].

Here we designed the ONs to target the c–Myc IRES RNA and block the binding of c–Myc interactions with the translation system (ribosome) and to stop the synthesis of the c–Myc protein. With a DNA sequence of 16 bp (base pair) in length it can form a unique duplex within the genome and the possibility of the same being found in other places is less than 1%. According to this selectivity, we designed the oligonucleotide with approximately 25 bases to bind with the mRNA. As the standard DNA can bind with mRNA, we synthesised the natural DNA for the first experiments. As the natural DNA will not be stable in the cell due to digestion with nucleases, we also synthesised modified oligonucleotides to make the DNA resistant to digestion, and also make a more stable DNA–RNA duplex. In addition, our aim is not to recruit RNase for digestion of the mRNA but to block translation by interfering with the ribosome, in particular by using alternative initiation steps; this is illustrated further below.

1.4.1 SiRNA

Since 1998, RNA interference (RNAi) was founded to be possible to control the gene expression[28] as RNAi can inhibit the transcription level by activating a sequence–specific RNA degradation process (posttranscriptional gene silence). [29] This has driven the development of siRNA for the past years. As siRNA can be used to inhibit the certain gene in many genetic diseases, it has attracted much attention being a potential therapy.

Especially siRNA can be adopted as a new tool to study the function of single gene both in both vivo and in–vitro [30].

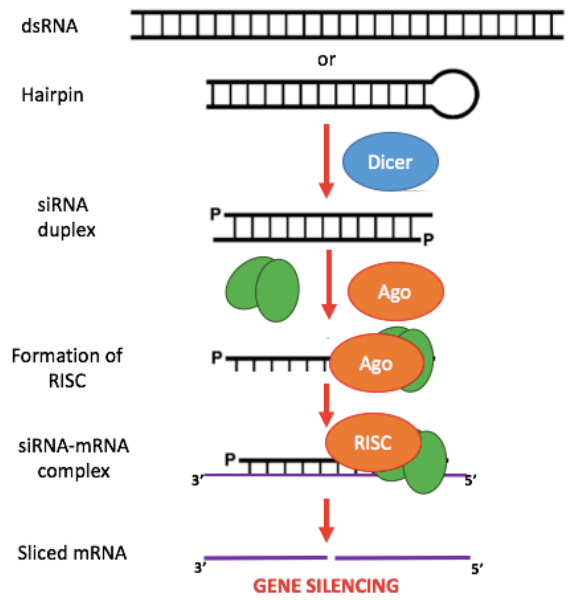


Figure 1–7 Mechanism of siRNA

SiRNA is a short strand double strand ONs, which can cause gene silencing through repression of transcription[31]. For the long strand double strand RNA (may come from hairpin, complementary RNAs and RNA dependent RNA polymerases), it is cleaved by Dicer (an endo–ribonuclease). The cleaved dsRNA forms siRNA, which enables the molecules to form the RNA–induced Silencing Complex (RISC) with other proteins. When siRNA becomes part of the RISC, the duplex siRNA will unwind to form a single strand siRNA. As the single strand RNA can recognize the complementary RNA, which is also the targeting RNA. This can induce mRNA cleavage. The mRNA is recognized as abnormal and degraded by the RNAse. Consequently, the target gene is silenced via this process.

1.5 Modified oligonucleotide

1.5.1DNA structure

DNA is the genetic material to store the information in all organisms. Though out the

single–crystal x–ray analyses, three known DNA family, A,B and Z have been proved through at least one example [32].

The A–DNA is right handed, vertically condensed, antiparallel duplex. The bases are towards the helical axis, which forms a tube–like structure. As the nucleobases mostly locate on the surface of the helix, the minor groove is shallow, while the major groove is wide and narrow. This kind of tertiary structure is mainly adopted by RNA–RNA duplex because of the 2'—OH group on the ribose induces a 3'–endo conformation. This structure is also adopted in RNA–DNA heteroduplexes.

The B–DNA is the most common one adopted in DNA structure. It's a right–handed, antiparallel double helix with a narrow minor groove and a wide major groove. The deoxyribose adopts a 2'–endo conformation in the B–DNA structure. Both grooves are deep as the bases are aligned near the centre of the duplex and almost perpendicular to the helical axis.

Z–DNA is a left–handed, antiparallel duplex with a repetitive unit of two nucleotides (C and/or G).

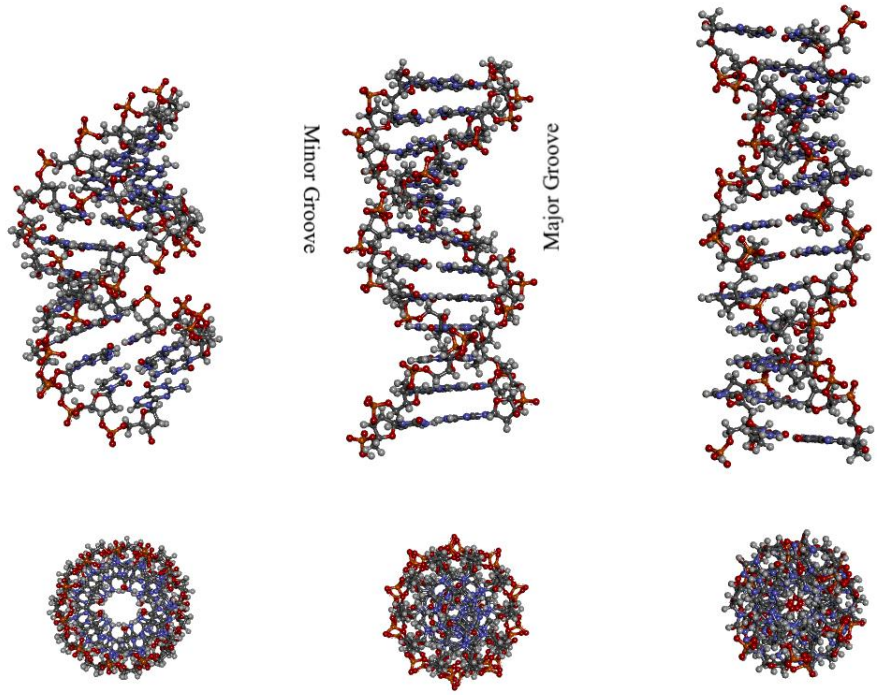


Figure 1.5–1 From left to right, A–DNA, B–DNA, Z–DNA

1.5.2 Modified nucleic acid

The standard ONs used as antisense therapy are in late clinical testing[24, 33]. However the standard DNA ONs are found to have the demerits including immune stimulation, delivery problems and off–target effect [34]. As the gene shares the partial homology, there is the possibility that siRNA target the unexpected gene. While this could be harmful and poisonous for the treated projects. Study showed that the chemical modification of the oligonucleotides can reduce the 'off–target' transcript silencing[35].

So the modified ONs have been attempted, as the modification give the antisense ON higher binding affinities, greater stability and lower toxicity[36]. One of the most common approaches is to substitute the phosphodiester (PO_4) with phosphorothioate (PS) throughout the sequence. Moreover, an O–methyl group (2'–O–Me) can be used to prolong the half–life of the ONs [34]. Adding a methylene bridge connecting the 2'–oxygen with the 4'–carbon of the ribose ring will increase the affinity of the duplex; this modification is

known as locked nucleic acid (LNA) as the ribose is locked in the RNA like conformation (see below).

In this essay, we adopt LNA modified ONs, phosphorothioate ONs and the combination of the two modification.

1.5.2.1 LNA

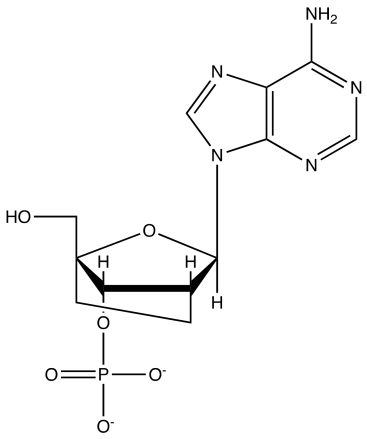


Figure 1.5–1 Structure of LNA modified nucleoside

Adopting oligonucleotides for therapeutic application is now a popular trend. While this novel therapy is dependent on improvement in molecule bio–stable, specificity and delivery. To address this issue, we designed the new RNA–like high affinity analogue, which can form the duplex with the fragment of mRNA to stop the further processes.

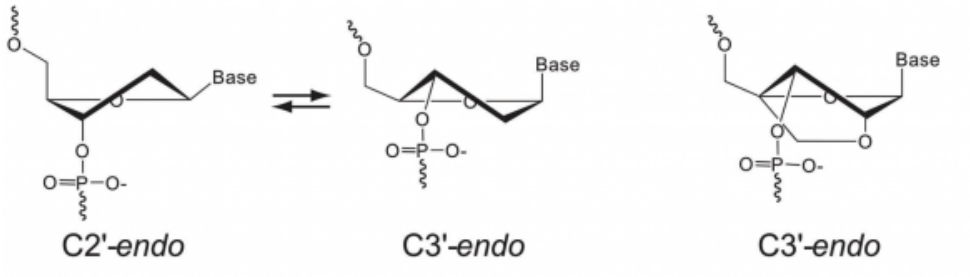


Figure 1.5–2 From left to right, the structure of DNA preferred conformation, RNA preferred conformation and locked nucleic acid (LNA)

LNA is a relatively well–established type of nucleic acid with a bridge from the 2'–OH to the 4'–C, which locks the ribose in the 3'–endo conformation and restricts the flexibility of the ribofuranose ring, creating a rigid bicyclic formation. This makes the LNA–DNA hybrid

duplex more A–DNA like, which will be more stable due to the matched conformation of the sugar moiety. This confers enhanced assay performance and an increased breadth of applications [37]. The LNA is also resistant to nuclease degradation.

1.5.2.2 Phosphorothioate DNA

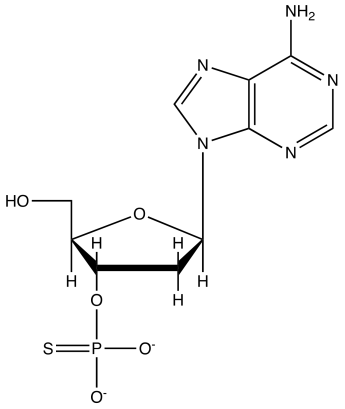


Figure 1.5–3 Structure of phosphorothioate modified nucleoside

Many modifications at phosphate group have been recently considered to use in the antisense approach to regulate the gene expression. On one hand, it will be easier to transfer into the target cells. As the cell membrane is a highly impermeable barrier of the lipid bilayer, the phosphorothioate modification can help the ONs deliver into the cells. On the other hand, the phosphorothioate can prolong the lifetime of the ONs in the exonuclease–rich environment. The antisense phosphorothioate oligonucleotides is highly stable to bind mRNA and trigger RNAse H activity, which can result the hydrolysis of the DNA/RNA duplex[21].

1.5.2.3 Phosphorothioate LNA

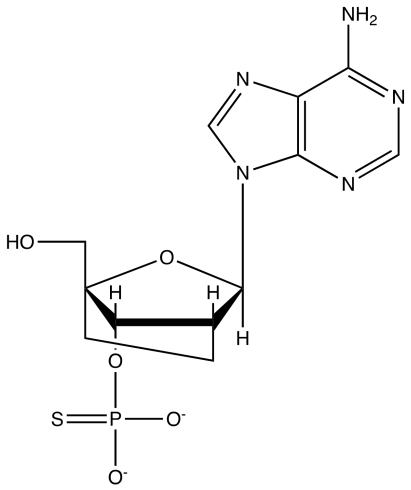


Figure 1.5–4 structure of phosphorothioate LNA modified nucleoside

Here the phosphorothioate LNA is designed to optimize the function of the ONs. Also it will be worth to compare with the single modification to see the superimposed effect.

1.6 Oligonucleotide synthesis

Oligonucleotides can be synthesized by different methods, but the phosphoramidite is most widely used way to synthesise artificial DNA in the laboratory. It can be mainly divided into the following steps: detrytilation, activating and coupling, capping, and oxidation. Then the synthesized oligonucleotides will be cleaved from the solid support and deprotected using concentrated ammonium hydroxide. The purification process depends on the quality of the crude DNA but is usually necessary. This can involve reverse phase HPLC, polyacrylamide gel electrophoresis, or affinity chromatography.

1.6.1 Standard DNA synthesis

The whole process of synthesis the DNA is based on the mechanism of the DNA synthesis circle. This mainly consists of the 4 steps: activation and coupling, capping, oxidation and detrytilation.

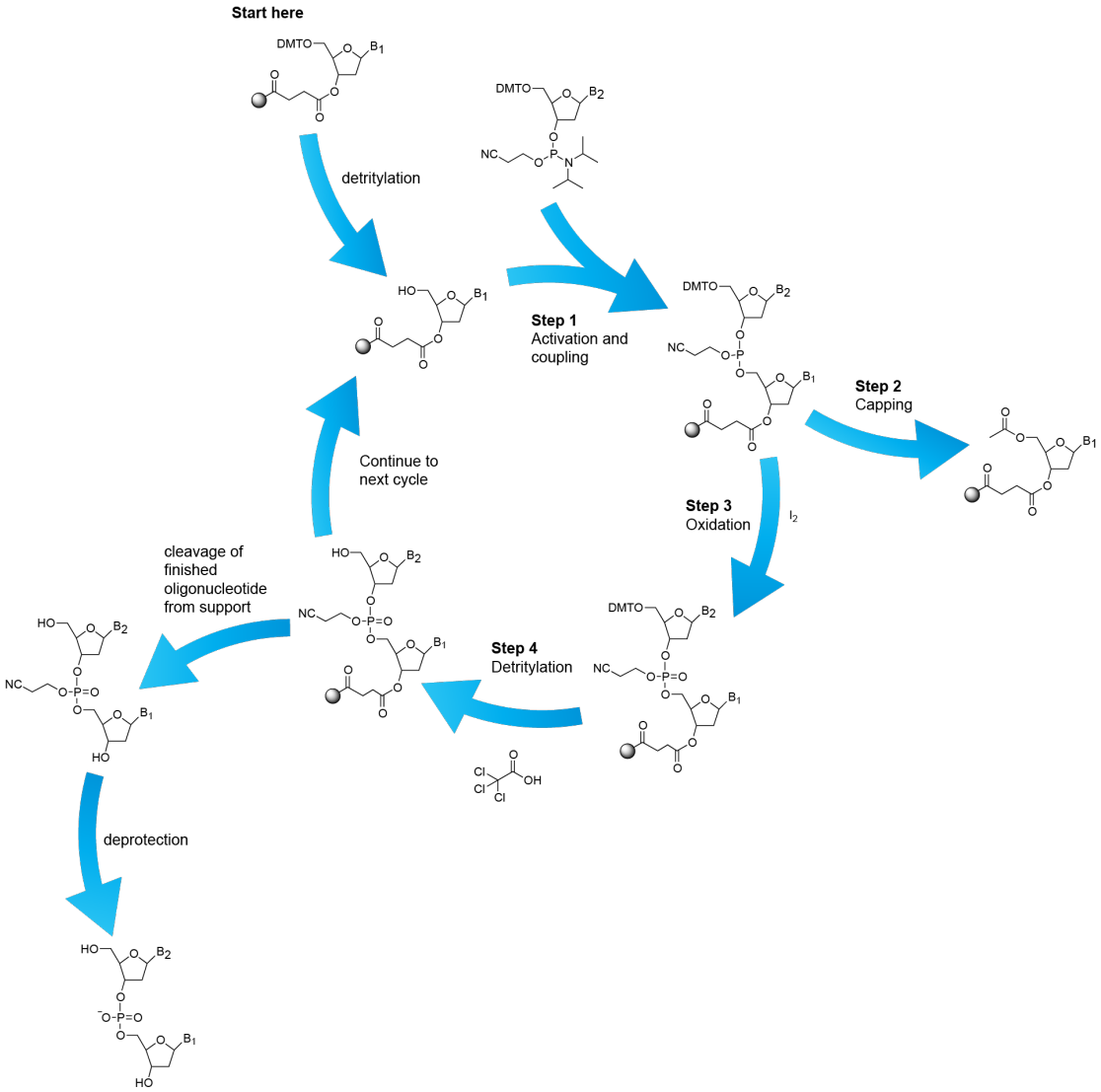


Figure 1.6–1 synthesis circle of DNA synthesis, adopted from the on–line book of Prof. Tom Brown from ATDbio.

–Detrytilation of the support–bound 3'–nucleoside

The nucleoside that is bound to the solid support has a 5'–DMT protecting group, to prevent the polymerization during the resin functionalization. This DMT needs to be removed (detrytilation) from the solid support, so that the next nucleoside can be attached to the 5'–position of the one on the solid support. This is achieved with acid (trichloroacetic acid).

