DEVELOPMENT OF A NUCLEIC ACID-BASED ISOTHERMAL DIAGNOSTIC TEST FOR BORDER DISEASE WHICH CAUSES LOSSES IN ANIMAL HUSBANDRY

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ABSTRACT

DEVELOPMENT OF A NUCLEIC ACID-BASED ISOTHERMAL DIAGNOSTIC TEST FOR BORDER DISEASE WHICH CAUSES LOSSES IN ANIMAL HUSBANDRY

Border disease is viral infection of ruminants, and it is associated with abortions, stillbirth, and birth of persistently infected (PI) lambs. It has a great potential to cause an outbreak and it is declared as one of the notifiable diseases of ruminants by World Organization for Animal Health (OIE). Border disease poses a threat against ruminant farming industry by causing major economic losses. Since there is no treatment or vaccine against border disease virus (BDV), early diagnosis and early isolation of infected animals is necessary. RT-qPCR is the gold-standard method for BDV identification, but it can only be applied by trained personnel in a laboratory with expensive instruments. There is a need for a point-of-care (POC) test, specifically designed for BDV. This thesis study aimed to develop a nucleic acid-based loop mediated isothermal amplification (LAMP) technique for BDV identification. LAMP is a nucleic acid identification technique that can be performed using 4-6 primers at a constant temperature with a *Bst* DNA polymerase. Firstly, multiple alignment of BDV sequences across the world was performed and most conserved region of genome was detected as 5'UTR. Then, three LAMP primer sets 1, 2a and 2b were designed to target 5'UTR. Designed primer sets were optimized in terms of temperature, fluorescent dye, primer mix, Mg^{2+} and enzyme concentration. After designation of optimum conditions, limit of detection (LOD) was determined for each primer set and their performances were compared. All primer sets have LOD equals to $2x10^4$ copies/μl. Overall, primer set 1 and 2b has higher sensitivity and specificity compared to primer set 2a, therefore they are more suitable to be used for BDV identification with LAMP.

ÖZET

HAYVANCILIKTA KAYIPLARA NEDEN OLAN BORDER HASTALIĞI İÇİN NÜKLEİK ASİT TEMELLİ İZOTERMAL TANI KİTİ GELİŞTİRME

Border (sınır) hastalığı, küçükbaş hayvanlarda görülen ve düşükler, ölü doğumlar ve persiste enfekte (PI) kuzuların doğumu ile ilişkilendirilen bir viral enfeksiyondur. Yakın temasla yüksek hızda bulaşan virüs (BDV), küçükbaş hayvancılık endüstrisi için büyük bir ekonomik tehdit oluşturmaktır. Bu nedenle, Dünya Hayvan Sağlığı Örgütü (OIE) tarafından bildirilmesi zorunlu hastalıklardan biri olarak görülmektedir. BDV'ye karşı bir tedavi veya aşı bulunmadığından, enfekte hayvanların erken teşhisi ve erken izolasyonu, hastalığın yayılmasını önlemenin tek yoludur. Bu sebeple, sahada BDV tanımlaması için kullanılabilecek hızlı ve ekonomik bir tanı testine ihtiyaç duyulmaktadır. Bu tez çalışması, BDV tespiti için bir nükleik asit bazlı bir izotermal amplifikasyon (LAMP) tekniği geliştirmeyi amaçlamıştır. LAMP, DNA iplikçiklerini ayırma kabiliyeti olan bir *Bst* DNA polimeraz ile sabit bir sıcaklıkta, 4-6 primer kullanılarak gerçekleştirilebilen bir yöntemdir. Bu tez çalışmasında dünyanın farklı bölgelerinden izole edilmiş BDV genomlarının çoklu sekans dizilimleri gerçekleştirildi ve genomun en çok korunan bölgesinin 5'UTR olduğu tespit edildi. Ardından, 5'UTR'yi hedeflemek için LAMP primer set 1, 2a ve 2b tasarlandı. Tasarlanan primer setleri sıcaklık, floresan boya konsantrasyonu, primer karışım konsantrasyonu, Mg^{2+} konsantrasyonu ve enzim konsantrasyonu açısından optimize edilmiştir. Optimum koşullar belirlendikten sonra her bir primer seti için gözlemlenebilme sınırı (LOD) belirlendi ve performansları karşılaştırıldı. Tüm primer setlerinin gözlemlenebilme sınırı 2 $x10^4$ kopya/µl olarak belirlendi. Primer set 1 ve 2b, primer set 2a'ya kıyasla daha yüksek duyarlılığa ve özgüllüğe sahip olması nedeniyle LAMP ile BDV tanımlaması için kullanılmaya uygun görüldü.

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LIST OF ABBREVIATIONS

BD:	Border Disease
BDV:	Border Disease Virus
PI:	Persistently Infected
BVDV-1:	Bovine Viral Diarrhea Virus-1
BVDV-2:	Bovine Viral Diarrhea Virus-2
CSFV:	Classical Swine Fever Virus
ORF:	Open Reading Frame
UTR:	Untranslated Region
OIE:	World Organization for Animal Health
LAMP:	Loop Mediated Isothermal Amplification
PCR:	Polymerase Chain Reaction
RT-PCR:	Reverse Transcriptase Polymerase Chain Reaction
RT-qPCR:	Reverse Transcriptase Quantitative Polymerase Chain Reaction
RNA:	Ribonucleic Acid
cDNA:	Complementary Deoxyribonucleic Acid
DNA:	Deoxyribonucleic Acid
FIP:	Forward Inner Primer
BIP:	Backward Inner Primer
F3:	Forward Outer Primer
B3:	Backward Outer Primer
LF:	Loop Forward Primer
LB:	Loop Backward Primer

Tm: Melting Temperature

ΔG: Gibbs Free Energy

NCBI: National Center for Biotechnology Information

µl: Microliter

LOD: Limit of Detection

kb: Kilobase

bp: Base-pair

dNTP: Deoxyribonucleotide Triphosphate

$MgSO_4$: Magnesium Sulphate

mins: Minutes

ELISA: Enzyme Linked Immunosorbent Assay

CHAPTER 1

INTRODUCTION

Border disease (BD) is a viral infection that is caused by a pestivirus called "Border Disease Virus (BDV)". It is associated with abortions, stillbirths, and birth of weak "hairy-shaker" lambs in flocks. Primary hosts of the BDV are ruminant species sheep (*Ovis aries*) and goats (*Capra hircus*) (Nettleton et al. 1998). However, border disease can cross species barrier by infecting all members of *Artiodacytla* such as other domestic ruminant species cattle (*Bos taurus*) and pigs (*Sus scrofa*); and also wildlife species such as Pyrenean chamois (*Rupicapra pyrenaica*), reindeer (*Rangifer tarandus*), wisent (*Bison bonasus*), alpaca (*Lama pacos*) and llama (*Lama glama*) (Kawanishi et al. 2014; Becher et al. 1997; Ueli Braun et al. 2019; U. Braun et al. 2014; Giangaspero et al. 2006; Caruso et al. 2017; Serrano et al. 2015; Danuser et al. 2009; Vilcek et al. 2010). It is reported for the first time as a disease of lambs in the border counties of England and Wales, in 1959. Due to international trade of livestock it is now distributed worldwide (Hughes, Kershaw, and Shaw 1959). Border disease can be easily transmitted and poses a significant risk for pregnant ewes and flock health. Currently, there is no treatment or vaccine against border disease. Therefore, future outbreaks can cause major economic losses in sheep and goat farming industry.

1.1 Border Disease Virus (BDV)

1.1.1 Genomic Features of Border Disease Virus (BDV)

Border disease virus (BDV) belongs to *Pestivirus* genus of *Flaviviridae* family. *Pestiviruses* are enveloped, single-stranded and positive-sense (+) RNA viruses. They have spherical shape with around 50 nm diameter. BDV genome is nearly 12.300 bp long and has a single long open reading frame (ORF) that is flanked by 3' and 5' untranslated regions (UTR) (Figure 1.1). ORF encodes approximately 3900 amino acid long polyprotein. Polyprotein is processed by viral and cellular proteases into 12 mature

proteins. For instance, N3 serine proteases recognize NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B cleavage sites within the polyprotein (Nettleton et al. 1998; Becher, Orlich, and Thiel 1998). Produced mature proteins consist of eight non-structural proteins N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B; and four structural proteins C, E^{rns}, E1 and E2 (Figure 1). C is a nucleocapsid core protein while E^{rns}, E1 and E2 are envelope glycoproteins. N^{pro} has protease activity and E^{rns} has RNase activity. These two proteins are specific to *Pestivirus* genus and they play an important role to cope with host defence mechanisms (Smith et al. 2017; Piras et al. 2020).

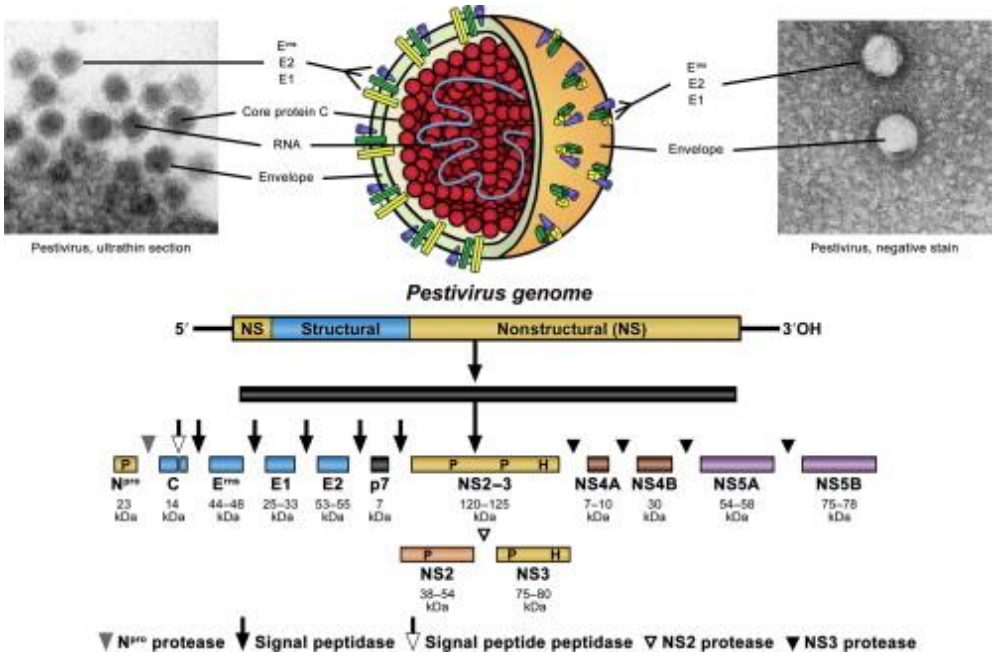

Figure 1.1 Structure and features of border disease virus (BDV).

(Tautz, Tews, and Meyers 2015)

1.1.2 Taxonomy of *Pestivirus* genus and Border Disease Virus

Pestivirus genus previously classified into four major species Bovine Viral Diarrhea Virus 1 (BVDV-1), Bovine Viral Diarrhea Virus 2 (BVDV-2), Border Disease Virus (BDV) and Classical Swine Fever Virus (CSFV). BVDV-1 and BVDV-2 infects cattle, while BDV infects ruminants and CSFV infects pigs. However, pestiviruses can easily cross species barrier and might infect members of *Artiodactyla*. This previous classification was made considering the species of the infected hosts and clinical

symptoms of the disease. However, discovery of novel virus isolates in unusual species such as giraffes, rats, pigs incapacitated the present classification system (Righi et al. 2021; Smith et al. 2017). Therefore, recently a new sequence-based taxonomy was proposed for *Pestivirus* genus. Four major species BVDV-1 is named *Pestivirus A*; BVDV-2 is named *Pestivirus B*; CSFV is named *Pestivirus C* and BDV is named *Pestivirus D*. Considering the newly discovered virus isolates, 7 novel pestivirus species are proposed: *Pestivirus E* (pronghorn pestivirus) isolated from antilops; *Pestivirus F* (Bungowannah virus) isolated from pigs; *Pestivirus G* (giraffe pestivirus) isolated from giraffes; *Pestivirus H* (Hobi-like pestivirus) isolated from bovine animals; *Pestivirus I* (Aydin-like pestivirus) isolated from small ruminants; *Pestivirus J* (rat pestivirus) isolated from rats and Pestivirus K (atypical porcine pestivirus) isolated from pigs (Smith et al. 2017; King et al. 2018). Additionally, virus isolates of sheep and goats which is called "Tunisian sheep pestiviruses*"* could not be included into classifications because their complete genome sequences are unavailable, but it is highly possible that they represent a novel group of pestivirus species as well (Ciulli et al. 2017).

Phylogenetic analyses of collected BDV field isolates grouped BDV into 8 different genotypes (BDV-1, BDV-2, BDV-3, BDV-4, BDV-5, BDV-6, BDV-7 and BDV-8). 5'UTR, N^{pro} and E2 sequences were used for characterization of BDV isolates through sequence comparison in phylogenetic analyses. 5'UTR is approximately 400 bp long and it is the most conserved region of the pestivirus genome and mostly used for primer design in RT-PCR studies. N^{pro} gene is specific to pestiviruses while E2 is essential for viral entry to host cells (Righi et al. 2021).

Pestivirus I (Aydin-like pestivirus) and Tunisian sheep pestiviruses are found to be phylogenetically distinct from BDV and are not included into current BDV genotypes even though they are infecting small ruminants and causing similar symptoms to border disease. Recent studies even show that these viruses are genetically closer to CSFV than to BDV (Ciulli et al. 2017; Postel et al. 2015; Oguzoglu et al. 2009).

1.2 Border Disease (BD)

1.2.1 Clinical Symptoms and Transmission

Border disease has two routes of transmission: vertical and horizontal. Horizontal transmission occurs through oro-nasal route. Nose-to-nose contact with nasal secretions, respiratory droplets, semen and droppings of infected animals can cause transmission of the virus (Løken 1995; Oğuzoğlu 2012). Horizontal transmission is the cause of acute infections in herds. Acute infections take 1-2 weeks and symptoms vary from mild to severe or sometimes infection can be completely unnoticed. Most common symptoms are mild such as high fever and mild leukopenia ((OIE) 2017). Border disease infection can also cause immunosuppression of infected animals and they become susceptible to other infections as well (Oğuzoğlu 2012). On the other hand, infection of pregnant ewes causes vertical transmission via placenta, and it has serious consequences for the fetus. Gestation of lambs take approximately 150 days and immune competence of the fetus starts to develop between 60-85 days. Infection at late gestation, which is after day 85, mostly result with birth of healthy, normal lambs since their bodies can neutralize BDV by producing antibodies. If fetus gets infected in the earlier phases of gestation before having a mature immune system, 50% of pregnancy will result with either abortion or stillbirth; while 50% will result as birth of so-called “persistently-infected (PI)” lambs (Nettleton et al. 1998). PI lambs will be born as tolerant to border disease virus. Since they are exposed to the virus without having an immune competence, they cannot produce antibodies against BDV. They can be serologically characterized being antigen positive but antibody negative for BDV. They carry BDV in all of their organs and they will spread the virus to the herd throughout their lives. (Sandvik 2014). Clinical signs of PI lambs are variable and might change depending on the time of gestation and amount of virulence. Most common clinical manifestations of PI lambs are various central nervous system malfunctions due to myelin deficiency and “hairy-shaker” haircoat due to changes of wool follicles of skin. PI lambs have poor quality of life and a short lifespan. However, some PI lambs show no clinical signs and continue to live and breed in the herd while shedding the virus throughout the flock and gone unnoticed. Furthermore, their trade can cause spread of the disease to other flocks as well. Therefore, PI lambs are one of the most important sources of BDV transmission. Detection and isolation of PI lambs is key

for the control and surveillance of border disease (Nettleton et al. 1998; (OIE) 2017; Newcomer and Givens 2013).

1.2.2 Epidemiology

First BDV infection was reported in the sheep at the border of England and Wales in 1959, but due to international trade of livestock it is now globally distributed. BDV seroprevalence is known to be around 30-98% worldwide (Righi et al. 2021). BD cases reported in many European countries such as Austria (Krametter-Froetscher et al. 2007), France (Dubois et al. 2008; Vilcek et al. 2014), Germany (Becher et al. 2003), Ireland (O'Neill, O'Connor, and O'Reilly 2004), Italy (Peletto et al. 2016; Giammarioli et al. 2015; 2011), Netherlands (Orsel et al. 2009), Slovakia (Lešková et al. 2013), Spain (Vega et al. 2015; Marco et al. 2015; Valdazo-González, Álvarez, and Sandvik 2008), Switzerland (Stalder et al. 2005; 2017) and the United Kingdom (Nettleton et al. 1998; Becher et al. 2003); as well as in other countries such as Algeria (Feknous et al. 2018), Australia (Becher et al. 1997), China (Mao et al. 2015; Li et al. 2013), India (Mishra et al. 2016), Iran (Hemmatzadeh et al. 2016), Israel (Nettleton et al. 1998), Japan (Kawanishi et al. 2014), Mexico (Gómez-Romero et al. 2018), Morocco (Fassi Fihri et al. 2019), New Zealand (Vilček et al. 1998), Tunisia (Thabti et al. 2004) and the USA (Sullivan, Chang, and Akkina 1997). BDV-3 is the most frequent genotype in Europe and Italy hosts the highest number of BDV genotypes among all countries (Figure 1.2). However, more epidemiological studies need to be conducted worldwide to fully understand the global distribution of border disease (Righi et al. 2021).

Animal husbandry is one of the important incomes of Turkey's economy and thus, economical losses in sheep and goat farming cannot be tolerated. Border disease threatens flock's health and reduces yield by causing abortions and stillbirth. Therefore, regular screening of the herds against BD is essential for early isolation of infected animals and prevent the spread of the disease. There are many studies about the presence of pestivirus infections of sheep and goats in different geographical regions of Turkey (Burgu et al. 2001; Okur-Gumusova, Yazici, and Albayrak 2006; Oguzoglu et al. 2009). Pestivirus seroprevalence is detected as 78.5% in Afyonkarahisar (Gür 2009); 45.87% in Aydın (Ural and Erol 2017); 30.58% in Balıkesir (Alpay, Öner, and Yeşilbağ 2018); 64.6% in Burdur (Hasircioğlu, Kale, and Acar 2009); 53.57% in Bursa (Alpay, Öner, and Yeşilbağ

2018); 12.7% in Çanakkale (Alpay, Öner, and Yeşilbağ 2018); 52.63% in Erzurum (Okur-Gumusova, Yazici, and Albayrak 2006); 62.5% in Hatay (Doğan 2021); 22.1% Kahramanmaraş (Doğan 2021); 74.56% in Kars (Yilmaz, Yildirim, and Coskun 2014); 32.26% in Kayseri (Okur-Gumusova, Yazici, and Albayrak 2006); 74.51% in Kırıkkale (Azkur et al. 2011); 50.64% in Tokat (Okur-Gumusova, Yazici, and Albayrak 2006); 75.9% in Van (Tutuncu et al. 2011). Recently, BDV-1 genotype is detected in wild boars of Turkey for the first time and indicates the possibility of BDV transmission btw. wildlife species in domestic ruminants sharing common pastures (Saltik, Kale, and Atli 2021).

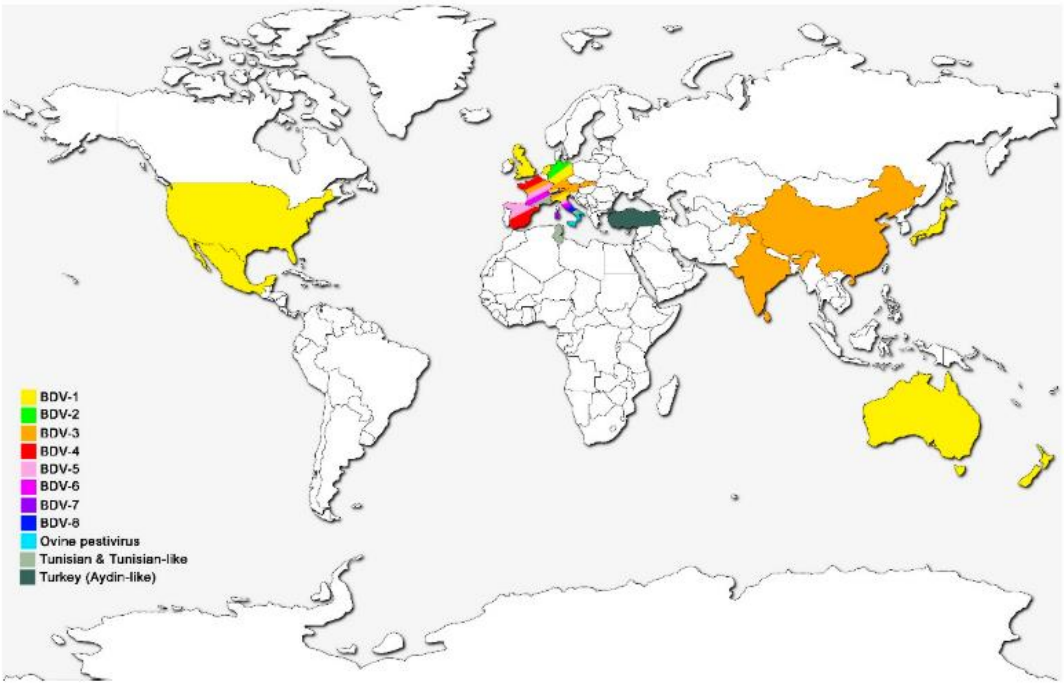

Figure 1.2 Global distribution of border disease (BD).

(Righi et al. 2021)

Most of the studies in Turkey are focused on pestivirus presence rather than BDV; because it is known that both BDV and other pestivirus species BVDV-1 and BVDV-2 can infect sheep, goats and cattle. However, even there is a commercially available vaccine against BVDV and ongoing eradication programmes, it is not the case for BDV. There is no currently available vaccine and treatment against border disease. Therefore, BDV seroprevalence in all regions of Turkey must be determined to understand its spread in Turkish flocks. It is essential to start eradication programmes against the disease (Yilmaz, Yildirim, and Coskun 2014).

Recent phylogenetic studies of Oğuzoğlu and colleagues determined a novel pestivirus species in Turkey. They isolated "Aydın/04" and "Burdur/05" pestivirus isolates from the sheep and goat farms with abortion history. Even though these pestivirus species cause clinical symptoms similar to BDV, phylogenetic analyses and antigenic characterization studies showed that they are novel species and genetically closer to CSFV than BDV (Oguzoglu et al. 2009; Postel et al. 2015). These isolates are called "Aydin-like" and recently classified as *Pestivirus I* (King et al. 2018). It is known that Aydin-like pestivirus is currently circulating in Turkey and its seroprevalence in the country must be extensively investigated with further studies (Oguzoglu et al. 2009).

