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SELECTING 2'FY RNA APTAMERS AGAINST SARS-CoV-2 PARTICLE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MERİÇ ÖZTÜRK

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ABSTRACT

SELECTING 2'FY RNA APTAMERS AGAINST SARS-CoV-2 PARTICLE

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Corona Virus Disease – 19 (COVID-19) is caused by infection of SARS-CoV-2 from other humans and animals, which results in clinical symptoms like fever, cough, breathing difficulties headache, muscle pain, and diarrhea. Further symptoms can be life threatening clinical conditions such as pneumonia, cardiovascular and rarely neurological complexities. According to studies, estimated fatality rate of the disease is about 4 %; reported cases-fatality rate is about 2.3 %. Early diagnosis is crucial to prevent further fatal symptoms. Although PCR tests are widely used, their success rate is about 60% and getting results of the test can take hours to days. Thus, more effective, and faster diagnostic tool is required. Aptamers are small nucleic acids that can be selected in vitro bind to their cognate targets with a high specificity and affinity. They can have either ssDNA or RNA backbones and they fold naturally into their unique three-dimensional shape. Aptamers can also be further modified to enhance their structural stability without any loss of affinity. These properties make them useful for both therapeutic and diagnosis applications. In this study, we successfully generated SARS-CoV-2 binding aptamers that will be further optimized for COVID-19 diagnosis.

Keywords: Aptamer, SELEX, RNA, SARS-CoV-2, COVID-19

ÖZ

SARS-CoV-2 PARÇACAIĞINA KARŞI 2'FY RNA APTAMERLERİN SEÇİLİMİ

Öztürk, Meriç
Yüksek Lisans, Moleküler Biyoloji ve Genetik
Tez Yöneticisi: Prof. Dr. Ayşe Gül Gözen
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Yeni koronavirüs hastalığı (COVID-19) SARS-CoV-2 virüsünün insanlar ya da diğer hayvanlar tarafından enfekte olunması sonucu oluşur ve ateş, öksürük, soluma zorlukları, kas ağrısı ve ishal gibi klinik semptomlara sebep olur. İleri seviyedeki semptomlar hayati tehlike oluşturabilecek akciğer yangısı, dolaşım sistemi ve nadir de olsa nörolojik sorunlar olabilir. Çalışmalara göre tahmin edilen ölüm oranı % 4; kayıt altına alınmış vakalar göz önüne alındığında ise ölüm oranı % 2,3'tür. İleri seviyedeki ölümcül semptomların oluşmasını engellemek için hastalığın erken teşhisi son derece önemlidir. PCR testleri yaygın şekilde kullanılsa da bunların başarı oranı %60 civarındandır ve sonuçların elde edilmesi saatlerce veya günlerce sürebilmektedir. Dolayısıyla, daha daha etkili ve hızlı teşhis için kullanılacak yöntemlere ihtiyaç duyulmaktadır. Aptamerler, in vitro seçilip, hedef moleküllerine yüksek afinite ve özgüllükle bağlanabilen küçük nükleik asit molekülleridir. Tek zincirli DNA veya RNA yapıda olabildikleri gibi, sekanslarına özgün üç boyutlu yapılar kazanabilmektedirler. Aptamerler yapısal kararlılıklarını arttırmak için, afinitelerinde herhangi bir kayıp olmaksızın modifiye edilebilirler. Bu özellikler aptamerleri terapi ve teşhis amaçlı uygulamalar için kullanılabilir kılmaktadır. Bu çalışmada, SARS-CoV-2 parçacıklarına bağlanacak ve ileride optimize edilip COVID-19 hastalığının teşhisinde kullanılabilecek aptamerleri başarıyla elde ettik.

Anahtar Kelimeler: Aptamer, SELEX, RNA, SARS-CoV-2, COVID-19

To my beloved family…

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INTRODUCTION

Wuhan Municipal Health Commission in China declared a group of pneumonia cases without any fatality on the 31st of December 2019. This is the official date of the beginning of the outbreak which kills 3,85 million people until now (June 19, 2021). After a series of unfortunate decelerations, finally, the Coronavirus Disease (COVID-19) outbreak was characterized as a pandemic on the 13th of March 2020 (WHO 2021) by the World Health Organization (WHO).

COVID-19 is one of the historical pandemics for humanity among many different ones. Having much more technological and biological opportunities to combat a virus makes this pandemic more historical because still we are not able to overcome after over a year. Also, at the beginning, unfortunately, top health officials underestimated using masks that later turned out to be the key tool to prevent the spread of the SARS-CoV-2 virus. This made the pandemic situation much more difficult to control.

Like any other diseases, early diagnosis of COVID-19 can prevent the hospitalization and ventilation of patients (Xu et al. 2020). From the beginning of the pandemic, PCR tests have been preferred because of its accessibility and lower cost (Ren et al. 2020). However, processing and waiting time for the results is the major drawback of the technique, which negatively affects the success rate of the intervention to patients. Thus, alternative tests preferable to PCR have been developed. Lateral Flow Immunoassays (LFI), ELISA Tests and Loop-mediated isothermal amplification

(LAMP) are some of the tests developed during pandemic based on experiences and results coming from earlier SARS outbreak (Helmy et al. 2020).

Finally, after serious lockdowns and occurrence of second waves in various countries, several companies including Pfizer-BioNTech (Polack et al. 2020), Johnson & Johnson (Sadoff et al. 2021), Moderna (Jackson et al. 2020), Sinovac (Gao et al. 2020) and Astra Zeneca (Voysey et al. 2021) developed COVID-19 vaccines simultaneously (Kaur and Gupta 2020; Prüβ 2021). First COVID-19 vaccine was approved by the United Kingdom's Medicines and Healthcare products Regulatory Agency (MHRA 2020) on the 2nd of December 2020. So far (June 19, 2021), 748 million people have been vaccinated across the world from this approval.

1.1 Brief History of Virology

1.1.1 Discocery of Viruses

Although the first written record dates back to 3700 B.C., humans were probably exposed to viruses many more years ago. The story begins with trigger of placenta formation in mammals by endogenous retroviruses. Analysis of repetitive elements originated from these viruses shed light on the evolution of such structures which mammals should be grateful to (Sha et al. 2000).

A hieroglyph found in Memphis portrays a temple priest faced with a disease that has identical symptoms of paralytic poliomyelitis, which is the first written record in the virology history (McCollum et al. 2014). Moreover, the mummified body of Pharaoh Ramses shows that he had pustular lesions on the face, a typical symptom of smallpox. He died in 1196 B.C (McCollum et al. 2014).

However, discovery of viruses as a disease-causing agent is only dating back to the late 1800s. The first virus was identified in 1898 simultaneously by Dimitri

Losifovich Ivanovski and Martinus Beijerinck (Lecoq 2001; Zaitlin 1998). They observed the tobacco mosaic virus (TMV) that causes the disease in tobacco plants and can pass through the smallest pore without losing its infectivity. Meanwhile in Germany, Friedrich Loeffler and Paul Frosch discovered an agent related to foot-and-mouth disease using the same filter method. Viruses can infect not only human and plant cells, but also bacteria. This phenomenon was later discovered in 1917 by Frederick Twort and Felix d'Herelle and such viruses were named as bacteriophages (Taylor 2014).

Major discoveries about viruses were carried out in the 1930s and subsequent decades until today. Salvador Luria and Max Delbruck used viruses as model systems and discovered their structures, genetics and life cycles. In 1939, the first electron micrograph of TMV was constructed by Gustav Kausche, Edgar Pfankuch, Helmut Ruska and Ernst Ruska (Kausche, Pfankuch, and Ruska 1939). Further discoveries of viruses generally based on invention of much more effective tools and methods based on genomics and bioinformatics like single virus genomics and virus metagenomics. Such developments shed light on the dark side of viruses and viral diseases and thus provide novel technologies to develop drugs and vaccines to eradicate some outbreaks (Taylor 2014).

1.1.2 Viral Outbreaks

Smallpox is one of the earliest viral outbreaks and official reports date back to ancient India (1500 B.C.). It is speculated that the virus originated from ancient Egypt, and it was endemic for at least 2000 years. The virus was introduced via Egyptian traders to India and then China and Japan (Barquet and Domingo 1997). Smallpox was caused by the highly contagious Variola virus, and its mortality rate was about 30%. The first vaccine in the world was developed to combat this disease by Edward Jenner in 1798 (Barquet and Domingo 1997). According to the records, smallpox killed 300 million people in the 20th century and approximately 500 million people

died from this disease in the last 100 years before its eradication (Geddes 2006). After vaccination of most of the population, in 1980, WHO announced the worldwide eradication of the virus (WHO 2007).

The first "global outbreak" which humanity has faced is *The Spanish flu* caused by the H1N1 strain of the influenza virus. It occurred between 1918 and 1920, while World War I was continuing, and it is believed that massive military movement between countries contributed to the spread of the virus. Mortality rate of the disease was 10-20% and close to 100 million people were killed by the virus all over the world (Spreeuwenberg, Kroneman, and Paget 2018). Same strain of the virus caused another outbreak, Swine Flu, in the beginning of the 21st century. Between 2009 and 2010, it infected 10% of the population and it is estimated that 150,000 to 500,000 people have died (WHO 2015b).

Acquired Immune Deficiency Syndrome (AIDS) is one of the deadliest disease and pathogenic agent of AIDS is human immunodeficiency virus (HIV) (Simon, Ho, and Abdool Karim 2006). About 40 million people over the world carry HIV and survive with AIDS and 1.7 million people acquired HIV in 2019. Although effective treatment was developed, it is estimated that 690,000 of them died from AIDS in 2019. It emerged in early 1980s in the USA and so far a total of 76 million people have been infected and 33 million have died (UNAIDS 2020).

On the 16th of November 2002, atypical pneumonia was reported in Guangdong, China. Then, on March 12, 2003, WHO declared a global alert for a severe form of pneumonia from China, Vietnam and Hong Kong (Peiris 2003). The disease was called *Severe Acute Respiratory Syndrome (SARS)*. This outbreak was prevented in July 2003 after a set of precautions. The syndrome causing virus named SARS-Coronavirus (CoV), it infected around 10,000 people all over the world and 1,000 people died (Yang et al. 2020).

After the SARS, in 2012, *Middle East Respiratory Syndrome (MERS)* emerged and is still ongoing in the Middle East (Yang et al. 2020). MERS is caused by a different strain of coronaviruses and its fatality rate is around %35 without any treatment and vaccine. Since September 2012, over 2500 people have been infected and over 900 people have died (European Centre for Disease Prevention and Control 2021; Yang et al. 2020).

More recently, *Ebola* and *Zika* virus outbreaks get public attention. Ebola emerged in 2013 and lasted until 2016, 28,000 people got infected and 11,300 of them died. On the other hand, after its first outbreak in the island of Yap in 2007, Zika re-emerged in 2014 in Micronesia and was identified in 2015 in Brazil. Different than other viruses, Zika causes microcephaly in unborn children of infected mothers (Sayres and Hughes 2020). The mosquito-transmitted disease was reported in a total of 86 countries to date (WHO 2018). Nevertheless, no treatment or vaccine is available for disease prevention.

The most current and the last pandemic is *COVID-19*. On the 31st of December 2019, Wuhan Municipal Health Commission in China declared a group of pneumonia cases without any fatality. This was the official date of the beginning of the outbreak which killed 3,430,000 people until now (21 May 2021). WHO characterized the COVID-19 outbreak as a pandemic on March 13, 2020. The disease-causing virus is named as SARS-CoV-2 and several vaccines have been developed and effective treatment strategies are still under development since then.

1.1.3 Discovery of Coronaviruses

The history of the genus of the COVID-19 causing virus, human coronaviruses, began in 1965. Tyrell and Bynoe discovered a virus in samples taken from the respiratory tract of a common cold patient. They named this virus as B814 (Tyrrell and Bynoe 1966). Meanwhile, Hamre and Procknow cultured a virus in tissue culture

provided by students with colds. They called the virus 229E (Hamre and Procknow 1966). Another group reported multiple virus strains from the human respiratory tract (McIntosh et al. 1967). Because the viruses were grown in organ cultures, they are named OC. Electron microscopy studies from organ cultures infected with OC, B814 and 229E viruses suggested that they share similar structures characterized by medium size (65-125 nm) particles with membrane coat and club-shaped surface structures. It was also observed that transmissible gastroenteritis viruses, mouse hepatitis and infected bronchitis are similar in structure as others. Afterall, this new strain of viruses named as coronaviruses due to having crown-like surface projection and later this strain officially accepted as a new genus of viruses (Kahn and McIntosh 2005).

Detailed further studies on pathology of the respiratory coronaviruses revealed a variety of respiratory illnesses associated with these viruses, especially upper respiratory infection with pneumonia in children and adults. They also cause asthma in infants and chronic bronchitis in adults. Besides humans, animal studies showed that coronaviruses were identified in cats, calves, dogs, turkeys, chickens and pigs. More recently, it was observed that civets, bats and camels also carry these viruses (Kahn and McIntosh 2005).

1.2 Pathogenesis of Coronavirus Associated Diseaes

Coronaviruses, belonging to the *Coronaviridae* family in the *Nidovirales* order, are positive sense single strand RNA viruses with size of 65-125 nm diameter. They have four subgroups: alpha, beta, gamma, and delta. MERS-CoV, SARS-CoV and SARS-CoV-2 are zoonotic coronaviruses, and they belong to *betacoronavirus* group, with higher fatality rate.

1.2.1 Systemic Acute Respiratory Syndrome (SARS)

After emerging in Guangdong Province in China, November 2002, SARS rapidly spread across Asia then all over the World. The first pandemic of the 21st century has a fatality rate of about 10%. Unlike other human coronaviruses, which cause mild symptoms, the novel coronavirus (SARS-CoV) causes severe symptoms mainly in the lower respiratory tract. General view of the origin of the first infection is coming from Himalayan palm civets, although no exact result was found about its origin.

SARS is transmitted among humans via airborne routes and physical contact. Infected patients show symptoms such as cough, fever, and malaise. In some cases, patients may develop diarrhea and nearly 90% of patients suffer from loss of smell and taste. While roughly 70% of the patients have persistent fever and shortness of breath right after infection, 30% of them show improvement in a week (Peiris et al. 2003). The striking outcome was significantly higher death rate among older people (>60 years of age), about 43%, than for younger people with 6.8% (Donnelly et al. 2003; Gu and Korteweg 2007).

Histopathology studies indicated SARS patients have lungs with diffuse alveolar damage (DAD). This is a result of both viral infection and immune pathogenetic factors. In most cases, the lungs of patients show massive consolidation due to the formation of fibrous tissue. Extended course of disease causes a higher rate of fibrous organization (Lang et al. 2003). SARS lungs also display these set of features: collapse of alveoli, desquamation of alveolar cells, hyaline membrane formation, and extensive edema. Imaging modalities like chest radiography can provide better results to predict correct clinical signs and settings to symptom-based therapy. Some studies argue that, besides lung, gastrointestinal tract, liver, bone marrow and brain may get affected by SARS-CoV (Gu and Korteweg 2007).

SARS-CoV uses Angiotensin-Converting Enzyme 2 (ACE2) receptor as an entrance route into host cells (Li et al. 2003). This functional receptor is present mostly on the luminal surface of tracheobronchial and alveolar epithelium. (He et al. 2006). General distribution of the ACE2 is also convenient, considering infected organs and cells. However, abundant presence of the receptor on endothelial and smooth muscle cells not infected by virus shows that ACE2 receptor is not the only a necessity for infection (Hamming et al. 2004). Moreover, although there is no ACE2 receptor on the brain, cases in which brain cells being infected were observed. These contradictions suggest other unknown mechanisms potentially play crucial roles in SARS-CoV infection (Gu and Korteweg 2007; Hung et al. 2003).

1.2.2 Middle East Respiratory Syndrome (MERS)

Between March 2012, when MERS first reported in Saudi Arabia, and November 2015, 1611 cases were reported with 579 deaths. In these dates, MERS-CoV spread to the United Arab Emirates, and then South Korea and active cases are still present in the Middle East. Origin of the disease is reported as dromedary camels in the Middle East including United Arab Emirates, and Saudi Arabia (WHO 2015).

Similar to SARS, shortness of breath, chest pain, abdominal pain, cough, vomiting, diarrhea, and fever are the clinical symptoms of MERS. Reported cases include fatality mostly from patients above age of 60. Fatality rate of the disease was much higher than SARS, and it was about 30%. Similar to SARS, MERS spreads human to human via airborne (Van Den Brand, Smits, and Haagmans 2015).

Radiology of MERS patients shows that pulmonary consolidations. Hospitalized patients have airspace and interstitial opacities, which indicate that MERS is mainly a lower respiratory tract disease. Thus, a large number of severely ill patients need mechanical ventilation (Al-Tawfiq et al. 2014). Computed tomography (CT) results

indicate that pneumonia having airspace involvement with ground-glass opacities in the subpleural and peribronchovascular regions. However, unlike SARS, a limited case showing development of fibrosis was observed (Ajlan et al. 2014). This suggests that mechanisms of disease to be different. Besides, some studies argue that, although it is a respiratory tract disease, systemic infection can occur in MERS patients (Al-Tawfiq et al. 2014).

