DEVELOPING A LAMP PCR BASED DIAGNOSTIC TEST FOR CRIMEAN CONGO HEMORRHAGIC FEVER VIRUS

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ABSTRACT

DEVELOPING A LAMP PCR BASED DIAGNOSTIC TEST FOR CRIMEAN CONGO HEMORRHAGIC FEVER VIRUS

Crimean-Congo hemorrhagic fever infection is one of the most common tick-borne viral infections, non-infectious in animals and fatal to humans with a mortality rate around 40%. The high mortality rate and lack of vaccines or drug treatment point to the potential danger of infection. The aim of this thesis was to detect 21 complete S-segment Crimean-Congo hemorrhagic fever virus strains originating in Turkey using Fluorimetric Loop Mediated Isothermal Amplification (LAMP) method, which is one of the PCR-based isothermal amplification methods for detecting viral infections. Conserved regions of the 21 strains of the CCHFV observed in Turkey were determined by sequence alignment and LAMP primers for these conserved regions were designed. DNA of CCHFV Ank-2 (GeneBank accession number: MK309333) strain was used and optimum LAMP reaction conditions were determined by changing the temperature, primer amount and $MgSO_4$ concentration. LAMP results were compared with qPCR, which is considered the gold standard. The detection limit for LAMP and qPCR was $2x10^6$ copies/µl. In the study, the CCHFV LAMP primer set-1 gave similar results to the qPCR primer set. The CCHFV LAMP primer set-4 performed better by turning positive 9 minutes earlier than the qPCR primer set. This results indicate LAMP is an alternative method for detecting CCHFV but reveals the necessity of improving the sensitivity of the test.

ÖZET

KIRIM KONGO KANAMALI ATEŞİ VİRÜSÜNÜN LAMP POLİMERAZ ZİNCİR REAKSİYONU BAZLI TANI TESTİNİN GELİŞTİRİLMESİ

Kırım-Kongo Kanamalı Ateşi (KKKA) enfeksiyonu kene kaynaklı en yaygın viral enfeksiyonlardan biridir. Enfeksiyonun %10-%40 ölüm oranına sahip olması ve onaylanmış kesin bir tedavisinin olmaması, temas yoluyla bulaşması hastalığın tehlike boyutunu göstermektedir. Enfeksiyonun hızlı tespiti için PCR bazlı, bir saatten kısa sürede sonuç verebilen Florometrik Döngü Aracılı İzotermal Amplifikasyon (Florometrik-LAMP) yöntemi kullanıldı. Çalışmada, Türkiye'de görülmüş 21 bütün S segment KKKA virüsünün suşunun sekans dizilimi kullanılarak hizalama çalışması yapıldı ve korunaklı bölgelere uygun LAMP primerleri tasarlandı. LAMP yönteminin testi için KKKA virüsünün Ank-2 (GeneBank Accession number: MK309333) suş DNA'sı kullanıldı. LAMP primerleri için, sıcaklık, primer miktarı ve $MgSO_4$ konsantrasyonu değiştirilerek optimum koşullar belirlendi. Sonuçlar, altın standart yöntem olarak kabul edilen qPCR ile karşılaştırıldı. Çalışmada LAMP ve qPCR için saptama limiti $2x10^6$ kopya/µl olarak belirlendi. CCHFV LAMP primer seti-1 qPCR primer seti ile benzer sonuçlar verirken CCHFV LAMP primer seti-4 qPCR primer setinden daha erken sinyal vererek daha iyi performans gösterdi. Sonuçlar LAMP metodunun qPCR yöntemine alternatif olabileceğini desteklemekle birlikte testin hassasiyetinin geliştirilmesi gerekliliğini de ortaya koymuştur.

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LIST OF ABBREVIATIONS

CCHF: Crimean Congo Hemorrhagic Fever

CCHFV: Crimean Congo Hemorrhagic Fever Virus

LAMP: Loop Mediated Isothermal Amplification

PCR: Polymerase Chain Reaction

RT-PCR: Reverse transcriptase Polymerase Chain Reaction

RT-qPCR: Reverse transcriptase Quantitative Polymerase Chain Reaction

qPCR: Quantitative (Real-Time) Polymerase Chain Reaction

cDNA: Complementary Deoxyribonucleic Acid

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

Tm: Melting temperature

ΔG: Free Energy

µl: Micro liter

LOD: Limit of detection

kb: Kilo base pair

TAE: Tris Acetate EDTA

dNTP: Deoxyribonucleotide triphosphate

$MgSO_4$: Magnesium sulphate

min: Minutes

ELISA: Enzyme Linked Immunosorbent Assay

IgM: Immunoglobulin M

IgG: Immunoglobulin G

FIP: Forward inner primer

BIP: Backward inner primer

LF: Loop forward primer

LB: Loop backward primer

CHAPTER 1

INTRODUCTION

CCHF virus infection is one of the most common tick-borne viral infections. Although the death rate varies from region to region, it can reach 80% (Kuehnert et al. 2021). There is no vaccine or therapeutic drug developed against infection and approved by the World Health Organization (WHO). Although it is can be fatal in humans, it has no disease-causing effect in animals. Therefore, animals play an important role in the spread of infection. While international and regional animal trade supports the spread of the infection, the migration routes of birds are also changing due to climate change. Changing migration routes can carry the infection to much wider regions around the world. (Kuehnert et al. 2021; De Liberato et al. 2018; Moraga-Fernández et al. 2021). Generally, people living in rural areas and dealing with livestock, veterinarians, as well as health workers who deal with infected people are at risk. Today, although Africa, the Balkans, the Middle East, and Asia are considered endemic regions. There are cases reported in northern countries such as Austria, Germany, and Switzerland (Fanelli and Buonavoglia 2021). Therefore, rapid identification is needed to monitor CCHF infection, early diagnosis and prevention of possible outbreaks. In the study, it was aimed to develop a diagnostic test with specific primers targeting the S segment of CCHF virus using Loop-Mediated Isothermal Amplification (LAMP), which is one of the popular isothermal amplification methods, does not require expensive devices, can be performed in one step, and gives results in a short time.

1.1. History

Crimean Congo hemorrhagic fever is a tick-borne viral infection caused by the Crimean Congo hemorrhagic fever virus. So far, the infection has been seen in many regions and countries; Northern Europe, Australia, America, Asia, Middle East (Iran, Iraq, Afghanistan, Pakistan, United Arab Emirates, Kuwait, Oman, Saudi Arabia, China,

Tajikistan, Uzbekistan, Kazakhstan, India), Africa (South Africa, Egypt), Mauritania, Kenya, Sudan, Democratic Republic of Congo, Chad, Nigeria, Niger, Senegal, Uganda, Tanzania) and many countries in Southeast Europe (Albania, Bulgaria, Turkey, Greece, Georgia, Russia, Kosovo, Spain) (Dai et al. 2021; Shahhosseini et al. 2021).

It was first seen in the present-day Tajikistan region in the 12th century. Later, in 1944, it was recorded as Crimean hemorrhagic fever with the illness of the people in the Crimea region and the Russian soldiers. In 1945, it entered the literature as tick-borne viral hemorrhagic fever. In 1969, it got its name by matching the virus first isolated from a sample taken from a sick child in the Congo region and was accepted as the Crimean Congo hemorrhagic fever virus (Endy 2020).

1.1.1. Structure and Features of CCHF Virus

Crimean Congo hemorrhagic fever virus is a virus with negative sense RNA, belonging to the Nairovirus genus of the Bunyaviridae family. The virus genome has three segments: S (small), M (medium), and L (large). The S segment is about 1.6 kb long and is responsible for encoding the nucleoprotein, the M segment is about 5.3 kb long and is responsible for coding for glycoprotein, and the L segment is about 12.1 kb long and is responsible for coding for RNA-dependent RNA polymerase (Hawman and Feldmann 2018; Lukashev et al. 2016; Ozdarendeli et al. 2010).

The S segment is involved in the oligomerization of the nucleoprotein (NP), which is involved in encapsulating viral RNA and forming a helical structure (Bente et al. 2013). NP is also known as ribonucleoprotein. Genome replication, with viral polymerase, helps initiate mRNA transcription and is therefore a very important protein in the virus's life cycle (Moming et al. 2018; Shayan et al. 2015). The M segment plays a role in immunity and pathogenicity. It encodes GP38, GP160, GP85 helper glycoproteins as well as Gn and Gc glycoproteins. Glycoproteins reffect to virus formation and the ability of the virus to infect so the M segment often targeted in vaccine studies (Freitas et al. 2020; Shayan et al. 2015). In particular, the Gc protein is the primary binding protein of the virus and is thought to play a role in the entry of the virus into the cell (Erickson et al. 2007).

The L segment contains a single open reading frame (ORF) and its near the N-terminus is the ovarian tumor protease (OUT) domain. This area is thought to play a role in the formation of viral-RNA dependent RNA polymerase (RdRp), which is involved in viral genome transcription and replication (Tchesnokov et al. 2020; Zivcec et al. 2016). In addition, CCHFV's L segment is longer than the L segment of other viruses in bunyaviridae family supports the idea that it may be involved in coding other proteins. (Shayan et al. 2015).

When CCHFV strains are evaluated in terms of genetic diversity, the L, M and S segments differ by 22%, 31% and 20%, respectively (Serretiello et al. 2020; Shahhosseini et al. 2021). The reason for this diversification is thought to be the long evolutionary history of Hyalomma ticks, which is the main reservoir of CCHF virus (Bente et al. 2013).

1.1.2. Distribution of CCHF in The World and Turkey

CCHF is considered to be one of the most common tick-borne viral diseases and is one of the priority diseases monitored by the World Health Organization. Ticks of the genus Hyalomma, which are considered the main vector of the virus, use many wild and domestic animals as hosts. Rabbits, ground-feeding birds, sheep, goats, cattle are the main hosts of these ticks. The major effect in the spread of the infection is seen as bird migration. The changing climate due to global warming has affected the migration map of birds, and it has begun to be seen in places where there was no infection before. In 2014, an infection was detected in a 70-year-old patient in England (Lumley et al. 2014). Most recently, the presence of Hyalomma ticks, which is the main vector for the virus, was detected in Australia, Germany, Sweden, Belgium, Netherlands, Slovenia, Switzerland, France and Italy (Chitimia-Dobler et al. 2016; Fanelli and Buonavoglia 2021; Grandi et al. 2020; Kwak 2018; Mancuso et al. 2019) and the genome of the virus was obtained from the tick in the study conducted in Italy (Mancuso et al. 2019). While the mortality rate, which is one of the factors that make the infection important, varies between 5-40% worldwide, it reaches 80% in some regions (Yen et al., 1985; Yilmaz et al., 2008).

In the map published by the World Health Organization in 2017 (Figure 1.1), the presence of Hyalomma ticks and the cases seen, the case density are expressed using different colors. On the map, Turkey stands out as one of the countries with the highest number of cases.

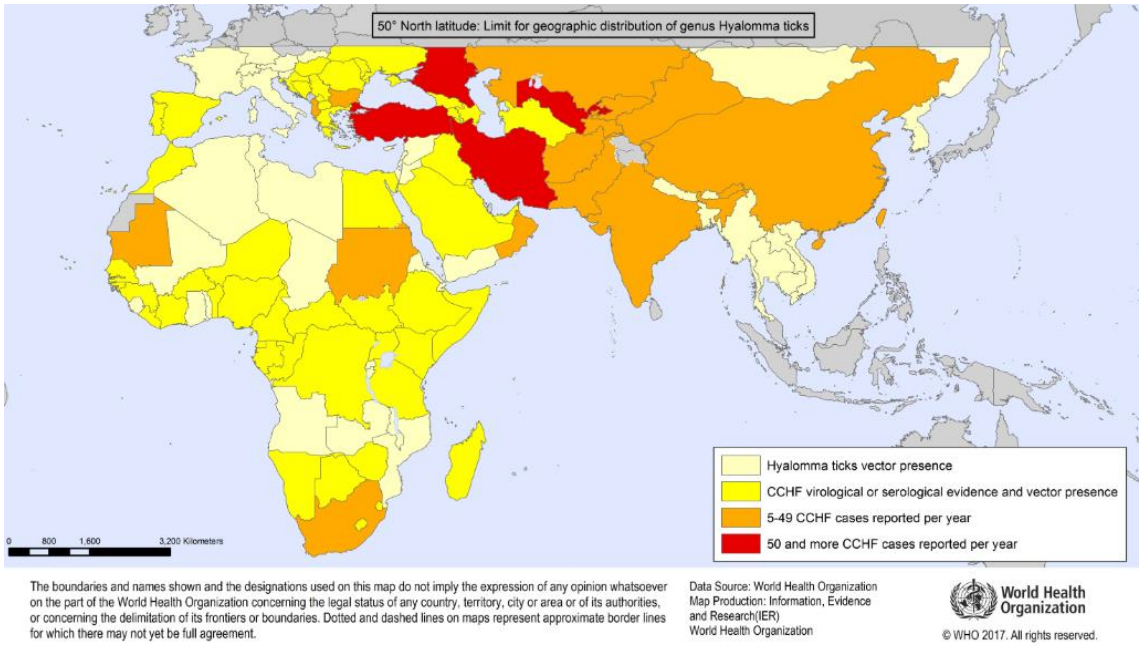


Figure 1.1 Geographical Distribution of Crimean-Congo Hemorrhagic Fever (Source: Url 1)

In Turkey it is mandatory to report CCHF infection cases since December 2003. In the last 15 years, there have been more than 10,000 cases reported by the Turkish Ministry of Health. Most of the cases are seen in the Black Sea region and Middle-Anatolia, especially in Yozgat, Çorum, Sivas, Kastamonu, Samsun, Bingöl. Apart from these provinces, it has spread to wider geographical areas such as Aydın, Çanakkale, Balıkesir, Kırklareli, Burdur, Isparta, Diyarbakır, Tunceli between 2003 and 2014 (Leblebicioglu et al. 2015).

1.1.3 Virus-Tick-Host Relationship

Considering the life cycle of Hyalomma ticks, which are seen as the main reservoir of CCHF virus, it is thought that the first step begins with the virus-tick interaction. With the tick's blood sucking, the virus passes to the epithelial cells in the intestine of the tick and spreads to various tissues of the tick from there.

It has been determined that the viral load is the highest especially in reproductive cells and salivary glands (Dickson and Turell 1992). The larvae that emerge from the eggs prefer small animals to feed, such as rabbits, mice and ground-feeding birds. When they pass from larva to adult, the nymphs stay on the host for a while. During this period, the virus passes through saliva to the host or to other larvae or adult ticks that they feed with. Ticks that reach adulthood later prefer larger hosts. Mating and feeding takes place on sheep, goats and cattle. During this period, the virus is transmitted to the host, and the virus is transmitted to other ticks during mating. In this way, the virus spreads quickly. The transmission of virus among ticks during feeding is called transstadial, and the transfer of virus to eggs by female ticks is called transovarial transmission (Gargili et al. 2017; Anna Papa et al. 2017). While infected ticks feed on animal hosts, the transmission of the virus to other ticks without infecting the host is called non-viremia spread (Aslam et al. 2016).

1.2 CCHF Infection and Transmission, Necessary Precautions

1.2.1 Clinical Symptoms of Infection

CCHF infection is examined in four main parts. Incubation, pre-hemorrhagic period, hemorrhagic period and convalescence period. The incubation phase is an asymptomatic process. The incubation period, which starts with a tick bite or entry of the virus into the body by other means, varies between about 3-7 days.

The route of transmission may affect the incubation period. There are specimens with an incubation period of 2-3 days with tick bites, and 5-6 days for contamination with infected blood or tissue (Swanepoel et al. 1987, 1989). In the pre-hemorrhagic stage, some symptoms begin to appear but are not distinctive for CCHF. Symptoms such as headache, nausea, diarrhea, high fever are seen in the patients. Symptoms may persist for 1-7 days. In addition, inflammatory redness can be seen in the white part of the eyes. Especially in this period, whether the patients are from the risky area for CCHF and whether they are related to animal husbandry becomes important in terms of diagnosis (Schwarz, Nsanze, and Ameen 1997; Swanepoel et al. 1989). The hemorrhagic period lasts 2-3 days.

It is shorter than other phases. However, heavy bleeding is observed in the patient during this period. Bleeding is seen especially in soft tissues, inside the mouth, nose, gums, abdominal cavities, uterus, urinary tract, intestines. In addition, bleeding occurs on the surface of the skin due to damage to the epithelial cells in the capillaries. In some cases, even cerebral hemorrhage can be seen. This is the stage where deaths occur (Ergonul et al. 2006; Swanepoel et al. 1989). Patients who survive the hemorrhagic period enter the recovery period. It usually begins 10-20 days after the onset of the disease. In this period, complaints such as irregular pulse, tachycardia, difficulty in breathing, hair loss, dry mouth, visual impairment, memory loss are observed in the patients. Although the recovery period varies from person to person, it can take up to 1-5 years (Ergönül et al. 2004; Schwarz, Nsanze, and Ameen 1997).

1.2.2. Transmission and Protection

CCHF virus is asymptomatic in animals but causes serious infection in humans with the possibility of death. Animals do not show any symptoms, but they generate antibodies against the virus, so it is very difficult to notice and the virus can spread quickly and insidiously. It can become widespread in the region undetected for a long time and can cause an epidemic when people are infected. Therefore, it is closely related to the seropositivity status in animals (Bente et al. 2013). It is transmitted to humans through Hyalomma tick bite or direct contact with infected blood or bodily fluids. Hospital-acquired cases are also quite common.

Groups at risk include shepherds, farmers, slaughterhouse workers, veterinarians, health workers (A Papa 2019). There are some points to be considered in order to prevent virus-borne infection and to prevent further infections.

CCHF virus control is one of the most important factors. Since it is asymptomatic in animals, the infection can spread quickly without being noticed by ticks. For this reason, CCHF infection controls should be carried out in animals going to slaughter. These controls can be applied during the quarantine period of approximately 14 days before going to slaughter. Another important factor is the uncontrolled animal trade across borders. To prevent this, various sanctions should be applied. Border controls should be tightened. And also, animals should be subjected to various health checks and health certificates of the animals should be requested (Harxhi et al. 2005; Leblebicioglu et al. 2015).

In order to reduce the risk of transmission of the virus to humans from ticks or animals, it is recommended that people wear long-sleeved clothes, especially in agricultural areas and areas where animal husbandry is common. Especially with the warming of the weather, humans become the target host for ticks. In any case of tick attachment, it is recommended to go to the nearest health institution and avoid individual intervention. In addition, if there is a febrile illness in people coming from areas where ticks are common, health workers should be informed. Animals coming from endemic areas must be quarantined and passed the necessary tests before slaughtering, and those working in the slaughter houses must wear protective clothing and gloves. In addition, the skin of the animals should never come into contact with the internal organs in order to prevent a possible risk of contamination. Animal wastes should never be thrown into open areas such as streams, and methods such as sanitary landfills or anaerobic digestion should be used (Leblebicioglu et al. 2015; Mostafavi et al. 2013). Another important risk is healthcare workers. In particular, healthcare professionals responsible for the care and treatment of CCHF patients should take the necessary awareness training and precautions (Conger et al. 2015).

Healthcare professionals responsible for patient care are more likely to get infections from patients who have bleeding in the mouth, nose, gums, vagina and injection sites.

For example, 8.7% of workers exposed to infected blood in a hospital and 33% of those exposed to needle stick injuries had infections (Ergönül 2006; van de Wal et al. 1985). If CCHF virus has come into contact with an open wound, the injured area should be washed with soap and water immediately, and it is recommended to start using antiviral drugs in addition (Maltezou and Papa 2011).

Treatment of CCHF cases is generally supportive treatments. An approved treatment is not yet available. Mostly, antiviral drugs that are not specific to CCHF virus are used in treatments. Ribavirin and favipiravir are commonly used drugs. Although there is no definite evidence about the effectiveness of ribavirin against CCHF virus, it is used in case of direct or indirect exposure of healthcare workers to the virus. Favipiravir is considered more promising as an RNA inhibitor.

However, drug development studies are still ongoing. On the other hand, there is no approved vaccine against CCHF virus. Although there are different studies in the literature, there is no vaccine that can be used yet (Mazzola and Kelly-Cirino 2019). In the literature, a vaccine developed based on the CCHFV Turkey-Kelkit06 strain provided 80% protection in a mouse model with high viral load. In another study, the Ankara (MVA) strain-based vaccine provided protection for 14 days after infection when targeting the glycoproteins of the virus, but had no protective effect when targeting the S segment (Buttigieg et al. 2014). Although a vaccine targeting glycoproteins for use in livestock is under evaluation. Especially the high variation in segment sequences makes vaccine development studies very difficult (Canakoglu et al. 2015).

1.3. CCHF Virus Diagnostic Tests

Laboratory tests are needed for the definitive diagnosis of CCHF. These tests include antigen capture enzyme-linked immunosorbent assay (ELISA), real-time polymerase chain reaction (RT-PCR), virus isolation, and antibody detection by ELISA (IgG and IgM). Laboratory diagnosis of a patient with a complaint consistent with CCHF symptoms can be determined by viral antigen detection (ELISA antigen capture), RT-PCR or virus isolation in the acute phase of the disease.

Usually, the onset of the disease may not always be correct, and therefore different approaches are used together for the diagnosis of CCHF infection. Currently, detection of viral RNA along with detection of IgM antibodies is the diagnostic method used in most reference laboratories (Raabe 2020; Tezer and Polat 2015). Early diagnosis of CCHF is very important for patient treatment and prevention of possible infections. Today, nucleic acid tests (real-time RT-PCR, RT-PCR, RT-qPCR), which are accepted as the gold standard for early diagnosis, are used. In order to carry out these tests, trained personnel, real-time thermal cycler device and appropriate laboratory environment are required, so the tests cannot be performed everywhere. In Turkey, these tests are carried out in training and research hospitals, university hospitals, private branch research hospitals, which are known as tertiary health institutions.

Turkey Public Health Institution Microbiology laboratory, Samsun Public Health laboratory, Erzurum Public Health laboratory, Bozok University Faculty of Medicine laboratory are the reference laboratories where the tests are applied.

1.3.1 Virus Isolation

Virus isolation, one of the diagnostic methods, is a laboratory technique for detecting whether virus samples infect various cell lines such as Vero, LLCMK2, SW-13, CER, BHK-21. In this type of tests, in the first 3 days after symptoms on set are suitable for successful result and virus isolation takes an average of 1-10 days (Bahrikarehmi and Yiğit 2020). However, in some cases it needs to be confirmed with nucleic acid tests or ELISA tests. CCHFV requires uncommon BSL-4 level laboratories due to its high risk of transmission and is not a preferred method due to its impracticality in practice. Also, since it requires high viremia, it is suitable for use only for the first week of infection (Mazzola and Kelly-Cirino 2019; Tezer and Polat 2015).

1.3.2 Serological Tests

Serological tests for CCHF detection are virus-specific antigen or antibody (IgG and IgM) capture tests. Viral antigen tests are suitable for use for approximately the first 5 days after the onset of symptoms. In the first 5 days after infection, viremia is mostly seen at high levels in the blood. As the viremia level decreases in the following days, the use of antigen tests becomes more difficult. When the antibody response during infection is evaluated in studies using ELISA and indirect immunofluorescence (IF) tests, IgM antibodies are seen in the body an average of one week after symptoms appear, and IgG antibodies a few days later. Viral antigen tests are less complex than the viral culture method and have a shorter time to result. It also does not require sophisticated instruments, but when the level of antibodies in the blood rises, the result of the antigen test may be affected and the result may be uncertain.

Antibody tests are far from being the right choice for early diagnosis, as the level of antibodies remains high for a long time in the body during and after the infection (Raabe 2020; Vanhomwegen et al. 2012).

