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Master's Degree Thesis

Development of whole genome sequencing methodology for studying the genetic diversity of TBEV in Norway: prevalence estimation and improvement of the amplification methods for whole genome sequencing

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14th February 2024, Oslo

Kawsar Bibi,

Abbreviations

ATP	Adenosine triphosphate
APS	Adenosine phosphosulfate
bp	Base pair
C	Capsid
cDNA	Complementary deoxyribonucleic acid
CNS	Central nervous system
Ct	Threshold cycle value
DMUK1	DMUK 400bp overlapping primer
dNTPs	Dinucleotide triphosphate
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
dUTP	Diuracil triphosphate
E	Envelope
EPP	Estimated pool prevalence.
F	Forward
FBS	Fetal- Bovine Serum
IPC	Internal positive control
Kb	Kilobases
KUPA1	KUPA 400bp overlapping primers
M/ M-protein	Membrane/ Membrane-protein
MM	Mastermix
MEM	Minimum Essential Medium
MIR	Minimum infection rate
ml	Milli-liter
mM	Milli-molar
MSIS	Norwegian surveillance system for communicable diseases
N	Nymph
NC	Nucleocapsid
NCRs	Non-coding regions

ng	Nano gram
NGS	Next generation sequencing
NIPH	Norwegian Institute of Public Health
nt	Nucleotide
ONT	Oxford nanopore technology
PC	Positive control
PCR	Polymerase chain reaction
poly (A)	Polyadenylated
prM/prM-protein	Pre-membrane protein
PPi	Inorganic pyrophosphate
R	Reverse primer
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
RT real-time PCR	Reverse transcription real-time polymerase chain reaction
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
SSIV	Superscript ^{TM} IV revers transcription kit
TBE	Tick-borne encephalitis
TBEV	Tick-borne encephalitis virus
TBEV-EU	Tick-borne encephalitis virus European
TBEV-FE	Tick-borne encephalitis virus Far Eastern
TBEV-Sib	Tick-borne encephalitis virus Siberian
TBF	Tick-borne flavivirus
WGS	Whole genome sequencing
µl	Micro-liter
µM	Micro-molar
µg	Micro-gram

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Abstract

The tick-borne encephalitis virus (TBEV), classified as an arbovirus within the Flavivirus genus, is responsible for tick-borne encephalitis. Ticks serve as main vector for TBEV. TBE is a serious disease condition in humans and several animal species. TBE has been a rising health concern in recent decades, not just in Europe but increasingly all over the world. The number of TBE cases seems to be increasing over time in Europe including Norway. Thus, the main purpose of the present study was to estimate the TBEV prevalence at Kilen, Mandal in southern Norway from questing nymphs collected in 2021. This study also addresses an amplification method and troubleshooting techniques to develop a method for WGS of low viral loads of TBEV in nymph samples.

In the present study a total of 518 nymph pools were screened and analyzed for TBEV. An overall TBEV prevalence of 0.7 % was estimated which is different from prevalence in 2018 and 2019 for the same site, when no TBEV was detected. This increase and change in prevalence indicated the monthly and yearly variation of TBEV prevalence in endemic regions of Norway.

The present study was also aimed to develop a WGS method of low virus load TBEV samples but, the study faced issues in obtaining desired 400bp PCR product for WGS method. Several amplification and troubleshooting experiments i.e., Inhibition tests and dilution tests were conducted out to explain the lack of required 400bp PCR product from nymph cDNA samples for WGS.

The result showed that there was no inhibition in the samples tested and the viral load in the samples may be too low to produce PCR product by the current PCR conditions. Spike-in methods were found helpful in improving PCR products. The study also found that cDNA generated by both the reverse transcription technique, High-Capacity cDNA, and Superscript IV cDNA, performs well with small fragment length overlapping primer pairs of the TBEV-Eu strain.

In conclusion, this study shows that there is a monthly and yearly variation of TBEV in Kilen Mandal. The developed amplification and troubleshooting procedures from this study may be further investigated to obtain an improved and desired PCR products for sequencing of whole viral genome of TBEV samples with low viral load.

1. Introduction

1.1 Vector and vector borne diseases.

The World Health Organization (WHO) defines vectors as biological organisms that can spread contagious diseases to people and animals. Most vectors are hematophagous (blood-feeders) and either transmit or pick up the pathogen while feeding (Lane, 1993). While consuming blood from an infected host, these vectors contract dangerous microorganisms (bacteria, viruses, or parasites), and during their subsequent blood meal, transfer the microbes into a new host, disseminating vector-borne diseases (WHO, 2017). Millions of cases of sickness are brought on each year by vector-borne diseases, which cause a burden on worldwide public health. Several vector-borne diseases are spreading because of introductions into new areas or shifting endemic disease patterns (Laaksonen *et al.,* 2017). More than 17% of all infectious diseases are vector-borne illnesses, and they pose a growing threat to humans and animal around the world (Holzmann, 2003; WHO, 2017).

1.1.1 The vector; ticks (*Ixodes ricinus*)

Ticks are a group of arthropod vectors, which transfer wide range of infections (Fig 1). They have effects on both human and animal health, and they have economic implications particularly in Southern Hemisphere nations. Many of these disease transmissions in Europe are linked to *Ixodes ricinus* (*I. ricinus)* sometimes known as the castor bean tick (Fig 1), and it is this tick that is most detected in Norway (Charrel *et al.,* 2004; Granström 1997). *I. ricinus,* a little hard tick belonging to the family Ixodidae, transmits a wide range of infections of significant medical and veterinary significance (Medlock *et al.,* 2013). The member of the Ixodidae family includes 224 species and is the largest tick genus in the world Dobler*et al.,* 2019). *I. ricinus* is the most noteworthy tick in Europe due to its widespread distribution throughout ecosystems and the range of transmitted infections, including Borrelia (which causes Lyme borreliosis) as well as the tick-borne encephalitis virus, (Boulanger, *et al.,* 2019). The spread of tick-borne encephalitis in central Europe and the recognition of *I. ricinus* as the carrier of the virus triggered investigation on *I. ricinus* in Europe. (Grey *et al.,* 2021).

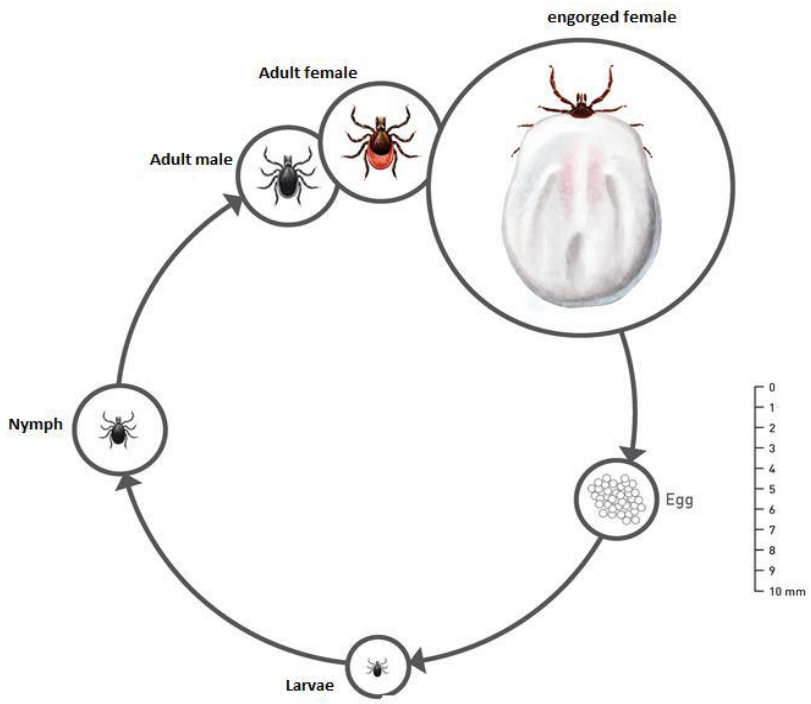

Fig 1. Image showing the life cycle of I. ricinus tick*; an egg, larva, nymph, an adult male and female.* (Made by Hallvard Elven and Preben Ottesen, Norwegian Institute of Public Health*).*

1.1.2 Distribution of *I. ricinus*

The *I. ricinus* is the dominant species in Europe, particularly in Scandinavia, which represents the northernmost extent of its distribution area. (Jaenson *et al.*, 2012). They favor environments in shady deciduous forests with dense vegetation that maintains high ground surface humidity (Pfäffle *et al.*, 2013). The subtypes of Borrelia (*Borrelia burgdorferi* sensu lato*/ Borrelia miyamotoi*), Ricketsiales (*Neoehrlichia mikurensis/Anaplasma phagocytophilum*), Babesia (*Babesia venatorum/Babesia divergens/Babesia capreoli)*, and tick-borne encephalitis virus (TBEV) (Kjelland *et al.*,2018).

The most northern viable population of *I. ricinus* is documented at Dønna, located in Nordland County, Norway, north of Brønnøysund, at around 66°N (Hvidsten *et al.*, 2020). The geographic distribution of ticks in Norway extends by the coastline, starting from Hvaler nearby the border with Sweden in Østfold County. While this geographical area exhibits high population densities, neighboring regions may have widely different population densities *(*Vikse *et al.*, 2020). I. ricinus

may be discovered at altitudes that are at least 1000 meters above the level of the sea (De Pelsmaeker *et al.,* 2021). In addition, *I ricinus* ticks have sporadically been discovered in inland regions and north of the polar circle (Hvidsten *et al.,* 2020).

1.1.3 Life and host cycle of *I. ricinus*

Ixodes ricinus has four life stages: an egg, a larva, and three active parasitic stages (nymph, adult tick). Under ideal conditions, each stage of the parasite takes one year to molt and proceed into the next life cycle. Questing ticks may sense the presence of a potential bloodmeal host by crawling onto plants in grassland, forests, or brushland. By extending their legs into the air and displaying their sensing organ, the Haller's organ, they sense what animals are nearby. This organ on the dorsal side of the legs contains chemo-, thermo-, and photoreceptors (Sonshine & Roe, 2014). When a host brushes against plants, a powerful stimulus is used to activate the tick's claws, which then attach onto the host's fur and subsequently they begin to consume blood (Sonshine & Roe, 2014). It has been suggested that *I. ricinus* has a three-host life cycle (Fig 2). When a larva initially hatches, it will hunt for and consume bloodmeal from smaller animals like as mice, other small mammals, birds, and lizards. After eating a full meal, the larva will fall to the ground and moult into a nymph. The hungry nymph will search for and take a bloodmeal from a medium-sized host, such as birds, rabbits, or foxes, before molting into an adult tick and falling to the ground. Before reproducing sexually, adult ticks feed on larger hosts like roe deer, red deer, moose, cows, and sheep (Fig 2). The female tick mates with the male tick that is already attached to the host. The female drops to the ground, where the engorged fertile female lays a few thousand eggs before continuing the gonotrophic cycle until she runs out of eggs and dies (Sonshine & Roe, 2014).

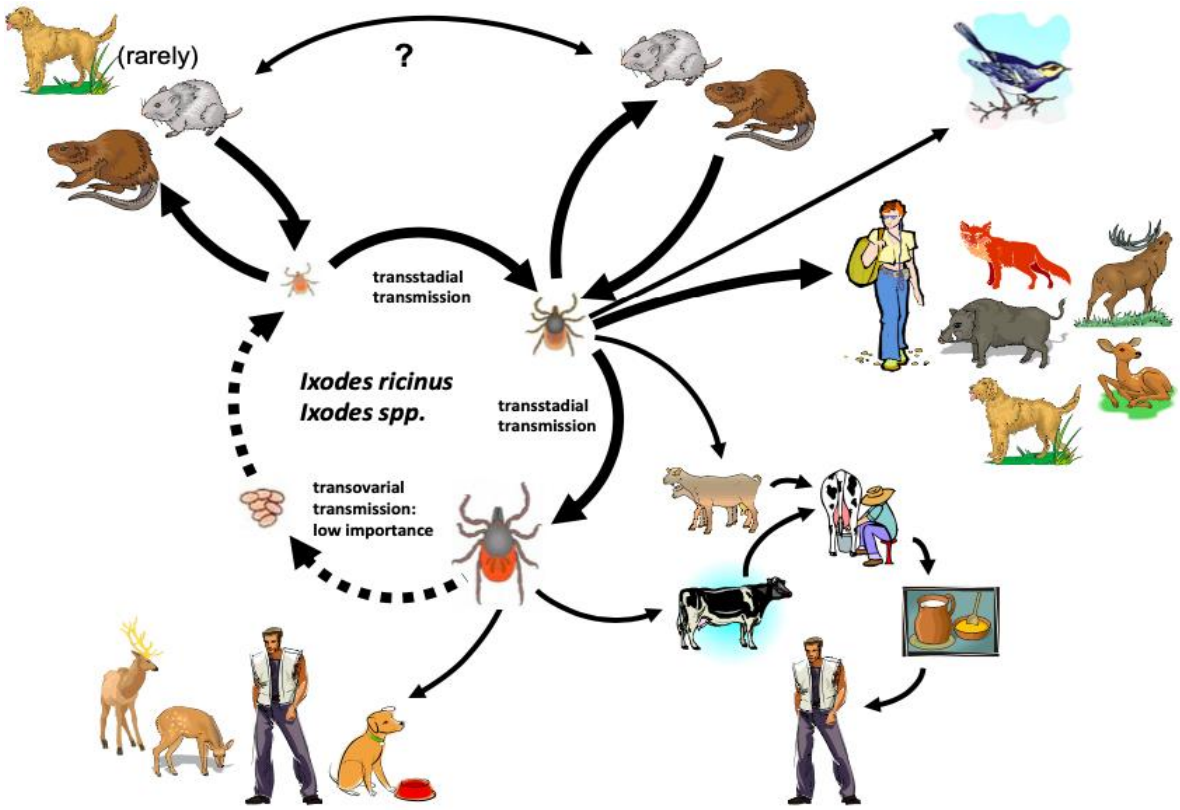

Fig 2. The diagram depicts the life cycle, host cycle and three transmission routes: 1) transovarial transmission, in which the virus may spread from adult females to their progeny. 2) transstadial transmission is the transfer from nymphs to adults via accidental hosts. 3) Co-feeding is the least essential transmission method. Each reservoir host plays a significant role in tick life cycle and TBEV transmission. (Dobler *et al.*, 2012).

1.2 Tick-borne encephalitis virus (TBEV)

The tick-borne Encephalitis virus (TBEV), classified as an arbovirus within the Flavivirus Genus, is responsible for tick-borne encephalitis. This virus exhibits three distinct subtypes characterized by genomic sequences: the European (TBEV-Eu), Siberian (TBEV-Sib), and Far Eastern (TBEV-FE) subtypes (Ecker *et al.,*1999). The distinction between TBEV subtypes is based on differences in vector competence, geographic distribution across Europe and Asia, and their impact on human pathogenicity (Carpi *et al.,* 2009). Particularly, the TBEV-European subtype is common in Norway, and *I. ricinus serves* as its primary vector (Vikse *et al.,* 2020; Gritsun *et al.,* 2003). The

phylogenetic distinction of TBEV-Eu is evident in Norway, Sweden, and Denmark. The first detection of a Norwegian TBEV strain in a patient's serum was in 1997(Skarpaas *et al.*, 2004).

Phylogenetic study indicates that TBEV-Sib and TBEV-FE are distinct lineages from TBEV-Eu, with common ancestry stretching back thousands of years (Lindquist, 2014). These two sub-types evolved earlier than the TBEV-EU subtype. These two subtypes show more variability than the TBEV-EU subtype (Tonteri *et al.*,2013).

1.2.1 TBEV genomic structure

TBEV particles are smooth and spherical, with a diameter of 50 nm, like other flaviviruses (Kuhn *et al.*, 2002). The TBEV genome, a positive-strand RNA of around 11 kilobases, encodes a single polyprotein that undergoes co- and post-transcriptional processing, resulting in three structural proteins (SP) and seven non-structural proteins (nSP) (Demina *et al.*, 2018). The TBEV virion is composed of three structural proteins: the envelope (E), membrane (M), and capsid (C), as well as a positive-sense single-stranded RNA genome. A nucleocapsid (NC) forms when genomic RNA and multiple copies of the C protein combine (Fig 3). This NC is surrounded by the viral membrane, which is made up of host-derived lipids and contains 180 copies of the M and E proteins (Füzik *et al.*, 2018).

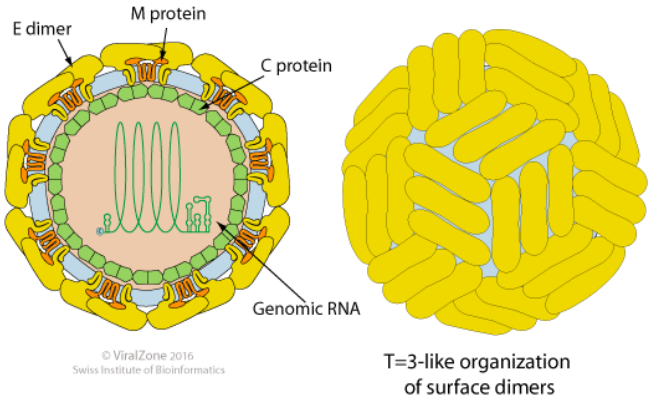

Fig 3. Image illustrating Flavivirus with its genetic material in the center. The spherical enveloped virion of TBE is enclosed by three primary structural proteins: the envelope region (E dimer), the membrane area (M protein), and the capsid region (C protein),(Picture Citation: Viral Zone, https://viralzone.expasy.org/by_species/43.)

1.2.2 TBEV hosts and transmission.

Ticks serve as reservoirs for TBEV because of their ability to transmit the virus both transovarian and transstadial throughout their life cycle. Ticks that have been infected with TBEV will carry it with them for the rest of their lives (Nuttall & Labuda, 2003). A suitable reservoir host is an animal that gets infected with TBEV and allows the virus to circulate in its bloodstream without causing host death, enabling ticks to feed on it and get infected. Small animals, such as rodents and insectivores, are believed to be the primary reservoir hosts. They are widely distributed, have narrow home ranges, and can be easily trapped, are often infested by ticks (Bakhvalova *et al.*, 2009; Michelitsch *et al.*, 2019). Therefore, rodents may serve as potential indicators for evaluating TBEV distribution and prevalence. Migratory birds, serving as both competent and incompetent hosts, may transfer TBEV to ticks by viraemic or non-viraemic routes (Waldenström *et al.*, 2007). Humans are passive dead-end hosts, playing no active part in the natural maintenance of TBEV (Dantas-Torres *et al.*, 2012; Bogovic & Strle 2015).

1.2.3 Tick-borne encephalitis disease

The tick-borne encephalitis disease is caused by the tick-Borne Encephalitis virus (TBEV). They can cause severe acute and chronic neurological diseases (Conze *et al.*, 2021). It is a serious disease condition in humans and several other animals (Gritsun *et al.*, 2003). The majority of TBEV infections are asymptomatic, do not cause significant neurological disorders, thus most cases go undetected (Hofhuis *et al.*, 2021). Virulence and disease symptoms differ amongst viral subtypes. TBE caused by the European virus subtype usually appears as meningitis, encephalitis, or meningoencephalitis, and in two-thirds of infected individuals, clinical symptoms are often biphasic, with a flu-like viremic first phase and either an asymptomatic- or severely symptomatic second phase. There is no therapy for TBEV, however symptoms assessments are sometimes used to guide treatment (Kaiser, 2008).

After a tick bite, the virus incubates for about eight days before entering the viremic phase, which lasts around five days. During this period, the virus may be detected in the serum, and patients may have symptoms like as fever, body pains, fatigue, nausea, and headache. An asymptomatic phase occurs after the viremic period and lasts around seven days. For around 65-70% of people, the illness course stops after the first phase; however, for the remaining 30-35%, the virus invades the

CNS, resulting in the neurological phase. Secondary symptoms of meningitis or encephalitis include fever, headache, tremor, paralysis, and altered consciousness (Fig 4). Acute sequelae impact 30% of patients in the secondary phase, and antibody testing is the only diagnostic tool available at that moment. The incubation time for infections acquired by the alimentary channel is shorter (Blom *et al.,* 2018; Ruzek *et al.,* 2019; Lindquist & Vapalahti 2008).