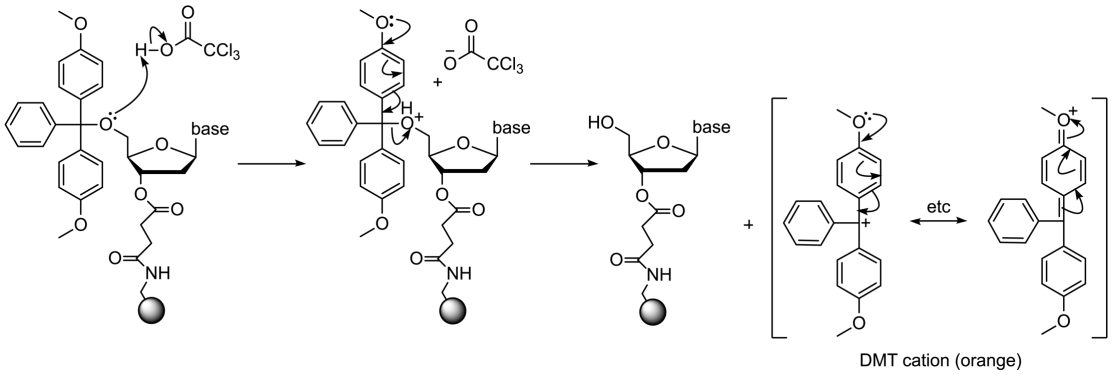


Figure 1.6–2 mechanism of acid–catalyzed detrilation of a DMT–protected nucleoside phosphoramidite

–Activation and Coupling

After the detrytilation, the nucleoside is ready to bind with the next base and gradually form a long strand of nucleotide. Nucleoside phosphoramidite is mixed with activator and dissolved in acetonitrile. The diisopropylamino group in monomer nucleoside is protonated by the activator, and thereby converted to a leaving group and being displaced by attack of 5'–hydroxyl group. Then the new phosphorous–oxygen bond is formed.

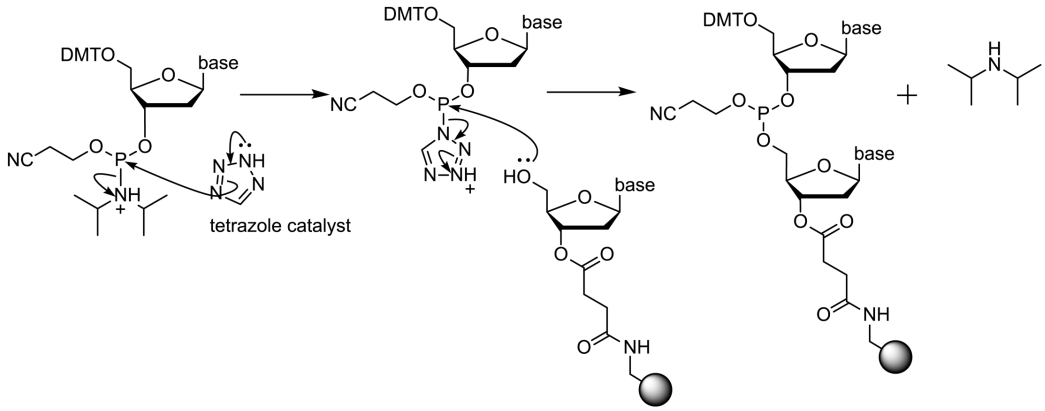


Figure 1.6–3 mechanism of phosphoramidite coupling reaction

–Capping

This step is required to block any unreacted 5'–hydroxyl groups. There is a chance that not all the nucleosides couple to the oligonucleotide bound to the solid support. The new batch of oligonucleotides could then bind to those "free" 5'–hydroxy groups on the solid support.

This would create oligonucleotides of different lengths and sequence. To avoid this problem, a capping step is introduced. During this step two capping solutions are used: N–methylimidazole and acetic acid anhydride, converting the unreacted 5'–hydroxy groups to acetate esters and blocking (capping) them from further reaction.

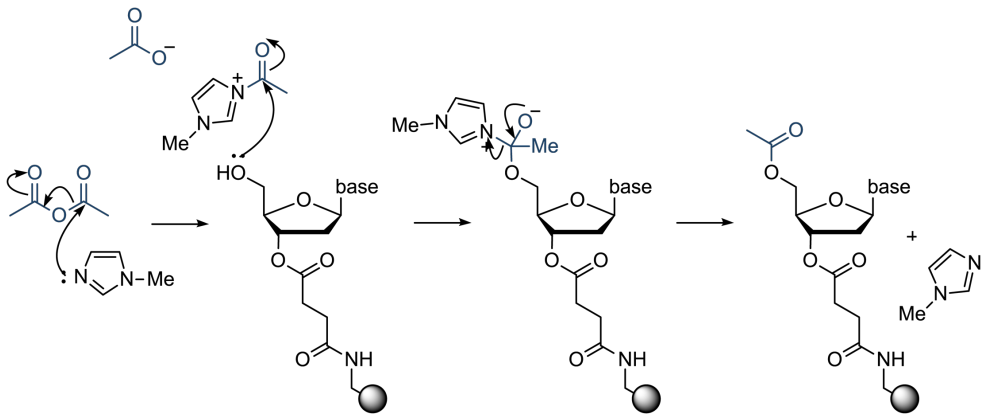


Figure 1.6–4 mechanism of capping step in phosphoramidite

–Oxidation

In the coupling step a phosphite–triester (P(III)) is formed. While the phosphite–triester is unstable in acidic conditions, so it needs to be converted to a stable phosphate triester of P(V) before the next round of the circle. This is done by iodine oxidation in the presence of pyridine and water to convert P(III) to P(V).

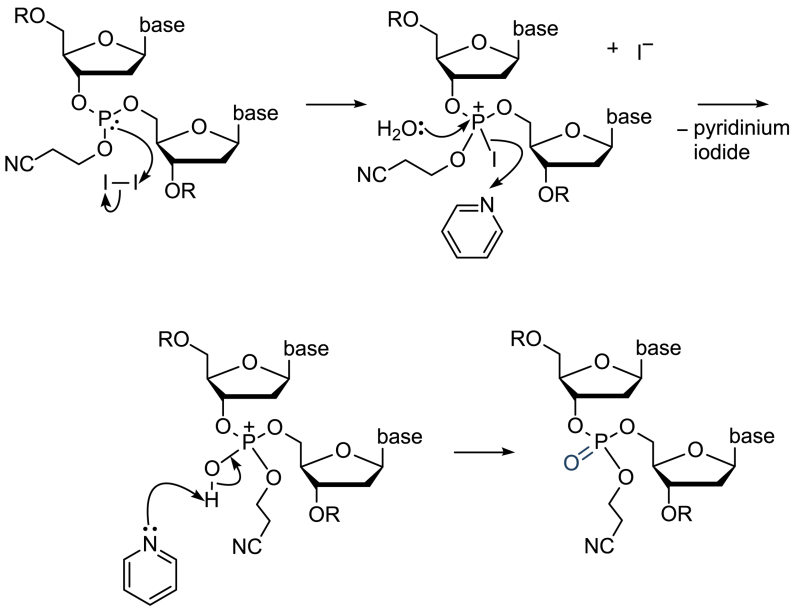


Figure 1.6–5 mechanism of the oxidation in phosphoramidite

– Detrytilation

The protecting group of the oligonucleotide at the 5'–end of the chain needs then again to be removed for the next coupling step. Only when it is removed will the primary hydroxyl group react with the next nucleoside. Deprotection is done with trichloroacetic acid in dichloromethane. The resulting orange colour of the released trityl group can be used to monitor the previous coupling efficiency through recording its absorbance.

1.6.2 Modified oligonucleotide synthesis

As the ONs are becoming popular in antisense therapy, synthesis of the optimized ONs attracts more attention in recent year. This encourages the reagent to help to synthesis the modified ONs through the solid phase DNA synthesiser.

1.6.2.1 Phosphorothioate DNA synthesis

To synthesis the phosphorothioate ONs, the key point is the replacement of the oxygen by sulfur. A number of reagents have been designed and tested in recent years for sulfurization of phosphorus(III) compounds from which those based on the 1,2,4–dithiazole skeleton appear to be advantageous alternatives to existing sulfurizing reagents.

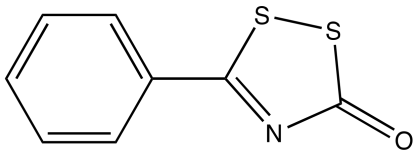


Figure 1.6–6 structure of 1,2,4–dithiazole–5–ones

The reaction involves rate–limiting nucleophilic attack of phosphorus on sulphur producing a phosphonium salt intermediate which then quickly decomposes to the corresponding product containing a P=S group[38].

1.6.2.2 LNA phosphoramidite synthesis

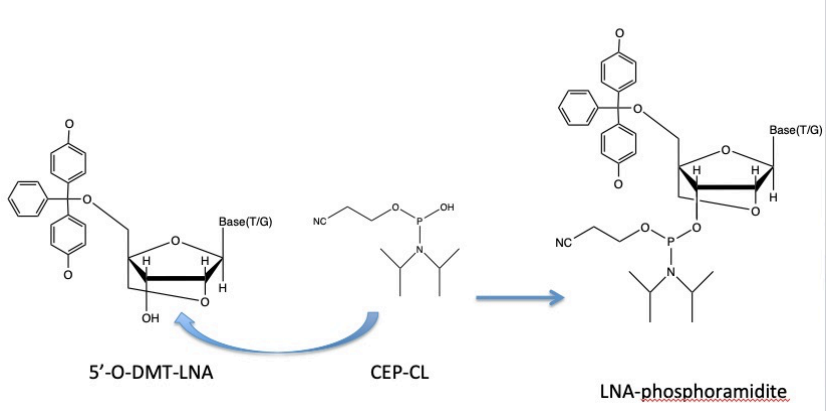


Figure 1.6–7 the mechanism of synthesizing the LNA phosphoramidite

LNA–DNA chimeras containing phosphodiester linkages can be assembled by standard DNA synthesizers using 2–cyanoethyl DNA phosphoroamidites and 2–cyanoethyl LNA phosphoroamidites. Compared to the standard DNA synthesis protocols, LNA requires longer coupling time and oxidation time to make sure the efficiency. When the LNA has been assembled, it is removed from the solid support as the 5′–end–*O*–dimethoxytrityl (DMT) protected LNA oligomer by treatment with concentrated aqueous ammonia that also removes the phosphate and nucleobase protecting groups.

1.7 Purification

The purification can be carried out using different methods. Here the Glen–Pak method is adopted, as it will produce a product pure enough for ONs transfection. As with Glen–Pak™ cartridges utilize the 5’ DMT retained on an oligonucleotide to specifically bind full–length sequences to the support. During the purification process, the failure sequences are eliminated and the DMT is subsequently removed, allowing for elution of purified product.

Aim of the work

As antisense therapy is used in many fields, here we designed the oligonucleotides to bind with the IRES of the c–Myc to block the process of translation, so as to inhibit the formation of c–Myc protein. As this work is following the result from previous experiments, the possible sequences have already been selected by my supervisors Eugen and Tilman and my colleague Ane.

1. Synthesis the standard and modified ONs.
2. Transfect the cells with different ONs. Optimize the protocol with better transfer reagent, functioning concentration, transfecting time, etc.
3. Check the viability of cells with flow cytometer.
4. Compare the results in different modification group.
5. To address the mechanism of the inhibition process on protein expression, using Western Blots to determine whether the protein levels have decreased.

Experimental work

3.1 Oligonucleotide synthesis

Solid–phase synthesis is carried out on a solid support held between filters, in columns that enable all reagents and solvent to pass freely. Expedite 8900 is the model of the DNA synthesiser being used for all the ON synthesis. The nucleotide phosphoramidites, solvents, and reagents are ordered from Link Technologies (UK) or Tides Service (Germany).

3.1.1 Standard oligonucleotides synthesis

The standard ONs are synthesized by the DNA Synthesizer with 1 μmole scale.

Then followed by manually wash with 1ml of concentrated aqueous ammonia for one hour to cleave from the polymer support. The mixture is transferred to a screw cap tube. And one last wash with 500 μL concentrated aqueous ammonia to get most of the ONs also goes into the screw cap tube. Deprotect the strands by heating up at 40°C for at least 6 hours or room temperature overnight.

The purify process follows the Glen Pak Cartridge protocol.

3.1.2 Phosphorothioaite DNA synthesis

The reagent is Sulfur42 (3–phenyl–1,2,4–dithiazoline–5–one), bought from SIGMA–ALDRICH. Reagent Sulfur42 is dissolved in anhydrous acetonitrile with the concentration of 0.1M in the synthesis vial and set on the DNA synthesiser. Ensure the chamber is sufficiently primed. Enter the sequence. Adjust the synthesis protocol 1. from ‘coupling–capping–oxidation’ to ‘coupling–sulfurization–capping ’, 2. use three equivalents sulphuring reagent, 3. set the reaction time with 60 seconds. Then start the reaction. The bright orange solution shown after the oxidization is a potential symbol for successful synthesis.

The cleavage process is the same as the standard ONs. Also do freezing dry to preserve the ONs after the determination of UV and MS.

3.1.3 Locked nucleic acid synthesis

As the synthesis started from 3' and we have the material of locked nucleotides T and G, the protocol can only allow the standard bases to start with.

Take 1 equivalent (349 μmole) of 5'–O–DMT–T–LNA (or 5'–O–DMT–G–LNA) in the flask and dry it by vacuum overnight. Resolve the dried material into 5ml DCM with the spinner. Carry on the whole reaction in the 2 neck round–bottom flask with Argon atmosphere. Set the molecular sieve. 4 equivalent (1396 μmole) of DIPEA are added and mix for ten minute. Then use 3 equivalent (1047 μmole) CEP–CL to substitute the H in —OH group. Monitor the reaction with TLC with the running solution of DCM and 10%MeOH. Leave the reactant for approximately 2 hours until all the starting material run out. Add more CEP–CL when massive starting material remains.

Add 5mL of hexane and put the whole flask in the freezer or on the ice to lower down the temperature. The yellow oily compound was formed. Remove the hexane and use the Argon to purge off the remaining hexane. Resolve the yellow precipitate with 0.5mL acetonitrile and remove into the DNA synthesiser vial. Wash with 1.5ml acetonitrile and transfer to the vial as well. Gently shake the vial to mix the solution.

3.1.4 Phosphorothioate locked nucleic acid synthesis

Combine the process of phosphorothioate ONs and LNA ONs. Modify the whole strand with the thio by setting the sulphuring 42 reagent and change the protocol. Using the LNA phosphoramidite to make the LNA nucleotides.

3.1.5 Purification by Glen–Pak

Glen–Pak DNA Purification Cartridge	1 set
HPLC Grade Acetonitrile	1mL

2.0M Triethylamine Acetate (60–4110–XX, TEAA, pH7)	
100 mg/mL Sodium Chloride	2mL
Salt Wash Solution (5% Acetonitrile in 100mg/mL Sodium Chloride)	2ml
2% Trifluoroacetic Acid (TFA)/Water	1ml
Water	1ml
50% Acetonitrile/Water containing 0.5% ammonium hydroxide	1ml

Concentrate the deprotected ONs by evaporate the ammonia solution to approximately 1ml. Mix the deprotected ONs with 100mg/ml sodium chloride with the scale of 1:1. Load the mixture into the glen–pak cartridge. Use the syringe and needle to take the solution to wash the cartridge, following the turn and dilution among the table. The last step of 50% acetonitrile with water containing 0.5% ammonium hydroxide is to collect the purified ONs. Then preserve the ONs in the 1.5ml screw–cap tube.

After the purification, freeze dry is used to remove the solution, so as to preserve the ONs longer. Normal drying ways are not adopted as the modification of the ONs could make the strands easier to degrade. In order to be meticulous and precise, the ONs are left in the –80°C freezer to become icy and then use the vacuum evaporator to dry the solution.

3.2 UV–VIS spectroscopy

In order to determine the concentration and purity of ON strands, UV–VIS spectrometer is used to determine the absorbance around 260nm. As general knowledge described, the absorbance of the ON strand has a comparably stable ratio of A260/A240=A260/A280=1.6. If the peak is left shift, it could result by not thoroughly washed salt. Or right shift, may lead by protein as byproduct.

The measurement was carried in the UV–spectroscopy in University of Southampton.

Blank measurement is 300uL H_2O. The spectrometer scan from 0 nm to 300 nm. Scanning speed 1 nm per second.

Record all the readout and calculate the concentration. While all the A (absorbance) value need to be between 0.2–1.0 to make sure the reliability of the data.

According to Beer–Lambert Equation [39], which provides a relationship among absorbance(A), molar absorptivity (), path length (L), and molar concentration(c)

A=Lc

Here the cuvette used is L=1cm

is calculated through the website of ADTbio: https://www.idtdna.com/calc/analyzer

3.3 Mass spectrometry

Mass spectrometry is used to measure the purity of ONs.

Samples are submitted with 40uM in 20ul water to the University of Southampton mass spectrometry.

3.4 Tissue culture

3.4.1 Cell maintaining

Cells are growing in a 15 cm (diameter) dish with 7 ml growth medium. This consist of DMEM and 10% of FBS. DMEM is the basal medium for cell growth, which provide the basic amino acid, vitamins and etc. While the FBS is to deliver the nutrients and growth factors, also protect the cells from oxidative damage and apoptosis. DMEM and PBS are purchase from ThermoFisher.

Cells are split every three days. Remove the medium in the plate and wash of the dead cells and remaining medium with 7ml PBS. Then add 1.5ml Trypsin and incubate in the incubator to detach the cells. Check the cells under the microscope. When most of the cells detached from the plate, add 8ml growth medium to stop Trypsin and transfer the mixture in the 15ml centrifuge tube. Separate Trypsin from cells by centrifuging the mixture with 1500 rpm and discard the supernatant. Add 3 ml pre–heated growth medium and mix with cells. Take 1ml of the mixture in a new culture plate and add another 7ml pre–heated growth medium. Trypsin is ordered from Sigma–Alorich.

3.4.2 Cell freezing

Collect the cells with the concentration of 5–10 million per ml, which is the best of the cells to be frozen. Add 10% of DMSO to slow down the metabolism of cells. The rest consists of 50% FBS and 10% DMEM to maintain the cells. Use the freeze panes to freeze the cells in −80°C.

