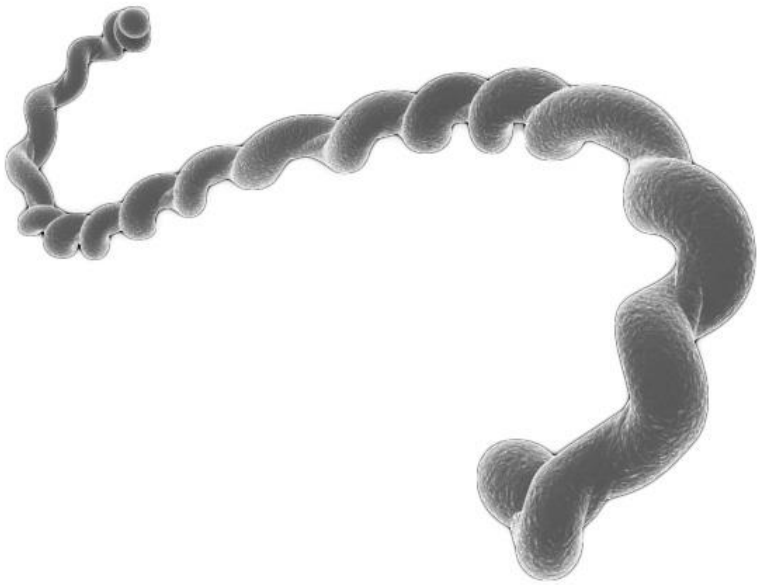
VALIDATION OF THE CURRENT RT-PCR ASSAY USED TO DETECT LEPTOSPIRA DNA

Bachelor Thesis in molecular and medical biology

In Cooperation with the Department of Microbial Surveillance and Research Staten's Serum Institution



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Front page Picture (1)

Resumé

Leptospirosis is a widespread zoonosis caused by the gram negative-like bacteria, *Leptospira.* The disease was first described in 1886 by the physician Adolf Weil and was named as Weil's disease. The bacteria can cause infection resulting in disease in both humans and animals with common outbreaks in Asia, Central and South America and parts of Europe. Leptospirosis mainly occurs in tropical and subtropical environments with heavy rain fall. A variety of factors may be important in the transmission of the disease such as climate and seasonal changes and the access of clean water. Thus, geographical locations and ecology systems may play an important role in the transmission of leptospirosis, the disease is often observed in rodents. Rodents act as a source of infection for other animals and human beings, since they can harbor the disease for many years without clinical manifestations. Generally, leptospirosis is found in humans when in contact with water contaminated with urine excreted from infected rodents. In addition, workers such as farmers, sewers and outdoor waters athletes are at higher risk of getting leptospirosis. Furthermore, the disease can cause major economic and ecological problems, since it can result in abortions, reduced milk production and death in mammalian animals such as cows, pigs, sheep, goats etc. The bacteria *Leptospira* is long, thin and helically coiled with high motile abilities. They are known for their axial flagellum that rotates and creates a clockwise movement that allows them to survive in inhospitable microenvironments. The bacteria consist of a double membrane structure of cytoplasmic and outer membrane, where the outer membrane is made up of lipoproteins and transmembrane proteins. These proteins are of great importance since their composition contributes to the different types of *Leptospira*. *Leptospira* can be classified into different species depending on their molecular structure. Their molecular structure is determined by genotypic classification with different genetic methods including DNA hybridization strategies and sequencing. The genotypic classification of *Leptospira* are very important since this contributes to the differentiation between the pathogenic, intermediate and non-pathogenic species. Furthermore, the species can also be classified into serovar, serogroups and strains with serological classification. However, these two classification strategies are incompatible since the serovar does not necessarily determine whether a *Leptospira* sp. is pathogenic or non-pathogenic and one serovar can belong to several *Leptospira* spp. and vice versa. This may cause confusion for clinicians and epidemiologists. Moreover, the most abundant
pathogenic *Leptospira* specie is known as *Leptospira Interrogans* and this specie has prevalently shown to cause disease in animals. *Leptospira Interrogans* contains over 200 serovar's including;

Bratislava, Hardjo, Hebdomadis and Pomona, where their genome comprises of 4.6 million base pairs. *Leptospira Interrogans*' genomes contain two 16S rRNA genes that are very important in genetic diagnostics and a gene for lipoprotein 32 (LipL32), which is the most dominant lipoprotein of the outer membrane. LipL32 has been proofed to be absent in non-pathogenic *Leptospira* species and is therefore thought to be a virulent factor only present in pathogenic and intermediate species. There are various of virulence genes and their correlated proteins that has been identified in Leptospira, however most of their functions are still not clear. The most common virulence factors are chemotaxis, motility and surface proteins that contributes to host invasion and tissue damaging. When the bacteria have entered the host, it can cause severe damage to the kidney's and lead to other fatal complications, if not discovered and treated. However, the disease is often difficult to recognize due to a wide variety of clinical symptoms ranging from mild flu like illness to severe symptoms as observed in Weil's disease. Leptospirosis is a variable disease and can be divided into different phases such as, incubation period, septicemic phase, interphase and immune phase.

The incubation phase begins from day 2-10 where the bacteria first entered the host. Consequently, if the bacteria are not discovered the patient will experience severe symptoms which is also called the immune phase. Therefore, culturing of *Leptospira* from patient urine samples are only possible if the samples are taken at the end of week one or the beginning of week two of the illness and blood samples are collected at the immune phase where antibody production is the highest and can be used in several diagnostic methods. The most common used diagnostic methods are serological and molecular genetics such as, MAT, ELISA and PCR. These methods each have their advantages and limitations, nevertheless PCR has shown to be more sensitive in the early phase of the disease. This method has a sensitivity of 28-96% in severe leptospirosis when applied to whole blood samples. Moreover, when testing on urine samples the sensitivity of PCR ranges from 42-86% in the acute phase of the disease but has shown to be highest in the late phase of the illness. To attain a high sensitivity when using PCR, samples must be obtained before or shortly after treatment of antibiotics since antimicrobials quickly remove *Leptospira* spp DNA. Furthermore, there are still an urgent need of PCR assays that must be validated and tested on relevant species and specimens of *Leptospira*. For this reason, the thesis is carried out to validate the current RT-PCR assay that is used for diagnosis of leptospirosis in humans and confirm that the assay can also detect *Leptospira* in animal samples at SSI. Moreover, RT-PCR has shown to be a quicker method and involves less risk of contamination than conventional PCR, during preparation. For this relevancy, a validation of the RT-PCR assay will be determined by generating four different studies which all are aimed to detect *Leptospira* DNA in

animal samples. The RT-PCR assay consist of two different master mixes a primary that targets *LipL32* and a confirmatory that targets *16S* genes in *Leptospira* spp. RT-PCR assay were only performed on extracted DNA from cultivated *Leptospira* strains, urine and histological tissue from pigs and dogs.

In study one, a primer blast and sequence alignment where performed on the primers LipL32 and 16S used to detect Leptospira DNA in the RT-PCR assay, along with their probe. The aim where to assure the primers ability to anneal to specific *Leptospira* strains of clinical relevance. The strains were; Bratislava, Hardjo, Hebdomadis and Pomona. The primer blast revealed that both primers were able to anneal to all the strains mentioned above. Additionally, the probes were localized in between the primers and were used as a fluorescent signal during the RT-PCR reaction. Furthermore, primer blast was also used to reveal if the primers had any possible homology with other prokaryotic and eukaryotic organisms. However, it was not obtainable to check for primer specificity for the forward and reverse primer collectively. Consequently, the forward and reverse were check separately which revealed several unintended targets. Moreover, the sequence alignment that was performed on the *16S* gene were performed on several strains and spp. of *Leptospira*. The alignment demonstrated great compliance of the probe, forward and reverse to each target sequence, except for Patoc I. In addition, the strain Patoc I belongs to the saprophytic spp. Of *Leptospira* and the alignment revealed that the strain had several nucleotide differences with the other strains and miss matches the probe, forward and reverse primer. In study two Patoc I was also not amplified during the RT-PCR reaction. In study two, the RT-PCR assay were tested on different sample material such as urine from pigs and histological tissue from pigs and dogs and ultimately several strains and ssp. of *Leptospira*. The assay where able to detect all strains and spp. of *Leptospira* that are relevant in the diagnosis of leptospirosis due to their pathogenic abilities. This by being able to discriminate between pathogenic and non-pathogenic spp. Of *Leptospira*. Moreover, the RT-PCR assay were also able to detect *Leptospira* DNA in animal samples such as kidney tissue from pigs and dogs. Nevertheless, the RT-PCR assay had conflicting results when analyzing urine from pigs. The LipL32 assay did not have any amplification in the 62-urine samples implying “negative” whereas the 16S assay did have amplification in the 62-urine sample implying “positive” for *Leptospira* DNA. These findings were confirmed in another study indicating that other prokaryotic organisms have been detected by the 16S assay. In addition, the same study executed sequencing on the PCR product conducted from the 16S assay and revealed Peptostreptococcus stomatitis and P. anaerobius to be amplified during the reaction.

However, in this project a further investigation was not carried out to confirm these findings. In study three, the aim was to examine the robustness of the assay by applying machine and human variation to the RT-PCR assay. Initially, an identical RT-PCR setup were generated and tested on two different RT-PCR machines. In addition, the setup where performed in triplicates by three different technicians.