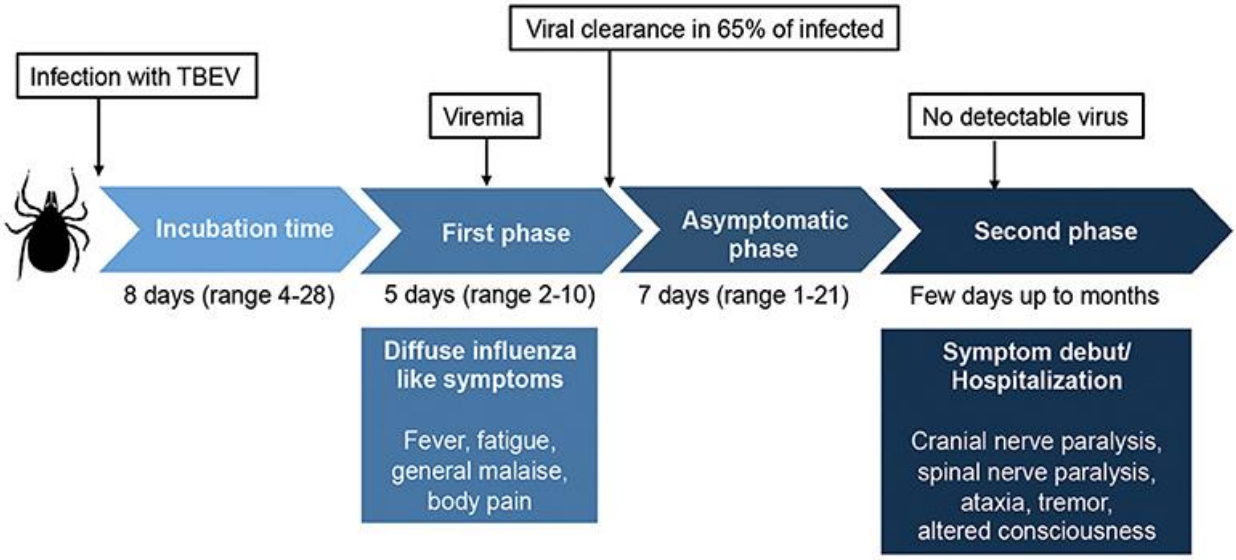

Fig 4. Schematic diagram shows the biphasic illness pattern seen in humans who are infected with the tick-borne encephalitis virus (TBEV) (Blom *et al.,* 2018).

1.2.4 TBEV prevalence and distribution in Norway and Europe

The tick-borne Encephalitis (TBE) has been a rising health concern in recent decades, not just in Europe but increasingly all over the world (Süss, 2008). TBEV is endemic in countries ranging from central and Eastern Europe to Siberia and parts of Asia, with an increasing detection of TBEV endemic areas, particularly in Asia, indicating the need for more information (Süss, 2011). The number of TBE cases seems to be increasing over time in Europe and in Norway. The prevalence of TBEV in questing nymphs in European endemic regions varied between 0.1% and 5.0% (Jensen *et al.,* 2004, Süss 2011). According to estimates from the European Centre for Disease Prevention and Control (ECDC), TBE cases ascended from twelve countries in 2000 to 25 in 2019, with approximately 3246 cases reported in the European Union (Wondim *et al.,* 2022). Europe have

three TBEV subtypes: European, Siberian, and Far Eastern (Ecker *et al.,* 1999). Eastern Europe is identified as a TBEV endemic region, with an increasing number of human TBE illnesses (Randolph, 2010). Similarly, TBE infections seem to be on an increase in Scandinavia and France, spreading into areas with no previous record of viral presence (Lamsal *et al.,* 2023; Fomsgaard *et al.,* 2009; Herpe *et al.,* 2007).

The Norwegian Surveillance System for Communicable Diseases (MSIS) has reported an increase in TBE cases (Health, 2022). TBE have been reported from coastal counties like Vestfold, Telemark, Agder, Buskerud, Akershus and Østfold in accordance with MSIS reports. TBE IgG antibodies was detected in blood donors from Østfold County (Larsen *et al.,* 2014). In northeastern Norway (Møre, Romsdal, and Trøndelag) TBEV prevalence in *I. ricinus* was 0.41% in nymphs and 3.08% in adults. (Paulsen *et al.,* 2015). MSIS statistics indicate that 202 TBE cases have been reported in Norway between 1998 and 2019, with many of them caused by tick bites in the southern coastal districts, as emphasized by (Health 2018; Marvik *et al.,* 2021). This suggests that TBEV is becoming more common in Norway than previously reported (Andreassen *et al.,* 2012; Paulsen *et al.,* 2019; Soleng *et al.,* 2018; Vikse *et al.,* 2020). The prevalence rates varied between 0% to 3.5% in nymphs (44,000 nymphs) and 0% to 20.6% in adults (3,404 adults), with an overall estimated average of 0.3% recorded between 2009 and 2016 (excluding 2010). Adult tick prevalence was highest in the former Rogaland and Vestfold County, although it was also high in other places such as Nordland, Agder, Vestfold, and Telemark County (Vikse *et al.,* 2020).

1.3 Methodological approaches for molecular analysis of TBEV

The signs and symptoms of tick-borne encephalitis virus (TBEV) disease may vary from asymptomatic to severe meningoencephalitis or meningoencephalomyelitis. Since infection usually becomes apparent after the virus has spread, relevant samples from patients of TBEV are exceedingly rare and, when they occur, are linked with low viral loads. Similarly, only a few research studies have directly sequenced the full TBEV genome from patient clinical samples (Zakotnik *et al.,* 2022). In a result, finding sensitive tools to identify these viruses is essential for accurate detection and research on both imported and native viruses. Even in natural viral foci, the prevalence of TBEV infection in tick populations is minimal (Pettersson *et al.,* 2014). When the disease is uncommon, pooling could be used to estimate the infection prevalence rate in a particular

area, making it more affordable than analyzing samples from individual animals (Cowling *et al.*, 1999).

1.3.1 Homogenization and the total RNA extraction

Tick samples must be mechanically disrupted before RNA extraction because of to their rigid shield. Before total RNA extraction, viral particles are made inactive by adding viral lysis solution, which releases viral RNA (Fitzpatrick *et al.*, 2021; Bastakoti, 2019). Typically, lysis buffer is given to samples after they have been homogenized using tungsten carbide beads, which breaks down tissues and cells and releases virus particles into the extraction medium (Gäumann *et al.*, 2010). The homogenized solution is centrifuged to remove any residual cells, tissues, and cell debris. Various techniques have been examined to extract total RNA or nucleic acids from various tick sample (Gäumann *et al.*, 2010).

1.3.2 Polymerase chain reaction (PCR)

There are various well-developed techniques for amplification of small region of genome, most of which consist of one or more PCR steps, such as nested-PCR (Puchhammer-Stöckl *et al.*, 1995), Hot-start PCR technique (Jansen *et al.*, 2021), and the touchdown PCR technique (Katargina *et al.*, 2013). Unlike to the conventional PCR approach, these techniques improve the efficacy and specificity of DNA PCR amplifications for library preparations and subsequently WGS. Polymerase chain reaction (PCR) is a familiar technique for detecting disease in ticks because it can amplify a small amount of viral genetic material (Schwaiger & Cassinotti 2003).

1.3.2a. Reverse transcription polymerase chain reaction (RT-PCR)

DNA polymerases that depend upon RNA use reverse transcription to convert RNA into DNA. Reverse transcriptase types are selected based on various characteristics, including low RNase H activity, maximum temperature stability, maximum processivity, maximum length of the single stretched cDNA, and resistance to reverse transcriptase inhibitors (Coffin *et al.*, 1997). Random hexamers were used as primers in various RT-PCR TBEV RNA research. Oligo dt primers must be avoided for the purpose to synthesize viral cDNA without causing host cell mRNA synthesis.

Depending on the required fragment size, several revers transcription polymerases can be used (Rio, 2014). To get a high yield of cDNA from short fragments, the High-Capacity revers transcription kit (Invitrogen, city, state) can use while, for longer fragments, superscript IV kit (Applied Biosystems, city, state) use.

1.3.2b. Real-time PCR for TBEV detection

Due to several diagnostic challenges, we used a real-time reverse transcription polymerase chain reaction (RT-PCR) assay based on TaqMan chemistry to detect TBEV RNA. Real-time RT-PCR depends on reverse transcription, followed by PCR amplification. The TaqDNA polymerase's 5′-3′ nuclease activity cleaves the TaqMan probe in PCR. The TaqMan probe has a fluorescent reporter dye at the 5′ end and a fluorescent quencher dye at the 3′ end (Fig 5). Increased fluorescence is caused by the reporter dye being cleaved during PCR; RT-PCR methods have previously been used for successfully identifying TBEV RNA in ticks and patient samples. This is precisely relative to the amount of PCR products. (Schwaiger & Cassinotti 2003). The current study used an in-house RT-realtime PCR TBEV technology developed by Torstein Tengs of the Norwegian Veterinary Institute for the Norwegian Institute of Public Health in Norway. The PCR method for TBEV detection is extremely specific and sensitive. It can be used to identify a 54-bp region of the Norwegian and European TBEV strains using primers that detect the TBEV E gene (1662-1715 nt) at the 3' ends of the TBEV genome.

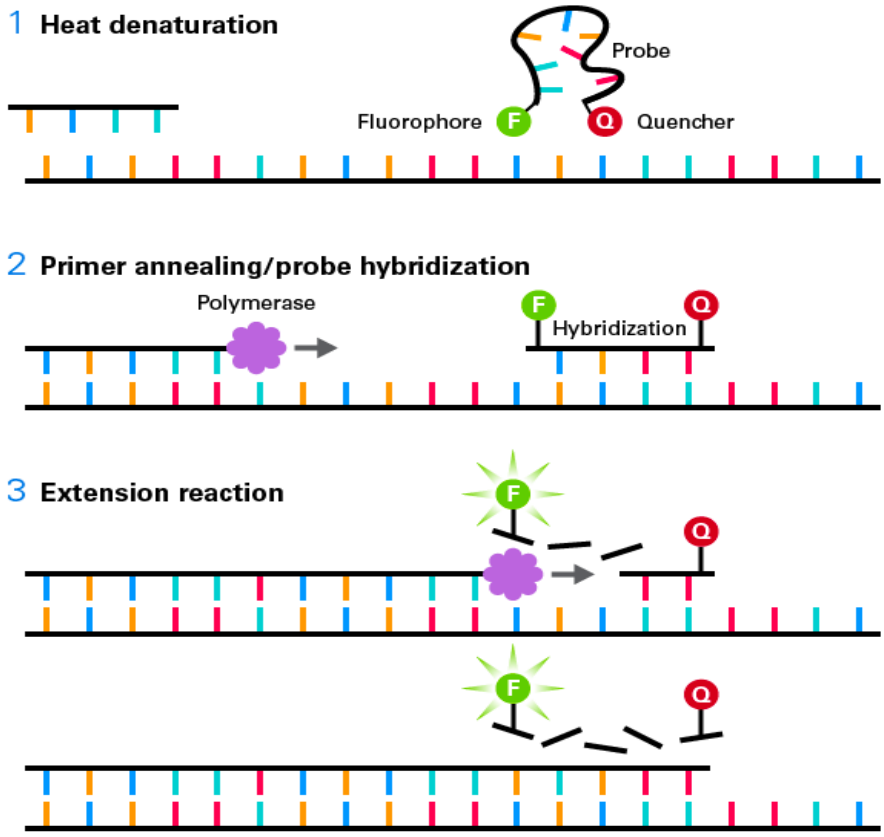

Fig 5. Diagram demonstrating the basic RT process, real-time with probe binding. The three important steps are denaturation, annealing, and extension of a new strand employing Taq polymerase in the 5'-3' orientation (TakaRa Bio, https://www.takarabio.com/learning-centers/real-time-pcr/overview/one-step-rt-qpcr-kits).

1.3.3 Pyrosequencing

Pyrosequencing is the first alternative technology for sequencing small PCR fragments (less than 100 bp) after the traditional Sanger sequencing method. This sequencing approach depends on the detection of pyrophosphate produced by enzymes during the incorporation of nucleotides by DNA polymerase (Fakruddin *et al.*, 2012) (Fig 6). In pyrosequencing, nucleotides are eliminated in two methods. Pyrosequencing is classified into two types: solid phase, which uses a three-coupled enzymatic process with washing stages, and liquid phase, which uses a cascade of four enzymes without any washing steps (Gharizadeh *et al.*, 2001).

Pyrosequencing takes place in the liquid phase utilizing four enzymes: luciferase, apyrase, DNA polymerase, adenosine triphosphate (ATP) sulfurylase, and substrates adenosine 5 phosphosulfate (APS) and luciferin. Nucleic acid polymerization is the first stage in the enzymatic cascade, releasing inorganic pyrophosphate (PPi) after polymerase incorporates the nucleotide (Fig 6). ATP-sulfurylase converts the liberated PPi into ATP, providing luciferase with the energy required to oxidize luciferin to generate light (Ronaghi 2001). A pyrogram's light signal appears as a peak signal that increases with the number of nucleotides incorporated (Gharizadeh *et al.*, 2007). Given that the additional nucleotides are known, the template sequence may be determined. Pyrosequencing has not been utilized for genome sequencing due to read length limitations, although it is used for confirmatory and de novo sequencing (Ronaghi 2001).

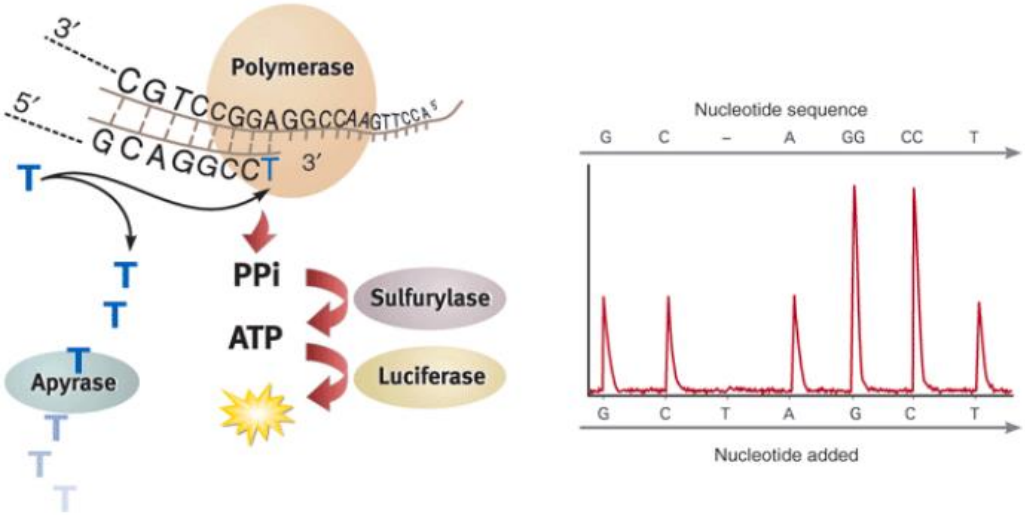

Fig 6. Figure showing graphical representation of the pyrosequencing process. The Fig on the left shows a newly produced strand along the template DNA polymerase, with dNTPs added independently. An integrated dNTP causes the release of ppi, which is turned into a light signal by several enzymes. The pyrogram on the right illustrates the integrated nucleotides as peaks, revealing the nucleotide order of the sequence (England & Pettersson, 2005).

1.3.4 Whole genome sequencing

Whole genome sequencing of viral genomes directly from clinical samples is essential for identifying disease-causing genetic variants, especially those responsive to positive selection pressure because of host interaction (Herbeck *et al.*, 2011). Whole genome sequencing has made it easier to investigate outbreaks of new diseases and analyze genetic data in real time. It provides essential information on virus transmission, dissemination, and evolution, which is required to successfully manage viral epidemics (Gardy & Loman 2018; Dudas *et al.*, 2017).

Whole genome sequencing (WGS) is a powerful technique that may discover several genetic differences in a single test with high sensitivity without the need for physical separation of responses in separate tubes (Datta *et al.*, 2015). Whole genome sequencing (WGS) methods provide more data processing capacity at lower costs than the traditional Sanger capillary electrophoresis sequencing technique, which is considered as a first-generation sequencing technology. Furthermore, WGS technologies allow for large-scale genomics research, including the analysis of whole populations. Significant advances in next-generation sequencing (NGS) have enabled scientists to sequence whole genomes at less expense and in a shorter period (Park & Kim, 2016). Several commercially available platforms make it easier to sequence viral genomes, including 454 pyrosequencing, SOLiD sequencing, SMRT (Pacific Biosciences), Ion torrent sequencing, Illumina sequencing (MiSeq and HiSeq) and Nanopore sequencing technology (Oxford Nanopore Technologies) (Slatko *et al.*, 2018).

1.3.4a Illumina sequencing

All the NGS platforms follow the same steps for library preparation, amplification (template), sequencing, read visualization, and data analysis, they vary in how each step is completed. One example is the Illumina sequencer, which uses solid-phase amplification. This procedure involves priming and extending single-stranded templates, which results in bridge amplification of each immobilized sample using neighboring primers. After numerous rounds of annealing, extension, and denaturation, a considerable number (about 200 million) of molecular clusters are formed. During sequencing, all four nucleotides are delivered simultaneously, each tagged with a different fluorescent dye. These nucleotides are intended to inhibit DNA synthesis after incorporation and

may be identified by examining the colors of the added bases (Fig 7). Sequencing is completed by removing the dye and rebuilding the 3'-hydroxide group (Beerenwinkel *et al*., 2012)

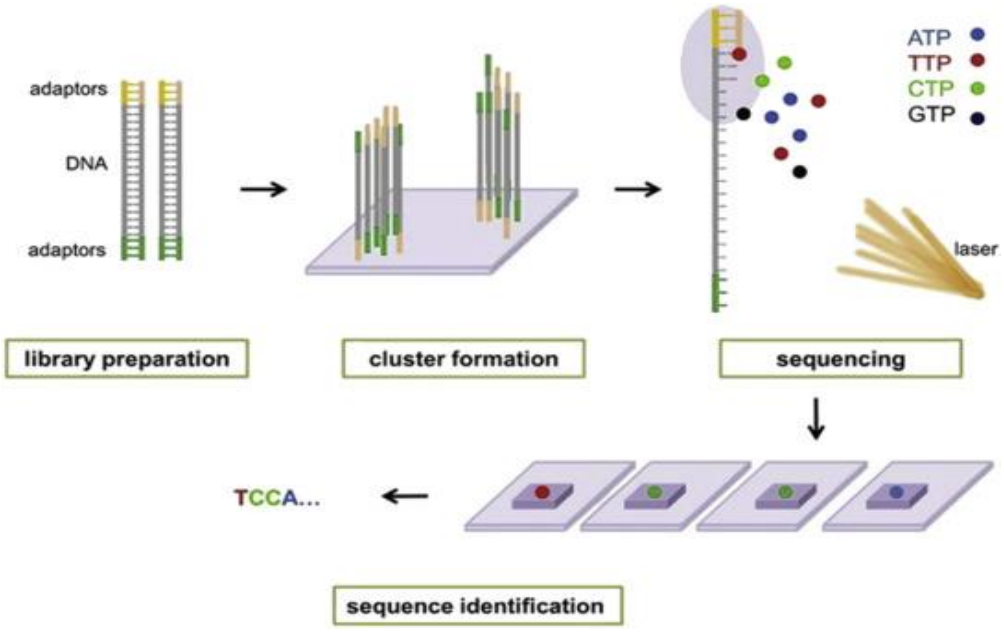

Fig 7. Schematic diagram illustrating MiSeq Illumina sequencer, NGS platform. The fundamental steps in Illumina sequencing are library preparation, cluster formation, sequencing, and sequence identification (Raghavendra & Pullaiah, 2018).

1.3.4b Oxford Nanopore Technology:

ONT is a sequencing method that use nanopores to analyze individual molecules. This technology use nano-sized openings built into a thin membrane structure to identify charge changes when charged biological molecules smaller than the nanopore pass through the hole. Thus, nanopore technology can identify and analyze individual molecules like amino acids, DNA, and RNA (Deamer & Akeson, M. 2000; Lin *et al.*, 2021).

The nanopore sequencing approach is typically classified into three steps: library preparation, sequencing process, and basecaller. The library preparation must be done before the nanopore sequencing procedure can begin. Regardless of whether the DNA fragments are sheared or not, they should be repaired. During the repair process, the repaired connector forms a DNA-protein

complex with either a polymerase or a helicase. The DNA strand to be sequenced is combined with replicas of the processive enzyme. The enzyme binds to a leader strand that is not linked with another at the end of the DNA template as the DNA-protein complex approaches the nanopore. It then separates the two strands of DNA and threads one of them through the nanopore. When an unzipped DNA strand passes through the nanopore one base at a time, a single molecule with high specificity can hinder the flow of electric current (Fig 8). Current signals may be utilized to verify the nature of the base. During the bascaller step, the flow of nucleotides through the nanopore causes differences in charge and structure, resulting in small disruptions in the measured current. Algorithms for deep learning may be used to turn electrical impulses into DNA sequences (Lin *et al.*, 2021).