Border disease is a big threat for sheep and goat production because it reduces the lambing rate of infected herds. Due to high transmission rates of BDV, it carries a great risk to cause epidemic outbreaks in flocks which causes major economic losses in the industry. "Aveyron strain" in BDV-5 genotype is responsible from one of the major border disease outbreaks. It is first isolated in 1984 from the Aveyron region of France and associated with a high mortality disease (Vilcek et al. 2014). In 1997, Aveyron strain caused a horizontal BDV infection in Spanish lambs and since it has high mortality, 70% of the herd is lost (Vega et al. 2015). It is known that border disease not only infect domestic ruminants, but also threatens life of wildlife species. In 2001, BDV-4 genotype caused border disease outbreak in Pyrenean chamois (*Rupicapra pyrenaica*) in Spain. Mortality rate was so high that Pyrenee population reduced 80% and due to dramatic loss, species were on the edge of extinction. Considering previous outbreaks with high mortality rates, BDV carries a great potential to cause outbreaks among domestic and/or wildlife ruminant species in the close future (Marco et al. 2015; Serrano et al. 2015).

1.2.3 Disease Control

Border disease is mostly not monitored and there are no international eradication campaigns against BDV. It is declared by World Organisation for Animal Health (OIE) as one of the notifiable terrestrial diseases that threatens livestock health ((OIE) 2017). For disease control, there is a need for international eradication campaign similar to BVDV, especially in Europe (Oğuzoğlu 2012). There is no commercially available vaccine or treatment against border disease. Killed and live attenuated vaccines against BVDV are used in sheep for protection against BDV, considering they are both pestivirus

species. However, recent study of Meyer and colleagues showed that BVDV vaccination is insufficient for preventing fetal infections. It is possibly due to antigenic differences of BVDV and BDV. Therefore, there is an urgent need for development for BDV vaccine and mass vaccination of susceptible herds for border disease eradication (Meyer et al. 2021). There are certain precautions to take for disease control. Firstly, PI lambs are one of the most important infection sources of BDV and since they are BDV antibody negative, serological tests will be insufficient for their detection. Regular screening of the herds for PI lambs, especially before breeding season using nucleic acid detection methods will enable their detection. After identification of PI lambs, they must be isolated to protect the rest of the herd, especially pregnant ewes. Before national and international animal trades, newly purchased animals must be tested against BDV to prevent spread of the disease nationally and globally. It is known that BDV can cross species barrier to affect other domestic ruminants such as cattle, pigs as well as wildlife species. It must be avoided co-housing of sheep, goat and cattle in crowded farms to prevent interspecies transmission of BDV (Løken 1995; Nettleton et al. 1998). Grassing of sheep and goat herds in common pastures with wildlife species can cause transmission of BDV to one another. Wild-life species such as Pyrenean chamois (*Rupicapra pyrenaica*) can be susceptible to border disease and possible outbreaks can threaten the biodiversity. Therefore, such interactions between domestic and wildlife ruminants must be controlled and avoided to protect both side (Saltik, Kale, and Atli 2021).

1.2.4 Diagnostic Techniques

Frequent abortions, stillbirths, and birth of weak “hairy-shaker” lambs in sheep or goat herd indicates presence of BDV infections. In such cases, there are different methods for diagnosis of border disease. Virus isolation is one of the common methods used for BDV determination. Almost all BDV isolates are non-cytopathogenic in cell culture so virus isolation can be properly applied. In this method, collected animal samples (whole blood, serum, semen or tissue) are co-cultivated with BDV-susceptible cell lines to detect they can infect them or not. Ovine cells, semicontinuous fetal lamb muscle (FLM), whole embryo or sheep choroid plexus are preferred cell lines for BDV isolation. This method is very sensitive but can yield results 7 days after culturing. Overall, virus isolation is time

consuming, labour-intensive and give results later than desired time (Nettleton et al. 1998; (OIE) 2017).

Enzyme- linked immunosorbent assay (ELISA) is another common method for BDV antigen detection from blood (washed leukocytes) or tissue samples (spleen). Mab (monoclonal antibody) capture ELISA can be used, especially for detection of PI animals in sheep (Fenton et al. 1990). It is sensitive as virus isolation and more practical and can be applied for testing a large group of animals. However, it might cause to get false-negative results in PI lambs younger than two months. Because these newborn lambs take colostral antibodies with colostrum milk for 2 months and these antibodies can mask presence of BDV antigen in blood in that time. It is not a desired outcome since early diagnosis and isolation of PI lambs is essential to preserve health of the herd. This method is also not sensitive enough to the detect antigen directly from blood, it is only applicable to washed and lysed leukocytes which is a time consuming and labour-intensive step. There are no commercially available ELISA antigen kits on the market against BDV. Commercially available kits against BVDV such as IDEXX BVDV Ag/Serum Plus Test (IDEXX, Switzerland) and IDEXX Bovine Viral Diarrhea Virus Antigen Point-of-Care Test (IDEXX, Switzerland) are used in the field for detection of BDV antigen as well. However, considering antigenic differences of BVDV and BDV; they are not recommended to be used for BDV identification (Nettleton et al. 1998; (OIE) 2017; Fenton et al. 1990). It is reported in many studies that BVDV antigen ELISA kits have low sensitivity and inadequate to detect BDV antigen (Mishra et al. 2016; Kittelberger and Pigott 2011; Strong et al. 2010)

Serological antibody detection tests such as virus neutralization and Mab capture ELISA can be used to identify BDV antibody in blood serum. Choosing the reference virus strain for virus neutralization test is difficult because BDV has eight genotypes indicating its antigenic heterogeneity. Therefore, it is recommended to use more than one reference strain and they must be chosen considering locally distributed strains. Generally, pestivirus-negative lamb kidney or testis cells are preferred for cultures. Virus neutralization also can be labour-intensive, requires trained personnel and very time-consuming since it results approximately in 7 days.

Another method for BDV antibody detection is Mab capture ELISA. Fenton and colleagues developed an ELISA which uses two pan-pestivirus mAbs to detect NS2 and

NS3 non-structural proteins of BDV (Fenton et al. 1991). On the other hand, commercially available Mab ELISA test kits are developed to detect both BVDV and BDV; such as LSIVetTM Ruminant BVD/BD p80 Serum/Milk ELISA Kit (LSI, France), ID Screen® BVD p80 Antibody One-Step (IDvet, France), ID Screen® BVD p80 Antibody Competition (IDvet, France) and CIVTEST BOVIS BVD/BD P80 (Hipra, Spain) (More et al. 2017). These tests designed to capture either E2 (p80) or E^{rns} envelope glycoproteins of these pestiviruses. However, E2 is one of the coding regions and it is highly variable having high mutation rates. Considering heterogeneity of BVDV and BDV isolates, using these tests are incapable of identifying antibodies against BDV in the field (Oğuzoğlu 2012; Mirosław and Polak 2020). Overall, these serological tests are insufficient to identify acute BDV infections. Instead, they can only reveal animal's immunity against the disease by measuring the antibody response and can be used to understand global distribution of border disease. Furthermore, PI animals are known to be antibody negative but antigen positive, hence serological tests cannot be used to detect PI animals either ((OIE) 2017; More et al. 2017).

OIE criteria designate the "gold-standard" test for BDV identification as nucleic acid detection by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). RT-PCR is more reliable for BDV identification because it has higher sensitivity and specificity compared to other diagnostic techniques. Blood, serum, or fixed tissue samples of suspected animals can be used for testing. It is faster than previously described BDV detection methods, but it requires preliminary RNA extraction step which can be time-consuming. All experiments should run using a qPCR instrument which is very costly. Also, single-step close-tube reactions should be applied by trained personnel to avoid contamination (More et al. 2017).

A nested, two-step RT-PCR assay was designed for specific BDV detection. First PCR step identifies all pestiviruses using consensus primers while second step uses BDV-specific primers to differentiate BDV (Fulton et al. 1999). Then, another nested but one-step, closed-tube RT-PCR assay was developed to differentiate pestivirus species using different fluorescent probes (McGoldrick et al. 1999). Most widely used one-step RT-PCR assay for BDV detection designed by Vilcek and Paton (2000). In the study, PBD1 and PBD2 primers designed based on most conserved 5'UTR region of BDV genome (Vilček and Paton 2000). A one-step, multiplex RT-PCR test is developed to differentiate BDV from BVDV-1 and BVDV-2 and also enables detection of all three pestiviruses

simultaneously (Willoughby et al. 2006). La-Rocca and Sandvik (2009) developed a one-step duplex TaqMan RT-PCR by designing one former and two reverse primers based on 5'UTR and different TaqMan probes to identify BDV and BVDV-1//BVDV-2 (La Rocca and Sandvik 2009). Recently, another study used previously designed primers (La Rocca and Sandvik 2009) and novel probes to improve real-time RT-PCR for BDV identification and optimized this assay to be applied to ear notch samples of animals. This improvement simplified the sampling process and more adaptive to field applications (Kalaiyarasu et al. 2019).

Overall, all real-time RT-PCR tests were more practical and rapid while having high sensitivity and specificity compared to virus isolation and ELISA assays. Since they can detect both residual nucleic acid and viral agent, they can be used to investigate acute infections and cause of abortions and stillbirths from the tissue samples as well. Real-time RT-PCR assays are not affected by the presence of colostral antibodies in the sample, therefore they can be used for early identification of PI lambs. However, considering the heterogeneity of BDV, assay primers must be designed in a way to cover all BDV subtypes. Even it is rapid compared to other detection techniques, real-time RT-PCR should be applied in laboratories by trained personnel using a qPCR machine, which is far from field conditions and can be very costly and labour intensive ((OIE) 2017; More et al. 2017).

1.3 Isothermal Amplification Techniques

Isothermal amplification techniques are widely used recently as an alternative to polymerase chain reaction (PCR) for nucleic acid amplification. These techniques are preferred, especially for point-of-care testing because isothermal amplification enables rapid, highly sensitive and specific amplification in a constant temperature (Boonbanjong et al. 2022).

PCR technique is one of the greatest discoveries of molecular biology and inventor Kary B. Mullis has been awarded with a Nobel Prize in 1993 (Soroka, Wasowicz, and Rymaszewska 2021). It has many applications in forensics, medical diagnostics, and genetic testing. PCR enables the amplification of even single-molecule DNA or RNA genetic material by creating millions of copies in a short time. A regular PCR reaction includes complementary primers to target gene, DNA template, thermostable DNA

polymerase enzyme, buffer solution and deoxyribonucleotides (dNTPs). PCR reaction has three main steps. First step is denaturation where the reaction is heated to 94℃ for separating the target double-stranded DNA strands into single strand. Second step is the annealing step, reaction temperature is decreased to 45-60℃ and primers can bind (anneal) their complementary regions at the target genome. Last step is called extension or elongation, reaction is heated to 72℃ so that thermostable DNA polymerase enzyme attaches primer-binding sites and starts to extend the target from primer binding site by adding dNTPs 5' to 3' direction. PCR has exponential growth, and these three steps are repeated 20-40 times which is called cycling to produce millions of DNA copies rapidly. Taq DNA polymerase is isolated from *Thermis aquaticus* bacteria and it is the most widely used thermostable DNA polymerase for PCR. RNA can also be amplified using PCR. Since it is a single-stranded molecule, it should be converted to double-stranded cDNA first using reverse transcriptase enzyme. This reaction is called reverse transcriptase (RT)-PCR (Canene-Adams 2013).

Isothermal amplification techniques have certain advantages compared to PCR. Firstly, they can be conducted in constant temperature while PCR requires temperature change through cycling and a thermocycler. Thermocycler is an expensive instrument while constant temperature can be easily arranged using simple and cheaper instruments such as a dry-heater or a water-bath. Since temperature is constant, isothermal amplification methods use special DNA polymerase enzymes that has strand displacement ability for denaturation process. RNA templates can also be amplified with isothermal amplification systems with addition of a reverse transcriptase step. Overall, these assays are cost-effective because they have simpler protocols that can be applied without trained personnel and without the need of expensive instruments. Also, they are convenient for point of care and on-site testing. They can be used in remote hospitals, outpatient clinics, veterinary clinics and even in the houses. Accessible and cost-effective assays play an important role for early diagnosis and rapid isolation of infected individuals, especially for infectious diseases, during pandemics like COVID-19 (Boonbanjong et al. 2022; Zanoli and Spoto 2013).

There are various isothermal amplification methods such as Loop-Mediated Isothermal Amplification (LAMP), Exponential Isothermal Amplification (EXPAR), Recombinase Polymerase Amplification (RPA), Exponential Rolling Circle Amplification (E-RCA), Exponential Strand Displacement Amplification (E-SDA),

Nucleic Acid Sequence-Based Amplification (NASBA), Helicase Dependent Amplification (HDA) and Whole Genome Amplification (WGA). LAMP method stands out among other isothermal amplification techniques with its high specificity, rapidity, simplicity, and easy interpretation of the results (Y. Zhao et al. 2015).

1.3.1 Loop-Mediated Isothermal Amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is one of the most popular isothermal techniques for nucleic acid amplification. It is first described by Notomi and colleagues (2000) as a simple, rapid, highly specific and cost-effective nucleic acid amplification protocol (Notomi et al. 2000). LAMP protocol requires no cycling, therefore there is no need to use a thermal cycler which is quite expensive. Instead, LAMP experiments run at constant temperature around 60-65°C and simple instruments such as a dry heater or a water bath is sufficient to provide experiment conditions. In order to maintain high specificity, LAMP technique uses 4-6 primers instead of 2 primers used in a regular PCR reaction. These primers recognize specific regions of the target gene and reassures the amplification of only target sequence. There is no denaturation step in LAMP reactions. Therefore, rather than regular Taq DNA polymerase of PCR; a polymerase with stand displacement ability is used for LAMP. *Bst* DNA polymerase isolated from *Bacillus stearothermophilus* bacteria, and it is the most favoured DNA polymerase for LAMP applications. LAMP reactions usually take up to 1-1.5 hours, product formation is much faster than conventional PCR and RT-qPCR. LAMP can be used for both DNA and RNA detection. Reverse transcriptase enzyme can be added to the reaction for RNA identification, and it is called RT-LAMP. LAMP can be optimized to be a closed-tube, single-step reaction which decreases the risk of contamination. It can be optimized to be conducted with any sample such as blood, serum, saliva; and sometimes without the need of RNA/DNA extraction step. Eliminating this step saves time, decreases the contamination risk and prevents the loss of genetic material during extraction steps. (Soroka, Wasowicz, and Rymaszewska 2021; Boonbanjong et al. 2022; Hayashida et al. 2015).

LAMP reaction contains 4-6 primers: two internal primers forward internal primer (FIP) and backward internal primer (BIP); two external primers forward primer (F3) and backward primer (B3); and the optional loop primers forward loop primer (FL) and

backward loop primer (BL) (Figure 1.3 A). LAMP reaction has two main steps: first step is the formation of dumbbell structure (Figure 1.3 B) and second step is the exponential nucleic acid amplification (Figure 1.3 C).

Dumbbell shape structure formation includes multiple steps. Firstly, backward inner primer BIP hybridizes to its complementary sequence on the target DNA and strand displacing DNA polymerase elongates the complementary strand. Secondly, outer primer B3 hybridizes to its complement, B3c, at 3' end of target DNA. Then, DNA Pol displaces the strand synthesized in the first step and synthesizes a new complementary strand. In the third step, forward inner primer FIP binds to its complementary fragment at the displaced strand of second step and DNA Pol elongates the rest of the strand starting from 3' end of F2 site. In the fourth step, forward outer primer F3 hybridizes to its complementary region, F3c, at the 3' end of target DNA. Then, DNA polymerase displaces the previously synthesized strand and synthesizes the complementary strand. The displaced strand in the fourth step includes complementary regions (F1-F1c and B1-B1c) at the ends. Once these regions bind to each other, dumbbell structure is formed. Dumbbell shape structured DNA contains many different primer-binding regions, including regions for optimal loop primers and this structure serves as template for further amplification. Exponential amplification of this self-priming template through strand displacement activity of DNA polymerase results in formation of double-stranded DNA with different lengths. These variable length double-stranded DNA fragments are the products of LAMP reaction and known as concatemers (Figure 1.3).

There are several detection techniques to visualize products of LAMP. LAMP has exponential product formation, and it is faster than PCR. 10^9 copies of the target can be produced less than 1.5 hours, which is 100 times higher and much faster than conventional PCR. Intense product formation enables to detect LAMP reaction results with naked eye. In a LAMP reaction, there is a by-product which is called magnesium pyrophosphate ($Mg_2P_2O_7$). It is formed by the interaction of Mg^{2+} ions in the reaction mix and pyrophosphate ($P_2O_7^{4-}$). Pyrophosphate is the side product generated after each dNTP addition during DNA strand synthesis. Accumulation of magnesium pyrophosphate ($Mg_2P_2O_7$) turns reaction mixture into opaque and precipitates in the tube are so concentrated that they can be seen by naked eye. This accumulation forms "turbidity" and an inexpensive turbidimeter instrument can be used for real-time monitoring as well (Soroka, Wasowicz, and Rymaszewska 2021; Bodulev and Sakharov 2020). Colorimetric

readouts are one of the useful end-point LAMP detection methods. Hydroxynapthol blue (HNB) and phenol red are commonly used dyes for colorimetric LAMP interpretation. HNB changes solution colour from violet to blue in terms of positivity. Colour change is affected by the decrease of Mg^{2+} concentration of the reaction mixture which happens due to magnesium pyrophosphate formation as a by-product during LAMP reaction. Phenol red colour change is affected by the pH of the LAMP reaction mixture. Another LAMP detection technique is fluorimetric readout. DNA intercalating dyes such as SYBR Green I, Eva Green, Syto 9, Picogreen can be used for fluorimetric detection. These dyes emit the light in a certain wavelength when they bind to double-stranded DNA, and they can be used for real-time monitoring of LAMP fluorescence signal. Agarose gel electrophoresis is a traditional method that can be used to confirm LAMP results as well. When loaded into gel, LAMP products are expected to form various bands since they have different lengths. However, this method is time-consuming and opening the tube for loading the gel can create a cross-contamination risk (Soroka, Wasowicz, and Rymaszewska 2021; Dhama et al. 2014).

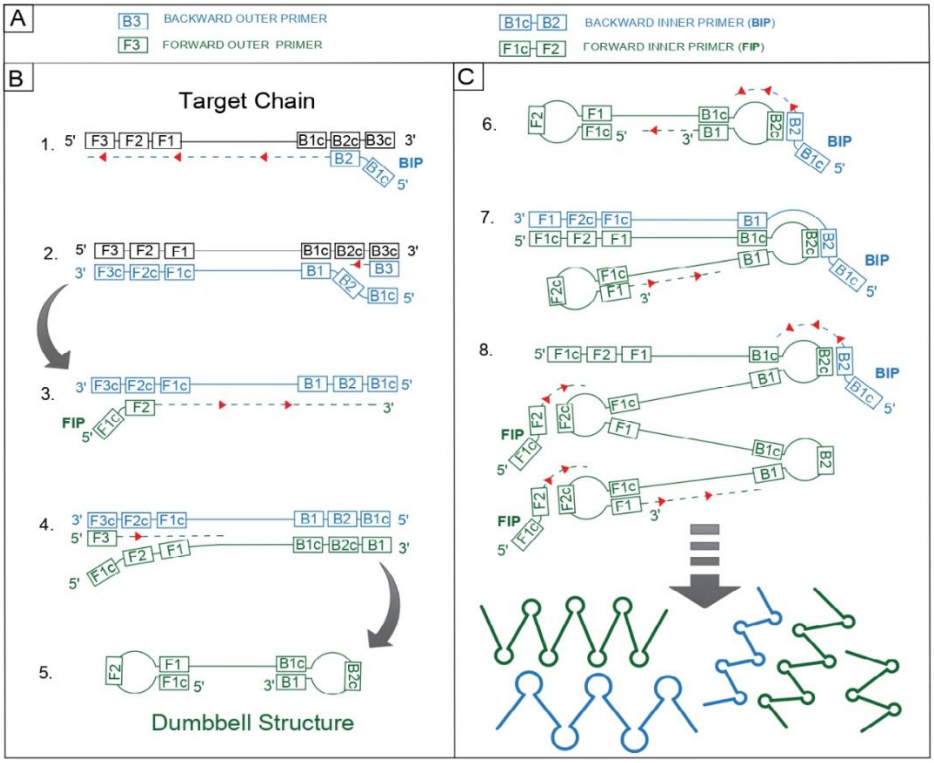

Figure 1.3 Principle of LAMP reaction.

LAMP and RT-LAMP has many practical diagnostic applications for detection of human viral pathogens such as Chikungunya virus (Parida et al., 2007), dengue virus

(Teoh et al. 2013), Ebola virus (Oloniniyi et al. 2017), hepatitis C virus (Quoc et al. 2018), hepatitis C virus (Nyan and Swinson 2016; N. Zhao, Liu, and Sun 2017), human immunodeficiency virus (HIV) (Hosaka et al. 2009), human papillomavirus (Hagiwara et al. 2007), human cytomegalovirus (HCMV) (Xiaoli Wang et al. 2015), influenza viruses (Nakauchi et al. 2014; Bao et al. 2015; Imai et al. 2007), Japanese encephalitis virus (Toriniwa and Komiya 2006), Middle East respiratory syndrome coronavirus (MERS-CoV) (Lee et al. 2017), severe acute respiratory syndrome (SARS) (Poon et al. 2004), West Nile virus (Parida et al., 2004) and Zika virus (Xuan Wang et al. 2016). There are also various multiplex LAMP methods has been developed. Yaren and colleagues established a multiplex LAMP test to identify Chikungunya, Dengue and Zika viruses transmitted by mosquitos to human (Yaren et al. 2021).

Experiencing the recent COVID19 pandemic pointed out the importance of affordable, approachable, fast, and accurate point-of-care (POC) testing, especially in underdeveloped countries. LAMP provides fast and reliable results, therefore spread of the infection can be prevented with early isolation. Interest in LAMP studies drastically increased with COVID19 pandemics and various RT-LAMP protocols have been developed and optimized for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) diagnosis (Chow et al. 2020; Mohon et al. 2020; Yan et al. 2020; Lau et al. 2020). Overall, these tests have limit of detection (LOD) around 10 copies/ml while a conventional RT-qPCR has LOD equals to 100 copies/ml, indicating higher analytical sensitivity. Also, they worked with 100% specificity by identifying only SARS-CoV-2 as viral agent when tested with closely related viruses. There are also specifically designed, portable devices for POC applications of LAMP. Lucira™ and Detect™ devices are developed for at-home COVID19 testing with nasal swabs and results will be interpreted with a phone application. These consumer-friendly tests can also be applied in field hospitals, schools, workplaces and places without access to laboratories (Mannier and Yoon 2022).