The results obtained were satisfying by which no significant variance was observed regarding machine or human variation to the RT-PCR assay. In study four, the goal sought to investigate the sensitivity and to set a detection limit of the RT-PCR assay. This was achieved by generating a serial dilution of the positive *Leptospira* control in a range from 10^3-10^8. The results revealed that the assay where able to detect Leptospira DNA down to a 10^5-10^6 sample dilution, however detection of the10^6 sample dilution was inconsistent due to the low amount of DNA in the dilution. To possible prevent this issue, the concentration of target DNA could be increased in the DNA extraction or by counting Leptospira cells per in a sample dilution. However, this project did not investigate the exact number of *Leptospira* cells the RT-PCR assay where able to detect, due to several complications that implies with count of *Leptospira* cells such as: risk of getting infected, complication in cultivating live cultures of *Leptospira* and difficulties by counting live cells due to high motility. Overall the results obtained in this project concludes that the assay LipL32 and 16S collectively, demonstrate high performance when detecting several *Leptospira* strains and spp., discriminating between pathogenic and nonpathogenic spp. of *Leptospira*, detecting *Leptospira* DNA in different animal tissue such as pig and dog's kidney tissue, capabilities to withstand both machine and human variation and ultimately, detecting *Leptospira* DNA as far to a 10^5-10^6 sample dilution. Therefore, these findings highlight the RT-PCR assay to exhibit favorable attributes in the detecting of *Leptospira* and thereby, determination and diagnosis of leptospirosis. Moreover, within the last decade major progress has been made in research and detection of *Leptospira*. However, important knowledge is still missing due to incomplete and delayed whole genome sequencing of all *Leptospira* spp. Therefore, there an urgent need for knowledge regarding the molecular structures and virulence factors of *Leptospira*. This comprehensive knowledge can help and assist the fully understanding of *Leptospira's* ability to cause infection in human and animals. Thereby, eventually elevate the methods used to detect *Leptospira* and to optimize the diagnosis of leptospirosis.

Abstract

To date serval detection methods has been utilized in the diagnostics of leptospirosis. It is often difficult to determine leptospirosis since it has shown to exhibit various clinical symptoms ranging from mild flue like manifestations to more severe as kidney and multi-organ failure and in worst cases death. In addition, Leptospirosis has also shown to mimic other diseases which moreover, complicates the diagnostics. The detection methods used today are either based on serological methods such as ELISA and MAT or genotypic methods such as PCR. Both detection methods are highly relevant in the diagnosis of leptospirosis depending on the state of the disease. Consequently, PCR has shown to be most sensitive in the early stages of illness whereas serological methods have greater sensitivity in later stages. This project is a collaboration with Staten's Serums Institution to expand the current RT-PCR assay used to detect *Leptospira* DNA that are pathogenic in mammalian animals. The current RT-PCR assay LipL32 and 16S are today applied to only human samples such as blood, cerebrospinal fluid and urine and the desire is to expand the assay to also include animal samples. Therefore, the current RT-PCR assay has been tested on several parameters. The results obtained in this project has demonstrated that the LipL32 and 16S assays have abilities to detect several *Leptospira* strains and spp. Including detection of *Leptospira* DNA in kidney tissue from pigs and dogs. Moreover, the LipL32 and 16S assays has illustrated to discriminate between pathogenic and nonpathogenic *Leptospira* spp. Nevertheless, the 16S assay appeared to be less specific by detecting what seemed to be other bacteria's in urine samples from pigs.

Abstrakt på Dansk

Hidtil anvendes adskillige detekteringsmetoder i diagnosticeringen af leptospirose. Det er ofte vanskeligt at diagnosticere leptospirose, da sygdommen har udvist forskellige kliniske symptomer, der spænder fra mild forkølelses lignende manifestationer til mere alvorlige som nyrer og multiorgan svigt og i værste fald død. Hertil kommer, at leptospirose også har vist sig at efterligne andre sygdomme, som desuden komplicerer diagnostikken. De påvisningsmetoder, der anvendes i dag, er enten baseret på serologiske metoder såsom ELISA og MAT eller genotypiske metoder, såsom PCR. Begge påvisningsmetoder er meget relevante ved diagnose af leptospirose afhængigt af sygdommens forløb. Dertil har PCR vist sig at være mest følsomme i de tidlige stadier af sygdom, mens serologiske metoder har større følsomhed i senere stadier. Dette projekt er et samarbejde med Statens Serums Institut, for at udvide det nuværende RT-PCR assay anvendt til at detektere *Leptospira* DNA, der er patogen hos pattedyr. Den nuværende RT-PCR assay LipL32 og 16S anvendes i dag kun til humane prøver, såsom blod, spinalvæske og urin, og ønsket er at udvide assayet til også at omfatte dyreprøver. Derfor er det nuværende RT-PCR assay blevet testet på adskillige parametre. Resultaterne opnået i dette projekt har vist, at LipL32- og 16S assay har evner til at detektere adskillige *Leptospira* stammer og spp. Herunder detektion af *Leptospira* DNA i nyrevæv fra svin og hunde. Desuden har LipL32- og 16S-assay vist sig at diskriminere mellem patogene og ikke-patogene *Leptospira* spp. Ikke desto mindre syntes 16S assay at være mindre specifik ved at detektere, hvad der syntes at være andre bakterier i urinprøver fra svin.

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Indholdsfortegnelse

Preface

This project is an experimental Bachelor thesis within Molecular and Medical Biology and is carried out in the fall 2018 semester at Staten's Serum Institution and Roskilde University. This following thesis is intended for an audience with a bachelor's level understanding of biology, particularly molecular and medical biology. The initial work provides information about the current RT-PCR assay that is used for diagnostic purposes of leptospirosis at SSI.

Acknowledgements

We would like to give a huge thanks to our supervisor Håvard Jenssen (PhD) at Roskilde University (RUC), for providing us with guidance and invaluable input towards our project. We would also like to state our thanks and appreciation for Karen Angeliki Krogfelt (PhD), Randi Føns Petersen (PhD) (section manager) and Øystein Angen (PhD) at the Department of Microbial Surveillance and Research Staten's Serum Institution for not only helping us with our project but allowing us to use their laboratory facilities to aid our investigations. Furthermore, we would like to thank Gitte (technician) and Ole Skovgaard (PhD) for providing us with invaluable help that have impacted this project. Their time and effort spent has been of great importance. Finally, a big thanks to RUC. We would like to thank RUC for allowing us to study such an interesting field within our bachelor's degree.

Reading guide

The following reading guide is intended to deliver a concise understanding of how the ensuing project should be read. Gene, bacteria and specie nomenclature are empathized in italics throughout the text. Furthermore, the most repeated terms are listed in the abbreviation list and figure citations are empathized in bold.

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Abbreviation list

MAT	Microscopic agglutination test
RT-PCR	Real-time polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
LipL32	Lipoprotein 32
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
SPP	Species
SP	Specie
SSI	Staten's Serum Institution
IK	Internal control

Introduction

To date leptospirosis is one of the most widespread zoonosis, with an estimate of over one million cases reported each year worldwide (2).The disease is caused by the pathogenic bacteria *Leptospira* and has shown greater incidence in tropical and subtropical areas with heavy rainfall. *Leptospira* can cause infection in both humans and animals but specific mammalian spp. such as rodents, can harbor the disease for many years without clinical manifestations. Rodents are a major and common reservoir for *Leptospira* and act as a source of infection for other animals and human beings (3). The epidemiology of leptospirosis can cause significant economic and ecological problems, since it can result in abortions, stillbirths, infertility, failure to thrive, reduced milk production and death in animals such as cows, pigs, sheep, goats etc. (4). These major problems have received significant publicity and resurgent the interest in leptospirosis (5). Generally, leptospirosis is an underdiagnosed disease because of the nonspecific symptoms early in the illness. For these purposes, several diagnostic approaches have been developed for early and definitive diagnosis of leptospirosis. An early definitive diagnosis allows clinicians to improve supportive treatments and provide more optimal prognosis of the disease (6). Nevertheless, even a late diagnosis of leptospirosis is significant for both patient and society, since it can define groups at risk for leptospirosis and determine the epidemiology of the disease. Therefore, methods with high sensitivity and specificity are sought for in the diagnosis of leptospirosis. The most common diagnostic approaches used are serological methods such as microscopic-agglutination test (MAT) and enzyme-linked immunosorbent assay (ELISA). However, these methods have shown to be less sensitive in the acute phase of the disease and in notably diluted test samples. Furthermore, MAT is also complex and difficult to standardize which therefore requires a more robust, sensitive and specific method. For the last 20 years, polymerase chain reaction (PCR) has been proved to be more sensitive and specific for early detection of *Leptospira* compared to serology. However, there are several limitations that still needs to be tested such as the specificity of the assay since *Leptospira* spp. cannot be completely differentiated due to the *16S ribosomal ribonucleic acid* (RNA) gene that is present in different organisms. Additionally, the sensitivity of the assay may also have some limitations depending on the phase of the disease and on the amount of deoxyribonucleic acid (DNA) present in a test sample. Furthermore, there is an urgent need of PCR assays that must be validated on all relevant spp. and specimens of *Leptospira*. With this knowledge, it is of relevance to validate PCR assays and potentially optimize the performance of the assay in the diagnosis of leptospirosis.