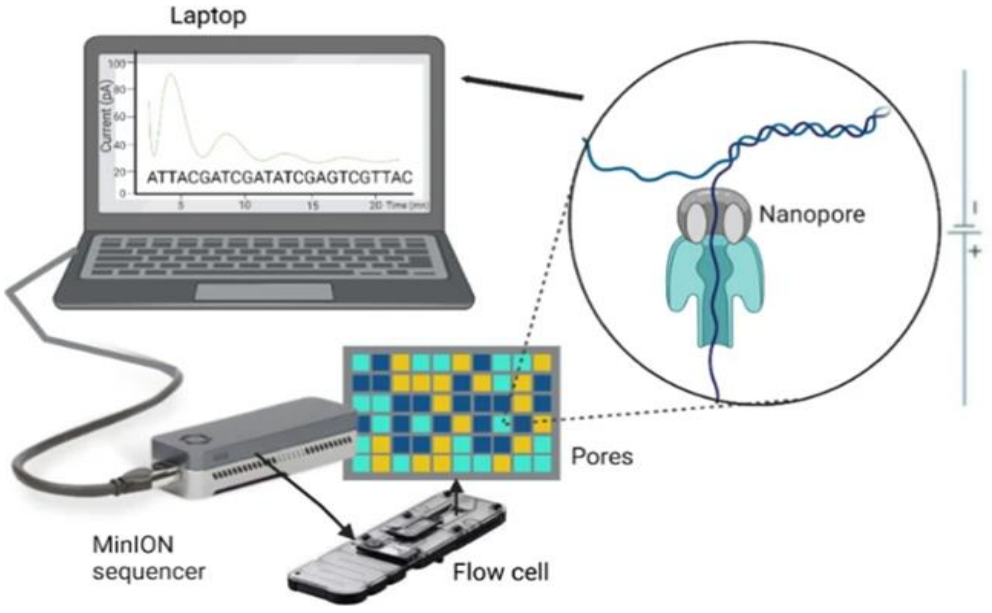

Fig 8. Image displaying the components of MinION device's which includes the flow cell, nanopore, real-time computer access, and process management. The nanopore membrane is made up of active green holes, inactive blue pores, and recoverable yellow pores (Wasswa *et al.*, 2022).

1.3.5 PCR Inhibition test

Strong PCR inhibitors can hinder PCR amplifications in the targeted region, resulting in false negative results in the sample of interest (Mackay *et al.,* 2002). Positive sample amplification is underestimated when suspected inhibitors are included in the PCR process (Demeke & Jenkins 2010; Pettersson *et al.,* 2014). Potential PCR inhibitors can come from tick tissues or debris collected with ticks (Schwartz *et al.,* 1997). Excess ethanol in the sample may potentially act as an inhibitor (Schrader *et al.,* 2012). Although most research have focused on the operation of polymerase enzymes, inhibitory activities may impact other components of the PCR process. Proteinases, phenol, and detergents may denature polymerase, whereas inhibitors, which are reversible effects of heme (the precursor to hemoglobin), can block the enzyme's active site (Alaeddini, 2012).

It is possible to indirectly analyze the presence of PCR inhibitory compounds by determining the quantity and quality of the PCR products using a variety of methods, including gel electrophoresis, dot blots, high-pressure liquid chromatography, and calorimetric tests (Alaeddini, 2012). A series of known quantities of the internal standard can be used to detect PCR failures and semi-quantitatively monitor PCR inhibition. This may be achieved by checking for amplification failures in the internal standards. Quantitative real-time PCR is the recommended method for detecting PCR inhibitors (Kontanis *et al.,* 2006). Alternatively, a more typical strategy is to add an exogenous internal positive control (IPC) fragment in the same multiplex reaction as the genomic product (Swango *et al.,* 2006; Swango *et al.,* 2007).

1.4 Aims and objectives.

Previous research undertaken by the Norwegian Institute of Public Health (NIPH) have examined the occurrence of TBEV in tick samples obtained from different regions of Norway. There are no studies available describing the TBEV strains from these regions and based on results from previous master thesis there are no methods available detecting TBEV from samples with low viral load. Therefore, the main aim of this study was to get insight about the genetic diversity and variants of TBEV in ticks from Mandal in southern Norway, with an overall objective to develop a whole genome sequencing method by Nanopore technology or Illumina sequencing for low viremic TBEV positive samples.

The specific aims of this study are:

1. Screening and estimation of TBEV prevalence in ticks collected in 2021 from Kilen, Mandal Norway.

2. Developing a method of whole genome sequencing for TBEV Viral genome from the same samples.

2.Materials and Methods

2.1 Study Area

Questing nymphs analyzed in this study were collected monthly in 2021 in Kilen (Mandal), in Lindesnes municipality, located in the western part of Agder County with **UTM**: 32V 6430577 411721, (**Longitude:** 58°00′N, **Latitude:** 07°30′E). The sampling area is 50-150 meter from the seashore and has a typical coastal climate (Fig 9).

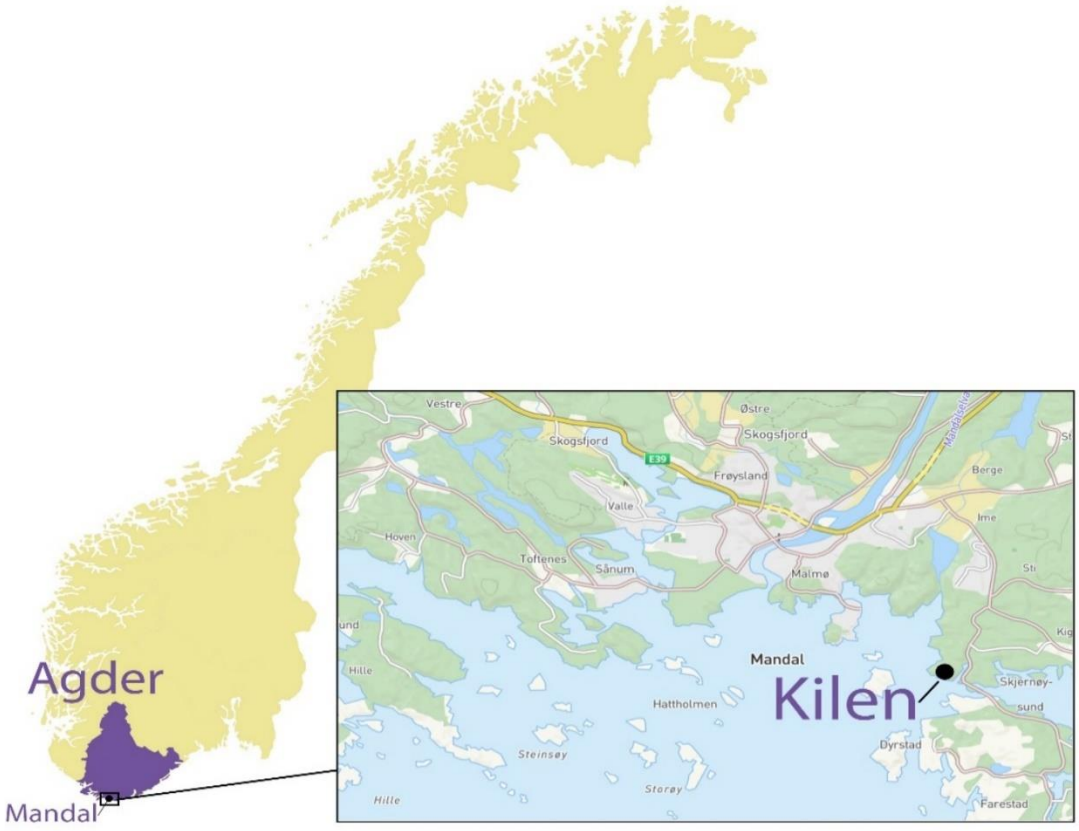

Fig 9. Map showing the study site for ticks' collection. Ticks for this study were collected in 2021 at the study site in Kilen (black pin) in Lindesnes municipality, located approximately 3.8 km from Mandal center, in the western part of Agder County (Map from © Kartverket and Norkart (Heidi Lindstedt, The Norwegian Directorate of Health).

2.2 Collection of ticks

The sampling was performed by dragging a white flannel cloth (1.05 × 1.15 m), with a lead weight in one end along the ground. Ticks were removed gently with tweezers (Fig 10A). Nymphs were pooled in groups of 10 in each tube. The tubes with ticks were stored and transported back to the laboratory in a cooling bag with crushed ice and placed in a -80°C freezers until further analysis. The climatic condition at the sampling site were logged using Tinytag PLUS TGP-4500 data logger, which were located at the tick-sampling site in Kilen, (Mandal) in Agder County, covered by the ACS-5050 Solar Radiation Shield to protect the data logger from direct sunlight and precipitation (Fig 10B). The shields were attached to an iodine spear of metal which was screwed into the ground. Temperature and relative humidity were measured every hour, approximately 30 cm above the ground.

Fig 10. Images showing ticks collection procedure. (A) Collection of ticks from the Flagg by tweezer. (B)*Microclimatic loggers on right hand at the local sampling site registering temperature and humidity.*

2.3 Study population

A total of 5180 nymphs (518 pools) were collected during 7 months in 2021 (Table 1).

Table 1. Table showing collection Month, date of collection, and No. of I. ricinus *nymphs collected for the study. In total 5180 nymphs were collected monthly in snow free season.*

Month	Date of Collection	Number of nymphs
April	**29.04.2021**	**740**
May	**27.05.2021**	**740**
June	**27.06.2021**	**740**
July	**19.07.2021**	**740**
August	**20.08.2021**	**740**
September	**19.09.2021**	**740**
October	**15.10.2021**	**740**

2.4 Experimental Design

The experimental work was divided into two parts:

1. Screening for the presence of TBEV by extraction, revers transcription, RT-Real-time PCR, verification by pyrosequencing and estimation of TBEV prevalence (Fig 11).

2. Development of a whole genome sequencing (WGS) method of TBEV by overlapping primers as described by Quick et al., (2017). cDNA reverse transcribed by SSIV from confirmed positive samples was analyzed by overlapping primers (DMUK1=400bp, KUPA1=400bp) to obtain the whole genome of the virus (Fig 11).

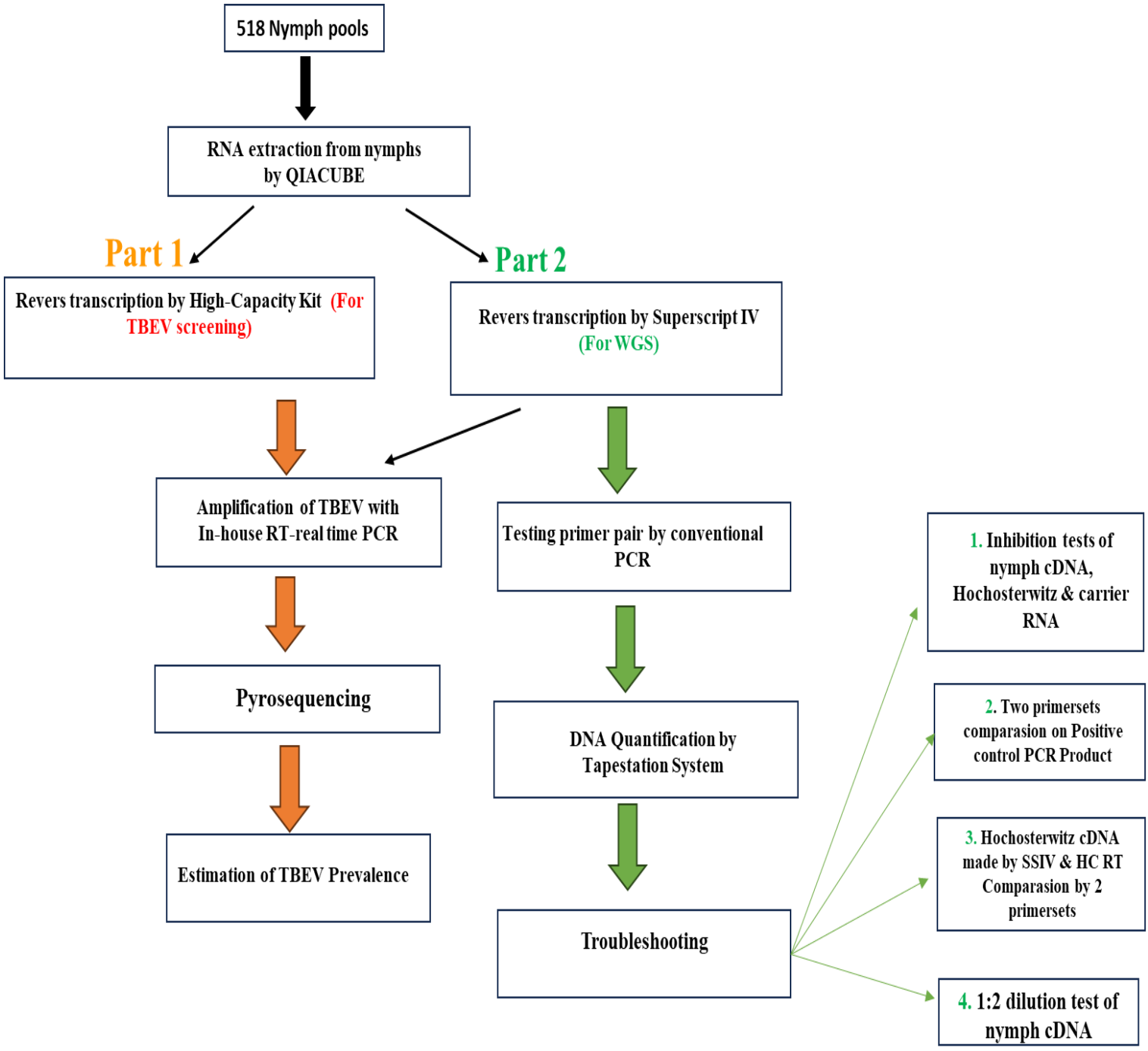

Fig 11. Experimental design demonstrating the steps, scheme, and workflow of this study to perform screening of TBEV from nymph pools and estimate TBEV prevalence and develop an amplification method for whole genome sequencing of low viral load samples of TBEV.

2.4.1 Screening and estimation of TBEV prevalence

2.4.1.1 Homogenization of tick samples

A total of 5180 nymphs in 518 pools were homogenized and analyzed for TBEV .A pool of 10 nymphs and 500 µl MEM were homogenized by MP FastPrep®-24 bead beating grinder and lysis system (MP biomedicals, Santa Ana, California USA CAT NO. SKU:116004500) at Program: CY-24x1, for 60 seconds with six stainless steel beads, FastPrep-24™ Lysing matrix S (MP products, city state) (size 3.175 mm, cat no. SKU:116925050-CFAfter Homogenization samples were centrifuged at 14,000 RPM for 5 minutes to remove cell debris and tissue remains. The supernatants were collected and divided into two archive tubes each containing 220 µl homogenate. One tube was for cultivation of virus and was added 22 µl (10%) of Fetal Bovine serum (FBS) for long time storage and stored at -80°C. The second tube were added 220 µl RLT buffer and used for lysis and immediate RNA extraction **(Appendix 1A**).

2.4.1.2 RNA extraction from Ticks by QIACUBE

Total RNA (TRNA) was extracted from the second tube containing 220 µl homogenate and 220 µl RLT lysis buffer (with 1% β-mercaptoethanol, Qiagen, CA, USA). RNA Extraction was performed in a QIAcube extractor (Qiagen, Inc., Valencia, CA, USA) by RNeasy RNA mini kit according to the manufacturer`s protocol (Qiagen, Inc., Valencia, CA, USA). The purified RNA was eluted twice in 2x 30 µl of Tris-elution buffer (1mM, pH 8.4). A total of 60 µl of TRNA sample was acquired (**Appendix 1A)**

2.4.1.3 Reverse Transcription

2.4.1.3 A. High-Capacity cDNA revers transcription for TBEV screening

The extracted RNA was immediately reverse transcribed into cDNA by the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Forster City, CA, USA) according to manufacturer`s protocol **(Appendix 2A).** A 15 µl of master mix (10X RT random primers, 10X RT buffer, 25X dNTP mix (100 mM), multiscribeTM reverse transcriptase (200 U/µl), RNase inhibitor (20 U/µl), and nuclease-free water) **(Appendix 2B**) was mixed with 5 µl extracted TRNA. The reverse transcriptase reaction was performed on a 2720 Thermal cycler (Applied biosystems, Thermo Fischer Scientific Corporation, Foster city, California, USA) with temperature setup as; 25°C for 10 mins, 37°C for 120 mins, 85°C for 5 mins and 4°C for infinity **(Appendix 2C**).

TBEV Hochosterwitz RNA (10^{-1} to 10^{-8}) isolated from a TBEV Hochosterwitz strain were used as a positive control, and water was used as a negative control in the PCR reaction.

2.4.1.3 B. Superscript IV revers transcription for WGS amplicon analysis

Superscript ™ IV revers transcription kit (SSIV) (Thermofisher Scientific, City, State) was used to reverse transcribed the extracted TRNA into approximately 15 kb long cDNA according to the manufacturer protocol (**Appendix 2A**). Two master mixes, yellow MM1 and green MM2 were prepared. Yellow MM1 (final volume of 7 µl) was prepared by mixture of 6X random primers, dNTP (10 mM) and nuclease-free water, to which 5 µl of extracted TRNA was added and heated at 65°C for 5 mins followed by ice cooling for 1 min (**Appendix 2D).** Green MM2 (final volume of 8 µl) was prepared by mixing superscript IV RT (200 U/µl) enzyme, 5x SSIV buffer, Dithiothreitol (DTT, 0.1M) and RNAse inhibitor (40 U/µl), (**Appendix 2D**). MM2 which was then added to MM1. The reverse transcription was performed on the the final volume of 20 µl reaction (15 µl MM+5 µl RNA) using the following thermal condition: 23°C for 10 mins, 55°C for 10 mins, 80°C for 10 mins in an Eppendorf gradient thermal cycler number 11381 (Eppendorf, Hamburg, Germany) (**Appendix 2E**). The obtained cDNA was preserved at 4°C for Pyrosequencing.

TBEV Hochosterwitz RNA (10^{-1} to 10^{-8}) isolated from a TBEV Hochosterwitz strain were used as a positive control, and water was used as a negative control in the PCR reaction.

2.4.1.4 Amplification of TBEV with in-house RT-real time PCR

In-house RT- Real-time PCR was used to amplify TBEV specific cDNA according to the protocol used by Torstein Tengs at the Norwegian Veterinarian Institute and modified at NIPH (Andreassen *et al.*, 2012) **(Appendix 3A)**. The Mastermix was prepared as a mixture of 50 mM MgCl2, 10X AB buffer, dNTP (25mM), 320F forward primer (25 uM), 373R reverse primer labelled with biotin (25 µM), 339 probe (25 µM), platinum taq polymerase (20U/ µl), and RNase free water with a total volume of 22 µl, to which 3 µl of cDNA were added (**Appendix 3B).** A 54 basepair (bp) fragment of the TBEV envelope gene were amplified with TaqMan primers (TBEV-320F and TBEV-373 Biotin and probe (TBEV-339) accustomed for the Norwegian strain of EU-TBEV according to Andreassen et al., (2012) **(Table 2)**. For each run all nymphal cDNA samples were run along with a 10-fold serial dilution of TBEV Hochosterwitz strain cDNA as positive controls and nuclease free water as negative control on a Rotor-gene Q instrument (Qiagen, Hilden, Germany). The TaqMan amplification condition were 95°C for 120 seconds, 48 cycles at 95°C for 15 secs, 60°C for 45 secs and 72°C for 30 secs, and at 4°C for infinity **(Appendix 3D)**. The PCR results were analyzed by the Rotor-gene 29 Q Series software version 2.3.1, and positive PCR products were marked and stored at -20°C for further verification by pyrosequencing.

Table 2: Primers and probes used for in-house real-time PCR for TBEV detection (Andreassen et al., 2012).

Primer	Sequence (5'-3')	GenBank accession no.
TBE 320F	5' - GGGAGCGCAAAACTGGAA-3'	U27495.1
TBE 373R Biotin	Biotin-5' –TGAGGAGCCCCAAATTCAAC-3'	U27495.1
TBE 339 probe	FAM-5'-AACGCAGAAAGAC-3'-MGB	U27495.1

2.4.1.5 Pyrosequencing for confirmation of TBEV Positive nymph pools

The positive samples from In-house Real-Time RT-PCR were verified by pyrosequencing according to manufacturer Sequence analysis (SQA) protocol (Andreassen, *et al.,* 2012) on a PyroMark Q48 system (Qiagen, Hilden, Germany) and PyroMark Q48 advanced reagents (Qiagen, Germany)(Andreassen *et al.,* 2012) **(Appendix 4)**. TBEV 320F **(Table 2)** was used as sequencing primer. Evaluation of results was preformed visually by comparing patterns from previously sequenced positive PCR samples revealed on the pyrogram and compared to a standard positive control (TBEV-Hochosterwitz). Samples with 70% sequence similarity with the positive control were regarded positive. Samples that were proven true positives were used later to develop method for the whole genome sequencing of TBEV.