MERS-CoV uses dipeptidyl peptidase 4 (DPP4, CD26) receptor expressed in many tissues including kidney, intestine and respiratory tract. Higher tissue tropism of MERS makes it more dangerous, considering the wide tissue distribution of the receptor (Meyerholz, Lambertz, and McCray 2016). Moreover, MERS-CoV triggers pro-inflammatory cytokines but inhibits the production of innate antiviral cytokines. Delayed pro-inflammatory response and inhibiting innate immunity results in MERS being more deadly than SARS (Van Den Brand et al. 2015; Mubarak, Alturaiki, and Hemida 2019).

A study demonstrated that chronic lung disease increases the expression of DPP4 in the respiratory tract and makes the patients more vulnerable to the MERS disease (Van Den Brand et al. 2015). Another study argues that some inflammatory conditions, such as asthma, may increase in the airways and thus, the patients with chronic lung disease need more care (Van Den Brand et al. 2015).

1.2.3 Coronavirus Disease (COVID-19)

COVID-19 is one of the historical pandemic humans have seen until now. First case was reported in December 2019, and case numbers reached 166 million and death numbers reached 3,430 thousand people. According to studies, estimated fatality rate of the disease is about 4%; reported cases-fatality rate is about 2.3%, which indicates that COVID-19 is less deadly than SARS and MERS diseases. However, it spreads more rapidly than other two diseases. Also, some studies observed the genetic

material belongs to SARS-CoV-2 in fecal samples, which argues that the digestive tract may be a transmission route (Sheleme, Bekele, and Ayela 2020).

Clinical symptoms of COVID-19 are very similar to SARS disease, including fever, cough, breathing difficulties (80% of patients) and some nonspecific symptoms, such as headache, muscle pain, and diarrhea. Similar to other two coronavirus diseases, COVID-19 is a great risk for people over 60 having a fatality rate of 10-25% (Pan American Health Organization 2020). Incubation period of the virus is 5 days, similar to MERS-CoV and SARS-CoV (Esakandari et al. 2020; Sheleme et al. 2020).

CT scans show that mild COVID-19 pneumonia is initiated primarily in lower lobs, observed as small, subpleural, unilateral or bilateral glass opacities. In the course of disease progression, mild pneumonia develops into a crazy-paving pattern and following consolidations. These lesions are absorbed with residual glass opacities and subpleural parenchymal bands after two weeks (Awulachew et al. 2020). Histopathology examinations on the lungs of patients suggest other nonspecific symptoms like edema, diffuse alveolar damage (DAD), and multinucleated giant cells (Deshmukh et al. 2021).

SARS-CoV-2, also, uses ACE2 receptors to infect the host. High sequence identity (76%) between these two viruses may explain why they use the same receptor to enter (Verdecchia et al. 2020). In lungs, the virus inhibits ACE2 activity via spike proteins and causes lung injury, besides entering the cell. Even the isolated viral spike protein can induce down-regulation of ACE2 receptors and then causes an increase of angiotensin2 in the lungs and precipitation of pulmonary inflammatory lesions (Verdecchia et al. 2020). In addition to lungs, ACE2 receptor is found in gastrointestinal system, circulatory system, urogenital system, central nervous system, and immune system cells. Combination of a wide distribution of ACE2 receptors in the body and a wide range of tropism ability of the SARS-CoV-2 makes COVID-19 a very dangerous disease for humans (Hikmet et al. 2020).

1.3 Biology of Coronaviruses

1.3.1 RNA Viruses

Virus families are grouped depending on their types of genetic material, which can either be DNA or RNA (Gelderblom 1996). DNA viruses can have double or single stranded DNA; positive sense single stranded RNA (ssRNA(+)), RNA viruses can have double stranded RNA or negative sense single stranded RNA (ssRNA(-)) (Gelderblom 1996). While DNA-dependent DNA polymerase is used by DNA viruses for replication, genetic material of ssRNA(+) viruses serves like an mRNA that can be directly translated by the infected host. Unlike ssRNA(+) viruses, prior to translation, ssRNA(-) viruses must convert their genetic material into positive sense using RNA-dependent RNA polymerase (Gelderblom 1996).

Replication mechanisms and genomes of RNA viruses show huge diversity. With their small genome size (3 to 32 kb), four distinct classes are defined as RNA viruses, only one of them shared with DNA viruses. DNA viruses contain only two classes despite their genome size being three times longer than RNA viruses (Baltimore 1971).

Replication of RNA viruses is totally dependent on RNA-dependent RNA polymerase that lacks proofreading mechanisms, which results in low fidelity RNA synthesis with higher mutation rate. This feature is counterbalanced by formation of a large population size of their progeny. This makes RNA viruses easily adaptive to new environments but limits their ability to expand genome by error threshold. That is, they must keep the mutation rate below a limit to allow virus survival (Gelderblom 1996).

1.3.2 Nidovirales

Nidovirales is one of the RNA viruses' orders. They have positive ssRNA as their genome. All of its members have lipid envelope with different architectures that protects the internal nucleocapsid structure and a number of viral surface proteins present on the surface. This group can be divided into six different families: *Coronaviridae, Arteriviridae, Ronidovirineae, Tobanivirdae, Mesnidovirineae* and *Monoviridae* (Gorbalenya et al. 2006; Snijder et al. 2003).

Nidovirus genome contains untranslated region (UTR) at its 5' and 3' ends. These regions include array of multiple genes with different numbers. Open Reading Frame 1a (ORF1a) and ORF1b are the largest 5' ORFs for all *Nidoviruses* and they occupy up to three-quarters of the genome. Subunit of replicase machinery is encoded by these two ORFs. Downstream ORFs of ORF1b encode envelope and nucleocapsid proteins and in some groups, these ORFs may encode additional structural and non-structural proteins. (Gorbalenya et al. 2006).

Nidovirus have four major branches having different virion architecture. *Coronaviruses, Mesnidoviruses* and *Arteriviruses* have spherical virions (Regenmortel et al. 2000); and *Ronivirus* particles are rod-shaped (Spann, Vickers, and Lester 1995). *Coronaviridae* virions bulge out over the envelope, which is called peplomers which provides characteristic crowns shape of the virus. Prominent peplomers are also abundant on *Ronivirus* envelopes and *Arterivirus* surface. *Coronaviruses* and *Toroviruses* have spike (S) and membrane (M) proteins in their envelopes (Gorbalenya et al. 2006; Weiss, Steck, and Horzinek 1983); GP5 and M for *Arteviruses*; gp116 and gp64 proteins for *Roniviruses* (Gorbalenya et al. 2006). Shared M and S envelope proteins between *Coronavirus* and *Toroviruses* indicates that possible common origin (Regenmortel et al. 2000). Although they have different sizes, all S proteins in four branches have a globular domain and a stem portion with heptad repeats as a coiled-coil structure. *Arteriviruses*' four glycoproteins are

recruited as two different structures: (I) GP5 - M disulfide-linked heterodimers and (II) GP2 - GP3 - GP5 heterotrimers. The membrane topology of M proteins of the virus contains outside-located amino-terminus and inside-located carboxy-terminus (Siddell et al. 2005).

The nucleocapsid includes only nucleocapsid protein (N) in all *Nidoviruses* (Almazán, Galán, and Enjuanes 2004), although the N proteins probably not evolutionarily related (Regenmortel et al. 2000). Unlike its *Arterivirus* counterpart, this protein is essential for coronaviruses for efficient genome replication and RNA synthesis (Molenkamp et al. 2000).

Nidovirus particles have other different proteins which are essential for some nidoviruses. For instance, E protein is ubiquitous in *Coronavirus* and *Arterivirus*es (Gorbalenya et al. 2006) and essential for *Arteriviruses* (Snijder and Meulenberg 1998), but not for all *Coronaviruses*. E gene deletion result in blocking of virus maturation, preventing virus release and spread and reduction of virus titers. The E protein of *Coronavirus* plays role in modifying membrane permeability via recruiting ion channels in the virion envelope (Gonzalez and Carrasco 2003). To facilitate the virus release, this protein may also modify the host cells plasma membrane (Wilson et al. 2004).

Nidoviruses infect the host cell using the receptors on the cell surface. This attachment is mediated by surface glycoproteins of the virus. This event results in releasing of the nucleocapsid into the cytoplasm. The genome of virus is uncoated and host ribosomes initiate the translation of two replicase ORF and eventually membrane bound replicase/transcriptase complex (Gorbalenya et al. 2006). This complex directs the genomic and sub-genomic RNA synthesis and mediates the viral protein expression. Envelopment of nucleocapsid structure, which follows the newly-formed genome and nucleocapsid protein association, results in formation of new virus particle (Ziebuhr, Snijder, and Gorbalenya 2000). Nucleocapsid

envelopment occurs through insertion of the envelope proteins into intracellular membranes and targeted to the membranes between Golgi complex and the endoplasmic reticulum, which is the site of virus assembly. In there, proteins come together with nucleocapsid, and it triggers the virus budding. Finally, the virions move out of cell following the exocytotic pathway (Lai and Cavanagh 1997; Ziebuhr 2004).

1.3.3 Coronaviruses and SARS-CoV-2

Icosahedral symmetric coronavirus (CoV) particles are approximately 125 nm in diameter with a 26–32 kb RNA genome. CoVs belong the family Coronoviridae composed of Letovirinae and Orthocoronavirinae. Orthocoronaviridae includes genera of Alphacoronavirus (αCoV), Betacoronavirus (βCoV), Gammacoronavirus (γCoV), Deltacoronavirus (δCoV) (Helmy et al. 2020; Lai and Cavanagh 1997; Lai, Perlman, and Anderson 2007).

CoV can infect domestic and wild animals; while γ- and δ-CoVs infect birds, α- and β-CoVs target mammals. Human coronavirus (HCoV), named B814, was discovered from patients who have common cold symptoms in 1960 (Tyrrell and Bynoe 1965). Since then, seven different HCoVs have been discovered: 229E, NL63, HKU1, OC43, SARS, MERS, SARS-CoV-2 (Helmy et al. 2020). SARS-CoV belongs to the lineage B βCoV, the causative agent of the pandemic originated in China (Drosten et al. 2003), while MERS-CoV belongs to the lineage C βCoV (Zaki et al. 2012). New virus, SARS-CoV-2 was determined as a member of lineage B βCoV (Chan et al. 2020). Phylogenetic analysis shows that SARS-CoV-2 belongs to the Nidovirales order, Coronaviridae family, Betacoronavirus genus, and Sarbecovirus subgenus (N. Chen et al. 2020). Genome similarities between SARS-CoV-2 and Bat CoV RaTG13, SARS-like CoV ZXC21, and SARS-CoV are 96.3%, 89%, and 82% respectively (Helmy et al. 2020).

1.3.3.1 Genome Organization

Coronaviruses' genome has a 3′ poly (A) tail and 5′ cap structure, which provides mRNA like structure for replicase polyproteins' translation. While, two-thirds of the genome is the non-structural proteins (nsps), only 10 kb of it consists of structural and accessory proteins (Fehr and Perlman 2015). Untranslated region (UTR) is present in the 5′ and it contains stem loop structures for replication and transcription (Brown et al. 2007); the structures for RNA synthesis are in the 3' UTR (Goebel et al. 2007). Although the accessory proteins are mostly nonessential for *in vitro* replication, some play crucial roles in viral pathogenesis (Fehr and Perlman 2015).

1.3.3.2 Virion Structure

Coronaviruses contain four main structural proteins encoded within 3' end: nucleocapsid (N), spike (S), envelope (E), and membrane (M) proteins. The S protein is around 150 kDa and highly N-glycosylated. It belongs to class I fusion protein and plays role in accessing to the ER by utilizing an N-terminal signal sequence (Fehr and Perlman 2015). More importantly, the trimeric S glycoprotein plays role in the virus-host cell attachment (Beniac et al. 2006). Also, unique spike structure on the virus is made up by trimeric complex (Delmas and Laude 1990). S1 and S2 polypeptides are generated upon the cleavage of S by a host cell furin-like protease (Abraham et al. 1990); while one of them recruits the receptor-binding domain (RBD), other makes up the stalk of the spike molecule, respectively. (Fehr and Perlman 2015; de Groot et al. 1987).

M protein is the most abundant protein in the virions and it gives the overall shape of the virion via three transmembrane domains. It is around 30 kDa and it has N-terminal ectodomain and C-terminal endodomain that lie into the particle (Armstrong et al. 1984). The M protein may adopt two conformations as a dimer in

the virion, which promotes membrane curvature and binding to the nucleocapsid (Fehr and Perlman 2015; Neuman et al. 2011).

The E protein is composed of C-terminal endodomain and N-terminal ectodomain and has ion channel activity as a transmembrane protein (DeDiego et al. 2007; Godet et al. 1992) It also helps virus assembly and release. Although it is a structural protein, the deficiency of E protein is not always lethal (Nieto-Torres et al. 2014).

Nucleocapsid of coronavirus is composed of only N protein with a C-terminal domain (CTD) and a N-terminal domain (NTD). These domains serve as a capability of RNA binding, despite using different mechanism to bind (Chang et al. 2006; Hurst, Koetzner, and Masters 2009). High phosphorylation of N protein triggers the structural changes which increase the affinity against viral RNA versus nonviral RNA (Stohlman and Lai 1979). The genomic packaging signal and the transcription-regulating sequences (TRSs) are N protein substrates (Molenkamp and Spaan 1997; Stohlman et al. 1988). Non-structural protein 3 (Nsp3) is an important agent as the replicase. Interaction of N protein with M and Nsp3 serve capability of viral genome binding to the replicase-transcriptase complex (RTC) and finally encapsulation of genome into viral particles (Fehr and Perlman 2015; Sturman, Holmes, and Behnke 1980).

Some βCoVs have hemagglutinin-esterase (HE) protein that acts as a hemagglutinin and contains acetyl-esterase activity. Having the protein increases ability of virus spread through the mucosa and helps S protein-mediated cell entry (Klausegger et al. 1999).

1.3.3.3 Cell Entry and Replication

In order to activate the endocytic route, S protein of SARS-CoV-2 needs proteolytic processing. Host proteases transmembrane serine protease 2 (TMPRSS2), furin and

cathepsin L participate in the cleavage of S protein to S1 and S2 (Hoffmann et al. 2020). Studies show that, in several tissues TMPRSS2 is highly expressed and co-expressed with ACE2, which explains tissue tropism of the virus (Hu et al. 2021).

The S1 subunit is divided into C- and N-terminal domains. CTD contains the RBD which helps to the virus for cell entry and plays crucial role in antibody neutralization (Bosch et al. 2003). The main functional motif in RBD is the receptor binding motif (RBM) composed of two regions which form the interface between the S protein and hACE2. As reported, RBD sequence of S protein lies between the residues 331 and 524 (Tai et al. 2020). Five residues of the motif differs in SARS-CoV-2 than in SARS-CoV, resulting in stabilization of the two virus-binding hotspots on the surface of hACE2 (Huang et al. 2020). A four-residue motif of RBM in SARS-CoV-2 has a more compact conformation than in SARS-CoV, and it increases the success of the contact with hACE2 through N-terminal helix (Y. Huang et al. 2020). These features of RBD in SARS-CoV-2 enhance the affinity of virus to hACE2 receptor when compared to SARS-CoV.

The S2 subunit contains fusion peptide (FP), cytoplasmic domain fusion (CT), heptad repeat 1 (HR1), heptad repeat 2 (HR2) and TM domain. S2 goes through conformational changes by inserting FP into the host membrane following the binding of RBD to ACE2 (Millet and Whittaker 2018). FP is exposed the HR1 domain and this interaction stimulates the HR2 and HR1 trimer binding to form 6-HB (Xia et al. 2020). Newly formed structure, 6-HB, brings the viral envelope and cell membrane together and initiate the viral fusion and entry (Y. Huang et al. 2020).

The viral RNA release to the cytoplasm follows the entry event. RNA translation in the cell generates pp1a and pp1b replicase polyproteins which are cleaved by proteinases encoded by virus into small proteins. In the replication, ribosomal frame shifting occurs during translation and it produces multiple copies of RNA species both genomic and sub-genomic to encode for relevant viral proteins. Virion assembly

occurs at Golgi complex and endoplasmic reticulum by viral RNA and viral protein interaction. Finally, newly formed virions are secreted out of the cells through vesicles (Kumar et al. 2020).

SARS-CoV-2 also may bind to ACE2 of cat, civet, rabbit, dog, ferret, and rhesus monkey. Having wide range of hosts put the animal studies into very important role in COVID-19 diagnosis and treatment. Besides, because of different susceptibilities of different animals provide information to understand the pathogenesis of SARS-CoV-2 infection and transmission dynamics (Hu et al. 2021).

1.4 Diagnosis of COVID-19

Similar to other diseases, early and rapid diagnosis is the most important factor of COVID-19 control. Clinical symptoms, serological and molecular diagnosis, case history and computed tomography are some of the criteria for rapid diagnosis of the disease. Although first cases are seen on the 31st of December 2019, WHO declared laboratory testing guidance with precautions for specimen collection, packaging, shipment and steps for detecting viral genes on the 2nd of March 2020 (WHO 2020). According to guidance, anal and oral swabs, blood and bronchoalveolar lavage fluid (BALF) are the bests way to collect samples for diagnosis (Helmy et al. 2020; Zhang et al. 2020).

Serological tools, such as immunoglobulin G (IgG) and M (IgM) ELISA Detection kits, have extensively been used as their levels were increased in COVID-19 patients (N. Chen et al. 2020). For instance, detection of SARS-CoV-2 IgG through ELISA was developed (Sapkal et al. 2020). That robust and reproducible technique has 92.37% sensitivity and 97.9% specificity. Despite wide use of ELISA kits, the fastest and most sensitive tests for the virus determination were based on the nucleic acid detection. Nested and Real-Time RT-PCR (qPCR) assays were developed and became available with successful diagnosis of the first positive

cases in Japan (Corman et al. 2020). So far, E, RdRp, N and S genes are the main targets for PCR-based tests (Helmy et al. 2020).