Although there are commercial ELISA kits developed for research use, none of them have been approved as a clinical diagnostic test. For example, VectoCrimea-CHF ELISA (Vector-Best, Novosibirsk, Russia) when tested for different geographical regions showed 87.8% sensitivity and 98.9% specificity for IgM antibodies, 80.4% sensitivity and 100% specificity for IgG antibodies (Baniasadi et al. 2019). Another example, the Crimean-Congo fever mosaic 2 immunofluorescence assay (IFA), showed 93.9% sensitivity and 100% specificity for IgM antibody detection, and 100% specificity and 86.1% sensitivity for IgG antibody. In the lateral flow test, which was developed as another serological method, it showed a sensitivity of 39.7% in the IgM antibody detection test performed on CCHF samples taken from different geographies (Vanhomwegen et al. 2012)

1.3.3 Molecular Diagnostic Tests

Molecular tests are polymerase chain reaction (PCR) based tests. The polymerase chain reaction allows to obtain a million-fold copy from a small amount of DNA within 1-2 hours. The synthesis step in PCR consists of three main parts, denaturation, hybridization, which is the step in which the primers are attached to the target chain, and the extension part, where the primers are extended by the polymerase and the chain is completed. Amplification is exponential as the products formed at the end of each synthesis step serve as a template for the next synthesis step.

The PCR reaction mix contains DNA template, two primers, Taq polymerase enzyme, mix containing four different deoxyribonucleotide triphosphates (dNTPs) and buffer. The mixture put into PCR tubes is placed in a thermal cycler and the required temperatures are provided at each step during the synthesis (Garibyan and Avashia 2013; National Laboratory of Enteric Pathogens Laboratory Centre for Disease Control 1991). In the denaturation phase, the temperature rises up to 94-95°C.

At this stage, the double-stranded DNA, which is the target chain, is separated by breaking the hydrogen bonds and provides the formation of two single-stranded chains. During the hybridization phase, the temperature is lowered and usually takes place at 40-70°C. It is the step in which the primers bind to the single-stranded target chain. At high temperature, hydrogen bonds are formed again between the primers and the target chain when the temperature is lowered. Because the primers are shorter than the complement chain of the target, they are easier to bind. The extension step takes place at 72°C due to the Taq DNA polymerase enzyme. Elongation occurs from the 5' end to the 3' end, from the point of attachment of the primers, by the polymerase using deoxyribonucleotide triphosphates by the extension of the primers.

Molecular tests are for detecting virus RNA. Phylogenetic studies based on the S segment of the CCHFV genome divided into 7 main genotypes. However, these groups have a lot of variation within themselves, so tests targeting a certain strain group may not detect strains in other geographical areas, if the virus concentration in the tested sample is outside the sensitivity of the test applied, it may not be detected, so the tests have to be repeated, moreover, in order to apply molecular tests, sensitive.

Generally not suitable for field applications as devices and a sterile environment are required. In addition, if there are other hemorrhagic fever viruses in the area, the test should be distinctive for CCHFV. Therefore, although molecular testing is a sensitive diagnostic method, several factors such as the patient's country of origin, the testing environment, the type of CCHFV strain tested, and the virus concentration used affect the test results (Escadafal et al. 2012). Due to the wide genetic variation of CCHFV, it makes detection of all strains of the virus difficult and presents difficulties with primer or probe design. Therefore, assays are often designed to detect a specific region in the S-segment of CCHFV, the most conserved region of the genome (Burt 2011). Most of the tests are suitable for a certain number of strains and for a certain geographical area. Most of the tests published so far are suitable for a certain number of strains and for a certain geographical area. The most applied molecular test method is real time PCR tests and is seen as the gold standard. In a study published in 1996, CCHFV was detected on patient samples from the United Arab Emirates using real-time RT-PCR (Schwarz et al. 1996), followed by RT-PCR testing on patient samples from South Africa (Burt 2011).

In the 2000s, real time RT-PCR test was applied on patient samples taken from Kosovo and the limit of detection level was determined as 2,779 virus genome equivalent/ milliliter(Drosten et al. 2002). In a study published in 2005, one step real-time test was applied on patient samples taken from the Eastern Anatolia region and it was able to detect at a concentration level of 10^2 genome equivalent/milliliter (Yapar et al. 2005). In 2006, one step real-time testing was performed on patient samples in Kosovo and detected the virus genome at the lowest concentration of 30 PFU/ml (Duh et al. 2006). In three different studies published in 2007, 18 strains from Uzbekistan were targeted using Real-Time PCR (Garrison et al. 2007), a total of 12 samples from Iran, Pakistan and South Africa were studied, and the lowest concentration of 1,164 copies/ml virus could be detected (Wölfel et al. 2007), also studied with samples from Albania (Anna Papa et al. 2007).

In a study in 2009 in which one step RT-PCR and DNA macro array tests were used together, 6.3 copies of the virus genome per reaction were detected in the test performed with primers based on 18 strains on patient samples taken from Iran, Namibia, Pakistan, South Africa (Wölfel et al. 2009).

In the study conducted with real-time RT-PCR for 18 strains, the virus concentration level was able to detect 5 copies of the virus genome per reaction (Atkinson et al. 2012). In the study published in 2013, based on 4 strains, RT-PCR and DNA microarray tests were used together, and 10^5 -10^6 PFU/ml cDNA was detected in patient samples taken from the Balkans and the Middle East (Filippone et al. 2013). In 2014, real-time RT-PCR test was able to detect the lowest concentration of 33-100 fg/µl with primers based on 1 strain (Fajfr et al. 2014). In one of the 2 separate studies with real time PCR in 2014, 11 genomes were detected per reaction in the test based on 8 strains from Turkey (Jääskeläinen et al. 2014), and in the other test performed on samples taken from animals and ticks in India, the lowest 7.6 copies/µl genome was found. was detected (Kamboj et al. 2014).

In the multiplex RT-PCR test performed for 3 strains from China, Senegal and the Democratic Republic of Congo in 2015, the lowest detection amount was 190 copies/ml (Das et al. 2015). In another study with one step real-time PCR, the lowest 20 genomes per reaction were detected in patient samples from Iran (Zahraei et al. 2016).

With the new generation sequencing published in 2017, multiplex tick samples collected from Aegean Mediterranean and Central Anatolia in Turkey were studied (Brinkmann et al. 2017). In studies published in 2018, the lowest detection level was determined as 2 copies/µl (Sas et al. 2018) with the primers created by selecting one strain for 6 genotypes using Real time PCR, while the lowest detection level was determined as 256 PFU/ml for the 16 strains used in the experiment in the other study (Koehler et al. 2018).

1.4. Isothermal Amplification Methods

Recently, the necessity and interest in rapid diagnostic tests have gained importance with the Covid-19 pandemic. Rapid diagnostic tests play a key role in the management of epidemics, in preventing and monitoring the spread, in differentiating the errors that require antibiotics in order to prevent unnecessary antibiotic use, or in determining which type of antibiotic should be used. Today, serological-based and nucleic acid-based diagnostic tests are available and continue to be developed.

Nucleic acid-based assays include PCR amplification. Therefore, PCR method is used as the gold standard in diagnosis. It is used in studies such as identification of various pathogens, determination of disease-causing genes, determination of genetic characterizations, etc. (Özay and McCalla 2021; Zanoli and Spoto 2012).

However, PCR is generally the method that uses sensitive devices such as a thermal cycler suitable for use in laboratory environments, and therefore results are obtained in more than 1 hour. It is not very suitable for use in the field, small health institutions, polyclinics, places where specialists are not available, especially in suspicious diseases that require diagnosis in a short time. Apart from the PCR method, there are isothermal amplification methods that are increasing in popularity. It has many advantages compared to PCR. The most important advantage of isothermal amplification methods is the amplification at constant temperature. It is the polymerase it uses that provides this. This polymerase has strand-displacement activity and does the job of different temperature steps in PCR.

In addition, it has equal sensitivity with PCR and results can be obtained in a shorter time. In addition, some isothermal methods can directly amplify RNA without DNA and have high tolerance to bio inhibitors found in clinical samples. Isothermal amplification methods defined and used in the literature Nucleic Acid Sequence-based Amplification (NASBA), Loop-mediated Isothermal Amplification (LAMP), Strand Displacement Amplification (SDA), Recombinase Polymerase Amplification (RPA) and Rolling Circle Amplification (RCA), Exponential Isothermal Amplification (EXPAR), Helicase Dependent Amplification (HDA), Whole genome Amplification (WGA) (Van Ness, Van Ness, and Galas 2003; Zhao et al. 2015).

In the thesis study, LAMP method, one of the isothermal amplification methods, was used. LAMP method has prominent advantages over other isothermal methods. It amplifies 10^9 fold in less than an hour, is more specific than other isothermal methods, the amplification result is visible to the naked eye, it is tolerant to contamination and bio inhibitors in the samples.

1.4.1 Loop Mediated Isothermal Amplification (LAMP)

The LAMP method uses 4 main primers targeting six regions on the target genome for amplification, uses a uniform enzyme operating at constant temperature and performs amplification in a single step. The method can be used for both DNA and RNA targets. For RNA targets, the AMV transcriptase enzyme is used in addition to DNA polymerase. Amplification is easily visible in a single tube without an additional imaging method.

During DNA amplification, pyrophosphate is released into the medium with each base added. The broken pyrophosphate combines with the magnesium ions in the reaction and forms a magnesium pyrophosphate precipitate. Since the product formed in the LAMP method is too much, this precipitate creates a visible dementia.

The LAMP polymerase chain reaction consists of two steps. The reaction works with 4 or 6 primers. There are inner primers (FIP and BIP), outer primers (F3 and B3) and optional loop primers (LF and LB) that can speed up the reaction (Fig. 1.2 A). It replicates a conserved region of 200-300 nucleotides in the target organism genome.

The LAMP polymerase chain reaction is shown in Figure 1.2. In Figure 1.2(B), BIP from the internal primers first binds to the complementary region and Bst DNA polymerase synthesizes the complementary strand of the target strand (step I). The outer primer B3 then binds to the B3c region of the target chain (step II). Bst DNA polymerase cleaves the duplex DNA synthesized in the first step and the target DNA complementary strand is synthesized from the B3 primer.

In the first step, the FIP primer is attached to the synthesized chain starting with BIP and the complement chain is synthesized (step III). The outer primer F3 is then linked to the chain synthesized in the first step from the F3c region. Bst DNA polymerase cleaves the duplex DNA formed in the third step, complementary strand synthesis begins with the F3 primer (step IV).

The chain synthesized in the third step creates the dumbbell structure seen in the fifth step by pairing the F1-F1c and B1-B1c regions. Thus, the first step of the RT-LAMP reaction is completed. The second stage, shown in Figure 1.2(C), proceeds through the dumbbell structure. FIP, BIP and, if available, loop primers (LF, LB) are used and exponential amplification takes place.

In the sixth step, DNA synthesis starts at two points, the B1c-B1 handle region of the dumbbell and the loop region to which the BIP primer is attached. Since Bst DNA polymerase is able to cleave duplex DNA, DNA synthesis continues until the end of the chain. Several different primers from the stem and loop regions can be attached to the DNA strands produced in the next steps. In this way, DNA polymerization proceeds exponentially. The produced chains form cauliflower structures of different lengths.

In the seventh step, the chain formed by the extension of the BIP primer is separated and released by the chain extended over the dumbbell structure. The released BIP extension chain turns into a dumbbell structure thanks to the F1-F1c and B1-B1c regions on it and becomes the target chain for the FIP primer. Thanks to the F1-F1c regions at the end of the chain, which are simultaneously extended over the dumbbell structure, the end of the chain takes the form of a loop and continues to extend from the F1 primer. In the eighth step, BIP and FIP primers are attached to the loop structures on the elongated chain and map the chain simultaneously, so that a large amount of amplicons of different lengths are obtained in a short time. In addition to the FIP and BIP primers, loop primers (LP) can also be used to speed up the reaction in the second step.

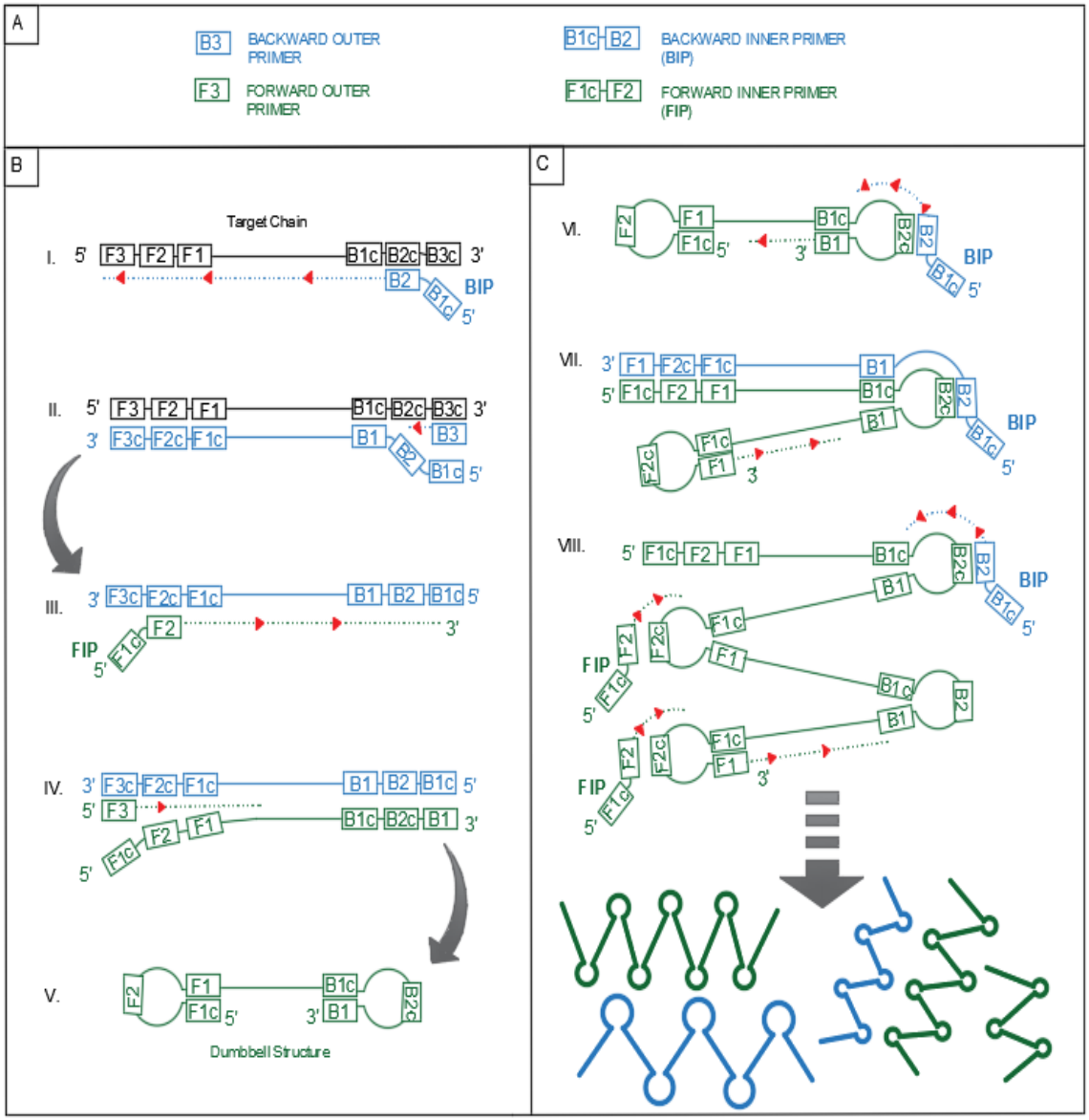


Figure 1.2 Principle of LAMP PCR Method

In the literature, there are examples of successful detection of many pathogens using the LAMP method. The first example in which the Reverse Transcriptase-LAMP method was used for CCHF virus detection is from Sudan. In the study, LAMP primers were designed by targeting the S segment genome of Sudan strain Alfulah-3 (GQ862371). Results were also compared with nested PCR.

As a result of the study, the detection limit for Alfulah-3, Alfulah-4, Abyei and Lagawa strains was found to be 50 viruses, and the results showed 100% agreement when compared with nested PCR.

The primers had high specificity as they did not show any interaction with the Rift Valley, Dengue, Yellow Fever virus and other hemorrhagic fever viruses seen in Sudan (Osman et al. 2013).

As sensitive and successful results as PCR results were obtained on fungi, parasites, viruses and bacteria. LAMP method was used to detect Mycobacterium tuberculosis (Bentaleb et al. 2016; Sreedeep et al. 2020), Streptococcus pneumonia (Takano et al. 2019; Xia, Guo, and Zhou 2014), Bordetella pertussis from human respiratory pathogens (Fujino et al. 2015; Kamachi et al. 2017). Also, it successful results were obtained in the detection of Salmonella typhi (Abdullah et al. 2014; Fan et al. 2015; Kaur et al. 2018), Campylobacter jejuni (Babu et al. 2020; Kreitlow et al. 2021; Pham et al. 2015) and Campylobacter coli (Kreitlow et al. 2021; Pham et al. 2015; Yamazaki 2013), Helicobacter pylori (Bakhtiari et al. 2019; Zheng et al. 2020), Listeria monocytogenes (Tirloni et al. 2017; Zhan et al. 2019) from food pathogens. It was used in the detection of parasites such as for the diagnosis of malaria caused by Plasmodium spp (X. Chen et al. 2021; Jang et al. 2021). In addition it was used for Leishmania parasite (Nzelu, Kato, and Peters 2019; Ruang-Areerate et al. 2021), Trichomonas vaginalis (Khurana et al. 2020; Reyes, Solon, and Rivera 2014), Strongyloides stercolaris (Fernández-Soto et al. 2016; Watts et al. 2019). In addition, it has been proven that the LAMP method can be used for early detection of Aspergillus flavus, A. parasiticus, A. nomius fungal species in humans (Ferrara et al. 2020; Luo et al. 2014). It has also been used in the detection of various viruses. Dengue virus (Lopez-Jimena et al. 2018; Yaren et al. 2017), Influenza viruses (Bakre et al. 2021; Kim et al. 2016), Hepatitis C and Hepatitis B (C.-M. Chen et al. 2020; Hongjaisee et al. 2021), Ebola virus (Bonney et al. 2020; Sabalza et al. 2018), Zika virus (Lamb, Bartolone, and Chancellor 2020; Silva, Pardee, and Pena 2019), Middle East Respiratory Syndrome virus (MERS-CoV) coronavirus (Huang et al. 2018; Lee et al. 2016) samples could be detected by LAMP method.

1.4.2. Whole Genome Amplification (WGA)

The whole genome amplification (WGA) method appears as the first multiple displacement amplification. The method uses random primers and DNA polymerase. It usually requires an additional step for DNA denaturation.

The reaction takes place at a constant temperature of 30-37°C. It takes about 10-16 hours for amplification and can produce products up to 10-70 kb in length. However, since the method amplifies the entire genome, its specificity is low compared to other isothermal amplification methods (Dean et al. 2002; Zanoli and Spoto 2012).

1.4.3. Strand Displacement Amplification (SDA)

It uses SDA (strand displacement amplification), DNA polymerase and NEase enzymes and 4 primers for amplification. DNA denaturation requires an additional step at 95°C. Then the reaction takes place at a constant temperature of 37 °C. It can produce up to 10^7 copies of analytes in an average of 2 hours. In the method, after the denaturation step, the primers in the primer hybridized DNA chain are extended by polymerase to form the double-strand DNA chain. The chains extending from the primers containing the nicking region are separated and serve as a template for the other primer pair. In this way the amplification takes place exponentially (Shi et al. 2014; Walker et al. 1992).

1.4.4. Helicase Dependent Amplification (HDA)

Helicase dependent amplification (HDA) method uses DNA polymerase, two primers and helicase, which is a DNA-binding protein. The reaction takes place at a constant temperature of 60-65 °C. On average, 10^7 copies of the analyte can be amplified in 2 hours. This method is suitable for amplification of kilobase-long target regions.

In the reaction, the double strand DNA is cleaved by the helicase and primers are attached to the cleaved strands. The DNA chain is doubled by extending the primers from their 3 ends with polymerase, and as a result of this process, two double-strand DNAs are obtained. This process is repeated continuously and exponential amplification takes place. Unlike PCR, HDA has a lot of background noise and the primers have a high probability of dimerization with each other. Therefore, false positive results may occur (Barreda-García et al. 2018; Vincent, Xu, and Kong 2004; Zhao et al. 2015).

1.4.5. Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA) method uses DNA polymerase, recombinase and two primers. In the presence of primers that can bind to the target site during the reaction, the recombinase double-strand decompose the DNA and in this way the primers bind to the complementary portion. Then the polymerase extends the 3 ends of the primers to make the chain a double strand and creates the DNA that will form the template for the next step. It causes the amplification to be exponential by forming the double-strand DNA strand in the same way in the two primers. The reaction takes place at a constant temperature of 37-42 °C. It can produce 10 genomic DNA copies in 1.5-2 hours on average. However, excessive background signal is considered as a disadvantage and accordingly additional reagents are used (Kersting et al. 2014; Lobato and O'Sullivan 2018; Shen et al. 2011).

1.4.6. Nucleic Acid Sequences Based Amplification (NASBA)

The nucleic acid sequences based amplification (NASBA) method uses AMV reverse transcriptase, RNase H, T7 DNA dependent RNA polymerase enzymes and 2 primers. It allows multiple copies of RNA to be produced from RNA. The method takes place in two steps. The first step takes place at 65°C and in this step, DNA is produced from RNA and an RNA-DNA hybrid structure is formed using primers.

Then the second step of the reaction takes place at about 41 °C. In the second step, RNase H disrupts the RNA chain and RNA is produced from single-stranded DNA by T7 RNA polymerase. Here the antisense of the target chain is produced and accumulates in amplification. The reaction takes an average of 2 hours and approximately $10^6 - 10^9$ copies of the analyte can be produced. The disadvantage of this method is that it has two steps and requires 3 different enzymes (Compton 1991; Md et al. 2012).

1.4.7. Rolling Circular Amplification (RCA)

The Rolling Circular Amplification(RCA) method uses phi29, Bst DNA polymerase and a probe with a sequence complementary to the target sequence with the NEase enzymes. When the reaction was first introduced, it was working at 30-37^{0}C, and then it was made to work at 60^{0}C. In its working principle, the 5 and 3 ends of the probe have the sequence that complements the target sequence. Therefore, when hybridizing with the target, the probe takes the C shape and is joined by interned DNA ligase. Amplification occurs by attaching primers to the probe array, which has become a circular molecule (Fire and Xu 1995; Murakami, Sumaoka, and Komiyama 2009; Zeng et al. 2013). The reaction can take an average of 1-3 hours to produce up to 10^3 copies of the analyte. In this method, the probability of miss-hybridization between probe and primers is quite high, and the reaction takes a long time (Bodulev and Sakharov 2020; Zhao et al. 2015).

1.4.8. Exponential Isothermal Amplification (EXPAR)

The Exponential Isothermal Amplification (EXPAR) method uses two probes that contain a nicking recognition site and bind to the target sequence. The reaction takes place at a constant temperature of 55-60^{0}C. It uses DNA polymerase and NEase enzymes. In its working principle, the target chain hybridizes with the complementary one of the 2 probes.

From the 3 ends of the probe, the polymerase begins to extend and completes the target chain. As the elongated probe leaves the nicking region, the target chain also leaves the probe (Jia et al. 2010; Van Ness, Van Ness, and Galas 2003).

As a result of repeated hybridization and separation in this way, 10^8 copies are produced in an average of one hour. However, this method contains a high probe concentration, so the probability of false positive results as a result of dimerization is high. Usually a denaturation step is recommended as the first step (Bodulev and Sakharov 2020; Zhao et al. 2015).

CHAPTER 2

AIM OF DISSERTATION

CCHF infection is a tick-borne viral disease with a wide geographical distribution and a high mortality rate. The absence of an approved vaccine and specific drug treatment of the infection increases the mass danger pointed out by the World Health Organization. Early diagnosis is important to prevent the spread of infection and to keep the patient alive. Although the current nucleic acid tests are sensitive, getting the test result takes more than an hour. Tests can only be run in certain test centers prolonging the diagnosis and increase the likelihood of loss of life.