Various LAMP protocols are proposed to detect bacterial pathogens of humans such as *Bordetella pertussis* (Fujino et al. 2015), *Klebsiella pneumoniae* (Nakano et al. 2015), *Mycobacterium tuberculosis* (Kaewphinit et al. 2017; Kumar et al. 2014), *Streptococcus pneumoniae* (Seki et al. 2005). A LAMP protocol is used for malaria diagnosis by identification of *Plasmodium* species as well (Zhang et al. 2017; Britton et

al. 2016). A specific LAMP has been established to detect bacteria species responsible from hospital-acquired pneumonia (Vergara et al. 2020).

LAMP methods are widely used in food industry to identify food-borne pathogens that can cause diseases in humans. LAMP tests are available for *Campylobacter* species (Pham et al. 2015), *Enterococcus faecalis* (Martzy et al. 2017), *Escherichia coli* (Ramezani et al. 2018), *Helicobacter pylori* (Yari et al. 2016), *Listeria monocytogenes* (Ye et al. 2015) and *Salmonella typhi* (Zhuang et al. 2014). LAMP protocols are widely used for identification of important plant pathogens and for distinguishing GMO-plants as well (Soroka, Wasowicz, and Rymaszewska 2021; Bhat, Aman, and Mahfouz 2022).

LAMP has many applications for identifications of animal pathogens such as poultry, ruminants and swine (Mansour et al. 2015). LAMP protocols are established for ruminant diseases such as Akabane virus (Qiao et al. 2013), bovine herpesvirus-1 (BoHV-1) (Pawar et al. 2015; El-Kholy, Abdelrahman, and Soliman 2014), bovine leukemia virus (Komiyama et al. 2009), (BLV) bovine rotavirus (BRV) (Xie et al. 2012), bluetongue virus (BTV) (Mulholland et al. 2014; Mohandas et al. 2015), caprine arthritis-encephalitis virus (CAEV)(Huang et al. 2012; Balbin et al. 2014) capripoxvirus (Das, Babiuk, and McIntosh 2012; Batra et al. 2015), foot and mouth disease virus (FMDV) (Madhanmohan et al. 2013; Reid et al. 2014), peste des petits ruminants virus (PPRV) (Mahapatra et al. 2019; Rajko-Nenow et al. 2019) and rift valley fever virus (RVFV) (Peyrefitte et al. 2008; Le Roux et al. 2009).

Currently, there is no LAMP protocol against BDV identification for border disease diagnosis. LAMP tests are designed for another pestivirus species, BVDV which is closely related to BDV. Developed tests are designed LAMP primers based on 5'UTR. Their results were compatible with gold-standard RT-qPCR technique by showing high specificity and sensitivity (Mungthong et al. 2021; Fan et al. 2012; Aebischer et al. 2014).

1.4 Aim of the Study

This study aims to develop a nucleic acid-based isothermal diagnostic test for BDV identification using LAMP technique. Developed test can be applied as a point-of-care (POC) test without the need of trained personnel with simple instruments. Accurate results will be reached less than 1.5 hours and can be interpreted easily. For this purpose,

multiple alignment of BDV sequences around the world were used to detect the most conserved region of the genome. After the determination of conserved region as 5'UTR, LAMP primer sets were designed to target that region. Finally, optimum conditions and limit of detection (LOD) of designed primer sets were determined to be used in LAMP experiments.

CHAPTER 2

MATERIALS AND METHODS

2.1 Multiple Sequence Alignment of BDV Genomes

Complete and partial DNA sequences of BDV genome were obtained from NCBI Virus database. There are totally 197 BDV sequences available: 13 complete and 184 partial sequences. These sequences were examined considering their collection date, geographical region, and host organisms. Among those, the ones isolated from domestic and wild ruminant species (sheep and goats) and more up-to-date sequences were chosen for multiple alignment. In total, 136 BDV sequences (11 complete and 125 partial) were used for multiple alignment. Table 1.1 demonstrates the accession numbers and detailed information of the BDV sequences used for multiple alignment. Multiple alignment was performed using UniPro UGENE programme (multiplatform open-source bioinformatics software) (http://ugene.net/). A consensus sequence is formed by UniPro UGENE based on the multiple alignment of BDV sequences. This consensus sequence represents all sequences used for multiple alignment and shows the frequency of A, T, G and C bases for each nucleotide position. Capital letters indicate conservation while lowercase letters indicate variation in the sequence. "-" indicates the gaps in the sequence, while "+" indicates highly variable positions. Since BDV has a very large genome which is equal to nearly 12300 nucleotides, it is examined by dividing it into 2000 base-long consensus sequences. Figure 2.1 represents the consensus sequence which includes 1-2000 nucleotides of BDV genome.

Table 2.1 BDV sequences used for multiple alignment

	Accession number	**Geographic location**	**Host species**	**Collection date**
	NC_003679	Germany	*Ovis aries*	1998
	MT648677	Germany	*Ovis aries*	2000

(cont. on the next page)

Cont. of Table 2.1

Complete Sequences	MG649392	Italy	*Rupicapra rupicapra*	2015
	MF102260	Switzerland	*Bos taurus*	2006
	MF102261	Switzerland	*Bos taurus*	2011
	MF102262	Switzerland	*Bos taurus*	2015
	KJ463422	USA	*Ovis aries*	2014
	KJ463423	USA	*Ovis aries*	2014
	GU270877	Andorra	*Rupicapra rupicapra*	2002
	GQ902940	Denmark		2010
	JX428945	Turkey	*Ovis aries*	2014
Partial Sequences	MT432532	United Kingdom	*Sus scrofa*	2012
	MT823310	United Kingdom	*Sus scrofa*	2012
	MH908078	Switzerland	*Bos taurus*	2009
	MH908079	Switzerland	*Bos taurus*	2009
	MH908080	Switzerland	*Bos taurus*	2010
	MH908081	Switzerland	*Bos taurus*	2010
	MH908082	Switzerland	*Bos taurus*	2008
	MH908083	Switzerland	*Bos taurus*	2011
	MH908084	Switzerland	*Bos taurus*	2012
	MH908085	Switzerland	*Bos taurus*	2012
	MH908086	Switzerland	*Bos taurus*	2012
	MH908087	Switzerland	*Bos taurus*	2012
	MH908088	Switzerland	*Bos taurus*	2012
	MH908089	Switzerland	*Bos taurus*	2012
	MH908090	Switzerland	*Bos taurus*	2013
	MH908091	Switzerland	*Bos taurus*	2015
	MH908092	Switzerland	*Bos taurus*	2015
	MH908093	Switzerland	*Bos taurus*	2016
	MH395751	Turkey	*Ovis aries*	2016
	MH395752	Turkey	*Ovis aries*	2016
	MG725337	Italy	*Rupicapra rupicapra*	2015

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Cont. of Table 2.1

	MG725338	Italy	*Rupicapra rupicapra*	2015
	LT629121	Spain	*Rupicapra pyrenaica*	2002
	LT629122	Spain	*Rupicapra pyrenaica*	2002
	LT629123	Spain	*Rupicapra pyrenaica*	2002
	LT629124	Spain	*Rupicapra pyrenaica*	2002
	LT629125	Spain	*Rupicapra pyrenaica*	2002
	LT629126	Spain	*Rupicapra pyrenaica*	2002
	LT629127	Spain	*Rupicapra pyrenaica*	2002
	LT629128	Spain	*Rupicapra pyrenaica*	2002
	LT629129	Spain	*Rupicapra pyrenaica*	2002
	LT629130	Spain	*Rupicapra pyrenaica*	2009
	KX573913	Italy	*Rupicapra rupicapra*	2015
	KT327869	China	*Ovis aries*	2015
	KT327870	China	*Ovis aries*	2015
	KT072634	Italy	*Capra hircus*	2014
	LM999985	Italy	*Ovis aries*	2014
	LM999986	Italy	*Ovis aries*	2009
	LM999987	Italy	*Ovis aries*	2012
	LM999988	Italy	*Ovis aries*	2009
	LM999989	Italy	*Ovis aries*	2010
	LM999990	Italy	*Ovis aries*	2013

(cont. on the next page)

Cont. of Table 2.1

	KC176358	Italy	*Capra hircus*	2011
	KC176359	Italy	*Capra hircus*	2011
	KC859383	France	*Rupicapra rupicapra*	2011
	KC859384	France	*Rupicapra rupicapra*	2010
	KC859385	France	*Rupicapra rupicapra*	2010
	KC859386	France	*Rupicapra rupicapra*	2010
	HF567456	Spain	*Sus scrofa*	2007
	JQ994198	Switzerland	*Ovis aries*	2002
	JQ994199	Switzerland	*Ovis aries*	2006
	JQ994200	Switzerland	*Ovis aries*	2006
	JQ994201	Switzerland	*Ovis aries*	2001
	JX683184	China	*Capra hircus*	2012
	JX437132	China	*Capra hircus*	2012
	JX437133	China	*Ovis aries*	2012
	JQ951954	China	*Capra hircus*	2012
	HE818617	Spain	*Rupicapra pyrenaica*	
	HE818618	Spain	*Rupicapra pyrenaica*	
	HE818619	Spain	*Rupicapra pyrenaica*	
	HE818620	Spain	*Rupicapra pyrenaica*	
	HE818621	Spain	*Rupicapra pyrenaica*	
	HE818622	Spain	*Rupicapra pyrenaica*	
	HE615083	Andorra	*Rupicapra pyrenaica*	2009

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Cont. of Table 2.1

	HE615084	Andorra	*Rupicapra pyrenaica*	2009
	HE615085	Andorra	*Rupicapra pyrenaica*	2009
	EU887952	United Kingdom	*Bos taurus*	2006
	EU887953	United Kingdom	*Bos taurus*	2006
	EU887954	United Kingdom	*Bos taurus*	2007
	EU887955	United Kingdom	*Bos taurus*	2008
	EU887956	United Kingdom	*Bos taurus*	2008
	FN397676	Spain	*Rupicapra pyrenaica*	2006
	EU224227	Austria	*Bos taurus*	2006
	EU636998	Germany	*Ovis aries*	2001
	EU636999	Germany	*Ovis aries*	2001
	EU637000	Germany	*Ovis aries*	2008
	EU637001	Germany	*Ovis aries*	2004
	EU637002	Germany	*Ovis aries*	2005
	EU637003	Germany	*Ovis aries*	2007
	EU637004	Germany	*Ovis aries*	2005
	EU637005	France	*Rupicapra pyrenaica*	2006
	EU477593	France	*Rupicapra pyrenaica*	2004
	AM905918	Spain	*Rupicapra pyrenaica*	2006
	AM905919	Spain	*Rupicapra pyrenaica*	2005
	AM905920	Spain	*Rupicapra pyrenaica*	2005
	AM905921	Spain	*Rupicapra pyrenaica*	2005
	AM905922	Spain	*Rupicapra pyrenaica*	2006

(cont. on the next page)

Cont. of Table 2.1

	AM905923	Spain	*Rupicapra pyrenaica*	2006
	AM905924	Spain	*Rupicapra pyrenaica*	2006
	AM905925	Spain	*Rupicapra pyrenaica*	2006
	AM905926	Spain	*Rupicapra pyrenaica*	2006
	AM905927	Spain	*Rupicapra pyrenaica*	2006
	AM905928	Spain	*Rupicapra pyrenaica*	2006
	AM905929	Spain	*Rupicapra pyrenaica*	2006
	AM905930	Spain	*Rupicapra pyrenaica*	2004
	AM905931	Spain	*Rupicapra pyrenaica*	2005
	AM905932	Spain	*Rupicapra pyrenaica*	2005
	AM905933	Spain	*Rupicapra pyrenaica*	2005
	EF693999	France	*Ovis aries*	2006
	EF694000	France	*Ovis aries*	2006
	EF694001	France	*Ovis aries*	2006
	EF694002	France	*Ovis aries*	2006
	EF694003	France	*Ovis aries*	2006
	AM765800	Spain	*Rupicapra pyrenaica*	2005
	AM765801	Spain	*Rupicapra pyrenaica*	2005
	AM765802	Spain	*Rupicapra pyrenaica*	2005

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Cont. of Table 2.1

	AM765803	Spain	*Rupicapra pyrenaica*	2005
	AM765804	Spain	*Rupicapra pyrenaica*	2006
	AM765805	Spain	*Rupicapra pyrenaica*	2006
	AM765806	Spain	*Rupicapra pyrenaica*	2006
	AM765807	Spain	*Rupicapra pyrenaica*	2006
	DQ275622	Spain	*Ovis aries*	2004
	DQ275624	Spain	*Ovis aries*	2004
	DQ275625	Spain	*Ovis aries*	2004
	DQ275626	Spain	*Ovis aries*	2004
	DQ898291	France	*Rupicapra pyrenaica*	2004
	DQ898292	France	*Rupicapra pyrenaica*	2002
	DQ898293	France	*Rupicapra pyrenaica*	2003
	DQ898294	France	*Rupicapra pyrenaica*	2003
	DQ898295	France	*Rupicapra pyrenaica*	2003
	DQ361067	Spain	*Ovis aries*	2002
	DQ361068	Spain	*Ovis aries*	2002
	DQ361071	Spain	*Ovis aries*	2002
	DQ361072	Spain	*Ovis aries*	2002
	DQ361073	Spain	*Ovis aries*	2002

1-70: ggttta+aggggagttcctg+tccc+ttaagaaattggg+gtttcaaatttttaaattggactagggtcc

71-140: cctcctagcgacggccgaaccgggttagccatacccgtagtaggactagcaaacgggaggactagccatc

141-210: gtggtgagatccctgagcggTctaAgccCtGaGTACaGggcAGTCgTCAgTaGTTCgACaCtaacttggt

211-280: ttttct+taGtCTcGAgtatGCtAcGTGGACGAGGGCatGCCCaaGAcacgcttTaaccccggCgGGGGGT

281-350: CGccggGgtgaAaacatCctaattccggGtgtTgGgattACAgCCTGATAGGGtGcTGCAGAGgCCcacg

351-420: cataggctagtataaaaaatctctgctgtacatggcacatggagttgaacaagtttgaacttttatacaa

421-490: aacaagtaaacaaagaccagtaggggttgt+gaaccagtctacgactcagcgggtaacccccctatatggt

491-560: gaaagaag+acaatacacccgcaatccaccctgaaactaccacat+acagaggagtagccgaggtgataa

561-630: caaccctgaaggatttgcc+aggaaaggagactgcaggagtgggaacca+cgaggcccagtgagtggcat

631-700: atacatcaaaccaggaccagtcatataccaggattacaagag+ccagtgtaccatagagcaccactggag

701-770: ct+ttcactgaggcacaattctgtgaggtcacaaagaggat+gggagagt+actggtagtgatggcaagt

771-840: tgtaccacct+tacgtttgctccgatgggtgcatattg+tgaaactggcaag+aggactgggaatgcagt

841-910: gctaaagtggacacataacatcctggattgtccactgtgggtgacaagctgctctgatgacaacaaaaac

911-980: aaaggggccg+t+gcaagaaaccagatagggtcaagcgcggagcaatgaaaatcacccccaaggagagtg

981-1050: agaaagatagcaaaaccaaaccacccgatgcaactatagtagtagaaggagtgaaataccaggtgaagaa

1051-1120: gaaaggaaaggtgaaggg+aagaacac+caagatggcctataccacaacaaaaacaaaccacc+gaatca

1121-1190: aggaaaaaattggagaaagcactgctggcttgggctattat+gcaattctcatgtggca+cccgta+cac

1191-1260: c+gaaaacataacccagtggaacctaagtgacaatgggactaatggtatccaacatgtaatgtaccaaag

1261-1330: aggtgtgaacaggagtttacatggtatttggccggaaaagatatgcacaggagtaccaac+cacctggca

1331-1400: acagacacagaactgaaaggaatccaggggatgatggatgccagtga+aaaaccaactacac+tgttgca

1401-1470: gacttcagaggcatgagtggaacaaacatgggtggtgtaactggtataacataga+ccgtggatatggtt

1471-1540: gatgaataaaacccaggctaacctgacagagggacctccaga+aaagagtgcgc+gttac+tgcaggttt

1541-1610: gacaaagaagc+gat+taaatgt+gtgacacaggccagggacagaccaaccactctaacagggtgcaaga

1611-1680: aagggaagaaattttcttttgcgggtatggt+atcgagggcccttgtaatttcaatgtatctgtggagga

1681-1750: catactatttggagacaatgaatgtagtaac+tgttccaggacacagccct+tatgtagtggatggggt+

1751-1820: accaacaccgttgagaatgctagacaaggggctgcaaaactgacatcctggctggggaagcagttgggta

1821-1890: taatggggaa-aaact-gaacataagag-aa-ac-tggttcgg-gc--atgccca-tcgcc-tattg-aa

Figure 2.1 2000 base-long consensus sequence representing 136 BDV sequences worldwide.

2.2 Design of LAMP Primers

LAMP primers against BDV were designed using the consensus sequence formed based on multiple sequence alignment results (Figure 2.1). BDV has a large genome, therefore most conserved region of the genome was determined as target for LAMP primers. Primer Explorer V5 software (Eiken Chemical Co. Ltd., Tokyo, Japan) (http://primerexplorer.jp/lampv5e/index.html) is the most widely used tool for designing LAMP primers and it was used to design BDV LAMP primers in this study. Programme evaluates the consensus sequence and design candidate primer sets against the target based on design parameters.

First parameter of LAMP primer design is melting temperature (T_m) of the primers. Optimum Tm value for F1c and B1c should be around 64-66°C; for F2, B2, F3 and B3 around 59-61°C; and for LF and LB around 64-66°C. T_m values of primers should be close to each other, maximum difference can be 5°C. Second parameter is the GC content of the primers, it should be around 50-60% for each primer. GC content directly affect T_m values and they are associated with the stability of primers. Higher GC content causes higher T_m values which complicates denaturation of DNA. If GC content is low, T_m will be low as well and primers will be less stable.

The stability of the 5' and 3' ends of primers was examined as another criterion for LAMP primer design. For optimum stability, the 3' ends of primers F2, B2, F3, B3, LF and LB; and the 5' ends of F1c and B1c primers should have a free energy (ΔG) equals to -4 kcal/mol or less. As ΔG of the reaction decreases, binding of primers to target will be better. Also, DNA synthesis by DNA polymerase starts at the 3’ end of primers, therefore they must have a certain level of stability.

Another important parameter is the distance between primers. The distance between 5’ end of F2 to 5’ end of B2 should be 120-160 bp long. Distance from 5’ end of F2 to 5’ end of F1; and from 5’ end of B1 to 5’ end of B2 should be 40-60 bp. Distance of this region is particularly important because loop primers are designed targeting this region. Finally, distance between F2-F3 and B2-B3 primers should be 0-60 bp long (Figure 2.2).

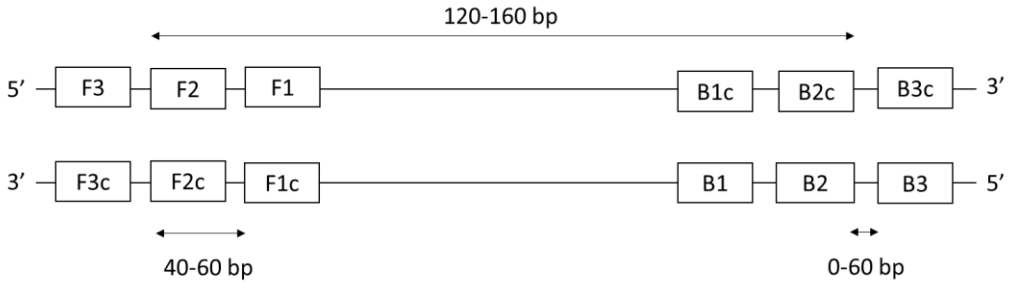


Figure 2.2 Locations of LAMP primers.

LAMP reaction contains 4-6 primers rather than 2 primers of conventional PCR. Therefore, it is more complex and has more parameters than PCR primer design. Candidate primer sets formed by the programme was evaluated based on their suitability to design parameters and three primer sets were chosen to setup and optimize BDV LAMP experiments.

Chosen primer sets were examined for possible secondary structure formation. If primers have complementary sequences within itself or another copy of the same primer, they might form stable secondary structures or homodimers. If primers have complementary sequences with other primer, they might form heterodimer structures. Heterodimer, homodimer or hairpin structure formation reduces possibility of product amplification and creates false-positive results. IDT Oligo Analyzer software (IDT, USA) (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/) is used to examine secondary structures of candidate LAMP primers.

Lastly, chosen LAMP primers were searched in the NCBI database using the NCBI BLAST tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if they are complementary to nucleotide sequences of *Artiodacytla* members. *Artiodacytla* comprises of domesticated and wildlife ruminant species and any complementarity of primers to ruminants might reveal false-positive results. NCBI BLAST tool is also used to compare designed primers with other pestiviruses and ruminant viral pathogens such as foot and mouth disease virus (FMDV), blue tongue virus (BTV), peste de petits ruminant virus (PPRV) and rift valley fever virus (RVFV) to determine their specificity to BDV and eliminate the risk of false-positive results.

2.3 LAMP Reaction Setup and Optimization

Designed three primer sets primer set 1, primer set 2a and primer set 2b; and template BDV DNA for LAMP experiments were synthesized by Integrated DNA Technologies (IDT, USA). As target DNA, 350 bp-long synthetic g-Block (IDT, USA) is used, and it comprises 50-400 bp of BDV DNA sequence. Results of LAMP reactions were interpreted monitoring the time-dependent fluorescent signal change with a real-time qPCR instrument, LightCycler® 96 (Roche Life Science, Switzerland). LAMP fluorescent dye (50X) (New England Biolabs, UK) is used as a DNA intercalating dye for real-time tracking of fluorescent signal. It has the same properties with SYBR Green I, its emission maximum is at 516 nm wavelength (SYBR/FAM channel).

A standard, 25 µl LAMP reaction setup includes: 2.5 µl from isothermal amplification buffer pack (10X) (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 500 mM KCl, 20 mM $MgSO_4$ and 1% Tween® 20) (New England Biolabs, UK), 2.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/ml) (New England Biolabs, UK), 1.5 µl from magnesium sulfate ($MgSO_4$) solution (100 mM) (New England Biolabs, UK), 3.5 µl from deoxynucleotide (dNTP) solution mix (10 mM) (New England Biolabs, UK), 0.5 µl from LAMP fluorescent dye (10X) (New England Biolabs, UK), 2 µl template DNA and rest is DNase/RNase-free dH_2O. Primer sets were prepared as 10X stock solutions. 10X primer set 1 includes six primers: 16 µM FIP/BIP, 2 µM F3/B3 and 5 µM LF/LB. 10X primer set 2a includes five primers: 16 µM FIP/BIP, 2 µM F3/B3 and 5 µM LB. 10X primer set 2b includes five primers: 16 µM FIP/BIP, 2 µM F3/B3 and 5 µM LB.

Each experiment is performed with a negative control (NC) which contains all components of the LAMP reactions but instead of template DNA, it contains same amount of DNase/RNase-free dH_2O. All experiments were performed in laminar flow cabinet to reduce the risk of contamination. All reaction components were stored at -20°C and dissolved on ice before use.