1.4.1 Serological Methods

1.4.1.1 Antibody Detection Methods

Both adaptive and innate immunity are important for combating SARS-CoV-2 infection. Role of the adaptive immunity is creating immune memory to prevent further infection by the same virus and its number one component is B cell-mediated immunity, also known as humoral immunity. It helps the virus removal and prevents reinfection by generating memory immune response (A. T. Huang et al. 2020). In the course of infection, virus specific antibody response is generated by the B cells, which includes IgM, IgG and IgA. These antibodies are seen 7-14 days after infection and their presence continues weeks after virus removal (Zhao et al. 2020). While IgM is a marker for early-stage infection, higher IgG levels are seen during late stages or post-recovery. The N and the S proteins are the most common targets for antibodies (Zalzala 2020).

1.4.1.2 Lateral Flow Immunoassays (LIFs)

Li and coworkers developed an LFI that can determine the presence of IgM and IgG antibodies against SARS-CoV-2 within 15 minutes in patient's blood. 128 negative patients and 397 PCR-confirmed cases serve as the samples, and sensitivity and specificity of the test was reported as 88.66% and 90.63% respectively, regardless of the infection stage of patients (Z. Li et al. 2020).

As another type of the LFI, sensitivity, and specificity of immunochromatography (IC) was reported as 34.2% and 98% respectively (Imai et al. 2020). The antibody IgM was observed in 27.8% of patients within the first week, 48.0% within one to

two weeks and 95.8% more than two weeks. In addition, the IgG antibody observation rates were 3.3%, 8.0% and 62.5%, respectively. Moreover, evaluation indicated the sensitivity of CT scans and CT scans with the IC assay for symptomatic (74.3% and 82.4% respectively) and asymptomatic patients (57.9% and 68.4% respectively) (Imai et al. 2020; Zalzala 2020).

Four companies' tests have been evaluated as of July 2020 and reported that determination rate for SARS-CoV-2 antibodies increased as the days after symptom onset passed (Zalzala 2020). Using N and S antigens, the specificity and sensitivity of the four tests were compared and the report indicated that the specificity of the tests was nearly identical during the course of infection. However, the sensitivity was changed depending on the infection time; 41% to 52% within 14 days after onset and reaching 100% after 21 days by increasing over time (Wu et al. 2020).

1.4.1.3 ELISA Tests

ELISA is a cheap technique to determine antibodies against an antigen in human serum (Zalzala 2020). ELISA tests that are approved by US Food and Drug Administration (FDA) are "The COVID-19 ELISA IgG Antibody Test (Mount Sinai Laboratory, New York, NY, USA; only use at Mount Sinai Laboratory)"; "VITROS Immunodiagnostic Products Anti–SARS-CoV-2 Total and IgG Reagent Packs (Ortho Clinical Diagnostics, Rochester, NY, USA)"; and "LIAISON SARS-CoV-2 S1/S2 IgG (DiaSorin, Stillwater, MN, USA)" (Ching, Chang, and Nerurkar 2020). It was reported that IgG and IgA specificity levels of the ELISA against recombinant S1 protein of SARS-CoV-2 were 91.9% and 73% respectively (Jääskeläinen et al. 2020).

As a variant of ELISA technique, microsphere immunoassay (MIA) allows detection of using combination of several antigens (Zalzala 2020). MIA is composed of magnetic carboxylated microspheres and antibodies against the antigen. When virus'

antigen interacts with microspheres, fluorescently labelled secondary antibody gives signal and diagnosis occurs. Both techniques are more sensitive and specific than LFI, but they need a longer time to detect the virus than ELISA (five hours for ELISA and three to eight hours for MIA) (Ching et al. 2020).

1.4.2 Molecular Methods

1.4.2.1 Nucleic Acid Detection Based Tests

Nucleic acid detection-based tests were most widely used method for the diagnosis of viral agents. These methods were used in SARS and MERS outbreaks for diagnostic purposes. In these methods, RT-PCR is the most preferable technique with its high specificity and success rate (Zalzala 2020). The technique contains five main steps: i) lysis of virus, ii) viral RNA purification, iii) cDNA production, iv) amplification of cDNA with virus specific primers v) and optical detection (Corman et al. 2020).

Right after the sequence of the SARS-CoV-2 genome became available, different RT-PCR assays with different primers were developed (Jung et al. 2020). These primers target different regions of virus genome and thus the sensitivity of the assays differs. One study reported that primers belonging to E and N2 genes serve better sensitivity to COVID-19 diagnosis. Another study compared seven primers for the N gene and three primers for Orf1 gene. Comparison also evaluated three different reaction temperatures (55, 58, 60°C) and resulted that Orf1 specific primer shows the best performance without non-specific amplification or cross-reactivity for other coronaviruses (Jung et al. 2020).

1.4.2.2 Loop-mediated Isothermal Amplification

As a technique for DNA amplification, RT-LAMP is utilized for RNA detection (Notomi et al. 2000; Zalzala 2020). Unlike PCR composed of different steps with different temperatures, constant temperature is used in LAMP. In a study, specificity of the technique was evaluated by nasopharyngeal swab samples taken from patients with SARS-CoV-2 infection, and results shows that sensitivity and specificity were 100% and 97.6% respectively. Another study reported that specificity and sensitivity of the RT-LAMP are 100% compared to RT-PCR (C. Yan et al. 2020). Having simplicity in application and low cost, this method has potential to be used as a simple screening assay for detection of viral infections (Zalzala 2020).

1.4.2.3 CRISPR-Cas Based Methods

The CRISPR-Cas, prokaryotic immune system, provides immune defense to genetic materials, which belong to different agents. CRISPR-Cas12 and CRISPR-Cas13 systems were used for the diagnosis of different viral infections in combination with LAMP (Gootenberg et al. 2017; Zalzala 2020). This rapid, inexpensive and sensitive nucleic acid detection method has high potential to find use in COVID-19 diagnosis.

For this purpose, a CRISPR-Cas12–based assay developed for detection of SARS-CoV-2 called the SARS-CoV-2 DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) (Curti, Pereyra-Bonnet, and Gimenez 2020). The CRISPR SARS-CoV-2 Kit (Sherlock BioSciences, Cambridge, MA, USA) was approved to use by the FDA in the emergency conditions (Guglielmi 2020; Zalzala 2020).

1.5 Aptamers

New understanding of nucleic acid – protein interactions were reported by human immunodeficiency virus (HIV) and adenovirus (AV) studies in 1980s. In these

researches, scientists revealed that viruses produce small ribonucleic acids (RNA) molecules that can bind viral or cellular proteins with high specificity. Similarly, other studies focusing on RNA structures figured out that small RNAs could fold into defined three-dimensional (3D) structures (Song, Lee, and Ban 2012). After all, three separate groups obtained small nucleic acid molecules developing an *in vitro* selection strategy (Ellington and Szostak 1990; Robertson and Joyce 1990; Tuerk and Gold 1990). They named these molecules as "aptamer" and their selection strategy as "Systemic Evolution of Ligands by Exponential Enrichment (SELEX)".

Aptamers are single stranded RNA or deoxyribonucleic acid (DNA) molecules that can bind their targets with high specificity and affinity with applications ranging from sensors to therapeutics (Ilgu et al. 2019; Keefe, Pai, and Ellington 2010; Lakhin, Tarantul, and Gening 2013; Marrazza 2017) Aptamers have significant advantages over antibodies. One advantage is their reusability, resulting from regeneration capability of nucleic acids with little activity loss. Antibodies, on the contrary, can be used only few times before their functionality is lost, which is caused by their protein structure and irreversible denaturation. Moreover, compared to protein-based molecules, aptamers are more stable and various modification and optimization options make them preferable to use in biological samples (Zhang, Lai, and Juhas 2019).

1.5.1 Systemic Evolution of Ligands by Exponential Enrichment

Enrichment of oligos is achieved by Systemic Evolution of Ligands by Exponential Enrichment (SELEX) method simulating evolution *in vitro*. Conventional SELEX is composed of three main steps: i) incubation, ii) separation and iii) amplification. The pool used for incubation contains around 10^{15} random oligonucleotides (60-100 base long) with unique 3D structures formed by noncanonical intramolecular interactions, sugar packing, stacking and base pairing. Their specificity to a particular target is provided by unique 3D structure of the oligos, provided by these interactions. This

pool incubated with a target of interest (protein, small molecule, virus particle etc.) for a chosen period of time and after removal of unbound nucleic acids, the first step is completed. Separation (ii) and amplification (iii) of bound oligonucleotides follow the first step. Separation method is differed depending on the type of the oligo pool used and SELEX type choose (Fan et al. 2008). If ssDNA has streptavidin beads, alkaline treatment can separate single stranded oligo from the beads and using ethanol precipitation, bound oligos can be saved. If the target is bound on nitrocellulose membrane and oligos do not have any label, Urea treatment and ethanol precipitation can provide bound oligos (Huang and Niu 2019). For ssDNA oligos, asymmetric PCR, size separations on denaturing urea polyacrylamide gel electrophoresis, Lambda exonuclease digestions other options to have bound oligos (Marimuthu et al. 2012). Amplification is carried out by PCR or RT-PCR depending on the type of nucleic acid; DNA or RNA, respectively. The protocol follows these three steps for several (average of 6 to 10) rounds. After the final round, to identify the selected oligos either Sanger (Komarova and Kuznetsov 2019), next-generation sequencing (NGS) (Tolle and Mayer 2016) or High Throughput Sequencing (HTS) (Komarova, Barkova, and Kuznetsov 2020) is employed (Ilgu et al. 2019; Sharma, Bruno, and Dhiman 2017). Then, comparing aptamers sequence-structure motifs using different bioinformatic tools, potential target recognition sites can be deciphered (Dao et al. 2016; Hoinka et al. 2012; Pei, Slinger, and Meyer 2017; Sullivan et al. 2019).

In vitro selection can take weeks to months. To make it faster, an alternative SELEX method, photochemical SELEX (PhotoSELEX) was suggested (Golden et al. 2000). These aptamers display higher specificity, because of having the ability to form a photoinduced covalent bond with their targets. Also, selecting aptamers in fewer selection rounds makes the technique faster than the traditional SELEX methods (Tombelli, Minunni, and Mascini 2005; T. Wang et al. 2019).

In addition to the conventional and Photo-SELEX, structure-switching SELEX has recently become popular (Oh et al. 2010). In this approach, the pool is annealed to a complementary region and to separate the nucleic acid oligos from the complementary sequence, they are required to change their structure when they interact with the target during SELEX. This way, it is ensured for the change in structure upon ligand bound (Oh et al. 2010). Other alternative SELEX methods have been developed including capillary electrophoresis (CE)-SELEX, Cell-SELEX, and *in vivo* SELEX as summarized in Table 1.1 (Kumar et al. 2019). After aptamers are selected, they may be further modified to improve their specificities and affinities by a number of approaches that, in total, can be referred to as maturation (Komarova and Kuznetsov 2019).

Table 1.1 SELEX Types

SELEX Type	**Features**
PhotoSELEX	*Light sensitive oligonucleotides are excited by UV and increased their affinity* (Golden et al. 2000).
Cell-SELEX	*Instead of the molecules, whole cell is used in SELEX. It is generally used for cell surface targets* (Sefah et al. 2010).
In vivo SELEX	*Oligonucleotide pool is given to living animal* (Sola et al. 2020).
In silico SELEX	*Computer programs are used to predict tertiary structure, affinity, and target interaction of aptamer candidates* (Wondergem, Schiessel, and Tompitak 2017).
CE-SELEX	*Capillary electrophoresis is used to select high-affinity aptamers and it reduce the process from weeks to days* (Mosing and Bowser 2009).

Spiegelmer Technology	*After selection, aptamers are synthesized as unnatural L-nucleotides which increase their stability* (Eulberg and Klussmann 2003).
Structure Switching SELEX	*To get the nucleic acids from the complementary sequence, they are required to change their structure when they interact with the target during SELEX. This way, it is ensured for the change in structure upon ligand bound* (Oh et al. 2010).
Robotic Assisted-SELEX	*Robotic platforms perform the selection procedure without any manual interruption. It reduces the process less then 2 days* (Breuers et al. 2019).
RAPID-SELEX	*It is a conventional SELEX method without amplification. Every round Kd values are measured, and enriched aptamers are sent to HTS* (Szeto et al. 2013).
GO-SELEX	*In conventional SELEX protocol, unbound oligos are adsorbed onto graphene oxide (GO)* (Nguyen et al. 2014).
Conditional SELEX	*This method enables selection of aptamers only working in the presence of a regulator molecule* (Smith and Gold 2002).
Tailored SELEX	*It is primer-free selection of aptamers. After each round pool is ligated with primers. This method prevents primers from binding to target* (Vater et al. 2003).
SPR-SELEX	*Desired target is immobilized on SPR chip and oligo pool injected on biosensor chip* (Dausse et al. 2016).
Chimeric SELEX	*It is used two different oligo pool to get chimeric aptamers, which have diverse features* (Burke and Willis 1998).

Sol-gel SELEX	*Desired target immobilized on microfluidic device* (Ahn et al. 2011).

1.5.2 Structure of Aptamer

Being nucleic acid in nature, aptamers have negative charge and an "ionic atmosphere" that is ideal for target interaction. This microenvironment attracts various ions. Molecule structure and target binding are affected by this electrostatic interactions and change of charge distributions (Yu et al. 2017). Also, RNA molecules have a pentose sugar with hydroxyl group (- OH) at the 2' position; DNA molecules have a hydrogen atom (-H) instead. Having 2'-OH makes RNA more susceptible to depolymerization at neutral pH, result of polyvalent metal ions causing transesterification. Moreover, the 2'-OH provides more options for metal ion binding and more structural (Hou et al. 2001; Ilgu and Nilsen-Hamilton 2016; Velema and Kool 2020). Metal ion interactions are essential for RNA folding and stabilization so RNA can perform all its biological functions. Based on this general knowledge about nucleic acids, structures of selected aptamers are dependent on microenvironment of SELEX (Hianik et al. 2007). Temperature and ingredients of selection buffer such as ions, and their concentrations (ionic strength) and pH are key components of the environment (Catuogno and Esposito 2017; Ilgu and Nilsen-Hamilton 2016; Komarova and Kuznetsov 2019; Zhuo et al. 2017) Therefore, aptamer features and affinities depend on buffers and temperature of selection environment and these two components require close attention during SELEX.

Because of their chemical composition, aptamers are direct target of nucleases present in many biological systems. Having -OH group makes RNA more susceptible to nucleases and because of nuclease susceptibility, RNA is more chemically labile in high pH and temperature than DNA (Ilgu and Nilsen-Hamilton 2016). This situation can be handled by post selection chemical modifications. Even though the modifications make aptamers more stable under particular conditions,

they may decrease specificity and affinity of them. It is better to use chemically substituted nucleotide analogs during selection, such as using 2'-floropyrimidines (2'FY) during RNA SELEX (Allerson et al. 2005).

Finding out secondary and if possible tertiary structural motifs for aptamers is essential to analyze target interaction which provide detailed information to develop aptamers with higher affinity and specificity to a particular target (Ilgu et al. 2019). To examine secondary motifs in the tertiary structures of aptamers, including the G-quadruplex, internal bulge, hairpin structure, nuclear magnetic resonance spectroscopy and X-ray crystallography techniques can be used (Buglak et al. 2020; Elskens, Elskens, and Madder 2020; H. Y. Li et al. 2020; Radom et al. 2013). However, both techniques are expensive and effortful; thus, computer algorithms which estimate lowest free-energy structures based on aptamer sequences are preferred (Lu, Bussemaker, and Olson 2015; Parisien and Major 2008). Although it makes secondary structure prediction of oligos without so much effort, this technique still development phase and so results are not reliable than experimental outcomes (Afanasyeva, Nagao, and Mizuguchi 2019; Buglak et al. 2020; Chojnowski, Waleń, and Bujnicki 2014; Hendrix, Brenner, and Holbrook 2005; Hermann and Patel 1999; Sullivan et al. 2019).

1.5.3 Use of Aptamers

1.5.3.1 Diagnostic Purposes

As new public health issues appeared, invention of novel diagnostic techniques has become important. Scientists desire to find novel and close to perfect methods to detect biomarker of interest to support precision medicine and early diagnosis which plays crucial role for treatment of many diseases, mainly cancer and neurodegenerative diseases (Banerjee and Nilsen-Hamilton 2013; Kulabhusan, Hussain, and Yüce 2020; Moulick and Bhattacharjee 2020; Zou et al. 2019) The

method should be sensitive, reusable, stable and nonreactive in biological samples, easy to demonstrate, quick and cheap (Akki and Werth 2018; Forouzanfar et al. 2020; Ilgu et al. 2019; S. R. Yan et al. 2020) Antibody-based biosensors have been developed to meet this need for a long time. However, because of their lower cost, better stability, reusability, and the aforementioned advantages (see Table 1.2), aptamers have become preferable agents rather than antibodies.