In the study, it was aimed to develop a LAMP based method for detecting CCHFV strains previously observed in Turkey. 21 full length S segment genome sequences of CCHFV previously reported in Turkey were aligned using the Unipro UGENE software program. Conserved regions in aligned sequences were identified and primers suitable for these regions were designed. Optimization experiments were performed using the DNA of CCHFV Ank-2 (MK309333) strain for the LAMP method. The detection limit for LAMP was determined under optimum conditions. The results were compared with qPCR, which is considered the gold standard method. The potential of the LAMP method for detecting CCHFV infection was evaluated by comparing LAMP and qPCR results.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sequence Alignment of CCHFV

We obtained the strains seen in Turkey from NCBI's nucleotide database. In particular, strains containing the full length S segment sequence were selected. Accession numbers of the strains used for alignment were: GQ337053, KY362517, DQ211649, MF511217, MF511216, MF511215, MF511214, MF511213, MF511212, MF511211, MF511210, MF511209, MF511208, MF5112207, KR092375, KR092376, KR092377, KR092378, MN811033, MN811034, MN811035. Unipro UGENE (free open-source cross-platform bioinformatics software) (Source: Url 2) program was used for alignment. It was performed in the UGENE program using 21 strains sequences and a set of sequences was obtained (Figure 3.1).

The full length S segment of CCHFV genome is 1673 bp long. Each letter on the aligned sequence represents the nucleotide on the aligned sequence represents. Capital letters indicate conserved nucleotides, while lowercase letters indicate the presence of different nucleotides corresponding to that position.

1-64: tctcaaagaaacacgtgccgcttacGCCCAcaGTGTTcTCTTGAGTGTctGCAAAATGGAaAAC

65-116: AAgATCGAGGTGAACAGCAAaGAtGAgaTGAACAAaTGGTTTGAgGAGTTtA

117-167: AaAAgGGAAATGGaCTTaTGGACACtTTCACAAaCTCcTACTCCTTTTGTG

168-222: AgAATgtaCCaAAtcTGGAtAagTTTGTGTtCCAgATGGCCAGcGCcACtGATGA

223-275: TGCACAgAAGGAcTCCATCTATGCATCgGCtcTgGTgGAaGCaACCAAGTTcT

276-330: GtGCaCCcATATATGAaTGTGCtTGGGTcAGcTCtACtGGcATtGTgAAGAAgGG

331-384: gCTTGAGTGGTTcGAGAAGaAttCAgGaACcATcAAaTCtTGGGATGAGAaCTA

385-438: tgCTGAGCTgAAGGTTGAtgTTCCcAAAATAGAaCAACTtgCCAatTAcCAaCA

439-491: GGCTGCtCTcAAGTGGAGgAAgGACATAGGTTTcCGTgTCAAtGCaAACACgg

492-546: CaGCctTaAGCAACAAaGTcCTTGCAGAaTAtAAaGTcCCTGGcGAaATtgTGaT

547-601: GtCTGTtAAaGAaATGCTGTCaGACATGATtAGaAGgAGGAATcTaATtCTcAAc

602-655: AGggGCgGTGatGAaAAtCCACGcGGcCCAGTgAGCCgTGAaCAtgTgGAgTGG

656-709: TGcAGGGAaTTtgTcaAaGGcAAgTAcATCATGGCtTTCAATCCACCTTGGGGg

710-762: GACATCAACAAaTCAGGcCGtTCaGGaATaGCACTTGTtGCAACaGGCCTTGC

763-815: CAAGCTtGCAGAgACcGAGGGgAAAGGaGTtTTtGAcGAaGCTAAGAaGACcG

816-869: TGGAgGCtCTCAAtGggTAttTgGAcAAgCAcAGgGAcGAaGTtGACAAaGCAA

870-923: GTGCTGACAgCATGaTaACAAaCCTcctaAAgCAcATTGCcAAaGCaCAaGAGC

924-978: TtTAtAAaAAtTCATCTGCtCTTCGTGCaCAaGGtGCACAGATTGACACcCCtTT

979-1033: CAGCTCgTTtTAcTGGCTCTACAAgGCcGGtGTgACtCCaGAgACcTTcCCaACt

1034-1085: aTCTCaCAgTTCCTtTTcGAaCTgGGgaAGCAgCCAAGGGGgACCAAaAAAA

1086-1136: TGAAaAAgGCaCTcCTgAGCACTCCaATGAAgTGGGGgAAGAAaCTtTATG

1137-1186: AGCTCTTTGCtGATGACTCTTTcCAGCAGAACAGaATCTAcATGCAcCCT

1187-1237: GCtGTGtTgACAGCcGGtAGaATcAGtGAaATGGGTGTCTGCTTTGGAACa

1238-1290: ATcCCtGTtGCcAAtCCtGAtGAtGCtGCtCAgGGATCTGGACAtACcAAgTC

1291-1342: CATtCTcAAcCTtCGgACAAgCACaGAGaCCAACAAtCCaTGcGCCAaGACa

1343-1395: ATTGTcAAatTaTTtGAaATcCAaaAaACAGGaTTTaACATAcAGGAcATGGA

1396-1448: CATtGTaGCcTCTGAGCACcTGCTGCAcCAaTCccTtGTTGGcAAGCAgTCtC

1449-1500: CaTTcCAaAATGCCTACaAcGTcAAGGGcAAtGCCACCAGtGCCAACATcAT

1501-1565: CTaaaactcaAGGtGtTtcAcAtTCagCtttTCtcctcCtGcATcAcTaCTtaCAGtTatgActA

1566-1645: tTAAtcAcgtTTatTttAAttgCttatgtAaTgctgttttgctaattttatcttgctatctttcatttcaaatacttaaa

1646-1673: gggctgtgcggcaacgatatctttgaga

Figure 3.1 Consensus sequence generated from sequence alignment of 21 CCHFV strains observed in Turkey downloaded from NCBI database. The sequence is 1673 bp long.

3.2. Primer Design for LAMP

LAMP primer design is more complex than PCR primer design, therefore special software is required. LAMP primer design programs use some parameters. There are several open source software that can be used in LAMP primer design, the most preferred software is Primer Explorer (Source: Url 3). Primer Explorer uses the parameters of distance between primers, melting temperatures of primers, secondary structures and dimer structures, stability of primer ends, percent GC of primers while designing primers. Since the LAMP reaction works at a constant temperature, each parameter has a certain value range. The distance between the primers is shown in Figure 3.2. Being longer or shorter can prevent amplification, make it difficult for the polymerase to open the double strand, so these ranges should not be exceeded.

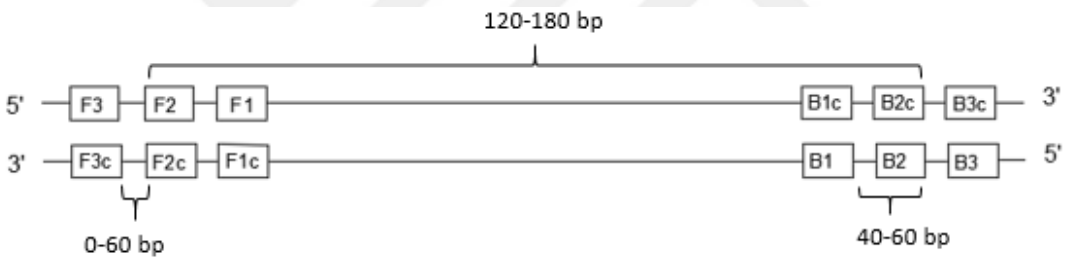


Figure 3.2 Distance between LAMP primers region

Tm value of primers is another important factor. The value of the F2/B2 primers should be between 59-61^{0}C. The Tm value of the F1/B1 and F3/B3 primers should be between 64-66^{0}C. Since there are FIP/BIP primers that are expected to bind first in the working principle, the Tm values of the F2/B2 primers contained in these primers should be lower than the other primers.

Secondary structures and primary dimer structures are not desirable for LAMP operating at constant temperature. Therefore, each primer obtained was checked using the Oligo Analyzer Tool of IDT (Integration DNA Technologies) (Source: Url 4).

Another important parameter is the stability of the ends of the primers. Since the ends of the primers are the starting point in DNA synthesis, they must have appropriate stability. The stability of the 3' ends of the F1c/B1c primers and the 5' ends of the F2/B2 and F3/B3 primers are expected to be maximum -4 kcal/mol.

The percentage of GC that affects the Tm values of the primers is also an important parameter. The GC percentage should be between 50%-60%. Since high GC ratio will increase the Tm value, primer binding sites that do not exceed this ratio should be selected.

In the study, based on the parameters mentioned for LAMP primer design, primer sets obtained from Primer Explorer and primer sets obtained from the USA with the contributions of our collaborator researcher Dr. Özlem YAREN (Foundation for Applied Molecular Evolution).

Because Primer Explorer allows to load a limited number of strain sequences, 21 strains used in the alignment were loaded in portions to design primers in the study. Strains numbered MN811033, MN811034, MN811035 were evaluated separately as they were incompatible in alignment. Primers were obtained for these strains only in Primer Explorer. The resulting primers were evaluated for where they fit in the sequences of other strains. Primer sets corresponding to sheltered areas were re-evaluated for use in the experiment. The sequence amplified by each primer set was checked using NCBI Blast (Source: Url 5). The Blast tool gives us the result of whether there is a match between the nucleotide sequences of the organisms in the database. NCBI Blast results were evaluated for the specificity of the primers. It was checked whether there was a match with the tick genome and whether there was a match with Dengue, Yellow Fever, Zika viruses.

3.3. Primer Design for qPCR

The regions amplified by the LAMP sets decided in Figure 2.3 are shown on the alignment sequence. qPCR assay design of IDT, Primer Quest Tool (Source: Url 6) was used to design qPCR primers that could recognize these regions.

For each sets they were loaded the sequences amplified by the LAMP primers into the program and for the using intercalating dye it was selected the suitable option from the design options.

Each of the primer sets was checked for dimer structures and hairpin structures in the Oligo Analyzer Tool. In NCBI Blast, the tick genome was evaluated for matches with virus genomes other than CCHF virus.

tctcaaagaaacacgtgccgcttacGCCCAcaGTGTTcTCTTGAGTGTctGCAAAATGGAa
AACAAgATCGAGGTGAACAGCAAaGAtGAgaTGAACAAaTGGTTTGAgGAGTTtA
AaAAgGGAAATGGaCTTaTGGACACtTTCACAAaCTCcTACTCCTTTTGTGAgAAT
gtaCCaAAtcTGGAtAagTTTGTGTtCCAgATGGCCAGcGCcACtGATGATGCACAgAA
GGAcTCCATCTATGCATCgGCtcTgGTgGAaGCaACCAAGTTcTGtGCaCCcATATAT
GAaTGTGCtTGGGTcAGcTCtACtGGcATtGTgAAGAAgGGgCTTGAGTGGTTcGAG
AAGaAttCAgGaACcATcAAaTCtTGGGATGAGAaCTAtgCTGAGCTgAAGGTTGAtgT
TCCcAAAATAGAaCAACTtgCCAatTAcCAaCAGGCTGCtCTcAAGTGGAGgAAgGA
CATAGGTTTcCGTgTCAAtGCaAACACggCaGCctTaAGCAACAAaGTcCTTGCAGAa
TAtAAaGTcCCTGGcGAaATtgTGaTGtCTGTtAAaGAaATGCTGTCaGACATGATtAG
aAGgAGGAATcTaATtCTcAAcAGggGCgGTGatGAaAAtCCACGcGGcCCAGTgAGC
CgTGAaCAtgTgGAgTGGTGcAGGGAaTTtgTcaAaGGcAAgTAcATCATGGCtTTCAA
TCCACCTTGGGGgGACATCAACAAaTCAGGcCGtTCaGGaATaGCACTTGTtGCAA
CaGGCCTTGCCAAGCTtGCAGAgACcGAGGGgAAAGGaGTtTTtGAcGAaGCTAAG
AaGACcGTGGAgGCtCTCAAtGggTAttTgGAcAAgCAcAGgGAcGAaGTtGACAAaGC
AAGTGCTGACAgCATGaTaACAAaCCTcctaAAgCAcATTGCcAAaGCaCAaGAGCTt
TAtAAaAAtTCATCTGCtCTTCGTGCaCAaGGtGCACAGATTGACACcCCtTTCAGCT
CgTTtTAcTGGCTCTACAAgGCcGGtGTgACtCCaGAgACcTTcCCaACtaTCTCaCAgT
TCCTtTTcGAaCTgGGgaAGCAgCCAAGGGGgACCAAaAAAATGAAaAAgGCaCTcC
TgAGCACTCCaATGAAgTGGGGgAAGAAaCTtTATGAGCTCTTTGCtGATGACTCT
TTcCAGCAGAACAGaATCTAcATGCAcCCTGCtGTGtTgACAGCcGGtAGaATcAGtG
AaATGGGTGTCTGCTTTGGAACaATcCCtGTtGCcAAtCCtGAtGAtGCtGCtCAgGGA
TCTGGACAtACcAAgTCCATtCTcAAcCTtCGgACAAgCACaGAGaCCAACAAtCCaT
GcGCCAaGACaATTGTcAAatTaTTtGAaATcCAaaAaACAGGaTTTaACATAcAGGAc
ATGGACATtGTaGCcTCTGAGCACcTGCTGCAcCAaTCccTtGTTGGcAAGCAgTCtC
CaTTcCAaAATGCCTACaAcGTcAAGGGcAAtGCCACCAGtGCCAACATcATCTaaaa
ctcaAGGtGtTtcAcAtTCagCtttTCtcctcCtGcATcAcTaCTtaCAGtTatgActAtTAAtcAcgtTT
atTttAAttgCttatgtAaTgctgttttgctaattttatcttgctatctttcatttcaaatacttaaagggctgtgcggcaacgatatcttt
gaga

Figure 3.3 qPCR primer design to target regions of LAMP primers. Cyan colored area on the sequence indicate the sequence amplified by the CCHFV-1 primer set, yellow colored area on the sequence indicate the sequence amplified by the CCHFV-2 primer set, Orange colored letter on the sequence indicate the sequence amplified by CCHFV-3 primer set, Gray colored area sequence indicate the sequence amplified by CCHFV-4 LAMP primer set respectively.

3.4. Template DNA Synthesis for Primers

3.4.1. cDNA Synthesis

Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used for cDNA synthesis. Kit contains that random hexamer primer (100 µM), oligo(dT) primers 100 µM, nuclease free water, Revert Aid reverse transcriptase (200 U/µl), RiboLock RNase inhibitor (20 U/µl), 10Mm dNTP mix, 5X Reaction buffer (250 mM Tris-HCL (ph:8.3), 250 mM KCL, 20 mM MgCl2, 50 mM DTT), Forward GAPDH Primer 10 µM, Reverse GAPDH Primer 10 µM, Control GAPDH RNA 0.05 µg/µL.

In the experiment, CCHF virus RNA samples that are sent by Professor Aykut ÖZKUL from Ankara University Veterinary Faculty Virology Department and Harun ALBAYRAK from Ondokuz Mayıs University Veterinary Faculty were used. The accession number of CCHF virus RNA sample used for cDNA synthesis was MK309333.

A total reaction of 20 µl was prepared. In the reaction, 12 µl of mixture was prepared by adding 1 µl of random hexamer primers (100 µM), 4 µl of RNA sample and 7 µl of nuclease free water, and incubated at 65°C for 5 minutes. Following, by adding 4 µl 5X reaction buffer,1 µl Ribolock RNase inhibitor (20 U/µl), 2 µl 10 mM dNTP mix, 1 µl Revert Aid M-MuLV Reverse Transcriptase (200 U/µl), the reaction was completed to 20 µl and the mixture was mixed, it was incubated at 42°C for 60 minutes and finally incubated at 70°C for 5 minutes, the reaction was terminated.

3.4.2. PCR

CCH-S all forward and CCH-S all reverse primers designed to amplify the cDNA synthesis product and the whole S segment were used in the PCR reaction. PCR reaction was performed using Phusion High-Fidelity DNA polymerase Kit (Thermo Scientific). The kit contains Phusion DNA Polymerase (2 U/µL), 5X Phusion HF Buffer, 5X Phusion GC Buffer, 50 mM MgCl2 solution, DMSO.

For full length S segment obtaining, the PCR reaction was prepared in a total of 50 µl. Mixture, 31.5 µl nuclease free water, 10 µl 5X Phusion HF buffer, 1 µl 10mM dNTP mix, 0.5 µl Phusion High fidelity DNA polymerase, 2 µl cDNA product, 2.5 µl CCH_S all forward primer (10 mM), 2.5 µl CCH_S all reverse primer (10 mM) and its PCR steps were shown in Table 3.1. And also, for obtaining CCHFV-2 LAMP primer set target region, reaction was repeated using 1µl cDNA product, 1 µl Q1-forward primer(10X) and 1 µl Q4-reverse primer (10X). PCR steps were shown in Table 3.2.

Table 3.1 PCR steps of Obtaining full length S segment

	Temperature	Time	Cycles
Initial Denaturation	98^0C	30 s	1
Denaturation	98^0C	10 s	30
Annealing	65.3^0C	20 sec	
Extension	72^0C	35 sec	
Final extension	72^0C	10 min	1
Hold	4^0C	forever	1

Table 3.2 PCR steps of Q1-Forward and Q4-reverse primer

	Temperature	Time	Cycles
Initial Denaturation	98^0C	30 s	1
Denaturation	98^0C	10 s	30
Annealing	62^0C	20 sec	
Extension	72^0C	20 sec	
Final extension	72^0C	10 min	1
Hold	4^0C	forever	1

Annealing temperature in PCR steps was chosen according to Tm values of primers. It was calculated as specified in the protocol and annealing temperature for the CCH_S all primers were 65.3°C and Q1-Forward, Q4-reverse primer were 62°C.

The results of the PCR reaction were loaded onto 1.5% agarose gel and the results were examined. For 1.5% agarose gel, it was dissolved by adding 0.6 g of agarose into 40 ml of 1X TAE buffer and heated in the microwave and 2 µl of Safe View dye was added to the agarose gel that had cooled to room temperature. Painted gel poured into the tank. After the gel has frozen, 1X TAE Buffer (100 ml TAE Buffer contains 5 µl Safe View dye) was poured on it, just enough to cover the gel. Then, the samples were loaded on the gel were prepared. 10 µl of the PCR sample was taken, 2 µl of loading dye (6X) was added to it and added to the gel wells. For the ladder, 1 µl of loading dye (6X), 3.5 µl of water was added onto 1.5 µl ladder and loaded onto the gel. It was run at 60 volts for 60 minutes.

Then, Gel Extraction Kit (Geneaid) was used for DNA isolation from agarose gel. The kit includes DF buffer, W1 buffer (ethanol is added), Wash Buffer, Elution Buffer, DF columns, 2 ml collection tubes. Extraction was performed following the kit protocol. Nanodrop (8 channels, Thermo Scientific) device was used to measure the amount of DNA in the obtained product. The measurement result was 16.26 ng/µl (8.9×10^9 copies/µl).

3.5. Fluorimetric LAMP

Bst 2.0 WarmStart DNA (NEB) polymerase Pack was used in the experiment. Bst 2.0 Warm Start DNA polymerase, Isothermal Amplification Buffer Pack, Magnesium Sulfate ($MgSO_4$) Solution (100 mM) are included in the pack. In addition, dNTP mix (NEB), LAMP fluorescent dye (50X), nuclease free water were used. It is stated to have the same properties as the LAMP fluorescent dye Sybr green. Experimental results were obtained in real time from the Roche Real Time Light Cycler 96 device. Three sets of LAMP primers were used for the LAMP Fluorescent assay, CCHFV-1, CCHFV-2, CCHFV-4. In the experiment, the reaction components were completely thawed on ice at room temperature, then the components were added on ice.

In experiment to determine the optimum dye concentration was performed. From 50X dye, we prepared 90 µl dye at 10X concentration, added 18 µl 50X dye and 72 µl nuclease-free water. The experiment was performed with primer sets CCHFV-1, CCHFV-2 and CCHFV-4 and synthetic templates of primer sets. 0.2X and 0.5X dye concentrations were tested in a 25 µl reaction. A 25 µl reaction was generated for each primer set. Reaction mix containing 0.5X dye concentration, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1.25 µl dye (10X) (final concentration is 0.5X), 1 µl Bst 2.0 Warm Start DNA polymerase, 2 µl (10^6 copies/ul) synthetic template (each primer set has its own synthetic template), contains 10.75 µl nuclease free water. For negative control, water was used instead of synthetic template.

25 µl reaction containing final concentration 0.2X dye, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 2.5 µl primer mix (CCHFV-1, CCHFV-2, CCHFV-4), 2 µl synthetic templates (10^6 copies/µl), 11.5 µl nuclease free water.

The experiment was tested with different template concentrations and 0.2X dye concentration. For primer sets CCHFV-1, CCHFV-2, CCHFV-4 25 µl of reaction mixture was prepared. The dye concentration was 0.2X dye for each. Mixture, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11.5 µl nuclease free water, 2.5 µl primer mix, 2 µl synthetic template($10^6, 10^5, 10^4, 10^3, 10^2$, 10 copies/µl) included.

The experiment was repeated using 3.5 µl of primer mix. Mixture, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 10.5 µl nuclease free water, 3.5 µl primer mix, 2 µl synthetic template($10^6, 10^5, 10^4, 10^3, 10^2$, 10 copies/µl) included.

The LAMP experiment was tested with a double-strand full length S segment template and 2.5 µl of primer mix at 65°C. Reaction mix for each set contained, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11.5 µl nuclease free water, 2.5 µl primer mix, 2 µl double-strand full length S segment template(10^6-10^4 copies/µl). For CCHFV-2 LAMP primer set, experiment was repeated with using double-strand T2 DNA template that was extracted from RNA sample. In experiment two same template concentrations were used. In the experiment, temperature was increased to 67°C and it was repeated with using double-strand full length S segment.

The experiment was tested at different temperatures for the CCHFV-1 and CCHFV-4 primer sets. 63^0C, 65^0C and 67^0C were used. Reaction mix for each set contained, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11.5 µl nuclease free water, 2.5 µl primer mix, 2 µl double-strand full length S segment template(10^6 copies/µl). For CCHFV-1 primer set, experiment was repeated with 2.5 µl and 3.5 µl primer mix amount at 67°C respectively. For 65°C, the experiment was repeated with CCHFV-1 primer set under the 2.5 µl primer mix amount, 8 mM $MgSO_4$. For CCHFV-4 primer set, it was repeated at 63°C with using 2.5µl and 3.5µl primer mix amount. For 65°C, the experiment was repeated with using 2.5 µl primer mix amount and 8 mM $MgSO_4$.

The experiment was repeated at 65^0C with 3.5 µl primer mix for primer sets CCHFV-1 and CCHFV-4. Mixture, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$, 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 10.5 µl nuclease free water, 3.5 µl primer mix, 2 µl double-strand full length S segment template (10^6 copies/µl) included.

In the experiment, 6mM, 8mM and 10mM $MgSO_4$ concentrations were tested using 2.5 µl primer mix at 65^0C. Each reaction mixture totaled 25 µl. For 6mM $MgSO_4$ concentration, 2.5 µl 10X isothermal amplification buffer, 1 µl $MgSO_4$ (100mM), 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 12 µl nuclease free water, 2.5 µl primer mix, 2 µl of Full length S segment template(10^6 copies/µl) was used. For 8 mM $MgSO_4$ concentration, 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$(100 mM), 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11.5 µl nuclease free water, 2.5 µl primer mix, 2 µl Full length S segment template(10^6 copies/µl) was used. For 10mM $MgSO_4$ concentration, 2.5 µl 10X isothermal amplification buffer, 2 µl $MgSO_4$(100 mM), 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11 µl nuclease free water, 2.5 µl primer mix, 2 µl of Full length S segment template(10^6 copies/µl) was used.

CCHFV-1 and CCHFV-4 primer sets were tested with different template concentrations at 65^0C, using 2.5 µl primer mix amount and 8mM $MgSO_4$ concentration, and the limit of detection value was evaluated. Reaction mix 2.5 µl 10X isothermal amplification buffer, 1.5 µl $MgSO_4$(100 mM), 3.5 µl dNTP mix, 1 µl Bst 2.0 Warm Start DNA polymerase, 0.5 µl dye (10X), 11.5 µl nuclease free water, 2.5 µl primer mix, 2 µl double-strand full segment template($10^8, 10^6, 10^5, 10^4, 10^3$copies/µl) included.