In this thesis, the aim is to validate the current real-time PCR (RT-PCR) assay that is used for leptospirosis in humans at SSI and confirm that the current assay can also detect *Leptospira* strains that are pathogenic in mammalian animals. SSI routinely performs leptospirosis test in humans and for this relevancy, a validation of the sensitivity, robustness and primer quality of the assay is performed.

The validation of the RT-PCR assay will be determined by performing four different studies that includes the following:

Study 1: To evaluate the performance and quality of the current primers lipoprotein 32 (LipL32) and 16S used in the RT-PCR assay and possibly review homology with other prokaryotic and eukaryotic organisms, in primer blast and by sequence alignment.

Study 2: To validate the diagnostic sensitivity of the RT-PCR assay by including a broad panel of *Leptospira* strains and different *Leptospira* specimens such as urine and tissue.

Study 3: To validate the robustness of the RT-PCR assay by implying machine and human variation and analyze the data with statistical approaches.

Study 4: To validate the sensitivity of the RT-PCR assay by determining the detection limit in a 10-fold serial dilution of the positive *Leptospira* control sample.

Thesis statement

How is the performance of the current RT-PCR assay when detecting DNA of different *Leptospira* strains and spp. in urine and tissue samples obtained from mammalian animals and can the assay be validated?

Theoretical considerations

Initially, it was essential to find empirical data and reliable studies to support our theory section. The empirical data was collated by searching on scientific databases including: PubMed and NCBI. A strategical research was made on these databases by using different word combinations that were relevant for our thesis, such as: '*Leptospira*', 'leptospirosis' and 'diagnostic methods'.

In the interest of selecting the current and most relevant studies, some inclusion's criteria were established including research conducted within the last 10 years.

Methodical considerations

To collate the experimental data, four different studies were performed to give the most reliable data to resolve our thesis statement. The experiments were made by initially performing primer blast and sequence alignment on four Leptospira strains for pathogenic reasons, to determine the quality and performance of the primers LipL32 and 16S used in the RT-PCR assay. Moreover, after assuring the performance of the primers, *Leptospira* DNA was extracted from urine and tissue samples of animals. The DNA was used in the RT-PCR assay where two separate mister mixes were created and used to target the *LipL32* and *16S* genes to detect *Leptospira*. Four different RT-PCR assays were created with the aim to validate and assure the performance of the RT-PCR used to detect *Leptospira* DNA in samples. The studies executed were performed at SSI by using their laboratory facilities with their assistance.

Limitations

A limited time frame was set from February till June 2018 and the experiments were executed within three weeks at SSI. The project was limited to an amount of test samples which resulted in limited data to draw solid conclusions upon.

Theory

In this project, the theory given provides the fundamental background knowledge required to understand the methodology, results and conclusion. The theory includes a review of the structure and morphology of *Leptospira*. Moreover, the complex taxonomy of *Leptospira*, previously based on serology and recently modified by a genotypic classification is presented, and the clinical and epidemiological value of molecular diagnosis (PCR) is also given and described.

Leptospira structure and morphology

Leptospira bacteria are long, thin and helically coiled bacteria with looped ends. They are highly motile with a travelling speed up to 20µm in 2-3 seconds in ordinary media. The pathogenic spp. consist of an axial flagellum (7). Studies (8) have shown that *Leptospira* spp. can withstand osmotic changes, due to its high motility. The flagellum rotates and creates a clockwise propel or wave movement, as seen in **figure 1.1**. They have an average diameter of 0.1µm with a helical amplitude of 0.1-0.15µm and a length of 6-20µm. *Leptospira* are gram-negative like bacteria consisting of a double membrane structure of cytoplasmic and outer membrane. The cytoplasmic membrane contains peptidoglycan that are overlain by the outer membrane. The outer membrane is made up of lipoproteins and transmembrane proteins, and the composition of these proteins may be important for adhesion of Leptospira to the host tissue. In addition, the outer membrane contains lipopolysaccharide (LPS) which contributes in the difference of the numerous serovar's of *Leptospira* spp. (8).

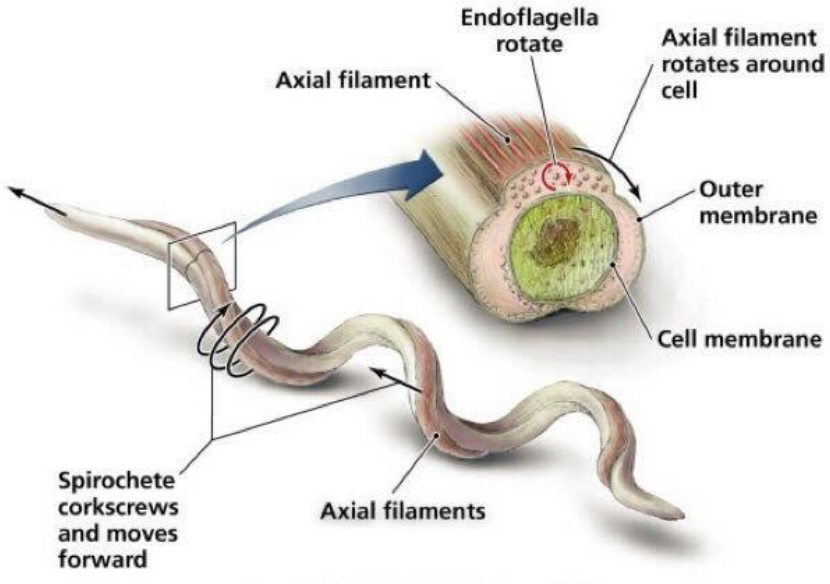

Figure 1.1. The structure of *Leptospira* bacterium and the rotation of the axial flagellum (filament).(9) The bacteria consist of an outer membrane, cell membrane and axial filament. The axial flagellum rotates and creates a clockwise propel movement. The bacterium has an average diameter of 0.1µm.

Growth conditions of *Leptospira*

Several media's can be used to culture and grow *Leptospira* strains including serum or albumin media. However, Leptospira are typically cultivated in Ellinghause-McCullough-Jonhson-Harris media (EMJH) and is a slow growing bacterium (10). The media is usually composed of rabbit serum, vitamins B1 and B2, ammonium salts and fatty acids that are metabolized by beta oxidation. Most pathogenic Leptospira have an optimum growth temperature between 28-30º C, with an optimal pH value between 7.2-7.6 (11). Visualization of Leptospira is best done with dark field microscopy (12).

The early discovery of *Leptospira* and Weil's disease

Leptospira is a bacterium that can cause an infection called leptospirosis and was first officially reported in 1886 by the German physician Adolf Weil. The physician described the infection as a specific type of jaundice accompanied with renal failure. Subsequently, the disease was named after the physician as Weil's disease (13). Prior to Adolf Weil, the etiology of the disease was unknown however, *Leptospira* appeared to be infectious in aquatic environments. *Leptospira* was first officially visualized with a histological silver-staining technique by Stimson in 1907 as seen in **figure 1.2.** Stimson named the organism *Spirocheta Interrogans* due to its spiral tale that is a characteristic mark of the bacterium (13).

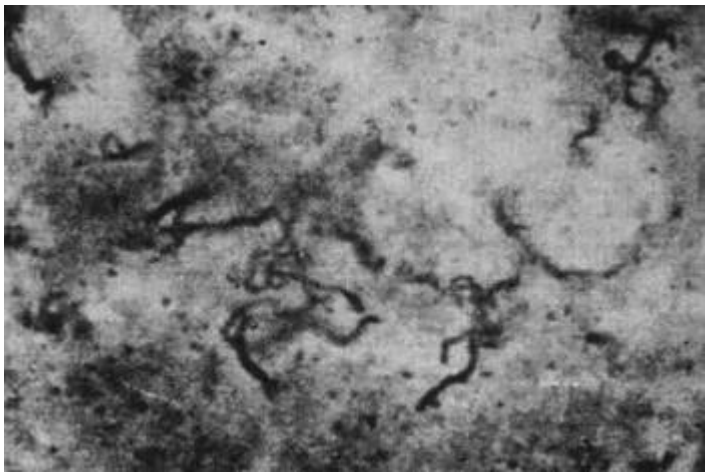

Figure 1.2 Stimson's original discovery of *Spirocheta Interrogans* from Kidney tissue of a Weil's disease victim (13).

In the following decades, major progressions were made in the understanding of leptospirosis. By 1950's many different *Leptospira* spp. Were discovered and it became clear that a classification system was necessary to differentiate between the different *Leptospira* strains. Noguchi was the first to propose the genus name *Leptospira* to allow differentiation from the Spirochete found in Weil's disease patients. He then published a systematic description of *Leptospira* morphology compared to the Spirochete. *Leptospira* are now put into the family of *Leptospiraceae* belonging to the class *Spirocheates* in the order *Spirocheatales* as seen in **figure 1.3** (13).