LAMP reaction setups prepared to determine the efficiency of three primer sets. Then, various parameters such as fluorescent dye concentration, temperature, primer concentration, Mg^{2+} concentration, presence of loop primers and enzyme concentration

of LAMP reactions were optimized for each primer sets. Finally, limit of detection (LOD) of each LAMP primer set for BDV identification was determined.

For fluorescent dye concentration optimization, efficiency of Eva Green (EG) and SYBR Green I (SG) dyes were compared. For this purpose, LAMP reactions were prepared for each primer set using both dyes. Reactions with 0.5X Eva Green dye contains 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 1.25 µl from EG dye (10X), 2 µl template DNA (10^8 copies/µl) and 10.75 µl DNase/RNase-free dH_2O. Reactions with 0.2X SYBR Green I dye contains 2.5 µl from 10X isothermal amplification buffer, 2.5 µl from 10X primer mix, 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^8 copies/µl) and 11.5 µl DNase/RNase-free dH_2O. All experiments were performed at 65°C for 90 minutes using RT-qPCR instrument. Results of LAMP experiments were compared, and optimum dye and its concentration was determined.

For temperature optimization, LAMP reactions were setup for each primer set and run at 63°C, 65°C and 67°C, respectively. A standard LAMP reaction was prepared using 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 11.5 µl DNase/RNase-free dH_2O. Triple positive control (PC) and one negative (NC) control was used for each experiment. Time-dependent fluorescent signal change was monitored for 90 minutes at different temperatures. Results of LAMP experiments were compared and optimum temperature for each primer set was determined.

For primer concentration optimization, LAMP reactions were setup for each primer set by increasing the primer mix concentration from 1X to 1.4X. 1X primer mix contains 1.6 µM FIP/BIP, 0.2 µM F3/B3 and 0.5 µM LF/LB. A standard LAMP reaction was prepared with 1X primer mix concentration as described previously. Then, LAMP reactions with 1.4X primer mix concentration was prepared with 2.5 µl from isothermal amplification buffer (10X), 3.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP

mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 10.5 µl DNase/RNase-free dH_2O. 1.4X primer mix contains increased concentrations of each primer: 2.24 µM FIP/BIP, 0.28 µM F3/B3 and 0.7 µM LF/LB. Triple PC and one NC was used for each experiment and experiments were performed at 65°C for 90 minutes. Results were compared and optimum primer concentration was determined for each set.

For Mg^{2+} concentration optimization, LAMP reactions with 6 mM, 8 mM and 10 mM Mg^{2+} was prepared for each primer set. A standard LAMP reaction has 8 mM Mg^{2+}: 2 mM comes from isothermal amplification buffer and 6 mM comes from $MgSO_4$ solution. Total Mg^{2+} concentration was arranged by changing the amount of $MgSO_4$ solution. A standard LAMP reaction was prepared with 8 mM Mg^{2+}. Then, LAMP reactions containing 6 mM Mg^{2+} was assembled with 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 12 µl DNase/RNase-free dH_2O. Lastly, LAMP reactions containing 10 mM Mg^{2+} was assembled using 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 1 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 2 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 11 µl DNase/RNase-free dH_2O. Triple PC and one NC was used for each experiment and experiments were performed at 65°C for 90 minutes. Results were compared and optimum Mg^{2+} concentration was determined for each set.

For enzyme concentration optimization, LAMP reactions containing 6 U, 8 U and 12 U *Bst* DNA polymerase enzyme concentration was prepared for each primer set. A standard LAMP reaction containing 8 U enzyme was prepared as described previously. Then, LAMP reactions with 6 U enzyme concentrations were made using 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 0.75 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 11.75 µl DNase/RNase-free dH_2O. After that, LAMP reactions containing 12 U enzyme were prepared with 2.5 µl from isothermal amplification buffer (10X), 2.5 µl from primer mix (10X), 1.5 µl from *Bst* 2.0 warm start DNA polymerase (8,000 U/m), 1.5 µl from $MgSO_4$ solution (100 mM), 3.5 µl from dNTP mix (10 mM), 0.5 µl from SG dye (10X), 2 µl template DNA (10^6 copies/µl) and 11 µl DNase/RNase-free dH_2O.

Experiments were performed at 65℃ for 90 minutes and results were compared to decide optimum enzyme concentration for each primer set.

Effect of the absence of loop primers were also investigated for each primer set. For this purpose, primer set 1, 2a and 2b were prepared without loop primers. Each set was containing only 16 μM FIP/BIP and 2 μM F3/B3 primers. Then standard LAMP reactions were prepared as described previously. Triple PC and one NC was used for each experiment and experiments were performed at 65℃ for 90 minutes. Results were compared with the results of reactions containing loop primers.

After the establishment of optimum conditions for each primer set in LAMP reactions, limit of detection (LOD) was determined for each primer set as an indicator of their sensitivity. For this purpose, standard LAMP reactions were prepared using 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies/μl BDV DNA as template, respectively. All reactions were run at 65℃ for 90 minutes. In order to improve LOD, temperature optimization and primer concentration optimization reactions were repeated for each primer set. Standard LAMP reactions were established as described previously, using 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies/μl BDV DNA as template. Experiments were performed at 65℃ for 90 minutes and effect of changing these parameters to LOD of primer sets was investigated.

CHAPTER 3

RESULTS

3.1 Multiple Sequence Alignment of BDV Genomes

BDV sequences across the world were evaluated based on their host species, isolated geographical region, and collection dates (Table 2.1). Then, 11 complete and 125 partial BDV sequences were chosen and downloaded from NCBI Virus database. Multiple alignment of these 136 BDV sequences was performed with UniPro UGENE programme. A consensus sequence representing the conserved and variable regions of BDV genome was created as a result. Since BDV has very large genome (12300 bases), multiple alignment results were separated into 2000 base-long consensus sequences. When consensus sequences were compared, it was decided that consensus sequence covering 1-2000 bp of BDV genome was the most conserved among all. Then, this region was examined in detail and the most conserved region was determined as 5'UTR which comprises between 1-400 nucleotides of the BDV genome. Therefore, 5'UTR of BDV genome was targeted for LAMP primer design. A representative image of multiple alignment results was shown in Figure 3.1, bar graph below represents the conservation of the genome and it shows that most conserved region is 5'UTR.

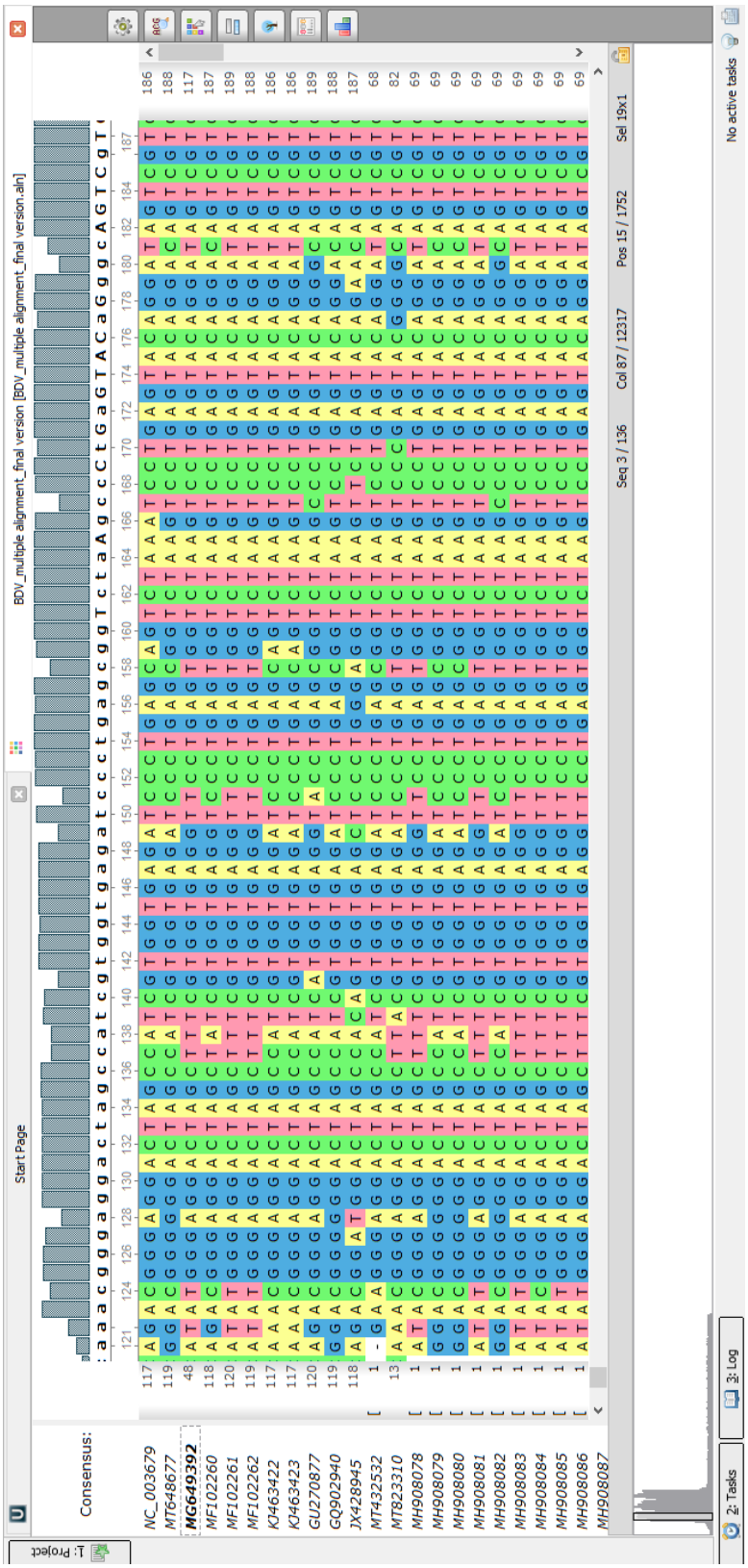


Figure 3.1 Representative image of multiple sequence alignment of BDV.

3.2 Design of LAMP Primers

According to multiple alignment of BDV sequences worldwide, most conserved region of the BDV genome was detected as 5'UTR. Therefore, 5'UTR was targeted for LAMP primer design for BDV detection. Consensus sequence that covers 5'UTR was fed into Primer Explorer v5 (EIKEN, Japan) tool and programme proposed various LAMP primer sets to target that region. Proposed candidate primer sets were evaluated based on their length, distance between primers, 5'and 3' end stability, T_m and GC content. As a result, three primer sets which are primer set 1, primer set 2a and primer set 2b were selected to target BDV in LAMP reactions (Table 3.1).

Primer set 1 contains six primers and targets between 87-332 nucleotides of BDV (Figure 3.2). Primer set 2a has five primers in total and targets between 146-340 bp of BDV genome (Figure 3.3). Primer set 2b includes five primers and targets between 158-340 bp of BDV sequences (Figure 3.4). Primers set 1 contains both loop primers LF and LB while primer set 2a and 2b only contains LB. It is because LF cannot be designed for these primer sets due to high variability of the BDV genomes. Loop primers are optional, they are not essential, but it is known that they speed up LAMP reactions and increases specificity.

BDV has at least eight subtypes. We aimed to design a LAMP test to detect all subtypes of BDV because spread of BDV and its subtypes around the world is yet poorly understood. For targeting more BDV sequences, some primers were modified by adding degenerate nucleotides. Degenerate nucleotide provides a mixture of nucleotides for that certain position to increase sequence coverage. Table 3.1 indicates the degenerate nucleotides of primers with red letters. K represents G or T, M represents A or C, R represents A or G, Y represents C or T, and W represents A or T.

Selected primer sets were analysed for possible secondary structure formations such as hairpins, homodimers, and heterodimers with IDT Oligo Analyzer software (IDT, USA). Results showed that possible secondary structures have T_m values lower than LAMP rection conditions (60-70°C) and ΔG values were higher than -9 kcal/mol which indicates that these structures are not stable enough and cannot cause non-specific binding and false-positives in BDV LAMP experiments. Finally, selected primers were compared with members of *Artiodacytla* which includes wild and domestic ruminant species, using

NCBI BLAST tool. Results proved that designed LAMP primers are not present in any *Artiodacytla* species genomes. Also, designed primers were compared with other pestiviruses and ruminant viral pathogens such as foot and mouth disease virus (FMDV), blue tongue virus (BTV), peste de petits ruminant virus (PPRV) and rift valley fever virus (RVFV). Results showed that designed primer set 1, 2a and 2b are specific to BDV only.

Table 3.1 Information of LAMP primer set 1, primer set 2a and primer set 2b

Primer Set	Primer	Primer Sequence	Dimer dG	Lentgh	5'-3' Position	5'dG	3'dG	GC(%)	Tm(℃)
Primer Set 1	F3	GAACCGGGTTARCCATACC		19	87-105	-5,79	-4,23	58	59,2
	F2	GTAGGACTAGCAKACGGGA		19	110-128	-4,43	-6,53	58	59,2
	F1c	TCAGGRCTTAGACCGCTCAGGK		22	151-172	-5,11	-5,7	56	65,1
	B1c	GTCTCGAGATGCTACGTGGACG		22	220-241	-5,53	-6,19	59	64,1
	B2	GTTTTCACCCYGRCGACC		18	277-294	-3,78	-7,03	61	60,2
	B3	AGCACCCTATCAGGCTGTR	-1,85	19	314-332	-6,24	-4,98	53	59,3
	LF	AYCTCACCACGAWGGCTAGT		20	131-150	-4,15	-4,81	50	60,1
	LB	GGCATGCCCAAGACWCRCTTTA	-2,09	22	244-265	-5,9	-4,09	55	65,7
Primer Set	**Primer**	**Primer Sequence**	**Dimer dG**	**Lentgh**	**5'-3' Position**	**5'dG**	**3'dG**	**GC(%)**	**Tm(℃)**
Primer Set 2a	F3	GAGRTYCCTGAGCGGTCTA		19	146-164	-4,1	-4,43	58	59,3
	F2	CTGAGTACAGGRCAGTCGT		19	169-187	-4,74	-5,57	58	60,4
	F1c	TCGTCCACGTAGCATCTCGAGA		22	221-242	-6,04	-5,29	55	64,6
	B1c	GGCATGCCCAWGACACGCTTTA		22	244-265	-5,9	-4,09	55	65,7
	B2	CAGGCTGTRATMCCAACACC		20	303-322	-6,08	-5,16	55	60,2
	B3	GCCTCTGCAGCACCCTAT	-2,01	18	323-340	-5,93	-4,41	61	60,9
	LF								
	LB	GGTCGYCRGGGTGAAAACA	-2,77	19	277-295	-7,03	-3,83	63	64,8
Primer Set	**Primer**	**Primer Sequence**	**Dimer dG**	**Lentgh**	**5'-3' Position**	**5'dG**	**3'dG**	**GC(%)**	**Tm(℃)**
Primer Set 2b	F3	YGGTCTAAGYCCTGAGTACA		20	158-177	-6,02	-4,13	55	60,2
	F2	GRYAGTCGTCAGTAGTTCG		19	179-197	-6,24	-5,18	58	59,3
	F1c	CGTCCACGTAGCATCTCGAGAC		22	220-241	-6,19	-5,53	59	64,1
	B1c	GGCATGCCCAWGACACGCTTTA		22	244-265	-5,9	-4,09	55	65,7
	B2	CAGGCTGTRATMCCAACACC		20	303-322	-6,08	-5,16	55	60,2
	B3	GCCTCTGCAGCACCCTAT	-1,5	18	323-340	-5,93	-4,41	61	60,9
	LF								
	LB	GGTCGCCGGGGTGAAAACA	-2,77	19	277-295	-7,03	-3,83	63	64,8

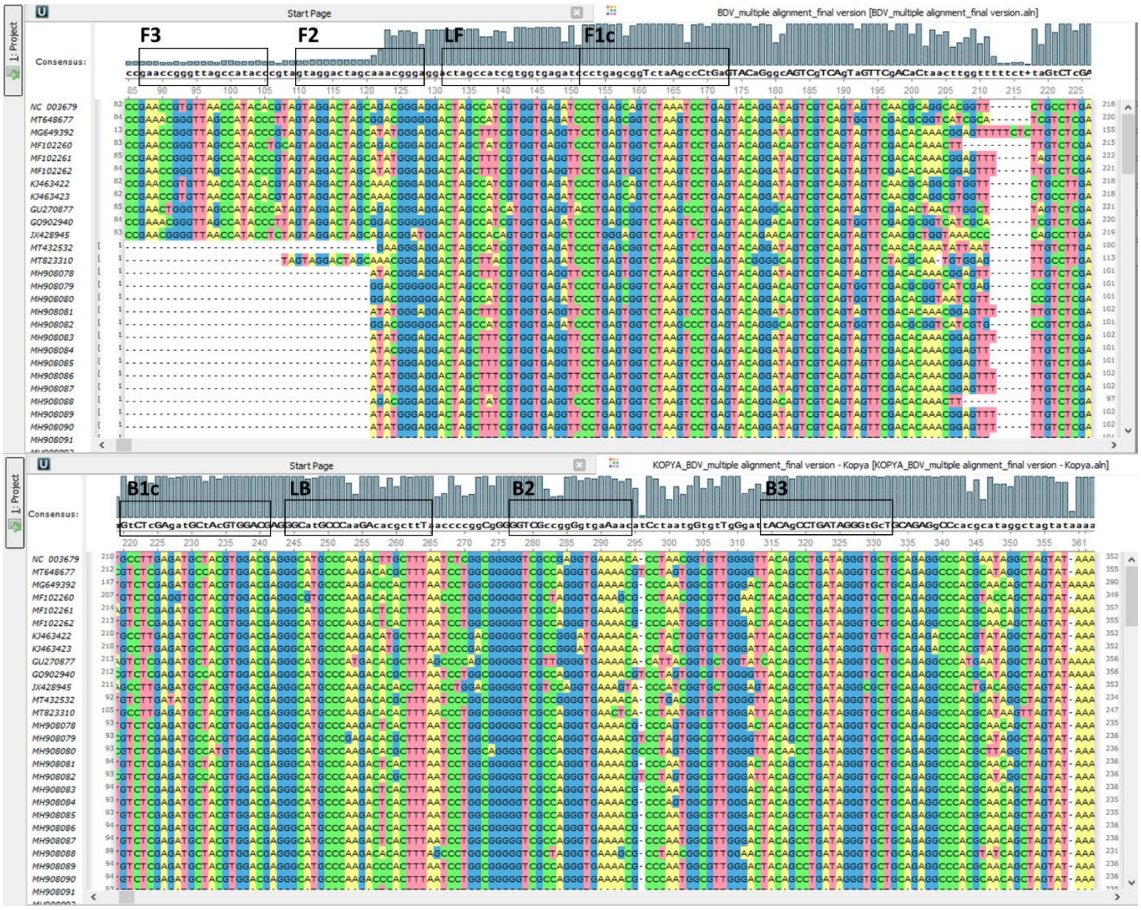
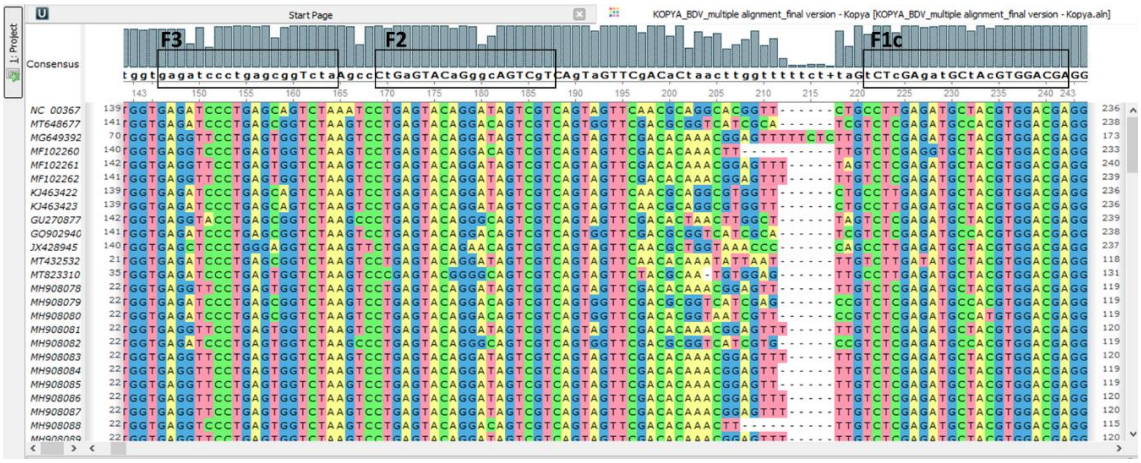


Figure 3.2 Display of primer set 1 primer locations on the BDV genome. Primer Set 1 targets between 87-332 bp of the BDV genome.



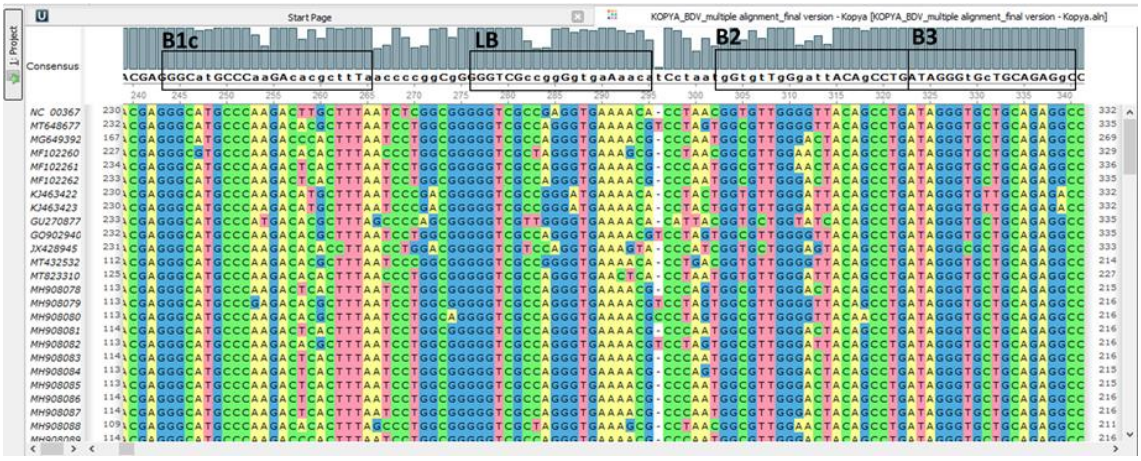


Figure 3.3 Display of primer set 2a primer locations on the BDV genome. Primer Set 2a targets between 146-340 bp of the BDV genome.