3.6. qPCR (Quantitative Polymerase Chain Reaction)

The Light Cycler 480 SYBR Green I Master kit (Roche) was used for the quantitative PCR reaction. The kit includes Light Cycler 480 SYBR Green I Master mix (2X), water. The master mix mainly contains Fast Start Taq DNA Polymerase and DNA double-strand-specific SYBR Green I dye. Reagents were thawed on ice; the master mix was especially protected from light as it contains dye.

A total of 20 µl of reaction was prepared for each qPCR primer pair. 20 µl reaction contains 10 µl 2X Master mix, 1 µl reverse primer (10 µM), 1 µl forward primer (10 µM), 6 µl water, 2 µl double-strand full length S segment template ($10^8, 10^6$copies/µl). The PCR program steps are shown in Table 3.3. It is recommended that the annealing temperature be chosen below the melting temperature of the primers at 5 °C.

Table 3.3 qPCR program steps

steps	**temperature**	**time**	**Ramp rate**	**Cycle number**	**Acquisition mode**
Preincubation	95^0C	5 min	4.4	1 cycle	None
Denaturation	95^0C	10 sec	4.4	45 cycles	none
Annealing	52^0C	20 sec	2.2		none
Extension	72^0C	10 sec	4.4		single
Melting curve	95^0C	5 sec	4.4	1 cycle	none
	65^0C	1 min	2.2		None
	97^0C	-	-		Continuous (5 per ^{0}C)
Cooling	40^0C	10 sec	1.5	1 cycle	none

CHAPTER 4

RESULT AND DISCUSSION

4.1. S Segment of CCHFV Alignment

21 CCHFV strains from Turkey containing the full length S segment sequence downloaded from NCBI database and aligned in the Unipro UGENE program. Full length S segment of CCHFV is 1673 bp long. A representative image of the alignment window in the UGENE program is shown in Figure 4.1. The gray bar above the sequence alignment represents how conserved the sequence is at that particular nucleotide position shown in the alignment below. The small black box in the bigger black box is representing the specific alignment region shown above. With this representation, it is possible to compare the specific genome region shown above with the full sequence in terms of sequence conservation. The presence of white bars indicates the variation at that position in the sequence. The longer the white bars, the more nucleotide difference in the column it belongs to. Result of aligning, conserved regions were determined and targeted for primer design.

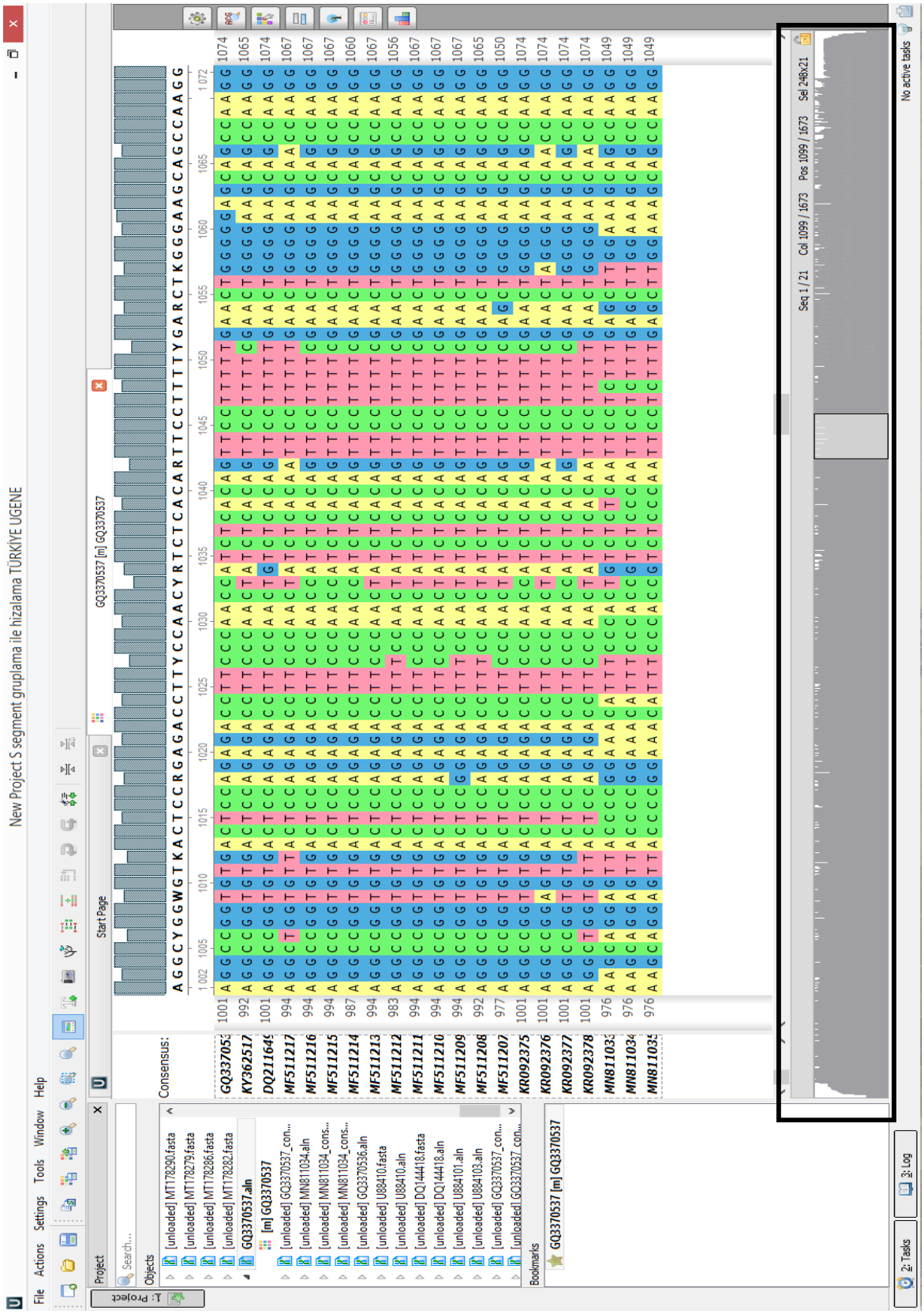


Figure 4.1 Sample Sequence Alignment in Unipro UGENE. Black frame indicates area graph of alignment result of full length S segment strains from Turkey

4.2. LAMP Primer Design

The stability, Tm values and GC% values of the 3' and 5' ends of the primers given by the Primer Explorer program were evaluated. In addition, each primer was evaluated by entering the '8mM' Mg^{+2} value in the Oligo Analyzer (Integrated DNA Technologies) Tool, the target type 'DNA' selected, and selecting 'default' as the parameter value. Primer sets targeting the conserved regions were selected and their secondary structures checked. While controlling the hairpin structures of the primers, it was desirable that the structures were degradable at 40^0C-45^0C and below. The ΔG values of the self-dimer and hetero-dimer structures formed were expected to be 7-8 times smaller than the ΔG value formed by the complement of the primers. At the same time, it was important that the primers did not recognize the tick genome or the genome of viruses from the same family in the NCBI database, as it was proof that they were specific to CCHF virus. In Table 4.1, primer sets obtained with strains other than MN811033, MN811034, MN811035 are shown. The primer sets provided by the Primer Explorer program for strains starting with MN are shown in Table 4.2. In tables, nucleotides expressed in red indicate the presence of nucleotides that differ at that location in alignment. The another primer group that was designed with collaborator Dr. Özlem YAREN is shown in Table 4.3. In the primer sets, which was designed using their software, red colors indicate different nucleotides at that position in alignment.

Primers that stood out in the evaluation were CCHFV-1, CCHFV-2, and CCHFV-4 primers designed with collaborator Dr. Özlem YAREN. Selected LAMP primer sets are shown in Table 4.4. The location of the LAMP primers on the MK309333 strain used in the experiment is shown in Figure 4.2, Figure 4.3, Figure 4.4, respectively. The sequence of strain MK309333 is 1371 bp in length and is partially reported in the NCBI nucleotide database. Therefore, the F3 primer of CCHFV-1 primer set could not be shown.

The aligned sequence of the region expected to amplify the LAMP primer sets (CCHFV-1, CCHFV-2, CCHFV-4) is shown in Figure 4.5, Figure 4.6 and Figure 4.7, respectively. In the figures, each color represents a nucleotide. As a result of the alignment, the different colors in each column represent the variation in the sequence. The regions shown in Figure 4.5, 4.6 and 4.7 are the most conserved regions within the full length S segment of CCHFV genome.

Table 4.1 Primer sets for strains except for MN811033, MN811034, MN811035. Red colors indicate presence of variation of primer sequences

	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
Set1	F3	CCAGTGAGCCGTGAACATG	19	629-647	-5.50	-4.21	-1.5	58	60.2
	F2	TGGAGTGGTGCAGGGAATT	19	648-666	-5.25	-4.01	-0.6	53	60.8
	F1	CCCCCAAGGTGGATTGAA	18	692-708	-5.96	-3.57	-7.2	53	55
	B1	CACTTGTTGCAACAGGCCTTGCCAAGCT	28	741-768	-4.66	-5.24	-4.7	54	72.1
	B2	CCACGGTCYTCTTAGCTTCG	20	799-818	-6.78	-6.03	-1.9	52,8	59.7
	B3	TGTGCTTGTCCAAATACCCA	20	829-848	-5.80	-5.09	-1	45	59.1

(cont. on next)

Table 4.1 (cont.)

	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
Set2	F3	CCAGTGAGCCGTGAACATG	19	629-647	-5.5	-4.2	-1.5	58	60.2
	F2	TGGAGTGGTGCAGGGAATT	19	648-666	-5.2	-4	-0.69	53	60.8
	F1	CCCCCAAGGTGGATTGAA	18	692-709	-6.8	-3.5	-7.2	56	58.3
	B1	TCAGGYCGTTCRGGAATAGCAC	22	722-743	-5.2	-4.9	-1.6	55	64
	B2	RACTCCTTTCCCCTCGGT	18	776-793	-4.8	-6	-0.8	56	59.3
	B3	CACGGTCYTCTTAGCTTCGT	20	798-817	-6.3	-5.1	-3	50	59.1
Set3	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
	F2	TGGAGTGGTGCAGGGAATT	19	648-666	-5.2	-4	-0.6	53	60.8

(cont. on next)

Table 4.1 (cont.)

Set 3	F1	TGTCCCCCCAAGGTGGATTGAA	22	692-713	-5.8	-3.5	-7.2	55	65.8
	B1	TCAGGYCGTTCRGGAATAGCAC	22	722-743	-5.2	-4.9	-1.6	55	64.3
	B2	ACTCCTTTCCCCTCGGTC	18	775-792	-5	-6.04	-0.8	61	59.7
	B3	CACGGTCYTCTTAGCTTCGT	20	798-817	-6.3	-5.1	-3	50	59.1

Table 4.2 Primer sets of MN811033, MN811034, MN811035 strains. Red colors indicate presence of variation of primer sequences

	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
MN set4	F3	TTGCACTTGTCGCAACCG	18	738-755	-5.5	-5.9	-5	56	60.6
	F2	GCAGAGACTGAGGGTAAAGG	20	770-789	-5.5	-3.6	-2.04	55	59
	F1	TTTATCAAGGTAATCCTTGAG	21	824-844	-2.7	-4.3	-8.6	33	52.2

(cont. on next)

Table 4.2 (cont.)

	B1	GAAGTCGACAAGGCAAGTGCT	21	854-874	-4.3	-5.6	-4.3	52	62.7
	B2	AGCCTTGGCAATGTGTTTCA	20	897-916	-5.5	-4.1	-4.5	45	60.4
	B3	GCACGAAGTGCAGATGAGTT	20	932-951	-6.5	-4.4	-6.4	50	60.3
	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
MN set5	F3	TTCAATCCACCTTGGGGC	18	692-709	-3.5	-7.2	-4.2	56	59
	F2	ATCAACAARTCAGGTCGCTC	20	713-732	-4.0	-6.2	-0.3	50	59.5
	F1	TCTCTGCCAGCTTGGCAAGG	20	757-776	-4.6	-5.8	-9.1	60	64.6
	B1	RGCTAAGAAGACTGTGGAGGCR	22	802-823	-4.9	-6.8	-2.0	59	64.6
	B2	GCCTTGTCGACTTCMTCTCT	20	848-867	-5.8	-4.4	-0.3	55	60.8
	B3	TCACCATGTTGTCAGCACTT	20	868-887	-5.4	-5.4	-2.2	45	59.4

(cont. on next)

Table 4.2 (cont.)

	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
MN set6	F3	CCAGTRAGCC GTGAACATG	19	629-647	-5.5	-4.2		58	60.3
	F2	TTGARTGGTG YAGGGAGTT	19	648-666	-5.2	-4.0	0.8	53	60.9
	F1	TGTCCCCCCA AGGTGGATTG AA	22	692-713	-5.8	-3.5	-7.2	55	65.9
	B1	TCAGGCCGTT CAGGAATAGC AC	22	722-743	-5.2	-4.9	-3.1	55	64.4
	B2	ACMCCTTTAC CCTCAGTC	18	775-792	-5.0	-6.0	0.4	61	59.7
	B3	CACAGTCTTC TTAGCYTCCT	20	798-817	-6.3	-5.1	1.1	50	59.1
	Primer name	Primer sequences	Length	Position of primer	5'ΔG	3'ΔG	hairpin ΔG	% GC	Tm
MN set7	F3	TCGAGGARG CTAAGAAGA CT	20	795-814	-5.8	-4.2	-2.7	50	59
	F2	AGGCRCTCA AGGATTACCT T	20	819-838	-7.7	-4.0	-3	50	61

(cont. on next)

Table 4.2 (cont.)

	F1	GTTGTCAGCACTTGCCTTGTCG	22	859-880	-4.6	-5.3	-2.5	55	64.2
	B1	ATTGCCAAGGCTCAAGAGCTCT	21	905-926	-5.4	-5.3	-4.4	50	64.2
	B2	TGAGCTGAAGGGAGTGTCA	19	967-985	-5.4	-5	-3	53	59.7
	B3	TTCCGGGGTAACTCCTGC	18	1004-1021	-6.1	-6.1	-5.1	61	60.4

Table 4.3 Primer sets designed by Dr.Özlem YAREN. Red colored letters indicate nucleotides combinations; R:A/G, Y:C/T.

	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCHF V-1	F3	GATCGAGGTGAACAGC	67	82	16	-1.9	56	59.8
	FIP	GGCCATCTGGAACACAAACTTTTT+GACTTATGGACACTTTCAC	129	147	19	-6.4	42	59.2
	F2	GACTTATGGACACTTTCAC	129	147	19	-1.7	42	59.2
	LF	CATTCTCACAAAAGGAGTAGGAG	173	151	23	-4.8	44	65.1
	F1	GTTTGTGTTCCAGATGGCC	190	208	19		53	65.4

(cont. on next)

Table 4.3 (cont.)

	B1	TGGARTCCTTCTGTGCATC	239	221	19		47	63.6
	LB	TGCATCGGCTYTRGTG	244	259	16	-2.9	62	65.1
	BIP	GATGCACAGAAGGAYTCCATTTTT+GGTGCRCARAACTTGG	282	267	16	-7.3	56	60.7
	B2	GGTGCRCARAACTTGG	282	267	16	-2.6	56	60.7
	B3	TGACCCAAGCACATTC	305	290	16		50	59.6
	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCHFV-2	F3	CTGGYGAAATTGTGATG	531	547	17	2	47	59.5
	FIP	GACAAATTCCCTGCACCACTTTTT+TAATTCTCAACAGGRGC	591	607	17	-3	47	59.3
	F2	TAATTCTCAACAGGRGC	591	607	17	0.3	47	59.3
	LF	CGCGTGGATTTTCAYCACC	626	608	19	-2.4	53	65.8
	F1	GTGGTGCAGGGAATTTGTC	652	670	19		53	65.4
	B1	CCCCAAGGTGGATTGAARG	708	690	19		53	64.5

(cont. on next)

Table 4.3 (cont.)

	LB	GGACATCAACAAATCAGGYC	709	728	20	-0.5	50	65.6
	BIP	CYTTCAATCCACCTTGGGGTTTTT+CAACAAGTGCTATTCCYG	749	732	18	-5.7	44	59.2
	B2	CAACAAGTGCTATTCCYG	749	732	18	-0.2	44	59.2
	B3	CAAARACTCCTTTYCCC	797	781	17	2	53	60
	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCHFV-3	FIP	GRCCTGATTTGTTGATGTCCTTTTT+GAGTGGTGCAGGGAAT	650	665	16	-6.5	56	61.6
	F2	GAGTGGTGCAGGGAAT	650	665	16	-0.6	56	61.6
	LF	GCCATGATRTACTTGCCTTTG	690	670	21	-0.4	48	66.3
	F1	GGACATCAACAAATCAGGYC	709	728	20		50	65.6
	B1	TTGGCAAGGCCTGTTG	765	750	16		56	63.4
	LB	CAGAGACCGAGGGRAAAG	771	788	18	0.1	61	65.1

(cont. on next)

Table 4.3 (cont.)

	BIP	CAACAGGCCTTGCCAATTTTT+TCTTAGCTTCGTCAAARAC	809	791	19	-5.3	42	60.8
	B2	TCTTAGCTTCGTCAAARAC	809	791	19	0.2	42	60.8
	B3	TGYTTGTCYAAATAYCC	846	830	17	0.5	47	60
	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCHFV-4	FIP	GTTCCAAAGCAGACACCCTTTTT+TCTAYATGCAYCCTGC	1173	1188	16	-3.8	56	61.7
	F2	TCTAYATGCAYCCTGC	1173	1188	16	-2	56	61.7
	LF	TCACTGATTCTACCRGCTG	1215	1197	19	0.5	53	65.2
	F1	GGGTGTCTGCTTTGGAAC	1219	1236	18		56	64.6
	B1	ATCCYTGAGCAGCRTC	1274	1259	16		62	64.5
	LB	CYAAGTCCATYCTCAACCTTC	1284	1304	21	0.5	48	65.1
	BIP	GAYGCTGCTCARGGATTTTTT+GTCTCTGTGCTTGTYC	1320	1305	16	-10	56	59.7
	B2	GTCTCTGTGCTTGTYC	1320	1305	16	-0.1	56	59.7
	B3	ATTGTCTTGGCRCATG	1344	1329	16	-2.5	50	60.9

Table 4.4 LAMP Primer sets that were selected. Nucleotide variations are shown with red letters for each primer sequences.

	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCH FV-1	F3	GATCGAGGTGAACAGC	67	82	16	-1.9	56	59.8
	FIP	GGCCATCTGGAACACAAACTTTTT+GACTTATGGACACTTTCAC	129	147	19	-6.4	42	59.2
	F2	GACTTATGGACACTTTCAC	129	147	19	-1.7	42	59.2
	LF	CATTCTCACAAAAGGAGTAGGAG	173	151	23	-4.8	44	65.1
	F1	GTTTGTGTTCCAGATGGCC	190	208	19		53	65.4
	B1	TGGARTCCTTCTGTGCATC	239	221	19		47	63.6
	LB	TGCATCGGCTYTRGTG	244	259	16	-2.9	62	65.1
	BIP	GATGCACAGAAGGAYTCCATTTTT+GGTGCRCARAACTTGG	282	267	16	-7.3	56	60.7
	B2	GGTGCRCARAACTTGG	282	267	16	-2.6	56	60.7
	B3	TGACCCAAGCACATTC	305	290	16		50	59.6

(cont. on next)

Table 4.4 (cont.)

	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCH FV-2	F3	CTGGYGAAATTGTGATG	531	547	17	2	47	59.5
	FIP	GACAAATTCCCTGCACCACTTTTT+TAATTCTCAACAGGRGC	591	607	17	-3	47	59.3
	F2	TAATTCTCAACAGGRGC	591	607	17	0.3	47	59.3
	LF	CGCGTGGATTTTCAYCACC	626	608	19	-2.4	53	65.8
	F1	GTGGTGCAGGGAATTTGTC	652	670	19		53	65.4
	B1	CCCCAAGGTGGATTGAARG	708	690	19		53	64.5
	LB	GGACATCAACAAATCAGGYC	709	728	20	-0.5	50	65.6
	BIP	CYTTCAATCCACCTTGGGGTTTTT+CAACAAGTGCTATTCCYG	749	732	18	-5.7	44	59.2
	B2	CAACAAGTGCTATTCCYG	749	732	18	-0.2	44	59.2
	B3	CAAARACTCCTTTYCCC	797	781	17	2	53	60

(cont. on next)

Table 4.4 (cont.)

	Primer Name	Primer Sequence	Start Pos	End Pos	Length	hairpin ΔG	GC %	Tm
CCH FV-4	F3	CCTGAGCACTCCAATG	1099	1114	16	-0.1	56	59.7
	FIP	GTTCCAAAGCAGACACCCTTTTT+TCTAYATGCAYCCTGC	1173	1188	16	-3.8	56	61.7
	F2	TCTAYATGCAYCCTGC	1173	1188	16	-2	56	61.7
	LF	TCACTGATTCTACCRGCTG	1215	1197	19	0.5	53	65.2
	F1	GGGTGTCTGCTTTGGAAC	1219	1236	18		56	64.6
	B1	ATCCYTGAGCAGCRTC	1274	1259	16		62	64.5
	LB	CYAAGTCCATYCTCAACCTTC	1284	1304	21	0.5	48	65.1
	BIP	GAYGCTGCTCARGGATTTTTT+GTCTCTGTGCTTGTYC	1320	1305	16	-10	56	59.7
	B2	GTCTCTGTGCTTGTYC	1320	1305	16	-0.1	56	59.7
	B3	ATTGTCTTGGCRCATG	1344	1329	16	-2.5	50	60.9

The concentration of FIP, BIP, F3, B3 and LP, LB in 10X primer mixture are 16 µM FIP, 16 µM BIP, 5 µM LF, 5µM LB, 2 µM F3, 2 µM B3, respectively.

TGGAACAATGGGTTTGAGGAGTTTAAAAAAGGAAATGGACTTATGGACACTTTCACAAA
CTCCTACTCCTTTTGTGAGAATGTACCAAATCTGGATAAGTTTGTGTTCCAGATGGCCAGC
GCCACGGATGATGCACAGAAGGACTCCATCTATGCATCGGCTCTAGTGGAAGCAACCAA
GTTCTGTGCACCCATATATGAATGTGCTTGGGTCAGCTCTACTGGCATTGTGAAGAAGGG

Figure 4.2 CCHFV LAMP primer set-1 on the MK309333 strain. Primers are placed between in 1-221 nucleotides and amplified part length is 239 bp. Colored parts express primer regions on the sequence. Yellow part indicates F2 primer, Red part indicates F1 primer, Gray part indicates complementary of B1 primer, Pink part indicates complementary of B2 primer, Cyan part indicates complementary of B3 primer.

TCCTTGCAGAATACAAAGTCCCTGGTGAAATTGTGATGTCTGTTAAAGAAATGCTGTCAG
ATATGATTAGAAGGAGGAATCTAATTCTCAACAGGGGCGGTGATGAAAATCCACGCGGC
CCAGTGAGCCGTGAACATGTGGAGTGGTGCAGGGAATTTGTCAAAGGCAAGTACATCAT
GGCTTTCAATCCACCTTGGGGGGACATCAACAAATCAGGTCGTTCGGGAATAGCACTTGT
TGCAACAGGCCTTGCCAAGCTTGCAGAGACCGAGGGGAAAGGAGTTTTTGACGAAGCTA

Figure 4.3 CCHFV LAMP primer set-2 on the MK309333 strain. Primers are placed between in 440-728 nucleotides and amplified part length is 267 bp. Colored parts express primer regions on the sequence. Green part indicates F1 primer, Yellow part indicates F2 primer, Red part indicates F1 primer, Gray part indicates complementary of B1 primer, Pink part indicates complementary of B2 primer, Cyan part indicates complementary of B3 primer.