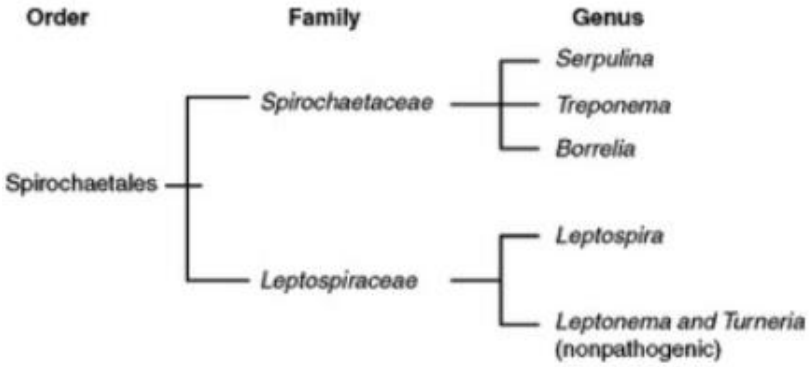

Figure 1.3. The earliest classification system divided in order, family and genus proposed by Noguchi (14).

Nevertheless, classification of the genus *Leptospira* has been complex and is still undergoing revision until date. Currently, two separate classification systems are used including the "gold standard" phenotypic classification based on serotyping and a genotypic classification based on deoxyribonucleic acid (DNA) relatedness (14).

Serological classification

In the traditional serologic classification, *Leptospira*'s are divided into two main spp. the pathogenic (*L. Interrogans*) sp. and non-pathogenic, saprophytic sp. (*L. Biflexa*). Since both spp. are morphologically similar, it is important to distinguish between them to prevent false positive results.

With serological classification, *Leptospira* spp. are classified into serovar's and organized in serogroups based on shared antigenicity or on the composition of LPS on the cell surface (14). This is done according to the microscopic agglutination test (MAT), where the pathogenic sp. comprises of 260 serovar's organized into 23 serogroups and the saprophytic of 60 serovar's organized into 28 serogroups (14,15) The serogroups have no taxonomic meaning but have been useful for initial serological diagnosis and epidemiological understanding (6). The serovar's however is of great importance sine it determines the outcome of the infection depending on the host (12). However, it has been found that there is a poor correlation between serovar's and their pathogenic and non-pathogenic abilities. The serovar does not necessarily determine whether a *Leptospira* sp. is a pathogenic or non-pathogenic sp., since one serovar can belong to several *Leptospira* spp. and vice versa (6). Therefore, studies have found a more accurate way to differentiate between the *Leptospira* spp. by genotypic classification. In genotypic classification the pathogenic and non-pathogenic serovar's are placed in the same spp. (5).

Genotypic classification

Within the last decade modern technology such as whole genome sequencing has facilitated to wider understanding of identification and characterization of *Leptospira* spp. (16).

In genotypic taxonomy and classification, *Leptospira* spp. are differentiated by genetic methods including DNA hybridization techniques and 16S rRNA sequencing (5) (17). Since then, over 200 *Leptospira* strains has been sequenced, which has first and foremost cleared up some miss-classification of different *Leptospira* strains. Subsequently, also revealed that *Leptospira* spp. not only consist of pathogenic and non-pathogenic strains but also a third intermediate group as seen on **figure 1.4** (16). This reclassification of *Leptospira* spp. Provides a strong foundation for future classification however, genetic classification is also problematic since its incompatible with serological classification and may cause confusion for clinicians and epidemiologists' (5).

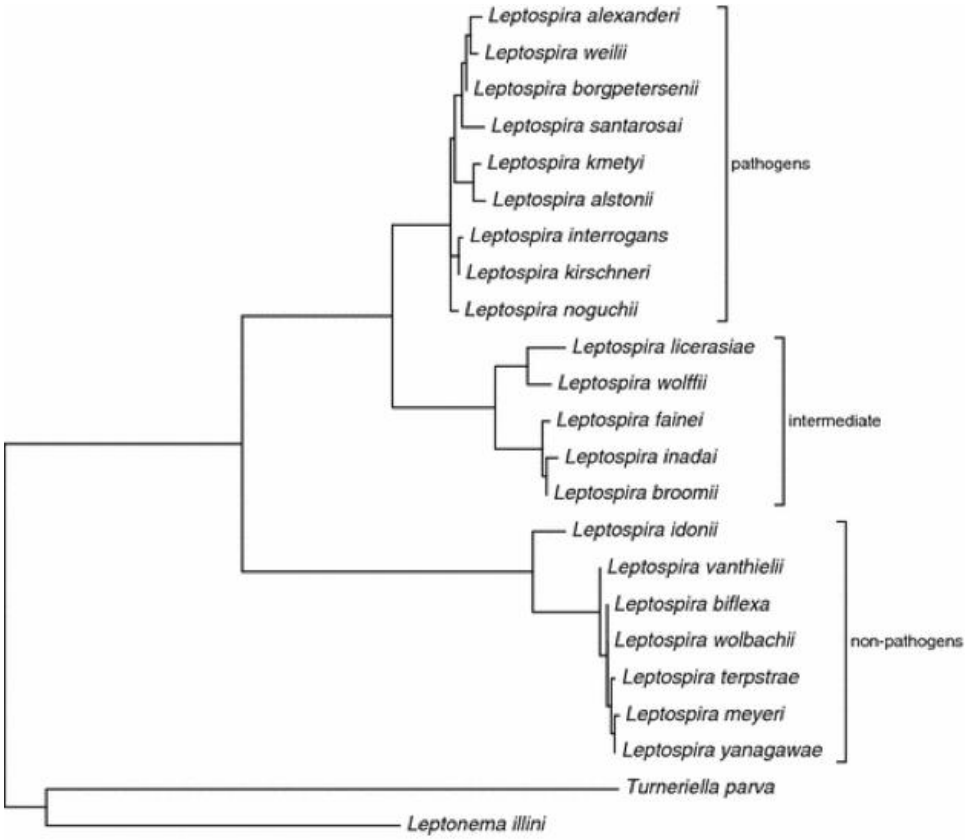

Figure 1.4. Genotypic classification system of *Leptospira* divided into pathogenic, intermediate and non-pathogenic spp. (16) (modified for relevancy to this study).

L. Interrogans molecular structure

Leptospira Interrogans is one of several pathogenic bacteria spp. that contains over 200 serovar's including; Bratislava, Hardjo, Pomona and Hebdomadis. Most pathogenic Leptospira spp. have a large genome consisting of two circular chromosomes with a total of 4.6 million base pairs (mbp). Chromosome I with 4.2 mbp and Chromosome II with 3.5 thousand bp (4). The *L. Interrogans* genome comprises a relatively large number of motility and chemotaxis genes (4). Furthermore, *Leptospira* genomes contain two *16S rRNA* genes, found on chromosome I, two *23S rRNA* genes and only one *5S rRNA* gene. Since Leptospira genomes vary in their molecular structure in pathogenic and non-pathogenic species, *16S rRNA* gene sequencing is a powerful method for identification in the clinical laboratory and offers a simplified approach to the identification of Leptospira spp. Another approach for diagnostic and identification of *Leptospira* spp. is the detection of the LipL32 (18). The intermediate- and pathogenic *Leptospira* spp. contain a lipoprotein LipL32, which is the

most abundant and dominant lipoprotein of the outer membrane. In addition, among saprophytic spp. LipL32 is absent and therefore is thought to be a virulent factor. (4)

Lipoprotein 32 in *L. Interrogans*

LipL32 is 32 kilo Dalton (kDa) and located on chromosome I of *L. Interrogans* with an estimate of 38.000 copies per cell. LipL32 make up 20 % of the outer membrane proteins pr. cell in Leptospira. Such high levels of LipL32 are potentially to demand a large metabolic cost for the cell, suggesting an important function of the protein (17,19,20). LipL32 exhibit the ability to bind to certain components in the extracellular matrix such as laminin, collagen and fibronectin. Studies has also shown that LipL32 possesses abilities to activate pathways that leads to production of pro-inflammatory cytokines (20,21). However, these studies have only been performed in vitro. In addition, strong evidence has suggested that Lipl32 may play an important role during infection due to the high expression of the gene. Additionally, the same studies have also shown that even when the gene is mutated, a lethal infection can still occur suggesting that Lipl32 is not an essential part of an infection (20). Consequently, Haake D.A (20) challenges the thought of LipL32 being located on the outer membrane, and suggesting that LipL32 appears to be in the periplasmic leaflet of the outer membrane;
"the abundance of LipL32 contributed greatly to its unfortunate misidentification as a surface lipoprotein"

Understanding of *Leptospira* virulence factors such as Lipl32, has lagged some way behind and resulted in an uncertain knowledge of how these factors may contribute to the pathogenic abilities of *Leptospira*. Therefore, more studies need to be conducted about the specific function and molecular structure of Lipl32 (21). The findings and understanding of Lipl32 and other surface exposed proteins may help in the understanding of the virulence of Leptospira and consequently Leptospirosis.

Leptospirosis

Leptospirosis is the most world spread zoonosis caused by the bacteria *Leptospira* (22). It can cause infection resulting in disease in both humans and animals. Observed from **figure 1.5**, the disease has become an emerging infectious disease with outbreaks in Asia, Central and South America and parts of Europe. Consequently, leptospirosis can result in acute kidney injury that also can lead to multiple organ failure and in worst cases death (3,21,23).