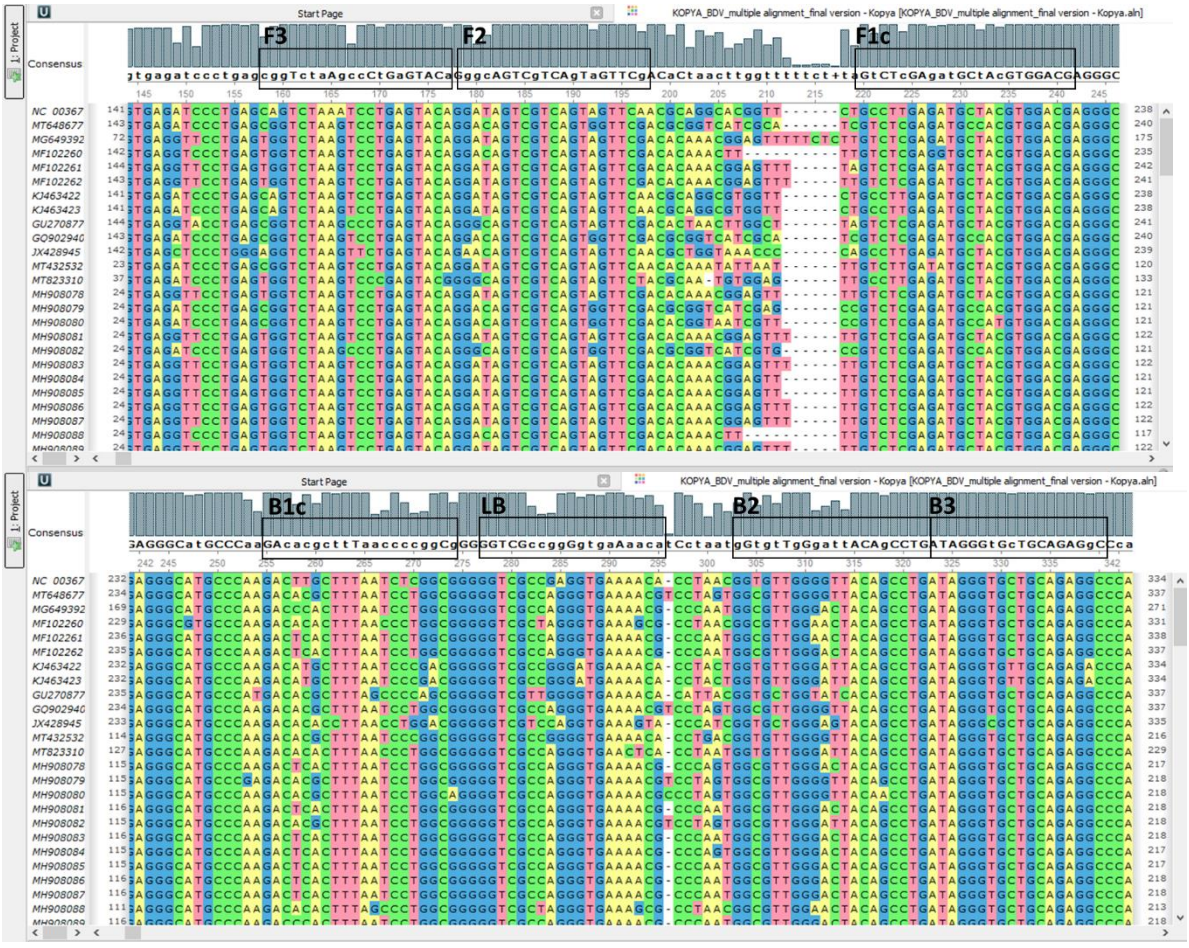


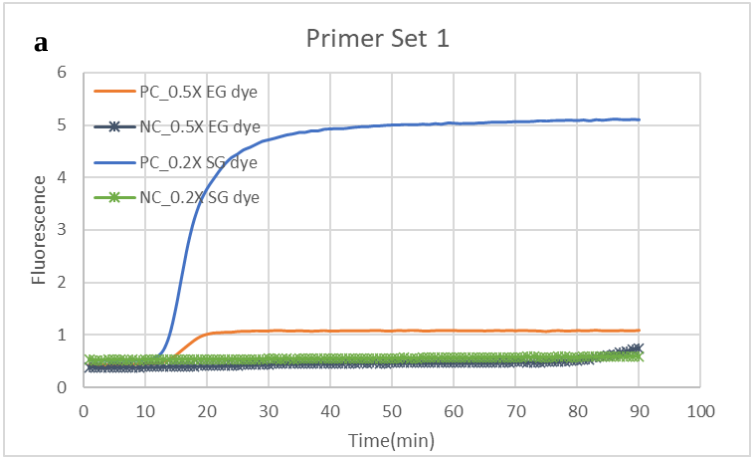
Figure 3.4 Display of primer set 2b primer locations on the BDV genome. Primer Set 2b targets between 158-340 bp of the BDV genome.

3.3 LAMP Reaction Setup and Optimization

Designed primer sets (primer set 1, primer set 2a and primer set 2b) were used to setup LAMP reactions for BDV detection. As a template, different concentrations of synthetic BDV template were used. Various parameters such as fluorescent dye concentration, temperature, primer concentration, Mg^{2+} concentration, presence of loop primers and enzyme concentration of LAMP reactions were optimized for each primer set. After the determination of optimum conditions, limit of detection (LOD) was determined for each primer set.

3.3.1 Fluorescent Dye Concentration Optimization

Firstly, efficiency of two fluorescent dyes SYBR Green I (SG) and Eva Green (EG), in LAMP reactions was investigated. For this purpose, LAMP reactions were performed using 0.2X SG dye and 0.5X EG dye for each primer set and performances of two dyes in LAMP reactions were compared. Reactions were prepared using 2 x 10^8 copies/µl BDV DNA as target and run at 65°C for 90 minutes. Despite being used in lower concentrations, 0.2X SG dye created a stronger signal than 0.5X EG dye in LAMP reactions (Figure 3.5). Results were consistent for all primer sets and thus, 0.2X SG dye was determined as optimum fluorescent dye concentration. In reactions containing 0.2X SG dye, 2 x 10^8 copies/µl BDV DNA was detected in 13 minutes with primer set 1; 19 minutes in primer set 2a and 14 minutes in primer set 2b (Figure 3.5).



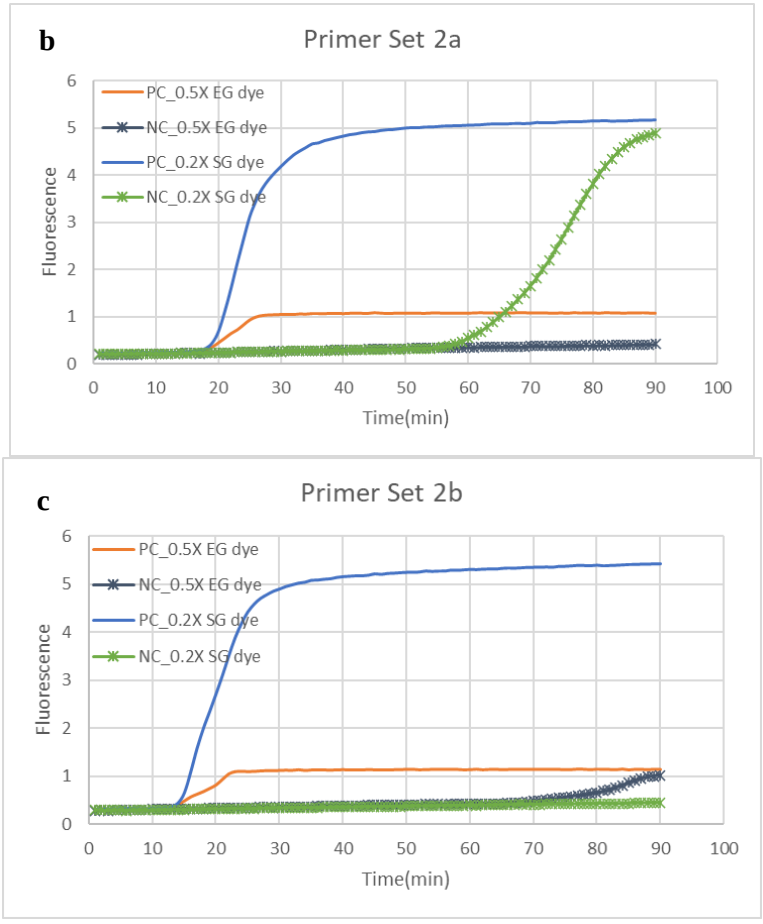


Figure 3.5 LAMP fluorescent dye concentration optimization. Efficiency of 0.2X SG dye and 0.5X EG dye was compared in a) primer set 1, b) primer set 2a and c) primer set 2b.

3.3.2 Temperature Optimization

Temperature is one of the most important criteria for LAMP reactions. It is known that *Bst* DNA polymerase works with 100% activity between 60-70°C. Most common temperature for LAMP reactions is 65°C. LAMP reactions were carried out at 63°C, 65°C and 67°C for all primer sets to determine optimum temperature of LAMP reactions. 2 x 10^6 copies/µl BDV DNA was used as template of all reactions and experiments were repeated three times for consistency.

In primer set 1, 2 x 10^6 copies/µl BDV DNA was amplified in average of 33 minutes at 63°C, 25 minutes at 65°C and 42 minutes at 67°C (Figure 3.6). Increasing and decreasing the temperature from 65°C drastically delayed the time-to signal for LAMP

reactions. There was no NC signal in any of the reactions. Overall, optimum temperature for LAMP reactions of primer set 1 was determined as 65°C.

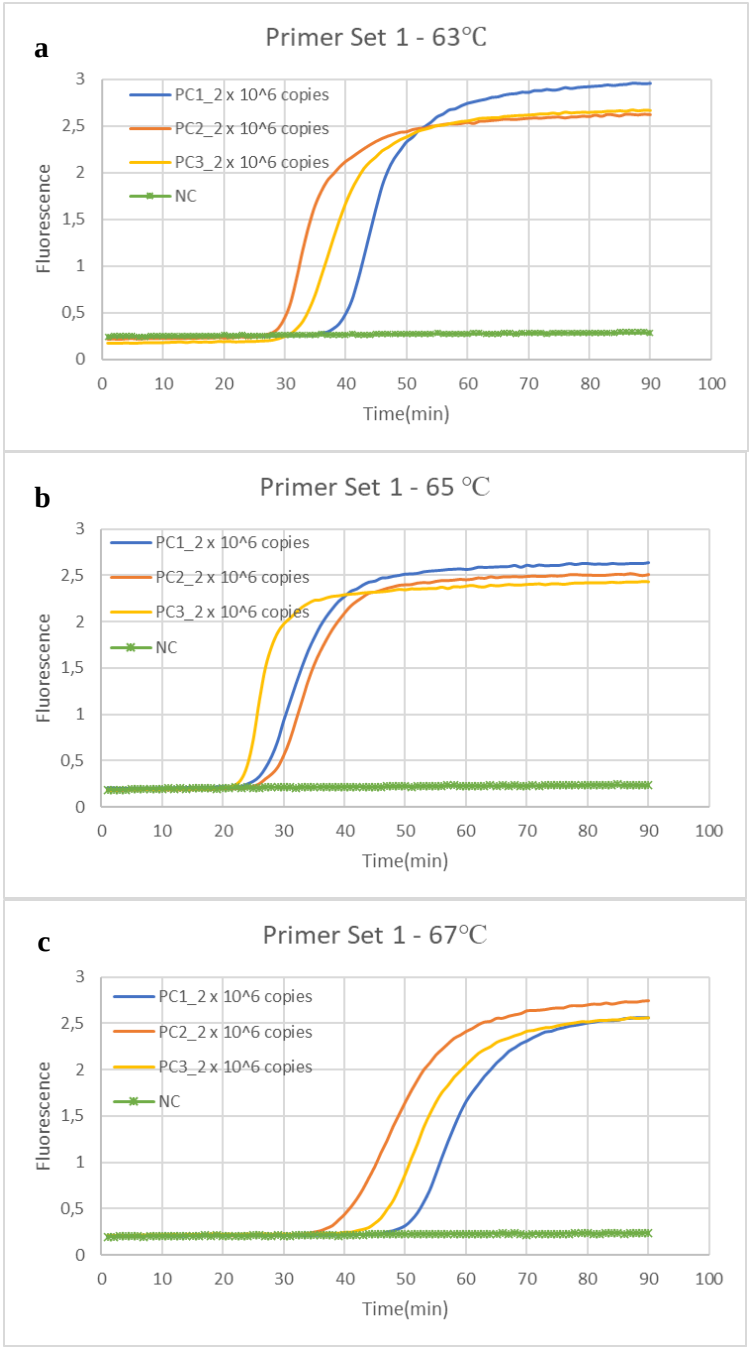


Figure 3.6 Primer set 1 LAMP temperature optimization. LAMP reactions were setup at a) 63°C, b) 65°C and c) 67°C.

In primer set 2a, 2 x 10^6 copies/µl BDV DNA was amplified in average of 38 minutes at 63°C, 33 minutes at 65°C and 65 minutes at 67°C (Figure 3.7). Earliest time to signal was detected at 65°C, thus it was determined as optimum temperature of primer set 2a LAMP reactions. However, it was noted that results were obtained between 25-38

minutes in repeated LAMP reactions at 65°C, which was highly variable and not consistent.

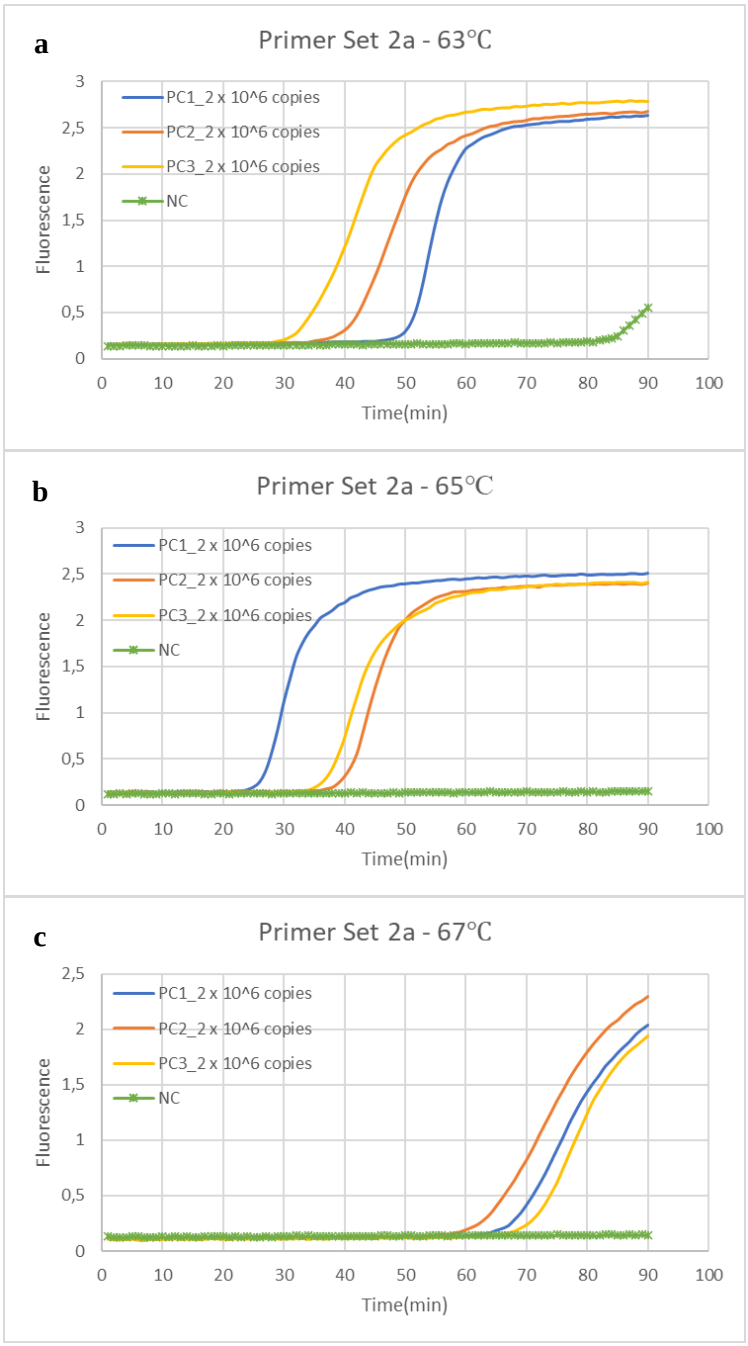


Figure 3.7 Primer set 2a LAMP temperature optimization. LAMP reactions were setup at a) 63°C, b) 65°C and c) 67°C.

In primer set 2b, 2 x 10^6 copies/µl BDV DNA was amplified in average of 34 minutes at 63°C, 33 minutes at 65°C and 48 minutes at 67°C (Figure 3.8). Earliest signals can be detected in LAMP both at 63°C and 65°Cfor this primer set. However, results of the repeated LAMP experiments were more consistent at 65°C, compared to 63°C.

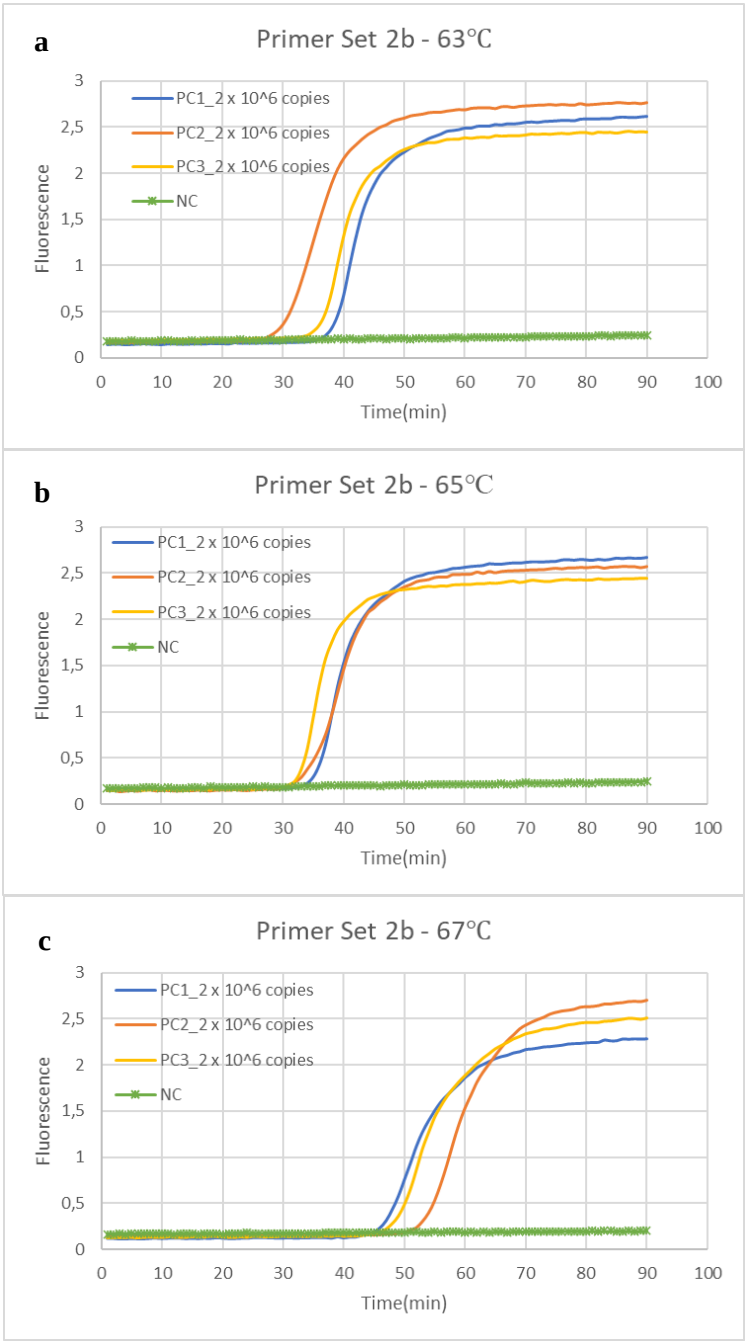


Figure 3.8 Primer set 2b LAMP temperature optimization. LAMP reactions were setup at a) 63°C, b) 65°C and c) 67°C.

3.3.3 Primer Concentration Optimization

Another important parameter for LAMP reactions is the primer mix concentration. It is recommended to use 1X primer mix which contains 1.6 µM FIP/BIP, 0.2 µM F3/B3 and 0.5 µM LF/LB. LAMP reactions were set up for primer set 1, 2a and 2b using 1X primer mix and 1.4X primer mix to observe the effect of increased primer concentrations

to the reactions. 1.4X primer mix includes increased concentrations of each primer: 2.24 µM FIP/BIP, 0.28 µM F3/B3 and 0.7 µM LF/LB. All reactions used 2 x 10^6 copies/µl BDV DNA as template. Each experiment was run at 65°C for 90 mins and repeated three times to provide consistency.

In primer set 1, reactions prepared with 1X primer mix concentration gave the signal in 25 mins in average while reactions with 1.4X primer mix signal was detected in 30 minutes (Figure 3.9). Increasing primer concentration from 1X to 1.4X delayed signal time approximately 5 minutes and an early false-positive signal was detected in 38 mins. Overall, 1X primer mix concentration was detected as optimum concentration for primer set 1.

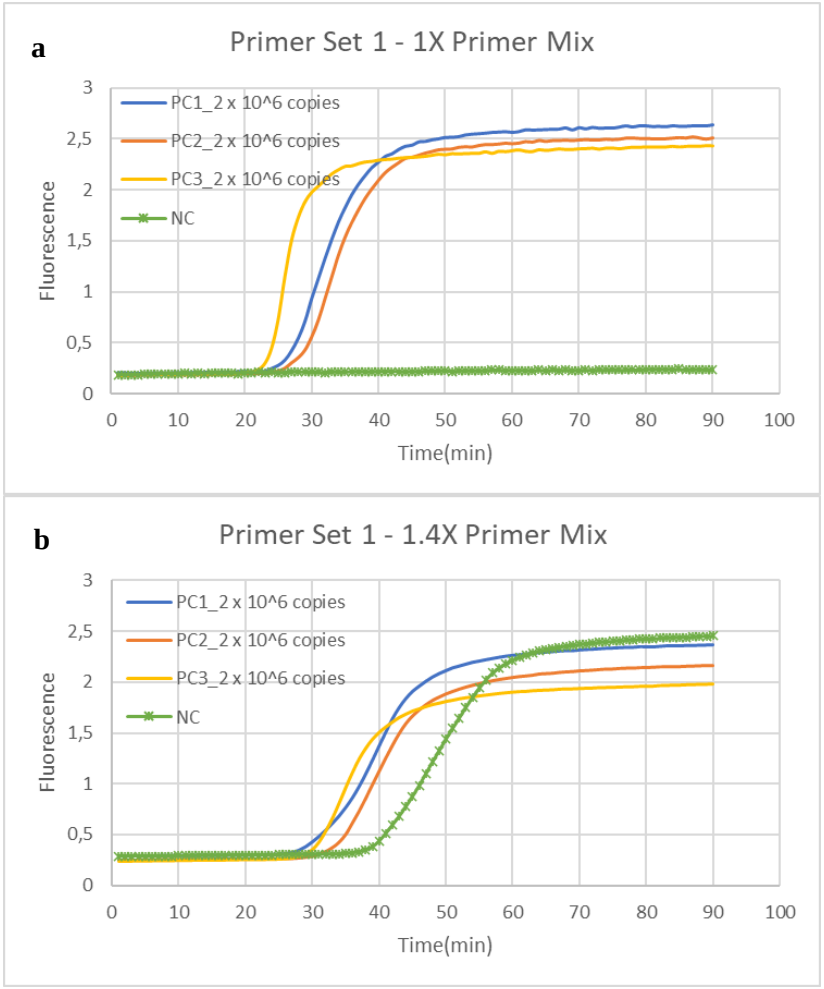


Figure 3.9 Primer set 1 primer concentration optimization. LAMP reactions were performed using a) 1X primer mix and b) 1.4X primer mix.