TTTTCGAACTGGGGAAGCAGCCAAGGGGGACCAAAAAAATGAAAAAGGCACTCCTGAGC
ACTCCAATGAAGTGGGGGAAGAAACTTTATGAGCTCTTTGCTGATGACTCTTTCCAGCAG
AACAGAATCTACATGCACCCTGCTGTGTTGACAGCCGGTAGAATCAGTGAAATGGGTGTC
TGCTTTGGAACAATTCCTGTTGCCAATCCTGATGATGCTGCTCAGGGATCTGGACATACCA
AGTCCATTCTCAACCTTCGGACAAGCACAGAGACCAACAATCCATGCGCCAAAACGATTG

Figure 4.4 CCHFV LAMP primer set-4 on the MK309333 strain. Primers are placed between in 950-1315 nucleotides and amplified part length is 246 bp. Colored parts express primer regions on the sequence. Green part indicates F1 primer, Yellow part indicates F2 primer, Red part indicates F1 primer, Gray part indicates complementary of B1 primer, Pink part indicates complementary of B2 primer, Cyan part indicates complementary of B3 primer and the sequence of the B3 primer differs from the nucleotides shown in red in the cyan region.

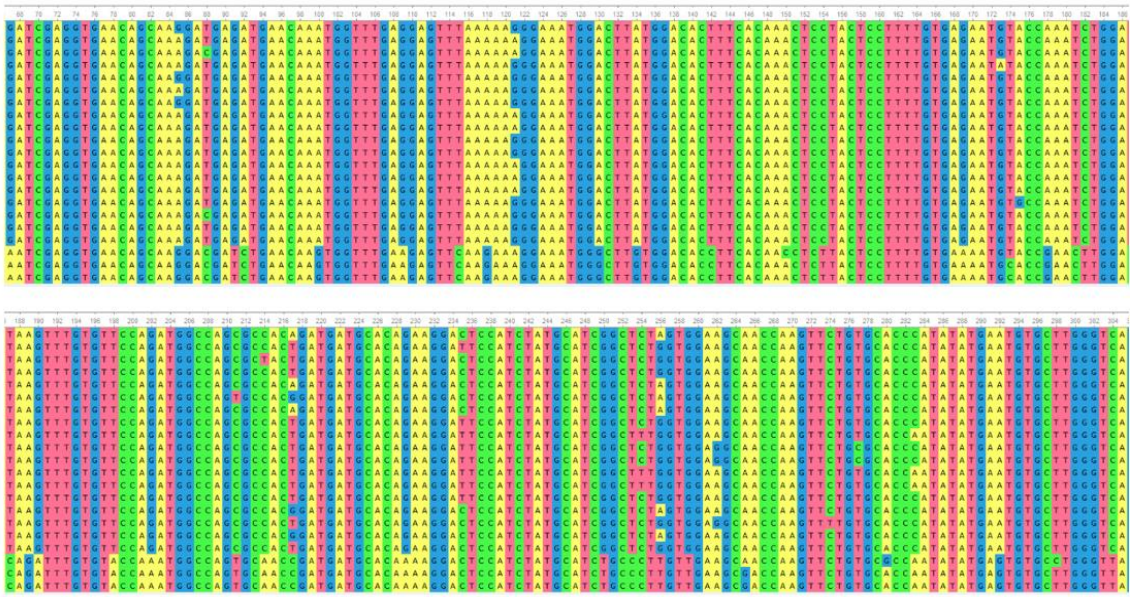


Figure 4.5 CCHFV LAMP primer set-1 target region on aligned CCHFV S Segment sequences. It is located between 67-305 nucleotides.

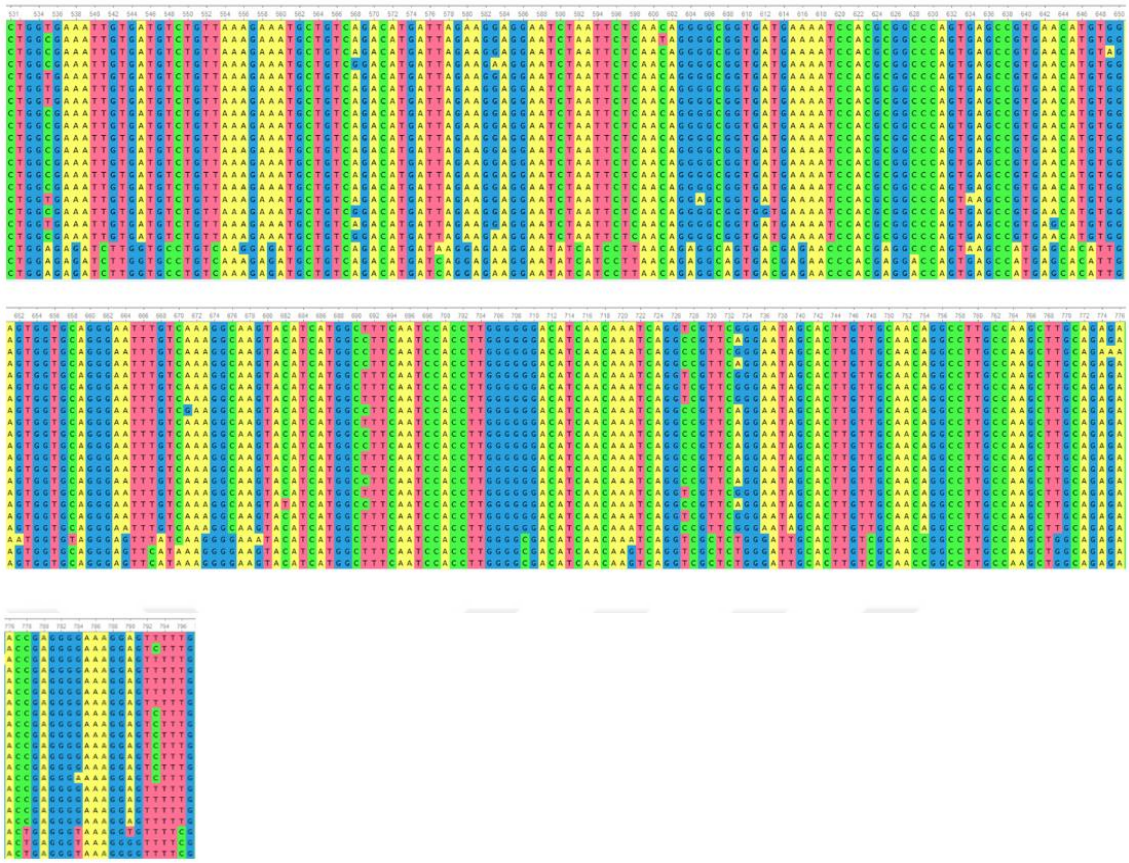


Figure 4.6 CCHFV LAMP primer set-2 target region on aligned CCHFV S Segment sequences. It is located between 531-797 nucleotides.

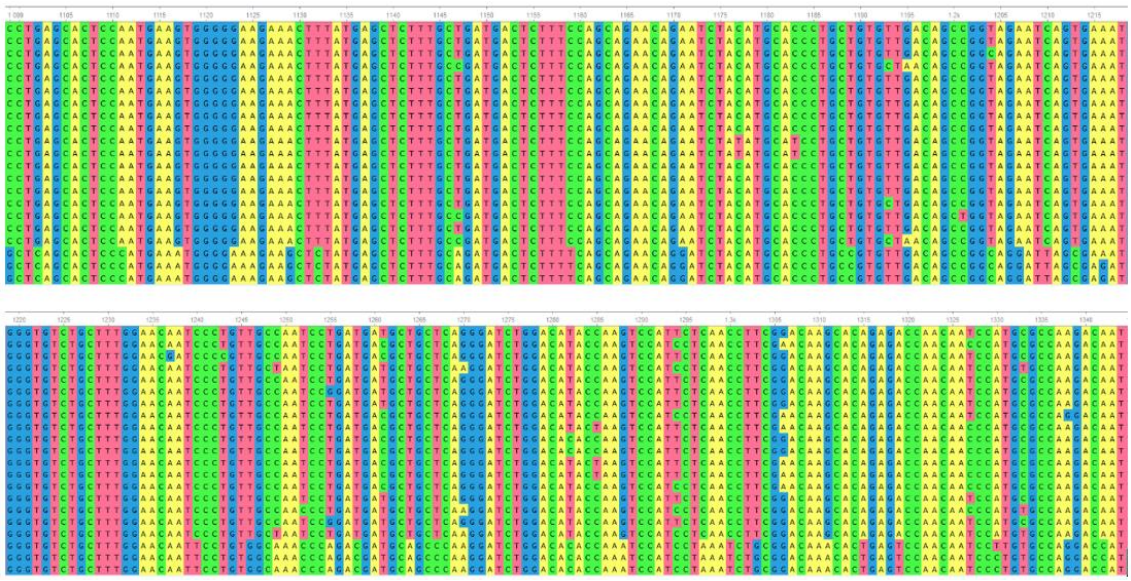


Figure 4.7 CCHFV LAMP primer set-4 target region on aligned CCHFV S Segment sequences. It is located between 1099-1344 nucleotides.

4.3. qPCR Primer Design

The ideal Tm values of the designed primers are considered to be between 60-62^{0}C, the ideal GC% ratio is expected to be close to 50%, and the primer sequence lengths should not exceed 18-30 bases. The dimer and hairpin structures and Tm values of the primers were checked in the Oligo Analyzer Tool (IDT). Dissolution of hairpin structures at a maximum of 40^{0}C was important in determining suitable primers. The results were analyzed under a concentration of 3mM Mg^{+2}, 0.5 mM dNTP, 0.2 µM oligo, 50 mM Na^+. The specificity of the primers was checked in the NCBI Primer Blast database. The sets obtained for qPCR are shown in Table 4.5, Table 4.6 and Table 4.7.

Q1 qPCR primer sets were evaluated and self-dimer ∆G values of set-1 primers were high, hairpin Tm values of set-2 primers were above 40°C, matched to NCBI tick genome for set-4 primers, Tm values of set-5 primers were above 40°C and self-dimer ∆G values were high. Therefore, set-3 primers were selected as the appropriate set. Q2 qPCR primer sets were evaluated and set 1 primers had high self-dimer ∆G values, set 5 primers gave a match to the tick genome in NCBI. The primers with the lowest percentage of GC in the other sets were set-3 primers and were determined as the appropriate set.

Q4 qPCR primer sets were evaluated and none matched the tick genome. Set-4 primers had high self-dimer ∆G values, so they were eliminated. Among the remaining sets, the primers with low Tm values and low GC percentage were set-3 primers. Set-3 primers were selected as the appropriate set. The sets shown in Table 4.8, Table 4.9 and Table 4.10 were selected.

Table 4.5 qPCR primer sets of region by CCHFV-1 primer set amplified

		Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Q1	Assay Set 1	Forward Primer	ACACTTTCACAAACTCCTACTCC	72	23	62.3	43.4	
		Reverse Primer	TATGGGTGCACAGAACTTGG	220	20	62.1	50	
		Product						149
	Assay Set 2	Forward Primer	TGAGATGAACAAATGGTTTGAGG	22	23	61.5	39.1	
		Reverse Primer	CCATCTGGAACACAAACTTATCC	140	23	61.3	43.4	
		Product						119
	Assay Set 3	Forward Primer	AGCAAAGATGAGATGAACAAATGG	14	24	62.1	37.5	
		Reverse Primer	AGTAGGAGTTTGTGAAAGTGTCC	92	23	62.3	43.4	
		Product						79
	Assay Set 4	Forward Primer	GATAAGTTTGTGTTCCAGATGGC	119	23	61.8	43.4	
		Reverse Primer	CACAGAACTTGGTTGCTTCC	212	20	61.3	50	
		Product						94
	Assay Set 5	Forward Primer	CCAAATCTGGATAAGTTTGTGTTCC	110	25	62.3	40	
		Reverse Primer	CAGAGCCGATGCATAGATGG	190	20	62.1	55	
		Product						81

Table 4.6 qPCR primer sets of region by CCHFV-2 primer set amplified

		Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Q2	Assay Set 1	Forward Primer	TGCTGTCAGACATGATTAGAAGG	31	23	62.2	43.4	
		Reverse Primer	AAATTCCCTGCACCACTCC	137	19	62.2	52.6	
		Product						107
	Assay Set 2	Forward Primer	GTGCAGGGAATTTGTCAAAGG	125	21	61.9	47.1	
		Reverse Primer	CAACAAGTGCTATTCCTGAACG	219	22	61.6	45.4	
		Product						95
	Assay Set 3	Forward Primer	AGGAGGAATCTAATTCTCAACAGG	51	24	61.8	41.6	
		Reverse Primer	ACTTGCCTTTGACAAATTCCC	150	21	61.6	42.8	
		Product						100
	Assay Set 4	Forward Primer	TGAGCCGTGAACATGTGG	103	18	62.0	55.5	
		Reverse Primer	GGCCTGATTTGTTGATGTCC	198	20	61.2	50	
		Product						96
	Assay Set 5	Forward Primer	GTCAAAGGCAAGTACATCATGG	138	22	61.5	45.4	
		Reverse Primer	CCTCGGTCTCTGCAAGC	252	17	61.6	64.7	
		Product						115

Table 4.7 qPCR primer sets of region by CCHFV-4 primer set amplified

		Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Q4	Assay Set 1	Forward Primer	GCCGGTAGAATCAGTGAAATGG	101	22	63	50	
		Reverse Primer	CTTGTCCGAAGGTTGAGAATGG	213	22	62.9	50	
		Product						113
	Assay Set 2	Forward Primer	GTGTCTGCTTTGGAACAATCC	123	21	61.6	47.6	
		Reverse Primer	TTGGTCTCTGTGCTTGTCC	225	19	61.7	52.6	
		Product						103
	Assay Set 3	Forward Primer	CTCTTTGCTGATGACTCTTTCC	41	22	60.9	45.4	
		Reverse Primer	CACCCATTTCACTGATTCTACC	125	22	60.8	45.4	
		Product						85
	Assay Set 4	Forward Primer	GGAAGAAACTTTATGAGCTCTTTGC	24	25	62.5	40	
		Reverse Primer	CAACAGGGATTGTTCCAAAGC	149	21	61.9	47.6	
		Product						126
	Assay Set 5	Forward Primer	GAAATGGGTGTCTGCTTTGG	116	20	61.4	50	
		Reverse Primer	ATTGTCTTGGCGCATGG	246	17	60.3	52.9	
		Product						131

Among the qPCR primers designed for the region amplified by the CCHFV-1 LAMP primer set, the most suitable one was shown in Table 4.8. In other primer pairs, the degradation of hairpin and dimer structures of some primers required a temperature of more than 45^0C.

Table 4.8 Q1 primer set was produced according to CCHFV-1 amplified region

	Type	Sequence	Start	Length	Tm	GC Percent	Amp licon
Assay Set 3 (Q1)	Forward Primer	AGCAAAGATGAG ATGAACAAATGG	14	24	62.1	37.5	
	Reverse Primer	AGTAGGAGTTTG TGAAAGTGTCC	92	23	62.3	43.4	
	Product						79

Primers targeting the region amplified by the CCHFV-2 LAMP primer set, the appropriate primer pair was shown in Table 4.9. The lower GC% rate among the other primer pairs, the absence of any match with the tick genome, and the deterioration of self-dimer structures below 40^0C were factors in the selection of the primer pair.

Table 4.9 Q2 primer set was produced according to CCHFV-2 amplified region

	Type	Sequence	Start	Length	Tm	GC Percent	Amp licon
Assay Set 3 (Q2)	Forward Primer	AGGAGGAATCTA ATTCTCAACAGG	51	24	61.8	41.6	
	Reverse Primer	ACTTGCCTTTGA CAAATTCCC	150	21	61.6	42.8	
	Product						100

Among the primer pairs targeting the region amplified by the CCHFV-4 LAMP set, the most suitable one was shown in Table 4.10. It was chosen because its Tm values were at 61^{0}C, hairpin and dimer structures were soluble below 40^{0}C, and that it did not match the tick genome or other virus genome as a result of the blast.

Table 4.10 Q4 primer set was produced according to CCHFV-4 amplified region

	Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Assay Set 3 (Q4)	Forward Primer	CTCTTTGCTGATGACTCTTTCC	41	22	60.9	45.4	
	Reverse Primer	CACCCATTTCACTGATTCTACC	125	22	60.8	45.4	
	Product						85

4.4. Template DNA Synthesis Suitable for Primers

4.4.1. cDNA Synthesis

Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific) was used for cDNA synthesis. Kit contains that random hexamer primer (100 µM), oligo(dT) primers 100 µM, nuclease free water, Revert Aid reverse transcriptase (200 U/µl), RiboLock RNase inhibitor (20 U/µl), 10Mm dNTP mix, 5X Reaction buffer (250 mM Tris-HCL (ph:8.3), 250 mM KCL, 20 mM MgCl2, 50 mM DTT), Forward GAPDH Primer 10 µM, Reverse GAPDH Primer 10 µM, Control GAPDH RNA 0.05 µg/µL.

4.4.2. PCR

It was aimed to obtain a full-length S segment as a result of the PCR reaction, and the reaction contained 2 µl of cDNA product, 2.5 µl of CCH S all forward primer (10 mM), 2.5 µl of CCH S, all using reverse primer (10 mM) (Table 4.11). Since the full S segment of CCHF virus is 1673 bp in length, a band was expected to be seen at this position in the gel. As expected, bands between 1500 bp and 2000 bp in Figure 4.9 indicate acquisition of the CCHF virus full-length S segment.

To obtain the target region of the CCHFV-2 LAMP primer set, 1 µl of cDNA product, 1 µl of Q1-forward primer (Table 4.8) and 1 µl of Q4-reverse primer (Table 4.10) were used in the PCR reaction. The region length to be amplified was expected to be 1.2 kb. In the agarose gel, the bands were visible although pale (Fig. 4.8). The PCR result was extracted from the 1.5% agarose gel for the double-stranded T2 template and the double-strand full-length S segment (Figure 4.8 and Figure 4.9).

Table 4.11 Primers for full length S segment

Primer	Sequence	Tm (^{0}C)	GC percent (%)
CCH_S all Forward	TCAAAGAAACACGTGCCGCT	66	50
CCH_S all Reverse	TCTCAAAGATATCGTTGCCGCA	65.3	45.5

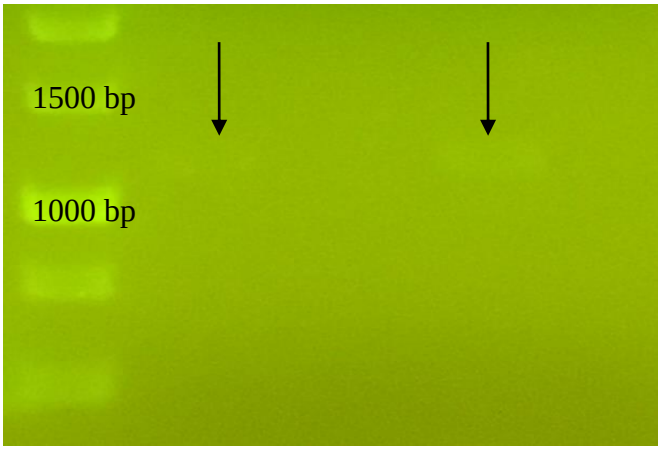


Figure 4.8 Double-strand T2 template of CCHFV on the Agarose gel. Black arrows indicate light bands of obtained 1.2 kb length double-strand T2 template

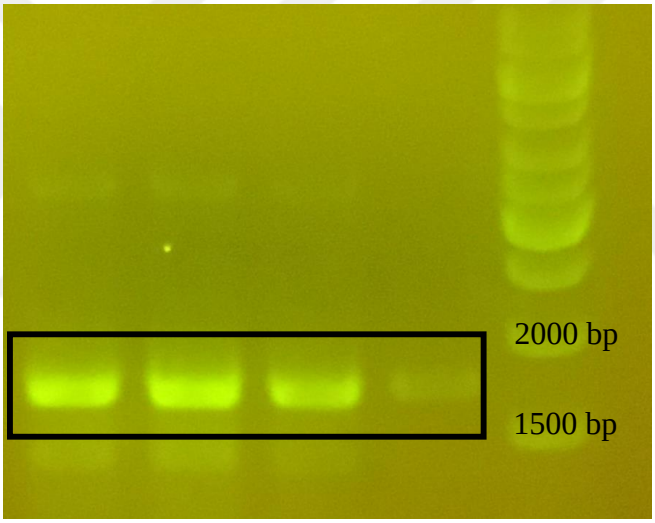


Figure 4.9 Full length S segment of CCHFV on the agarose gel

For extraction, double-strand full length S segment was extracted using the Geneaid Gel Extraction Kit protocol and DNA concentration was determined with the Nanodrop device. In the Nanodrop device, the Nucleic acid tab was selected when the screen was opened to measure, then the nucleic acid type was selected as DNA. First, 2 µl of water was used to optimize the measurement, then 2 µl of the DNA sample was placed at the point to be measured. Full length S segment concentration was measured as 16.26 ng/µl ($8.9x10^9$ copies/ul) and double-strand T2 template concentration was measured as 131.4 ng/µl ($1.04x\ 10^{11}$copies/ul).

4.5. Fluorimetric LAMP

In fluorimetric LAMP experiments, different dye concentrations, primer mix amount and minimum detectable template concentrations were tested with synthetic templates. The double-strand full length S segment of CCHF virus obtained from the RNA sample and for CCHFV-2 LAMP primer set, additionally double-strand T2 template were tested as a template in the experiments. Fluorimetric LAMP reaction was carried out at different temperatures, different primer concentrations and different $MgSO_4$ concentrations to determine the optimum reaction conditions. Tested reaction temperatures were 63^0C, 65^0C, 67^0C. 2.5 µl and 3.5 µl of 10x primer mix concentrations were tested. And also, 6mM, 8Mm, 10 mM $MgSO_4$ concentrations were tested to determine the optimum $MgSO_4$ amount for the reaction.

$MgSO_4$ supports the bond formed between the phosphate group of dNTPs and the 3'-OH end of the primer in polymerization, and also facilitates the binding of the primer to DNA by neutralizing the negative charges on the DNA.

Bst 2.0 Warm Start DNA (NEB) polymerase Pack was used in the experiment. Only three sets of LAMP primers were used for the LAMP Fluorescent assay, CCHFV-1, CCHFV-2, CCHFV-4.

4.5.1. Determination of Dye Concentration for LAMP Primer Sets

In experiment to determine the optimum dye concentration was performed. The experiment was performed with primer sets CCHFV-1, CCHFV-2 and CCHFV-4 and synthetic templates of primer sets. 0.2X and 0.5X Sybr Green dye concentrations were tested. For negative control, water was used instead of template. Each LAMP primer set (CCHFV-1 , CCHFV-2, CCHFV-4) reaction contained, for 0.2X Sybr Green dye concentration, 0.5 µl dye (10X), 2.5 µl primer mix, 8mM $MgSO_4$ and 2 µl synthetic templates (10^6 copies/µl). 0.5X Sybr Green dye concentration, 1.25 µl dye (10X), 2.5 µl primer mix, 8mM $MgSO_4$ and 2 µl synthetic templates (10^6 copies/µl). The graphs are shown in Figure 4.10, 4.11, 4.12, respectively.