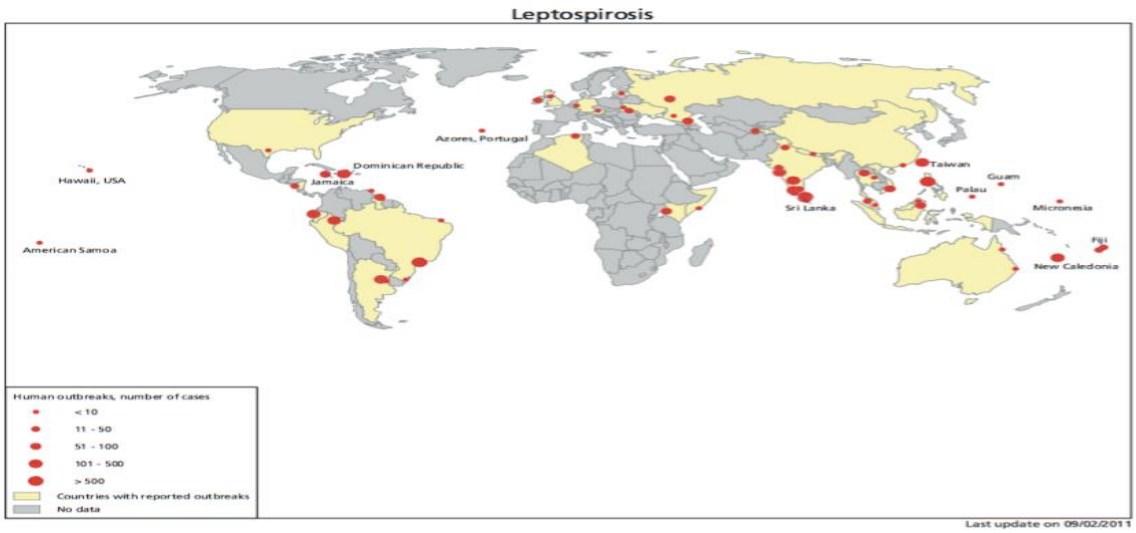

Figure 1.5. Leptospirosis human outbreaks worldwide. The countries with reported outbreaks are marked yellow and the countries with no data are marked grey (23).

Epidemiology and risk factors

Leptospirosis mainly occurs in tropic and subtropical environments with heavy rainfall. The disease is generally found in humans when in contact with water contaminated with urine excreted from infected rodents. Between "1994-2018" 200 cases of Leptospirosis have been registered in Denmark as seen in **figure 1.6**. Additionally, study (24) from 2012 shows that 2.3% out of 170 victims died of complications as a result of Leptospirosis, in Denmark from 1980-2012 (24). The people in the greatest risk of being infected are occupational groups such as farmers, sewer workers, coal miners, fishers and people that practices water sports (25).

A variety of factors may play a role in the transmission of the disease such as climate and seasonal changes, and the access of clean water sources. Thus, geographical locations and ecology systems

may play an important role in the spread of leptospirosis. The spread of leptospirosis is often observed in slum areas of developing countries due to interaction between infected rodents and humans (17).

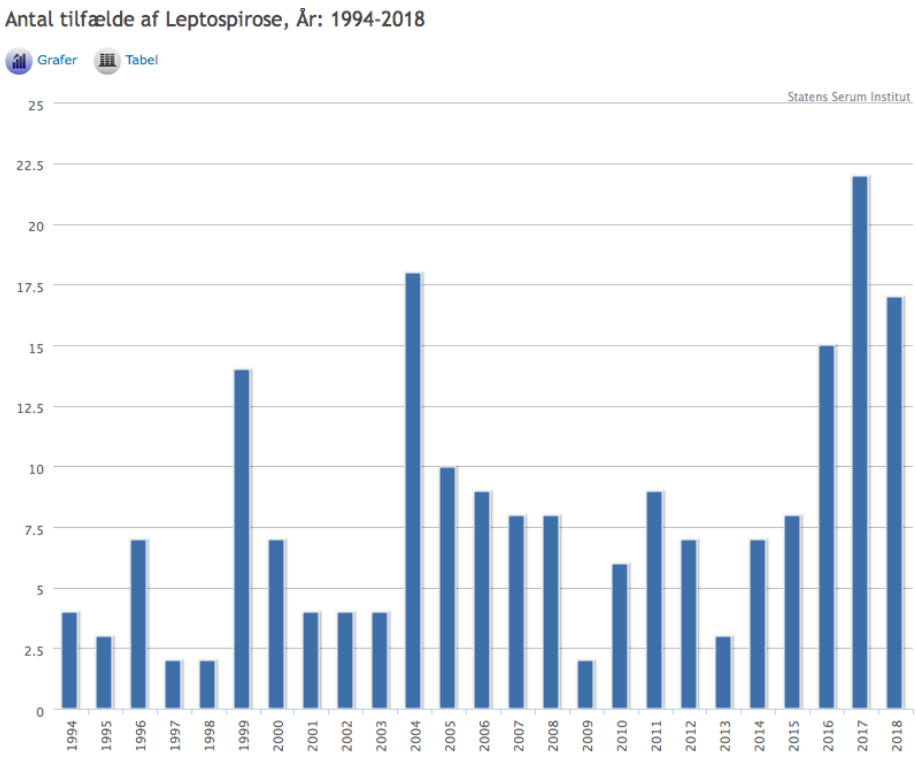

Figure 1.6. Graf illustrating the outbreaks of leptospirosis from 1994-2018 in Denmark, where the x axis equals each year and y axis equals incidences (26).

Leptospira can infect a human either through direct contact with infected animal's through tissue or body fluids such as urine or indirectly through contaminated water or soil. *Leptospira* can survive in soil with moisture content of >20% water and soil with pH around 5.5 and 7.6, in temperatures ranging from 4º C to 40º C. In addition, *Leptospira Interrogans* has shown to survive and stay virulent in water for up to 344 days (25).

Furthermore, studies have shown that *Leptospira* hosts can be classified into two types, a maintenance host and incidental host (27). The maintenance host is infected by *Leptospira* where the bacteria can reside in the renal tubule and multiply for some time in the kidney particularly the proximal tubules (21,28). The incidental host is generally infected by accident from direct or indirect contact with the maintenance host (5). One major maintence host are rodents which are common reservoirs of *Leptospira*. In addition, rodents are the only animal species that can throughout their life shed

Leptospira without clinical manifestations. Therefore, rodents are also designated as the primary source of infecting humans. Additionally, cattle and pigs excrete large amounts of *Leptospira* in their carrier state. All mammalian species can be infected and be harbor for *Leptospira* in their kidneys, though certain animals such as, cattle's, buffaloes, horses, sheep's, pigs and dogs are common maintenance hosts of *Leptospira* (3,13).

Disease phases and symptoms

Leptospirosis is often difficult to recognize due to a wide variety of clinical symptoms ranging from mild flu like illness to severe and sometimes lethal symptoms as observed in Weil's disease. Furthermore, leptospirosis has also shown to imitate other diseases such as dengue fever, thyroid, viral hepatitis and other viral hemorrhagic diseases which makes it difficult to identify (3,23).

Leptospirosis is variable and the disease can last up to several months or become chronical and lead to other complications long after the acute phase of the disease (24). The disease is divided into different phases to provide more accurate diagnostic and better treatment options for the patient. Before any symptoms, there is an incubation phase from 2-10 days where the bacteria first entered the host, as seen in **figure 1.7**. Hereafter, the beginning of the actual disease is called the septicemic phase and is characterized by mild symptoms that lasts a week, as seen in **figure 1.7.** Consequently, if the bacteria are not discovered the patient will experience severe symptoms which is also called the Immune phase. Usually, this is the beginning of week two after the first symptoms where antibodies are produced and *Leptospira* is excreted in the urine.

Therefore, culturing of *Leptospira* from patient urine samples are only possible if the samples are taken at the end of week one or the beginning of week two of the illness. Culturing of *Leptospira* is more complex since it can take a long time and sometimes Leptospira are unable to grow which makes it useless in the case of very ill patients. Additionally, blood samples are collected at the immune phase where antibody production is the highest and can be used in several diagnostic purposes/methods (3).

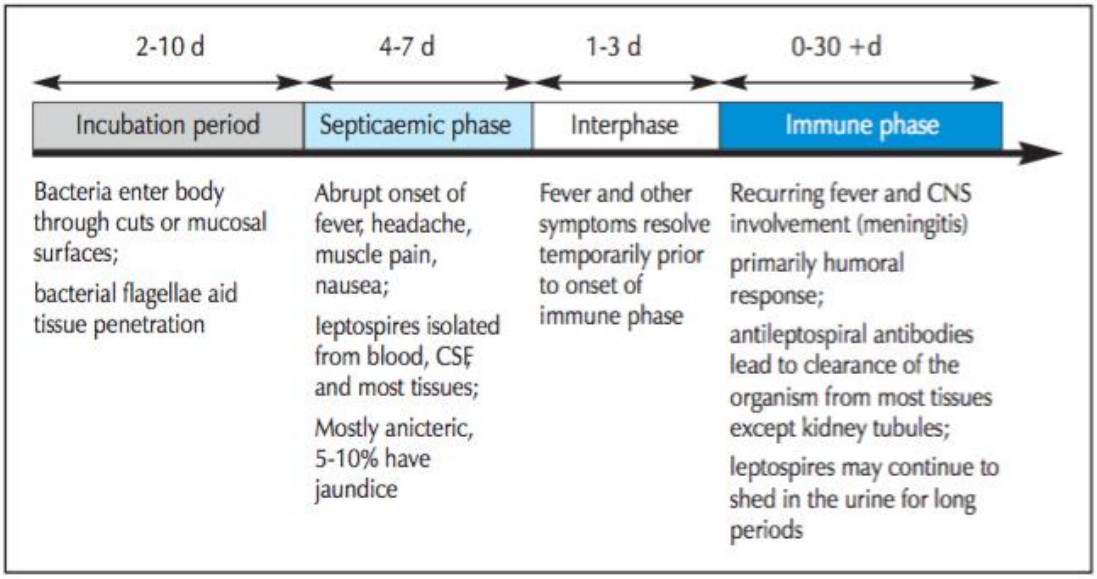

Figure 1.7. A timeline for the typical course of leptospirosis (3). The course is given in days and divided into different phases including symptoms that occurs during each phase.