3.4.3 Cell defrosting

The cells are defrosted in the room temperature. 10ml of growth medium is added and centrifuge to discard the DMSO. Seed the cells in the culture plate with growth medium. 10% FBS is added to help the cells to recover.

3.4.4 Cell counting

Take 80uL of cell homogenate and add 20uL of Trypan Blue to dye the cells. Count cells manually under the microscope.

3.5 Transfection

Day 0

Seed 15,000 cells per well in 24–well plate the day before transection. Add 500μl of growth medium in each well. Incubate at 37°C， 5% CO2, allow enough time (mostly overnight) for the cells attach to the bottom.

Day 1

45pmole of ONs are diluted in 100μl transfer medium. Then add 2μl of the transfer reagent (Opti–MEM, purchased from ThermoFisher) immediately homogenize by vortex for 10 seconds. Incubating in room temperature for 10minutes. While the complex is forming, remove the medium from the plate and add 500μl of the pre–heated medium. After that, mix the complex with the cells gently. Incubate the cells at 37°C at 5% CO2 for two days.

Day 3

1.For flow cytometry

Take out the entire growth medium in the flow cytometry test tube. Wash cell with 100µl PBS, preserve the PBS with growth medium. Detach the cell with 100ul Trypsin, incubate in the incubator for 3 minutes. Add 100µl MACS buffer to stop trypsin and remove to collect with growth medium. Wash with 100µl MACS buffer. Also transfer into the flow cytometry test tube. Centrifuge the test tube with 1500 rpm for 5 minutes, dispose the supernatant.

MACS Buffer	
Phosphate–buffered saline (PBS)	Solution
Calf serum	2%
EDTA	2mM

2. For western blot

Collect the cells with the same process as flow cytometry. Wash with 500 µl of PBS, centrifuge with 2500 rpm and discard the supernatant. Keep in the freezer –80°C.

3.6 Flow cytometry

Flow cytometry is a widely used method for analysing the expression of cell surface and intracellular molecules, characterizing and defining different cell types in a heterogeneous cell population, assessing the purity of isolated subpopulations and analysing cell size and volume. In order to use this method, label all the cells with fluorescent. The fluorescent enables the flow cytometer to identify the transfected and untransfected cells, because each will give a different signal. All the flow cytometry experiments are carried in Southampton General Hospital. The machine is BD FACSAria Flow Cytometer from company BD Bioscience(UK). The labelling material is 4′,6–diamidino–2–phenylindole(DAPI) purchased from Thermofisher. DAPI is a blue–fluorescent DNA stain, which binds the AT region in dsDNA. Because its high affinity to dsDNA, it is also used for counting cells, measuring apoptosis and sorting cells based on DNA content. Here DAPI is used to mark the dead cells.

When all the cells are collected from 24 well plate after the transfection. In order to keep

the cell viability stable during the experiment process, MACS buffer is used to maintain the cells.

Add 50–100 µl of MACS buffer and gently shake the samples to form a uniform solution. Add 1µl of DAPI into the sample and mix properly just before loading on the flow cytometry machine.

Prepare the control samples with 10 times volume of the experiment group. One with all the alive cells as control, the other 1:1 scale mixture of dead and alive cells. Staining with exactly the same conditions as the experiment group.

Use the control to adjust the parameter for side scatter (SSC) and forward scatter (FSC), choose the range for the cells. Change the parameter of violet laser until the graph shows a sharp clear peak at 102. Staining the mixture cells with DAPI. Fine adjustment to have separate peaks.

Load the samples analyse 20,000 cells to give a result.

3.7 Western blot and coomassive staining

3.7.1Protein extraction

RIPA buffer(1x)	
Sodium chloride	150mM
SDS	0.1%
Triton X–100	1.0%
Sodium deoxycholate	0.5%
Tris(pH=8.0)	50mM

Freeze cell lysate in –80°C overnight. Dilute 100µl of RIPA(10x) buffer and 100 µl of protein inhibitor in 800µl of water. Cells were on the ice during the whole process. Mix 20µl of the lysate mixture with cell pellet, use pipet up and down to break the cells. Vortex the samples every 5 minutes. After 20 minutes, centrifuge with 13,000 rpm for 15 minutes. Take the supernatant in new Eppendorf tubes and discard the pellet.

3.7.2 Protein quantification

A bicinchoninic acid (BCA) protein assay was done to calculate protein quantity in the samples. The assay was done using an appropriate 96 well plate. A standard linear connection was prepared using 25µl of known concentration BSA, which are 2000µg/ml, 1500µg /ml, 1000µg /ml, 750µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 25µg/ml and 0µg/ml. Dilute the samples 3µl into 30µl MilliQ water and take 25µl of the samples into the test wells. Add 200µl of the working reagent to each of the wells and gently shake 30 second to mix the samples. Cover the plate and incubate in 37°C for 30 minutes. Cool down to room temperature and measure the absorbance at 562nm on a plate reader.

3.7.3 Western blot and coomassive staining

The detection of c-Myc protein is done by western blot and coomassive staining. Running the proteins in the SDS-polyacrylamide gel to separate the different protein, incubate with the primary antibody to find the target protein. Attach the secondary antibody to show form the detectable signal and compare the intensity of the signal to draw the conclusion about the expression in protein level.

Separating gel(12%) for 15ml	
D.water	5.03ml
Tris-HCL(1.5M,pH=8.8)	3.75ml
Acrylamide gel solution (30%)	6.0ml
10%SDS	150µl
10%APS*	75µl
TEMED	7.5µl

Stacking gel(4%) for 15ml	
Acrylamide	1.98ml
0.5MTris-HCL(pH=6.8)	3.78ml
10%SDS	150µl

D.water	9ml
10%APS*	75μl
TEMED	15μl

*The APS is used to dehydrate. So add right before pulling into the gel model.

Making the gel with the listed recipe.

Mix the sample with loading bu♪er 1eq: 1eq. The total volume should be less than 40∟l to ♫t the gel well. Boil the samples at 95 ŹC for 5 minutes to denature the proteins.

Running buffer (10X)	
Tris –HCl (pH=8.5)	25 μM
SDS	0.1%
glycine	192mM

Set the gel in the tank filling with 1X running buffer. Load the samples in different wells. Run the gel in 150V for approximately one hour until the tracking dye reach the bottom.

For coomassive staining, the gels are washed with water and staining the gel in the coomassive blue to check whether there is the band shown in the relative protein size.

For western blot, the proteins are transferred from gels to the Nitrocellulose membranes via wet transfer.

Transfer buffer	
Glycine	2.93g
Tris–base (1.5M pH=8.3)	32ml
MeOH	200ml
Water	1L

Making the transfer bu♪er and preserve in -80 ŹC for 30 minute to cool down. Prepare the sandwich transfer cassette of pad-♫lter paper-membrane-gel-paper-pad. Set it in the tank and transfer with 2 hours with 230mA stable electric current.

Wash off buffer on the membrane with water and block the membrane with 5% milk in 0.5% PBS solution for one hour.

Washing buffer (1L scale)	
PBS	50g
TWEEN	1ml

Wash the membrane with washing buffer 3 times, 5minute every time. Incubate with the primary antibody with the dilution of 1:500 in 5% milk in 0.5% PBS in 4 ŹC overnight. The primary antibody is anti-C-MYC rabbit, purchased in Abcam

Wash with washing buffer 3 times, 5minutes each. And apply the secondary antibody anti–rabbit with the dilution of 1:2000 in 5%milk.

Wash the membrane to clean out the milk and secondary antibody and apply the HRP kit to trigger the signal and scan the membrane.

Attach the actin by incubate the membrane with actin dilute in 5% milk PBS solution with the dilution of 1:5000 for one hour. Wash off the milk and actin with washing buffer, and repeat the washing three times. Apply the HRP kit again to get the actin signal.

Result and discussion

4.1 Sequence selecting

The oligonucleotide is designed based on the secondary c–Myc IRES structure [16]. The five sequences are selected from different part of the IRES structure. Some are from the duplex to open the structure to inactive the function. And there is one designed to bind the transcription starting point. Also the bubble sequence is designed to target the bubble loop, so as to stop the transcription process.

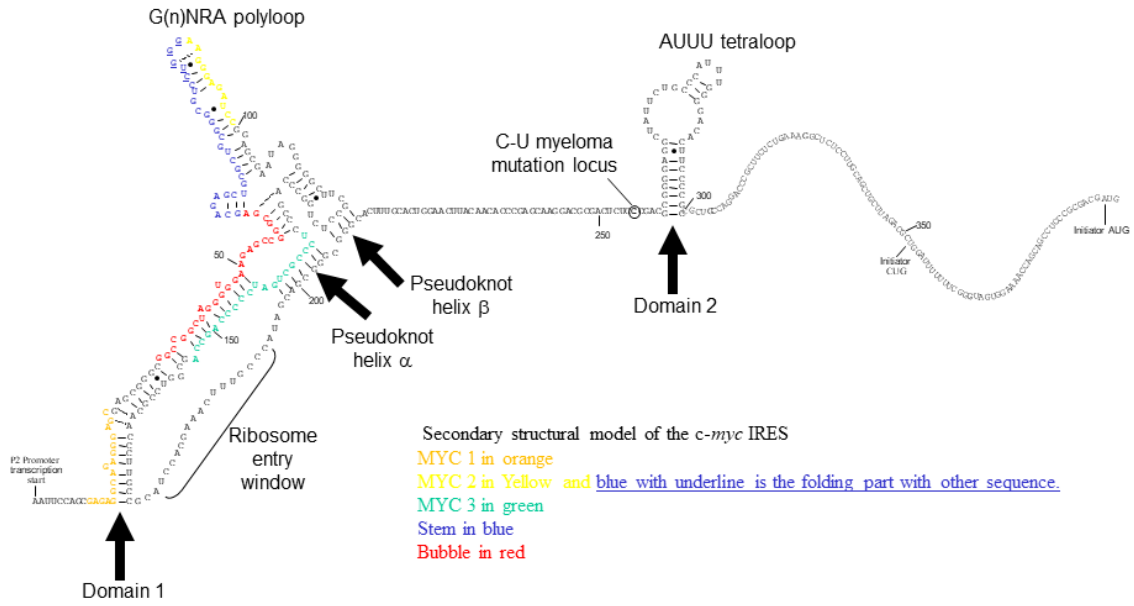


Figure 4–1 the secondary structure of C–MYC IRES. Five ONs are chosen from the fragment of the IRES structure, which are labeled in different colors.

4.2 Sequence modification

4.2.1 Standard ONs

The unmodified sequences are synthesised according the c–Myc IRES structure.

BUBBLE	5'–TCG CCC GGC TCT TCC ACC CTA GCC GGC C

STEM	5'–CCC AGG ACG CCC GCA GCG CAG CTC TGC T
MYC 1 *	5'–CTC TCC GTC TCC CTC GCT CG
MYC 2 *	5'–GCA GGA CCC TTC CCT CTA GG
MYC 3	5'–AGG GCG ACT AGG GGG TCG GT
CONTROL A	5'–ACT ACC GTT GTT ATA GGT GT

*Figure 4–2 Sequences chosen for the experiments. * As MYC 1 and MYC 2 doesn't work well, when comes to modified ONs, they are not involved.*

4.2.2 Phosphorothioate modified ONs

The sequences correspond to a fully modified phosphorothioate DNA (PS–DNA), where all phosphate groups contain sulphur.

BUBBLE	5'–TCG CCC GGC TCT TCC ACC TAG CCG GCC
STEM	5'–CCC AGG ACG CCC GCA GCG CAG CTC TGC
MYC 3	5'–AGG GCG ACT AGG GGG TCG GT
CONTROL	5'–ACT ACC GTT GTT ATA GGT GT

Figure 4–3 Phosphorothioate modified DNA.

4.2.3 LNA modified ONs

For the LNA modified ONs, LNA phosphoroamidites of G and T are designed to form the LNA–DNA chimera. This modification is not adopted for the whole strand because the LNA modifier is rather expensive, and fully modified LNA sequences could lead to issues about toxicity and solubility.

BUBBLE	5'–**T**CG CCC **G**GC **T**CT **T**CC ACC C**T**A GCC **G**GC C
sSTEM*	5'–CCC A**G**G AC**G** CCC **G**CA GC**G** CAG C**T**C TGC **T**C
MYC 3	5'–A**G**G GC**G** AC**T** AGG **G**GG **T**CG **G**T
CONTROL	5'–AC**T** ACC **G**TT **G**TT ATA **G**GT **G**T

Figure 4–4 LNA phosphorothiate modified DNA.

4.1 Quality of oligonucleotides

4.1.1 Standard ONs

	Standard MW	Mass spectrometry result peak	Extinction Coefficient L / (mole*cm)	Final volume (μmole)
Bubble	8398.4	8397.8	226200	0.9036
Stem	8496.5	8495.8	253100	0.9651

Figure 4–5 qualities of standard ONs in the aspects of MS result and yield (appendix figure 1–2).

According to the mass spectrometry result (data can be found in appendix figure 1 and 2), the standard ONs have the molecular weight mistake less than 1 mass unit. And the UV result shows the peak around 260nm. The ONs are synthesised with 1μmole scale, so yield is more than 90%.

4.1.2 Phosphorothioate ONs

	Standard MW	Mass spectrometry result peak	Extinction Coefficient L / (mole*cm)	Final volume (μmole)
Bubble	8832.1	8831.2	226200	0.826
Stem	8930.2	8929.3	253100	0.7988
MYC 3	6584.2	6582.9	199100	0.7314
Control	6443.2	6642.9	194600	0.8798

Figure 4–6 qualities of phosphorothioate ONs in the aspects of MS result and yield (appendix 3–6).

The mass spectrometry and UV results are quite reliable. The mass spectrometry results are shown in appendix, figure 3–6 respectively. With one step of phosphorothioate, the yield is not as good as standard one, but still with more than 70% efficiency.

4.1.3 LNA

	Standard MW	Mass spectrometry	Extinction Coefficient	Final volume (nmole)

		result peak	L / (mole*cm)	
Bubble	8566.5	8566.5	226200	56.87
Stem	8953.8	8953.9	253100	82.70
MYC 3*	6447.2	6646.3	199100	26.36
Control*	6278.1	6277.2	194600	22.36

Figure 4–7 qualities of LNA ONs in the aspects of MS result and yield (appendix 7–9)

*MYC 3 and control are synthesised with 1μmole solid support. After this we changed cartridge to improve the yield.

The LNA doesn't have very good yield. During the synthesis of the LNA phosphoramidite, we did see the yellow oily precipitate. But it wasn't very clear and colour was comparable whitish. So during the ONs synthesis, we saw light orange, which is supposed to be bright orange during the coupling process. So we inferred the coupling process was not very efficient. Then the 200 nmole solid support cartridge was adopted with the 1μmole protocol to maximize the coupling efficiency. And the mass results are available in appendix from figure 7–9.

The effect of stepwise yield (coupling efficiency) on overall percentage yield

Length (bases)	90%	95%	97%	98.5%	99.5%
10	38.7%	63.0%	76.0%	87.3%	95.6%
20	13.5%	37.7%	56.1%	75.0%	90.9%
50	0.6%	8.1%	22.5%	47.7%	78.2%

According to the table above, even the stepwise yield is 1% less; the overall outcome will be much lower. So for this synthesis, the finial yield was comparable low with the percentage of 15%–30%. However, for one transfection, only 45 pmole is required. So this ONs is enough for the experiment.

4.1.4 Phosphorothioate LNA

	Standard MW	Mass spectrometry result peak	Extinction Coefficient L / (mole*cm)	Final volume (nmole)
Bubble	8694.9	9000.15	226200	15.49
Stem	9403.5	9405.3	253100	17.52
MYC 3	6752.3	6751.8	199100	15.60
Control	6583.2	6852.8	194600	20.39

Figure 4–8 qualities of phophorothioate LNA ONs in the aspects of MS result and yield (appendix 10–12)

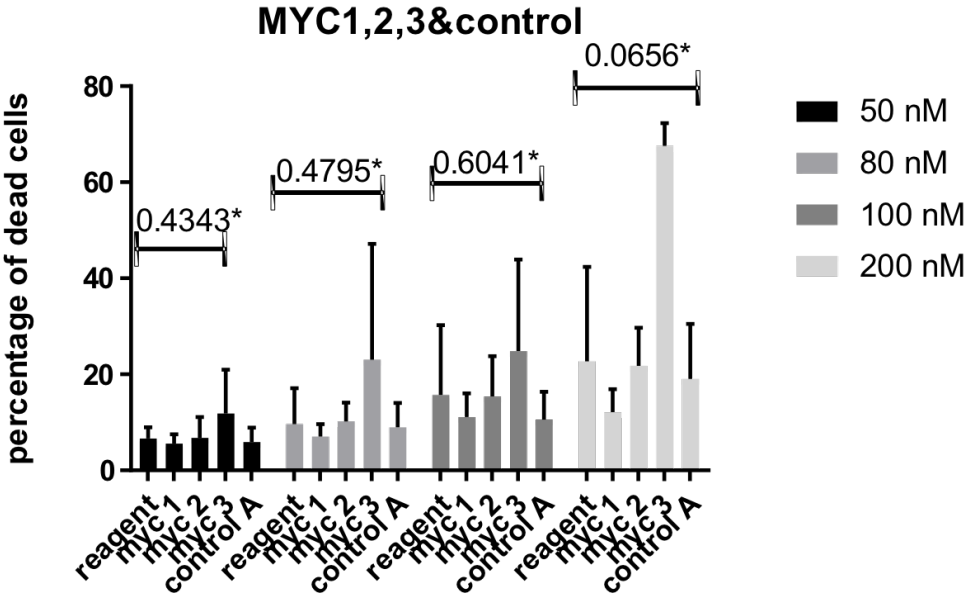
As the LNA phophoramidite shows an imperfect yield, this also has comparably lower output with similar amount of LNA. And the phophorothioate didn't affect the yield, as the reagent performs well. All the mass results are shown in the appendix, figure 10–12.