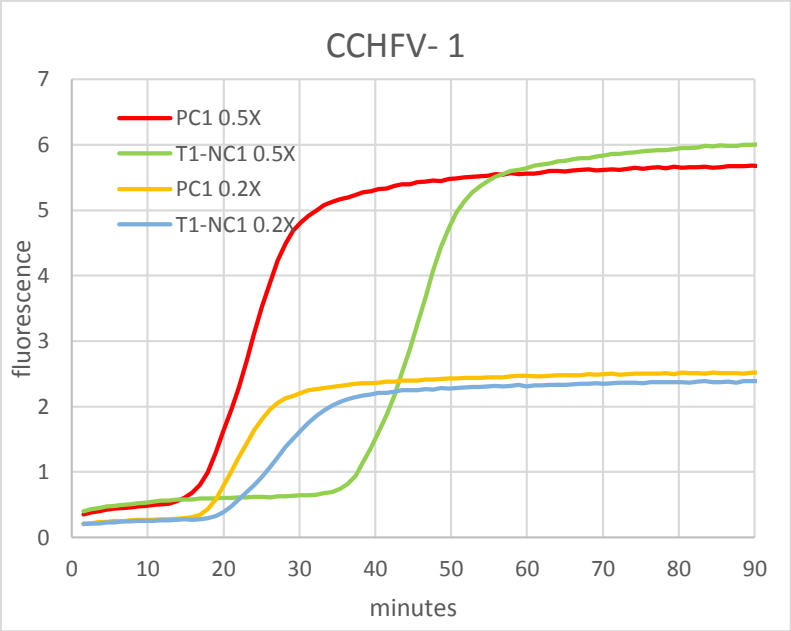


Figure 4.10 Result of CCHFV LAMP Primer Set-1 with 0.2X and 0.5X Dye Concentration

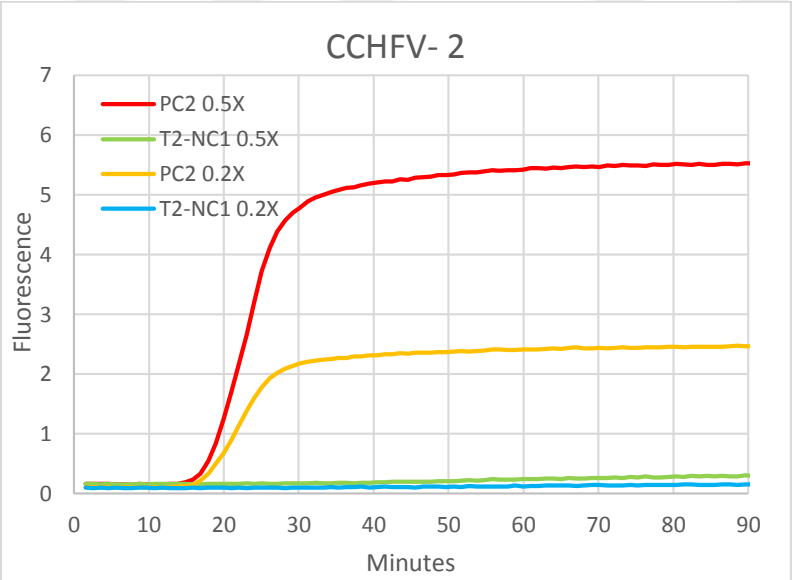


Figure 4.11 Result of CCHFV LAMP Primer Set-2 with 0.2X and 0.5X Dye Concentration

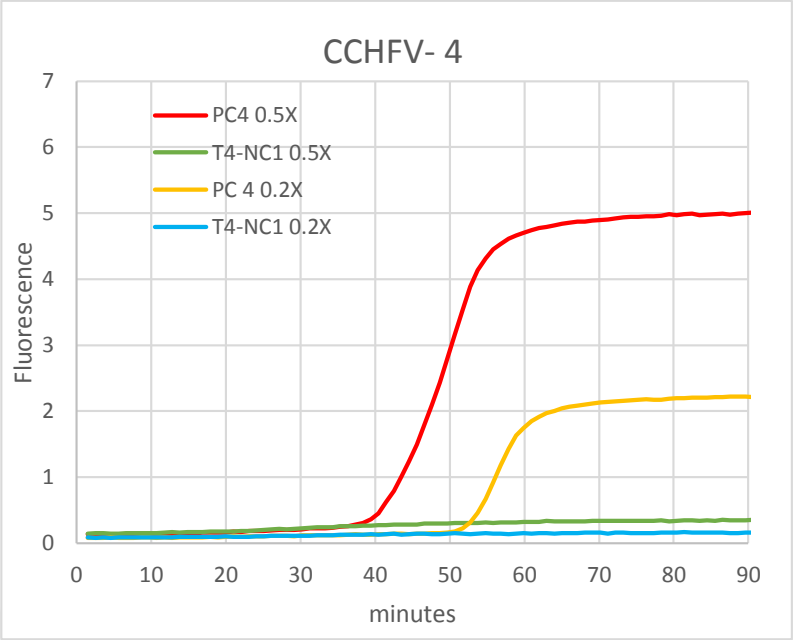


Figure 4.12 Result of CCHFV LAMP Primer Set-4 with 0.2X and 0.5X Dye Concentration

At both 0.5X and 0.2X dye concentrations detectable fluorescence signal from primer set-1 was observed around 18-20 minutes. There is almost no difference in signal times, so a concentration of 0.2X dye was considered suitable for the CCHFV-1 LAMP set. Signal from 0.2X dye negative control samples (Figure 4.10, blue curve) was around 20 minutes. This might be due to template contamination. The signal times for the CCHFV-2 LAMP set were quite close and the signal was seen at 18 minutes. Therefore, a dye concentration of 0.2X was chosen. For the CCHFV-4 LAMP set, the signal for both dye concentrations detected later than expected. The results were evaluated, 0.2X concentration was chosen for the LAMP primer sets.

4.5.2. Testing LAMP Primer Sets with Synthetic Templates

CCHFV-1, CCHFV-2, CCHFV-4 LAMP primer sets were tested at 65°C with different template concentrations. 0.2X dye concentration was used. For each LAMP Primer sets (CCHFV-1, CCHFV-2, CCHFV-4), mixture contained 2.5 µl primer mix, 8mM $MgSO_4$ and 2 µl synthetic template ($10^6, 10^5, 10^4, 10^3, 10^2$, 10 copies/µl).

The results are shown in Figure 4.13(a), (b), (c) respectively. Results demonstrated that in the CCHFV-1 set, for $2x10^6$copies/µl, the signal was seen in 15 minutes but the signal for other concentrations were seen after 1 hour. Especially the simultaneous occurrence of the signal for $2x10^4$ copies/µl and 20 copies/µl showed that the result is not reliable for these concentrations (Figure 4.13(a)). In CCHFV-2 LAMP set, for $2x10^6$ copies/µl concentration, the signal was taken in 15 minutes, but the signal of $2x10^5$ copies/µl was determined in 1 hour (Figure 4.13(b)). In the CCHFV-4 LAMP set, the signal was seen only for $2x10^6$ copies/µl and determined in 50 minutes (Figure 4.13(c)).

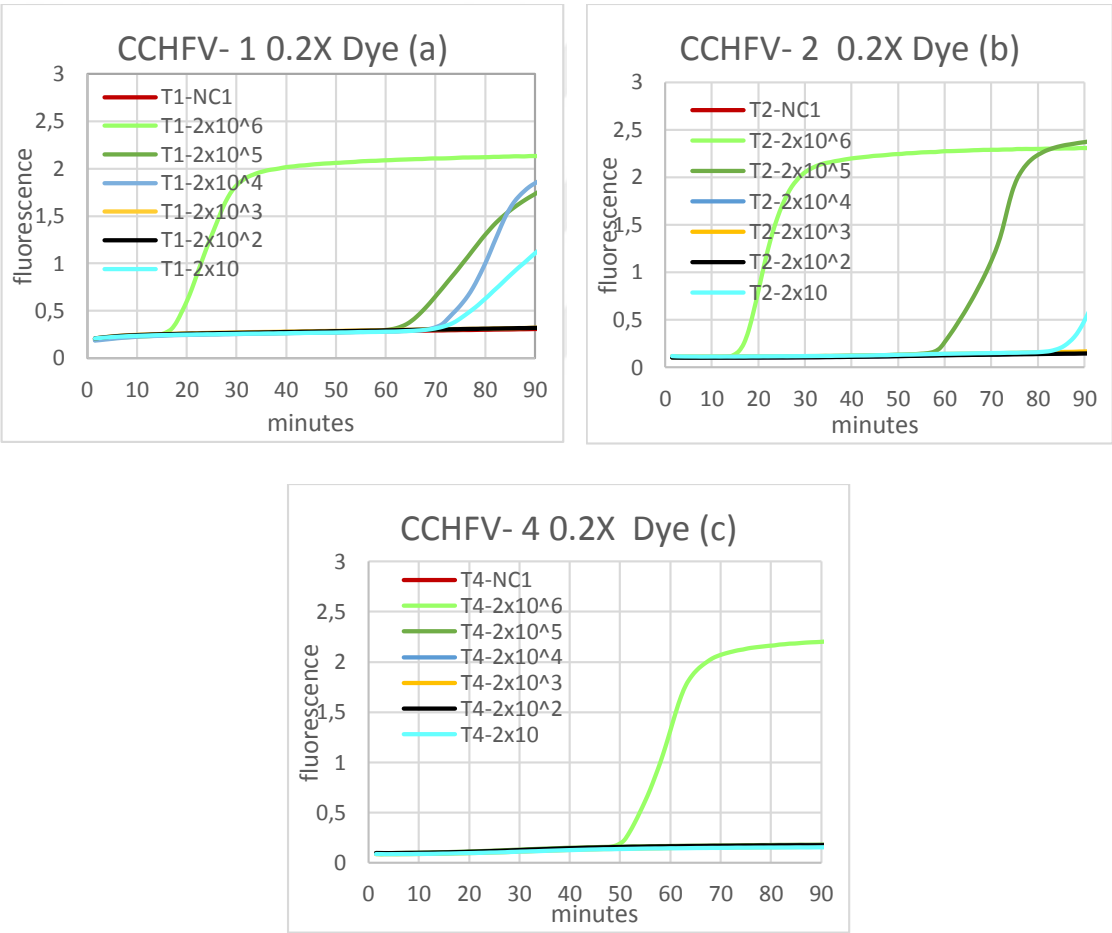
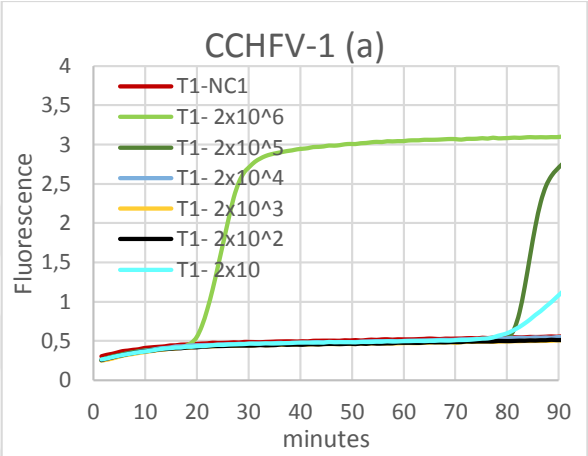


Figure 4.13 LAMP primer sets with synthetic templates. The 0.2X dye concentration with 65°C. (a) CCHFV-1 primer set result, (b) CCHFV-2 primer set result, (c) CCHFV-4 primer set result

4.5.3. Effect of Increased Primer Amount

In order to increase the performance of the experiment, the primer mix amount was increased from 2.5 µl to 3.5 µl. Reaction mixture contained, 3.5 µl primer mix, 2 µl synthetic template($10^6, 10^5, 10^4, 10^3, 10^2$, 10 copies/µl) . The resulting graphs are shown in Figure 4.14 (a), (b), 4.15 (a), (b), and 4.16 (a), (b), respectively.



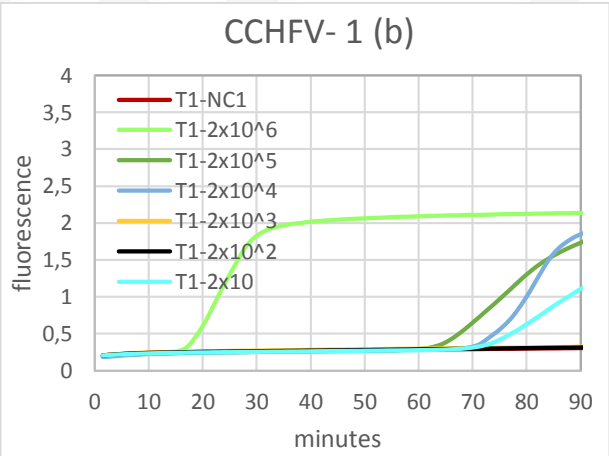


Figure 4.14 CCHFV LAMP primer set-1 result with different primer mix amount. Result of 3.5 µl primer mix amount (a) and 2.5 µl primer mix amount (b)

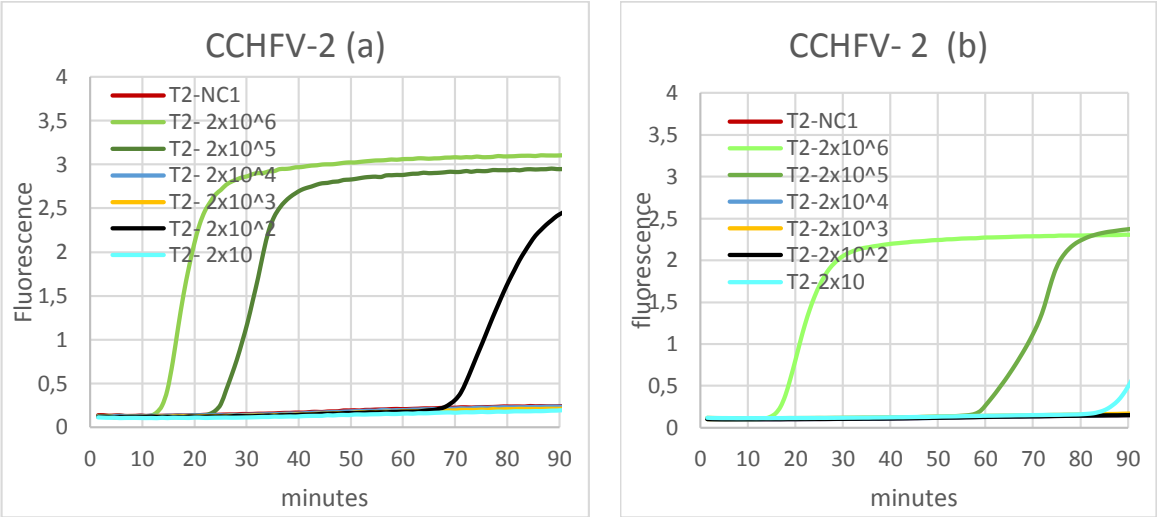


Figure 4.15 CCHFV LAMP primer set-2 result with different primer mix amount. Result of 3.5 µl primer mix amount (a) and 2.5 µl primer mix amount (b)

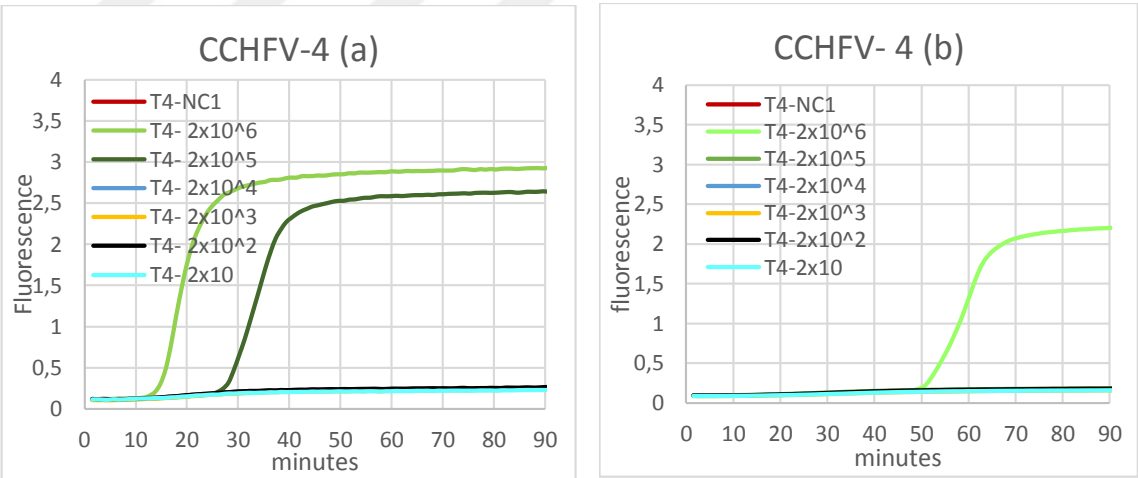


Figure 4.16 CCHFV LAMP primer set-4 result with different primer mix amount. Result of 3.5 µl primer mix amount (a) and 2.5 µl primer mix amount (b)

In increased the amount of primer mix, for the CCHFV-1 set, the signal was seen in 20 minutes for $2x10^6$ copies/µl, and it caused the signal to arrive 5 minutes later than the 2.5 µl primer mix amount. In the CCHFV-2 set, there was no affect the signal for $2x10^6$ copies/µl but for $2x10^5$copies/µl signal was seen in 22 minutes. It made a difference in the CCHFV-4 set, signaling in 15 minutes for $2x10^6$copies/µl and 25 minutes for $2x10^5$copies/µl.

As a result, 3.5 µl slowed the signal time for CCHFV-1 by 5 minutes, and signaled in a shorter time for CCHFV-2 and CCHFV-4. The desired sensitivity could not be achieved in the experiments with synthetic templates.

4.5.4. Testing LAMP Primer Sets with DNA Sample

The double-strand CCHF virus full length S segment template obtained from the RNA sample was tested at 65°C. For CCHFV-1, CCHFV-2, CCHFV-4 LAMP primer sets, reaction contained 0.2X Sybr Green dye, 8mM $MgSO_4$, 2.5 µl primer mix and 2 µl double-strand full length S segment template (10^6-10^4 copies/µl). The result graphs are shown in Figure 4.17 (a), (b), (c).

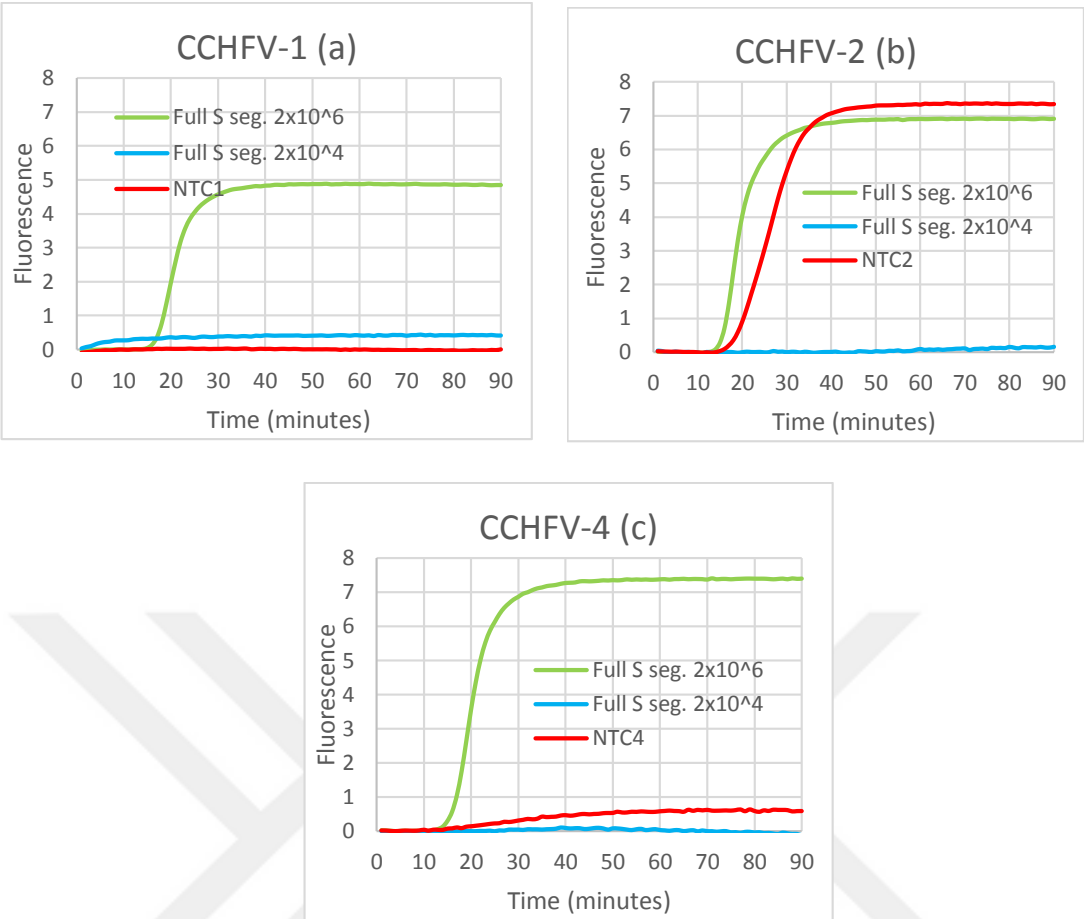


Figure 4.17 Results of LAMP primer sets with Full Length S segment. (a) CCHFV-1 primer set result, (b) CCHFV-2 primer set result, (c) CCHFV-4 primer set result

In experiment results, signal was seen in 15 minutes for CCHFV-1 LAMP set, CCHFV-2 and CCHFV-4 LAMP set. For CCHFV-2 LAMP set, also negative signal was seen in 15 minutes. For CCHFV-2 LAMP set, experiment was repeated at 65°C and presence of negative signal was evaluated.

4.5.5. Determination of Optimum LAMP Primer Sets

For CCHFV-2 LAMP primer set, experiment was repeated using double-strand T2 template that was extracted from RNA sample. Two same template concentrations that was $2x10^6$copies/µl were used. Reaction contained 0.2X Sybr Green dye, 8mM $MgSO_4$, 2.5 µl primer mix, 2 µl double-strand T2 template ($1x10^6$copies/µl) (Figure 4.18(a)) or full length S segment ($1x10^6$ copies/µl) (Figure 4.18(b)). In results, negative signal was seen for two experiments and also in Figure 4.18 (a) negative signal was seen earlier than the sample. Signals of samples were seen in long time as 35 minutes (Figure 4.18(a)) and 45 minutes, 50 minutes (Figure 4.18(b)), respectively.

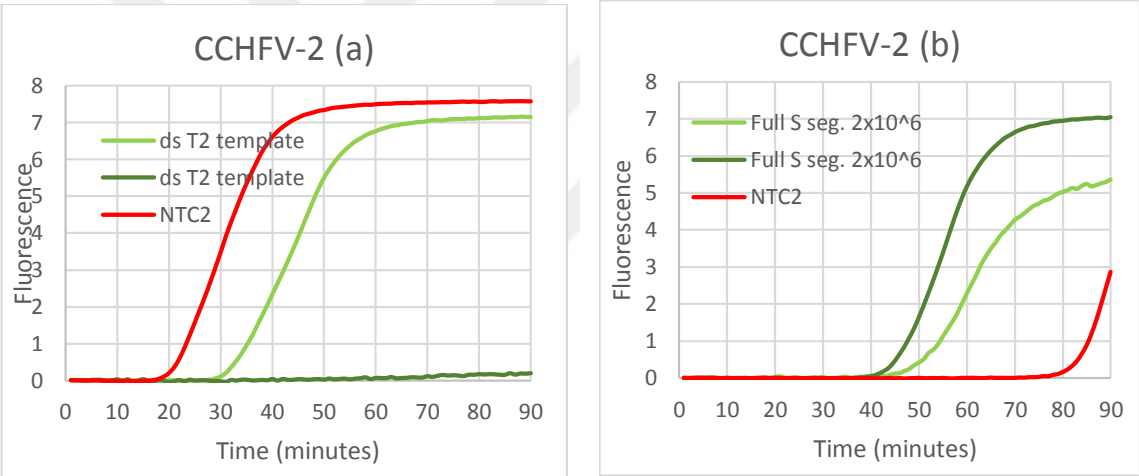


Figure 4.18 Results of CCHFV LAMP primer set-2 with in 65°C. 2.5 µl primer mix amount, 8mM $MgSO_4$ with double-strand T2 template (a) and full length S segment (b)

For eliminating negative signal, temperature was increased to 67°C and experiment was repeated using 8mM $MgSO_4$, 0.2X Sybr Green dye, 2.5 µl primer mix and 2 µl full length S segment ($1x10^6$copies/µl).