Pathology

The main mechanism of pathology is considered common across all *Leptospira* spp. and involves damage to the endothelial cells of small vessels (29). *Leptospira* enters the host through mucosa and broken skin, resulting in bacteremia. However, the mechanism of tissue damage is not fully understood (28). The spirochetes multiply in organs, most commonly the central nervous system, kidneys, and liver, seen in **figure 1.8**. They are cleared by the immune response from the blood and most tissues but persist and multiply for some time in the kidney tubule. In host animals, it appears that the environment in the kidneys is an optimal environment for the survival and multiplication of the bacteria. A few *Leptospira* strains can also colonize the corpus vitreous in the eyes and have shown to be associated with recurrent uveitis in both horses and humans (6).

Pathologically, *Leptospira* induces disease through a toxin mediated process by which causes a vascular injury also known as vasculitis. Additionally, the breakdown of the endothelial cells in the vessel increases permeability which is linked to the change of tight junction and/or apoptosis in the vessel endothelial cells (21). Additionally, *Leptospira* can cause abortions, stillbirths, infertility, failure to thrive, reduced milk production, and death in animals such as cows, pigs, sheep, goats, horses, and dogs (4). Furthermore, the severity of the disease depends on several factors that have not

been fully understood, but studies have shown a correlation between *Leptospira* serovar causing the infection and the severity of the disease (6).

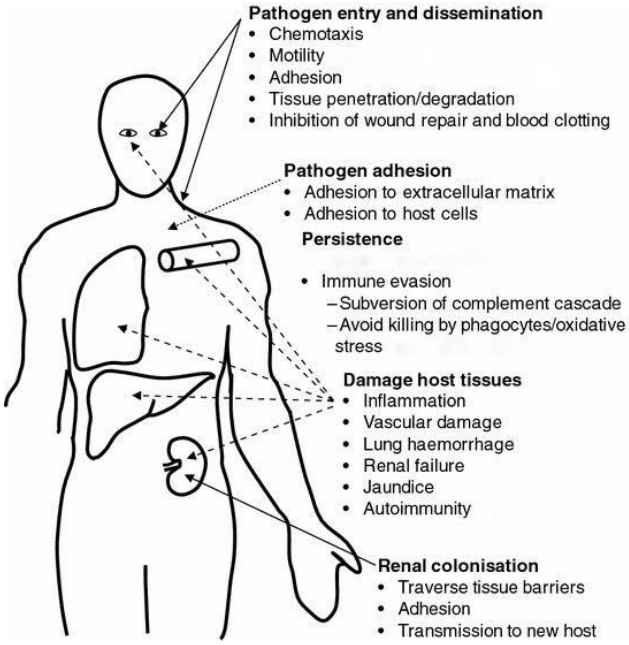

Figure 1.8. Pathogenesis of *Leptospira* and virulence mechanisms when in the human host (8) The pathogenesis and virulence mechanisms of *Leptospira* includes, pathogen adhesion, damage of hos tissue and renal colonization (modified for relevancy to this study).

Virulence factors of *L. Interrogans*

To date a various number of virulence genes and their proteins has been identified in *Leptospira* however, most of these functions are not fully covered. Among the known virulence's factors there are; chemotaxis, motility and either cell surface exposed proteins involving in attachment and host invasion or secretory proteins involving in tissue damaging (30). Subsequently, motility is an important survival mechanism beside to cause infection. In the environment, the ability to move is crucial for *Leptospira* to escape from inhospitable microenvironments example prolonged exposure to sunlight and move towards more favorable conditions. It is shown that *Leptospira* can move on viscous Matrices around 15µm/s and liquid surfaces (5µm/s). Furthermore, chemotaxis towards hemoglobin might lead the pathogen to reach entrance into the animal body in aqueous environments (25).

Prevention and treatment

Protective clothing and equipment to avoid contact with contaminated waters are some of the safety measures that can prevent leptospirosis. However, this can be difficult to establish in case of flooding in developing countries. Additionally, environmental control such as rodent- and flood- controls are difficult to implement (23). Moreover, leptospirosis can be treated with a wide range of antibiotics such as Doxycycline, β-Lactams like penicillin and amoxicillin among others. The choice of drug is depending on the severity and stage of disease. It is recommended in a person with severe disease to administrate penicillin G intravenous until oral tolerance of the drug. Additionally, many cases of late administration of antibiotics has shown efficiently to decrease mortality rates. Furthermore, supportive remedies should be administrated in parallel with antibiotic treatment to maintain fluid intake and electrolyte balance (11).

Vaccines are available for animals, but not for humans, however these vaccines available are serovar specific and therefore targeted against certain geographical areas where the serovar's cause leptospirosis (3,20,31,32)

Current Diagnostic strategies

Leptospirosis is frequently underdiagnosed since the infectious disease has often minimal to no clinical symptoms in the early stages (33). Therefore, it's important that diagnosis is based on laboratory tests rather than clinical symptoms exclusively (34). Laboratory diagnostic tests are broadly divided into two categories, the direct methods and indirect methods as seen in **figure 1.9**. The direct method aims to isolate or detect the actual *Leptospira's* and the indirect method aims to detect an immune response to *Leptospira* (antibodies) or to detect the antigens of *Leptospira*. In this section, a summary of the current relevant serological and genetic diagnostic methods will be given.

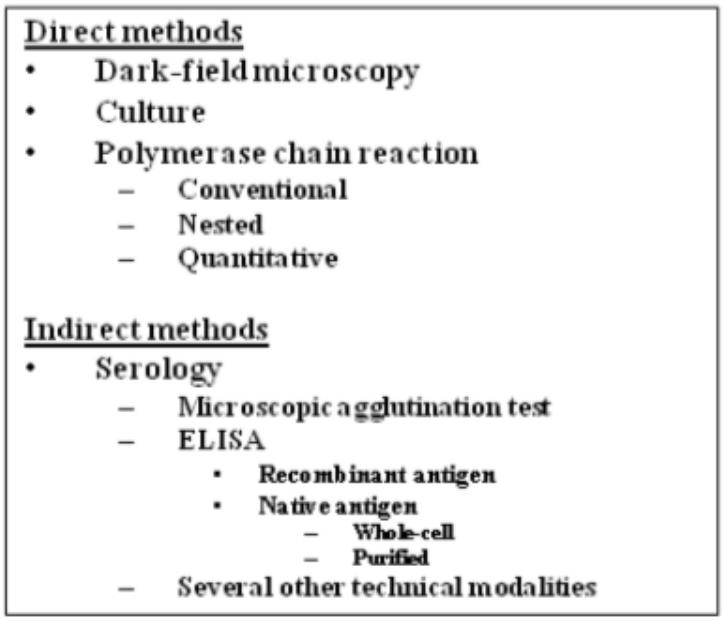

Figure 1.9. Overview of the current diagnostic methods used for *Leptospira*, divided into direct and indirect methods (6). The direct methods include; Dark-field microscopy, culture and several types of PCR. The indirect methods include; Serology such as MAT and ELISA.

Serological diagnostic

Serological diagnostic approaches are carried out to detect antibodies or antigens of *Leptospira* and will be described in this section. For more details in regards to MAT test is found in appendix 10.

Microscopic agglutination test (MAT)

The most common and widespread method used for diagnosis of leptospirosis is MAT which is also considered the "gold standard" in serology, until this present day. MAT is an immunological test that aims to detect both immunoglobulin M (IgM) and immunoglobulin G (IgG) classes of antibodies found in the patient serum. These antibodies are formed as an immune response to infection by *Leptospira* (35). Leptospira can be detected by MAT from day 10-12 after the onset of illness and sometimes later if specific antibiotics have been prescribed. This method has shown to have a sensitivity of 41 % during the first week, 82 % during the second to fourth week and 96 % beyond the fourth week of illness (36)

The MAT test is performed by a serial dilution of the patient serum and incubated with living cultures of different Leptospira serovar's in 30º C. Consequently, if the patient has been infected with Leptospira, the patient sera will react with the live Leptospira antigens and agglutination will occur due to antibody-antigen reaction. Subsequently, the agglutination of the test is observed by dark field microscopy as seen in appendix 10. A test sample is regarded positive for a given titer if 50 % or more of Leptospira have agglutinated. The sensitivity of detecting Leptospira in a test sample is increased by including a broad panel of live *Leptospira* serovar's. However, the maintence of a large panel of live pathogenic *Leptospira* standard cultures is costly, complex and the live organisms can create risk of infection to the laboratory technicians (35). Furthermore, it can be difficult to determine agglutination in very diluted test samples and if the patient received antibiotics in the early stages of the infection (17). For these reasons, a good diagnostic alternative to MAT has been sought for to eliminate or improve these drawbacks. A good serological alternative to MAT that has shown to detect *Leptospira* in the earlier stages of the infection is enzyme-linked immunosorbent assay (ELISA) (35).