4.2 Cell assay of transfection

All the plates are checked under the microscope before doing the flow cytometry. One day after the transfection, it's significant to see some cells flowing on the medium, which is possibly result from being transfected by the ONs. And the further precise data is measured by flow cytometer.

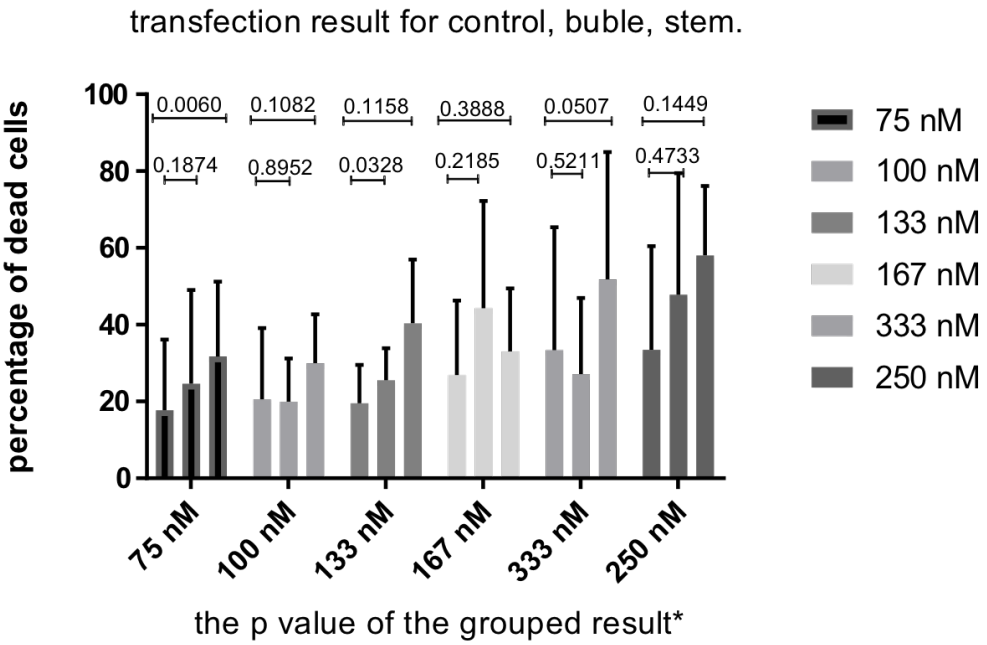
4.3 Flow cytometry

For the standard myc1, 2, 3, they are designed to target the IRES point as the picture shows. While only MYC 3 shows comparably significant affect to the cells. And the control sequence is a scramble sequence, which is supposed to have no impact to cells. From the standard ONs experiment, it seems to satisfy the requirement. So the control and MYC 3 are chosen to be used in further modified ONs experiments. All the data are coped via Prism 7.



*The data is P value for MYC 3, which means the reliability according to the triplicate of the experiments. The smaller number means more reliable. Comparably, when the concentration is 200 nM, it's obvious that the ONs are working well.

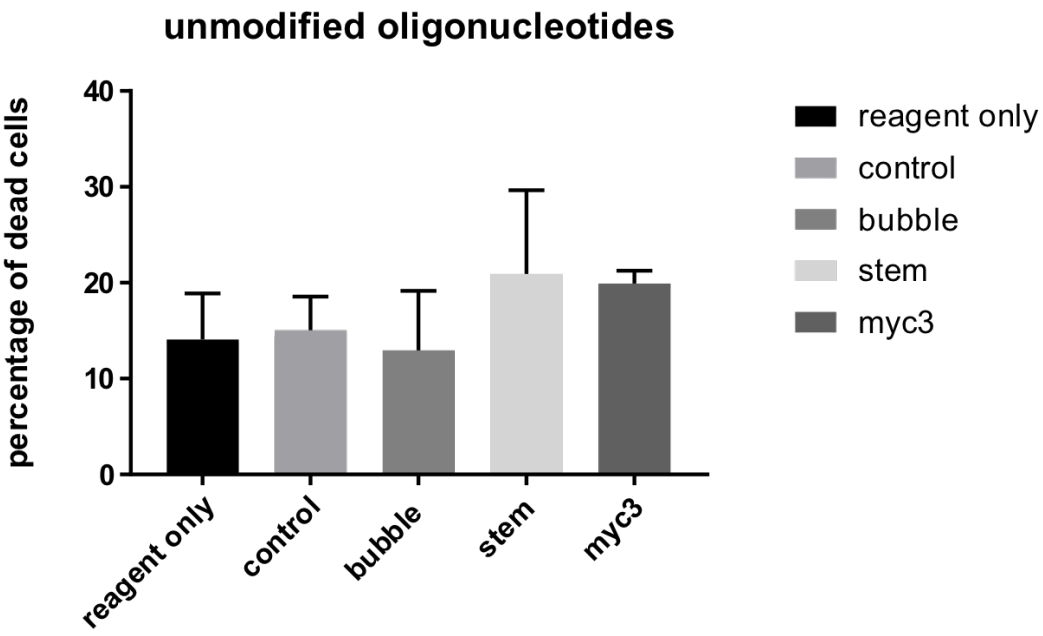
The original data is available in the appendix figure 13. With different concentrations and different oligonucleotides, the transfections were carried for two days to acquire the dramatic results. All the experiments with different parameters were repeated for three times in totally same conditions, so as to acquire reliable result.



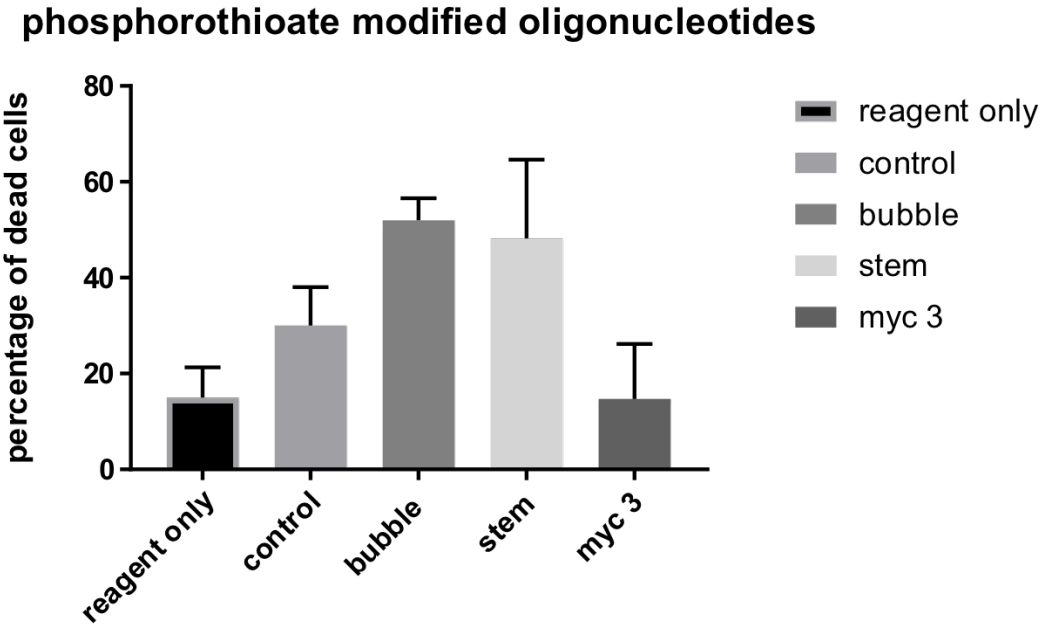
All the original results are shown in the appendix figure 14. For each condition, the experiments were carried five times to gain the final data to analyze. According to these primary results of the viability and performance stability, 4 of them are selected as the possible strands to carry on. So the control, which is the scramble sequence, bubble, stem and MYC 3 are chose to have more experiments. And the concentration of the ONs is set with 75nM. As this concentration has the comparably stable result also is very economic.

	Reagent	Control	Bubble	Stem	MYC 3
Standard ONs	14.08%	15.04%	12.94%	20.90%	19.90%
Phosphorothioate ONs	14.94%	30.02%	51.98%	48.18%	14.70%
LNA ONs	13.02%	69.74%	36.86%	56.1%	28.7%
Phosphorothioate LNA ONs	14.08%	52.84%	70.00%	48.66%	31.38%

Figure 4–9 the average death rate of the cells treated with 75nM ONs for 1 day (appendix figure 14).

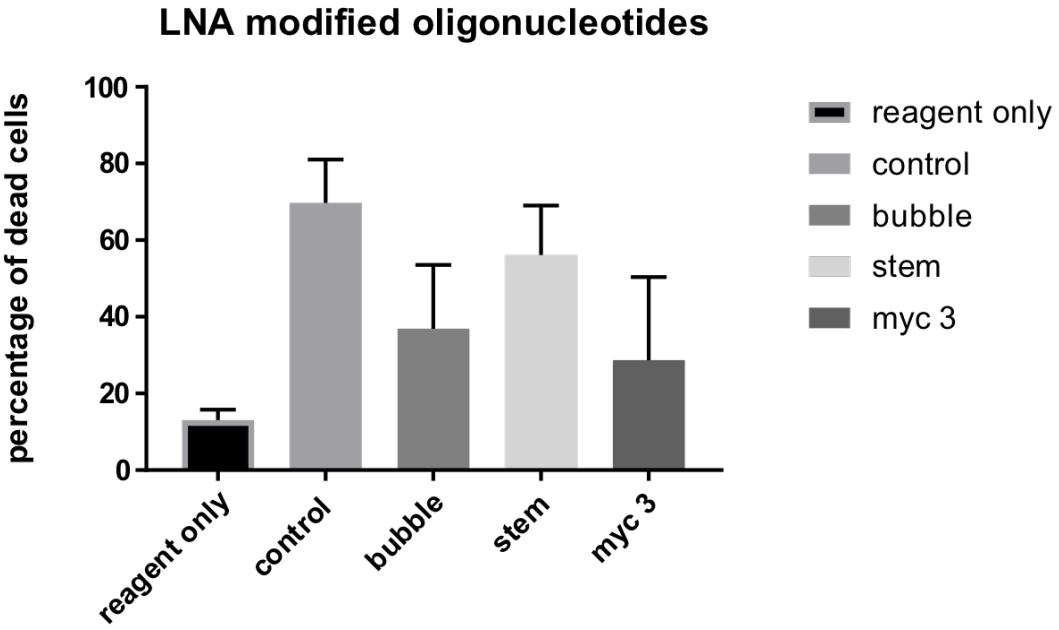


From the unmodified ON transfection result, stem seems worked most dramatic. Bubble didn't have very stable and obvious affect. The cell death rate is even comparably lower than the control group. It is unavoidable that the reagent is poisoner for the cells. Supposing the ONs may bind with the transfection reagent. But as the sequence doesn't work on hela cells, it just consume the transfer reagent.

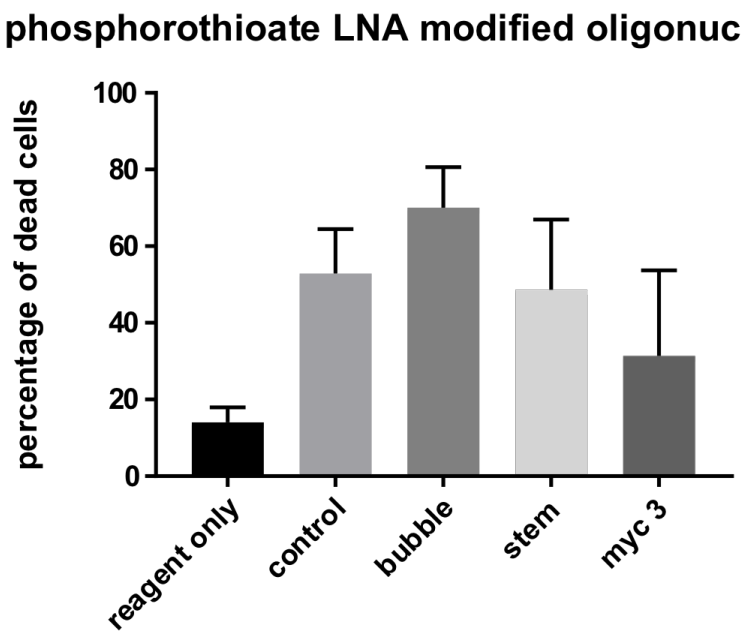


Here from the graph, control sequence is killing the cells, which is not in expectation. The control group is designed to not bind with the mRNA. However, here it's affecting the cell

viability. For the sequences of bubble and stem, they have dramatic different impact on the cell viability compare to the unmodified groups. So here we hypnosis, phosphorothioate modification is working on the improving the ONs function. But the final results require experiments in mRNA and protein level to prove.



Seen from the graph, obviously, the LNA modification is also enhancing the function of the ONs. But this data also proves that the control group needs to be reconsidered. It even had the most dramatic affect on the cell death rate among all the sequences. But compare to the standard ONs and the untransfected (reagent only), LNA modified ONs are having more effect on the cells.



Phosphotothioate LNA modified ONs are designed to have more dramatic impact on the cell viability. Here from the graph, the results are as the prediction. But this group compare with other

4.4 Western blot

First time using the cells from one transfection

Sample name	ug/ul	Total ug
1.00	0.32	6.42
2.00	0.19	3.76
3.00	0.28	5.60
4.00	0.38	7.65
5.00	0.23	4.58
6.00	0.19	3.76
7.00	0.36	7.24
8.00	0.43	8.67
9.00	0.32	6.42
10.00	0.19	3.76
11.00	0.17	3.35

12.00	0.13	2.54
13.00	0.23	4.58
14.00	0.15	2.94
15.00	0.14	2.74
16.00	0.15	2.94
17.00*	0.16	3.15
18.00*	0.15	2.94
19.00*	0.23	4.58
20.00*	0.13	2.54

*is the sample for trying, which is not valid for the final result.

In order to make sure the size of the protein, Coomassie staining was done to address the size of the protein. And here is the result.

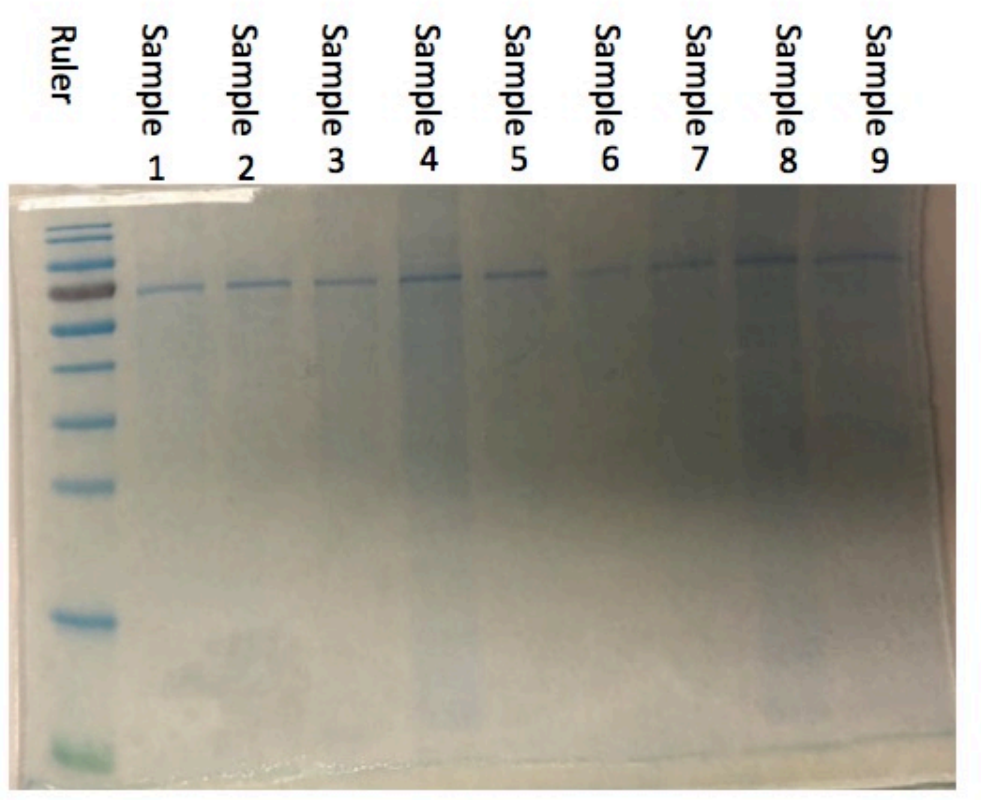


Figure 4 from left to right: ruler, sample 1, sample 2, sample 3, sample 4, sample 5, sample

6, sample 7, sample 8, sample 9. With 3μl of protein, the volume is approximately 0.5μg.