2.4.1.6 Estimation of TBEV Prevalence

Estimated pool prevalence (EPP) was calculated for each true TBEV positive tick pool from Mandal. The pooled prevalence was calculated by epitool epidemiological calculator with fixed pool size of 10 and perfect tests resulted in the EPP for each pooled samples using website https://epitools.ausvet.com.au/pooledprevalence. This method estimate depends on the theory that there is only a single infected individual in each positive pool when it is collected in an area where you expect the prevalence to be low. The EPP gives an estimate pooled prevalence of the uncertainty within the confidence interval (95%) associated with the prevalence estimations (Galfsky *et al.,* 2019).

2.4.2 Development of amplification and WGS Method for TBEV

2.4.2.1 Testing primer pair by conventional PCR

Superscript IV RT enzyme was used for cDNA synthesis as explained in section 2.4.1.3B. Eight positive TBEV nymph pools from sampling site S5 in 2021, N56, N220, N235, N236, N237, N240, N241, N309 with lowest possible Ct Values (expected higher viral load) **(Table 3)** that were verified positive by pyrosequencing were selected for further analysis and amplification of TBEV whole genome sequencing. Primer pairs giving rise to a PCR product of 400 bp named as DMUK1 (Designed by Urusha Maharjan) with concentration of 0.015 µM were used for amplification **(See Appendix 9 for DMUK1 sequences)**. DMUK1 were divided into two pools: Pool 1 comprised of DMUK 400bp odd primer pairs and Pool 2 comprised of DMUK 400bp even primer pairs. Each primer pool of 4.42 µl were mixed with 15.58 µl Masteremix for Q5® High Fidelity PCR kit (5X Q5 Reaction Buffer, 10 mM dNTPs, Q5 Hot Start High-Fidelity DNA Polymerase, 5X Q5 High GC Enhancer) (New England BioLabs® Inc., Cat. No. E0555L), 5 µl of selected positive TBEV nymph pool cDNA with final volume of 25 µl for PCR reaction. Positive control (10^{-3} dilution Hochosterwitz) and negative control (Water) were run along in conventional PCR **(Appendix 5A)**.

The PCR reactions were generated in a thermal cycler: 98°C for 30 secs followed by 40 cycles of 98°C for 15 secs and 65°C for 5 mins combined annealing and extension (**Appendix 5B)**. Reaction from both primer pools were mixed (final volume 50 µl) after PCR amplification.

Table 3. Table presenting TBEV nymph pools with Ct value selected for amplification and method.

Sample name	Collection Month	CT value
S5(21)-N56	April	34
S5 (21)- N220	July	31
S5(21)-N235	July	29
S5(21)-N236	July	33
S5(21)-N237	July	31
S5(21)-N240	July	32
S5(21)-N241	July	33
S5 (21)- N309	August	34

The cleanup of PCR products (40 µl) was carried out using the Agencourt AMPure XP beads (Beckman Coulter Life Sciences, Lakeview Parkway S Drive, Indianapolis, IN 46268, USA) according to the manufacturer's instructions. PCR products of 400 bp were purified by 1.8X volume of the PCR reaction. For further purification, 200 µl of 80% ethanol was utilized for washing. The 40 µl of DNA was then eluted using elution buffer (10 mM Tris-HCl, PH 8.0–8.5) and was transferred to new tube for further analysis (**Appendix 6)**.

2.4.2.2 DNA quantification of purified and unpurified PCR products using tapestation.

The Purified PCR products amplified by conventional PCR were quantified by tapestation analysis according to manufacturer protocol. Similarly, to check if the templates were successfully amplified, a quantification was performed by a virtual electrophoresis gel in Agilent 4200 Tapestation system (Agilent Technologies, Waldbronn, Germany) showing the measured peak heights of each PCR fragment as visual bands. The concentration and quality of dsDNA fragments was evaluated for whole genome sequencing **(Appendix 7)**.

To check if PCR amplified DNA was lost during the purification step, the concentration of unpurified PCR products was also quantified by tapestation to investigate the difference in concentration between and purified and unpurified PCR products.

2.4.2.3 Troubleshooting

A standard protocol as in section 2.4.2.1 was developed for further troubleshooting to find if there are inhibitory substances that may affect the activity of DNA amplification. RT-PCR products of cDNA synthesized by Superscript IV reverse transcription kit (section 2.4.1.3B) of nymph pools and of 10-fold serial dilutions 10^{-1} to 10^{-12} of Hochosterwitz TBEV strain were selected for PCR amplification. A 4.42 µl of overlapping DMUK1 primer pair of each pool (0.015 µM) were mixed with 15.58 µl Mastermix for Q5® High Fidelity PCR kit kit (5X Q5 Reaction Buffer, 10 mM dNTPs, Q5 Hot Start High-Fidelity DNA Polymerase, 5X Q5 High GC Enhancer) (New England BioLabs® Inc., Cat. No. E0555L) and 5 µl of respective cDNA with final volume of 25µl for each PCR reaction. The PCR reactions were generated in a thermal cycler: 98°C for 30 secs followed

by 40 cycles of 98°C for 15 secs and 65°C for 5 mins combined annealing and extension (**Appendix 5A)**. Reaction from both primer pools were mixed (final volume 50 µl) after PCR amplification.

Derived PCR Products were assessed with virtual electrophoresis gel in Agilent 4200 Tapestation system (Agilent Technologies, Waldbronn, Germany) according to manufacturer protocols **(Appendix 7).**

2.4.2.3A Inhibition test of Spike-in cDNA from PCR positive control with nymph pool cDNA

An inhibition test was performed to study if there are some inhibiting substances effecting the amplification of nymph pools from Mandal. A 2.5 µl of 10^{-8} dilution of PCR positive control cDNA (Spike-in), 2.5µl of nymph cDNA were mixed with Mastermix in final volume of 25µl for the PCR reaction **(Appendix 8).** A reaction with and without cDNA from the nymph pools, S5-237N, S5-240N, S5-241N were run and the experiment was performed according to standard method described in section 2.4.2.3

2.4.2.3B Inhibition test of Spike-in cDNA from PCR positive control with carrier RNA, comparing two revers transcription kit and two overlapping primersets.

To evaluate if the addition of carrier RNA into the cDNA of Hochosterwitz samples would provide different results compared to the previous samples spike-in with Mandal cDNA samples (2.4.2.3A) another spike-in test was performed with two overlapping primer sets; DMUK1 (0.015 µM) and KUPA1(0.015 µM) **(See Appendix 9 for DMUK1 and KUPA1 sequences)**. A 2.5 µl of PCR positive control cDNA, 2.5 µl of carrier RNA (310µg) were added to Mastermix with final volume of 25 µl reaction used for amplification.

This experiment was performed according to standard method described in section 2.4.2.3 with the following changes:

a) The cDNA of the Hochosterwitz sample generated by two different revers transcription kits (High-capacity and SSIV) were used to see whether both kits can produce amplicons.

b) Two overlapping primer sets DMUK1(0.015 µM) and KUPA1(0.015 µM) were used for PCR amplification of Spike-in Hochosterwitz PCR products

2.4.2.3C Test of overlapping primer pair (KUPA1) on nymph TBEV cDNA

Another short overlapping primer set KUPA1 400 bp in two pools; Pool 1(KUPA 400bp odd primers) and Pool 2 (KUPA 400bp even primers) that was designed for Larvik Tick samples were tested on nymph pool cDNA from Mandal. cDNA made by high-capacity reverse transcription kit were used, due to lack of SSIV cDNA samples to generate PCR products from TBEV Nymph pools. TBEV Nymph pools (N56, N220, N235, N237, N240, N241, N309), positive control (10^{-7} dilution Hochosterwitz) and negative control (Water) were selected for PCR reaction to generate amplicons. Experiment was performed according to standard method described in section 2.4.2.3.

2.4.2.3D Amplification of diluted cDNA samples from nymph pools

To study the effect of dilution on amplification in nymph cDNA samples (synthesized from Superscript IV reverse Transcription) of 3 nymph Pools 220N, 235N and 309N. Nymph pool cDNA of 1.25 µl was diluted in 1.25µl RNAse free water with the ratio of 1:2 (Final volume 2.5 µl) and were used for PCR amplification with overlapping primer pair DMUK1 (0.015 µM). Samples in the experiment were tested according to standard method described in section 2.4.2.3

3. Results

3.1 Screening and estimated prevalence of TBEV in nymphs from Mandal, Norway

In this study 5180 nymphs (518 pools) were analyzed. The TBEV screening was performed by in-house RT real-time PCR, confirmed by pyrosequencing, and were compared to the sequences of positive controls. A total 42 of 518 tick pool samples of nymphs were detected to be TBEV positive out of which 37 were confirmed positive by pyrosequencing **(Appendix 10)**. The overall prevalence of TBEV was 0.7%, with a monthly variation between 0.3-1.9%. The prevalence showed two peaks, one in July and a second peak in August **(Table 4)**.

To illustrate the real time PCR results, we are showing examples of a Rotor gene report (Fig 12) and a pyrogram of a TBEV sample 231N, positive control, and negative control (Fig 13). The green amplification curves (Fig 12) represent the 10-fold serial dilution of TBEV Hochosterwitz positive control from 10^{-3} to 10^{-5} and pink curves represent amplification curve for Tick samples from Mandal. The Ct values for the TBEV positive pools are ranging between 30 to 35 at a threshold level of 0.02 (Fig 12).

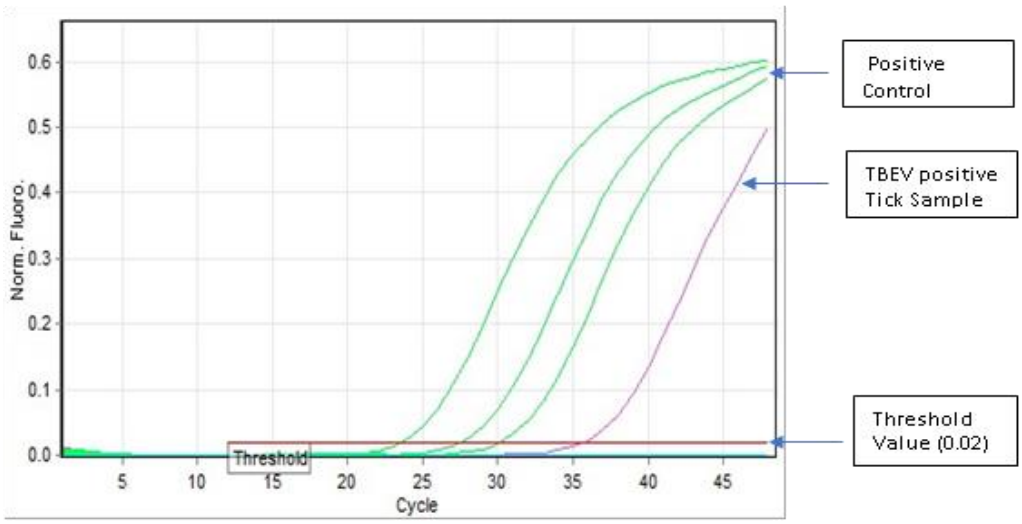

Fig 12. The PCR amplification curve was generated using Rotor gene Q-Software for TBEV positive tick samples. The report was generated by modifying the curves with a threshold value of 0.02, linear scales and slope correction. TBEV positive from mandal as shown as pink curve while TBEV positive control Hochosterwitz from 10^{-3} to 10^{-5} represented by green amplification curve.

The pyrogram shows an example of a successfully amplified TBEV positive tick samples 231-N that was confirmed positive by pyrosequencing compared to a positive Control (Hochosterwitz) and a Negative water Control (Fig 13).

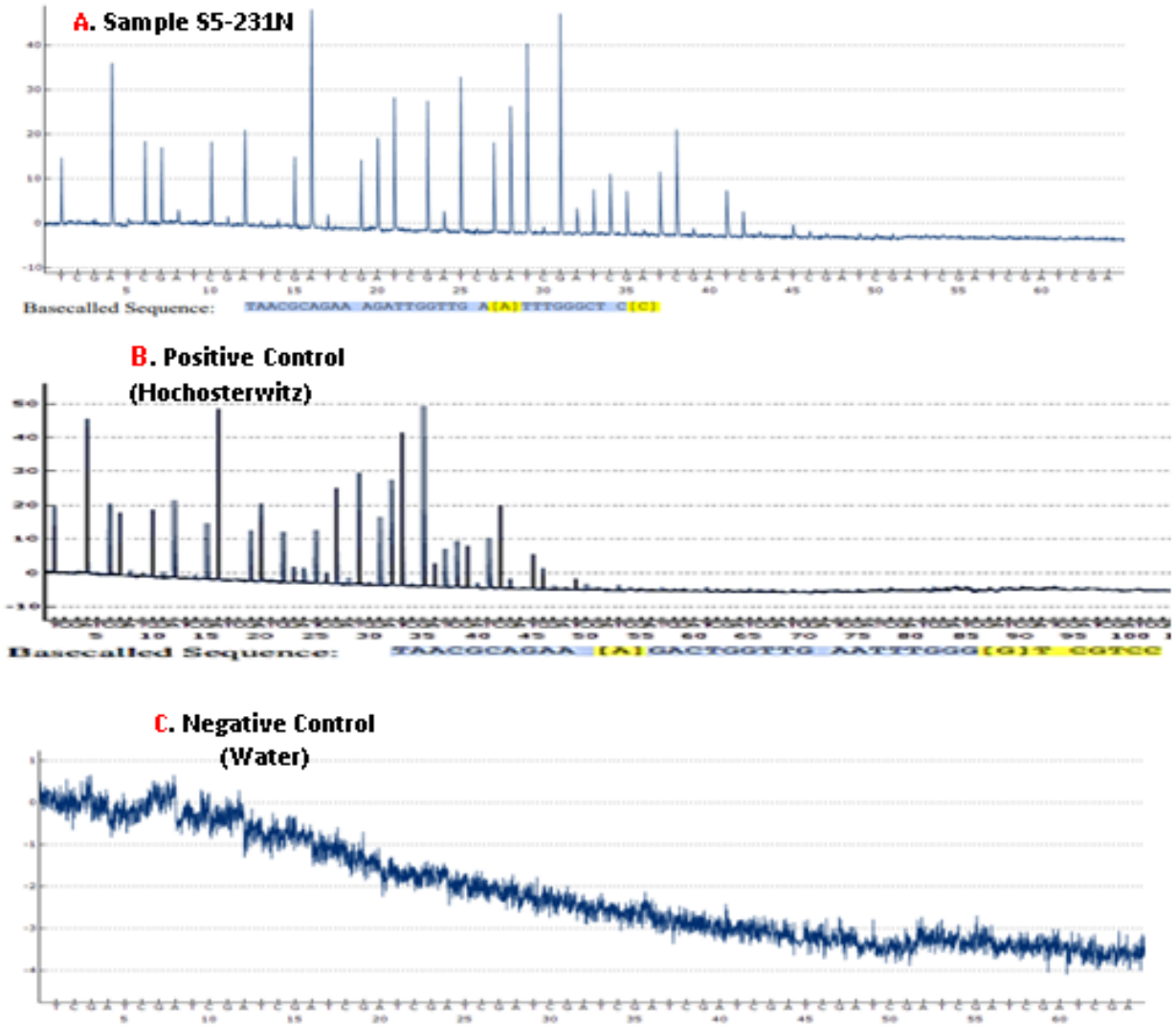

Fig 13. Pyrogram showing S5(21)231-N, one of the true TBEV positive tick samples. The row in the pyrogram indicates the cycles of the TCGA nucleotide pattern, whereas the column in the pyrogram represents the fluorescence of the nucleotide added. A) The sequence pattern of the TBEV positive sample, B) The sequence pattern for the TBEV positive control (Hochosterwitz). C) Nuclease-free water used in pyrosequencing as a negative control.

As shown in Table 4 the highest number (13 positive) of TBEV detected positive was from the month of July and a lowest from the month of May and September with only 1 positive each. The lowest Ct value of 29 was detected in nymph samples from the month of July while the other month had highest Ct range between 35 and 39. In Total, the acquired Ct values for the TBEV positive samples were all near to the highest detectable Ct value of the positive control (Hochosterwitz 10^{-10} dilution).

All the 13 positives from the month of July were confirmed true positive by pyrosequencing. Only 5 of total 42 was not confirmed after pyrosequencing while the remaining 37 had their true positivity confirmed by pyrosequencing.

The estimated pool prevalence (EPP) of the nymph pool showed an overall pooled TBEV prevalence of 0.7% (37 positive pools; 518 tested pools). The highest prevalence EPP was obtained from nymph pools collected in the month of July 1.9% (13 positive pools; 74 tested pools) followed by Nymph pools from August with 0.9% (7 positive pools; 74 tested pools) and October with 0.8% (6 positive pool; 74 tested pools) respectively.

Table 4. Detection of total number of TBEV positive tick samples from each month (Mandal,2021) by in-house RT real-time PCR, and confirmed positive by pyrosequencing with an overall and monthly estimated prevalence.

Collection month	No. of samples analysed	No. of PCR positive (Ct value range)	No. of confirmed (pyrosequencing)	Estimated prevalence
April	74	5 (34 – 39)	5	0.6%
May	74	1 (36)	1	0.3%
June	74	5 (35-36)	4	0.5%
July	74	13 (29– 37)	13	1.9%
August	74	10 (34-39)	7	0.9%
September	74	2 (35 – 37)	1	0.3%
October	74	6 (34 – 37)	6	0.8%
TOTAL	**518**	**42 (29-39)**	**37**	**0.7%(Overall)**

3.2 Development of amplification and WGS method for TBEV

After verification of positive nymph pools by pyrosequencing from Mandal samples, the aim was to establish a method for whole genome sequencing of TBEV strain with low viral load from Mandal. Several tests, including testing of primer for amplification (DMUK1 and KUPA1), quantification of PCR products and inhibition tests were performed prior to viral genome sequencing of TBEV by WGS.

3.2.1 Quantification and quality test of purified PCR products (DMUK1)

The Purified PCR amplicons were visualized by tape station analysis and comparison of virtual bands generated for DNA ladder by tape station was performed (Fig 14). A strong 400bp band was observed for positive control (10^{-3} dilution Hochosterwitz) indicates that primer pairs DMUK1 worked well to generate amplicons and no band for negative control (Water) was also proof for success of DMUK1.There were no 400bp fragments visualized by Tapestation for purified PCR products of the TBEV nymph pools. The absence of 400bp bands indicated that the viral load was too low to be amplified by DMUK1 Primer pairs. While weak bands for shorter fragments than 400bp for TBEV Nymph pools were also observed (Fig 14).

The concentration of dsDNA in the amplicons was quantified by tapestation which ranged between 0.95-6.39 ng/µl (Fig.14).

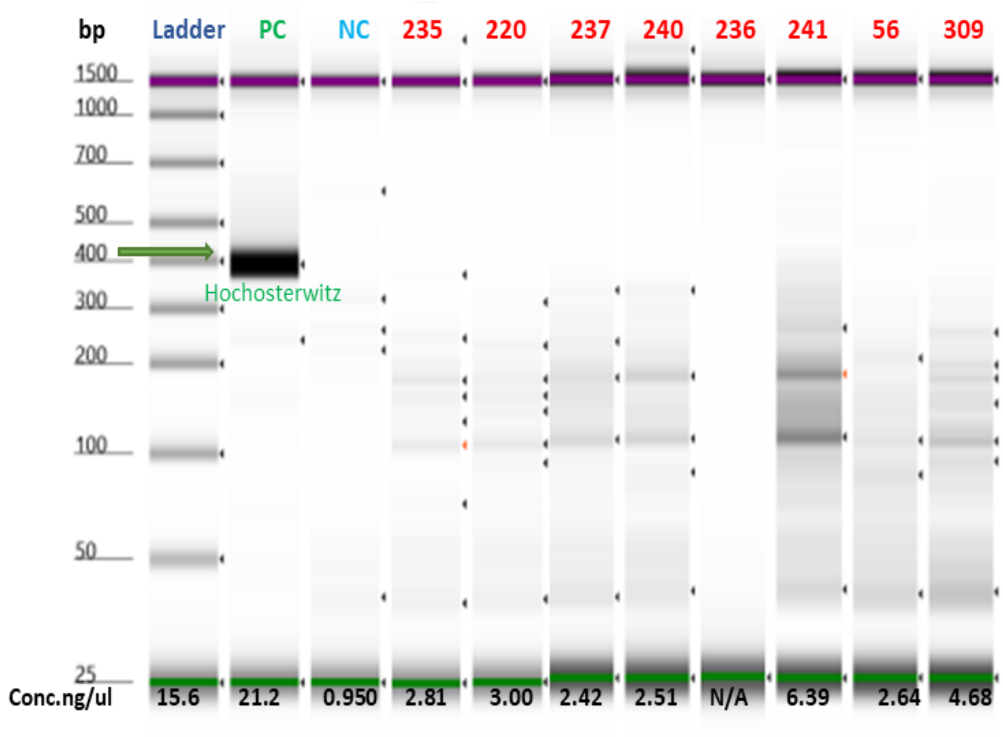

Fig 14. The Tape station (D1000) gave a result displaying the size of the Purified PCR amplicons and the concentration of double-stranded DNA (dsDNA) in the amplicons, measured in ng/µl. The lower marker is shown by a green line at the bottom, while the upper marker is shown by a purple line at the top. The ladder size is 1500bp. PC refers to the positive control marked by green arrow (Hochosterwitz), NC refers to the negative control (Water), and the numbers 235, 220, 237, 240, 236, 241, 56, and 309 represent the selected TBEV Nymphs pools from site S5(21).