In primer set 2a, reactions containing 1X primer mix concentration signals in 33 mins in average, while reactions with 1.4X primer mix signal was detected in 38 minutes

(Figure 3.10). Increasing primer concentration delayed the signal and cause to get a false-positive signal at 60 minutes. Therefore, optimum primer mix concentration was detected as 1X for this set.

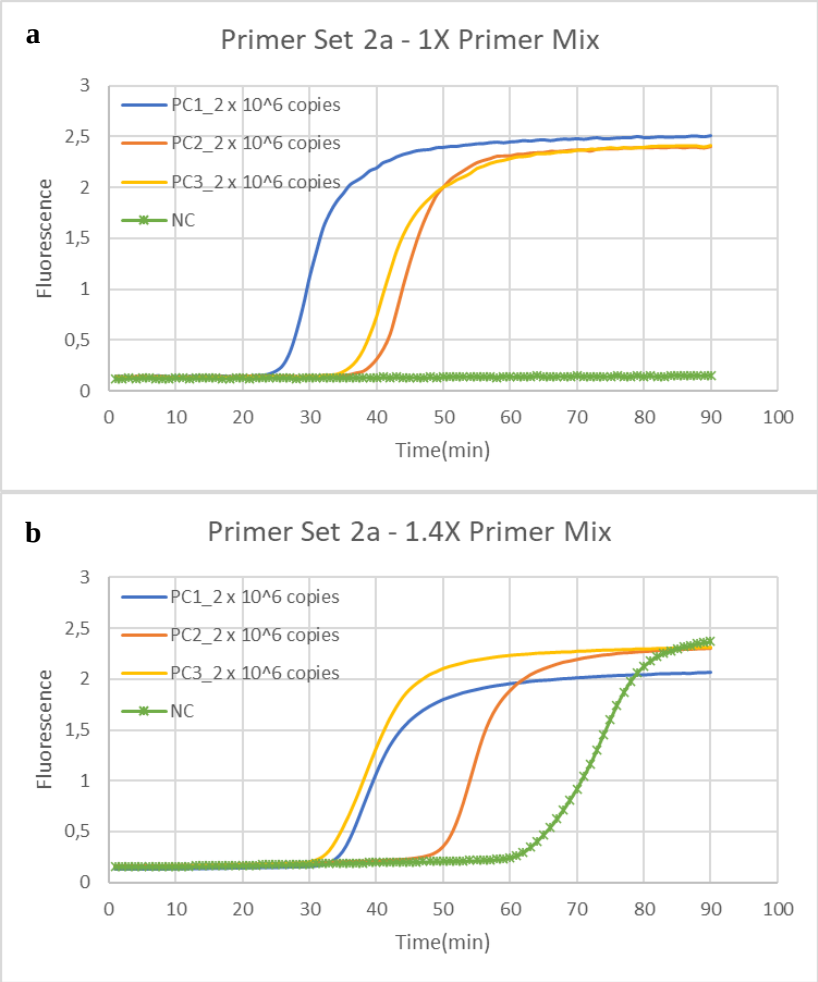


Figure 3.10 Primer set 2a primer concentration optimization. LAMP reactions were performed using a) 1X primer mix and b) 1.4X primer mix.

In primer set 2b, reactions containing 1X primer mix concentration signals in 33 mins in average, while reactions with 1.4X primer mix signal was detected in 29 minutes (Figure 3.11). Increasing the primer concentrations speed up the LAMP reactions, but results of reactions containing 1X primer mix were more consistent compared to reactions with 1.4X primer mix.

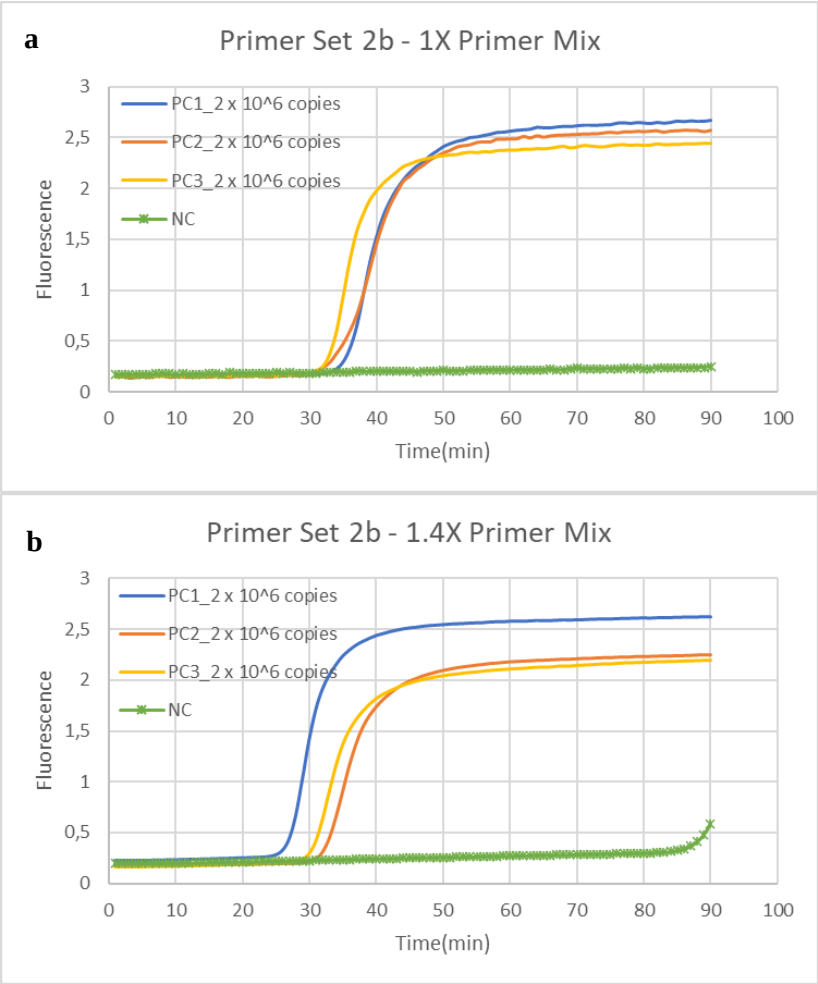


Figure 3.11 Primer set 2b primer concentration optimization. LAMP reactions were performed using a) 1X primer mix and b) 1.4X primer mix.

3.3.4 Mg^{2+} Concentration Optimization

Mg^{2+} concentration is a crucial factor that affects the results of LAMP. It is recommended to prepare LAMP reaction with 8 mM Mg^{2+}: 2 mM from isothermal amplification buffer and 6 mM from $MgSO_4$ solution. For observing the effect of total Mg^{2+} concentration change to LAMP reactions, 6 mM, 8 mM and 10 mM Mg^{2+} containing LAMP tests were performed for each primer set. All reactions were amplified 2 x 10^6 copies/µl BDV DNA at 65°C for 90 mins and experiments were repeated for three times.

For primer set 1, results of LAMP reactions with 6 mM Mg^{2+} was determined 16 minutes, reactions containing 8 mM Mg^{2+} detected in 25 minutes and reactions with 10 mM Mg^{2+} detected in 25 minutes in average (Figure 3.12). Overall, decreasing Mg^{2+}

concentration speed up the LAMP reactions while increasing has no effect. Therefore, optimum Mg^{2+} concentration was determined as 6 mM for primer set 1.

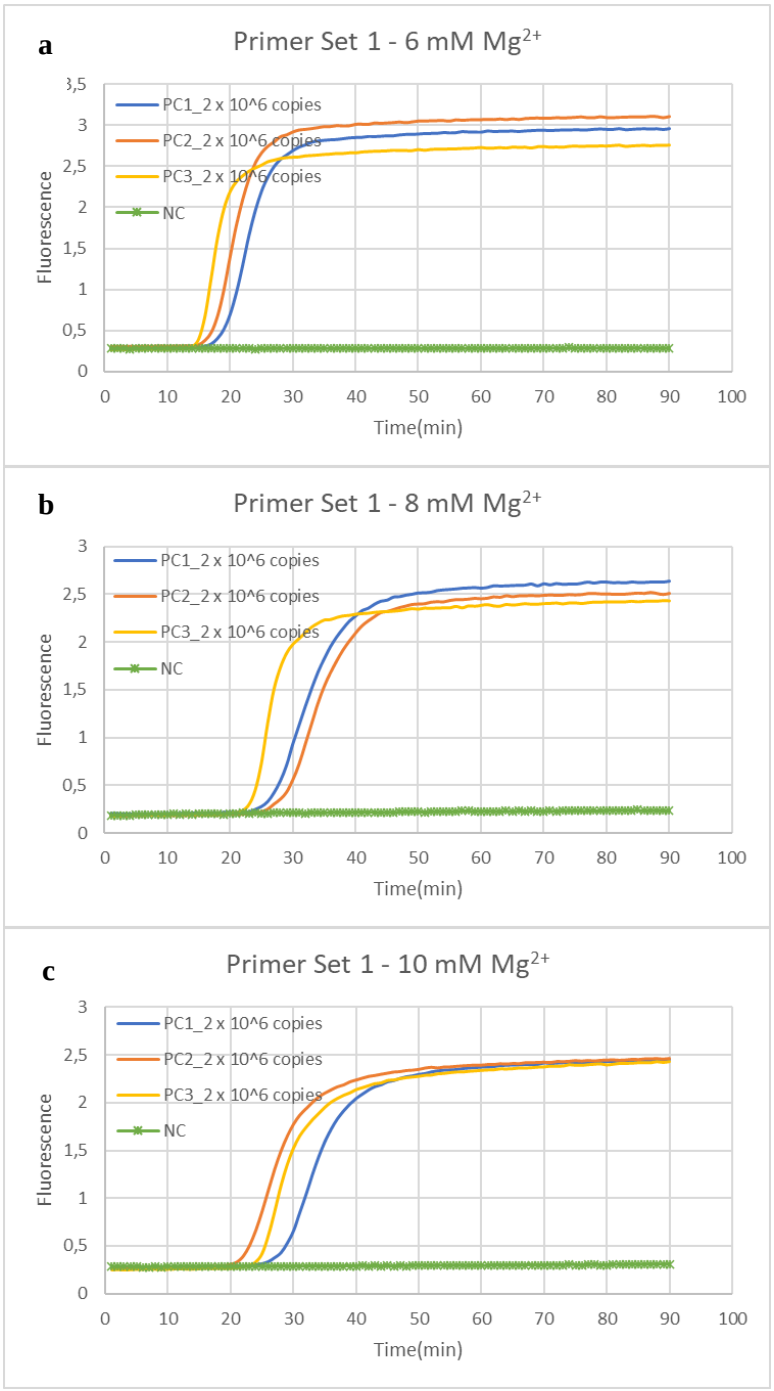


Figure 3.12 Primer set 1 Mg^{2+} concentration optimization. Reactions were prepared using a) 6 mM, b) 8 mM and c) 10 mM Mg^{2+} concentration.

For primer set 2a, LAMP reactions containing 6 mM Mg^{2+} resulted in 26 mins in average, 8 mM Mg^{2+} in 33 minutes and 10 mM Mg^{2+} in 35 minutes (Figure 3.13). Decreasing Mg^{2+} concentration accelerated the reactions while increasing the

concentration caused no dramatic effect. Therefore, 6 mM was determined as optimum Mg^{2+} concentration for primer set 2a.

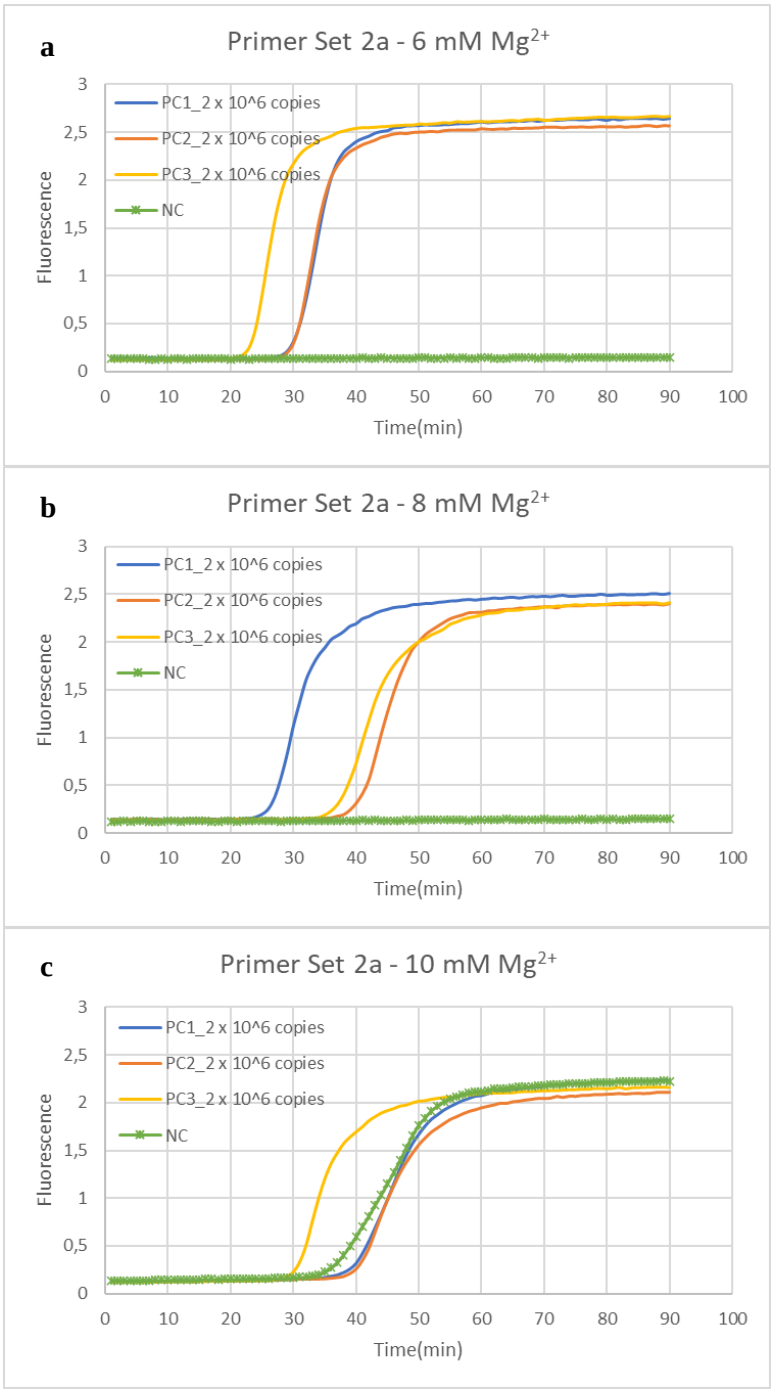


Figure 3.13 Primer set 2a Mg^{2+} concentration optimization. Reactions were prepared using a) 6 mM, b) 8 mM and c) 10 mM Mg^{2+} concentration.

For primer set 2b, LAMP reactions with 6 mM Mg^{2+} amplified the BDV DNA in 25 mins, reactions with 8 mM Mg^{2+} concentration resulted in 33 mins and reactions containing 10 mM Mg^{2+} signal was obtained in 23 minutes in average (Figure 3.14).

Earliest results were obtained in LAMP reactions containing 10 mM Mg^{2+} but, increasing the Mg^{2+} caused early false-positive signals. Therefore, optimum Mg^{2+} concentration for primer set 2b was determined as 6 mM Mg^{2+}.

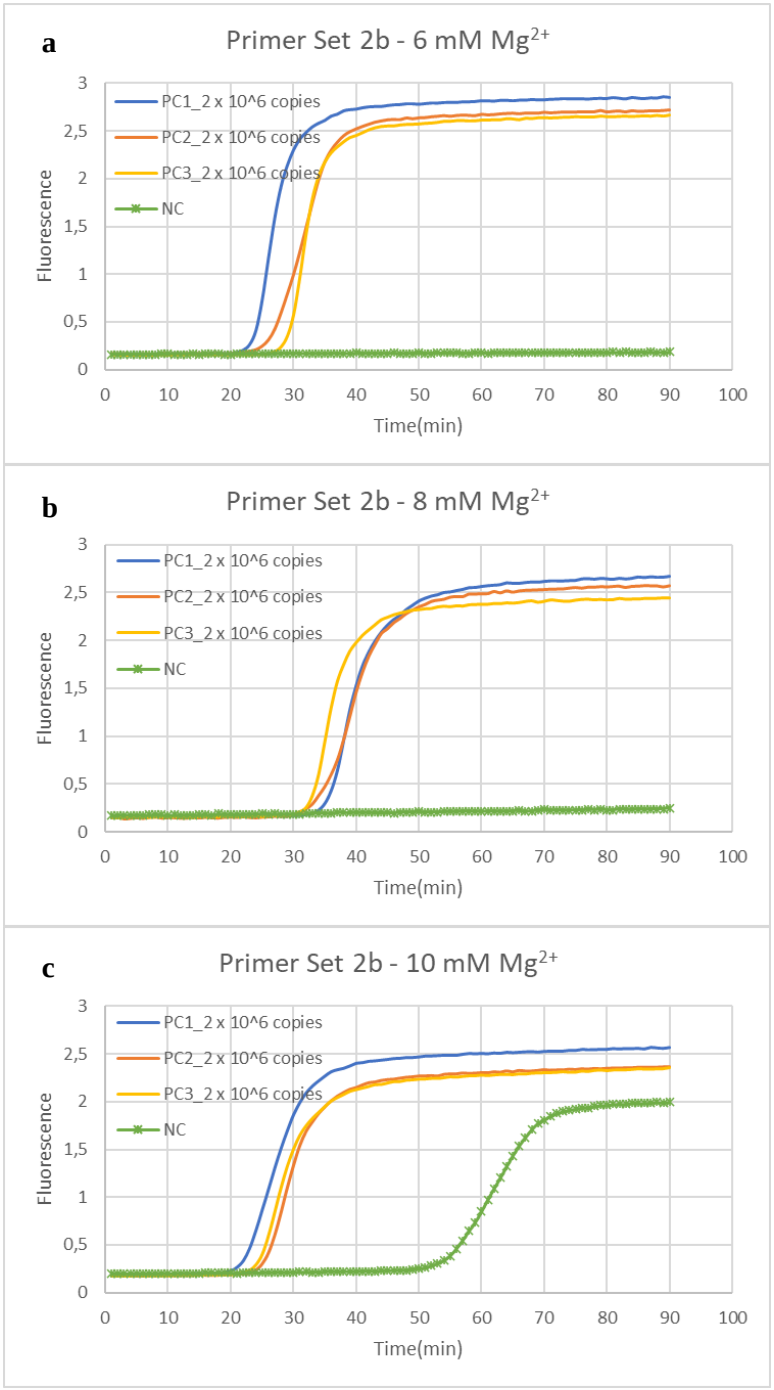


Figure 3.14 Primer set 2b Mg^{2+} concentration optimization. Reactions were prepared using a) 6 mM, b) 8 mM and c) 10 mM Mg^{2+}concentration.

3.3.5 Enzyme Concentration Optimization

Bst DNA polymerase has strand displacement property, and it is the most widely used enzyme for LAMP reactions. Adjustment of the enzyme concentration is known to be effective on the LAMP results. It is recommended to use 8 U enzyme in a standard LAMP reaction. LAMP reactions containing 6 U, 8 U and 12 U *Bst* DNA polymerase concentrations were prepared for each primer set to see the effect of enzyme concentration in LAMP reaction rate. All reactions were amplified 2 x 10^6 copies/µl template at 65°C for 90 minutes.

In primer set 1, results of LAMP reactions containing 6U enzyme obtained in 24 minutes, reactions with 8U enzyme resulted in 24 minutes and reactions containing 12 U enzyme resulted in 16 minutes (Figure 3.15). Decreasing the enzyme concentration has no impact on time to signal but, false-positive signal was observed in negative control. Increasing the enzyme concentrations accelerated the reactions but also caused getting an early false-positive signal in 44 minutes. Therefore, 8 U was determined as optimum *Bst* DNA polymerase concentration for primer set 1.

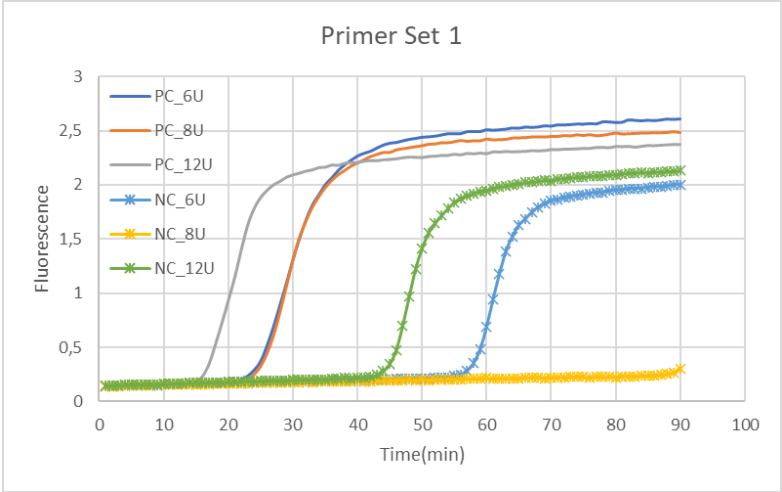


Figure 3.15 Primer set 1 *Bst* DNA polymerase enzyme concentration optimization.

In primer set 2a, LAMP reaction products containing 6 U *Bst* DNA polymerase enzyme was amplified in 38 minutes, reactions with 8 U enzyme were amplified in 38 minutes and reactions using 12 U enzyme resulted in 23 minutes. Decreasing the enzyme concentration has no impact in time to signal of the reaction. On the other hand, increasing the enzyme concentration accelerated the reaction but caused formation of an early false-

positive signal in negative control. Therefore, optimum *Bst* DNA polymerase enzyme concentration was maintained as 8 U for primer set 2a.