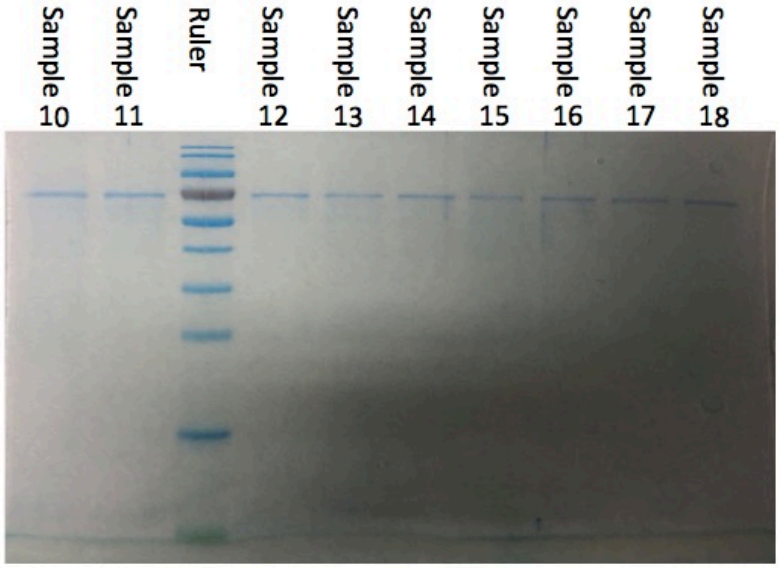


Figure 5 from left to right sample 10, sample 11, ruler, sample 12, sample 13, sample 14, sample 15, sample 16, sample 17, and sample 18. With 3μl of protein, the volume is approximately 0.5μg.

Here the band is 70kDa, which is possibly the housekeeping protein. And the C-MYC is supposed to be around 57kDa. So we need WB to detect more precisely.

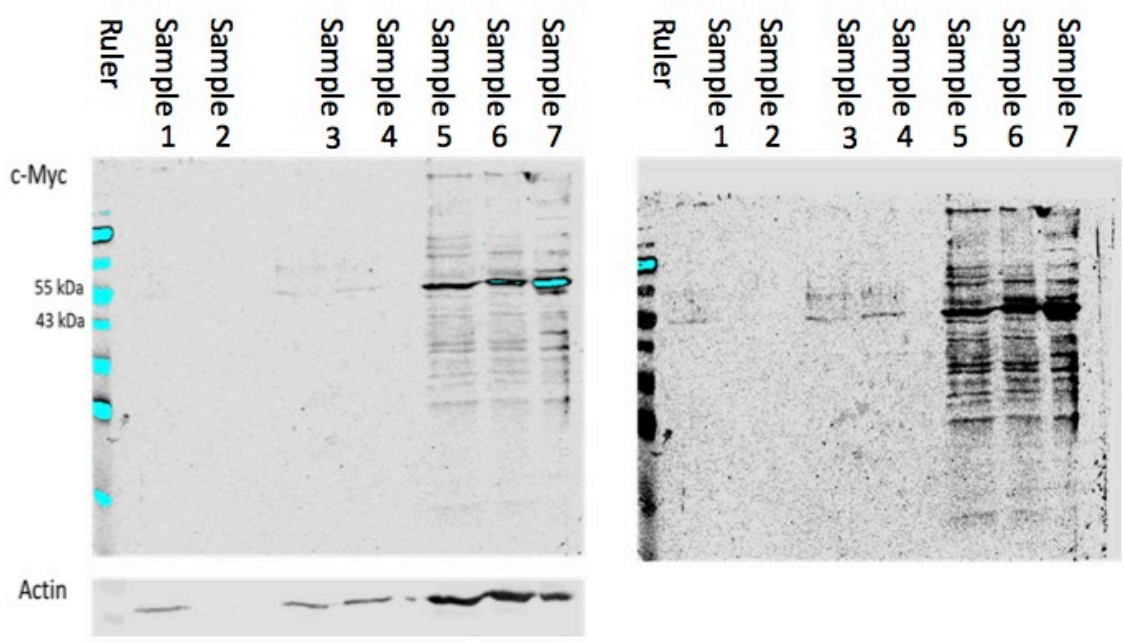


Figure 5 1–7 are 4μg sample 19,2 μg sample 20; 5μg HELA cell protein extraction 1; 5μg HELA cell protei extraction2; 20 μg HELA cell protein extraction;20μg HEK 293 cell protein extraction respectively. Picture enhanced intensity based on picture 1.

This is a trial experiment. As the protein extract is not enough, all the protein is loaded to see whether there is any signal. Here from the result we can see, there is a very week band around 53kDa, which is the proper size for c–Myc. As the band is not very significant and the intensity is not able to compare, this result suggests increasing the protein volume.

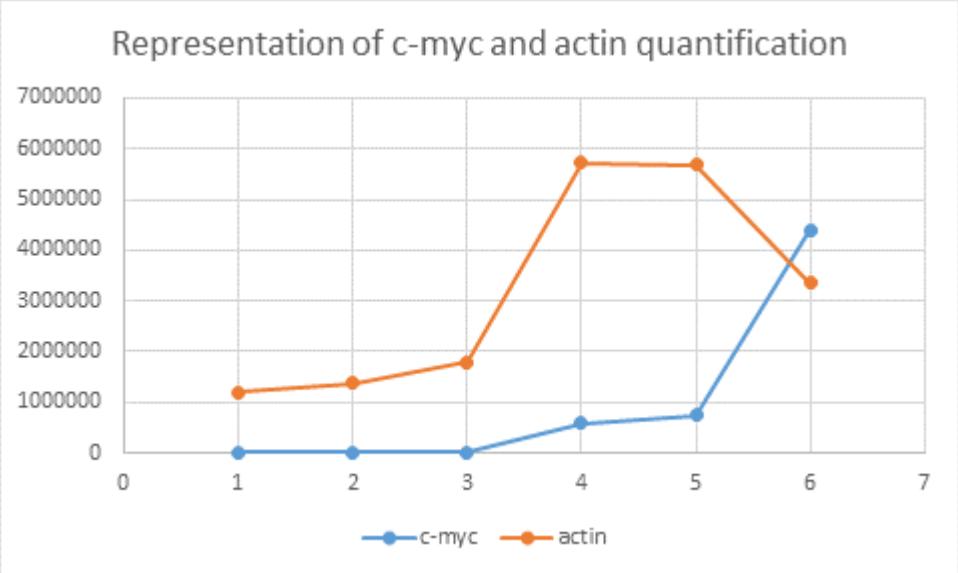


Figure 7 fluorescent intensity for different samples.

	1	3	4	5	6	7
c–myc	6589	7294	10687	588652	741392	4392759
actin	1198909	1385084	1781257	5720981	5693729	3351934

From the table we can see these data cannot be compared. As the ratio between C–MYC and actin is not consistent before and after dilution. So the concentration affects the experiments. In this case, the data is not available to be used to draw a reliable conclusion.

In order to get the reliable result, the cell transfection volume is increased and the second experiment is carried with three times more all the materials.

–protein quantification (BCA result)

With the total volume 20μl each sample.

	Absorbance at 526nm	*Concentration (μg/ml)*
1	0.248199999	137.8193
2	0.312400013	200.88415
3	0.168599993	59.62675
4	0.173099995	64.0472
5	0.339599997	227.60315
6	0.221100003	111.19845
7	0.267399997	156.6798
8	0.447899997	333.98825
9	0.145999998	37.42635
10	0.230199993	120.13755
11	0.207399994	97.7407
12	0.226699993	116.69945
13	0.176300004	67.1906
14	0.145999998	22.9863
15	0.230199993	82.80945
16	0.207399994	141.74855

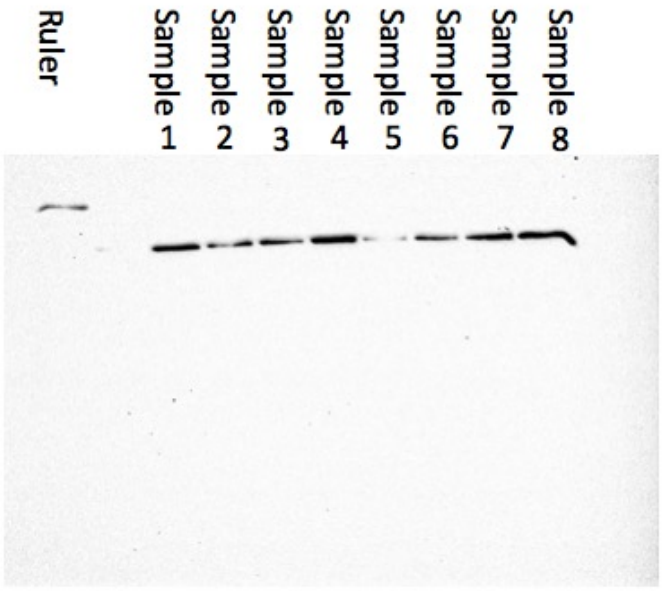


Figure 8 samples 1–8 respectively, the clear actin can be seen in the picture, but can't find the signal for C–MYC.

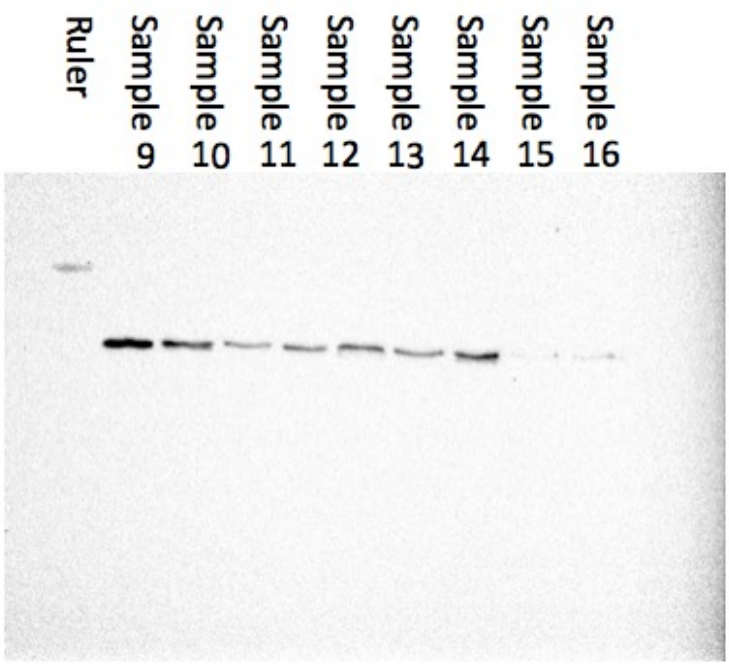


Figure 9 samples 9–16 respectively, the clear band of actin can be seen from the picture, but also can't see the signal from C–MYC.

4.5 Discussion

During this semester, the whole series of exploratory experiment has been done. From the

initial sequence selection till the final protein determination.

For the sequence selection, according to the secondary structure, 5 sequences are chosen. Among the 5 ONs, bubble, stem and MYC3 work more dramatically than the rest. The possible reason for this activity is currently unclear, but they seem to be better at decreasing the cell viability.

For the ONs synthesis, standard ONs and phosphorothioate ONs are efficiently synthesised. Whereas the LNA phosphoramidite was not very successful. This may be because of the CEP–CL was not of high enough quality. Or the reaction is not thoroughly completed before being precipitation was done. So the yield is not ideal, which can be adjusted and improved in the future experiments.

For the cell transfection, no reagent transfection has also been conducted. But the transfection is not successful. In this case, targeting the IRES structure with ONs (both standard and modified) require transfection reagent to deliver the ONs into the cells to function. And the modification can enhance the ONs' effect. The more modification, the effect is more dramatic. Comparing the phosphorothioate modification and LNA modification, there is not a very clear trend which one works better. Besides, the HELA cells express the c–Myc gene. However, after the transfection, these cells seem to undergo apoptosis. This is a potential signal that the c–Myc is inhibited.

For the western blot, at present, there is no reliable result. As the protein concentration is comparably low. Even with 20 μg of protein, there is still no significant band. This may be because the protein is extracted from the cells. So the protein in the cell membrane and organelle also involved, which make up much of the concentration for the overall protein concentration. But c–Myc is expressed at a very low level, which is too low to be detected. From the western blot with fluorescent secondary antibody, it is obvious that there is c–Myc, but whether the expression has been inhibited remains unsure.

Future work

In this semester, most time was spent to optimize the protocol and keep consistent on the transfection results. We got considerably similar results from flow cytometry, which give a promising proof for this project. While the protein level needs more work to draw a reliable conclusion. Also the RNA level should also be determined.

The transfection experiments should be done in a larger scale to extract enough protein and perform western blot for a triplicate to acquire consistent results. And for RNA level, qPCR is supposed to be done to acquire the result.
Moreover, the nanoparticle technology can also be adopted to enhance and optimize the function of ONs.

It is known that efficiency of the IRES element varies in different cells. Different from the siRNA binding with IRES structure could inhibit the function, while sometimes can activate the translation process. This concept is one of the reasons to design this project. Activating the protein is also on the list to be studied to confirm the IRES.

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This is my first time to study in a foreign country. I'm appreciate all the friends I made and everything they have done for me.

Finally, I am very grateful for all the support from my family.