Biosensors are diagnostic tools that provide particular and measurable signals formed after biomolecular interactions. These tools consist of a bio-recognition element (bioreceptor), binding to molecule of interest, and a transducer that provides detectable signal as a result of interaction. The success of any biosensor depends on affinity and specificity of its bioreceptor. Also, money saving and stable bioreceptors are generally preferred by manufacturers. In addition to these desired features, sensitive detection of bacteria, viruses, cancer cells, metabolites, biomarkers, toxins, drugs is possible (Chen et al. 2016; Kulabhusan et al. 2020; Z. Li et al. 2019; Zhou et al. 2017).

Fluorescently labeled aptamers in an optical biosensor as a recognition element were integrated onto solid supports for real-time analyte detection. Majority of these proof-of-principle level studies were performed using thrombin aptamers (TA), which have highly stable G-quadruplex structure and their target, thrombin, found in human serum (Avino et al. 2012; Deng et al. 2014; Derszniak et al. 2019; Drolet, Moon-McDermott, and Romig 1996). However, using less stable aptamers targeting less stable analytes in other aptasensors are likely to be challenging (Ozaki et al. 2006; Sun et al. 2018).

Table 1.2 Comparison of Antibodies and Aptamers

Features	**Antibodies**	**Aptamers**
Specificity	*High*	*High*
Affinity	*High*	*High*
Immunogenicity	*High*	*No humoral response*
Production	*In vivo*	*In vitro*
Cost	*High*	*Low*
Stability	*Unstable*	*Stable*
Potential targets	*Limited to immunogenic molecules*	*Wide range*
Affinity	*High*	*High*
Immunogenicity	*High*	*No humoral response*

1.5.3.1.1 Electrochemical Aptasensors

Binding of an aptamer having redox-active feature or changing the charge distribution on an electrode surface is the bases of electrochemical aptasensors (Ikebukuro, Kiyohara, and Sode 2004). Conductometric, amperometric, or impedimetric assays can be used for detection of analytes thanks to these types of aptasensors. Also, switch on/off mechanism can be used in these sensors via labeling aptamers with electroactive group, and taking advantage of structural constraints of aptamers (Ikebukuro et al. 2004). In these systems, structural change in the aptamer based on binding to desired analyte results in distance change of electroactive group from electrode surface, which causes signal change in terms of switch on /off (Ikebukuro et al. 2004). Electrochemical aptasensors are targeting wide range of molecules including thrombin, cocaine, adenosine, aminoglycosides, ATP, inorganic

ions, PDGF and immunoglobin E. Also, concentration of these targets can be accurately measured observing electrochemical changes (Díaz-Fernández et al. 2020; Ilgu and Nilsen-Hamilton 2016; Z. Li et al. 2019; Mishra, Sharma, and Mishra 2018)

One example for usage of structural constraint is using redox-active methylene blue (MB) as an aptamer label and electro-transfer communication agent with the electrode, which enables the demonstration of changes in aptamer conformation. For instance, an aptasensor having an MB-labelled TA was constructed immobilizing on an electrode (Sabzi et al. 2008). Structural flexibility of aptamer allowed the electrotransfer from MB to the electrode. Signal-off mode was activated when structural changes occurred which shields MB (Ilgu et al. 2019).

Using complementary oligonucleotide to one part of the aptamer to inactivate it is one alternative of the structural constraints, beside structural change upon target binding. In a study, a complimentary assist DNA was used to form a Y-type DNA structure by hybridizing it with biotin-tagged zeatin aptamer. At the terminals of DNA, this system included alkaline phosphate (ALP), which has catalytic activity resulting in p-nitrophenol (PNP) production. When the zeatin was in environment, the system was disrupted and oxidation signal from PNP was decreased (Zhou et al. 2018).

Aptasensors that can operate in a “signal-on” mode is preferred by several studies with different approaches. For example, a thiol group and as a redox label, electroactive ferrocene group was added both end of the thrombin aptamer. When ferrocene was closer to electrode surface result of stabilization of G-quadruplex structure via thrombin binding, system gave positive signal. This is resulting from electron transfer between ferrocene and electrode, caused by close proximity (Song et al. 2008).

Currently, as an alternative platform for aptasensors, nanoporous metal surfaces have been preferred (Jiang et al. 2021; Kashefi-Kheyrabadi and Mehrgardi 2013). Various aptamers having oxidative capabilities are used on these types of sensors in addition to a redox probe which causes electron transfer to the gold surface for effective detection of target via electrochemical impedance spectroscopy (EIS). As an example, 3,4-diaminobenzoic acid (DABA), which creates EIS signal via oxidation, can be linked to one half of ATP aptamer and oxidation property can be used to detect for analytes like bisphenol A which can participate redox reactions (Zhu et al. 2015). Also, different types of surfaces with nanoporous feature such as graphene oxide/Au, porous PtFe or PtTiAl ternary alloys can be used to detect different targets (Chekin et al. 2018; F. Li et al. 2019; Yan et al. 2013).

Hybridization of aptamers with other oligonucleotides can also be used on nanoporous gold surfaces to create gates over pores. In a study, highly specific aptamer for avian influenza virus H5N1 was hybridized with oligonucleotides bound to the surface. In the presence of the virus, the gates opened, and the aptamers were released. Cofactor for lactate dehydrogenase (LDH) layered below the gold surface. The substrates entered the pores after gates' opening and oxidation of NADH by LDH was detected using cyclic voltammetry (Yan et al. 2013).

1.5.3.1.2 Optical Aptasensors

Optical aptasensors use mostly fluorescent and colorimetric assay formats. Having different labeling options and easy aptamer synthesis make fluorescent detection preferred, which is also suitable for real-time detection (Gaudin 2020; Ghorbani et al. 2019; Ilgu et al. 2019; Zahra, Khan, and Luo 2021).

One strategy to convert aptamers into fluorescent signal providing agents is to place aptamer in a structure having fluorophores or a fluorophore and a quencher at its ends. It utilizes fluorescence resonance energy transfer (FRET), a system based on

energy transfer between donor and acceptor upon target binding. Disruption of the structure of system via separation of two ends and resulting in fluorescent signal is the basis of the operating mechanism of the FRET. Using quantum dots or organic fluorescent dyes can improve the performance of such a system. Another strategy is to design an aptamer with fluorophore in a structure with its complementary DNA with quencher. When the target binds to the aptamer, complementary oligonucleotide is separated, and the fluorescence signal is increased (Song et al. 2008).

Using optical aptasensors, simultaneous detection of several analytes is possible. As an example, different types of fluorophore aptamers were immobilized on a glass platform. Simultaneous detection of three of the cancer biomarker molecules (basic fibroblast growth factor, inosine monophosphate dehydrogenase and vascular endothelial growth factor) and thrombin was achieved (Gaudin 2020; Ilgu and Nilsen-Hamilton 2016; McCauley, Hamaguchi, and Stanton 2003; Zahra et al. 2021).

Another example to use of multiplexed sensors is a graphene oxide-based aptasensors. In a study, H1N1 detection was achieved using graphene-oxide based aptasensor, and in the system, SYBR Green 1 was used for signal amplification. Also, Pseudomonas aeruginosa was detected by a system composed of aptamers highly specific to the bacterium, also these aptamers were conjugated with photoluminescent carbon dots to get fluorescent signal. In this sensor, graphene oxidase was used as quencher (Sadeghi et al. 2018).

1.5.3.1.3 Microcantilever Aptasensors

Microcantilever aptasensors can be preferable because of their small testing volume, high scalability, and label free targeting features. High-throughput analysis can also be achieved even though this type of sensors cannot be easily multiplexed. These

aptasensors have been operated in dynamic and static modes. Aptamers are used for functionalization of one side of the microcantilever in the static mode which is more sensitive then dynamic mode in liquid environment (Luka et al. 2015). While target analyte is adsorbing onto functionalized side, surface stress is generated and the differences between stress of top and bottom surfaces results in bending of microcantilever. This bending can be positive (upward) or negative (downward) based on molecular interaction of analyte and aptamer. Bending results in displacement of the light beam, which is then detected by different techniques like optical, capacitive or piezoresistive. Biological analytes such as proteins and small molecules were detected by aptamer-based cantilevers with different sensory systems including interferometry and piezoresistivity (Kang et al. 2011; Kang, Nilsen-Hamilton, and Shrotriya 2008).

1.5.3.1.4 Acoustic Aptasensors

Quartz crystal microbalance (QCM) is one of the formats used in acoustic aptasensors. In this kind of sensors, light changes in the input and output is used to detects target binding which result in frequency or phase shift . Using surface acoustic eave (SAW) aptasensors which based on propagation of the acoustic wave, real-time quantification of proteins can be measured. As an example, a system including an array of sensor elements detecting human alpha thrombin or HIV-1 Rev peptide was developed (Schlensog et al. 2004).

Another technique that relies on a change in refractive index due to analyte-aptamer interaction is surface plasmon resonance (SPR) sensor (Tombelli, Minunni, Luzi, et al. 2005). Retinol binding protein-4 specific SPR sensors was found more sensitive than an antibody-based ELISA assay (Wilson et al. 2013). As an example, for detection of Bisphenol A (BPA), a U-shaped fiber optic SPR aptasensor was developed. This system was invented utilizing gold nanoparticles (AuNPs). AuNPs/ssDNA complex was formed result of the incubation of bare AuNPs with

BPA aptamer. Then, BPA binding broke the complex and causes to AuNPs aggregation. This aggregation enhance the refractive index of the solution in the aptasensor (Luo et al. 2016).

SPRs have three main limitations for cell detection. Nonspecific binding, resulting in the refractive index change, is the most common limitation, which can be overcome using reference flow cells (Cai et al. 2018). Another limitation is the sensing range with 200 nm compared to cells which have dimensions of micron ranges. This limitation can be fixed using long range SPR with depth of over 800 nm, which increase the sensitivity of the system. Low throughput is another drawback that was demonstrated to be easily overcome by SPR imaging technology (Faure-Perraud et al. 2011).

1.5.3.1.5 Surface-Enhanced Raman Spectroscopy (SERS) Sensors

To solve sensitivity issues in analyte detection, SERS-based sensors have been developed in diagnostic applications with extreme sensitivity and easy operation (Cong et al. 2020). Raman spectroscopy is dependent on incident light and illuminated target molecules and the energy transfer between them. As Raman signal is very week, generally two separate enhancement mechanisms are utilized: i) a chemical enhancement resulting from interaction between target molecule and the metal surface and ii) an electromagnetic enhancement caused by electromagnetic resonance formation near metal structures that results in large local fields (Pilot et al. 2019). Enhancement rate is found far higher in electromagnetic (enhancement factor of 10^{11}) than chemical enhancement (enhancement factor of 10^{3}). Thus, electromagnetic enhancement effect is preferred in the most SERS-based sensors (Pilot et al. 2019).

To increase matrix selectivity and Raman signal, antibodies, antimicrobial peptides, and aptamers can be used as recognition elements. Aptamers are the most suitable

agents among these with their stability, low cost and before mentioned advantages (H. X. Wang et al. 2019). As an example, a SERS system based on the reduction of $HAuCl_4$ via aptamer and graphene oxide surface to detect Hg^{2+} was developed. By electrostatic interaction, charged aptamer was adsorbed onto GO surface and Victoria blue 4R (VB4r) was used as a Raman probe. While Hg^{2+} was in the environment, aptamer-Hg complex was formed and aptamers were separated from the surface, resulting in increased SERS signal. The system achieves an LOD of 0.08 nM Hg^{2+} (Li et al. 2018)

Another example includes *Vibrio parahaemolyticus* detection. It uses sandwich method-based SERS system with target specific aptamers. Aptamers were immobilized onto polydimethylsiloxane film through hybridization with the Au NPs via Au-S bond. 4-mercaptobenzoic acid (4-MBA) was introduced to sandwich complexes of SERS as Raman detection molecule. Having high affinity, aptamers captured *Vibrio parahaemolyticus* and the signal increased. The presence rate of *Vibrio parahaemolyticus* showed a linear relationship with the intensity of 4-MBA signal (Shen et al. 2019).

Different techniques in SERS aptasensors with the Raman signal molecules were possible and they can be used in the detection of small molecules, microorganisms, tumor cells, toxins, and other functional molecules (H. X. Wang et al. 2019).

1.5.3.2 Therapeutic Purposes

Besides clinical diagnostics, aptamer can be used in therapeutic applications. Inhibiting target molecule, acting like receptor agonist or antagonist, activating related pathways and acting like drug delivery agents are some therapeutic strategies that aptamers serve (Song et al. 2012). Several aptamer studies are in the proof-of-concept state, some are undergoing clinical trials, and some are about to reach this step. However, only Macugen (pegaptanib) selected against vascular endothelial

growth factor (VEGF) has been approved by FDA in USA for its use in the treatment of vascular ocular disease (Song et al. 2012).

One of the key applications of aptamers is aptamer-based drug delivery systems. In these systems, aptamers are used as cargo carrier agents with higher specificity to a target cell or tissue. Binding of aptamers to the target cell receptor results in internalization of aptamers with desired cargo. However, this application is possible only using aptamers that can recognize specific cell surface proteins. Cell-based selection techniques, Cell-SELEX, were developed to select aptamers against receptor proteins when it is in natural form (Chen et al. 2016; Ilgu et al. 2019).

1.5.4 Aptamers Against Viruses

A variety of viruses has been used as targets of aptamers. Aptamers against Human Papillomavirus (HPV) (Leija-Montoya et al. 2014), Hepatitis C and B Virus (HCV and HBV) (Gao et al. 2014), HIV (Leija-Montoya, Benítez-Hess, and Alvarez-Salas 2016), Influenza Virus (Gopinath et al. 2006), Ebola Virus (Shubham et al. 2018) (Hong et al. 2019), Zika Virus (Argondizzo, Silva, and Missailidis 2021), Rift Valley Fever Virus (Ellenbecker et al. 2015), Dengue Virus (Chen et al. 2015), SARS-CoV-2 (Sun et al. 2021) are generated to detect or inhibit the virus.

Isolation of virus targeting aptamers is achieved by different SELEX methods. Advantage of using whole viral particles in SELEX is obtaining aptamers that interact with native viral protein structures on particle surface. Then, this aptamers can be used in diagnosis and therapeutics without any knowledge of nature or target and infection mechanism of virus (Leija-Montoya et al. 2016).

The first application based on whole virus approach was against Rous sarcoma virus (RSV). In this study, 19 RNA aptamers were isolated and five of them successfully neutralized the virus infection (Pan et al. 1995). This research was a pioneer of

aptamer applications in virology. It leads to the development of aptamers as diagnostic and therapeutic agents, particularly for viral diseases that need fast diagnostic or that result in chronic viral-induced conditions (Wandtke, Woźniak, and Kopiński 2015).

1.5.4.1 Human Immunodeficiency Virus Type I (HIV-I)

Aptamers against proteins of the etiological agent of AIDS, HIV-1, have been selected. These include reverse transcriptase (RT) (Nickens, Patterson, and Burke 2003), RNaseH (Andreola et al. 2001), integrase (Rose et al. 2019), TAR-element RNA (Kolb et al. 2006), nucleocapsid, Tat, Gag and gp120 (Leija-Montoya et al. 2016).

As an RNA virus, RT is needed to transform the genomic RNA of HIV-1 into double stranded DNA and provide the genetic material to start replication. Because of its nature, RT has been always the main target of AIDS treatment studies. Thus, dozens of DNA and RNA aptamers which can inhibit RT activity in cell culture have been selected with Kd between 25 pM to 30 nM (Wandtke et al. 2015).

Tat protein interacts with the trans-activation responsive (TAR) element and regulates the viral gene expression. Selected aptamer against this molecular (RNATat) is highly specific to their target and its binding affinity 100-fold higher than affinity of natural target of Tat (TAR-1 RNA) to Tat *in vivo* (Leija-Montoya et al. 2016). Using this aptamer, a molecular beacon with a fluorophore and a quencher was developed. In natural conformation of the beacon, the fluorophore and quencher stay close to each other and thus, signal is not produced. When the system faced with Tat, strand separation is triggered, which holds apart the two agent and allows fluorescence signal (Yamamoto and Kumar 2000).

HIV-1 uses CD4 surface receptors to infect human cells by its surface glycoprotein gp120. Thus, gp120 has potential to be a target for AIDS treatment. Therefore, several aptamers have been selected against gp120 to neutralize the virus. Aptamer B40 is one of them and results of its characterization shows that it is highly specific to HIV-1 R5 strain and it can neutralize the virus in vitro (Dey et al. 2005). Also, this aptamer demonstrates synergetic effects with anti-CD4 binding site monoclonal antibody and gp41 fusion inhibitor which suggests that B40 has a potential to use as an adjuvant (Mufhandu et al. 2012).

1.5.4.2 Human Papilloma Virus (HPV)

Having oncoprotein activity, HPV one of the main focus of virus studies. Aptamers targeting HPV proteins to block viral infection, inhibit the oncoprotein activity or diagnosis of the virus are developed by several research groups (Kim and Lee 2021; Leija-Montoya et al. 2016; Toscano-Garibay, Benítez-Hess, and Alvarez-Salas 2011; Trausch, Shank-Retzlaff, and Verch 2017; Valencia-Reséndiz et al. 2018).

HPV capsid is composed of L1 and L2 proteins (ratio of 5:1 respectively). Self-assembly of L1 can construct virus-like particles (VLPs). (Leija-Montoya et al. 2016). The first RNA aptamer that targets L1 protein was selected against HPV-16 VLPs a VLP broadly used in HPV studies. The aptamer Sc5-c3 contains hairpin structure which binds VLPs with higher affinity (0.05 pM) (Leija-Montoya et al. 2014). Specificity of the aptamer to VLPs suggests that it may provide a potential diagnostic agent against HPV infections and it can be used as a molecular for virus neutralization.