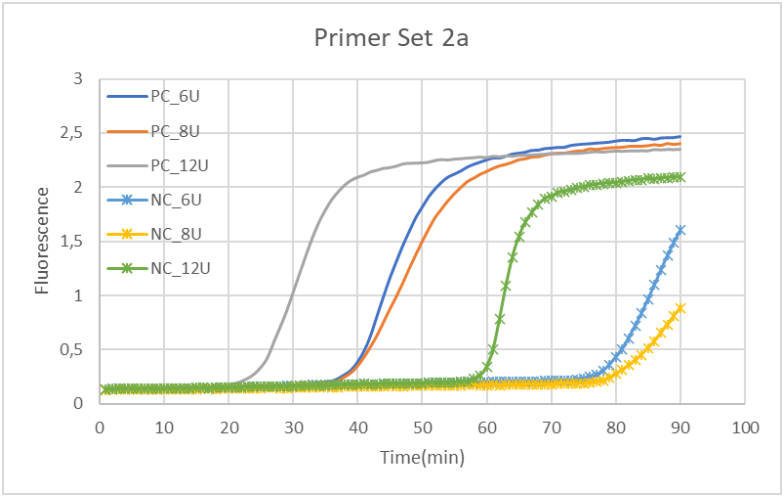


Figure 3.16 Primer set 2a *Bst* DNA polymerase enzyme concentration optimization.

In primer set 2b, LAMP reactions setup with 6 U *Bst* DNA polymerase enzyme was resulted in 26 minutes, reactions with 8 U enzyme signals were determined in 27 minutes and reactions containing 12 U enzyme has time to signal equals to 17 minutes (Figure 3.17). Decreasing the enzyme concentration did not affect the reaction rate but caused false-positive signal in 36 minutes. Increasing the enzyme concentration elevated the reaction rate but it also caused false-positive signals in 46 minutes. Therefore, 8 U *Bst* DNA polymerase enzyme concentration was determined as optimum for primer set 2b.

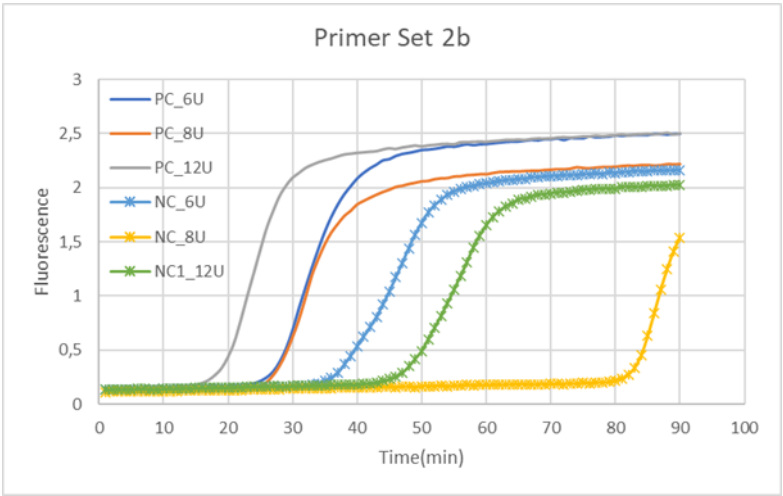


Figure 3.17 Primer set 2b *Bst* DNA polymerase enzyme concentration optimization.

3.3.6 Effect of Loop Primers

LAMP reactions are performed using 4-6 primers. Four primers are essential: FIP/BIP and F3/B3 primers. Other two primers LF/LB are loop primers, and they are optional for LAMP reactions. However, their use was recommended since loop primers speed up the reactions and also increases specificity of the test. The aim here was to investigate the effect of loop primers in LAMP reactions. For this purpose, LAMP reactions were set up for each primer set using a primer mix with and without loop primers. All reactions were run at 65°C for 90 minutes and experiments were repeated three times.

In primer set 1, 2 x 10^6 copies/µl BDV DNA was amplified approximately in 25 minutes with primer mix containing loop primers. On the other hand, same template was amplified in 45 minutes with reactions containing primer mix without loop primers (Figure 3.18). Absence of loop primers delayed time to signal about 20 minutes and caused false-positive signals in negative control.

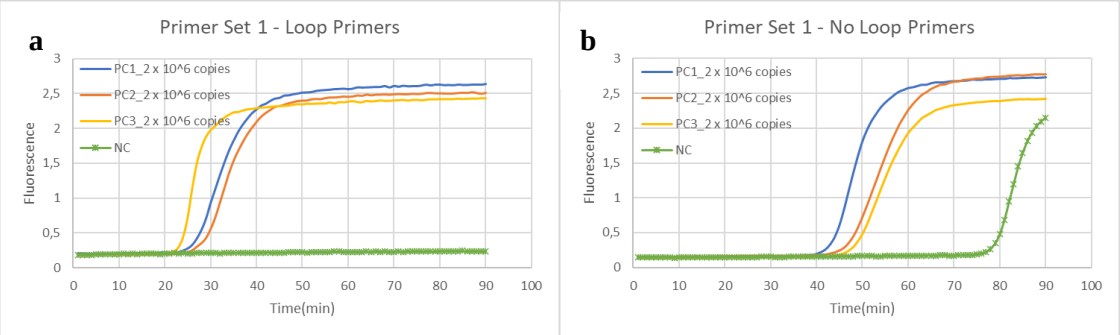


Figure 3.18 Effect of loop primers in LAMP reactions of primer set 1. LAMP reactions were performed a) with loop primers b) without loop primers.

In primer set 2a, 2 x 10^6 copies/µl BDV DNA was detected 33 minutes in average with reactions containing loop primers. Reversely, reactions performed without loop primers amplified the same target approximately in 53 minutes (Figure 3.19). Absence of loop primers in primer mix caused about 20 minutes delay in LAMP reaction rate and caused false-positive signals of negative control.

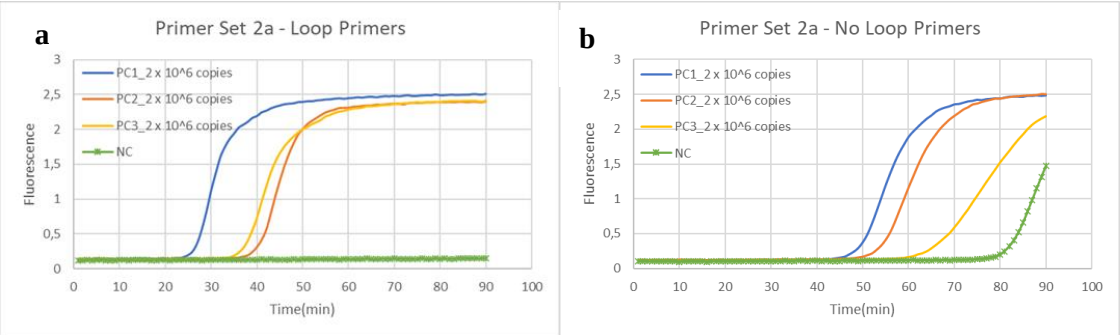


Figure 3.19 Effect of loop primers in LAMP reactions of primer set 2a. LAMP reactions were performed a) with loop primers b) without loop primers.

In primer set 2b, 2 x 10^6 copies/µl BDV DNA was amplified in 33 minutes in average with loop primers while same target was detected in 45 minutes with primer mix containing no loop primers (Figure 3.20). Absence of loop primers delayed time to signal for 22 minutes and caused arising of false-positive signals.

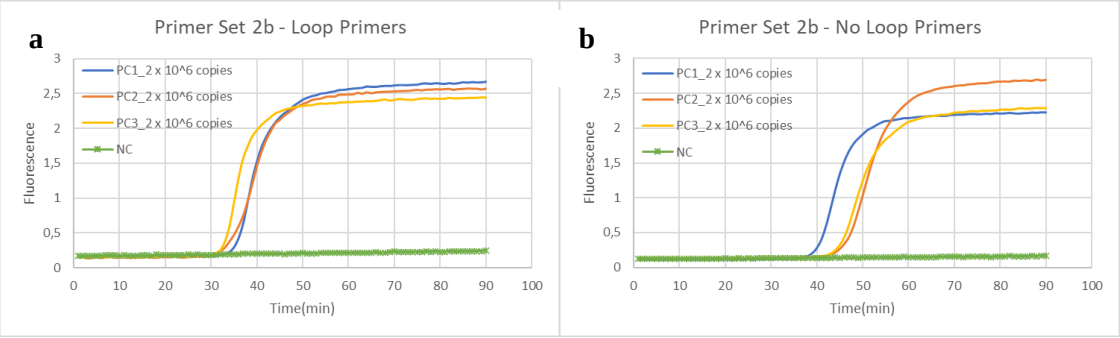


Figure 3.20 Effect of loop primers in LAMP reactions of primer set 2b. LAMP reactions were performed a) with loop primers b) without loop primers.

3.3.7 Limit of Detection (LOD) Determination

Determination of the limit of detection (LOD) was essential because it is an indicator of developed LAMP test's sensitivity. After completing the optimization studies and finding the optimum conditions for LAMP reactions of primer sets 1, 2a and 2b; LOD was determined for each primer set. For this purpose, LAMP reactions were setup to amplify 10^8 to 10^1 copies/µl BDV DNA, respectively. All reactions were performed using

0.2X SYBR Green I dye, 8 U *Bst* DNA polymerase enzyme, 1X primer mix with loop primers, 6 mM Mg^{2+} at 65°C for 90 minutes.

In primer set 1, 2 x 10^8 to 2 x 10^4 copies/µl BDV DNA was amplified within 22 minutes (Figure 3.21). Lower concentrations than 2 x 10^4 copies/µl BDV DNA could not be detected. Therefore, primer set 1 LOD was determined as 2 x 10^4 copies/µl BDV DNA.

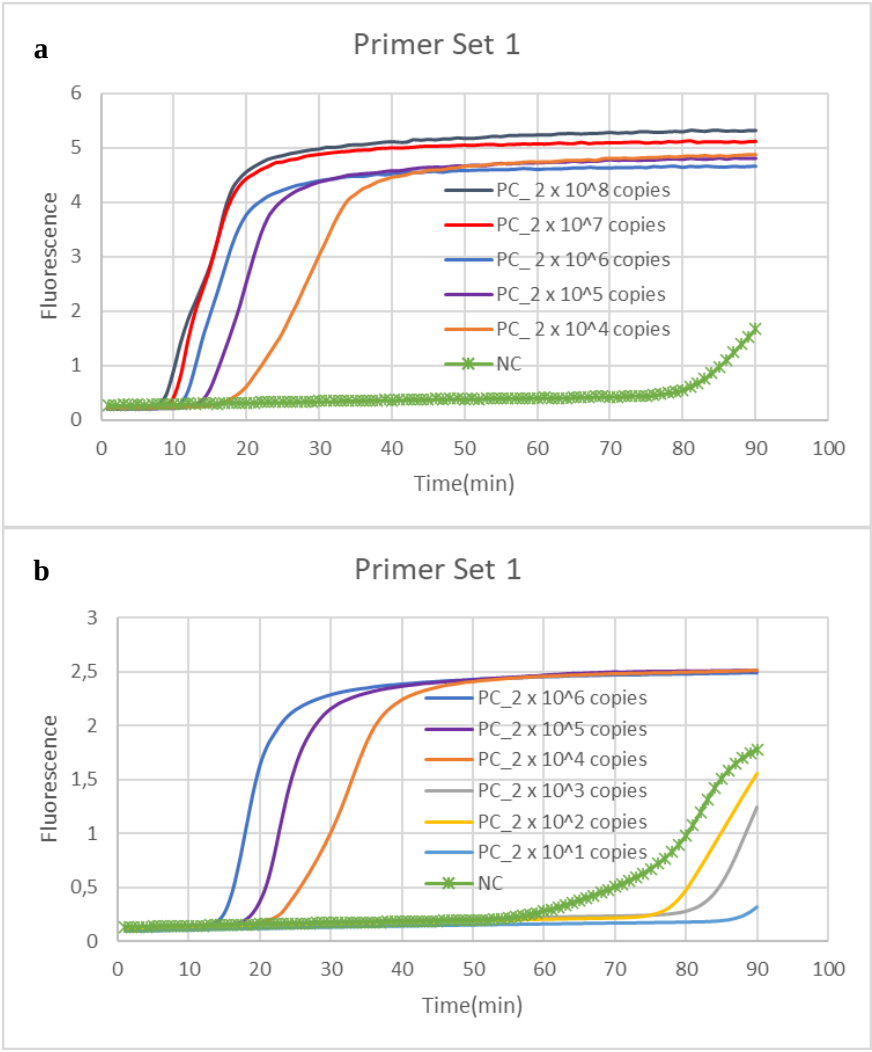


Figure 3.21 Primer set 1 limit of detection (LOD) determination in LAMP. Reactions were targeted a) 2 x 10^8 to 2 x 10^4 copies/µl BDV DNA and b) 2 x 10^6 to 2 x 10^1 copies/µl BDV DNA.

In primer set 2a, 2 x 10^8 to 10^4 copies/µl BDV DNA was amplified within 35 minutes (Figure 3.22). However, concentrations lower than 2 x 10^4 copies/µl BDV DNA

could not be detected and an early false-positive signal in 45 minutes was obtained in one of the negative controls. Furthermore, 2 x 10^4 copies/µl BDV DNA could not be detected at all in one of the experiments, which indicates inconsistency. Even 2 x 10^2 copies/µl BDV DNA was detected in 38 minutes, it is not reliable because there was no signal in reactions containing higher concentrations of the target. Overall, 2 x 10^4 copies/µl BDV DNA was detected as LOD of primer set 2a.

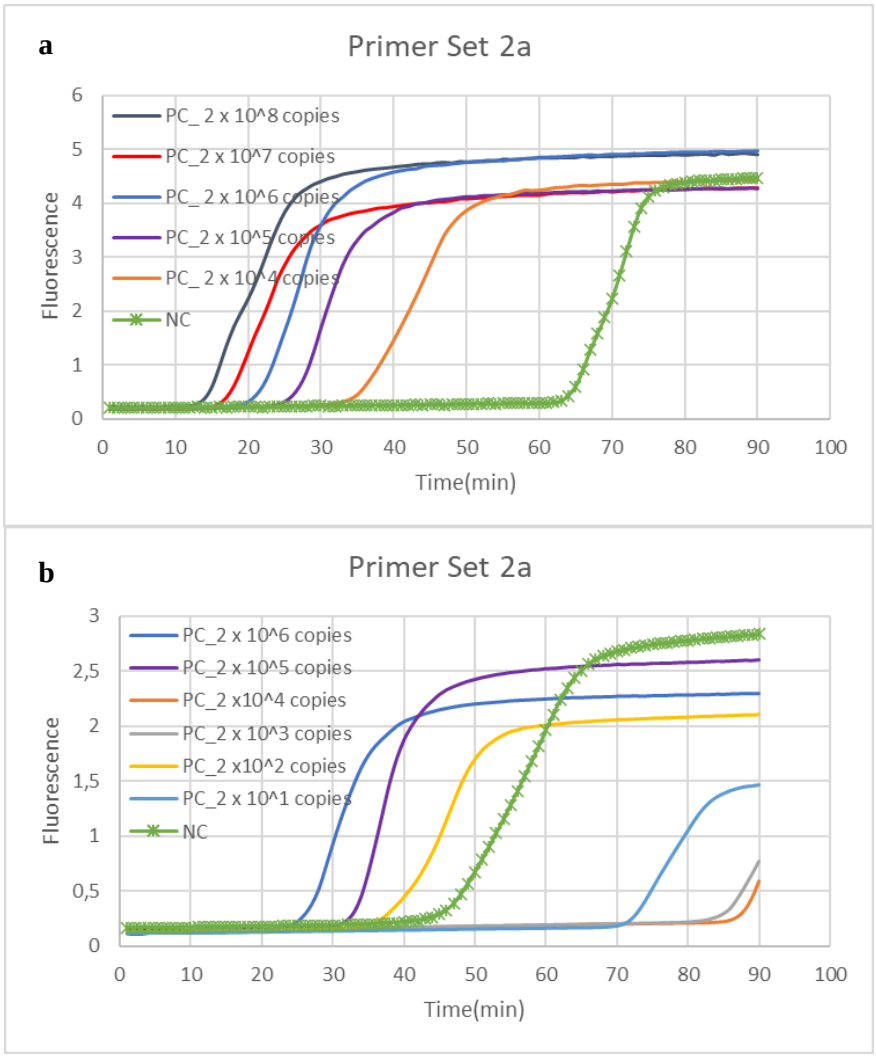


Figure 3.22 Primer set 2a limit of detection (LOD) determination in LAMP. Reactions were targeted a) 2 x 10^8 to 2 x 10^4 copies/µl BDV DNA and b) 2 x 10^6 to 2 x 10^1 copies/µl BDV DNA.

In primer set 2b, 2 x 10^8 to 2 x 10^4 copies/µl BDV DNA was amplified within 25 minutes (Figure 3.23). Concentrations lower than 2 x 10^4 copies/µl BDV DNA could not be detected in LAMP experiments. Even there are signals coming from 2 x 10^2 copies/µl

and 2 x 10^3 copies/µl BDV DNA containing reactions, these signals are late. Even it has lower concentration, 2 x 10^2 copies/µl BDV DNA was identified earlier than 2 x 10^3 copies/µl BDV DNA, which indicates that these results are not reliable. Overall, 2 x 10^4 copies/µl BDV DNA was determined as LOD of primer set 2b.

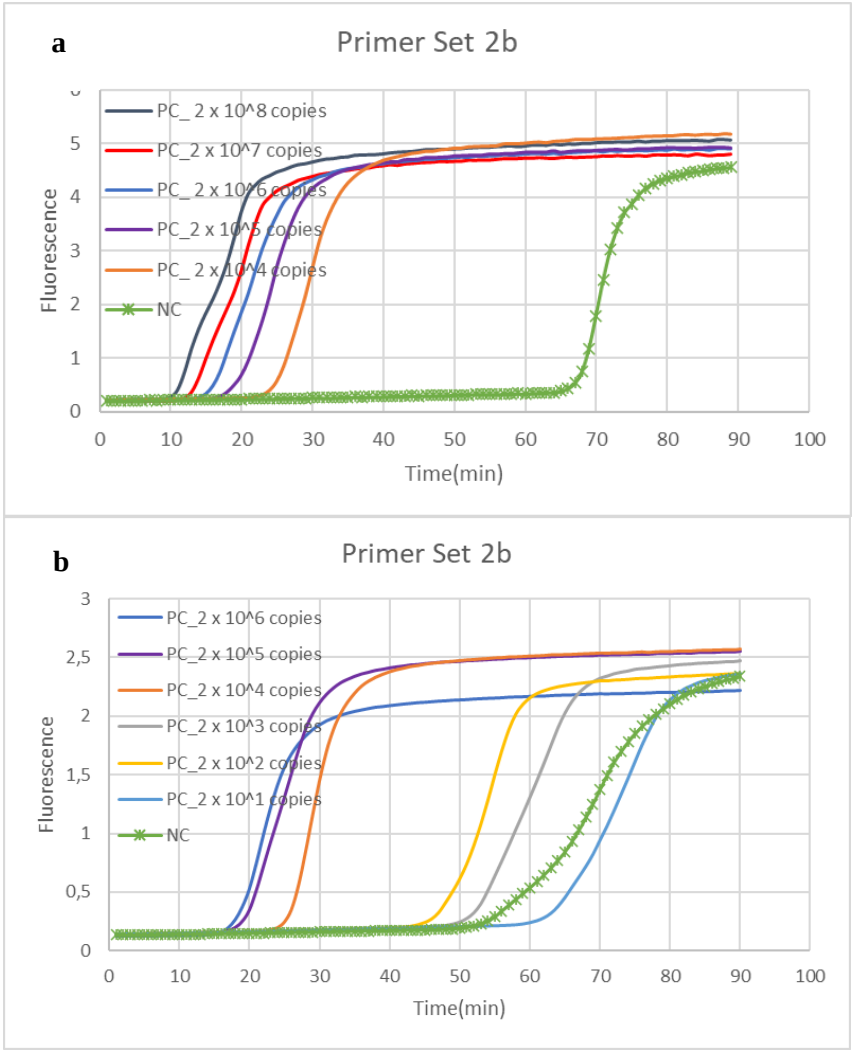


Figure 3.23 Primer set 2b limit of detection (LOD) determination in LAMP. Reactions were targeted a) 2 x 10^8 to 2 x 10^4 copies/µl BDV DNA and b) 2 x 10^6 to 2 x 10^1 copies/µl BDV DNA.

3.3.7.1 Temperature Optimization for Improvement of Detection Limit

Limit of detection (LOD) is the lowest amount of target DNA that can be detected by designed LAMP test. Low LOD indicates higher sensitivity. Determination of LOD was made in optimum conditions of LAMP for each primer set. Optimum temperature

for LAMP was determined as 65°C for all primer sets and LOD was detected as 2 x 10^4 copies/µl BDV DNA for primer set 1 and 2b. Here, LAMP reaction temperature was changed to 63°C, 65°C, 67°C and 70°C for primer set 1 and 2b to investigate if changing reaction temperature would improve the LOD of primer sets. Primer set 2a was eliminated due to inconsistent and highly variable results. As template, 2 x 10^6 to 10^1 copies/µl BDV DNA was targeted, respectively.

In primer set 1, 2 x 10^4 copies/µl BDV DNA (LOD) could be amplified in 22 minutes at 65°C. It was also detected in LAMP experiments performed at 63°C, but time to signal was 50 minutes, which was really late. Increasing and decreasing the reaction temperature did not improve the LOD, instead its signal was delayed in reactions performed at 63°C or completely lost in reactions performed at 67 and 70°C (Figure 3.24). Therefore, optimum LAMP reaction temperature was maintained 65°C for primer set 1.