3.2.2 Quantification and quality test of unpurified PCR products

Based on the result from section 3.2.1, further tests were performed to compare the concentration between unpurified PCR products. Further quantification of unpurified PCR products of these sample was conducted to study if there were a loss of PCR product due to the purification process of the 400 bp fragment. No 400bp fragments were detected by the Tapestation analysis of the unpurified PCR products. However, the presence of faint bands (with a size smaller than 400bp) of amplified PCR products appeared that indicated that the viral load in the samples is either too low to be detected or is being inhibited.

The concentration comparison revealed a difference between the purified and unpurified PCR products (Fig 14 & Fig 15). The concentration of dsDNA in the amplicons for unpurified PCR products ranged between 46.9-112 ng/µl while those for purified PCR product was 0.95-6.39 ng/µl.

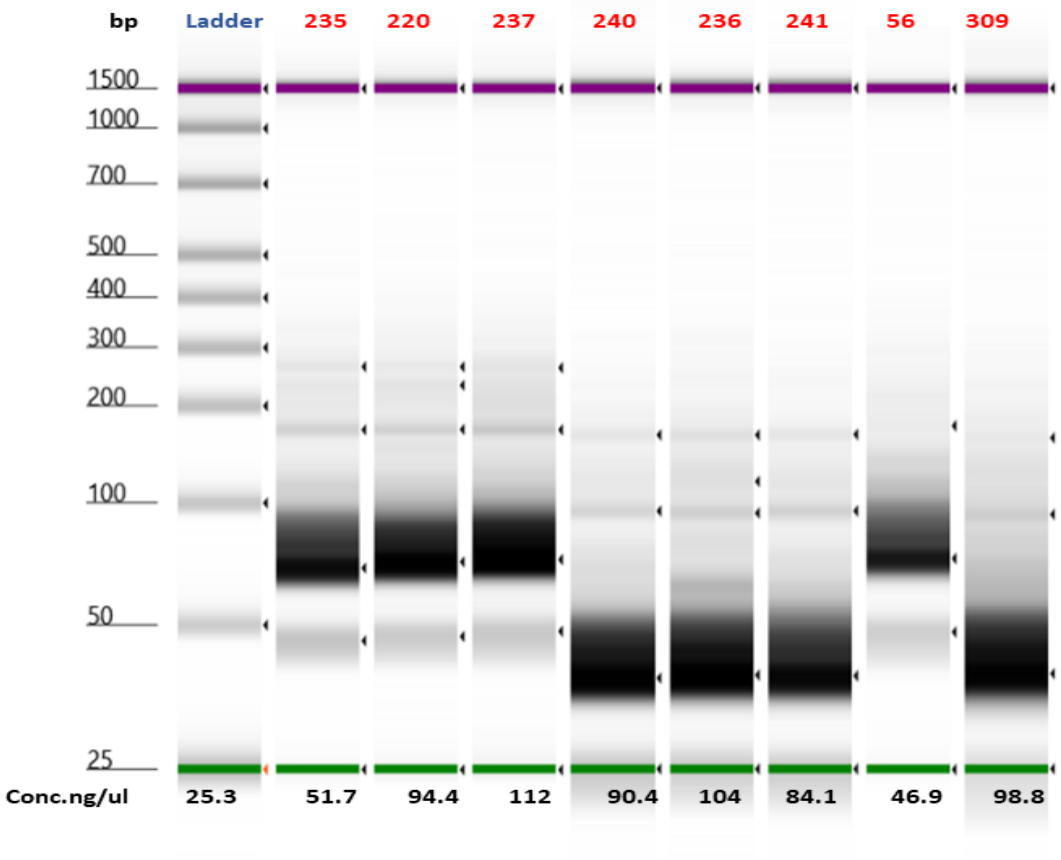

Fig 15. The Tape station result showing the size of the PCR amplicons and the concentration of dsDNA in the amplicons for unpurified PCR products. The presence of intensive bands indicates the occurrence of primer-dimers or non-specific binds in the samples prior to purification. The green line represents the lower marker, while the purple line represents the upper marker.

3.3 Troubleshooting

3.3.1 Inhibition test of Spike-in of Hochosterwitz with TBEV cDNA from nymphs

Considering that all the positive samples analyzed by real-time RT-PCR in the present study had high Ct-values (Table 3), it may indicate that the viral load in these samples may be too low. To assess if there are additional inhibitory effect of the PCR reaction, an experiment was conducted by including a study of spike-in TBEV Hochosterwitz strain. An amplified PCR products of TBEV Hochosterwitz strain serial dilution (10^{-1} to 10^{-12}) were quantified by tapestation. A band of 400bp with different intensities were observed for all serial dilutions of TBEV Hochosterwitz strain (Fig 16).

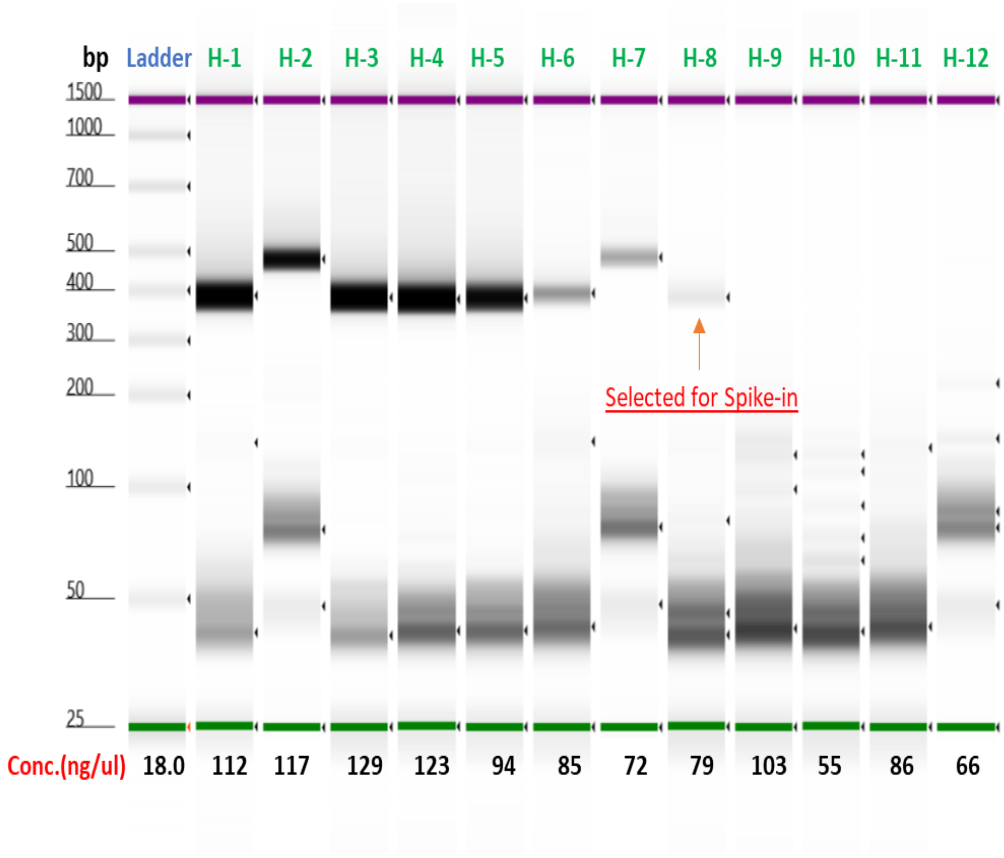

Fig 16. The Tape station visualizing a result for the quality test of Hochosterwitz dilutions.H-1 to H-12 denotes the Hochosterwitz dilutions, ranging from 10^{-1} to 10^{-12}. The red arrow is used to indicate the faint band sample (H-8) that was chosen for spike-in. The numbers shown at the bottom indicate the concentration (ng/ul) of double-stranded DNA (dsDNA) in the amplicons. The samples H-2, H-7, H-12 are shifted upward as compared to the ladder.

The positive control sample Hochosterwitz 10^{-8} dilution, shown as a faint band (Fig 16), was chosen for Spike-in with cDNA of TBEV Nymph pools (N237, N240, N241) to conduct an inhibition test. The selected samples were added with and without 10^{-8} dilution of TBEV Hochosterwitz strain cDNA from the for comparison. This allowed for the observation of inhibition of the positive control sample if there were any differences between the spiked-in samples and the samples without any added substance. Water was included as a negative control in the PCR reaction.

After comparing both results (Fig 16 & Fig 17), it was observed that there were markedly more prominent bands detected for Hochosterwitz 10^{-8} after it had been mixed (spike-in) with TBEV nymph pools. This indicates that there was no inhibition in the selected TBEV nymph pools. Upon observing the image, the samples without Hochosterwitz 10^{-8} exhibited weakened bands, indicating that the viral load in the original sample is too low to be detected by the amplification process without spike-in.

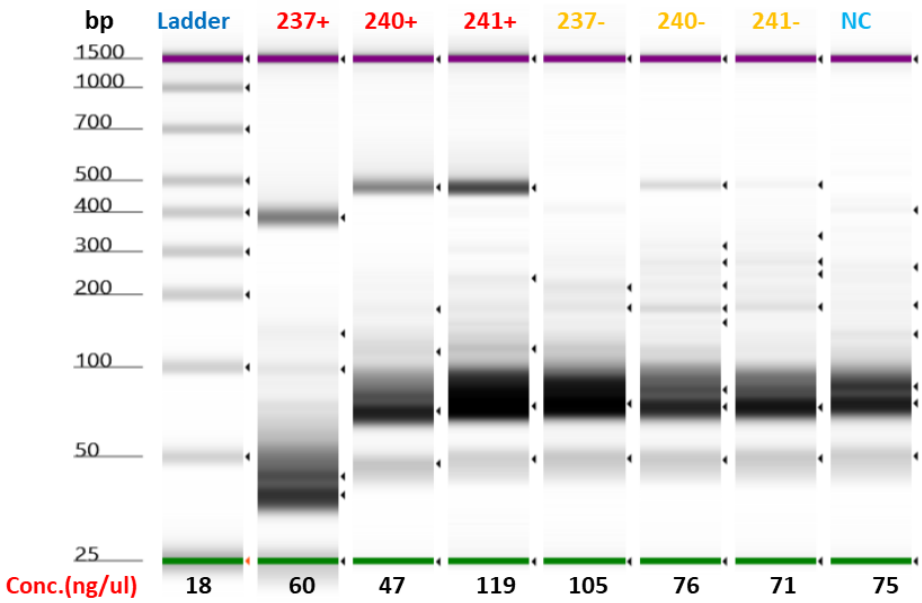

Fig 17. Result achieved from the Tape station, corresponding to a quality test performed on the Spike-in of Hochosterwitz with Mandal sample. The samples labeled as 237+, 240+, and 241+ were spiked with Hochosterwitz, while the samples labeled as 237-, 240-, and 241- were without any additional cDNA. The NC sample serves as a negative control for the PCR process. The green line represents the lower marker, while the purple line represents the upper marker. All samples except 237+ in lane 2 are shifted upward compared to ladder.

3.3.2 Inhibition test of spike-in of Hochosterwitz with carrier RNA, comparison of two reverse transcription kits and two primer sets

To evaluate if addition of spike-in Hochosterwitz strain with carrier RNA would provide same or improved PCR products as compared to spike-in of Hochosterwitz strain with Mandal cDNA samples (Fig 17) another test was conducted by using two primer sets, DMUK1 and KUPA1. The effect of the two different reverse transcription kits (High-Capacity and SSIV) were tested on the spike-in Hochosterwitz samples with carrier RNA.

Significantly more noticeable bands were seen in the Hochosterwitz dilutions when they were mixed (spike-in) with Carrier RNA indicated that spike-in may improve PCR products (Fig 18). The two different primer sets exhibited comparable and strong 400bp bands. DMUK1 seemed to be independent of the reverse transcription kits while the efficiency of KUPA1 was lower for SSIV bands compared to High-Capacity (Fig 18).

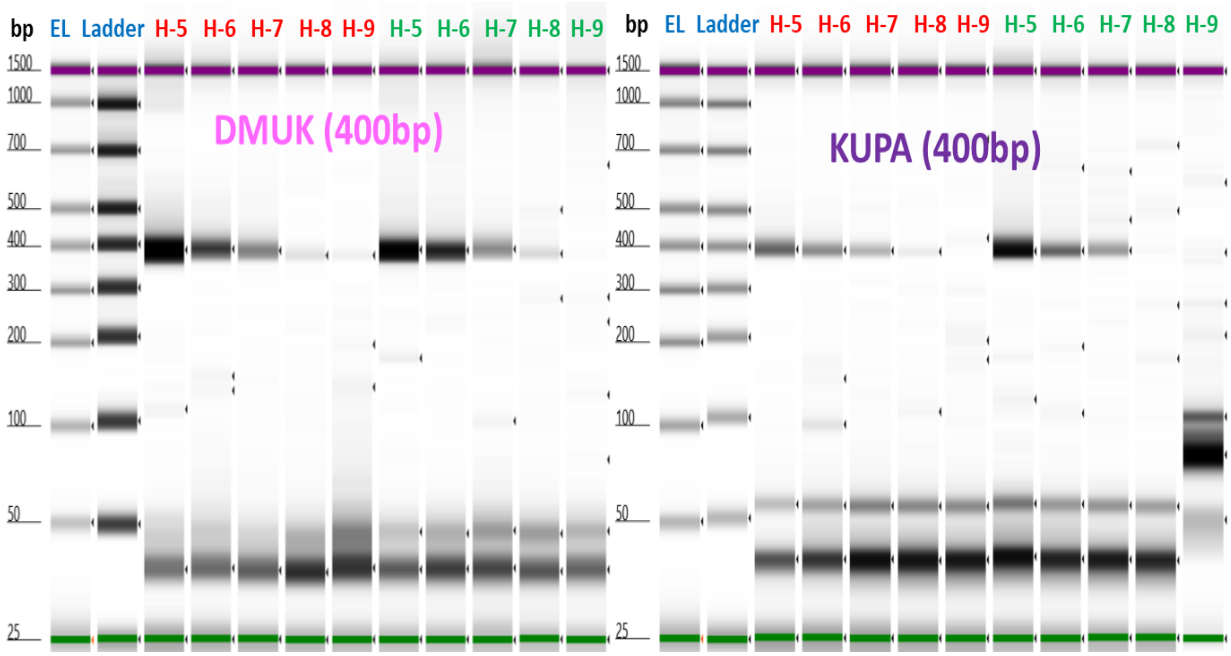

Fig 18. Image demonstrating the result of tapestation (Spike-in of Hochosterwitz with carrier RNA). Two primer pairs (DMUK1 on the right hand, KUPA1 on the left) are used. EL stands for electronic ladder; labelling (H-5, H-6, H-7, H-8, H-9) in red indicates Hochosterwitz DNA fragments made by the SSIV Kit; and green indicates Hochosterwitz DNA fragments produced by the High-capacity Kit. Sample H-9(green) on right hand figure in last lane is shifted upward compared to ladder.

3.3.3 Testing overlapping primer pair (KUPA1) on high-capacity PCR products (nymph cDNA)

By testing another short overlapping primer set KUPA1 on nymph cDNA, a strong 400bp band was observed for positive control (10^{-7} dilution Hochosterwitz) and no band for negative control (Water) was detected indicating that primers pairs (KUPA1) worked well to generate amplicons (Fig 19). No 400 bp fragments were detected for TBEV samples. Hence it was confirmed that both primer sets (DMUK1and KUPA1) performed well without any issue for the positive control (Fig 19).

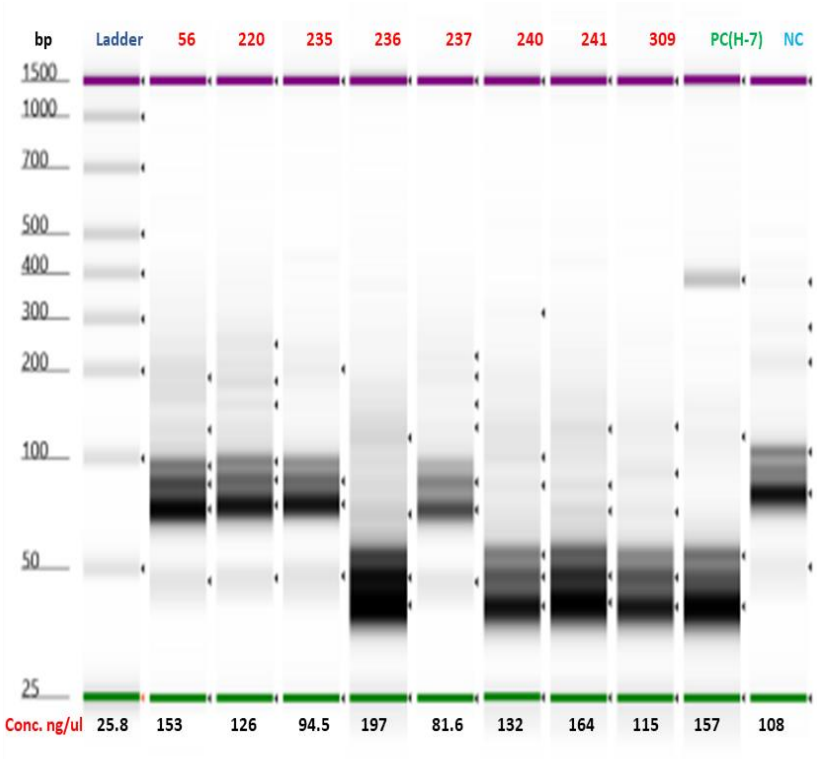

Fig 19. The Tape station visualizing a result from the quality test of High-capacity PCR products (since SSIV generated PCR products were finished) using KUPA 400bp Primer pairs. The result includes information on the size of the PCR amplicons and the concentration of dsDNA in the amplicons. Green line; lower Marker, purple line; upper marker. Samples 56,220,235,237 and NC are shifted upward compared to ladder.

3.3.4 Quantification and quality test of 1:2 dilution of nymph cDNA samples

To test if the PCR products were inhibited and would increase the yield of amplicons after dilution of the cDNA at a ratio of 1:2. The PCR products of TBEV Nymph pools (N220, N235, N309) amplified by DMUK1 primers were quantified by tapestation. An amplified PCR products of TBEV displayed a faint 400 bp bands only in sample 220 (Fig 20). And no difference was noticed for the other two samples from the tape station results.

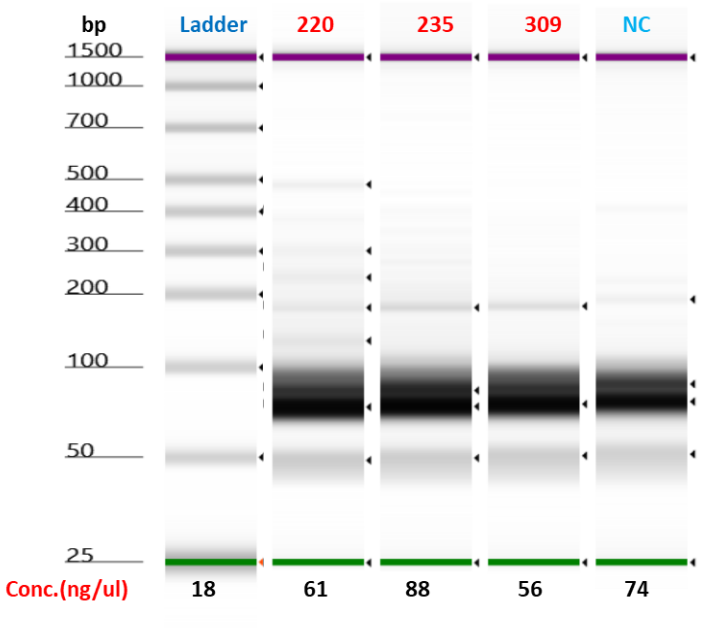

Fig 20. Image showing result obtained from a quality test conducted on diluted PCR products using DMUK 400bp Primer pairs. The test provides information on the size of the PCR amplicons and the concentration of double-stranded DNA in the amplicons. All the samples are shifted upward compared to ladder.

4.Discussion

According to previous studies and the MSIS report 2022 and 2018, the prevalence and incidence of TBEV are increasing in both endemic and non-endemic regions (Health 2018; Health 2022; Andreassen *et al.,* 2012; Soleng *et al.,* 2018; Vikse *et al.,* 2020). The objectives of this study were to investigate estimated prevalence of TBEV in questing nymphs and to develop a method of whole genome sequencing for TBEV from Kilen, Mandal collected in 2021.