Appendix

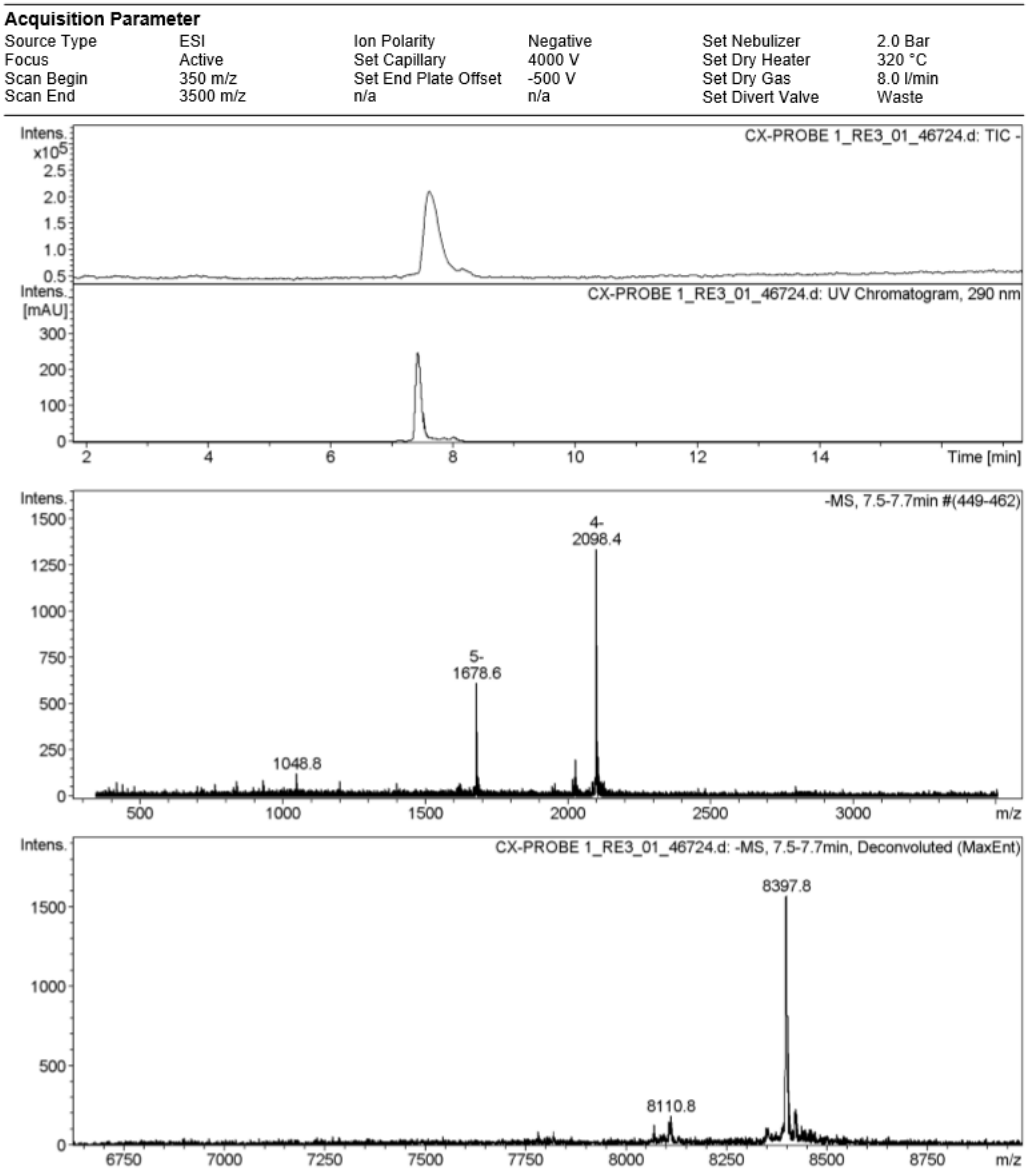

Figure 1 Mass spectrometry result for standard ONs of bubble

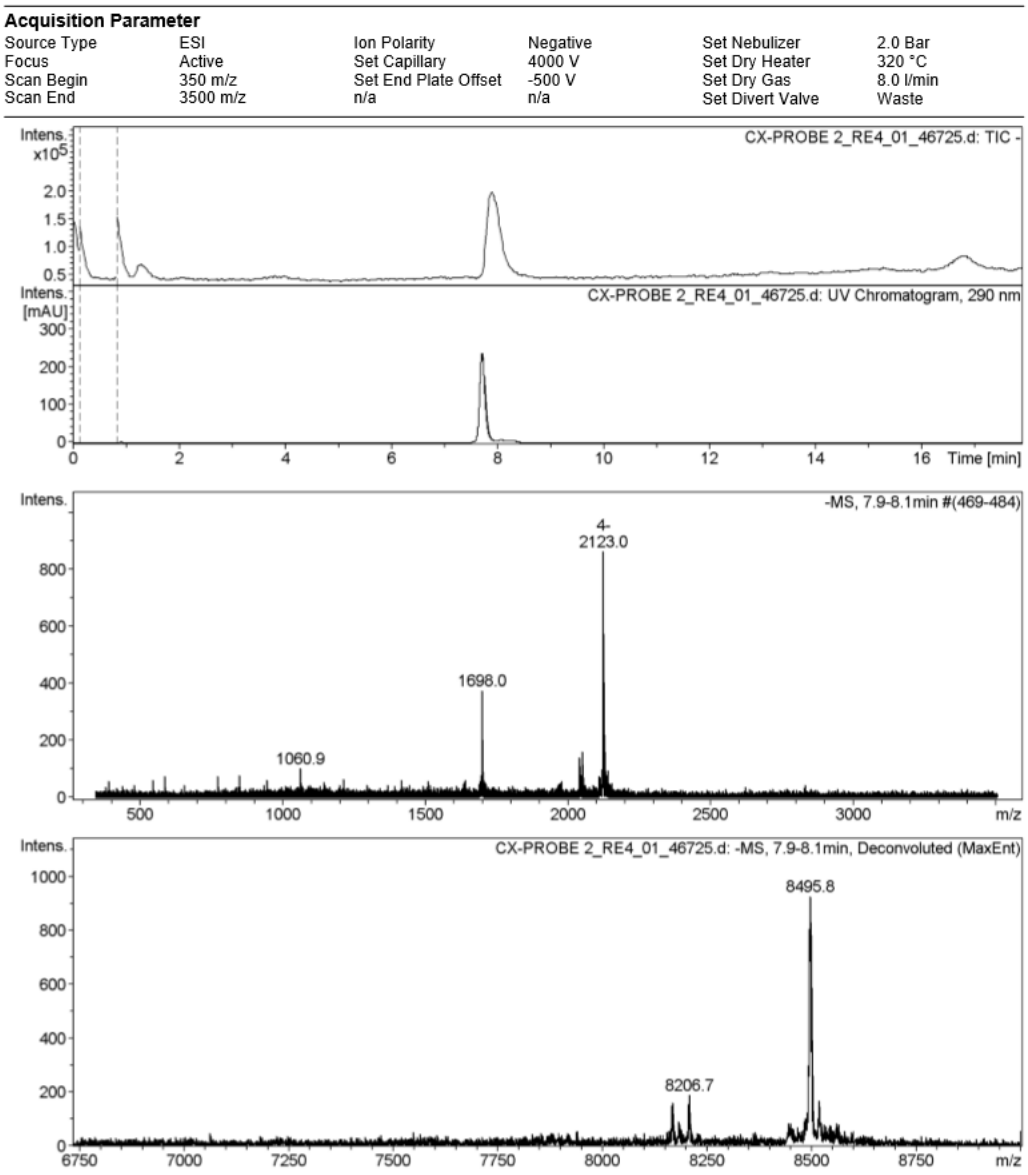

Figure 2 Mass spectrometry result for standard ONs of stem

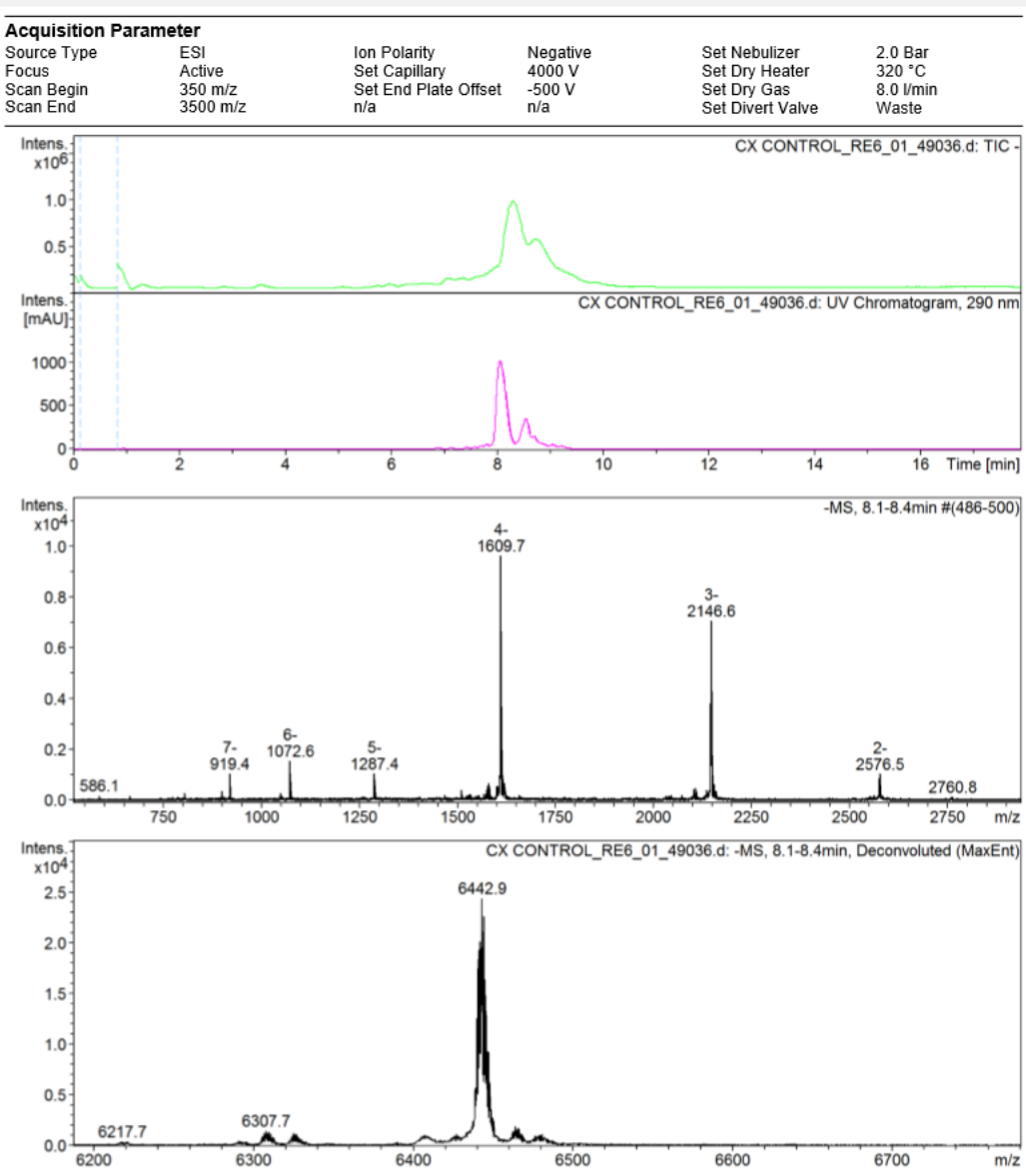

Figure 3 Mass spectrometry result for Phosphorothioate modified ONs of control

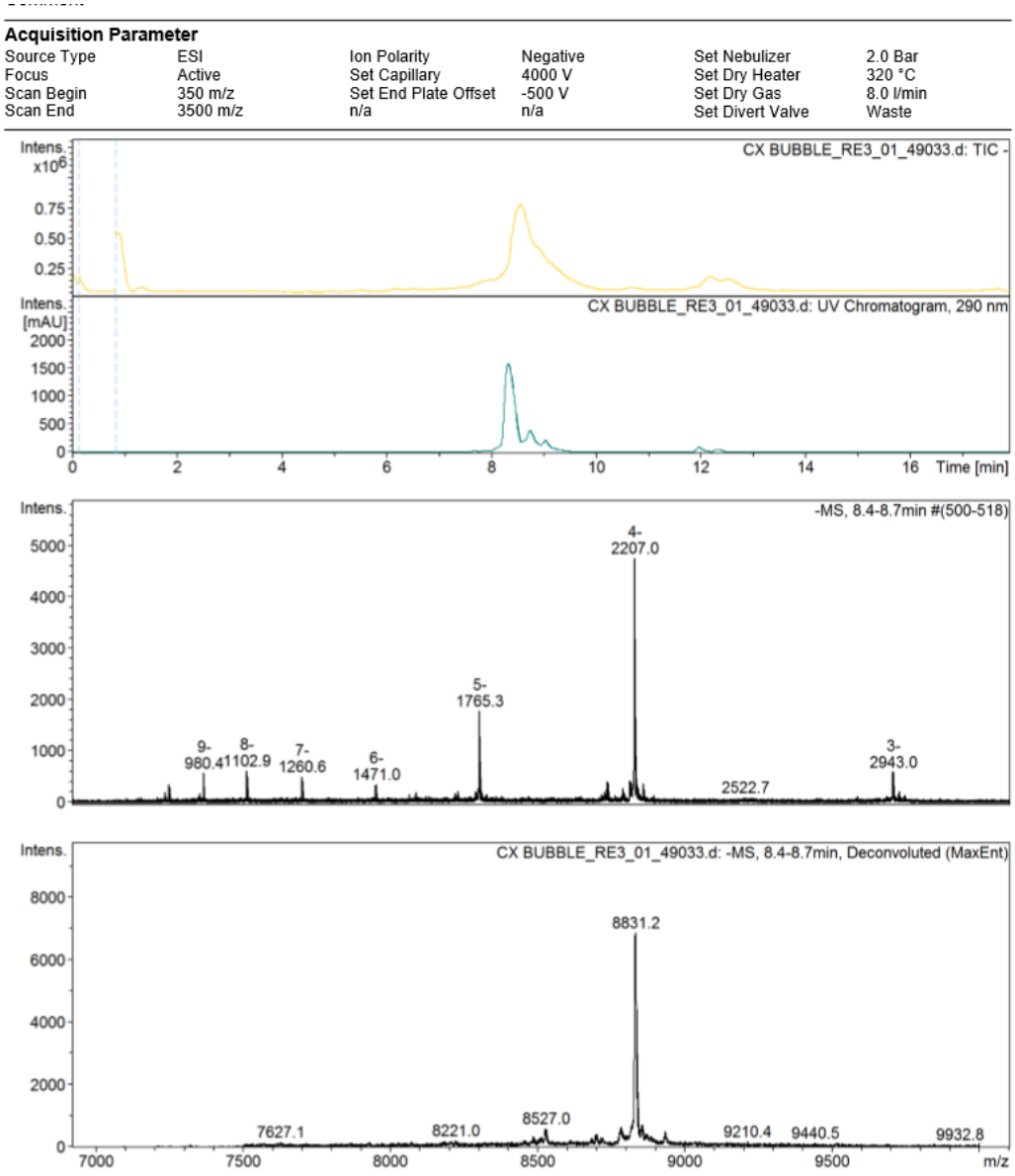

Figure 4 Mass spectrometry result for Phosphorothioate modified ONs of bubble

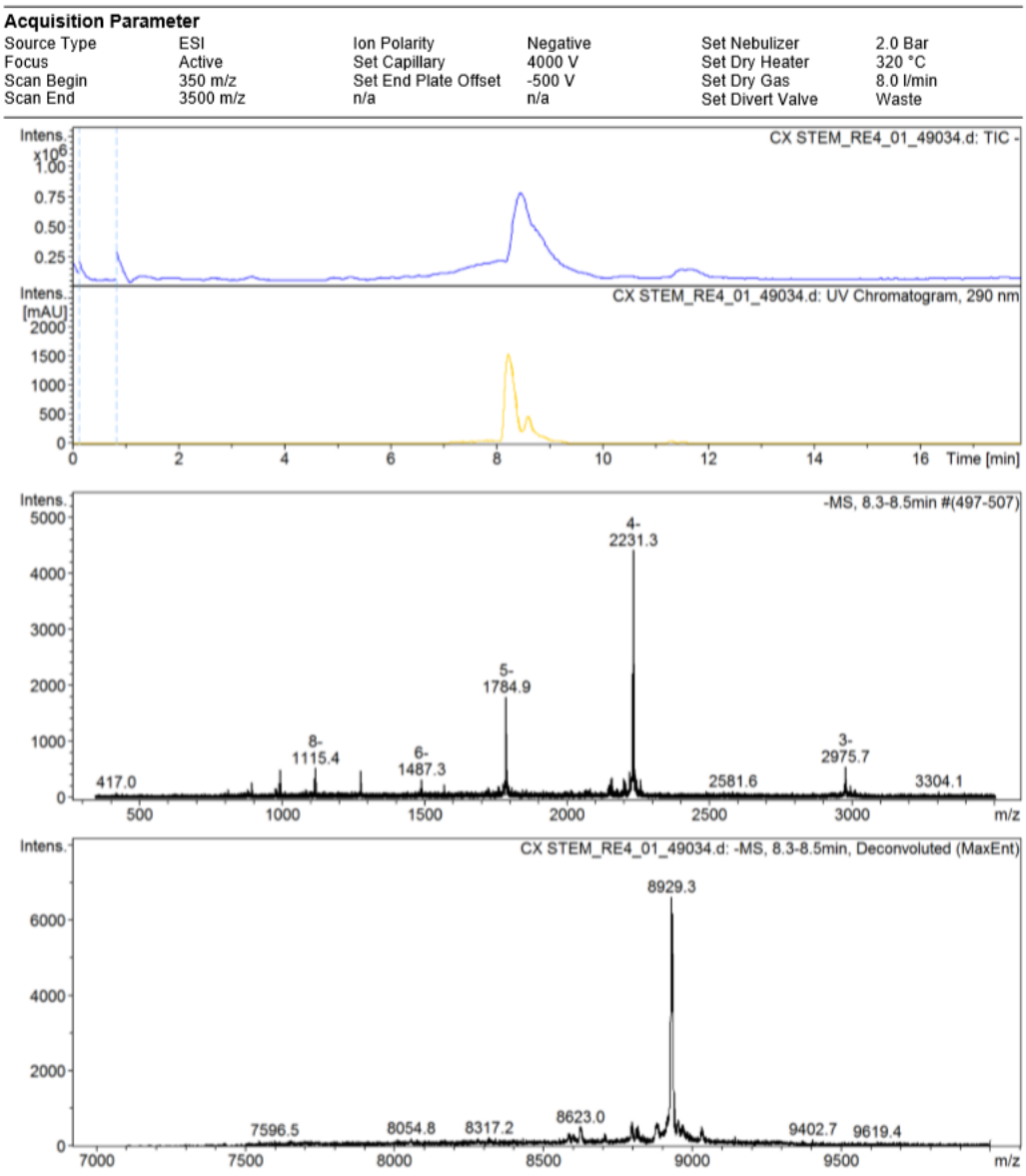

Figure 5 Mass spectrometry result for Phosphorothioate modified ONs of stem