Whole HPV infected cells also is used as a tool for aptamer selection. One of the tools is HPV-16 E6/E7 transformed human tonsillar epithelial cells (HTEC) used in a study based on cell-SELEX protocol to isolate RNA aptamers. Results of the study provides reasonable mechanism to deliver therapeutics into the HPV-16 associated

tumors (Gourronc et al. 2013). Another study generates aptamer that can recognize cell surface differences between HPV-transformed and non-tumorigenic cell lines through cell-SELEX method, which serves accomplished diagnostic tools for HPV-infected cells. Moreover, one of these aptamers, Aptamer 14, can enter the cell without interacting with cell surface proteins, which have high potential to be an agent in drug delivery systems (Graham and Zarbl 2012).

1.5.4.3 Hepatitis C Virus (HCV)

Small, enveloped virus HCV has linear positive sense ssRNA genome, seven nonstructural proteins and structural proteins C, E1 and E2. Causing to chronic liver disease puts the HCV main focus on virology research (Leija-Montoya et al. 2016).

Several HCV aptamers are selected against NS2, NS3, NS5 proteins and some IRES domains because of their importance in viral infection, replication, and proliferation. For instance, aptamers NS2-2 and NS2-3, isolated against NS2 protein, reduces infectious virus production. These HCV specific aptamers do not initiate innate immunity responses. Results suggest that NS2-2 aptamer disrupts NS2–NS5 interaction through binding the NS2 N-terminus, which results in antiviral activity (Gao et al. 2014).

Structural protein E2 is enveloped and direct interaction with CD81 receptor initiate the viral infection. Using CT26 cells, E2 specific aptamers were isolated via cell surface SELEX method. The aptamer ZE2 which exhibit high affinity and specificity to E2 was able to detect and neutralize HCV in vitro (Chen et al. 2009). Also, E1E2-6 inhibits host cell binding, which results in blockage of viral infection (Yang et al. 2013). Based on these two aptamers, a system was developed to detect immobilized infectious HCV particle in enzyme linked apto-sorbent assay (ELASA) (Park et al. 2013).

1.5.4.4 Influenza Virus

Severity of flu pandemics depend on type of the virus. While Influenza A virus (IVA) targets birds and mammals, influenza B (IVB) mainly infects humans. Although Influenza C (IVC) is less common, it also causes disease. Having different host targets, all types can infect human and subject for aptamer studies (Leija-Montoya et al. 2016).

Selected aptamer, P30-10-16, has higher affinity to HA protein and it can distinguish between A/Panama/2007/1999 and A/Aichi/2/1968 H3N2 subtypes (Gopinath et al. 2006). Further study determined that GNCNU pattern of aptamer as the minimal element needed to bind HA (Misono and Kumar 2005). Also, IVB types can be discriminated by aptamers selected using whole virus particle or purified proteins. HA of B/Johannesburg/05/1999 virus specific RNA aptamer can distinguish this subtype from other subtypes, while preventing viral infection by membrane fusion (Gopinath et al. 2006). In addition, two aptamers specific to HA protein of B/Tokyo/S3/99 and Jilin/20/2003 were generated and specificity of Tokyo aptamer is about 250ifold higher than commercial antibodies (Lakshmipriya et al. 2013).

1.5.4.5 SARS-CoV and SRAS-CoV-2

Since SARS pandemics is started, different groups working on aptamers focused on develop aptamers specific to SARS-CoV for diagnostic and therapeutic purposes. Although these studies were finished after end of the SARS pandemics, emergence of COVID-19 helped to relaunch aptamer researche working on interface of aptamers and SARS-CoV-2. In these works, spike and nucleocapsid proteins and NTPase/Helicase (nsP10) of SARS-CoV and SARS-CoV-2 were used as target. Also, aptamers selected against SARS-CoV are repurposed for SARS-CoV-2 detection and neutralization in novel coronavirus studies.

nsP10 of SARS-CoV unwinds duplex DNA performing helicase activity, which is important action for replication. Thus, this viral helicase has a potential to be a target for therapeutics in SARS. In a study, RNA aptamer called ES15 selected against nsP10 protein to inhibit its helicase activity, and results showed successful inhibition up to 85%. It was reported that aptamer ES15 binds the nsP10 stronger than natural substrate, dsDNA, of the helicase (Jang et al. 2008).

Another study aiming helicase activity of the virus reported that selected aptamers specific to helicase inhibit the enzymatic activity with different IC50 values. For instance, aptamers NG1, NG3 and NG8 inhibited the unwinding with IC50 values of 87.7, 120.8 and 91.0 nM, respectively. Also, these three aptamers are highly specific to the SARS-CoV helicase (Shum and Tanner 2008).

Another study focused on SARS-CoV targeted the nucleocapsid (N) protein of the virus. N protein is one of the most abundant structural proteins and has a potential to be a diagnostic marker for SARS and COVID-19. In this study selected RNA aptamer performs high affinity to N protein of SARS-CoV with Kd of 1.65 nM. Based on this aptamer, chemiluminescence immunosorbent assay and a nanoarray aptamer chip were constructed and nucleocapsid protein was detected at a concentration as low as 2 pg/mL in vitro. Also, further analysis reported that isolated aptamer recognized the C-terminal region of the protein with high specificity (Ahn et al. 2009).

N protein specific aptamers were used by Roh et al to develop a diagnostic tool. Different than above mentioned chip, this tool conjugate RNA aptamers with quantum dots (QDs). The recombinant SARS-CoV N protein was directly immobilized on the surface of a glass chip and QD-RNA conjugate specifically hybridized with the protein. Interaction between proteins and conjugate serve the optical signal variation which is the basis of detection. Detection limit of the optical QDs-based RNA aptamer chip is about 0.1 pg mL^{-1} (Roh and Jo 2011).

The same target was used in another study to develop diagnostic tool for SARS-CoV. The group, Cho et al, isolated 15 DNA aptamers and determined higher affinity aptamer against N protein using ELISA and identified an aptamer with Kd of 4.93 nM. Further analysis showed that the DNA aptamer detect the SARS-CoV nucleocapsid protein better than nucleocapsid antibody in vitro (Cho et al. 2011).

N protein sequences of SARS-CoV and SARS-CoV-2 is strongly conserved. Thus, it is not hard to say that the developed aptamers against SARS-CoV N protein could be, also, beneficial as therapeutic agents for COVID-19 (Parashar et al. 2020). A study was conducted for this purpose and the aptamer generated by Cho et al was found to bind N protein of SARS-CoV-2 with high affinity (Z. Chen et al. 2020).

Although there is not any aptamer study targeting spike protein of SARS-CoV in literature, Song et al isolated two aptamers, CoV2-RBD-1C and CoV2-RBD-4C, against RBD of S protein with a Kd of 5.8 nM and 19.9 nM, respectively. Simulated interaction modeling and competitive experiments indicated that these aptamers partially bind one site of RBD, which is the identical binding site of ACE2. Thus, besides being a diagnostic tool, these aptamers can be used in therapeutic purposes and in studies that shed light the mechanisms behind the coronavirus infection (Song et al. 2020). In another study, aptamer-based electrochemical assay was constructed using aptamer CoV2-RBD-1C. In this system S1 subunit of the spike protein was introduced into saliva and results showed that detection was possible down to 0.1 fg/mL in saliva sample (Zakashansky et al. 2020).

Several studies have been conducted for therapeutic purposes. Inhibition of human ACE2 receptor and receptor-binding domain of SARS-CoV-2 S protein (SRBD) interaction is a promising treatment strategy. It was reported that SRBD specific aptamer CoV2-6 can compete with ACE2 to bind SRBD. Further, truncated and circular bivalent version of this aptamer, cb-CoV2-6 was tested, and the results

showed that new aptamer could block the virus with an IC50 of 0.42 nM, it was highly stable in human serum (more than 12 hours) and was stored in room temperature for more than 14 days (Sun et al. 2021).

An interesting aptamer study against pseudo-SARS-CoV-2 particle reported that a selected aptamer, SP6, inhibited the virus infection without preventing SRBD and human ACE2 contact. In addition to providing a new molecule, this research opens up new ways to develop virus blockers independent of S protein or ACE2 receptor targeting (Schmitz et al. 2021).

Considering the aptamers' potential to detect very low amount of virus in different samples from different body fluids, they are very good candidates to use for COVID-19 diagnosis. Thus, in here, our aim is selecting 2'FY-RNA aptamers against SARS-CoV-2 particles to use diagnostic purposes.

CHAPTER 2

MATERIAL AND METHODS

2.1 Inactivated SARS-CoV-2 Particle Production

The particle was produced by a group supervised by Prof. Dr. Füsun Can in Koç University and sent to our laboratory into DMEM. The brief explanation of the protocol to produce the particle is below:

The sequences were pieced together via SPAdes program and the gene sequence of the virus (Gene bank number: MT675956 (B303)) can be reached through NCBI.

2.1.1 Concentrating the Virus

20 ml virus stock was first centrifugated at 400 g for 10 minutes to remove cell debris. Subsequently, supernatant was centrifugated at 100,000 g for 1,5 hour and pellet was dissolved in 600 µl of DMEM (no FBS, 1% Pen/Strep, %0.2 Amphotericin).

2.1.2 Inactivation

0.1% B-propiolactone (inactivation agent) was transferred into the concentrated virus. 16h incubation at 4°C then 2 hours at 37°C was done for inactivation.

2.2 Design of DNA Library and Primer for SELEX

Oligonucleotides used in the library were synthesized by Integrated DNA Technologies (IDT; Coralville, IA) or by Oligomer (Ankara, Turkey). The ssDNA library was synthesized by IDT with the sequence of GCC TGT TGT GAG CCT CCT GTC GAA (53 N) TTG AGC GTT TAT TCT TGT CTC CC. N mentions that an equimolar mixture of A, C, G and T.

The primers were used for PCR are: 5'-TAA TAC GAC TCA CTA TAG GGA GAC AAG AAT AAA CGC TCA A-3' (oligo484) and 5'-GCC TGT TGT GAG CCT CCT GTC GAA-3' (oligo485) to generate dsDNA template. The red bolded sequence indicates the T7 polymerase promoter region that is required during *in vitro* transcription (IVT) reactions to generate 2′FY-RNA pool (Shubham et al. 2018).

2.3 Synthesis of 2'FY RNA Pool

Synthesis of 2'FY-RNA pool (Shubham et al. 2018) was started with extension reaction using oligo484 (65 °C for 15 minutes). Reaction was applied using WizPure^{TM} Taq DNA Polymerase Kit (Wizbiosolutions, Seongnam, South Korea). Solution was incubated at 94 °C for 5 min followed by incubation at 65 °C for 15 min then at 72 °C for 99 min using the thermocycler (Thermo Scientific, Arktik Thermal Cycler). The dsDNA was observed by agarose gel (2%) in 1X TAE Buffer (50X: 242 g of Tris-base, 57.1 mL of Glacial acetic acid, 100 mL of EDTA (0.5 M, pH 8.0), distilled water up to 1000 mL) followed by purification procedure using a Qiagen PCR purification kit, and purified dsDNAs were quantified via spectrophotometer (BioDrop, Nanodrop Technologies).

2′FY-RNA was produced by IVT (Beckert and Masquida 2011) using the Apt-Get T7 transcription kit (Roboklon, Berlin, Germany). 2 nmoles of dsDNA was mixed with dNTP Mix (5 mM ATP, 5 mM GTP, 5 mM 2′F-UTP, 5 mM 2′F-CTP), 10X

reaction buffer and T7 polymerase (total volume of 25 µL for each tube). The mixture was incubated at 42 °C for 4 h. Then, digestion procedure was applied using 0.5 µl of DNAse I (GeneAll® Biotechnology, Seoul, South Korea) and incubating at 37 °C for 30 minutes.

2.4 RNA Precipitation

IVT product was precipitated using the following protocol: 0.143M Sodium Acetate added into elute samples in the rate of 1:10 (Sample:0.143M $C_2H_3NaO_2$). Solution was vortexed and 100% Isopropanol (ration of 1:1) was added. After vortexing, solution was frozen at -80 ^oC for overnight. Then, the samples were centrifuged at 13,000 g at 4^oC for precipitation. Then, formed pellet washed with 70% ethanol. Elution was completed via resuspending frozen samples by 50 µl of RNase free water.

2.5 SELEX

At the start of the SELEX, 5 µM of 2'FY-RNA was refolded in 1X PBS as refolding solution (0.137 M NaCl, 0.0027 M KCl, 0.01 M Na2HPO4, 0.0018 M KH2PO4) by heating to 95 °C for two minutes and cooled for an hour at room temperature. Then, the pool was incubated with $2.5x10^6$ of virus particle stored in DMEM without FBS for 15 minutes in an Eppendorf tube at room temperature. Incubation solution mixture (including 2'FY-RNA with virus particles) was put on nitrocellulose membrane pre-wetted with PBS and vacuumed. During vacuum step, nitrocellulose membrane washed with 15 mL 1X PBS to remove unbound oligos. The bound 2'FY-RNA sequences were eluted via 1M Urea by heating it to 55 ^oC for 15 minutes. Eluted oligos was precipitated following RNA precipitation protocol.

2.5.1 Reverse Transcription PCR (RT-PCR) and In vitro Transcription (IVT)

Precipitate was resuspended by 80 µl of RNase free water. Then, eluted 2'FY-RNAs amplified by reverse transcription - polymerase chain reaction (RT-PCR) (Bachman 2013). In reverse transcription step, the T7 polymerase sequence was annealed to 2'FY-RNA sequence by heating at 65 ^{o}C for 5 minutes and cooling at 4 ^{o}C for 5 minutes. Following this, reverse transcription reaction was started (heating samples to 65 ^{o}C for 1 hour, heating 85 ^{o}C for 5 min) using a thermocycler. Then, RT samples were amplified by PCR (Denaturation: at 93^{o}C for 3 min; Annealing: 93^{o}C for 30 sec., 65 ^{o}C for 1 min, 72^{o}C for 1 min.; Extension: 72 ^{o}C for 10 min) and products were separated on 1% agarose gel in 1X TAE Buffer (Appendix B). PCR products were cleaned up via PCR Purification kit (QIAGen, Germantown, MD, USA) and in vitro transcription (IVT) was performed using Apt-Get 2'F T7 Transcription Kit (Roboklon GmbH, Berlin, Germany) incubating solution at 42 ^{o}C for overnight (Appendix A).

For every round of SELEX, amount of 2'FY-RNA and virus particle were decreased about 20% to increase the selection stringency on oligos. Also, PCR cycles were optimized between 8-13 depending on the PCR products observed on the agarose gels. At fourth round, a counter selection was performed to get rid of membrane binding oligos. In total, 7 rounds were applied during the SELEX as shown in Table 2.1.

2.6 Competent Cell Preparation

E. coli BL21 cell line was inoculated to 3 mL LB at 37 ^{o}C for overnight. Then, inoculated cells were seeded to 200 mL LB until the OD600 reaches to 0.4-0.6. After OD reaches to the desired level, the culture was incubated on ice for 15 minutes. Then, cells were centrifuged at 3500 rpm for 5 minutes at 4^{o}C. Supernatant was

discarded and pellet was resuspended with 20 mL ice-cold Buffer1 (100 mM RuCl, 30mM CH_3CO_2K, 10 mM CaCl2 10, 15% Glycerol, pH is adjusted to 5.8 with dilute acetic acid and filter sterilized). Resuspended cells were incubated on ice for 15 minutes and the same centrifugation step was repeated. After second

Table 2.1 SELEX Conditions

Round	Oligo Pool (µM)	Target (particle x 10^7)	Incubation Time (min)	PCR Cycles
1	5	2.50	15	12
2	4.13	2.06	15	11
3	3.40	1.70	10	11
4	5.00	Counter SELEX	10	-
5	2.81	1.40	10	10
6	2.32	1.16	5	10
7	1.91	0.96	5	11

centrifugation, pellet was gently resuspended in 8 mL of ice-cold Buffer2 (75mM CaCl2, 10mM RuCl, 10mM MOPS, 15% Glycerol, pH is adjusted to 6.5 with 0.2 M KOH and filter sterilized). Then, 100 µL aliquots were incubated on ice for 30 minutes. After this incubation, aliquots were frozen on dry ice and stored at -80 ^oC for further use (Hanahan 1983).

2.7 PCR Cloning

For PCR cloning, CloneJET PCR Cloning Kit (Thermo Scientific; #K1232) was used following the protocol supplied with the kit. 7^{th} round PCR product of SELEX was used as PCR product in the blunting reaction set up (Table 2.2) The mixture was incubated at 70 ^oC for 5 min, and then the samples was chilled on ice. Ligation reaction including pJET1.2/blunt Cloning Vector (50 ng/µL) with ampicillin

resistance gene was set up and combined with the blunting reaction mixture (Table 2.2). The ligation reaction mixture was incubated at 22 ^{o}C for 5 minutes, and it was ready to perform transformation.