ELISA

The antibody that is detected during the first week of illness by *Leptospira* is IgM. The IgM is formed as an immune response targeting the LPS on the outer membrane of *Leptospira* and appears in the earlier stages of leptospirosis. Detection of IgM by ELISA assay has been widely used and has shown to be more sensitive than MAT (33). The IgM antibody can be detected from patient serum and from cerebrospinal fluid (CSF) of leptospirosis patients. Both micro titer-plate IgM-ELISA and commercial dipstick IgM dot-ELISA are used to detect antibodies in patient serum and CSF (37). *Leptospira* can be detected by ELISA from day 6-8, which is earlier than MAT and has shown to have a mean sensitivity of 60.1 % on sera collected within the first ten days of the illness (36,38). When detecting for IgM *Leptospira*. The patient sample will then be added to the 96-well plate and if IgM antibodies are present in the patient sample an antigen-antibody binding complex will form in the wells. In order to detect this reaction, an enzyme can be linked directly to the primary antibody (direct assay) or introduced through a secondary antibody (indirect assay). Addition of a specific substrate will then be added to the 96-well plate and a color change will occur in the wells. The quantity of the antigen can then be determined visually by spectrophotometry among other methods. Generally, ELISA is an easier approach in the detection of *Leptospira* and the assay can also be

automated. However, there are though limitations to this test. ELISA has shown to be less specific than MAT and the broadly reactive antigens used in this technique does not differentiate between serovar's. Furthermore, ELISA needs conformational test and validation by current existing methods, which takes a long time. For these reasons, newer and more specific diagnostics strategies are sought for (6).

Molecular genetic diagnostic

Genetic diagnostic tests can be carried out to detect the presences of DNA or genes from a given organism of interest, including *Leptospira*. These tests can offer a more sensitive detection of *Leptospira* while providing a definitive diagnosis in the early phase of the disease (39). Furthermore, there are several genetic diagnostic methods that carries out this aim but with different approaches (40). One of the methods that are used so far in genetic diagnostic of *Leptospira* is a conventional polymerase chain reaction (PCR). PCR detection of *Leptospira* is mainly based on specific target genes and the most widely used is *16S* or *23S rRNA* genes but other genes such as LipL32 have also been used.

PCR

In 1987 the use of PCR dramatically changed the detection of *Leptospira*, since the method has been proved to be more sensitive and specific for early diagnosis of *Leptospira* compared to serology (41). In humans, *Leptospira* can be detected by PCR from blood, urine and CSF samples collected during the septicaemic phase (first week) of leptospirosis as seen in **figure 1.10**.

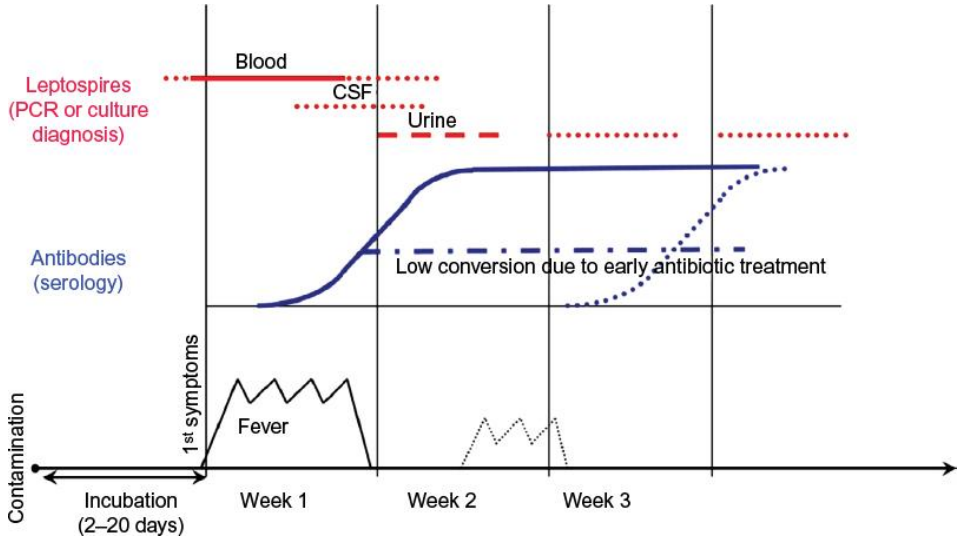

Figure 1.10. A course of the biological diagnosis of leptospirosis. This course is given in weeks and shows the different detection phases on blood, cerebrospinal fluid and urine samples. The course also represents the basic diagnostic principles that may be used for certain phases of the illness (42).

This method has a sensitivity of 28-96 % in severe leptospirosis when applied to whole blood samples. However, the sensitivity of PCR may vary depending on the phase of the disease and the applied sample material (43). When testing on blood samples, the sensitivity of PCR ranges from 50 % to 100 % and in urine from 42 % to 86% in the acute phase of the disease. However, when testing on urine samples in the late phase of the disease the sensitivity of PCR is highest (6). To ensure a high sensitivity when using PCR, samples have to be obtained before or shortly after treatment of antibiotics since antimicrobials quickly remove *Leptospira* spp. From the blood (43).

In recent years, several conventional PCR assays have been described for diagnosing of leptospirosis, but only a few assays have been properly validated for diagnostic use in clinical samples. Some of these conventional PCR assays are not sensitive enough to detect small amounts of DNA in a sample and in some cases unable to amplify certain spp. of *Leptospira*. Additionally, it has also been reported that certain conventional PCR assays are not able to differentiate between pathogenic (*L. Interrogans*) and non-pathogenic (*L. Biflexa*) spp. which can lead to false positive results. Moreover, there is a higher possibility of contamination of the sample material when using conventional PCR due to the transferring of samples. As a result, many reference laboratories have started to implement real-time PCR (RT-PCR) for diagnosing of leptospirosis, since this assay has shown better specificity and sensitivity than conventional PCR. Studies has also successfully detected pathogenic *Leptospira* spp. from histological tissue samples with RT-PCR (17).

However, there are several limitations that still needs to be tested such as a variety of components in a test sample that can potentially inhibit the RT-PCR assay. In addition, the specificity of the assay may also be questioned since *Leptospira* spp. cannot be completely differentiated due to the *16S rRNA* gene that is present in different *Leptospira* strains. Furthermore, the sensitivity of the assay may also have some limitations and must further be investigated.

Method

In this project, the method section is subdivided into four different studies as illustrated in **figure 2.1**. The aim of the overall studies is to validate the current RT-PCR assay used in the diagnosis of leptospirosis at SSI. The samples used in this project are from animals exclusively. For more details regarding the concentrations and volumes used in each study the protocols are found in appendix 1-9.

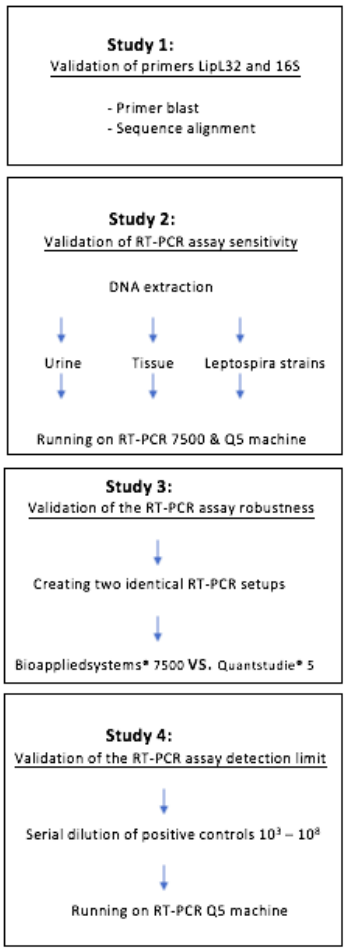

Figure 2.1. Flow diagram of the experimental studies. The flow diagram shows the process in which the laboratory work was generated and performed for each individual study: Study 1; Primer blast and sequence alignment were performed to validate the current primers. Study 2; PCR reaction mix was generated with a primary and confirmatory master mix and used to validate the sensitivity of the current RT-PCR assay. Study 3; Validation of the robustness of the RT-PCR assay by using 7500 and Q5 RT-PCR machines as comparison. Study 4; Validation of the detection limit for the RT-PCR assay by creating a serial dilution 10^3-10^8.

Study 1.

To validate the quality of the primers used, a primer blast and a sequence alignment was proceeded. The primers used to detect *Leptospira* at SSI are targeted against the genes LipL32 and 16S rRNA. The LipL32 gene is usually found in pathogenic Leptospira spp. and the 16S rRNA genes are found in all prokaryotes including pathogenic and non-pathogenic *Leptospira* spp. Therefore, the primers are referred to as LipL32 and 16S in this study and the sequences of the primers can be found in **table 2.1**. Moreover, a probe for both LipL32 and 16S primers are also included since these acts as a fluorescence signal during the RT-PCR reaction. Furthermore, the primers used in the RT-PCR assay were already designed and validated prior to this study nevertheless, a brief explanation of primer designing will be presented.