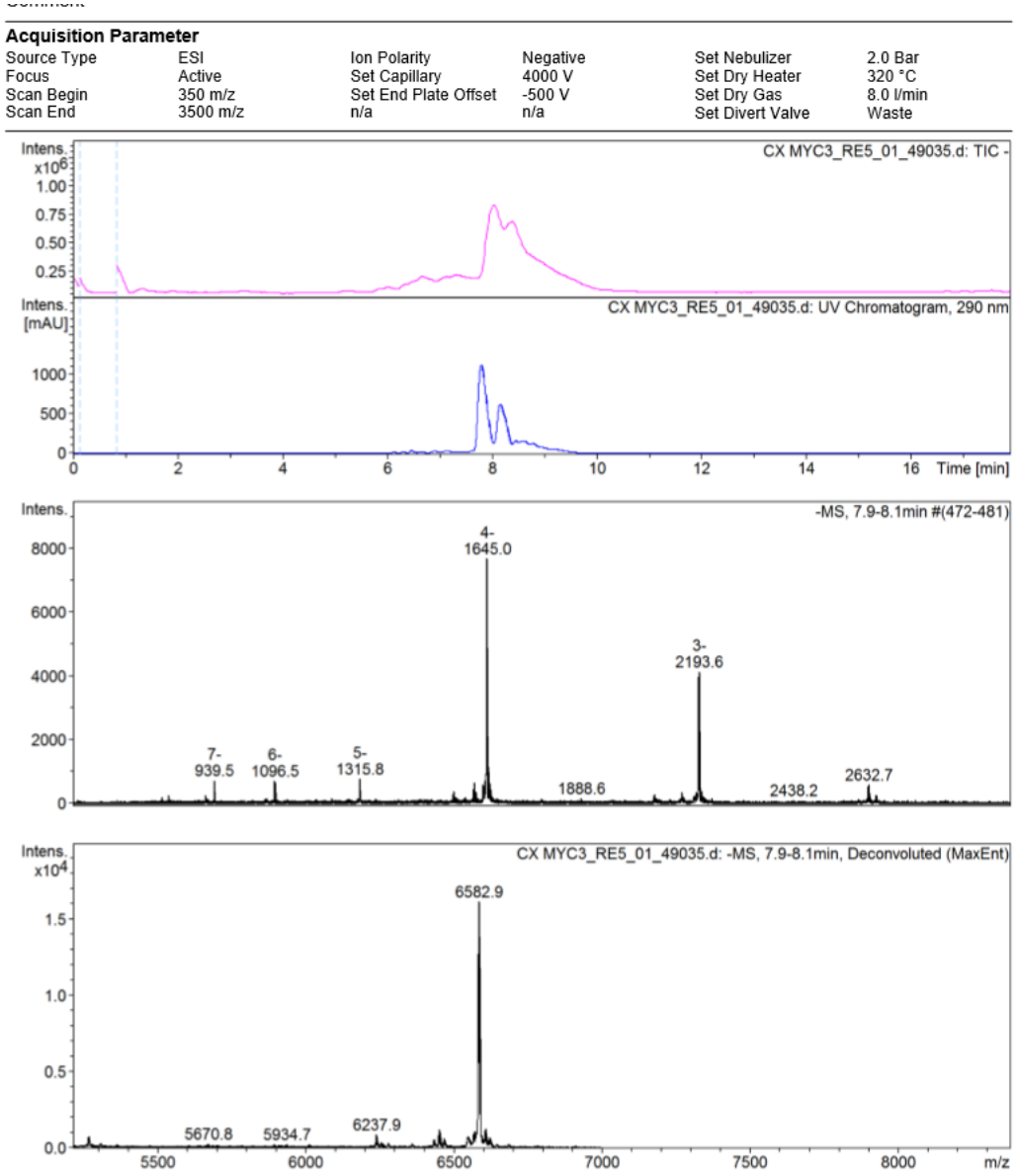

Figure 6 Mass spectrometry result for Phosphorothioate modified ONs of MYC 3

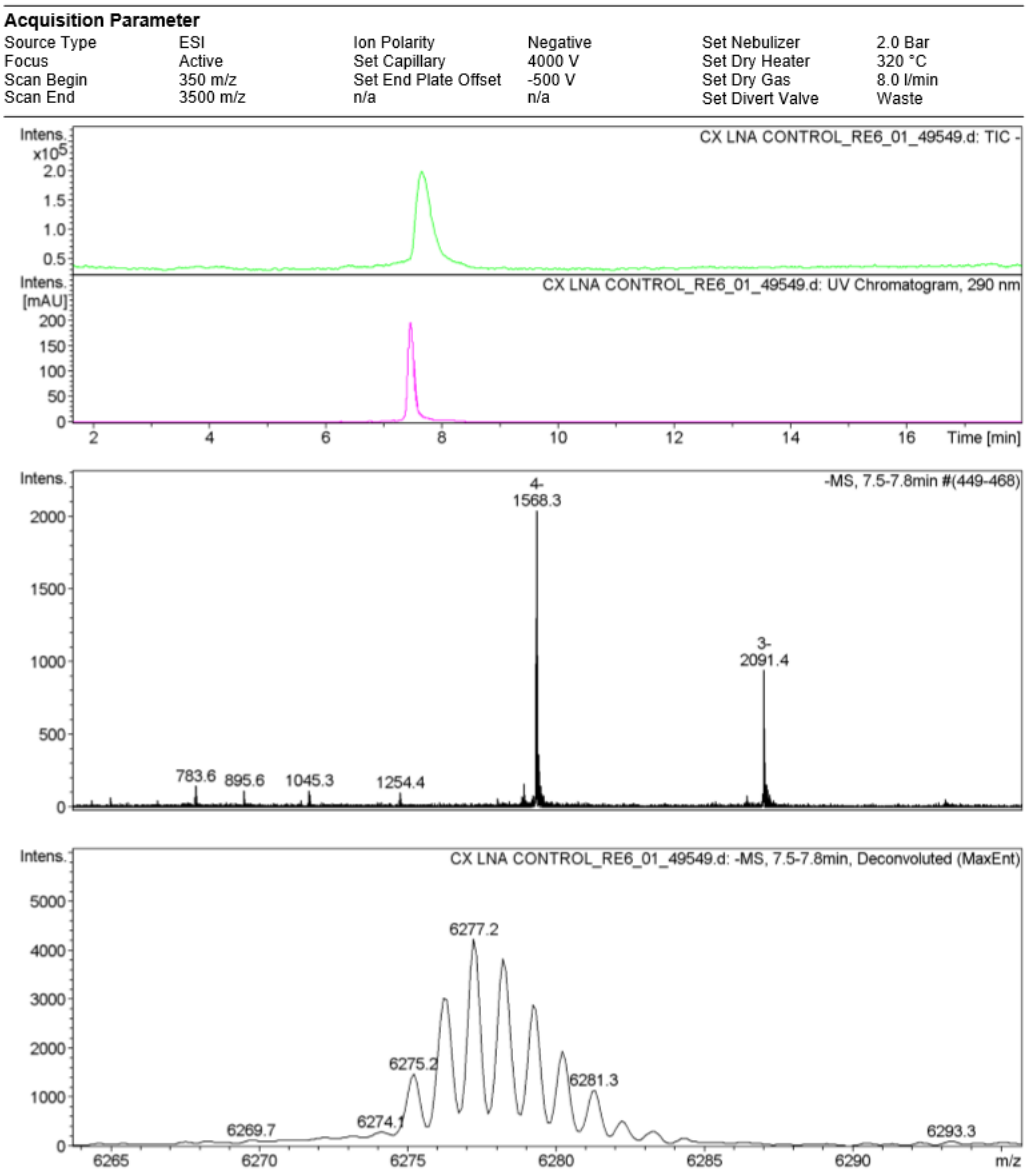

Figure 7 Mass spectrometry result for LNA modified ONs of control

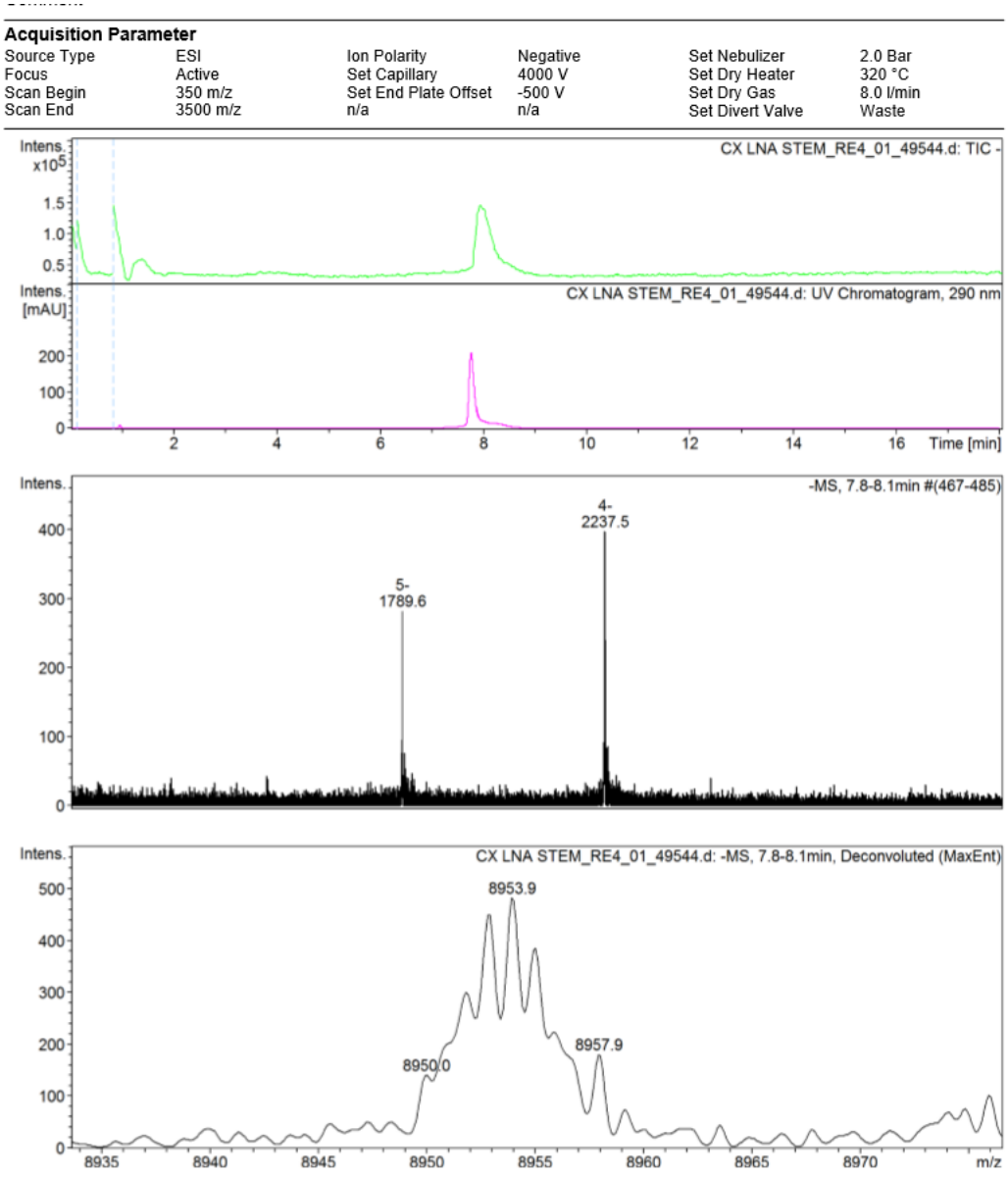

Figure 8 Mass spectrometry result for LNA modified ONs of stem

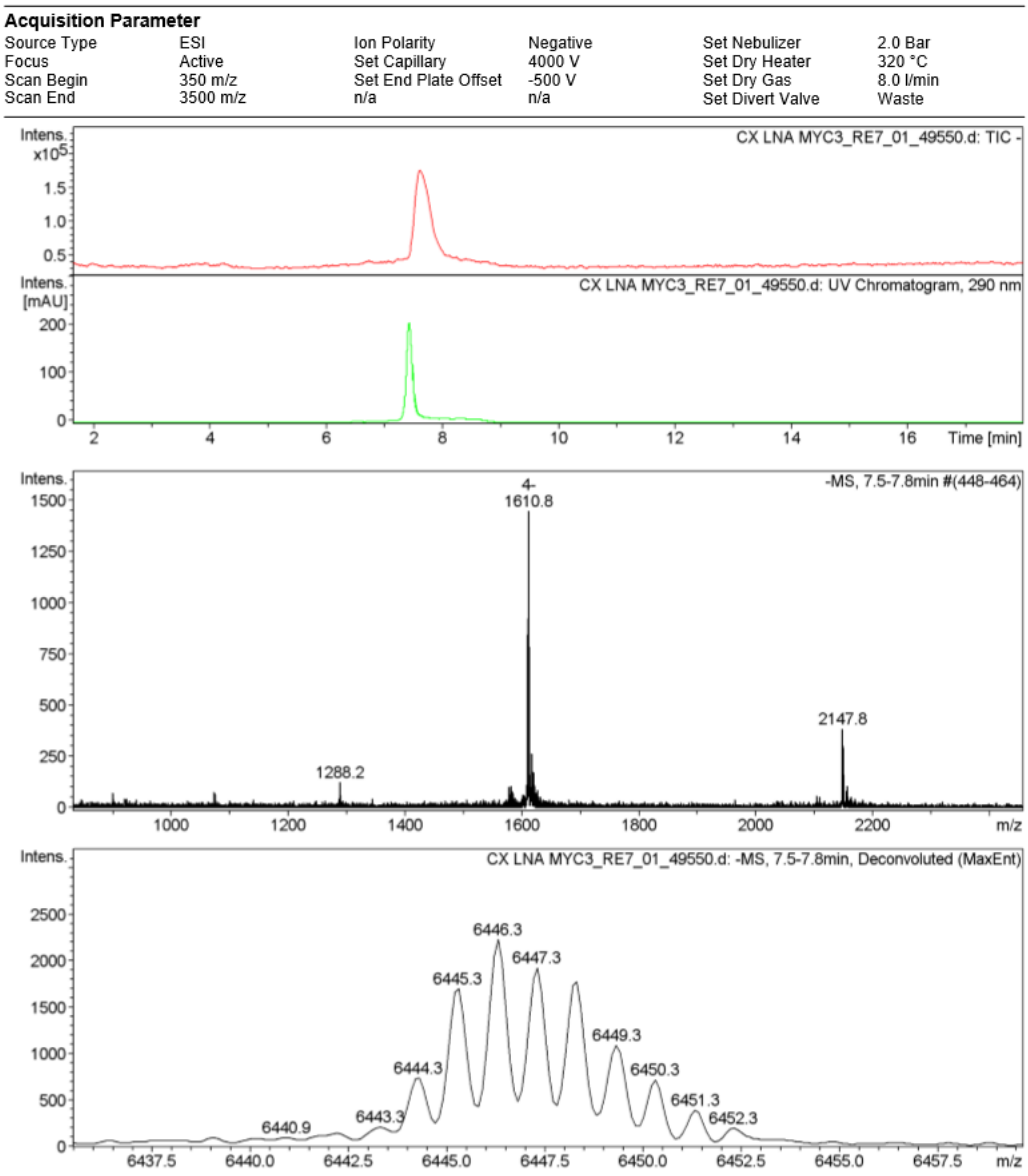

Figure 9 Mass spectrometry result for LNA modified ONs of MYC 3

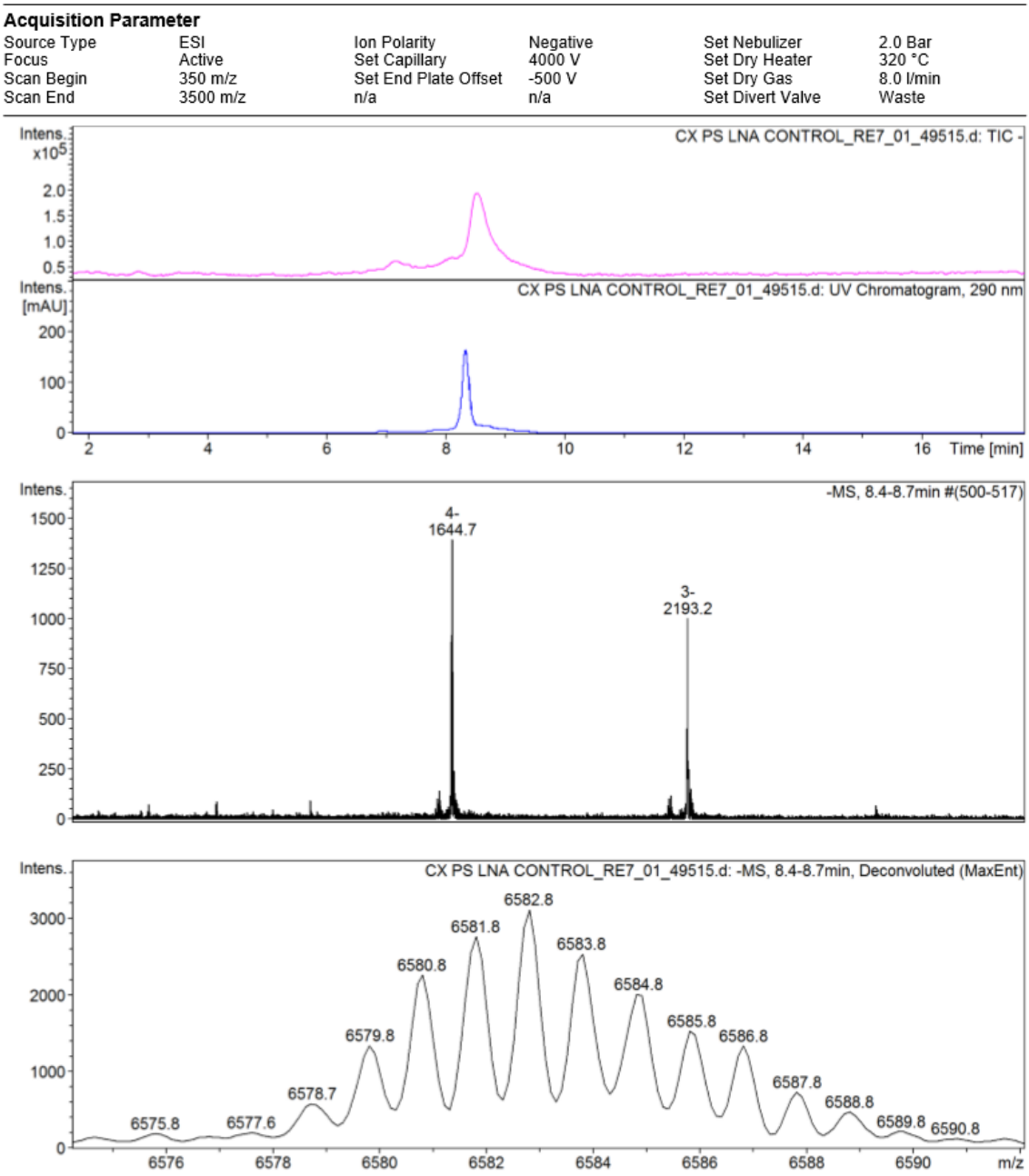

Figure 10 Mass spectrometry result for Phosphorothioate LNA modified ONs of control