Table 2.2 Blunting and Ligation Reaction Ingredients

Blunting Reaction Components	**Volume (µL)**
2X Reaction Buffer	10
7th Pool of SELEX	1
Blunting Enzyme	1
dH2O	6
Total Volume	18
Ligation Reaction Components	**Volume (µL)**
Blunting Reaction Product from above	18
pJET1.2/blunt Vector (50 ng/µL)	1
T4 DNA Ligase	1
Total Volume	20

2.8 Transformation

For transformation, *E. coli* BL21 cells were used. 120 µl of competent cells were thawed on ice. Then, 20 µl of ligation reaction was gently added and the tube was tapped for mixing. After incubation on ice for 40 minutes, cells were incubated at 42 ^{o}C for 45 seconds and immediately placed on ice for 10 minutes of incubation. After the heat shock, 880 µl sterile LB was added into the mixture and incubated at 37 ^{o}C in 210 rpm shaker for 1.5 hours (Hanahan 1983). Finally, 250 µl of incubated cells were inoculated on LB Agar plates containing 100 µg/mL ampicillin (in total, 4 plate) via spread plate method.

2.9 Plasmid Isolation

120 clones were picked for plasmid isolation, which was done using Miniprep Plasmid isolation kit (Machery-Negel Company, Catalog#740588.50) protocol and their concentrations were determined by BioDrop. After isolation, plasmids were directly used for Sanger sequencing (Sanger, Nicklen, and Coulson 1977).

2.10 Sanger Sequencing

Sanger sequencing (Sanger et al. 1977) was performed by INTERGEN company using reverse primer 5'-AAGAACATCGATTTTCCATGGCAG-3'.

2.11 Family Analysis and Secondary Structure Prediction of ProtoAatamers

Proto-aptamer sequences were aligned according to their full sequences for further analysis. The sequences were aligned using CLUSTAL 0(1.2.4) multiple sequence alignment (Sievers et al. 2011) and the phylogenetic tree constructed using ClustalW2 (Larkin et al. 2007). After prediction of 2-D structure of oligos using RNAfold webserver (Hofacker 2003) without changing default parameters, nine of the oligos were selected depending on their structures and sequence similarities and their templates were synthesized by IDT.

2.12 Aptamer Screening Assay

Synthesized oligos were annealed to form dsDNAs and then converted to 2'FY RNA via IVT reaction. IVT products were precipitated following RNA precipitation protocol.

Then, following SELEX protocol, 0.5 μM of RNAs were incubated with $1x10^5$ of virus particles stored in DMEM without FBS for 10 minutes in an Eppendorf tube at

room temperature. After collecting bound oligos, RT-PCR protocol with 13 PCR cycle was applied. PCR products were separated on 2% agarose gel in 1X TAE Buffer.

2.13 SARS-CoV-2 Binding Assay

After determination of better binding oligos, same protocol was followed to observe virus binding. The oligo was incubated with i) DMEM without virus (named as T_0); ii) DMEM with $2x10^4$ number of virus (named as T_2); iii DMEM with 10^5 number of virus (named as T_{10}). Same SELEX and RT-PCR protocols were followed (see section 12). PCR products were separated on 2% agarose gel in 1X TAE Buffer.

2.14 Truncation of Aptamer Candidates and SARS-CoV-2 Binding Assay

Aptamer candidate (Oligo3879) having two potential virus binding loops truncated as Oligo3899 and Oligo38901 using data from RNAFold web server (Hofacker 2003).

Truncated aptamers and DNA aptamer CoV2-RBD-4C were tested by following the same protocol above (see Section 13) including groups of T_0, T_2 and T_{10}.

2.15 3-D Structure Prediction and Docking

Using data from RNAFold web server (Hofacker 2003), 3-D structure prediction of truncated aptamers were created using RNAComposer web server (Biesiada et al. 2016).

Docking was applied using HDOCK (Yan et al. 2017) and Rosetta web servers (Lyskov et al. 2013). SARS-CoV-2 S protein structure was used as aptamer target (RCSB PDB data bank, ID: 6VSB). Aptamer – target binding interfaces were

generated using PyMol (DeLano n.d.) by selecting “3Å and close interaction” (Seeliger and De Groot 2010). Server provide 100 different results having different energies. The results with the lowest energies are used.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Selection of 2'FY RNA Aptamer Against SARS-CoV-2 Particle

3.1.1 Design of Oligonucletide Library and Primers

ssDNA library having constant primer binding regions and a central region with random sequences in the middle was used to construct a 2'Fluoropyrimidine (FY)-RNA pool (Shubham et al. 2018). Design of the pool is one of the critical steps to successfully select aptamers against the target of interest. Because PCR plays a key role in aptamer selection, the design of the primers is crucial for the initial library. Number of bases in forward and reverse primers should be in the range of 18-30 with melting temperatures (Tm) between 55°C to 70°C and within 5°C of each other (wu et al. 1991). Shorter primers anneal their targets more effectively than longer ones. The annealing temperature during PCR should be low enough to allow primers to bind to the template, but not so low to prevent formation of intramolecular hairpins and non-specific duplexes that may reduce reaction efficiency (Rychlik, Spencer, and Rhoads 1990). Thus, Tm between 55°C to 75°C was found ideal (Kämpke, Kieninger, and Mecklenburg 2001). Another consideration is the G and C composition as these bases construct strong hydrogen bond between each other and increase stability of the primer for higher yield of the PCR. However, repeating G or C bases can result in the primer-dimer formation. Thus, GC content of the primer should be in the range of 40-60%, and repeated G or C bases should be avoided (Benita 2003). In addition, balanced distribution of GC-rich and AT- rich domains is required to prevent self-dimers/hairpins or primer-dimers formations and increase efficiency of PCR (Arif and Ochoa-Corona 2013; Kumar and Kaur 2014). Thus, the

library used to synthesize 2'FY-RNA pool for aptamer selection against SARS-CoV-2 and primers were designed considering the facts explained above.

3.1.2 2'FY RNA Pool Synthesis

According to *in vitro* studies, half-life of the RNA aptamers in plasma is few seconds, in contrast to DNA aptamers having 30 to 60 min half-life (White, Sullenger, and Rusconi 2000). Having 2'-hydroxyl group (2'-OH), RNA molecules become chemically unstable and are susceptible to hydrolysis, especially in alkaline solutions, which results in shorter half-life. Moreover, the hydroxyls are targeted by prevalent endoribonucleases such as RNase-A that plays role in catalysis of RNA. Thus, RNA aptamers are generally modified at the 2'-position of pyrimidines (Patra et al. 2012). These modifications can be either applied during library design or after SELEX to increase stability. Modified RNA aptamers can equal or surpass the stability of DNA aptamers. Modifying aptamers after SELEX is costly and may result in affinity changes (Lakhin et al. 2013). Therefore, in this study, we generated the initial pool as 2'FY-RNA from dsDNA pool to select aptamers without having stability problems in their final applications.

3.1.3 SELEX

Aptamer selection was carried out using chemically inactivated SARS-CoV-2 particle in this study. The aim of using whole virus particles was selecting aptamers against glycoproteins of virus in their natural forms, which increase the success rate of aptamer use both *in vivo* and *in vitro* (Ohuchi 2012). Another criterion to increase the success rate is the content of selection buffer. As mentioned above, selection buffer directly affects aptamer structure, and hence, its affinity (Ilgu and Nilsen-Hamilton 2016). We designed the selection buffer similar to human serum considering the potential use in human body for therapeutic or in human serum out of the body for diagnostic purposes.

Unique 3D structures of aptamers provide higher affinity and specificity to them. Temperature is an important factor which affects aptamer folding into specific structures (Ilgu and Nilsen-Hamilton 2016). Thus, incubation temperature was arranged as room temperature considering further use of the aptamers *in vitro*.

Initially, 5 nmol 2'FY-RNA was incubated with $2.5x10^5$ of chemically inactivated SARS-CoV-2 particles (Figure 3.1). Then, every round of incubation, oligo and virus particle amounts, and incubation time were decreased gradually to increase to selection pressure for the success of the best binders. Table 2.1 summarizes the parameter changes.

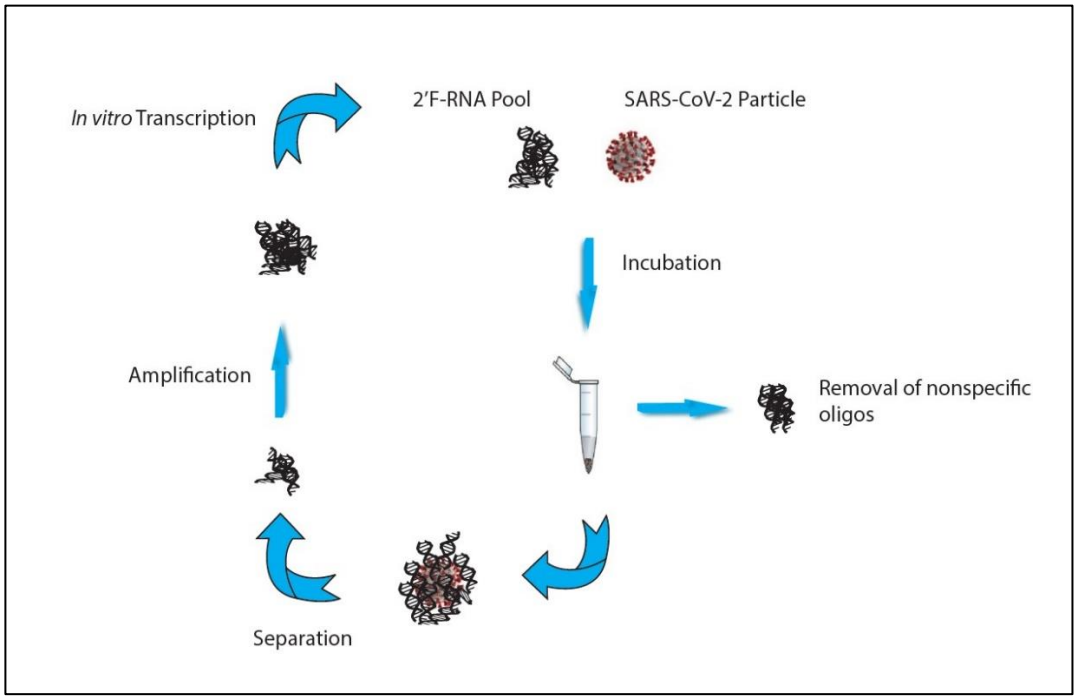


Figure 3.1. Schematic Organization of SELEX.

Another important parameter of SELEX is the numbers of PCR cycles. Optimization of PCR cycle in every SELEX round is performed to prevent formation the longer and shorter sequences and higher amplification of non-specific binders that

potentially decrease efficiency of PCR and SELEX (Musheev and Krylov 2006). Accordingly, we determined amplification cycles for every SELEX round applying PCR to only a small portion of the collected bound oligos and evaluating the result with agarose gels. When we reached desired cycle number, we applied the PCR for the rest of the sample which include reverse transcription product. Table 2.1 includes PCR cycle numbers and Figure 3.1 shows PCR results from every round of SELEX.

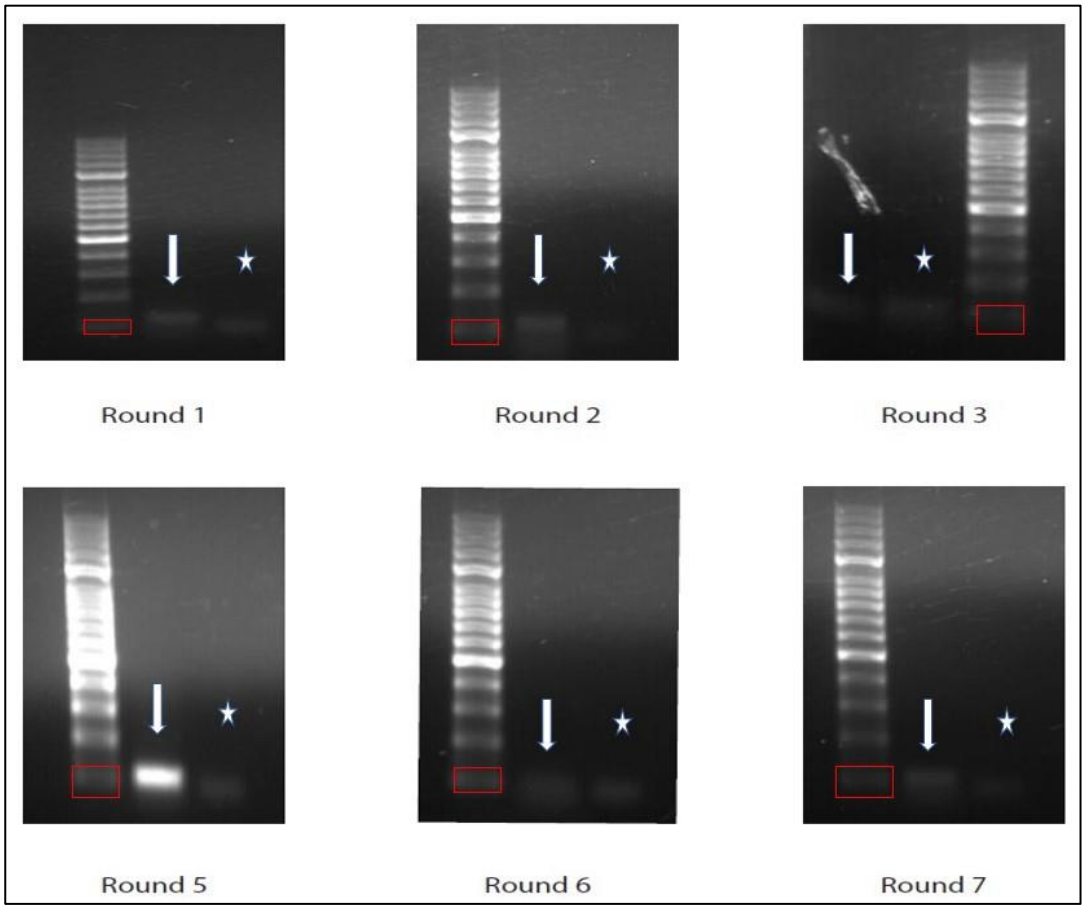


Figure 3.2. PCR Results of SELEX Rounds. While arrows indicate band of PCR results, asterisk indicates control band which is produced PCR of 117 nucleotide long sample.

3.2 Sequence Analysis

After SELEX is completed, PCR products of last round (7^{th} round) was ligated into pJET1.2/blunt Cloning Vector and plated using BL-21 cells onto four agar plates. Total of 134 colonies were picked from these plates randomly and homogenously. Plasmids were isolated from bacteria picked from these plates and sent for Sanger sequencing. Although next generation sequencing would have given better results, we preferred Sanger sequencing because of having less cost and giving fast results.

Despite the pros of Sanger sequencing technique, low efficiency in ligation and plasmid isolation can cause to missing or low signal that indicates wrong or missing sequences. Therefore, we obtained 84 of oligo 134 sequences after analyzing the signals and generated phylogenetic tree of sequences to analyze similarity of sequences using ClustalW OMEGA webserver (Larkin et al. 2007). The server provided a phylogenetic tree with three main branches based on sequence similarity (Figure 3.3). Then, we aligned some sequences from Branch-3 using CLUSTAL 0(1.2.4) (Sievers et al. 2011) multiple sequence alignment to check efficiency of the tree. Figure 3.4 indicates sequence similarities between oligos from Branch-3 and Figure 3.5 indicates their similarity rates. While oligos from same sub-branch shows higher similarity, oligos from different branch shows lower similarity as expected.