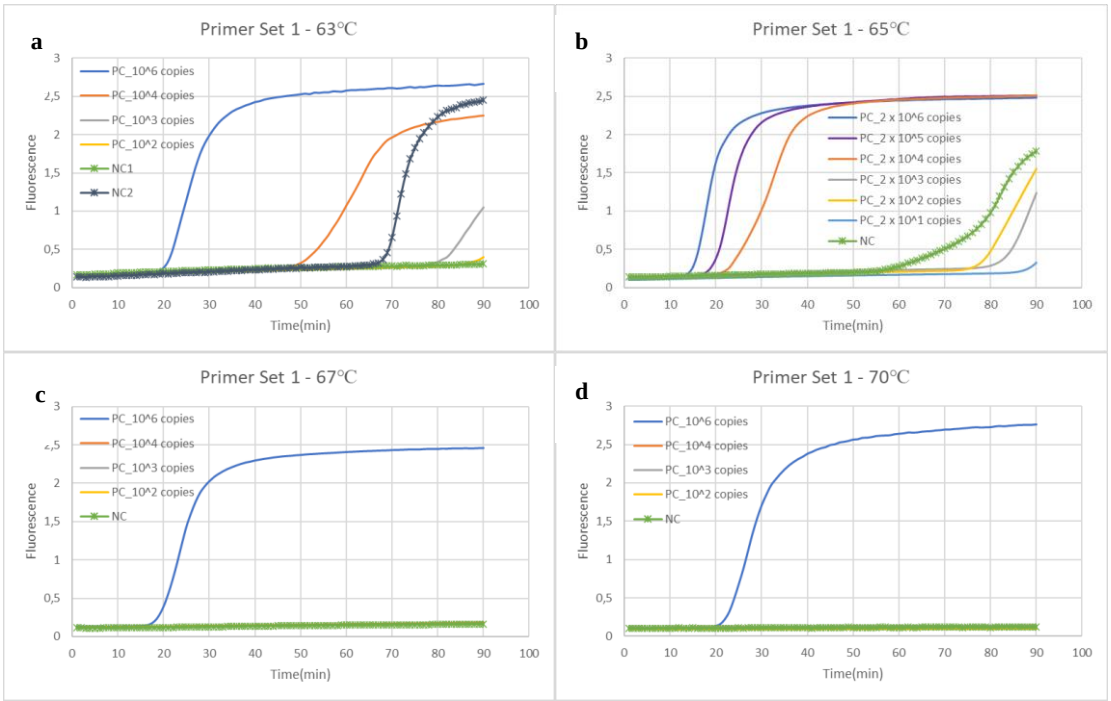


Figure 3.24 Temperature optimization of primer set 1 for LOD improvement. LAMP reactions were setup at a) 63°C, b) 65°C, c) 67°C and d) 70°C.

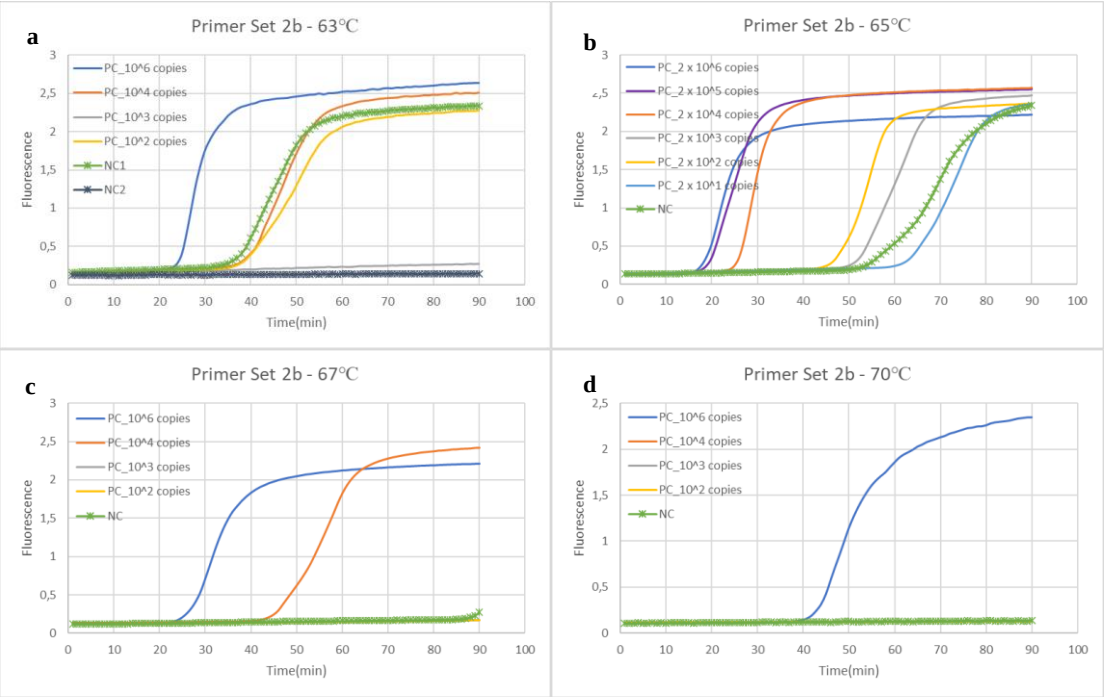


Figure 3.25 Temperature optimization of primer set 2b for LOD improvement. LAMP reactions were setup at a) 63°C, b) 65°C, c) 67°C and d) 70°C.

In primer set 2b, 2 x 10^4 copies/µl BDV DNA (LOD) was amplified in 25 minutes at 65°C. It was also detected in LAMP experiments setup at 63°C within 37 minutes, but at the same time a false-positive signal was obtained in negative control of the experiment which reduces the reliability of the results. 2 x 10^4 copies/µl BDV DNA (LOD) was amplified within 45 minutes at 67°C and signal was lost in experiments done at 70°C. Increasing and decreasing the reaction temperature could not improve the LOD, therefore optimum reaction temperature was maintained as 65°C for primer set 2b (Figure 3.25).

3.3.7.2 Primer Concentration Optimization for Improvement of Detection Limit

Optimum primer concentration for LAMP was determined as 1X primer concentration which contains 1.6 µM FIP/BIP, 0.2 µM F3/B3 and 0.5 µM LF/LB. It was intended to see if increased concentrations of LAMP primers can improve LOD of primer set 1 and 2b. For this purpose, LAMP reactions were setup using 1X and 1.4X primer mix and 2 x 10^6 to 10^1 copies/µl BDV DNA was targeted, respectively. 1.4X primer mix

contains 2.24 µM FIP/BIP, 0.28 µM F3/B3 and 0.7 µM LF/LB. All reactions were run at 65℃ for 90 minutes.

In primer set 1, increasing primer mix concentration from 1X to 1.4X caused no improvement in LOD of the LAMP reaction (Figure 3.26). Therefore, 1X primer concentration was maintained as optimum primer mix concentration for primer set 1.

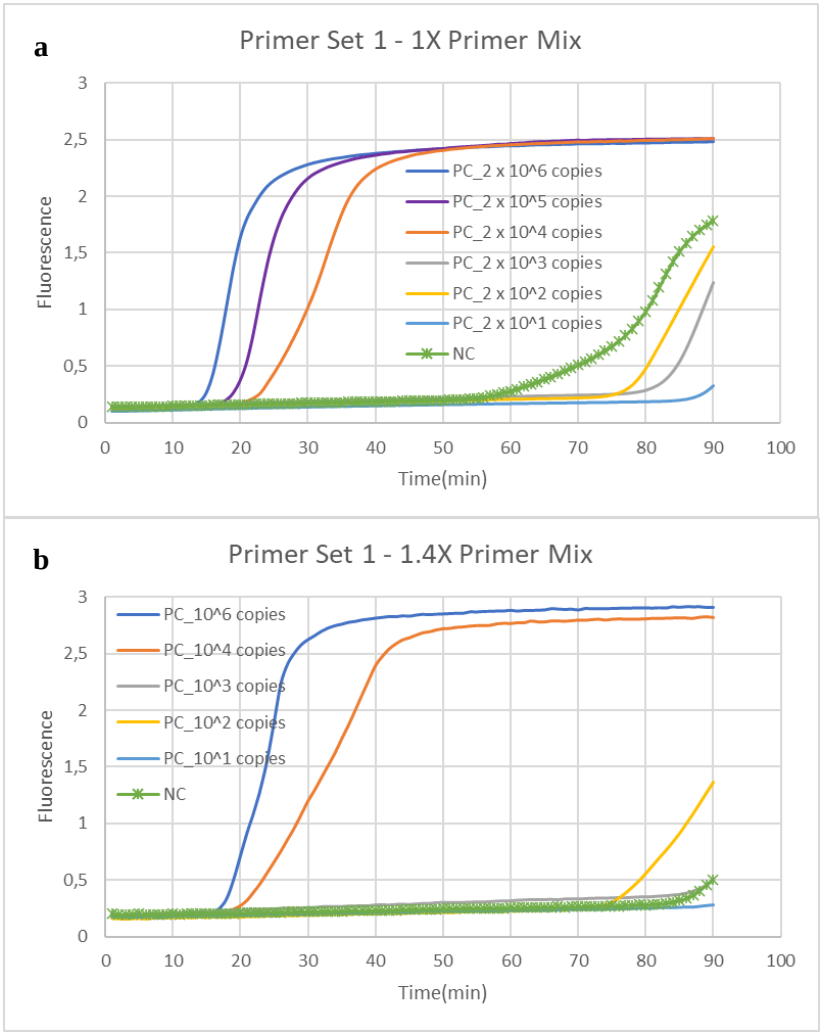


Figure 3.26 Primer concentration optimization of primer set 1 for LOD. LAMP reactions were performed using a) 1X and b) 1.4X primer mix.

In primer set 2b, increasing primer mix concentration from 1X to 1.4X caused 20 minutes delay of the of 2 x 10^4 copies/µl BDV DNA detection. Furthermore, when1.4X primer mix was used, 2 x 10^3 copies/µl was detected in 30 minutes, 14 minutes earlier than detection of 2 x 10^4 copies/µl which was not rational (Figure 3.27). Overall, 1X

primer mix concentration was maintained as optimum primer mix concentration of primer set 2b for LAMP.

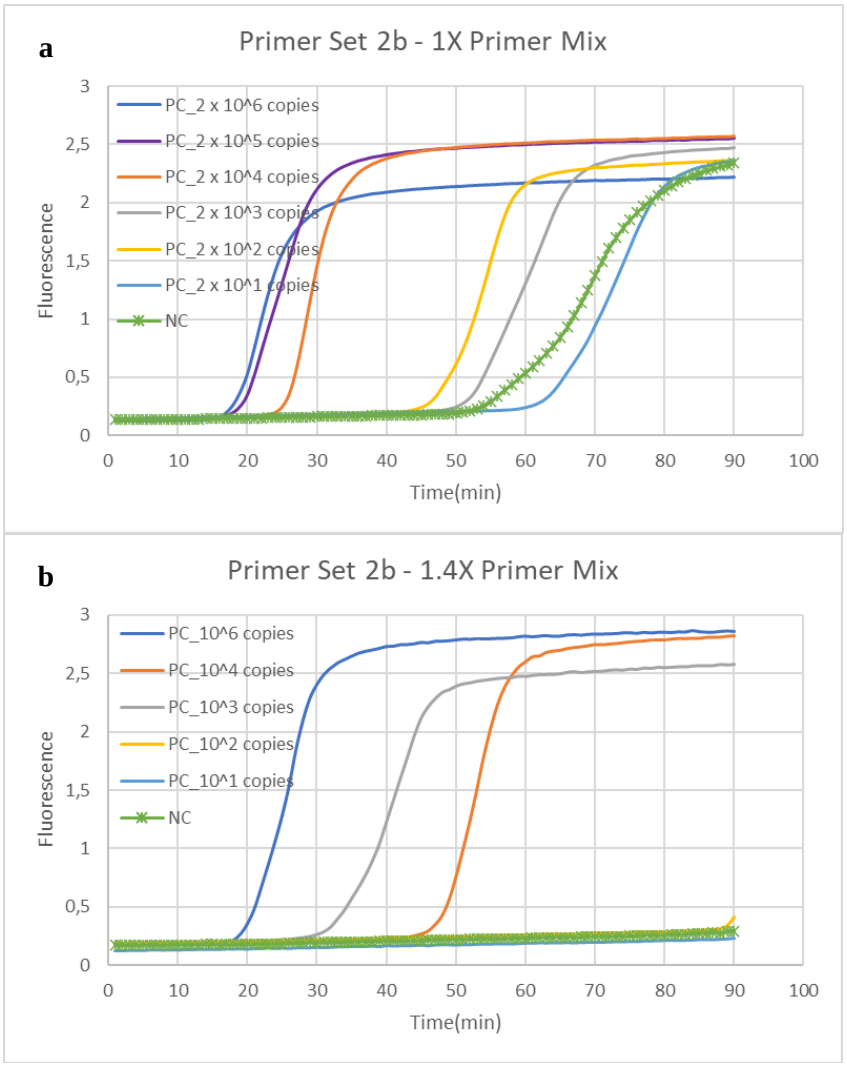


Figure 3.27 Primer concentration optimization of primer set 2b for LOD. LAMP reactions were performed using a) 1X and b) 1.4X primer mix.

CHAPTER 4

DISCUSSION

Border disease is a viral disease of small ruminants that causes abortions, stillbirths, and birth of persistently infected (PI) lambs. It is highly transmissible with close contact, and it can infect other farm animals such as cattle, pigs and even wildlife species. Its causative agent is a pestivirus species called border disease virus (BDV). Since there is no treatment or vaccine against border disease, early diagnosis is extremely important. Therefore, early isolation of infected animals will prevent the spread of the infection to the herd. There are various diagnostic methods for BDV detection such as virus isolation, ELISA antigen and antibody tests and RT-qPCR. RT-qPCR is a widely used nucleic-acid amplification technique and it is accepted as the "gold-standard" test for BDV identification due to its high sensitivity and specificity. However, it can only be applicable in a laboratory by trained personnel, and it requires expensive instruments such as qPCR machine. Since not every region has a fully equipped laboratory for RT-qPCR application, shipping animal samples to reference laboratories delays diagnosis. Therefore, there is a need for a simple, accurate, inexpensive, point of care test that can be applied in the field.

The aim of this thesis study was to develop a nucleic acid-based loop mediated isothermal amplification technique (LAMP) for BDV identification. LAMP tests can be applied in the field as a point of care test, without the need of trained personnel, laboratory environment and expensive instruments. It has a simple protocol and results can be interpreted easily by colour change or turbidity measurements.

For this purpose, available BDV genome sequences were downloaded from NCBI virus database and multiple sequence alignment was applied. There were 197 BDV sequences available: 13 complete and 184 partial sequences. Among them, 136 BDV sequences containing 11 complete and 125 partial sequences were selected for multiple sequence alignment. Sequences selected for multiple sequence alignment were mostly ruminant host species such as sheep, goats and wildlife species of mountain goats isolated within the last 20 years.

Recently, BDV was classified as *Pestivirus D* (King et al. 2018). "Aydin-like" pestivirus which is widely distributed, especially in Aegean part of Turkey was classified as *Pestivirus I* (King et al. 2018). Even Aydin-like pestivirus is genetically distinct from BDV species, it causes a similar clinic scenario to BDV infections and widely distributed in Turkey (Oguzoglu et al. 2009; Postel et al. 2015). Therefore, complete sequence of Aydin-like pestivirus (Accession Number: JX428945) was included into multiple sequence alignment studies. In this way, infection caused by this species can also be determined.

Multiple alignment results showed that most conserved region of the BDV genome is 5'UTR. It comprises of 0-400 base-long region of the genome and previously RT-PCR primers were designed based on this region for BDV detection due to its high conservation (Vilček and Paton 2000). Hence, 5'UTR of the genome was targeted in this study for BDV detection with LAMP.

Three primer sets were designed targeting 5'UTR of BDV genome which are primer set 1, 2a and 2b using Primer Explorer v5 (EIKEN, Japan) tool. Initially, programme proposed different primer sets to target 5'UTR. Among proposed primer sets, these three sets were selected based on their length, distance between primers, 5'and 3' end stability, T_m and GC content. Primer design is the most critical step while developing a LAMP test. It is more complex than designing conventional PCR primers because LAMP requires 4-6 primers instead of 2. Furthermore, these primers need to be designed to target at least 200 base-long conserved part of the genome. BDV has at least eight subtypes and its genome has approximately 74% overall conservation according to multiple sequence alignment results which indicates variability. Therefore, it was difficult to detect fully conserved 200 base-long regions to design LAMP primers. Due to the variability in BDV genome, some primers in primer set1, 2a and 2b includes degenerate bases. A degenerate base provides a mix of bases for a particular nucleotide position in the primer to increase the possibility to amplify target sequence. In this way, the variable positions of the target sequence can be amplified.

LAMP primer sets primer set 1, 2a and 2b were used to setup and optimize LAMP reactions. First, fluorescent dye concentration was optimized, and it was found that for all three primer sets 0.2X SYBR Green I dye was optimum. In this study, LAMP results were interpreted by following the real-time fluorescent signal that comes from fluorescent

dye, SYBR Green I. Fluorescent dye was added into the master mix and reaction was run as a closed tube reaction which decreases the possible risk of contamination.

Then, temperature optimization was made for LAMP. *Bst* 2.0 warm start DNA polymerase was known to have 100% activity at temperatures between 60-70℃. It is recommended to run LAMP reactions at 65℃, however there are LAMP tests that optimized to work better at different temperatures in this range as well (Tanner and Evans 2014). LAMP reactions were setup at 63℃, 65℃ and 67℃ to observe the effect of temperature change in LAMP results. In primer set 1, 2a and 2b most consistent and early signals were detected at 65℃ for 2 x 10^6 copies/µl BDV DNA amplification. Therefore, 65℃ was determined as optimum temperature of LAMP reactions for all primer sets.

For primer concentration optimization, primer mix concentrations was raised from 1X to 1.4X to observe the effect of increased primer amounts in LAMP reactions. 2 x 10^6 copies/µl BDV DNA was used as template. In primer set 1 and 2a, increasing the primer mix concentration delayed the signal and caused a false-positive signal in negative control. Negative control signal can be due to contamination. Another possibility is increasing the primer amount of the reaction which might cause undesirable non-specific interactions between primers that creates a false positive signal. In primer set 2b, increasing the primer concentration speed up the LAMP reactions and created no negative control signal while amplifying 2 x 10^6 copies/µl BDV DNA. However, when 1.4X primer set 2b mix was used to detect lower concentrations such as 2 x 10^4 copies/µl, time to signal was drastically delayed which indicates reduced sensitivity (Figure 3.27). Therefore, 1X primer mix concentration was determined as optimum primer concentration for all primer sets.

For Mg^{2+} concentration optimization, LAMP reactions were prepared with 6 mM, 8 mM, and 10 mM Mg^{2+} for all primer sets to see the effect of Mg^{2+} concentration on the reaction rate. Mg^{2+} ions come from $MgSO_4$ solution to the LAMP reaction mixture. Mg^{2+} concentration is the most important factor in LAMP optimization because *Bst* DNA polymerase enzyme is Mg^{2+} dependent. Also, Mg^{2+} ions are responsible from stabilizing the negatively charged phosphate backbone of DNA during primer and DNA strand interactions. Mg^{2+} ions function as cofactors of the phosphodiester bond formation during dNTP addition to primer's free 3'-OH site during DNA strand synthesis by *Bst* DNA polymerase. In all three primer sets, decreasing Mg^{2+} concentration to 6 mM speed up the

reactions and thus, 6 mM was detected as optimum Mg^{2+} concentration. Liu and colleagues stated that high amounts of Mg^{2+} might stabilize non-specific bindings of primers to non-target regions and cause false-positive signals by decreasing the specificity. Furthermore, high amount of Mg^{2+} in the reaction mix can stabilize target DNA strand further and might prevent denaturation (Liu et al. 2013). Results of BDV LAMP experiments agreed with this finding, increasing Mg^{2+} concentration to 10 mM delayed the signal and created false-positive signals.

For *Bst* DNA polymerase enzyme concentration, 6 U, 8 U and 12 U enzyme containing LAMP reactions were performed for each primer set. It was recommended to use 8 U *Bst* DNA polymerase enzyme for a standard LAMP reaction. Previous studies showed that concentrations lower than 8 U enzyme caused decreased signal in product yield in LAMP reactions (Nie 2005). Increasing enzyme concentration to 12 U speed up the LAMP reactions, but also created false-positive signals in negative control. Therefore, recommended amount 8 U/reaction was determined as optimum *Bst* DNA polymerase concentration for all three primer sets.

Loop primers are optional for LAMP reactions. They are recommended to be used for increasing the reaction speed and the specificity (Tanner and Evans 2014). In this study, primer set 1 contains two loop primers LF/LB while primer set 2a and 2b contains only one loop primer LB because LF could not be designed due to high variability in the sequence of that region. Overall, results of this study agreed with the findings and proved that absence of even one loop primer drastically delayed the signal of LAMP reactions and decreased specificity by creating false-positive signals in negative controls.

After determination of optimum conditions, limit of detection (LOD) in LAMP for all primer sets were determined. LOD is the most important indicator of developed LAMP test's sensitivity because it shows lowest amount of target DNA that can be possibly detected with the test. LOD for primer set 1 equal to 2×10^4 copies/µl and can be detected on average in 20 minutes. LOD for primer set 2b equals to 2×10^4 copies/µl and can be detected in 25 minutes on average. On the other hand, 2×10^4 copies/µl cannot be detected consistently with LAMP reactions using primer 2a. Primer set 2a has more variable results, late time to signal and caused more frequent false-positive signals in negative controls compared to other primer sets. Therefore, primer set 1 and primer set 2b are determined to be more suitable for BDV identification with LAMP.

CHAPTER 5

CONCLUSION

Border disease is a highly transmissible viral infection of ruminants, and it is associated with economic losses in sheep and farm industry. There is no cure or vaccine against border disease, therefore early diagnosis is essential to prevent the spread of the disease. In this thesis study, a nucleic acid-based LAMP test was developed for border disease virus (BDV) identification. LAMP is an isothermal amplification technique that can be applied without the need of a trained personnel or expensive instruments. Its simple protocol enables it to be performed as a point of care (POC) test that gives accurate results rapidly, in less than 90 minutes.

For BDV identification, BDV genome sequences from all over the world was used for multiple sequence alignment and multiple alignment results stated that most conserved region of the genome is 5'UTR. Then, three LAMP primer sets, primer set 1, 2a and 2b were designed targeting 5'UTR. Designed primers were used to setup and optimize LAMP reactions to improve the sensitivity and specificity of the developed test. LAMP optimization studies were focused on different parameters of the reaction such as optimization of fluorescent dye concentration, temperature, primer mix concentration, Mg^{2+} concentration and enzyme concentration. After determination of optimum LAMP reaction conditions for each primer set, their detection limit (LOD) was determined as an indicator of sensitivity. All primer sets have LOD equals to 2×10^4 copies/µl. Finally, when the performances of three primer sets in LAMP was compared, it was shown that primer set 1 and 2b has higher sensitivity and more consistent results compared to primer set 2a. Therefore, primer set 1 and 2b can be used to target BDV.

Future studies can be performed for optimization of developed LAMP tests for BDV RNA samples. For this purpose, reverse transcription step can be added into LAMP reaction (RT-LAMP) by including reverse transcriptase enzyme into the reaction mixture. Then, developed RT-LAMP test can be optimized with clinical samples of ruminants such as blood, serum, saliva, or semen to evaluate its usage in the field. Also, comparison of

developed LAMP test with “gold-standard” RT-qPCR technique can be applied to prove that LAMP can reach same analytical sensitivity while giving rapid results.

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