4.1 Screening and prevalence of TBEV in Mandal, Norway 2021

Kilen, Mandal Norway was previously identified as a hotspot for TBEV in ticks (Vikse *et al.,* 2020). In the present study, total 5180 nymphs collected in 2021 from Kilen, were analyzed for the prevalence of TBEV by an "In house" RT-real-time PCR and confirmed by pyrosequencing analysis. TBEV was detected in 42 nymph pools. The Ct values of nymph pools varied between 29 to 39, showing a low concentration of viral TBEV RNA. The Ct values of the positive control ranged from 20 to 33, which is within the same range as previously reported (Andreassen *et al.,* 2012; Soleng *et al.,* 2018; Vikse *et al.,* 2020).

Of the 42 positives, five were not confirmed by pyrosequencing. This lack of confirmation might be caused by cross-contamination with other samples or false-priming which can result from the presence of almost identical sequences, leading to false results when PCR run at low annealing temperatures (55°C) (Michael & Altshuler 2006). Alternatively, it is also possible that the viral concentration was low, and the amount of PCR product analyzed by pyrosequencing was lower than the detection limit of the pyrosequencing process. However, the use of positive controls will effectively reduce the occurrence of cross contamination (Tang *et al.,* 2016). The pyrogram of the TBEV positive nymph pools were compared to the positive control Hochosterwitz. The presence of a comparable sequence pattern between the samples and controls indicates a true TBEV positive. The current research based on the in-house RT real-time PCR method followed by pyrosequencing is the best suited method for tick screening and has also been adjusted for the Norwegian strain of TBEV-EU (Andreassen *et al.,* 2012).

The total estimated prevalence (EPP) of TBEV in *I. ricinus* nymphs from Kilen, Mandal 2021 in this study was 0.7% which was higher than found in earlier research at the same site showing from

0% to 1.2% and no TBEV was detected in 2018 and 2019 tick samples (Andreassen *et al.,* 2012; Vikse *et al.,* 2020; Haüsler, 2021; Diekmann, 2021). Furthermore, a study site near Mandal at the island of Hille, which is approximately 8 km from Kilen, also showed almost an undetectable range of TBEV prevalence between 2009 and 2014, and no TBEV was detected in 2014 (Andreassen *et al.,* 2012; Soleng *et al.,* 2018, Vikse *et al.,* 2020). These yearly variation in EPP within the same region is supported by previous studies in Germany which have shown a yearly variation in both TBEV prevalence as well as in TBEV cases (Borde *et al.,* 2019; Süss et *al.,* 1999). The prevalence in the present study of 0.7% was relatively similar to data from other endemic European locations. The prevalence of TBEV in *I. ricinus* ticks was between 0.1% and 5% throughout Europe, 0.28% in Scandinavia, and 0.53% in Norway (Andreassen *et al.,* 2012; Paulsen *et al.,* 2015; Pettersson *et al.,* 2014).

Previous investigations conducted on nymphs in southern Norway has identified areas where the TBEV is either absent or the prevalence is very low. Based on these investigations, an average prevalence of TBEV in both nymphs and adult ticks was found to be 0.3% (Kjelland *et al.,* 2018; Vikse *et al.,* 2020).

When compared to other locations in Norway, the total EPP of 0.7% in the current research was lower than that of Larvik, where the overall estimated prevalence calculated for TBEV in nymphs collected in 2021 was 1.0% (Maharjan, 2023). While in comparison it was relatively similar to TBEV prevalence (0.6%) of nymphs from Vest-Agder and Telemark (2014 and 2015) (Kiran, 2017). The occurrence of TBEV in ticks showed yearly and monthly variation between distinct locations and within the same sites (Andreassen *et al.,* 2012; Lamsal, 2017; Haüsler, 2021). Furthermore, another study also reveals that the prevalence of TBEV changes from one year to another (Süss *et al.,* 1999).

The results of our current study indicated a monthly variation in the estimated prevalence of TBEV in nymphs. There was a steady monthly variation in EPP, reaching its highest peak in July with EPP 1.9%. From March to July there was a gradual increase from 0.3% to 1.9% with a decrease and a second lower peak in October. Overall, the EPP was shown to be highest in beginning of summer months with a slow decrease to the end. Variation in estimated prevalence from each month could be because of climatic effect. Similar monthly variation in estimated prevalence was also detected for ticks collected in 2015 at Spjærøy when TBEV prevalence in nymphs raised from

May to July, and was reduced until September, and risen again in October (Lamsal 2017). Environmental factors such as temperature and humidity influence tick phenology and seasonal activity (Cayol *et al.,* 2017). Experimental evidence indicates that reduced humidity promotes the elimination of the virus from the tick's body (Naumov *et al.,* 1980), while higher temperatures and humidity may boost the viral concentration of TBEV in ticks (Daniel *et al.,* 2018; Danielova *et al.,* 1983). July and August are hot and humid month in Norway, which creates the perfect conditions for the growth of ticks and replication of the virus. A previous study conducted in endemic regions of southern Norway showed an association between TBEV prevalence and high humidity, as well as a low saturation deficit (Andreassen *et al.,* 2012). The nymph ticks observed throughout July and August are most probably caused by the growth of larval populations that start feeding as soon as the temperature becomes optimal (around 5-10°C) in the early spring (Randolph *et al.,* 2002).

Climatic variables influence the natural transmission cycle of TBEV (Randolph, 2008), and previous research has shown that environmental elements have a major effect on TBEV prevalence in natural environments (Randolph & Rogers, 2000; Randolph, 2001). Studies conducted on microclimatic parameters have shown that humidity has a crucial role in the growth and survival of ticks (Daniel *et al.,* 2015; Danielova *et al.,* 1983). Ticks are very susceptible to desiccation and need on a relative humidity of 80% at ground level to search for hosts and stay alive (Knülle, 1966; Pfäffle *et al.,* 2013). An extension of the vegetative season in Scandinavia has been recognized as an essential variable in the increasing abundance of ticks and their activity in Sweden and Norway. Estimation for the future indicated that the Nordic countries will be faced with a climate characterized by increased precipitation and higher temperatures, along with a lengthening of the period suitable for plant growth due to an effect of climate change, this tick suitable environment can lead to increase of TBEV (Lamsal *et al.,* 2023). This is supported by the findings in the present study findings where the prevalence for TBEV in Mandal increased from previous years probably because of change in climate. The climate was found to be warm and dry with less precipitation in 2019 when no TBEV was detected (Haüsler 2021).

4.2 Development of amplification method and WGS for TBEV

The second objective of the present study was to develop a method for whole genome sequencing of low-level viral load TBEV in ticks. Viruses have been subjected to genome sequencing to investigate the transmission of diseases during outbreaks. Appropriate genomic monitoring plays a significant role for successful surveillance of viral epidemics, since it may provide valuable insights into transmission, distribution, and evolution of viruses (Quick, *et al.,*2017). It is quite likely that there is a spread and distribution of many TBEV variants, which may be confirmed by whole genome sequencing analysis. Therefore, it is essential to use WGS for complete sequencing of viral genome to obtain an overview of newly developed virus strains. The rise in the rate and incidence of TBEV has led to an increased need for knowledge on the TBEV genomic sequence. The newly obtained TBEV sequences will improve the comprehension of the evolutionary processes and phylogenetic distribution of TBEV strains (Asghar *et al.,* 2017).

4.2.1 Quantification and quality test of purified PCR products using overlapping primers:

In the current study previously designed short overlapping primers, DMUK1, was used to amplify TBEV sequences in nymphs by a multiplex PCR method. A previous study recommended that shorter amplicons may be helpful when the viral load is low (Quick *et al.,* 2017). The approach of multiplex PCR generating overlapping amplicons was used to capture the whole genome sequence of clinical Zika virus samples (Quick *et al.,* 2017). Multiplex PCR involves the use of primers that lead to the amplification of unique DNA regions, both when used individually and in combination with multiple primers, all under a single set of reaction conditions (Markoulatos *et al.,* 2002). The multiplex PCR method offers benefits by decreasing reagent expenses and minimizing the likelihood of laboratory mistakes (Paulsen *et al.,* 2021). Studies performed with primer pools, which amplifies many small amplicons in a multiplex-PCR reaction, is a frequently used technique for genomic sequencing (Arana *et al.,* 2022).

Electrophoresis-based approaches by tapestation, Bioanalyzer, and GX Touch provide a means to visually evaluate the quality of the libraries and determine the concentration of dsDNA in the amplicons (Hussing, *et al.,* 2018). We utilized tapestation to evaluate the virtual length and dsDNA concentrations of PCR amplicons produced. With tapestation the PCR products from newly designed primer pairs DMUK1 was verified according to size evaluation. Proper quantification of

PCR products to make libraries is necessary to avoid variations in coverage when multiple samples are included in a single sequencing study.

In the present study the DMUK1 primer pairs produced an intensive 400bp band from the positive control (10^{-3} dilution Hochosterwitz), however, there were no bands from the nymph samples. Another short overlapping primer set, KUPA1, also gave strong 400 bp bands with the positive control (Hochosterwitz), while no fragments were seen for selected TBEV samples. This showed that the multiplex PCR reactions and primer sets are working well. The lack of desired PCR products from the selected TBEV samples can be explained by several factors: such as low viral load or presence of inhibitors.

4.2.2 Troubleshooting

To explain the lack of 400bp fragments in the purified samples we decided to test the unpurified PCR products to check if something was lost during the purification process. The quantification and quality test of purified and unpurified PCR products revealed a difference in the concentration of dsDNA. For all reactions, the DNA concentration before cleanup was higher than after indicating loss of excess primers and primer dimers during the purification procedure while the presence of unspecific shorter fragments and lack of 400 bp fragments, indicates that the virus level is low. This result is in accordance with the high Ct values obtained in the real time PCR reaction. Another possibility is the presence of PCR inhibitors. Studies have suggested that PCR inhibitors limit the efficiency of PCR reactions. Inhibitors of PCR often affect the PCR process by directly interacting with DNA or inhibiting the activity of thermostable DNA polymerases. DNA polymerases need certain cofactors, which may be targets of inhibition (Bessetti, 2007). PCR inhibitors like metal ions, ethanol, salts and EDTA may still be present in DNA samples, even after the process of extraction and purification. Research has shown that the presence of metal ions, when co-purified at precise amounts, might hinder the process of DNA amplification (Combs *et al.*, 2015).

4.2.2.1 Inhibition tests

a) In a previous study, assessment of PCR products by methods such as gel electrophoresis, dot blots, high-pressure liquid chromatography, and calorimetric tests, revealed that inhibitory compounds may indirectly affect the quantity and quality of PCR products (Alaeddini 2012). A frequent strategy to test for inhibition is to mix a positive sample with the test sample in the same multiplex reaction (Swango *et al.,* 2006; Swango *et al.,* 2007). In our study we tested if inhibition was the reason for the lack of 400bp bands by spike-in testing. Hochosterwitz strain (10^{-8} dilution) were mixed with selected nymph samples (S5-237N, S5-240N, S5-241N). The tapestation result visualized a stronger 400 bp band in the Hochosterwitz sample after being spiked with selected TBEV nymph pools than unspiked samples. This suggests that there were no inhibitory effects in the selected TBEV nymph pools, however, the viral load might be below the detection limit of the amplification process. A previous study on TBEV patients discussed the association of TBEV viral load and TBE detection in different phases of the disease (Saksida *et al.,* 2018).

b) To study whether spike-in of the Hochosterwitz strain with Carrier RNA are producing the similar or more products than spike-in with selected nymph samples as described above, Hochosterwitz cDNA were mixed with Carrier RNA. A study reported that carrier RNA has been used to recover degraded or small quantities of DNA (Shaw *et al.,*2009). Carrier RNA plays an essential role for accurately detecting small tandem repeats. Many recent techniques have been developed to possibly improve the recovery of DNA, such as using molecules like carrier RNA to enhance DNA amplification (Parys-Proszek, *et al.,* 2008). The spike-in test in the present study was performed using two primer sets (DMUK1 and KUPA1) and cDNA of Hochosterwitz made by two reverse transcription kits (High-Capacity and SSIV). When the Hochosterwitz samples were spiked with Carrier RNA, the strength of bands improved significantly, demonstrating that spike-in could improve the PCR reaction. This finding is in consistent with a previous study reporting that carrier RNA significantly increased DNA recovery in forensic samples (El-Shorbagy *et al.,* 2022). KUPA1 was less efficient than the DMUK1 in creating bands with the SSIV kit than High-Capacity kit. While DMUK1 was independent of reverse transcription kit. This indicated that the TBEV-Hochosterwitz strain is more related to central European strains (represented by DMUK1) than eastern strains (represented by KUPA1).

c) To see whether the primer set KUPA1(designed for TBEV Kumlinge strain) was able to amplify Mandal TBEV cDNA reverse transcribed by High-capacity kit, an amplification test was performed where 400 bp band was seen for the positive control (Hochosterwitz) but no 400 bp fragments were seen for nymph TBEV cDNA as expected, since the primer was not designed for Mandal TBEV.

4.2.2.2 Dilution test of nymph cDNA

To test if dilution of nymph samples may explain the lack of 400 bp fragments, a dilution experiment was performed. Several studies have raised concerns about the effect of inhibition during multiple steps of analysis, including the extraction and amplification steps, as well as possible contamination from the environment during sample processing (Greay *et al.,* 2018; Lejal *et al.,* 2020). A study suggested that low biomass samples such as nymphs are at more risk to contamination which might result in lowering the DNA quantity (Lejal *et al.,* 2020; Salter *et al.,* 2014. In this study, among all 1:2 diluted amplified TBEV PCR products, only one diluted sample produced weak 400 bp band visualized by the tape station. The current results show that diluting the samples can improve the quality of PCR products, but because just one dilution test was conducted in this study so, it is difficult to conclude that the samples lacking 400bp band were inhibited.

5.Conclusion

The main objectives of the present study were to investigate the prevalence of TBEV in confirmed TBEV positive nymph pools collected in 2021 from Kilen, Mandal, and to develop a WGS method for low virus load samples. The current study found a TBEV prevalence of 0.7%, which differs from samples of the same location in 2018 and 2019, when no TBEV was detected. This shift in prevalence from previous years may have been related to climate changes which indicated that there is a monthly and yearly variation of TBEV in Kilen, Mandal with an increase in TBEV prevalence demonstrated that TBEV is rising in endemic regions of Norway with low infection rates and prevalence. Furthermore, several amplification and troubleshooting experiments by two different overlapping primers were conducted out to obtain a required 400bp PCR product from nymph cDNA samples for WGS. The current investigation showed that cDNA generated by both the reverse transcription technique, High-Capacity cDNA, and Superscript IV cDNA, performs well with small fragment length overlapping primer pairs of the TBEV-Eu strain. In this research, the viral load in the samples were too low to produce PCR product by the current PCR conditions but, spike-in methods were found useful for improving PCR products. To conclude, the developed amplification and troubleshooting procedures in this work may be further investigated to obtain an improved and desired PCR products for sequencing of whole viral genome of TBEV samples with low viral load.

6. Future perspectives

The present study faced issues in obtaining desired PCR product for WGS method of low virus load TBEV samples from Mandal. It is important to continue studying TBEV, its primary vector, and hosts to estimate future climatic changes. And analyze more tick samples by different overlapping primer pairs to test if the Mandal TBEV strain has changed.

Future studies should aim to:

- Continue monthly sampling and analysis of ticks to study the prevalance and effects of climatic changes from year to year.
- Continue testing more short fragment length (400-500 bp) and long fragment length (2000 bp) overlapping primers with different concentration.
- Continue testing different dilutions of the cDNA and RNA samples by all designed primers for TBEV Eu-strain with different concentrations.
- Continue testing low viral load cDNA and RNA samples by spike-in with carrier RNA.

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Appendix

Appendix 1A: RNA extraction flow chart

1A: RNA EXTRACTION FLOWCHART

Appendix 2A: Reverse Transcription flowcahrt

Appendix 2A: Reverse Transcription High-capacity kit + Superscript IV (cDNA)

Appendix 2B: Master mix for reverse transcription using High-Capacity kit.

Table 1. Mastermix for reverse transcription using High-Capacity kit.

Components	**1x sample µL**
10X RT Random Primers	**2.0**
10X RT Buffer	**2.0**
25X dNTP Mix (100 mM)	**0.8**
MultiScribe™ Reverse Transcriptase	**1.0**
RNase Inhibitor (20 U/µl)	**1.0**
Nuclease-free H_2O	**8.2**
Total mix	**15 µL**

Add 15 µl MM+ 5 µl RNA = 20 µl total volume

Appendix 2C: High-Capacity PCR condition

Table. PCR condition for reverse transcription using High-Capacity kit.

Temperature	**Time**
25°C	10 mins
37°C	120 mins
85°C	5 mins
4°C	∞

Appendix 2D: Mastermix for reverse transcription using Superscript IV kit.

Prepare Mastermix 1(Yellow) first:

Table. Mastermix1 using Superscript IV Kit.

Component	1x ul reaction	Final concentration
Random hexamers (50ng/µl)	1	10 ng/µl
dNTP (10 mM)	1	2 mM
Nuclease-free water	5	-
Total	7 µl	-

Add 5 µl RNA to 7 µl of Master mix 1 and heat on a heating block at 65°C for 5 mins. Cool it on ice for 1 min.

Prepare Mastermix 2 (Green):

Table. Mastermix2 using Superscript IV Kit.

Component	1x ul reaction	Final concentration
RT-Superscript IV (200 U/µl)	1	40 U/µl
5x SSIV buffer	4	4X
Dithiothreitol (DTT, 0.1M)	1	0.02 M
RNAse inhibitor (40 U/ µl)	2	16 U/ µl
Total	8 µl	-

Add 8 µl of mastermix2 to mastermix1 containing RNA.

Appendix 2E: Superscript IV PCR condition

Table. PCR condition for reverse transcription using Superscript IV kit.

Temperature	Time
23°C	10 mins
55°C	10 mins
80°C	10 mins
4°C	∞

Appendix 3A: In-house Real-time RT-PCR Flowchart

Appendix 3A: Real-time In-house RT-PCR flow chart

Date _____ **No. of Ticks** _____ **from** _____ **To** _____

Appendix 3B: Mastermix for TBEV Real-time RT-PCR

Table. Mastermix for TBEV Real-time RT-PCR

Components	***1x sample µL***
50 Mm $MgCl_2$	***2.5***
10X AB Buffer	***2.5***
25X dNTP	***0.2***
25 pmol 320F	***0.25***
25 pmol 373R + Biotin	***0.25***
25 pmol 339 Probe	***0.3***
Pt-Taq enzyme[1]	***0.19***
RNase-free H_2O	***15.81***
Total mix	***22 µL***

[1]Platinum®Taq DNA Polymerase enzyme from Invitrogen™.

22 µl + 3 µl RNA = 25 µl total volume

Appendix 3C: 10X AB buffer recipe

10X AB buffer contains:

Table. 10X AB buffer components.

Component	**Quantity**
1M Tris pH 8.8	**75 ml**
$(NH_4)_2$ SO4	**20 ml**
Tween 20	**0.2 ml**
RNase free water	**4.8 ml**
Total	**100 ml**

Appendix 3D: PCR-condition for Realtime-RT PCR.

Table. PCR condition for Realtime-RT PCR.

Temperature	Time	Total cycles
95°C	**2 mins**	**1**
95°C	**15 secs**	**48**
60°C	**45 secs**	
72°C	**30 secs**	

Appendix 4: Pyrosequencing flowchart

Appendix 4: Protocol for Pyrosequencing

Before Experiment:

- Prepare, and print worksheets. Store worksheet in PyroMark software (Ordinær N:
- Virologi >MMR >Pyrosequencing >TBEV runs.
- Check if you have all the reagents and buffers.
- Make sure if you have Pyromark kits and where you have it.

Go to Clean room:

- Clean PyroMark cartridges using 200 µl MilliQ water two times (Follow machine instruction).
- Dilute TBEV 320 forward primer to 25 µM from 250 µM stock and take RNase-free water.
- Login into PC with your user ID. Go to PyroMark program.
- Press ⋈ > SEQ > Dispensation order > 60 (TCGA) > save/ (or open TBE method from N: drive if the method is already stored).
- New run (icon with →) > disc setup > right click on position for sample to add > open method (from N:) on the first cell and copy to remaining > give name for each disc position according to worksheet.
- Click on Primer loading > automatic > save.
- Save on pendrive as well: (Ordinær N: > Virologi >MMR >Pyrosequencing >TBEV runs > copy and paste on Kingston D: drive/pendrive).
- Put the pendrive into the machine.
- In the machine: Press Seq > press your file > press arrow to load.
- Follow the instructions of the machine.
- Put the absorption strip.
- Load reagents:
    - Substrate (S) and enzyme (E) is stored in the freezer.
    - Remaining reagents are inside the PyroMark advanced kit box in the freeze.