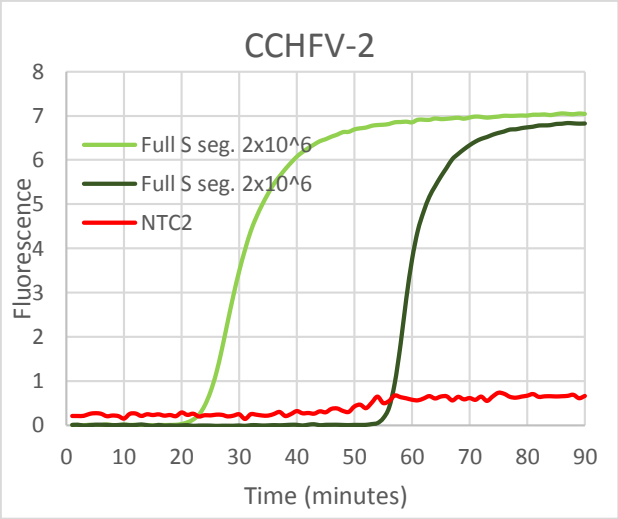


Figure 4.19 Results of CCHFV LAMP primer set-2 with in 67°C

Negative signal was not seen but two same samples signals were seen in 25 minutes and 55 minutes (Figure 4.19).

In the experiment with the Full length S segment, a signal was obtained for the $2x10^6$copies/µl concentration in 15 minutes for all sets, while no signal was observed for the $2x10^4$copies/µl. In CCHFV-2 set, negative signal was seen in two of three experiments (Figure 4.18(a) and Figure 4.17 (b)) and one of three assays signals were in 45 minutes (Figure 4.18 (b)). For 67°C, there was a difference of 30 minutes between signals from the same sample, not indicating a reliable result. The CCHFV-2 set was eliminated because the presence of a negative signal in the CCHFV-2 set and the temperature difference suppressed the negative signal, but did not produce a big difference for the positive result signal. The CCHFV-1 and CCHFV-4 sets were performed in the next experiments.

4.5.6 Determination of Optimum Temperature

The experiment was repeated at 63^{0}C, 65^{0}C, and 67^{0}C for the CCHFV-1 and CCHFV-4 primer sets. For each LAMP sets, reaction contained 8mM $MgSO_4$, 0.2X Sybr Green dye, 2.5 µl primer mix and 2 µl double-strand full length S segment template (10^6 copies/µl). The result graphs of CCHFV-1 primer set are shown in Figure 4.20 (a), (b), (c) respectively.

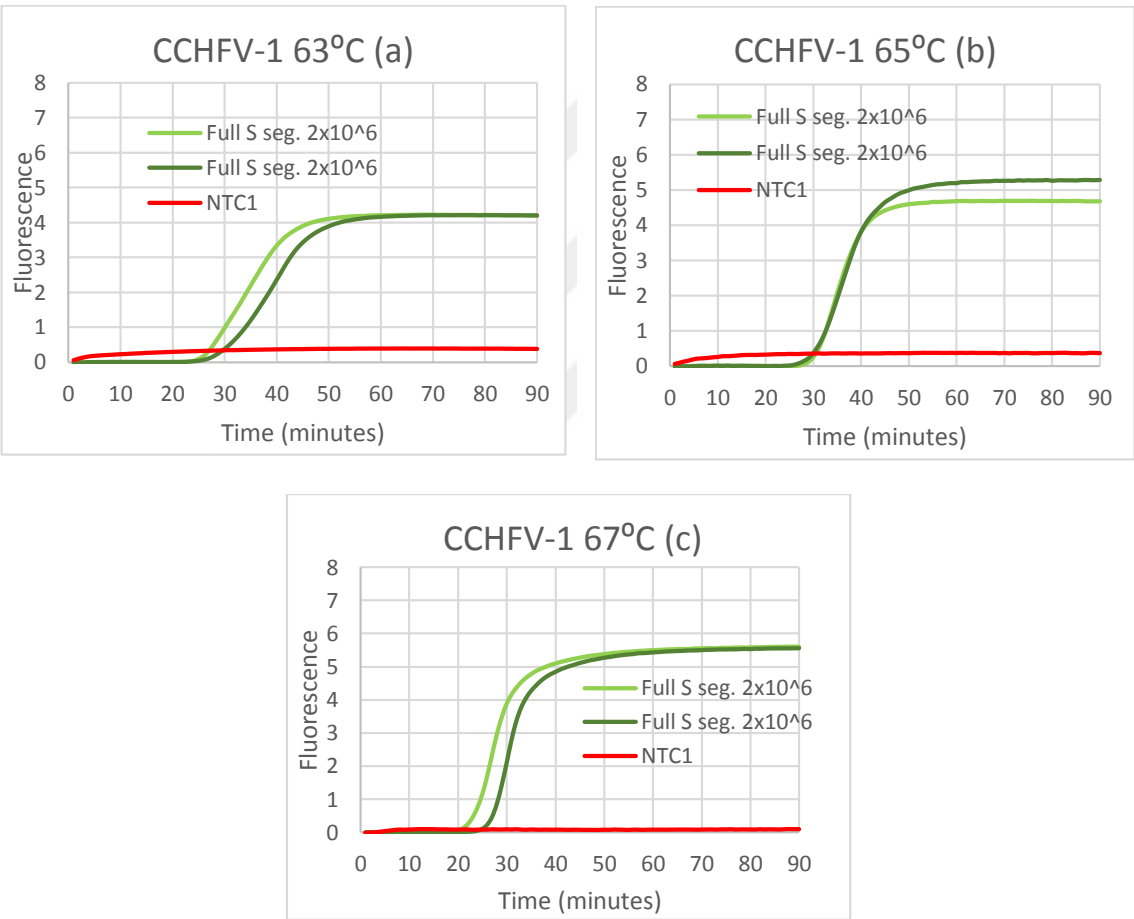
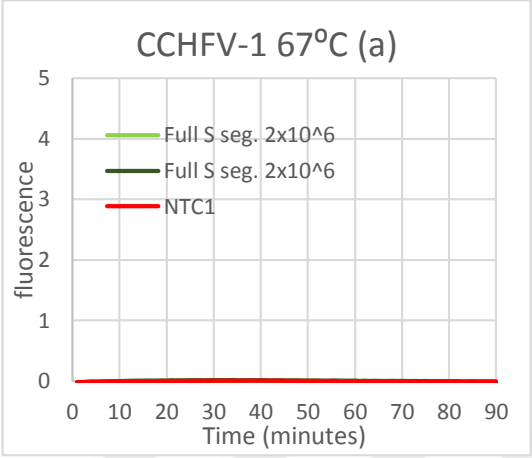


Figure 4.20 CCHFV LAMP primer set-1 result at different temperatures (a) 63°C, (b) 65°C, (c) 67^{0}C

In results for CCHFV-1 LAMP set, signal was seen in 28 minutes at 63°C, 30 minutes at 65°C and 25 minutes at 67°C, respectively.

Since the signal was determined at 67°C at the earliest, the test was repeated at 67°C and was used 2.5 µl and 3.5 µl primer mix, respectively (Figure 4.21 (a) and (b)).



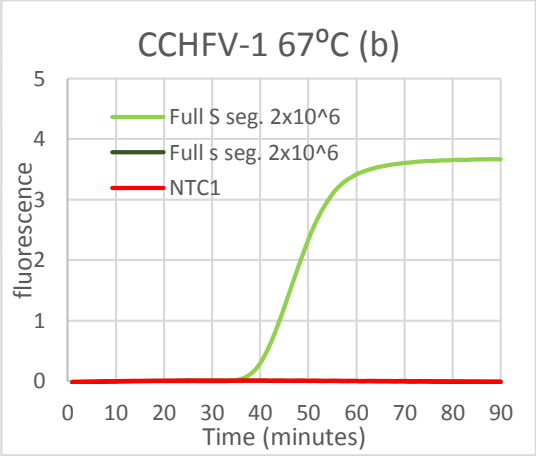


Figure 4.21 CCHFV LAMP primer set-1 with different primer mix amount in 67°C. Result with 2.5 µl primer mix (a) and 3.5 µl (b) primer mix amount

In experiment, sample signals were seen in 40 minutes with 3.5µl primer mix amount. Increased primer mix amount for 67°C prolonged the signal time and signal was not seen with 2.5 µl primer mix amount (Figure 4.21). So It was decided that 67°C was not suitable. CCHFV-1 LAMP primer set was repeated at 65°C. For 65°C, the experiment was repeated under the same conditions (Figure 4.22 (a) and (c)) and the total results are shown in Figure 4.22.

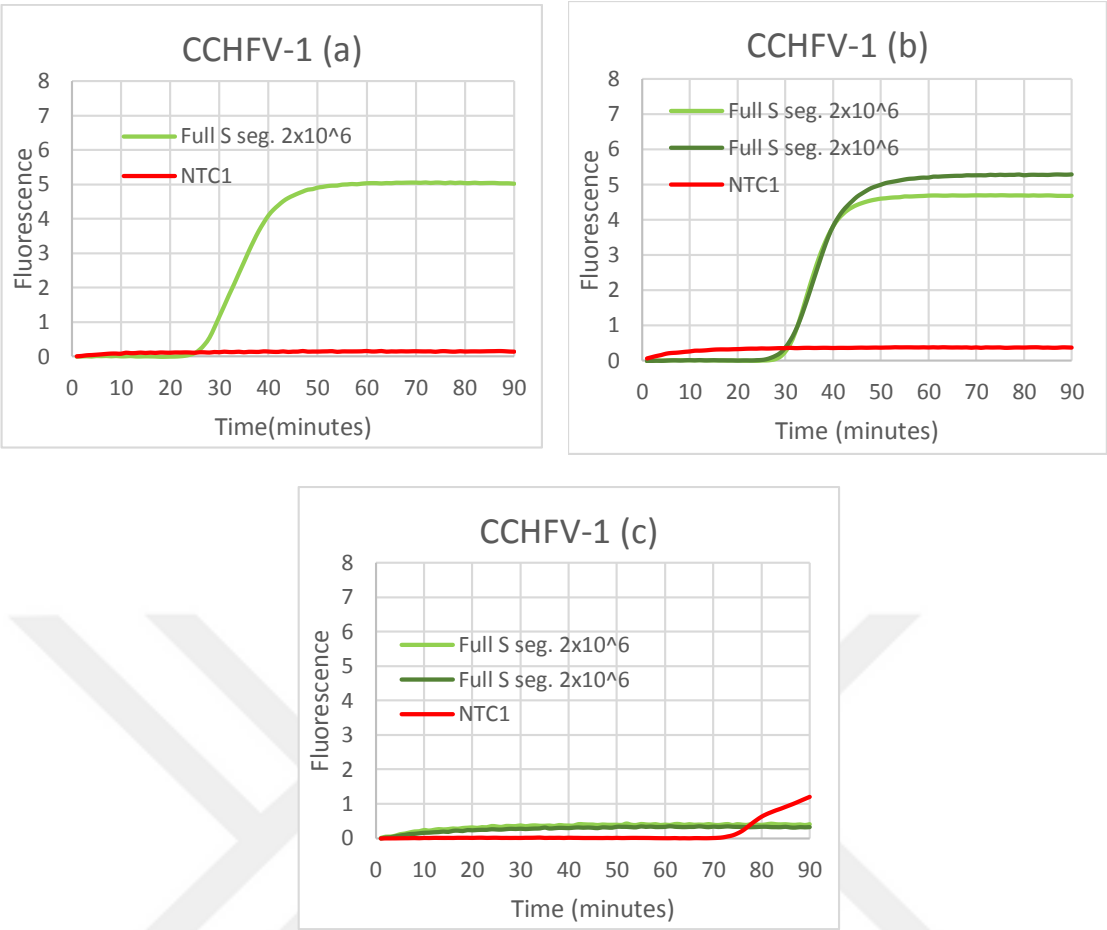


Figure 4.22 CCHFV LAMP primer set-1 result with 65^{0}C. It contains 2.5µl primer mix and 8mM $MgSO_4$

The results indicated that the signal was seen for 2x10^{6}copies/µl within 30 minutes. For the CCHFV-1 set, three different temperatures changed the signal time by 4-5 minutes. Since increased risk of negative signals due to nonspecific formations at low temperature, different temperatures (Figure 4.20) did not have a big effect, the recommended optimum temperature is 65^{0}C for Bst DNA polymerase, 65^{0}C was chosen as the optimum temperature for this set.

For CCHFV-4 primer set, three different temperatures were tested and results are shown in Figure 4.23 (a), (b), (c) respectively. Reaction contained 8mM $MgSO_4$, 2.5 µl primer mix and 2 µl double-strand full length S segment template(10^6 copies/µl).

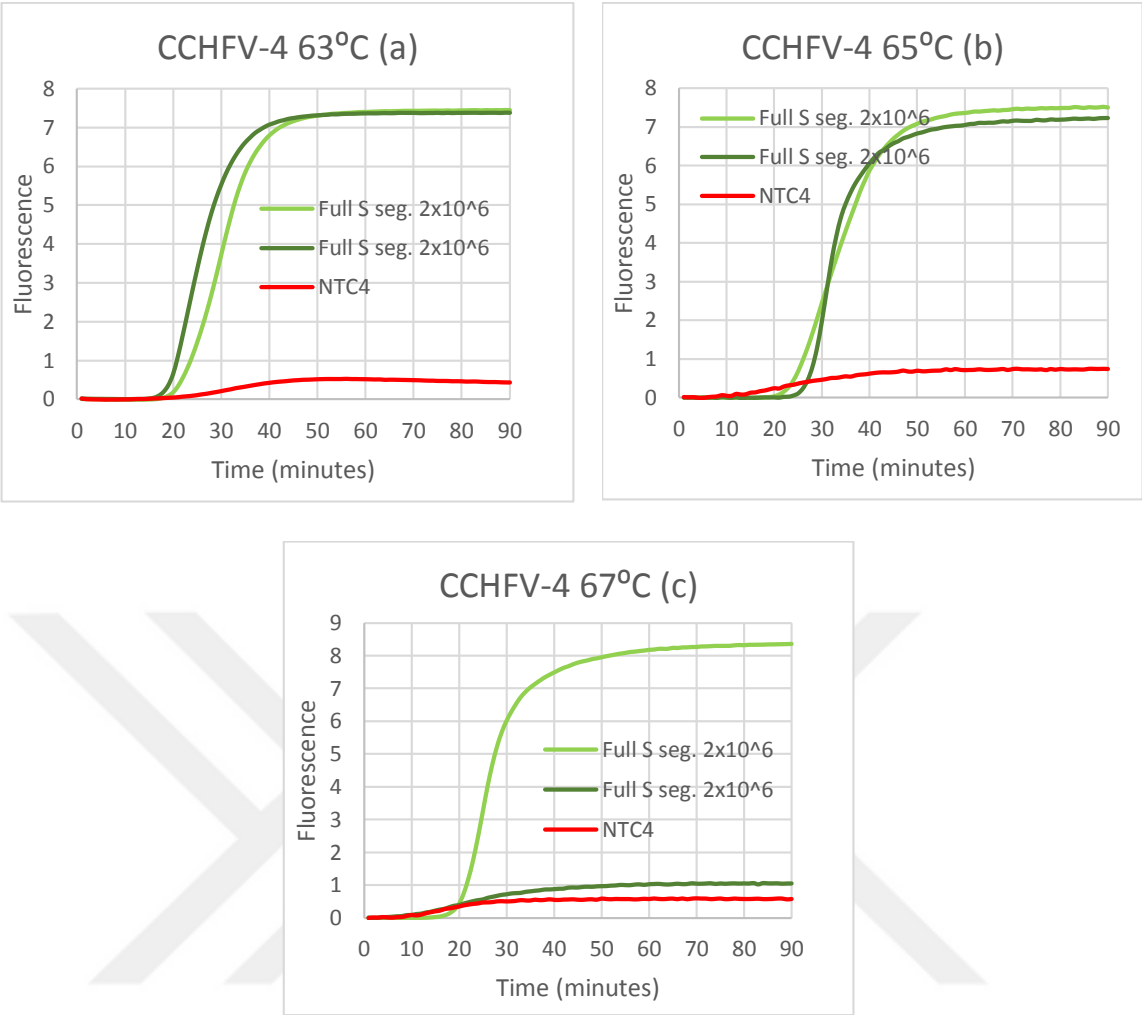
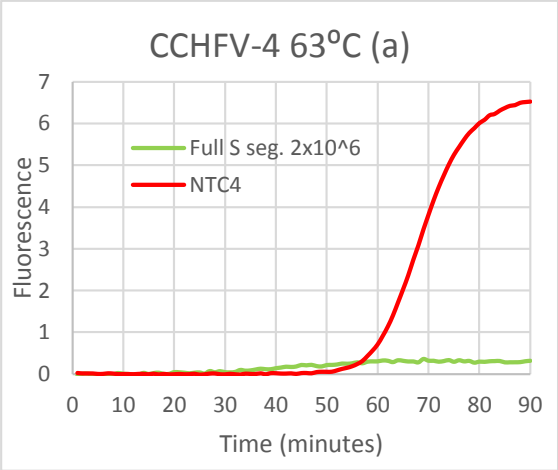


Figure 4.23 CCHFV LAMP primer set-4 result with different temperatures. Results of 2.5 µl primer mix amount and 8mM $MgSO_4$ at 63°C(a), 65°C (b), 67°C (c)

In experiment, signal was seen in 20 minutes at 63°C, 23-25 minutes at 65°C and 20 minutes at 67°C, respectively (Figure 4.23). And also in 67°C signal was seen for one of two samples so it was not chosen.

CCHFV-4 primer set was repeated at 63°C with using 2.5µl and 3.5µl primer mix amount, 8mM $MgSO_4$. For 3.5 µl primer mix amount, two samples were used that have same concentrations. Results are shown Figure 4.24 (a) and (b), respectively.



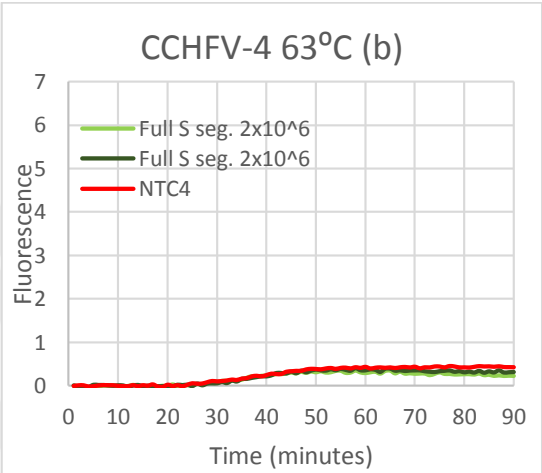


Figure 4.24 CCHFV LAMP primer set-4 with different primer mix amount at 63°C. Result of 2.5µl primer mix amount (a) and 3.5 µl primer mix amount (b)

In the experiment repeated at 63°C, although the amount of primer mix was increased, no signal was observed, in addition, a negative signal was observed.

For 65°C, the experiment was repeated under the same conditions (Figure 4.25 (a) and (c)) and the total results are shown in Figure 4.25.

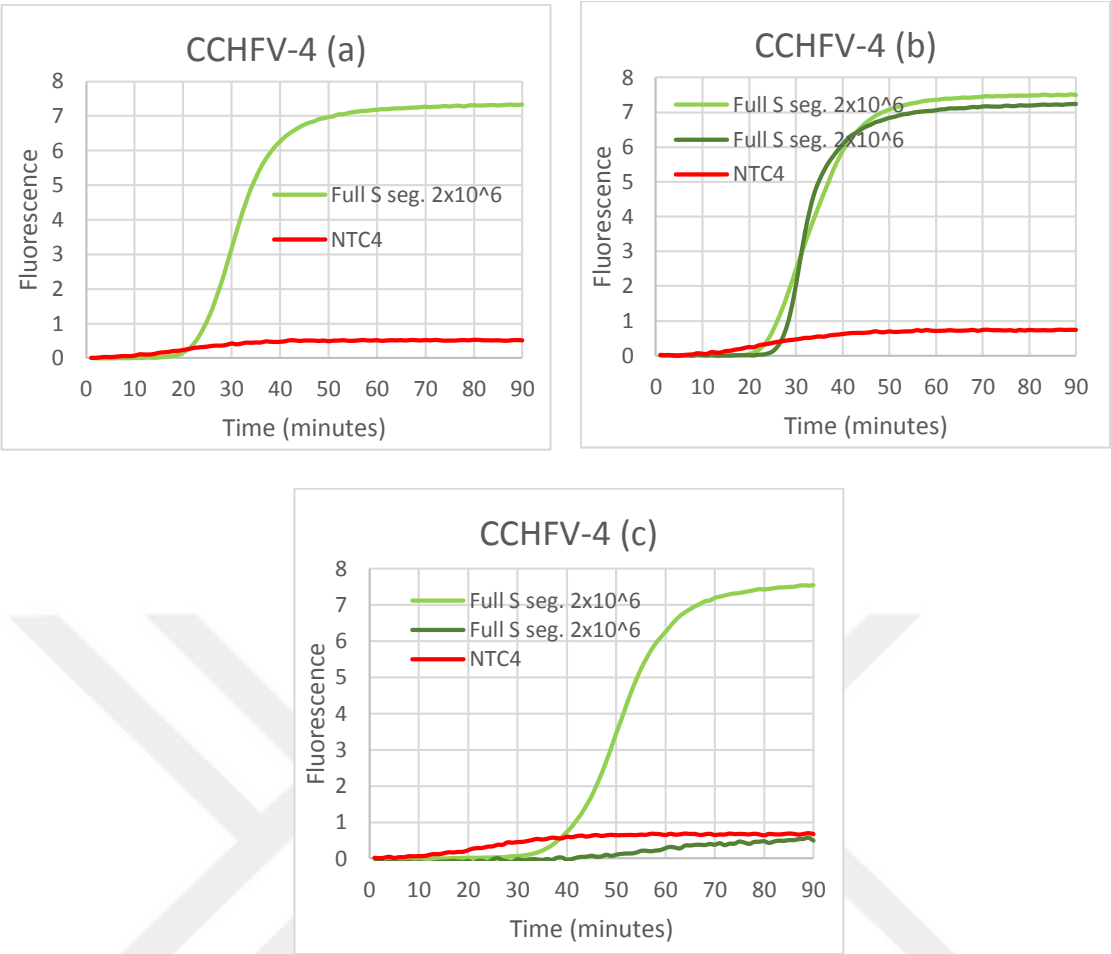


Figure 4.25 CCHFV LAMP primer set-4 result at 65°C. It contains 2.5µl primer mix amount and 8mM $MgSO_4$

The results indicated that the signal was seen for $2x10^6$copies/µl within 25 minutes (Figure 4.25 (a) and (b)) and within 35 minutes (Figure 4.25(c)). Signal was seen in 20-25 minutes two of three experiments. In the CCHFV-4 primer set, different temperatures were able to change the signal time by 3-4 minutes. Different temperatures (Figure 4.23) did not have a big effect and since increased risk of negative signals due to nonspecific formations at low temperature, the recommended optimum temperature is 65^0C for Bst DNA polymerase, 65^0C was chosen as the optimum temperature for this set.

4.5.7. Determination of Optimum Primer Amount

The experiment was repeated at 65^{0}C with 3.5 µl primer mix for primer sets CCHFV-1 and CCHFV-4. Mixture contained 0.2X Sybr Green dye, 8mM $MgSO_4$ 3.5 µl primer mix, 2 µl double-strand full length S segment template (10^6 copies/µl). The results are shown in Figure 4.26, Figure 4.27, respectively.

Using 3.5 µl primer mix in the experiment affected the signal time by 2-3 minutes on the CCHFV-1 primer set (Figure 4.26). A primer mix amount of 2.5 µl was considered appropriate, as it did not have a major effect. In the CCHFV-4 primer set, 2.5 µl primer mix was considered ideal for this set, as it affects the signal time by 2-3 minutes and does not make a big difference (Figure 4.27).