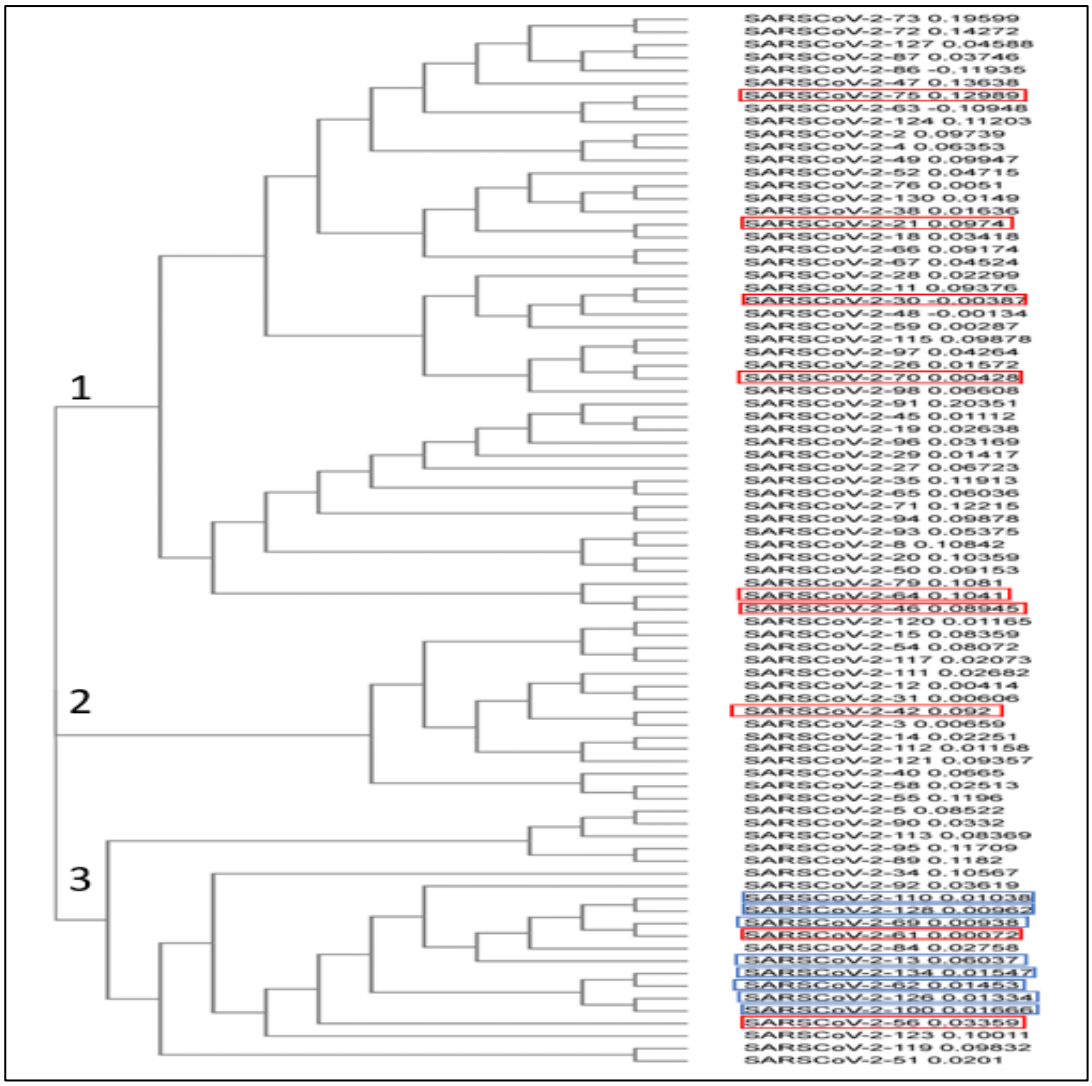
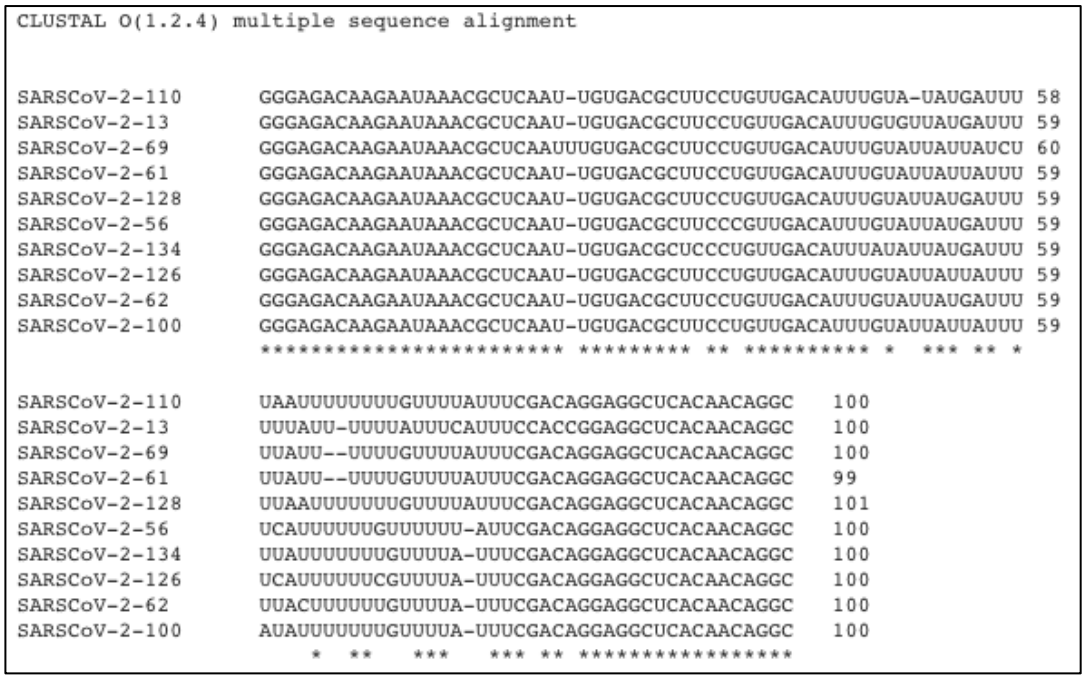


Figure 3.3. Phylogenetic Tree of Oligo Pool. Red squares indicate that oligos selected to tests; blue squares indicate that oligos selected to check similarity scores.



```
CLUSTAL O(1.2.4) multiple sequence alignment


SARSCoV-2-110       GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUA-UAUGAUUU 58
SARSCoV-2-13        GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUGUUAUGAUUU 59
SARSCoV-2-69        GGGAGACAAGAAUAAACGCUCAAUUUGUGACGCUUCCUGUUGACAUUUGUAUUAUUAUCU 60
SARSCoV-2-61        GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUAUUAUUAUUU 59
SARSCoV-2-128       GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUAUUAUGAUUU 59
SARSCoV-2-56        GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCCGUUGACAUUUGUAUUAUGAUUU 59
SARSCoV-2-134       GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUCCCUGUUGACAUUUAUAUUAUGAUUU 59
SARSCoV-2-126       GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUAUUAUUAUUU 59
SARSCoV-2-62        GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUAUUAUGAUUU 59
SARSCoV-2-100       GGGAGACAAGAAUAAACGCUCAAU-UGUGACGCUUCCUGUUGACAUUUGUAUUAUUAUUU 59
                    ************************ ********* ** ********** *  *** ** *


SARSCoV-2-110       UAAUUUUUUUUGUUUUAUUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-13        UUUAUU-UUUUAUUUCAUUUCCACCGGAGGCUCACAACAGGC   100
SARSCoV-2-69        UUAUU--UUUUGUUUUAUUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-61        UUAUU--UUUUGUUUUAUUUCGACAGGAGGCUCACAACAGGC   99
SARSCoV-2-128       UUAAUUUUUUUGUUUUAUUUCGACAGGAGGCUCACAACAGGC   101
SARSCoV-2-56        UCAUUUUUUGUUUUUU-AUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-134       UUAUUUUUUUGUUUUA-UUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-126       UCAUUUUUUCGUUUUA-UUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-62        UUACUUUUUUGUUUUA-UUUCGACAGGAGGCUCACAACAGGC   100
SARSCoV-2-100       AUAUUUUUUUGUUUUA-UUUCGACAGGAGGCUCACAACAGGC   100
                        *  **   ***   *** ** ******************
```

Figure 3.4. Multiple Sequence Alignment Results of Selected Oligos from Branch-3.

	CoV-2-110	CoV-2-13	CoV-2-69	CoV-2-61	CoV-2-128	CoV-2-56	CoV-2-134	CoV-2-126	CoV-2-62	CoV-2-100
SARSCoV-2-110	100	91.92	96.94	97.96	98	94.95	93.94	93.94	94.95	93.94
SARSCoV-2-13	91.92	100	90.91	91.92	94	88.89	89.9	88.89	91.92	89.9
SARSCoV-2-69	96.94	90.91	100	98.99	96.97	92.86	92.86	93.88	93.88	94.9
SARSCoV-2-61	97.96	91.92	98.99	100	97.98	93.88	93.88	94.9	94.9	95.92
SARSCoV-2-128	98	94	96.97	97.98	100	94	94	93	96	94
SARSCoV-2-56	94.95	88.89	92.86	93.88	94	100	92	94	93	92
SARSCoV-2-134	93.94	89.9	92.86	93.88	94	92	100	95	97	96
SARSCoV-2-126	93.94	88.89	93.88	94	93	94	95	100	96	97
SARSCoV-2-62	94.95	91.92	93.88	94.9	96	93	97	96	100	97
SARSCoV-2-100	93.94	89.9	94.9	95.92	94	92	96	97	97	100

Figure 3.5. Similarity Rates of Selected Oligos from Branch-3.

3.2.1 Secondary Structure Prediction

9 proto aptamers from three main branches are selected and the number of oligos selected from a particular branch was based on its size. Then, we obtained 2D structures of these oligos using RNAfold web server (Hofacker 2003). Simply, 2D structure of an oligo is determined by sequence of bases and their interactions between each other (Figure 3.6). Thus, as expected, while oligos from the same or close branches exhibit similar structures (e.g. Oligo3879/SARS-CoV-2-21 vs Oligo3887/SARS-CoV-2-75), oligos from different branches exhibit very different structures (e.g. Oligo3881/SARS-CoV-2-42).

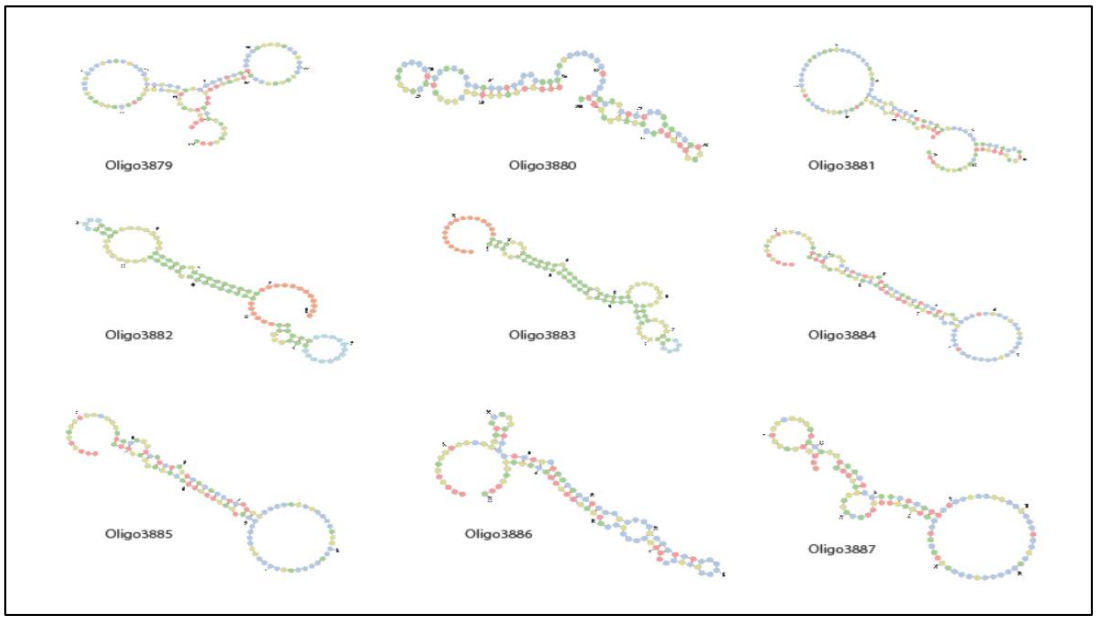


Figure 3.6. 2D Structure of Oligos Selected for Testing.

3.3 Characterization of Aptamer Candidates

3.3.1 Determination of Best Binders by SARS-CoV-2 Binding Assay

To check whether differences between binding affinity and specificity of oligos chosen from different branches and having different nucleotide sequences, we tested these nine oligos using diluted (1:10) virus particle. Results show that oligos 3880, 3885, 3886, 3887 and 3879 binds to the virus properly and efficiently (Figure 3.7).

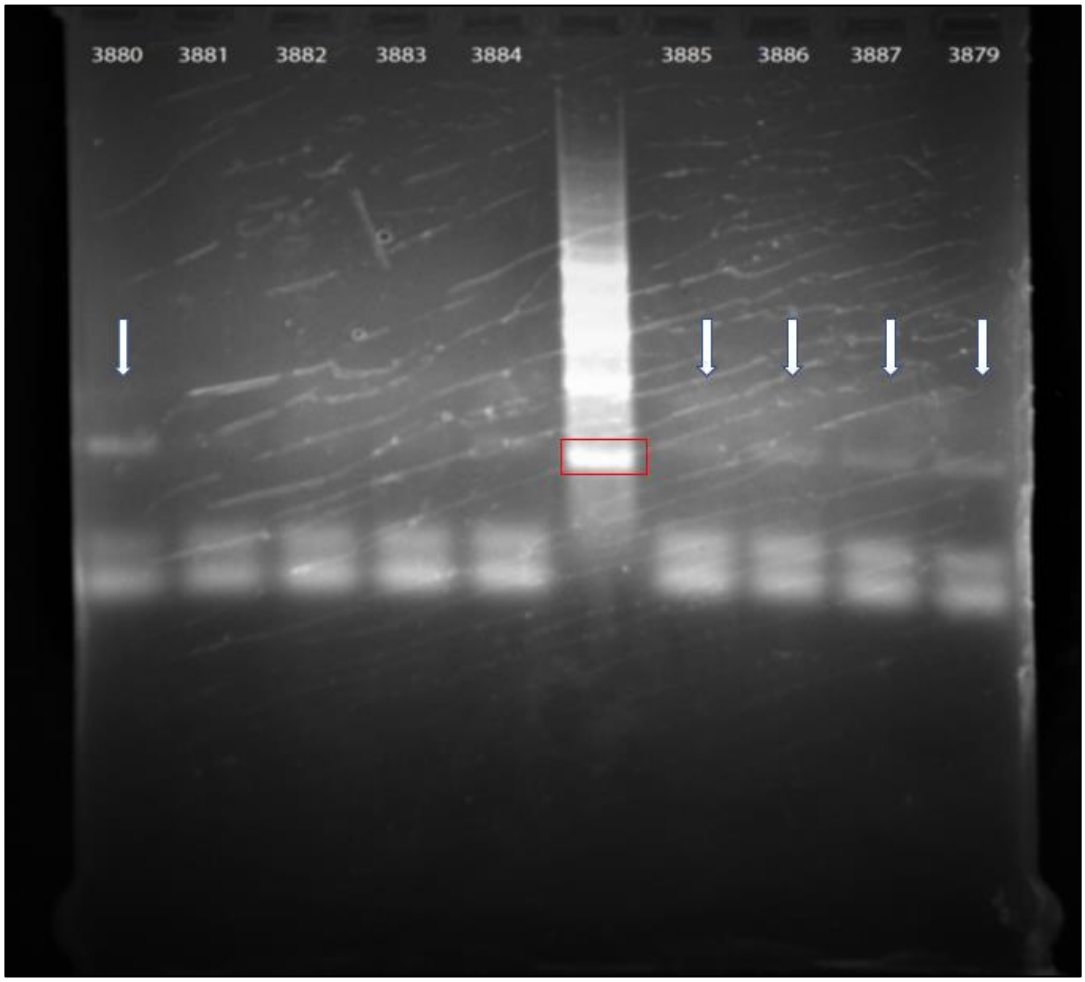


Figure 3.7. PCR Results of Oligos Selected for Testing. Red square indicates 100 nucleotide band of the ladder.

However, although we applied counter selection using only membrane to get rid of membrane binders, some oligos binding to membrane could be selected. Therefore, we arrange another binding assay to determine whether there are still membrane binding aptamer candidates via diluting the virus 0:10 (incubation without virus), 1:10 and 1:50. Results of this assay (Figure 3.8) shows that Oligo3879 and Oligo3887 exhibit specific binding to SARS-CoV-2 particle, and Oligo3879 exhibits best binding with ability to bind the virus diluted to rate of 1:50 (5.000 number of virus particle). Also, we observed that we have still aptamer candidates that can bind nitrocellulose membrane.

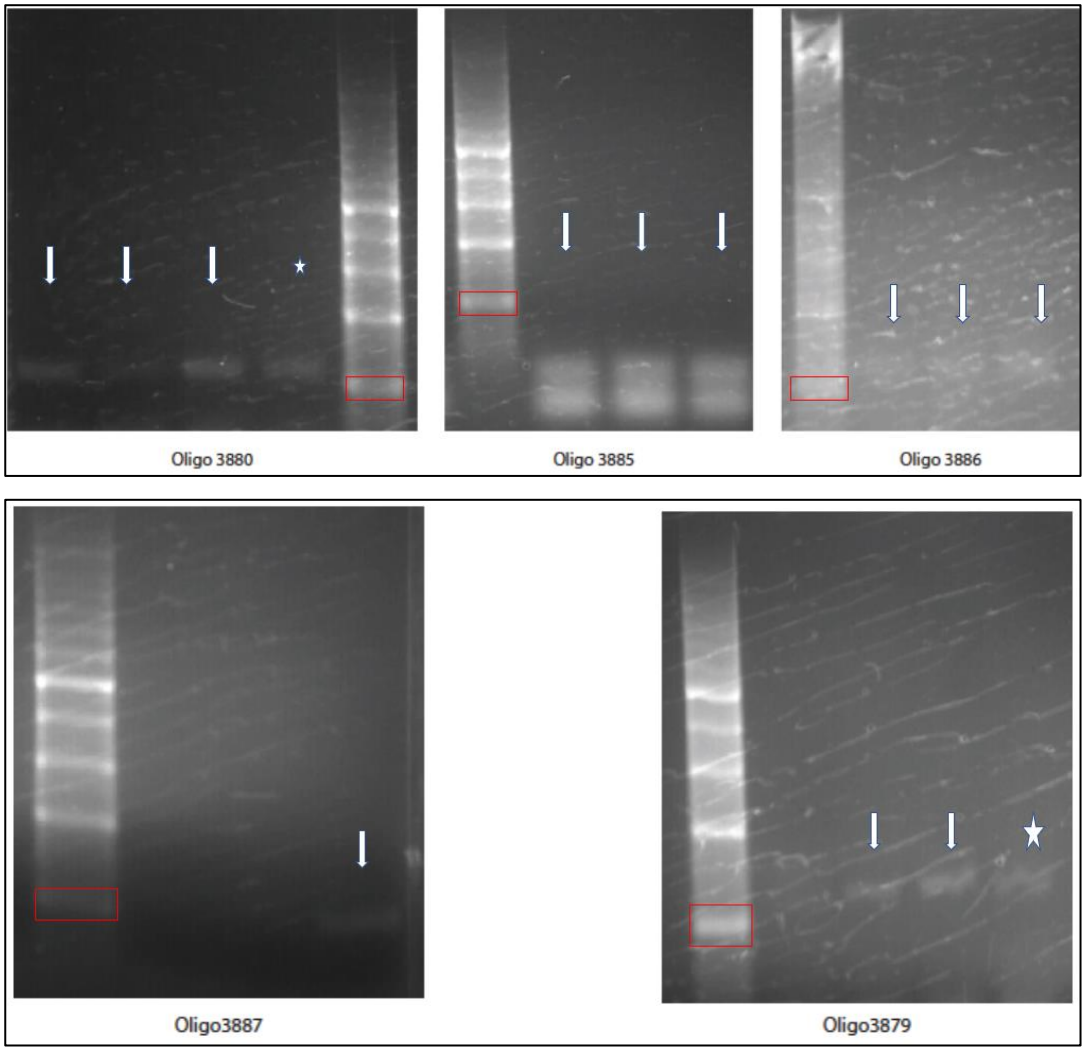


Figure 3.8. PCR Results of T_0, T_2 and T_{10} Tests of Aptamer Candidates.