- Dissolve E and S each with 660 µl PyroMark advanced annealing buffer.
- Swirl the mixture and don't vortex.
- Leave it in room temperature for 5-10 mins to fully dissolve it.
- Load all reagents on correct chambers according to the markings & follow instructions given by machine. Avoid air bubbles. Machine calculates reagents volume according to the sample size.
- Close chambers and lock the lid.
- Dilute primer to 4 µM. Machine calculates and gives you amount to load (for e.g., 15 samples require 90µl primer). Load it in chamber.
- Start the machine >follow instructions.
- In dirty workstation of 350 lab room: use arm protector, and add magnetic beads (3 µl) and sample (10 µl) into the white disc.
- On the disc mark the start and end sample position with marker. (For e.g., starting on position A2 and last sample on C2 position then mark them).
- Load sample first on the disc wells. Vortex the magnetic bead tube after every 3 loads to get the beads.
- Put the disc on machine by opening the screw. Close the screw and lid.
- Start the machine. The sequencing will end in 45 mins, and give real time result as well.

After Run Completion:

- Clean all PyroMark cartridges using 200 µl MilliQ water two times.
- Take out the absorption strip.
- Open lid of chambers and close the main lid of the machine. Turn the machine off.

Viewing and Result Analysis:

- Quality assessment: The disc overview in the Overview tab gives a quick overview of the quality assessments. Color bar shows quality assessments: Blue: passed, yellow: check, red: failed, and white: not analyzed.
- Check SEQ analysis results. Compare the obtained result with that of positive control and analyze sequences manually.
- Copy result from pen drive to PC and store in N: drive under TBEV runs.
- To generate a report, select the desired report from the Reports menu.
- Look for pattern "GTTGAATTTGGGGCTCCTCA"**.** If 70% or more nucleotides match, then the sample is true positive.

For example:

Sample Sequence:
GGGAGCGCAAAACTGGAACAACGCAGAAAGACTGGTTGAATTTGGGGCTCCTCA

TBEV-320 F

GGGAGCGCAAAACTGGAACAACGCAGAAAGACTGGTTGAATTTGGGGCTCCTCA

18 bp

Pyrosequence (pattern to look for in sample sequence to confirm true positive)

CAACGCAGAAAGACTGGTTGAATTTGGGGCTCCTCA-36 nucleotides

Appendix 5A: Protocol for Conventional PCR (DMUK 400bp Primerset)

- ✓ Bring cold metal block to clean room (put outside), Take another cold metal block inside from the fridge.
- ✓ Take TBEV Q5-Fidelity Kit, primer pools and RNase free water from fridge.
- ✓ Check quantity and concentration of all tubes, defrost all reagents, Vortex and spin them briefly.
- ✓ Prepare Mastremix for both primer pools (pool 1 and Pool 2) as per calculated in the recipe.
- ✓ Transfer MM and RNase free water on metal block to Lab 322A.
- ✓ Label PCR tubes and place them on cold metal block.
- ✓ Add 8.84 µl of respective sample cDNA and Master Mix to each tube.
- ✓ Transport the samples within the PCR-tubes to the PCR room in a cold metal block.
- ✓ Run the conventional PCR program as per required PCR condition.
- ✓ After the PCR run finished keep the samples in cold metal block in the fridge (4°C) overnight.

Mastermix for Conventional PCR

Table. Mastermix for Conventional PCR using DMUK (400bp) primer mix.

Component	**25 µl reaction**	**50 µl reaction**	**X µl Reaction**	**Final Conc.**
5X Q5 Reaction Buffer	5 µl	10 µl		1X
10 mM dNTPs	0.5 µl	1 µl		200 µM
Primers (Pool 1 or Pool 2)		8.84 µl		
Q5 Hot Start High-Fidelity DNA Polymerase	0.25 µl	0.5 µl		0.02 U/µl
5X Q5 High GC Enhancer	(5 µl)	10 µl		(1X)
Nuclease-Free Water	to 25 µl	14.66 µl		

*Add 5 µl cDNA of each sample to 45 µl of Master Mix

- **Note:** Gently mix the reaction. Collect all liquid to the bottom of the tube by a quick spin if necessary. Overlay the sample with mineral oil if using a PCR machine without a heated lid.

Appendix 5B: PCR condition for multiplex conventional PCR

PCR condition:

Table. PCR condition for multiplex conventional PCR using DMUK (400 bp) primer mix.

Temperature	**Time**	**Holds**
98°C	30 secs	1
98°C	15 secs	40
65°C	5 mins	
4°C	Infinity	-

TBEV positive cDNA (Superscript) selected for WGS.

Table: TBEV positive cDNA (Superscript) selected to be further amplified for development of WGS.

Sample	**Collection Month**	**CT value**	**Pyrsosequncing**
S5(21)-N235	July	28.94	Confirmed True
S5 (21)- N220	July	30.88	Confirmed True
S5(21)-N237	July	31.12	Confirmed True
S5(21)-N240	July	32.13	Confirmed True
S5(21)-N236	July	33.21	Confirmed True
S5(21)-N241	July	33.27	Confirmed True
S5(21)-N56	April	33.96	Confirmed True
S5 (21)- N309	August	33.96	Confirmed True
NC			
PC H-1			

Appendix 6. Cleanup /Purification of cDNA by Kapa Hyperplus Kit flowchart:

Step 1: Agencourt AMPure XP

Performing 1.8x Bead-bases cleanup for DMUK primers PCR product (40 µl) + AMPure beads (72µl) = 112µl Total volume (Mixing thoroughly by vortexing, and/or pipetting up and down multiple times)

Step 2: Binding

- Incubate tube at room temperature (RT) for 5-15 min (to bind DNA to the Paramagnetic beads)

Step 3: Separation

- Place the tube on a magnet to capture the beads (Incubate until the liquid is clear)
- Carefully remove and discard the supernatant.

Step 4: Ethanol Wash

- Keeping the plate/tubes on the magnet, add 200µl of 80% ethanol.
- (Carefully remove and discard the supernatant)
- Incubate tube on the magnet at RT for > 30 Sec Keeping the plate/tubes on the magnet, again add 200µl of 80% ethanol.
- Carefully remove and discard the supernatant.
- Incubate tube on the magnet at RT for > 30 Sec Dry the beads at RT (3-5 mins), or until all the ethanol has evaporated (NOTE: Over drying, the beads may result in reduced yield)
- Resuspend the beads.

Step 5: Elution Buffer

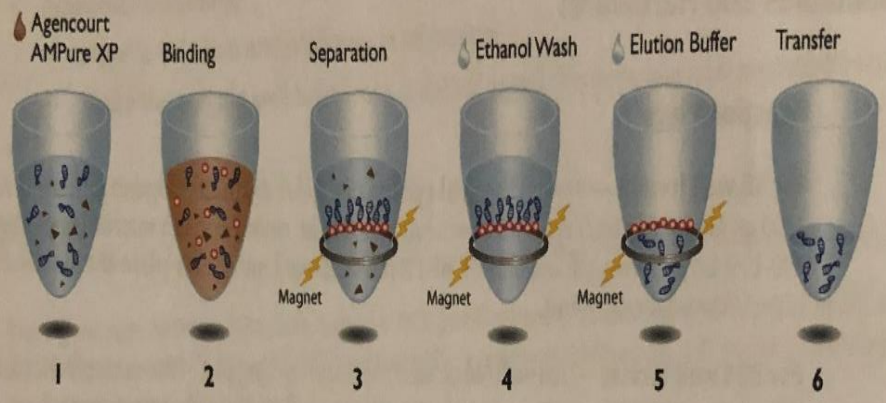
- Add 40 µl of elution buffer (10 mM Tris-HCL, PH 8.0 -8.5) to proceed with library amplification.
- Incubate the tube at RT (2 min) to elute the DNA off the beads.
- Place the tubes on magnet to capture the beads (Incubate until the liquid is clear)

Step 6: Transfer

- Transfer the clear supernatant to a new tube and proceed with Library Amplification
- Transfer 40 µl of supernatant for Library Amplification
- Keep it in the fridge for Library Preparation.

PCR Purification Process Overview

Figure 1 Workflow for PCR Purification



The workflow for the PCR purification process is as follows:

1. Add 1.8 µL AMPure XP per 1.0 µL of sample.
2. Bind DNA fragments to paramagnetic beads.
3. Separation of beads + DNA fragments from contaminants.
4. Wash beads + DNA fragments twice with 70% Ethanol to remove contaminants.
5. Elute purified DNA fragments from beads.
6. Transfer to new plate.

A detailed procedure for using a 96 well format to perform PCR purification can be found in the 96 Well Format Procedure section of this manual within PCR Purification Process Procedure.

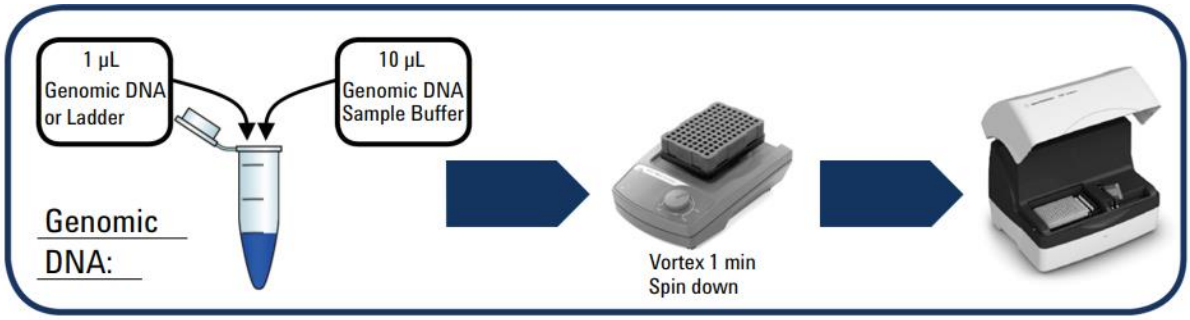
A detailed procedure for using a 384 well format to perform PCR purification can be found in the 384 Well Format Procedure section of this manual within PCR Purification Process Procedure.

Appendix 7: Protocol for Agilent 4200 TapeStation system (Quantification of amplicons & Verification of primer pairs)

Agilent 4200 TapeStation system is an automated platform for simpler, faster, and reliable electrophoresis. The Genomic DNA ScreenTape system is designed to analyze genomic DNA samples in size range from 200 bp to >60000 bp using Genomic RNA reagents (Ladder and Sample Buffer).

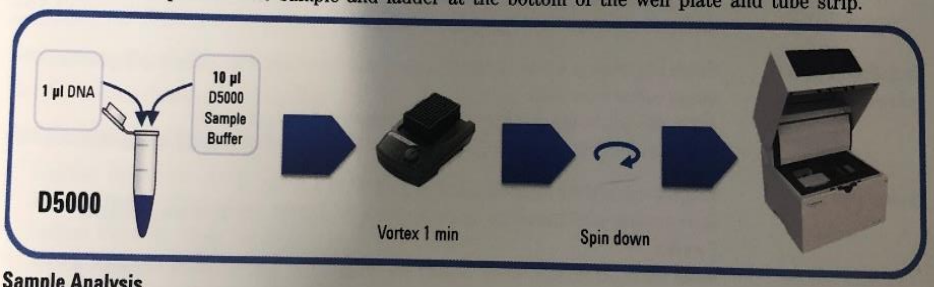
Protocol for Agilent 4200 TapeStation system:

- ✓ **Step 1**: Equilibrate the Genomic DNA reagents at room temperature for 30 mins.
- ✓ **Step 2**: Vortex the reagents before use.
- ✓ **Step 3**: Prepare ladder by mixing 10 µl Genomic DNA Sample Buffer with 1 µl Genomic DNA Ladder. (Note: Use a fresh ladder for each run. Electronic Ladder is not available for the Genomic DNA assay)
- ✓ **Step 4:** Prepare sample by mixing 10 µl Genomic DNA Sample Buffer with 1 µl genomic DNA sample (10 - 100 ng/µl). (Note: when pipetting small volumes ensure that no sample remains within the tip)
- ✓ **Step 5:** Spin down and then vortex the mix using IKA vortexer and adaptor at 2000 rpm for 1 min.
- ✓ **Step 6**: Spin down briefly to collect the contents at the base of the tubes.
- ✓ **Step 7:** Launch the 4200 TapeStation Controller Software.
- ✓ **Step 8**: Load Genomic DNA ScreenTape device and loading tips into the 4200 TapeStation instrument.
- ✓ **Step 9:** Load samples into the 4200 TapeStation instrument.
- ✓ **Step 10**: Click Start and specify a filename with which to save the results.



Agilent D5000 Assay Operating Procedure

1 Allow D5000 Reagents (5067-5589) to equilibrate at room temperature for 30 minutes.
2 Launch the Agilent 4200 TapeStation Controller Software.
3 Flick the D5000 ScreenTape device (5067-5588) and load it into the 4200 TapeStation instrument.
4 Place loading tips (5067-5598) into the Agilent 4200 TapeStation instrument.
5 Vortex reagents and spin down before use.
6 Prepare ladder:
   - For 1 – 15 samples: pipette 10 µL D5000 Sample Buffer (●) and 1 µL D5000 Ladder (●) at position A1 in a tube strip (401428).
   - For 16 or more samples: pipette 20 µL D5000 Sample Buffer (●) and 2 µL D5000 Ladder (●) at position A1 in a tube strip.
7 For each sample, pipette 10 µL D5000 Sample Buffer (●) and 1 µL DNA sample in a well plate (5042-8502) or a tube strip (401428).
8 Apply foil seal (5067-5154) to sample well plate and caps (401425) to tube strips with ladder or sample.
9 Mix liquids in sample and ladder vials using the IKA vortex at 2000 rpm for 1 min.
10 Spin down to position the sample and ladder at the bottom of the well plate and tube strip.



Sample Analysis

1 Load samples into the Agilent 4200 TapeStation instrument. Carefully remove caps of tube strips.
2 Place ladder in position A1 on tube strip holder in the 4200 TapeStation instrument.
3 Select required sample positions on the 4200 TapeStation Controller Software.
4 Click **Start**.
5 The Agilent Tapestation Analysis Software opens after the run and displays results.

Technical Support and Further Information

For technical support, please visit www.agilent.com/genomics/contact. Visit Agilent Technologies` web site. It offers useful information, support and current developments about the products and technology: www.agilent.com/genomics/tapestation.

Appendix 8: Flowchart for Inhibition (Spike-in of TBEV positive with H-8 Dilution of Hochosterwitz) test:

- Bring cold metal block to clean room (put outside), Take another cold metal block inside from the fridge.
- Take TBEV Q5-Fidelity Kit, primer pools and RNase free water from fridge.
- Check quantity and concentration of all tubes, defrost all reagents, Vortex and spin them briefly.
- Prepare Q-5 fidelity Mastremix for both primer pools (pool 1 and Pool 2) as per calculated in the recipe.
- Transfer MM and RNase free water on metal block to Lab 322A.
- Label PCR tubes and place them on cold metal block.
- Use sample 237N,240N and 241N for Spike-in test.
- Dilute Positive Control Hochosterwitz to 10-8 series.
- Mix 2.5 ul H-8 Dilution of Hochosterwitz with 2.5 ul of TBEV positive sample.
- Transfer 20ul of MM and 5ul spike-in mix to respective PCR tubes.
- Transport the samples within the PCR-tubes to the PCR room in a cold metal block.
- Run the conventional PCR program as per required PCR condition.
- After the PCR run finished keep the samples in cold metal block in the fridge (4°C) overnight.

PCR condition:

Table. PCR condition for multiplex conventional PCR using DMUK (400 bp) primer mix.

Temperature	**Time**	**Holds**
98°C	30 secs	1
98°C	15 secs	40
65°C	5 mins	
4°C	Infinity	-

Appendix 9: Sequences for overlapping 400bp DMUK1 and KUPA1 primer

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Line No	Cat No/ID No.	Description/Sequence	Amount (OD)
27	10336-022	DMUK2F \ AshildKAndreassen	1 Each (C03) 5.30
	W6643C03	TCCCTCTGAAACAGGCCACA	
28	10336-022	DMUK2R \ AshildKAndreassen	1 Each (C04) 6.70
	W6643C04	TGAGCCTTCCTGTTTCCCACA	
29	10336-022	DMUK3F \ AshildKAndreassen	1 Each (C05) 7.70
	W6643C05	GACCATAGATCAAGGAGAAGAGCC	
30	10336-022	DMUK3R \ AshildKAndreassen	1 Each (C06) 6.70
	W6643C06	TCCCCTGAGTACCAGTCACAAA	
31	10336-022	DMUK4F \ AshildKAndreassen	1 Each (C07) 6.60
	W6643C07	GTTCTGGTTGTGCTCCTGTGTT	
32	10336-022	DMUK4R \ AshildKAndreassen	1 Each (C08) 6.10
	W6643C08	TTTTTGCCTCACAAGCCGC	
33	10336-022	DMUK5F \ AshildKAndreassen	1 Each (C09) 8.10
	W6643C09	CAGTGTGTAAGAGAGATCAGAGTGA	
34	10336-022	DMUK5R \ AshildKAndreassen	1 Each (C10) 7.60
	W6643C10	GCAGAGCCAGATCATTGAACCA	
35	10336-022	DMUK6F \ AshildKAndreassen	1 Each (C11) 6.10
	W6643C11	CCGTCATCCTTGAGCTTGACAA	
36	10336-022	DMUK6R \ AshildKAndreassen	1 Each (C12) 6.90
	W6643C12	GAGAATGTGACTTCCATGACTACTGT	
37	10336-022	DMUK7F \ AshildKAndreassen	1 Each (D01) 9.00
	W6643D01	GATGAAAGGTCTTACGTACACAATGTG	
38	10336-022	DMUK7R \ AshildKAndreassen	1 Each (D02) 5.70
	W6643D02	TTGAACTCAGAAAGCCTCCAGC	
39	10336-022	DMUK8F \ AshildKAndreassen	1 Each (D03) 5.70
	W6643D03	GGGTTTTCCAAAAGACCAAGAAAGG	

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OSLO, 0456
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?shild Kristine Andreassen
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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
40	10336-022	DMUK8R \ AshildKAndreassen	1 Each (D04)	6.90
	W6643D04	GTGTCTCCGGGTAGTAGGCATA		
41	10336-022	DMUK9F \ AshildKAndreassen	1 Each (D05)	5.50
	W6643D05	AACGAATGGAGCTCCGCTGT		
42	10336-022	DMUK9R \ AshildKAndreassen	1 Each (D06)	5.70
	W6643D06	ACACTCACTTTGTCCTTCCGTG		
43	10336-022	DMUK10F \ AshildKAndreassen	1 Each (D07)	6.10
	W6643D07	AAAGTCTCCTGGAAAAGCTGGG		
44	10336-022	DMUK10R \ AshildKAndreassen	1 Each (D08)	5.90
	W6643D08	ATAACTCCGAGTCCACCACGT		
45	10336-022	DMUK11F \ AshildKAndreassen	1 Each (D09)	7.60
	W6643D09	TGACACAGGCACTTATATAGTTGAACT		
46	10336-022	DMUK11R \ AshildKAndreassen	1 Each (D10)	8.00
	W6643D10	GGTCATGGACTGGTCGTATTTCC		
47	10336-022	DMUK12F \ AshildKAndreassen	1 Each (D11)	7.50
	W6643D11	AGAGTGGCAAGGTTATCCCAGA		
48	10336-022	DMUK12R \ AshildKAndreassen	1 Each (D12)	6.40
	W6643D12	CAAACACAGCCTGGAGTAGCAT		
49	10336-022	DMUK13F \ AshildKAndreassen	1 Each (E01)	6.30
	W6643E01	TGGGATGGTCAGGATAGAGAGC		
50	10336-022	DMUK13R \ AshildKAndreassen	1 Each (E02)	5.20
	W6643E02	CGAAGCTACACTCAGGAAGCAT		
51	10336-022	DMUK14F \ AshildKAndreassen	1 Each (E03)	5.00
	W6643E03	GCTCTTGCTCATGGCTCTCAT		
52	10336-022	DMUK14R \ AshildKAndreassen	1 Each (E04)	5.20
	W6643E04	CGGCAACCAGCTGCATCTTT		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
53	10336-022	DMUK15F \ AshildKAndreassen	1 Each (E05)	6.90
	W6643E05	ATGATGCGACACACCTCCCA		
54	10336-022	DMUK15R \ AshildKAndreassen	1 Each (E06)	7.40
	W6643E06	AGACACCGTCCTTAACCTCGAA		
55	10336-022	DMUK16F \ AshildKAndreassen	1 Each (E07)	6.80
	W6643E07	ATGCTGAGGTCATCTCGAAGGT		
56	10336-022	DMUK16R \ AshildKAndreassen	1 Each (E08)	6.90
	W6643E08	ATTGGTATTGCCCCAAGCTTCC		
57	10336-022	DMUK17F \ AshildKAndreassen	1 Each (E09)	6.80
	W6643E09	AACAGTACAGGTCCATGCCTTC		
58	10336-022	DMUK17R \ AshildKAndreassen	1 Each (E10)	6.10
	W6643E10	TTGGAGCCAACACCAACGTT		
59	10336-022	DMUK18F \ AshildKAndreassen	1 Each (E11)	6.20
	W6643E11	GTCAGATCACAGTGCTGGACAT		
60	10336-022	DMUK18R \ AshildKAndreassen	1 Each (E12)	6.30
	W6643E12	AGGGTTCACTCTTACCAGGAGG		
61	10336-022	DMUK19F \ AshildKAndreassen	1 Each (F01)	6.70
	W6643F01	GCTGCCAGAGGTCATTTGTACA		
62	10336-022	DMUK19R \ AshildKAndreassen	1 Each (F02)	6.00
	W6643F02	GGCTTGATGTTTGTCCTCCCAT		
63	10336-022	DMUK20F \ AshildKAndreassen	1 Each (F03)	7.10
	W6643F03	CTGACTTTGTGGTCACGACTGA		
64	10336-022	DMUK20R \ AshildKAndreassen	1 Each (F04)	4.90
	W6643F04	TGGGTGAGAAGATGTCGGAAGT		
65	10336-022	DMUK21F \ AshildKAndreassen	1 Each (F05)	6.40
	W6643F05	GCCACCTTCTATGGACCAGAAC		