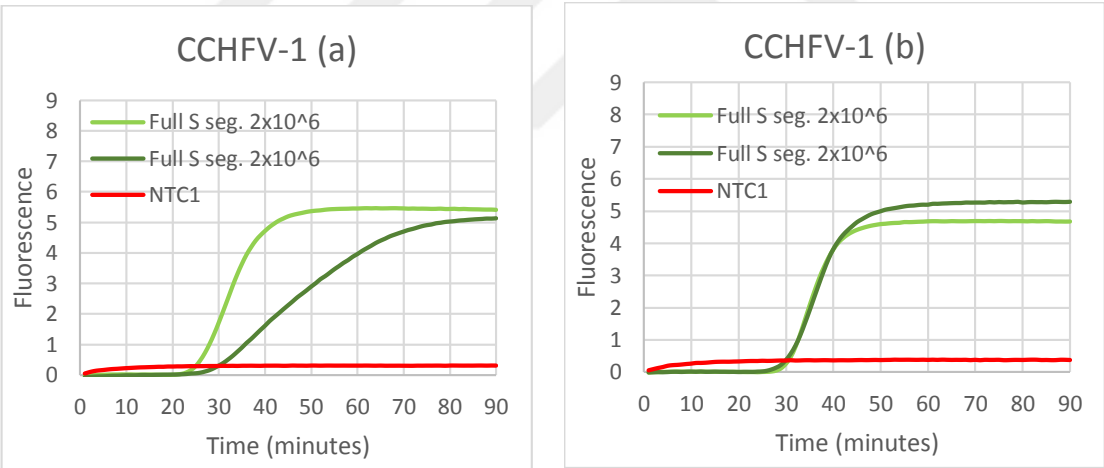


Figure 4.26 CCHFV LAMP primer set-1 with different primer mix amount at 65°C. Result of 3.5 µl (a) and 2.5 µl (b) primer mix result

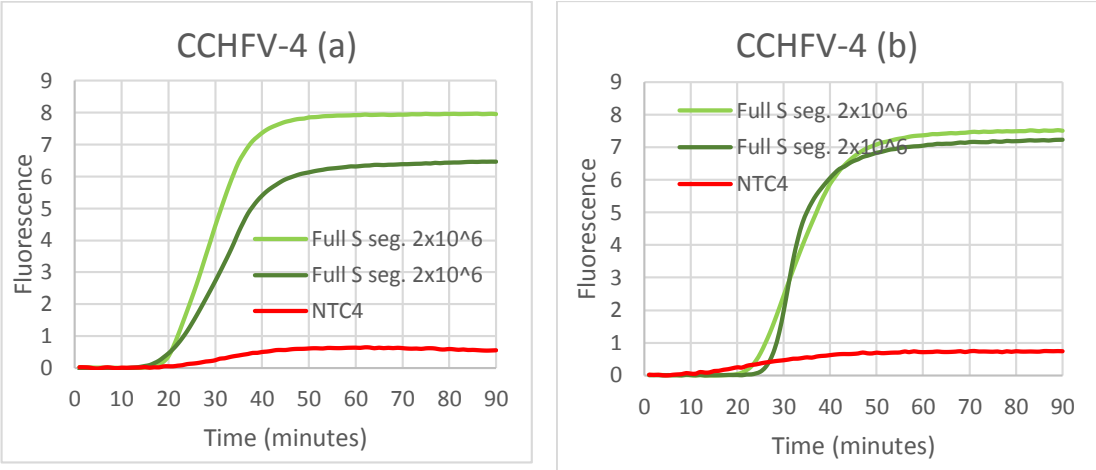


Figure 4.27 CCHFV LAMP primer set-4 with different primer mix amount at 65°C. Result of 3.5 µl (a) and 2.5 µl (b) primer mix result

4.5.8. Determination of Optimum $MgSO_4$ Concentration

In the experiment, 6mM, 8mM and 10mM $MgSO_4$ concentrations were tested using 2.5 µl primer mix at 65^{0}C. For 6mM $MgSO_4$ concentration, 1 µl $MgSO_4$ (100mM), 2.5 µl primer mix, 2 µl of full length S segment template (10^6 copies/µl) was used. For 8 mM $MgSO_4$ concentration, 1.5 µl $MgSO_4$ (100 mM), 2.5 µl primer mix, 2 µl full length S segment template (10^6 copies/µl) was used. For 10mM $MgSO_4$ concentration, 2 µl $MgSO_4$ (100 mM), 2.5 µl primer mix, 2 µl of full length S segment template (10^6 copies/µl) was used. The results of 6mM, 8mM, 10mM $MgSO_4$ concentrations for the CCHFV-1 primer set, are shown in Figures 4.28 (a), (b), (c), respectively.

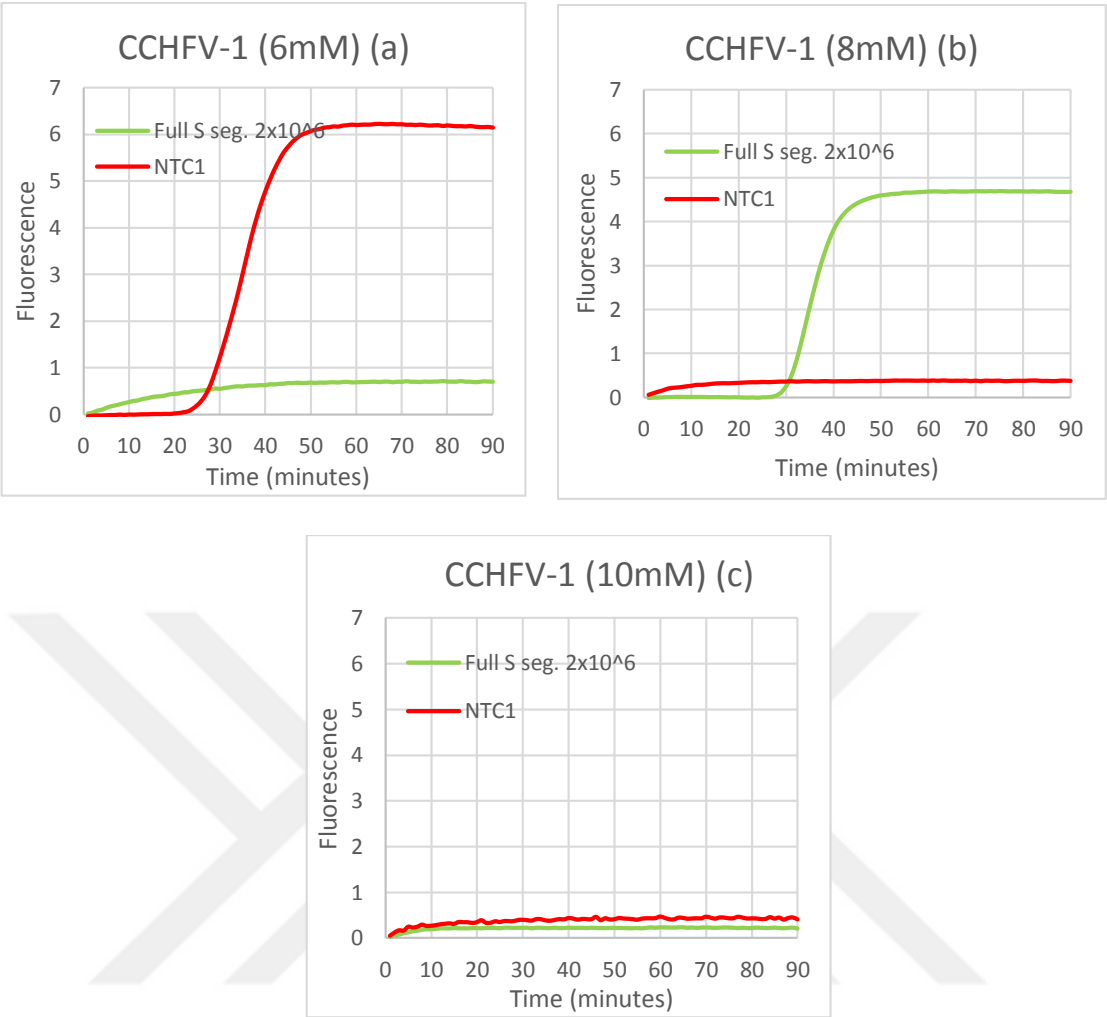


Figure 4.28 CCHFV LAMP primer set-1 with different $MgSO_4$ concentrations. Results with 6mM (a), 8mM (b), 10mM (c) $MgSO_4$

The results of 6mM, 8mM, 10mM $MgSO_4$ concentrations for the CCHFV-4 primer set, respectively, are shown in Figures 4.29 (a), (b), (c).

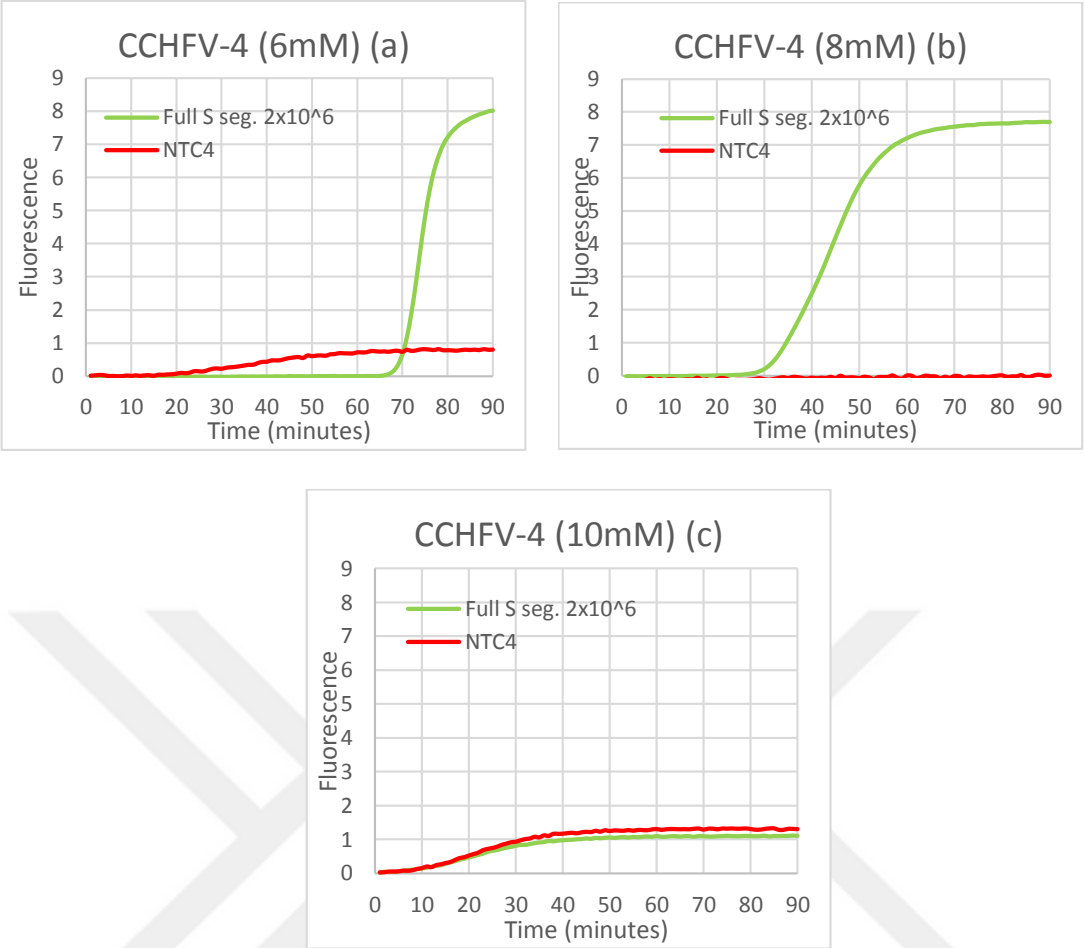


Figure 4.29 CCHFV LAMP primer set-4 with different $MgSO_4$ concentrations. Results with 6mM (a), 8mM (b), 10mM (c) $MgSO_4$

The effect of different $MgSO_4$ concentrations on the reaction was evaluated, only 8 mM $MgSO_4$ signal was seen in the CCHFV-1 set and 8 mM was chosen as the ideal concentration since the recommended concentration for the LAMP reaction is 8 Mm (Figure 4.28). For the CCHFV-4 primer set, the signal was seen at 6mM and 8mM, but the signal from 6mM was quite late. The optimum concentration was determined 8mM for CCHFV-4 LAMP set (Figure 4.29).

4.5.9. Determination of Detection Limit

CCHFV-1 and CCHFV-4 primer sets were tested with different template concentrations at 65^0C, using 0.2X Sybr Green dye, 2.5 µl primer mix and 8mM $MgSO_4$ concentration. As template concentrations 2 µl double-strand full length S segment template ($10^8, 10^6, 10^5, 10^4, 10^3$copies/µl) were used and evaluated. The result graphs are shown in Figure 4.30, Figure 4.31, respectively.

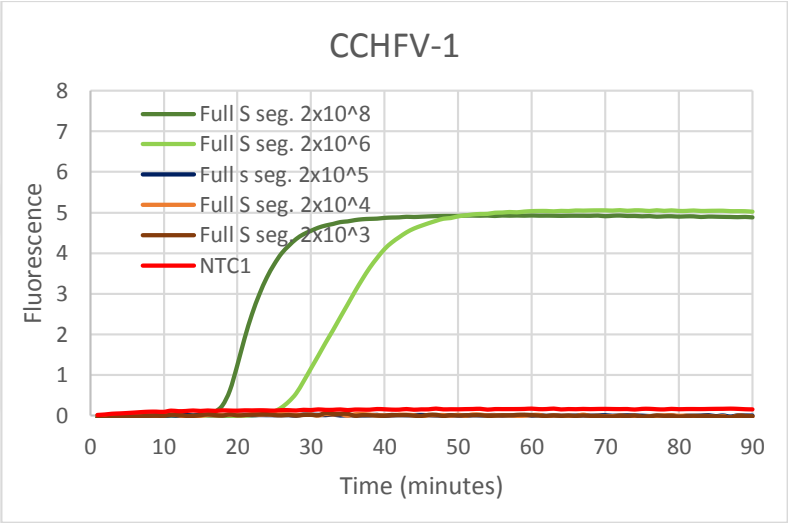


Figure 4.30 CCHFV LAMP primer set-1 limit of detection

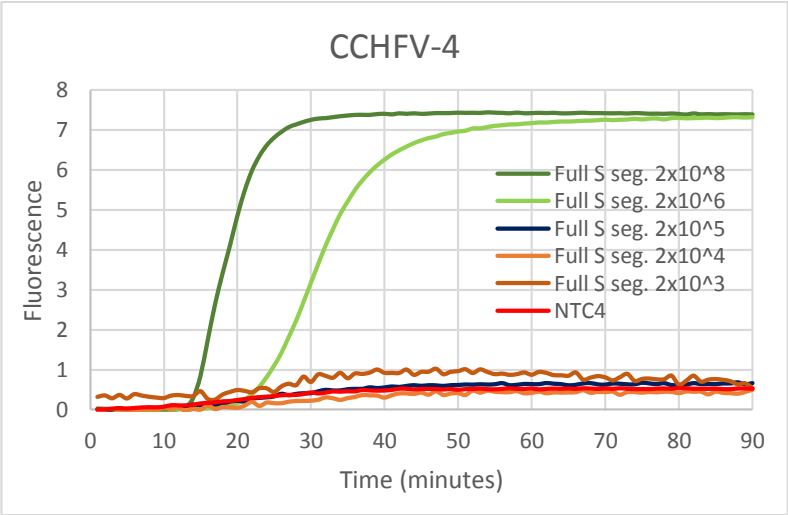


Figure 4.31 CCHFV LAMP primer set-4 limit of detection

The limit of detection values were determined for CCHFV-1 and CCHFV-4 sets, no signal was seen for concentrations less than $2x10^6$copies/µl.

Therefore, the limit of detection value was determined as $2x10^6$copies/µl in both sets. As a result, the ideal working conditions for both sets were determined as 2.5 µl primer mix amount and 8mM $MgSO_4$ concentration at 65^0C.

4.6. qPCR

To compare LAMP assay results with qPCR, the Light Cycler 480 SYBR Green I Master kit (Roche) was used. For each qPCR reactions contained 1 µl reverse primer (10 µM), 1 µl forward primer (10 µM) and 2 µl double-strand full length S segment template ($10^8, 10^6$copies/µl). The qPCR primers used in the reaction are shown in Table 4.12 and Table 4.13.

Table 4.12 Q1 primer pair that were selected

	Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Assay Set 3 (Q1)	Forward Primer	AGCAAAGATGAGATGAACAAATGG	14	24	2.1	37.5	
	Reverse Primer	AGTAGGAGTTTGTGAAAGTGTCC	92	23	2.3	43.4	
	Product						79

Table 4.13 Q4 primer pair that were selected

	Type	Sequence	Start	Length	Tm	GC Percent	Amplicon
Assay Set 3 (Q4)	Forward Primer	CTCTTTGCTGATGACTCTTTCC	41	22	60.9	45.4	
	Reverse Primer	CACCCATTTCACTGATTCTACC	125	22	60.8	45.4	
	Product						85

The graphs obtained as a result of the experiment are shown in Figure 4.32 and Figure 4.33 below.

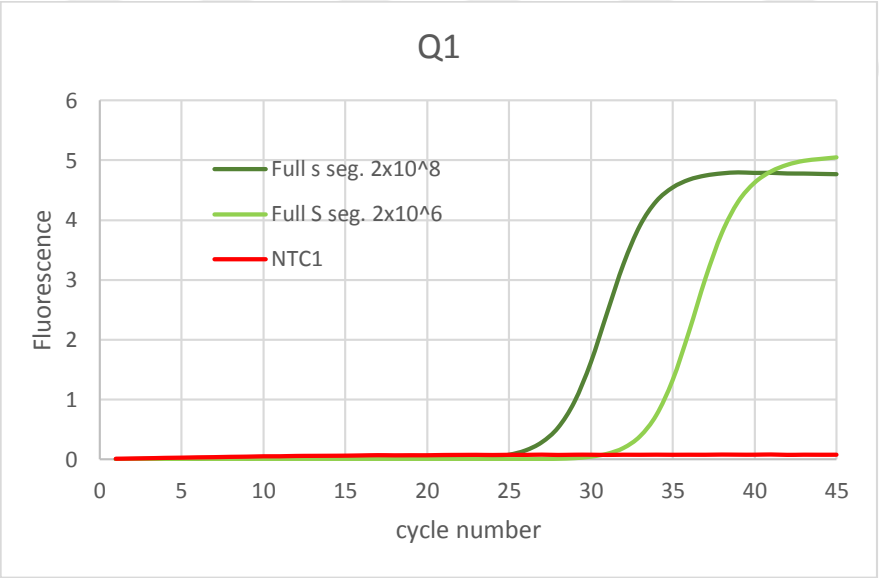


Figure 4.32 Result of qPCR assay with Q1 primer set

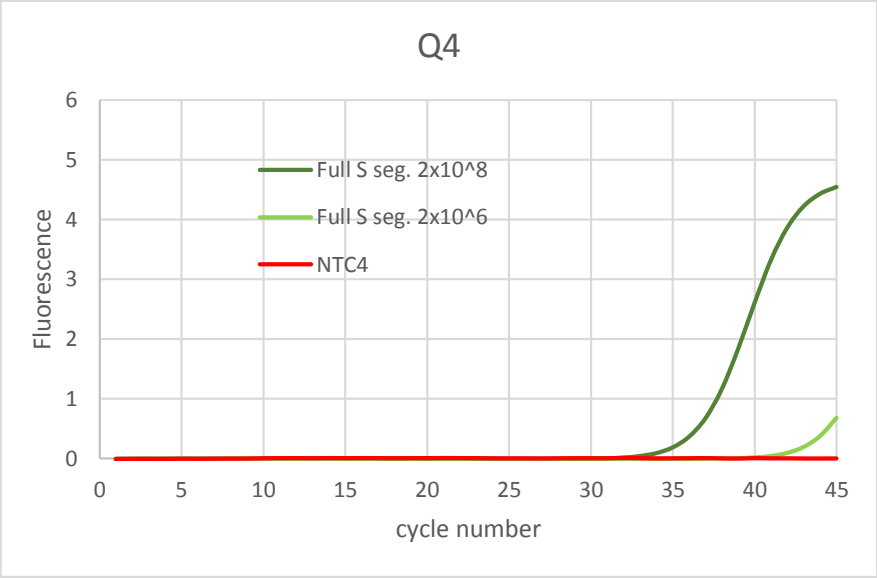


Figure 4.33 Result of qPCR assay with Q4 primer set

Each cycle corresponded to 40 seconds. 2 µl 10^8 copies/µl and 2 µl 10^6 copies/µl double-strand full length S segment were used as template concentrations. Signals from concentrations for the Q1 primer set was seen in 26th cycle and 32th cycle. Signals for the Q4 primer set were seen at 36th cycle and 42th cycle. Signals for the Q1 primer set were seen at late cycle numbers. Signals for the Q4 primer set were seen at later cycle numbers than the Q1 primer set results. Therefore, lower template concentrations were not tested for the Q1 and Q4 primer sets.

4.7. Discussion

Table 4.14 Resulting time of LAMP primer sets and qPCR primer sets

	$2x10^8$copies/µl	$2x10^6$copies/µl
LAMP CCHFV-1 primer set	18 minutes	25 minutes
Q1 Primer pair	18 minutes	22 minutes
LAMP CCHFV-4 primer set	15 minutes	22 minutes
Q4 Primer pair	24 minutes	28 minutes

Comparison of LAMP and qPCR experiment results for two different template concentrations are shown in Table 4.14. CCHFV-4 LAMP primer set signaled 9 minutes and 6 minutes before the Q4 qPCR primer set result for concentrations of 2x10^8 copies/µl and 2x10^6 copies/µl, respectively. Limit of detection value was 2x10^6copies/µl. The CCHFV-1 LAMP primer set gave results for 2x10^8 copies/µl at 18 minutes and had the same time as the qPCR result. For 2x10^6 copies/µl, the CCHFV-1 LAMP set gave results at 25 minutes, while the qPCR set gave results at 22 minutes and detection limit was 2x10^6copies/µl.

In the study, the limit of detection for CCHFV-1 and CCHFV-4 LAMP sets was determined as 2x10^6copies/µl, although lower titers were targeted. CCHFV-1 LAMP set were similar to qPCR, but the CCHFV-4 LAMP set outperforming the qPCR result.

The sequence information of the MK309333 strain tested in the study was limited, therefore, no control could be obtained about the compatibility of the forward outer primer of the CCHFV-1 set on the sequence (Figure 4.2). The possibility that the primer sequence differs from the target region may affect the sensitivity of the reaction, and may be considered as the reason for not decreasing to lower titers.

Similarly, 2 nucleotides from the sequence of the backward outer primer of the CCHFV-4 primer set differ from the target region of the strain (Figure 4.4 cyan part). This may have affected the detection limit of the CCHFV-4 set. It is clear that the high variation in CCHF virus strains complicates the primer design. The primers obtained in the study can be reviewed and the existence of more suitable sets can be checked.

CHAPTER 5

CONCLUSION AND FUTURE PERSPECTIVE

CCHFV-1 LAMP primer set was tested with 0.2X Sybr Green dye concentration at different temperatures (63°C, 65°C, 67°C) and 65°C was selected accordingly. The same set was tested with 2.5 µl and 3.5 µl primer amounts and 2.5 µl primer amount (F3:FIP:FL: 2:16:5, B3:BIP:LB: 2:16:5) was selected. The effect of different $MgSO_4$ concentrations (6mM, 8mM, 10mM) was evaluated and 8mM $MgSO_4$ was determined as optimum. CCHFV-4 LAMP primer set was tested with 0.2X Sybr Green dye concentration at different temperatures (63°C, 65°C, 67°C) and 65°C was selected as the optimum operating temperature. The same set was tested with 2.5 µl and 3.5 µl primer amounts and 2.5 µl primer amount (F3:FIP:FL: 2:16:5, B3:BIP:LB: 2:16:5) was selected. The effect of different $MgSO_4$ concentrations (6mM, 8mM, 10mM) was evaluated and 8mM $MgSO_4$ was determined as optimum. In LAMP and qPCR comparison, the detection limit for CCHFV-1 and CCHFV-4 LAMP primer sets was determined as $2x10^6$ copies/µl and it was same result as the qPCR detection limit. The CCHFV-1 LAMP primer set gave similar results to the qPCR results, but the CCHFV-4 LAMP primer set gave better results than the qPCR results.

Testing the LAMP method with a CCHFV RNA sample can be considered as the next step. The performance of primers can be evaluated using field-collected samples such as blood, serum, saliva, feces, which contain biological inhibitors and are known to contain CCHF virus. It can provide insight into both the clinical performance and the potential of the method for use in limited field conditions.

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