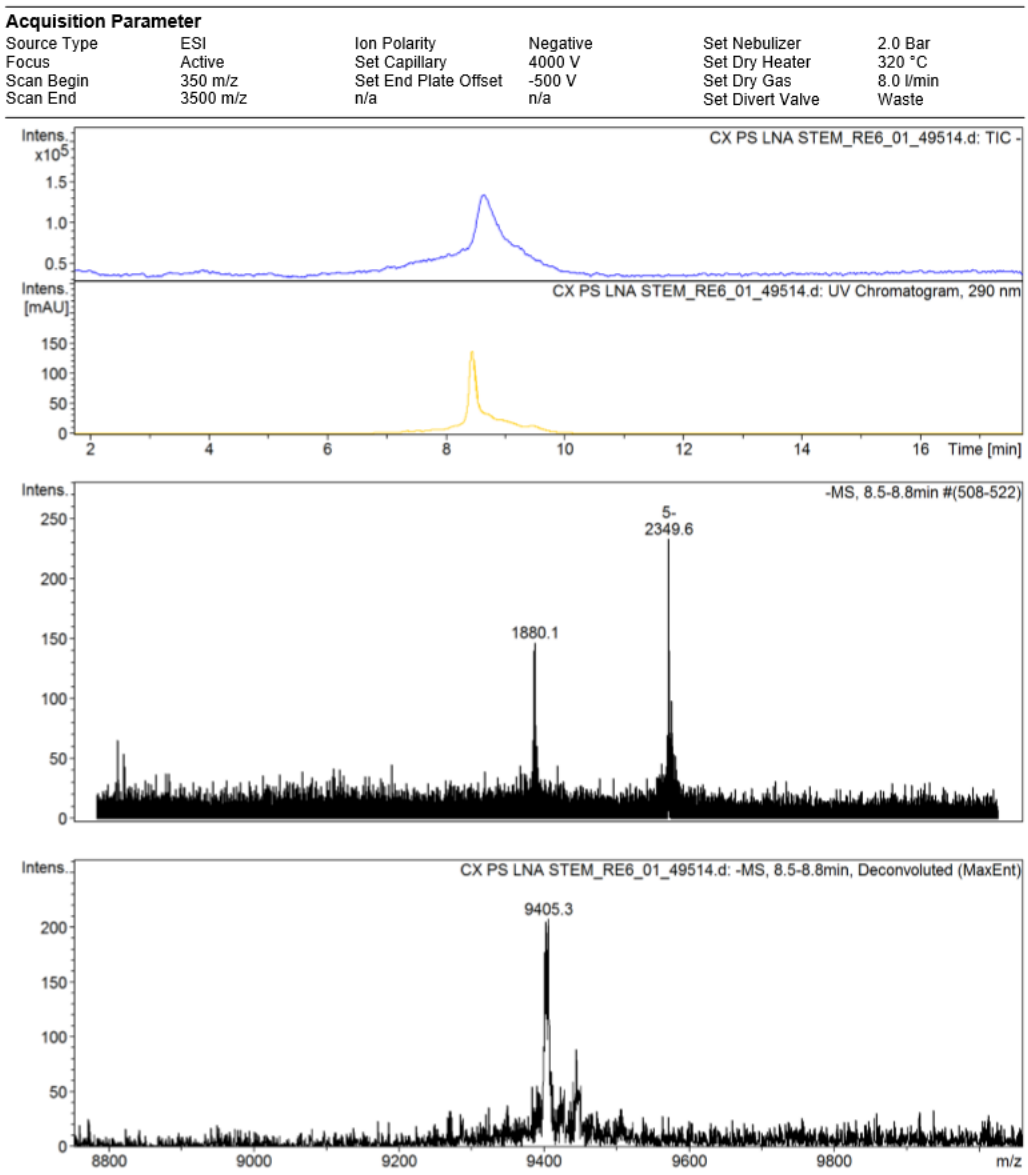

Figure 11 Mass spectrometry result for Phosphorothioate LNA modified ONs of stem

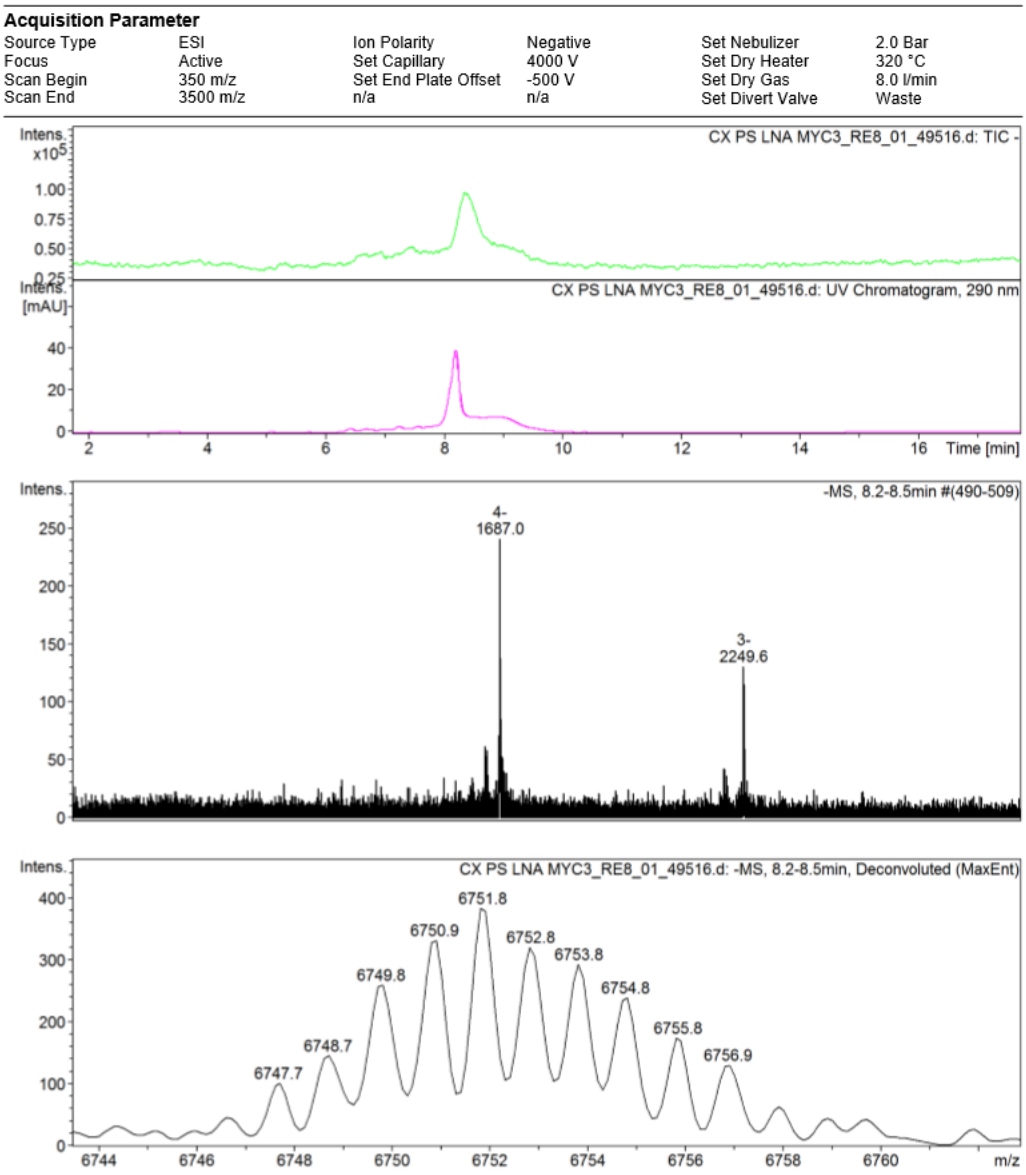

Figure 12 Mass spectrometry result for Phosphorothioate LNA modified ONs of MYC 3

2 days	No treatment	reagent only	MYC 1	MYC 2	MYC 3	Control
50 nM	2.30%	4.50%	3.50%	3.20%	4.90%	2.90%
80 nM	2.20%	4.00%	4.00%	14.10%	6.90%	5.10%
100 nM	1.70%	5.30%	5.40%	24.80%	10.30%	4.20%
200 nM	5.30%	14.20%	6.60%	19.10%	62.90%	6.30%
2 days	No treatment	reagent only	MYC 1	MYC 2	MYC 3	Control
50 nM	3.00%	9.10%	5.90%	5.40%	8.40%	5.80%
80 nM	7.40%	18.10%	8.70%	6.30%	11.40%	7.10%
100 nM	10.00%	32.30%	13.70%	8.70%	17.80%	12.00%
200 nM	30.90%	45.20%	15.20%	30.70%	67.50%	28.70%
2 days	No treatment	reagent only	MYC 1	MYC 2	MYC 3	Control
50 nM	4.60%	6.30%	7.30%	11.60%	22.20%	8.90%
80 nM	3.90%	6.70%	8.40%	10.10%	50.80%	14.70%
100 nM	5.80%	9.50%	14.20%	12.60%	46.40%	15.50%
200 nM	5.00%	8.60%	14.50%	15.60%	72.30%	22.00%

Figure 13 The flow cytometry result for the transfection of MYC 1, MYC 2, MYC 3 and control. Transfection time: two days, transfection reagent: 2μl, 3μl, 4μl 8μl oligofectamine.

Reagent	Control	Bubble	Stem	Myc 3
Unmodified				
16.8%	17.9%	22.7%	34.1%	19.2%
12.4%	12.5%	10.1%	14.0%	18.9%
9.1%	11.6%	6.6%	15.5%	
11.1%	13.6%	10.4%	15.2%	
21.0%	19.6%	14.9%	25.7%	22.2%
Ps-oligo				
26.0%	29.2%	51.4%	42.3%	25.5%
10.7%	16.7%	57.7%	32.1%	2.2%
10.8%	37.3%	50.4%	41.7%	15.4%
14.1%	33.2%	54.8%	49.3%	4.0%
13.1%	33.7%	45.6%	75.5%	26.4%
LNA				
10.7%	69.8%	48.6%	60.2%	22.6%
10.1%	86.0%	47.2%	61.5%	66.2%
12.7%	56.2%	51.0%	72.8%	26.8%
16.8%	73.8%	16.1%	42.4%	14.9%
14.8%(8.10)	62.9%	21.4%	43.6%	13.0%
Ps-lna				
19.8%	53.8%	61.9%	72.2%	66.4%
16.1%	56.9%	74.8%	29.4%	29.0%
11.5%	60.6%	74.0%	56.6%	36.9%
12.7%	60.3%	82.8%	54.4%	11.4%
10.3%	32.6%	56.5%	30.7%	13.2%

Figure 14 Result of flow cytometry of cell transfection. Transfection time: 1day. Transfection reagent: 2μ INTERFERin.

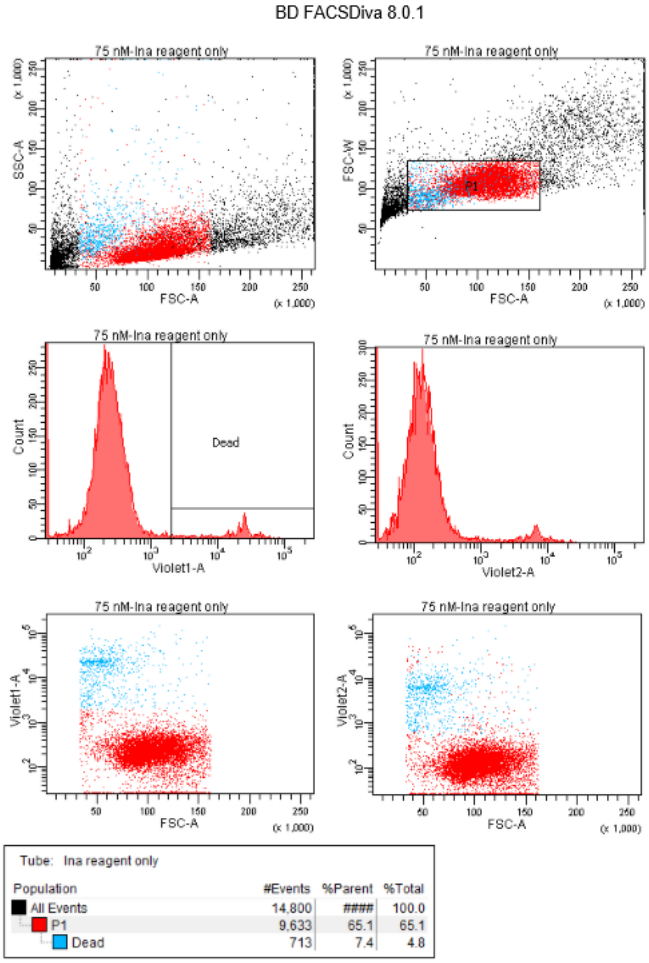

Figure 15 the flow cytometry result for reagent only as a control group.

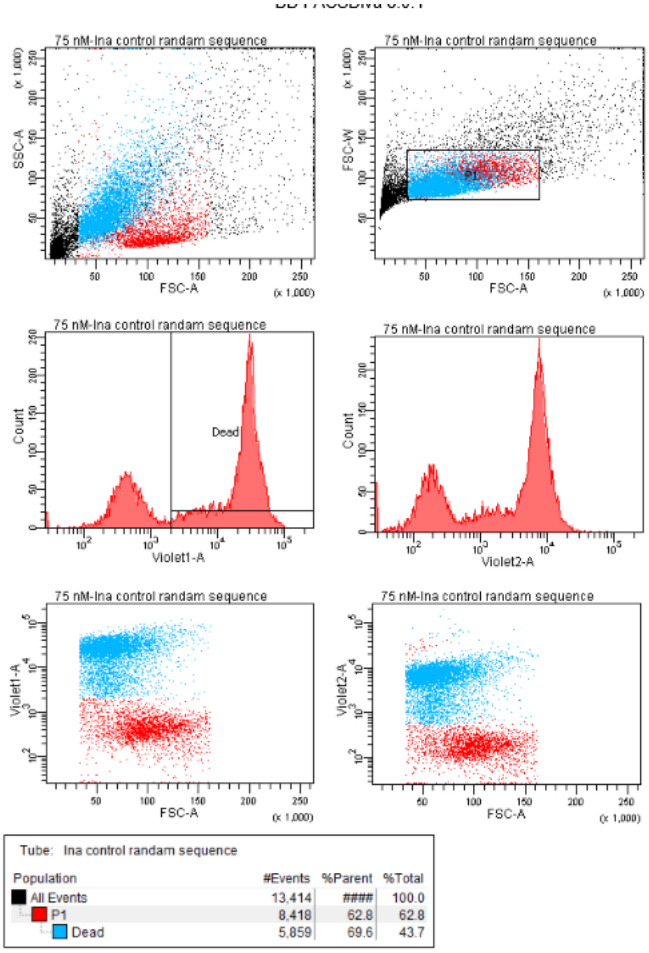

Figure 16 the flow cytometry result for LNA modified ONs of control, which is designed to be scramble sequence not binding with any structure.

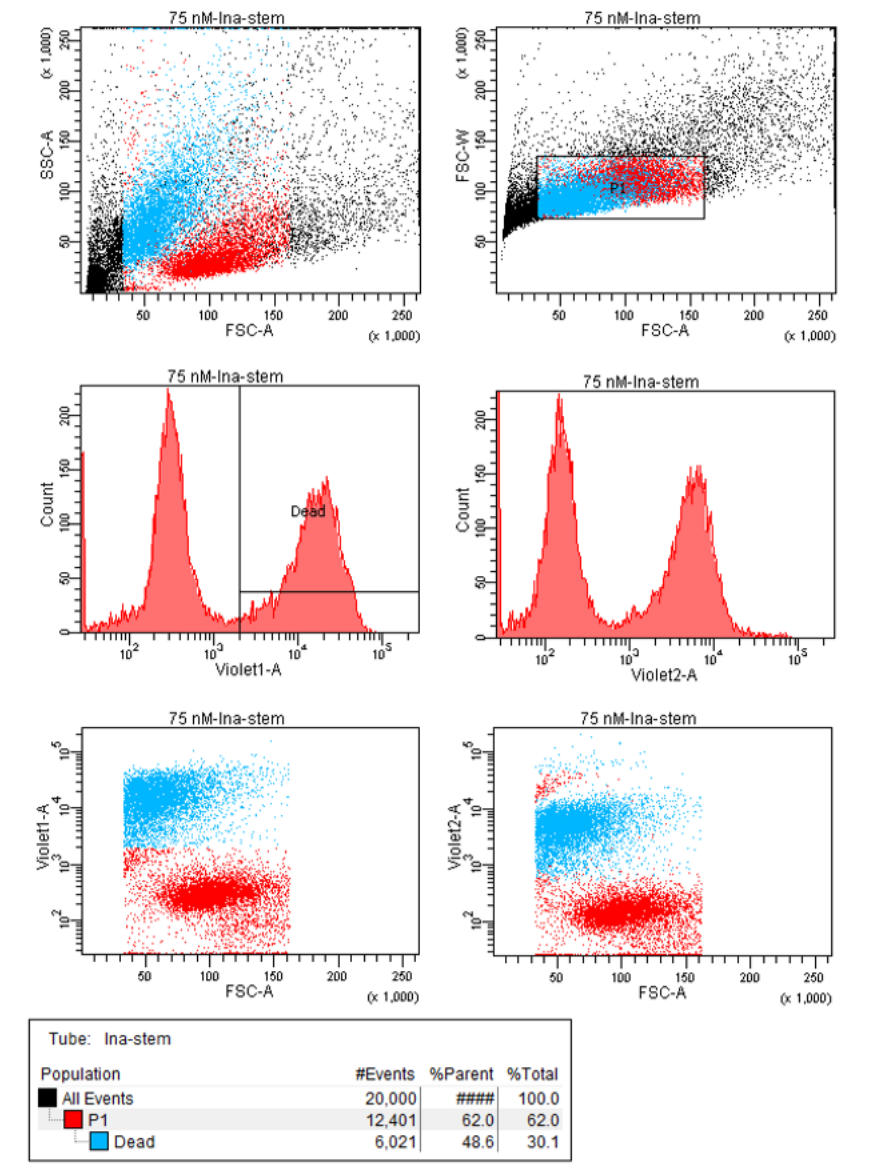

Figure 17 the result of flow cytometry of LNA modified ONs in the sequence of stem.