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Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
66	10336-022	DMUK21R \ AshildKAndreassen	1 Each (F06)	5.10
	W6643F06	CATCTGTGCCGCAAAAGCTC		
67	10336-022	DMUK22F \ AshildKAndreassen	1 Each (F07)	7.00
	W6643F07	GCACGCATGTTCAAAGAAGGAC		
68	10336-022	DMUK22R \ AshildKAndreassen	1 Each (F08)	6.10
	W6643F08	CACTCCGGCCATATTCCCAT		
69	10336-022	DMUK23F \ AshildKAndreassen	1 Each (F09)	6.00
	W6643F09	CGGACTTCAATCAGCCGTATGA		
70	10336-022	DMUK23R \ AshildKAndreassen	1 Each (F10)	6.40
	W6643F10	GTCTGCAGTTGGTGGATGATGT		
71	10336-022	DMUK24F \ AshildKAndreassen	1 Each (F11)	7.40
	W6643F11	AATCTCGGCCATGGAGTGAATG		
72	10336-022	DMUK24R \ AshildKAndreassen	1 Each (F12)	5.50
	W6643F12	TGATGACATCCCCATCCACCAT		
73	10336-022	DMUK25F \ AshildKAndreassen	1 Each (G01)	6.30
	W6643G01	TGTCTGGTCTGGAGGCTGAATT		
74	10336-022	DMUK25R \ AshildKAndreassen	1 Each (G02)	4.30
	W6643G02	ATCTCCAAGCGTGTCTCCCT		
75	10336-022	DMUK26F \ AshildKAndreassen	1 Each (G03)	5.80
	W6643G03	TGACACACTGTGGACGATGC		
76	10336-022	DMUK26R \ AshildKAndreassen	1 Each (G04)	6.30
	W6643G04	TCGGGATGCCGCATAATAGGA		
77	10336-022	DMUK27F \ AshildKAndreassen	1 Each (G05)	6.00
	W6643G05	TTGAGGAACGCGGATATGCC		
78	10336-022	DMUK27R \ AshildKAndreassen	1 Each (G06)	6.60
	W6643G06	GTTGGAATCTGTGCAGTGCTTC		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
66	10336-022	DMUK21R \ AshildKAndreassen	1 Each (F06)	5.10
	W6643F06	CATCTGTGCCGCAAAAGCTC		
67	10336-022	DMUK22F \ AshildKAndreassen	1 Each (F07)	7.00
	W6643F07	GCACGCATGTTCAAAGAAGGAC		
68	10336-022	DMUK22R \ AshildKAndreassen	1 Each (F08)	6.10
	W6643F08	CACTCCGGCCATATTCCCAT		
69	10336-022	DMUK23F \ AshildKAndreassen	1 Each (F09)	6.00
	W6643F09	CGGACTTCAATCAGCCGTATGA		
70	10336-022	DMUK23R \ AshildKAndreassen	1 Each (F10)	6.40
	W6643F10	GTCTGCAGTTGGTGGATGATGT		
71	10336-022	DMUK24F \ AshildKAndreassen	1 Each (F11)	7.40
	W6643F11	AATCTCGGCCATGGAGTGAATG		
72	10336-022	DMUK24R \ AshildKAndreassen	1 Each (F12)	5.50
	W6643F12	TGATGACATCCCCATCCACCAT		
73	10336-022	DMUK25F \ AshildKAndreassen	1 Each (G01)	6.30
	W6643G01	TGTCTGGTCTGGAGGCTGAATT		
74	10336-022	DMUK25R \ AshildKAndreassen	1 Each (G02)	4.30
	W6643G02	ATCTCCAAGCGTGTCTCCCT		
75	10336-022	DMUK26F \ AshildKAndreassen	1 Each (G03)	5.80
	W6643G03	TGACACACTGTGGACGATGC		
76	10336-022	DMUK26R \ AshildKAndreassen	1 Each (G04)	6.30
	W6643G04	TCGGGATGCCGCATAATAGGA		
77	10336-022	DMUK27F \ AshildKAndreassen	1 Each (G05)	6.00
	W6643G05	TTGAGGAACGCGGATATGCC		
78	10336-022	DMUK27R \ AshildKAndreassen	1 Each (G06)	6.60
	W6643G06	GTTGGAATCTGTGCAGTGCTTC		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
92	10336-022	DMUK34R \ AshildKAndreassen	1 Each (H08)	7.80
	W6643H08	TCCATACAGGAGAGATAGTCCCTG		
93	10336-022	KUPA4001F \ AshildKAndreassen	1 Each (H09)	7.30
	W6643H09	GGTCAAGAAGGCCATCCTGAAA		
94	10336-022	KUPA4001R \ AshildKAndreassen	1 Each (H10)	6.90
	W6643H10	CTGCATCCTTTCCTTCAGCTCT		
95	10336-022	KUPA4002F \ AshildKAndreassen	1 Each (H11)	8.50
	W6643H11	ATTACTCTGTTGGGGATGACGC		
96	10336-022	KUPA4002R \ AshildKAndreassen	1 Each (H12)	6.30
	W6643H12	TCCAGACCCATCCTTCAACTCT		
97	10336-022	KUPA4003F \ AshildKAndreassen	1 Each (B07)	5.10
	W6644B07	ACAAGGCGCTCAGTGCTGAT		
98	10336-022	KUPA4003R \ AshildKAndreassen	1 Each (B08)	6.60
	W6644B08	TACTCACGTGTCTTAGCAGGGT		
99	10336-022	KUPA4004F \ AshildKAndreassen	1 Each (B09)	5.50
	W6644B09	ATAACAGCTGAGGGGAAGCCT		
100	10336-022	KUPA4004R \ AshildKAndreassen	1 Each (B10)	6.10
	W6644B10	TTCCTCCCACTATGTGTCTCGT		
101	10336-022	KUPA4005F \ AshildKAndreassen	1 Each (B11)	6.40
	W6644B11	CGCCAACAAAATAGTGTACACGG		
102	10336-022	KUPA4005R \ AshildKAndreassen	1 Each (B12)	5.60
	W6644B12	GCCTTCAGTAACACTCCAGTCTG		
103	10336-022	KUPA4006F \ AshildKAndreassen	1 Each (C01)	7.70
	W6644C01	AGACTGGTTGAATTTGGGGCTC		
104	10336-022	KUPA4006R \ AshildKAndreassen	1 Each (C02)	6.00
	W6644C02	CTCTATGAAGCCACCTCCATTGTT		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
105	10336-022	KUPA4007F \ AshildKAndreassen	1 Each (C03)	6.10
	W6644C03	ACAAAGCCCTGTAGGATCCCA		
106	10336-022	KUPA4007R \ AshildKAndreassen	1 Each (C04)	6.00
	W6644C04	TCATATTCAGGCCCAACCAAGC		
107	10336-022	KUPA4008F \ AshildKAndreassen	1 Each (C05)	6.80
	W6644C05	TCCTTGGTGGTGCTTTCAACAG		
108	10336-022	KUPA4008R \ AshildKAndreassen	1 Each (C06)	7.40
	W6644C06	AGCCAGATTCAGTTCTGTGACC		
109	10336-022	KUPA4009F \ AshildKAndreassen	1 Each (C07)	7.90
	W6644C07	ACATTTGAGGAGGGAAGCTGTG		
110	10336-022	KUPA4009R \ AshildKAndreassen	1 Each (C08)	4.20
	W6644C08	TCCCATCACTCCTGTGTCACAT		
111	10336-022	KUPA40010F \ AshildKAndreassen	1 Each (C09)	5.70
	W6644C09	TTCACGGTGGCAGAATTCGG		
112	10336-022	KUPA40010R \ AshildKAndreassen	1 Each (C10)	6.90
	W6644C10	CACACTTGGCATTGATGGTAACG		
113	10336-022	KUPA40011F \ AshildKAndreassen	1 Each (C11)	7.70
	W6644C11	ATTCAGAACAGGTGAAAGGGCC		
114	10336-022	KUPA40011R \ AshildKAndreassen	1 Each (C12)	5.70
	W6644C12	CAAGCAGAGCGAGTACGACAAT		
115	10336-022	KUPA40012F \ AshildKAndreassen	1 Each (D01)	7.00
	W6644D01	GTGGCATTGTTTGTGGTCCTTG		
116	10336-022	KUPA40012R \ AshildKAndreassen	1 Each (D02)	4.50
	W6644D02	TCTTTCCTTCTGCCGTGCAAG		
117	10336-022	KUPA40013F \ AshildKAndreassen	1 Each (D03)	5.70
	W6644D03	GCCTTGAGGATCTCTGGAAATGG		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
119	10336-022	KUPA40014F \ AshildKAndreassen	1 Each (D05)	4.20
	W6644D05	CTCACCGAGGAAGACGATCTTTC		
120	10336-022	KUPA40014R \ AshildKAndreassen	1 Each (D06)	5.90
	W6644D06	CTCAGCATTTCCGTGAGCGT		
121	10336-022	KUPA40015F \ AshildKAndreassen	1 Each (D07)	6.20
	W6644D07	AATGATGGCTTTTTGGCTGCTG		
122	10336-022	KUPA40015R \ AshildKAndreassen	1 Each (D08)	8.10
	W6644D08	TGGACCTGTACTGTTTCACCTTTC		
123	10336-022	KUPA40016F \ AshildKAndreassen	1 Each (D09)	5.90
	W6644D09	TACCTACTGGGCTGATGTGAGG		
124	10336-022	KUPA40016R \ AshildKAndreassen	1 Each (D10)	6.80
	W6644D10	ATGTCCAGCACTGTGATCTGAC		
125	10336-022	KUPA40017F \ AshildKAndreassen	1 Each (D11)	7.40
	W6644D11	AAACTAATGAGACCTACGTCAGCAG		
126	10336-022	KUPA40017R \ AshildKAndreassen	1 Each (D12)	4.70
	W6644D12	CCATGATTGCCACCTCCCAAT		
127	10336-022	KUPA40018F \ AshildKAndreassen	1 Each (E01)	6.30
	W6644E01	CAATAGTCGACGTGATGTGCCA		
128	10336-022	KUPA40018R \ AshildKAndreassen	1 Each (E02)	5.80
	W6644E02	TCGAAGGTCTTGCTGTTCAAACA		
129	10336-022	KUPA40019F \ AshildKAndreassen	1 Each (E03)	6.30
	W6644E03	CCTTCGATTGCAAAAGGTGGTG		
130	10336-022	KUPA40019R \ AshildKAndreassen	1 Each (E04)	6.30
	W6644E04	GTTGTCAAGAAGTATTTGCGCCTC		
131	10336-022	KUPA40020F \ AshildKAndreassen	1 Each (E05)	6.60
	W6644E05	CAGGACGGACGAACAGATGAAT		

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CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
132	10336-022	KUPA40020R \ AshildKAndreassen	1 Each (E06)	6.00
	W6644E06	CTCTTTGAACATGCGTGCGTC		
133	10336-022	KUPA40021F \ AshildKAndreassen	1 Each (E07)	7.10
	W6644E07	GATCGAAGCTGGACATGGGAAG		
134	10336-022	KUPA40021R \ AshildKAndreassen	1 Each (E08)	6.10
	W6644E08	CTGATTGAAGTCCGGACGACAA		
135	10336-022	KUPA40022F \ AshildKAndreassen	1 Each (E09)	9.40
	W6644E09	GAGGCCTTTCTGACTATGGTTGAG		
136	10336-022	KUPA40022R \ AshildKAndreassen	1 Each (E10)	5.30
	W6644E10	ATTCGTCCATTCACTCCATGGC		
137	10336-022	KUPA40023F \ AshildKAndreassen	1 Each (E11)	6.70
	W6644E11	TTCTGGAGAAGACCAAGGCAGA		
138	10336-022	KUPA40023R \ AshildKAndreassen	1 Each (E12)	6.50
	W6644E12	AGACCTTGTGAGCTCTCTGTGT		
139	10336-022	KUPA40024F \ AshildKAndreassen	1 Each (F01)	4.40
	W6644F01	TTTGCTTGGCGGCATTCCAT		
140	10336-022	KUPA40024R \ AshildKAndreassen	1 Each (F02)	4.50
	W6644F02	TCGAAGCCAGAGTCTATGTCCA		
141	10336-022	KUPA40025F \ AshildKAndreassen	1 Each (F03)	5.30
	W6644F03	TGACACACTGTGGACGATGC		
142	10336-022	KUPA40025R \ AshildKAndreassen	1 Each (F04)	6.60
	W6644F04	GGATGCCGCATAATAGGACCAG		
143	10336-022	KUPA40026F \ AshildKAndreassen	1 Each (F05)	5.70
	W6644F05	TTGAGGAACGCGGATATGCC		
144	10336-022	KUPA40026R \ AshildKAndreassen	1 Each (F06)	6.50
	W6644F06	TGCTTCTATCACTTCTGGGCGA		

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Line No	Cat No/ID No.	Description/Sequence		Amount (OD)
145	10336-022	KUPA40027F \ AshildKAndreassen	1 Each (F07)	7.60
	W6644F07	GGAGCAATGGAAAAATAGGAACCC		
146	10336-022	KUPA40027R \ AshildKAndreassen	1 Each (F08)	5.00
	W6644F08	TACGGGTGTTCCTCGTCCAT		
147	10336-022	KUPA40028F \ AshildKAndreassen	1 Each (F09)	6.20
	W6644F09	GGACAAGGTGAAAGAACAAGACG		
148	10336-022	KUPA40028R \ AshildKAndreassen	1 Each (F10)	5.70
	W6644F10	GGCTGCATTTGATTTCACTTTTGC		
149	10336-022	KUPA40029F \ AshildKAndreassen	1 Each (F11)	7.40
	W6644F11	GGATTTTGGAACGACTGGCTCA		
150	10336-022	KUPA40029R \ AshildKAndreassen	1 Each (F12)	5.80
	W6644F12	AAGCTTATTCCCTCAACTCCAGC		
151	10336-022	KUPA40030F \ AshildKAndreassen	1 Each (G01)	5.50
	W6644G01	AGTTCGAGGCTCTTGGATTCCT		
152	10336-022	KUPA40030R \ AshildKAndreassen	1 Each (G02)	6.20
	W6644G02	TGAGGGTGTTAAGGGCATAGGT		
153	10336-022	KUPA40031F \ AshildKAndreassen	1 Each (G03)	6.50
	W6644G03	AGGCTGCATCATGGATGTCATC		
154	10336-022	KUPA40031R \ AshildKAndreassen	1 Each (G04)	5.40
	W6644G04	TCCTTCATCACTAGCTCGTGGA		
155	10336-022	KUPA40032F \ AshildKAndreassen	1 Each (G05)	5.80
	W6644G05	GAAGGACATTGGGGAATGGGAG		
156	10336-022	KUPA40032R \ AshildKAndreassen	1 Each (G06)	5.20
	W6644G06	AGGGTTGTCCAAAATCCACACC		
157	10336-022	KUPA40033F \ AshildKAndreassen	1 Each (G07)	6.30
	W6644G07	AGCTACTTCCATCGGCGAGA		

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Order Number		
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SOLD TO
FOLKEHELSEINSTITUTTET
FAKTURAMOTTAK DF?

POSTBOKS 4746 TORGARDEN
EHF:983744516
TRONDHEIM, 7468
NO
EHF:983744516

SHIP TO
FOLKEHELSEINSTITUTTET
AVD FOR MILJ?MEDISIN
FOLKEHELSA SENTRALLAGERET
LOVISENBERGGATA 8
?shild Kristine Andreassen
OSLO, 0456
NO
?shild Kristine Andreassen
10222617

CUSTOMER REP.	CUSTOMER P.O. NO.	CUSTOMER CONTRACT NO./ RELEASE NO.	PLACED BY:
CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence	Amount (OD)
158	10336-022	KUPA40033R \ AshildKAndreassen	1 Each (G08) 8.10
	W6644G08	AGATTATTGAGCTCTCCAGTCTGAG	

*** The following items are in production and will be shipped to you shortly ***

118	10336-022	KUPA40013R \ AshildKAndreassen	1 Each
		TGGGAGGTGTGTCGCATCAT	

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FOLKEHELSEINSTITUTTET
FAKTURAMOTTAK DF?

POSTBOKS 4746 TORGARDEN
EHF:983744516
TRONDHEIM, 7468
NO
EHF:983744516

SHIP TO
FOLKEHELSEINSTITUTTET
AVD FOR MILJ?MEDISIN
FOLKEHELSA SENTRALLAGERET
LOVISENBERGGATA 8
?shild Kristine Andreassen
OSLO, 0456
NO
?shild Kristine Andreassen
10222617

CUSTOMER REP.	CUSTOMER P.O. NO.	CUSTOMER CONTRACT NO./ RELEASE NO.	PLACED BY:
CKULKAR	60005450		chandrakant kulkarni

Line No	Cat No/ID No.	Description/Sequence	Amount (OD)
118	10336-022	KUPA40013R \ AshildKAndreassen	1 Each (A01) 8.60
	W6664A01	TGGGAGGTGTGTCGCATCAT	

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Appendix 10: All TBEV positive samples with Ct value from Realtime PCR:

Sample name	Collection Month	CT value from In-house Realtime PCR	Pyrosequencing result
S5(21)-N42	April	35.76	Confirmed True
S5(21)-N48	April	34.37	Confirmed True
S5(21)-N56	April	33.96	Confirmed True
S5(21)-N67	April	38.9	Confirmed True
S5(21)-N69	April	36.16	Confirmed True
S5(21)-N138	May	35.82	Confirmed True
S5(21)-N179	June	35.42	Confirmed True
S5(21)-N201	June	34.85	Confirmed True
S5(21)-N209	June	34.86	Confirmed True
S5(21)-N211	June	35.29	Confirmed True
S5 (21)- N218	June	47.24	Negative
S5 (21)- N220	July	30.88	Confirmed True
S5(21)-N222	July	35.82	Confirmed True
S5(21)-N228	July	34.03	Confirmed True
S5(21)-N229	July	34.82	Confirmed True
S5(21)-N231	July	36.68	Confirmed True
S5(21)-N235	July	28.94	Confirmed True
S5(21)-N236	July	33.21	Confirmed True
S5(21)-N237	July	31.12	Confirmed True
S5(21)-N240	July	32.13	Confirmed True
S5(21)-N241	July	33.27	Confirmed True
S5(21)-N242	July	34.06	Confirmed True
S5(21)-N277	July	35.64	Confirmed True
S5(21)-N278	July	36.56	Confirmed True
S5 (21)- N309	August	33.96	Confirmed True
S5 (21)- N317	August	35.88	Confirmed True
S5 (21)- N321	August	34.37	Confirmed True
S5 (21)- N322	August	36.26	Confirmed True
S5 (21)- N324	August	34.93	Confirmed True
S5 (21)- N325	August	35.34	Confirmed True
S5 (21)- N328	August	37.74	Confirmed True
S5 (21)- N337	August	38.61	Confirmed True
S5 (21)- N383	August	37.12	Negative
S5 (21)- N388	August	36.69	Negative
S5 (21)- N423	September	36.23	Negative
S5 (21)- N432	September	34.28	Confirmed True
S5 (21)- N437	October	34.72	Confirmed True
S5 (21)- N447	October	36.73	Confirmed True
S5 (21)- N473	October	35.8	Confirmed True
S5 (21)- N477	October	36.36	Confirmed True
S5 (21)- N481	October	36.38	Confirmed True
S5 (21)- N503	October	36.55	Negative