In theory, we expected that oligos exhibit similar structure should show similar binding pattern. The results were convenient with this theory; structure of the best binder aptamers have similar structure (Figure 3.9).

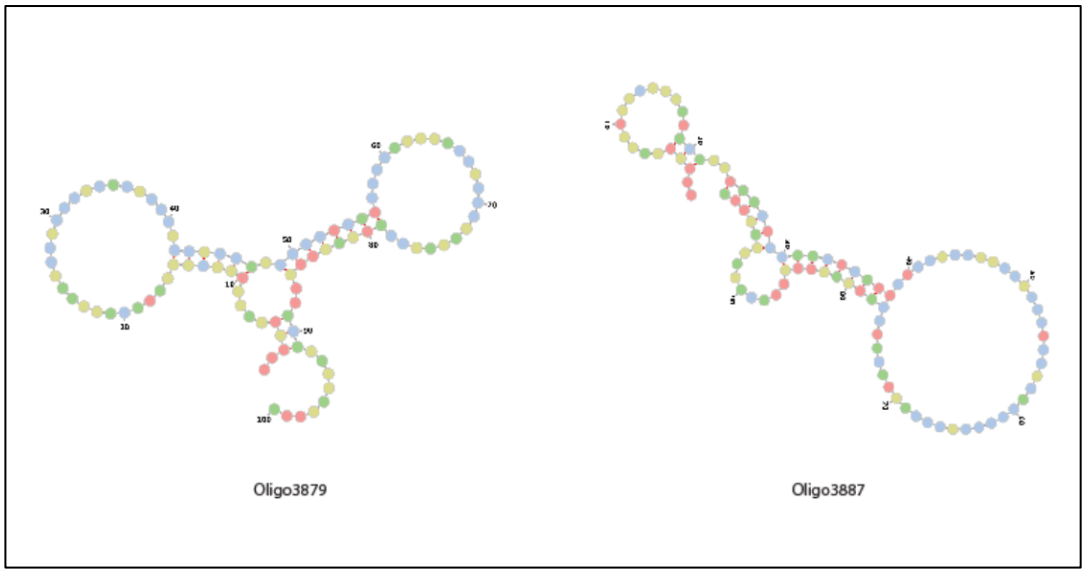


Figure 3.9. 2D Structure of Oligo3879 and 3887.

3.3.2 Truncation of Oligo3879

One of the most common optimizations of aptamers is the reduction of their size. This procedure may increase affinity and specificity of aptamer, besides produces shorter oligos which are synthesized easily and cheaper (Rockey et al. 2011). Also, determination of possible binding sites of aptamer sheds light on aptamer-target interaction. Considering all of these, aptamer truncation is essential step of any aptamer work (Adachi and Nakamura 2019).

Stem loops (hairpin loops) one of the common structures into which aptamers fold. These loops and nucleotides on the loops are critical structures for target recognition and binding (Feigon, Dieckmann, and Smith 1996). According to 2-D prediction results, our best binding aptamer, Oligo3879, has two different stem loop structure which we are expected to be virus binding site. Thus, we truncated this aptamer as

38SARS1A (right loop) and 42SARS2A (left loop) using results from RNA-Fold server.

3.3.3 SARS-CoV-2 Binding Tests for Truncated Candidates

To check whether truncation effects binding capability of the aptamer, we applied virus binding tests as applied earlier. Results indicated that both loops were better binding than full-length aptamer. Compared to 42SARS2A, 38SARS1A was found not to bind nitrocellulose membrane (Figure 3.10). That is, truncation of Oligo3879 increased binding affinity of the proto aptamer. As mentioned before, aptamers fold into unique 3D structures and these structures determined by base sequences and their interactions. As reported earlier (Adachi and Nakamura 2019; Rockey et al. 2011), presence of other sequences in addition to target binding region may affect the binding affinity and specificity of the aptamer. Thus, truncation may give better binding ability to the aptamer.

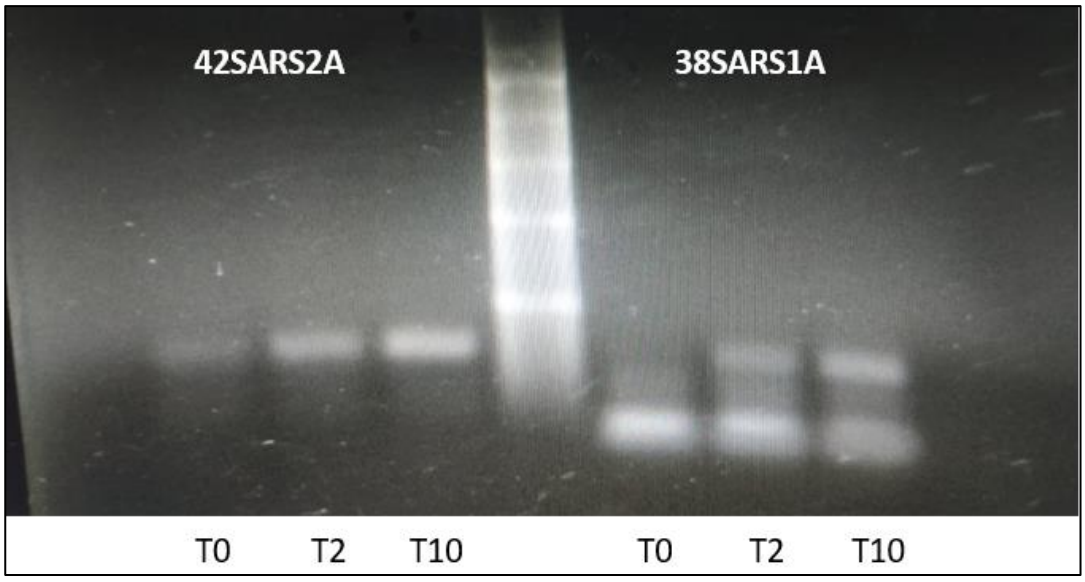


Figure 3.10. Binding Results of Truncated Aptamers. Lower bands are primer dimers.

We also compared these two aptamers with a previously established DNA aptamer, CoV2-RBD-4C, developed against SARS-CoV-2 receptor binding domain (Song et al. 2020). As mentioned before, SARS-CoV-2 has envelope covered with Spike protein. Thus, we estimated that our generated aptamers bind to the Spike protein. According to the results, we can clearly say that the aptamers, 38SARS1A and 42SARS2A, obtained in our studies bind to virus as good as CoV2-RBD-4C (Figure 3.11).

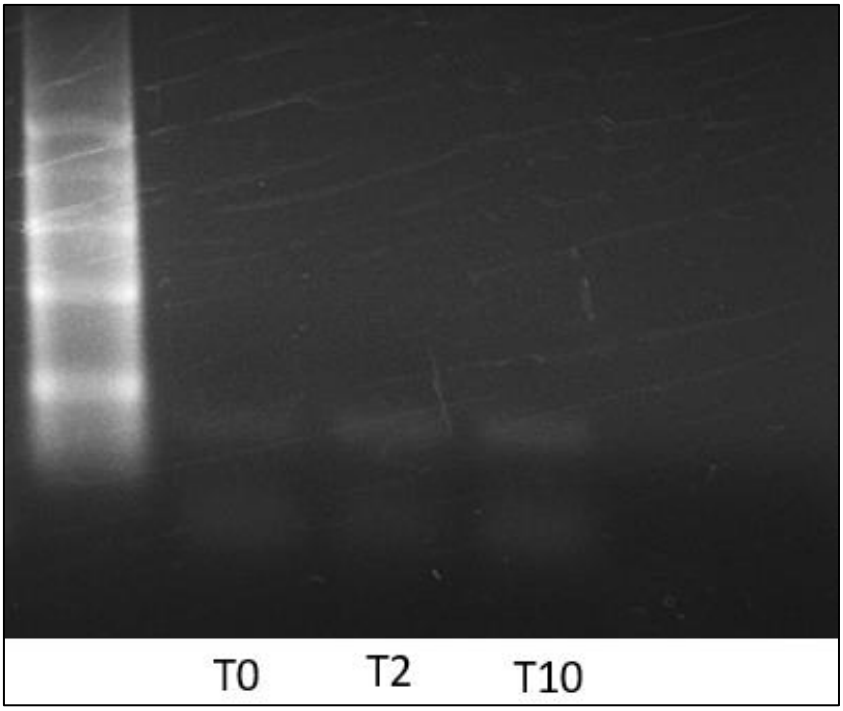


Figure 3.11. Binding Results of CoV2-RBD-4C.

3.3.4 Docking of Truncated Aptamers to Spike Protein of SARS-CoV-2

As previously mentioned, on its envelope SARS-CoV-2 has spike proteins that have ACE2 binding domain. Aptamer named CoV2-RBD-4C (Song et al. 2020) was selected against this protein and according to the docking results, this aptamer binds to a site called receptor binding domain (RBD) located very close to domain's ACE2

binding site. Considering this, we docked both of our aptamers to spike protein of SARS-CoV-2 to better understand aptamer-virus interaction.

Docking results showed that 38SARS1A binds far away from RBD whereas 42SARS2A has potential RBD binding interface (Figure 3.12).

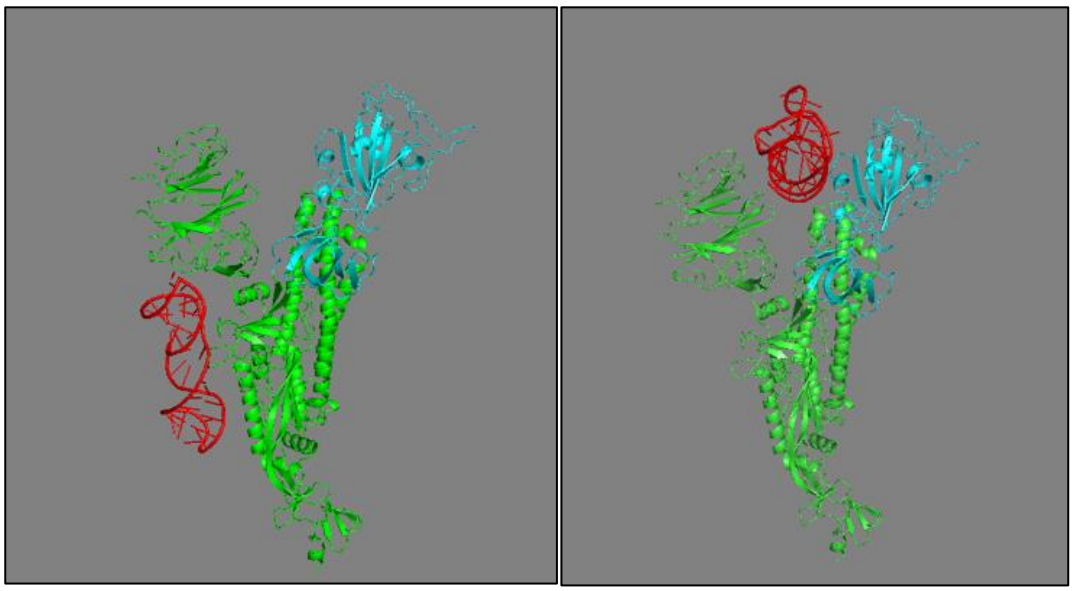


Figure 3.12. Docking Results of 38SARS1A and 42SARS2A, respectively. Aptamers are indicated as red color; RBD of S-Protein as blue.

As reported, RBD sequence of S protein lies between the residues 331 and 524 (Tai et al. 2020). Especially, ACE2-RBD binding occurs through Gln498, Thr500, Asn501, Lys417, Tyr453, Gln474 and Phe486 residues (Ali and Vijayan 2020). On the other hand, 42SARS2A binds RBD via Ala372, Ser371, Asn370, Val367 and Ser366. According to these results, 42SARS2A interacts with RBD of S protein but we cannot suggest that it directly binds to ACE2 binding site. (Figure 3.13). However, binding to these residues may change RBD structure and may affect ACE2 binding capability of the spike protein. Also, spike protein is present on the virus envelope as a trimer. Song et al (Song et al. 2020) applied docking using monomer spike protein and we followed the same protocol to compare their findings with ours.

It is possible that trimer version of protein poses different chemistry and structure, which may affect aptamer binding. Therefore, our results might need to be confirmed via wet lab experiments.

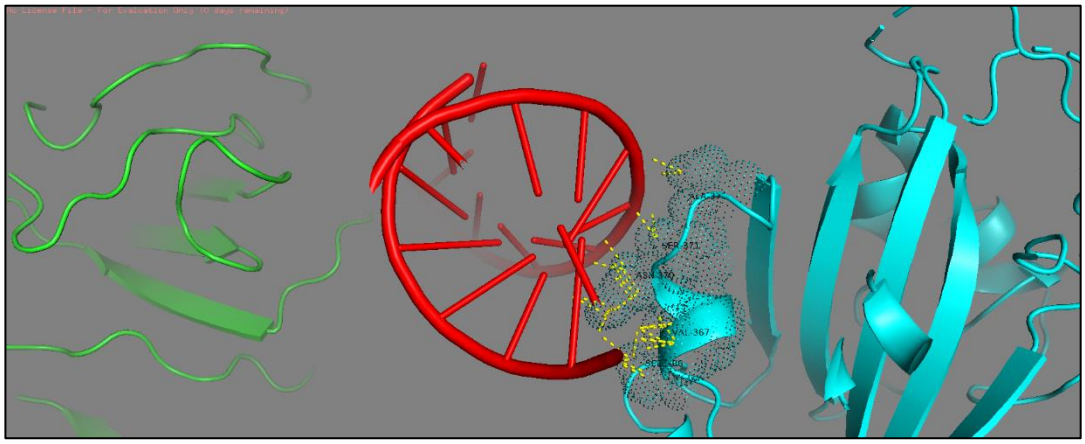


Figure 3.13. Potential Interactions of 42SARS2A and RBD of S-Protein. Aptamer is indicated as red color; RBD of S-Protein as blue color.

CHAPTER 4

CONCLUSION AND FUTURE PRESPECTIVE

With their current use and high potential, aptamers are popular biomolecules for diagnostic and therapeutic approaches. Although therapuetic applications are not much common, higher affinity and specificity makes them good biorecognition elements.

In this thesis, we were able to generate two different aptamers, 38SARS1A and 42SARS2A. Comparing aptamer CoV2-RBD-4C, 38SARS1A and 42SARS2A serve relatively better binding to SARS-CoV-2 particles. Use of natural conformation of virus particle in SELEX procedure might be the reason of this difference. As mentioned before, natural conformations of the target increase the chance of selecting aptamers efficiently.

Moreover, docking results indicate that, besides being used in a diagnostic tool, 42SARS2A may be used for therapeutic purposes because it potentially binds RBD of SARS-CoV-2 spike protein. On the other hand, our second aptamer 38SARS1A binds to far away from RBD. Unlike 42SARS2A binding site, binding site of 38SARS1A is not on the S-Protein/receptor interface. Thus, it is likely that 38SARS1A would be better for diagnostic agent.

Although selection procedure seems successful, binding efficiency should be re-checked using methods, such as ITC, which gives numerical affinity values. Moreover, wet lab experiments should be applied to observe whether 38SARS1A and 42SARS2A can inhibit spike protein/ACE2 activity.

Considering all of these results and future works, we can argue that our generated aptamers have potentials to be used in diagnostic kits and one or both of them may have a potential to be therapeutic agent/s.

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APPENDICES

A. In vitro Transcription Reaction

Reaction Component	Amount (µL)
5X Reaction Buffer	5
2'F-Py NTP Mix (25 mM)	1.5
DNA Template	1 µg
Apt-Get 2'F-T7 RNA Polymerase	0.5
dH_2O	Fill up to 25 µL
Total Volume (for each tube)	25 µL

B. Polymerase Chain Reaction (PCR)

Component	**Volume (µL)**
RT Product	20
Forward Primer- Oligo 484 (100 µM)	0.5
Reverse Primer- Oligo 485 (200 µM)	0.5
Wizpure™ 2X PCR Mix	21
Total Volume (each tube)	42
PCR Reaction	**Duration**
Initial Denaturation (93 degree C)	5 minutes
Denaturation (93 degree C)	30 seconds
Annealing (65 degree C)	1 minutes
Extension (72 degree C)	1 minutes
Final Extension (72 degree C)	10 minutes
Stop Reaction (4 degree C)	5 minutes - endless