Leptospira primer/probes	Sequence
LeptoF	CCC GCG TCC GAT TAG
LeptoR	TCC ATT GTG GCC GRA CAC
Probe Lepto A-1 (16S rRNA)	Fam-CTC ACC AAG GCG ACG ATC GGT AGC-BHQ-1
Lipl32 P	Fam-AAG TGA AAG GAT CTT TCG TTG C - MGB
Lipl 32F	AGA GGT CTT TAC AGA ATT TCT TTC ACT ACC T
Lipl 32R	TGG RAA AAG CAG ACC AAC AGA

Table 2.1. The nucleotide sequences for forward and reverse primers of both LipL32 and 16S, including the nucleotide sequence of LipL32 and 16S probes. The R indicates that at this position there is a purine (A or G) in the sequence.

Primer design

The primers designed prior to this study, are targeted against the *LipL32* and *16S rRNA* genes found in *Leptospira* spp. In general, there are few specific measures to consider in the construction of primers. Such measures include optimal primer specificity, melting temperature (Tm), binding capacity, and product size (75-200bp). The primer sequence should have an optimal length of 18-24

bp, as this is long enough for adequate specificity and short enough to anneal to the template. Moreover, the goal for the Tm should be 60°C and the content of guanine and cytosine should be around 50-60% to enable primer annealing at high temperatures. Furthermore, the primers must be checked for specificity and homology with other prokaryotic and eukaryotic DNA sequences this can be achieved with a primer BLAST (44).

Primer blast

The aim of primer blast is to investigate the performance of the primers and review possible unintended targets. Primer blast provides different information about the primers such as Tm, GC content, PCR product length and unintended targets. In study one the primer blast was performed on NCBI for mainly four strains; Bratislava, Hardjo, Hebdomadis and Pomona. These four strains were chosen because of their pathogenic impact on animals. To perform a primer blast, the genome sequences of the four strains were downloaded from NCBI in a Genbank format and uploaded to UGENE respectively. UGENE was used to find the annealing location of the primers to the sequence. This location was relevant since it determines the size of the PCR product. The PCR product size was used to limit the range when performing primer blast, since primer blast cannot analyze a file that exceeds 5000 bp Consequently, the range limit was then inserted into NCBI and a blast was achieved. As a note, whole genome sequences were only available of Bratislava and Hardjo and partial genome sequences for Hebdomadis and Pomona. Consequently, sequence alignment was performed.

Sequence alignment

The aim of performing sequence alignment was to ensure amplification of several *Leptospira* spp. when using the 16S primer during the RT-PCR assay. Sequence alignment was performed on the *16S* gene exclusively, since *LipL32* is only present in pathogenic *Leptospira* spp. The alignment was completed in ClustalX by downloading a FASTA format of the 16S gene sequence for several Leptospira strains that are relevant for SSI, due to the pathogenicity. The strains included are found in **table 2.2**. Moreover, the probe, forward and reverse primer where also aligned in ClustalX to examine any mismatched base pairs during annealing.

Genomspecies	Serovar	Serogroup	Strain
L. Biflexa	Patoc	Semaranga	Patoc I
L. Borgpetersenii	Ballum	Castellonis	Castellon 3
L. Fainei	Hurtsbridge	Hurtsbridge	H.B.6
L. Interrogans	Autumnalis	Autumnalis	Akiyami A
L. Interrogans	Bataviae	Bataviae	Swart
L. Interrogans	Bratislava	Australis	Bratislava
L. Interrogans	Canicola	Canicola	Hond Utrecht IV
L. Interrogans	Copenhageni	Copenhageni	M20
L. Interrogans	Hardjo	Serjoe	Hardjo prajitno
L. Interrogans	Hebdomadis	Hebdomadis	Hebdomadis
L. Interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
L. Interrogans	Iustralis	Australis	Ballico
L. Interrogans	Pomona	Pomona	Pomona
L. Interrogans	Pyrogenes	Pyrogenes	Salinem

Table 2.2. The *Leptospira* strains included in the studies listed in alphabetical order, including their correlating serovar, serogroup and genome spp.

Real time polymerase chain reaction

In this section, a presentation and explanation of the RT-PCR assay performed is demonstrated. RT-PCR was performed for study two, three and four and the following PCR principle is equivalent for all above mentioned studies.

The RT-PCR assays were performed by using Bioappliedsystems® 7500 (7500) and Quantstudio™ 5 (Q5) RT-PCR machines. The PCR setups were generated for each study, in a 96-well plate and all setups are illustrated in **figure 2.2**. The PCR setups were executed by creating two different master mixes; a primary and a confirmatory. The Primary master mix targets the *Lipl32* gene and the confirmatory targets the *16S rRNA* gene. Therefore, the primary master mix contains specific primers and probe for *Lipl32* gene, and the confirmatory master mix specific primers and probe for 16S rRNA gene. Additionally, common reagents are added to both master mixes such as gibco-water (DNase free water), immolase-buffer, dUTP mix, 50mM MgCl2, IK (intern control) 107, probe that targets IK control, glycerol 50%, ROX dye and immolase Taq (DNA polymerase).
Moreover, the immolase Taq was held on ice to prevent the immolase Taq from initializing the synthesis reaction before the samples were loaded to the RT-PCR machines.

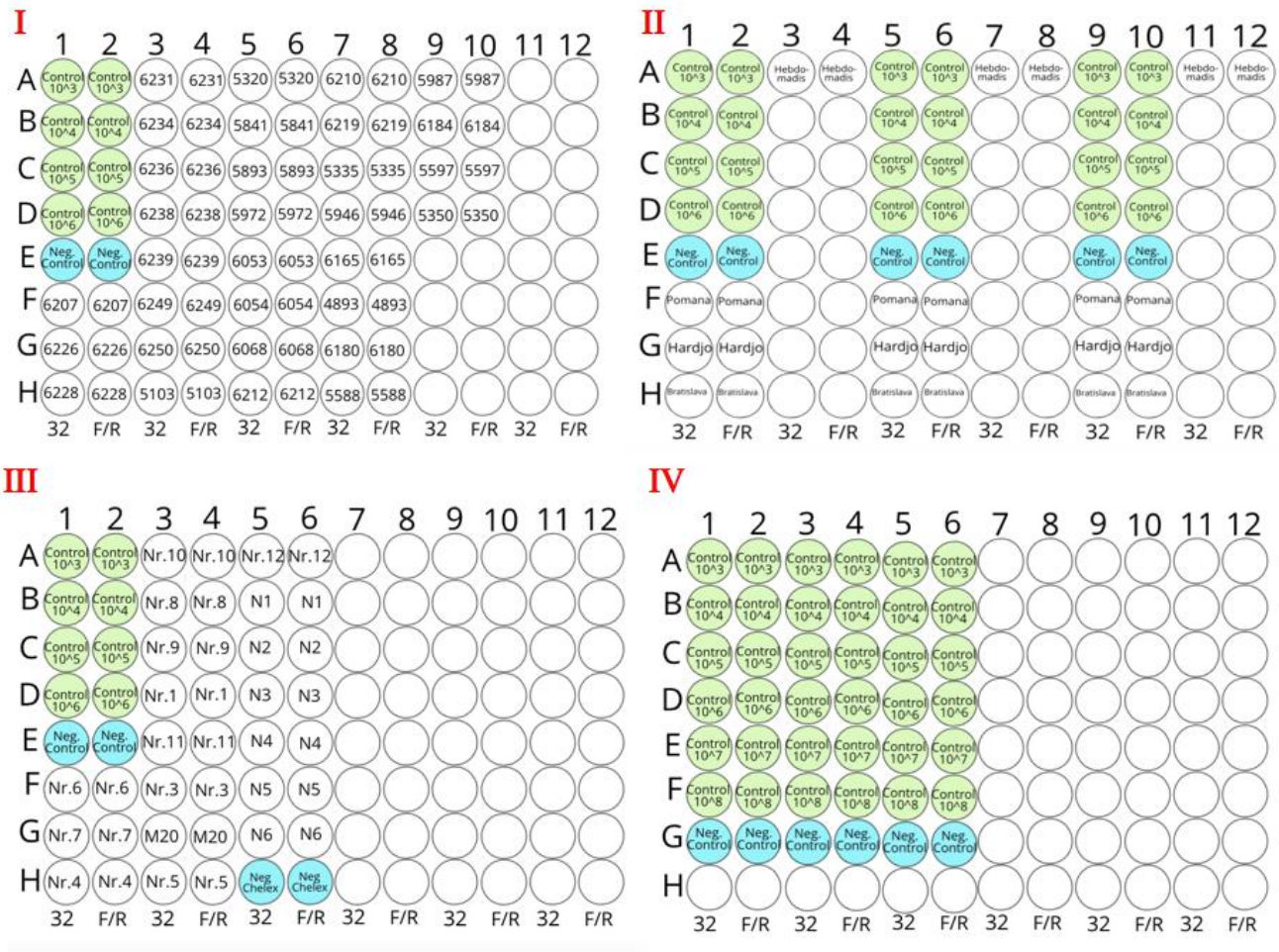

Figure 2.2. The RT-PCR 96-well setups for; I. Study 2; validation of RT-PCR sensitivity, II. Study 2; validation of RT-PCR sensitivity and Study 3; validation of RT-PCR robustness, III. Study 2; validation of RT-PCR sensitivity, IV. Study 4; Validation of detection limit. The setups contain positive and negative controls including primary and confirmatory master mixes named 32 and F/R (16S).

The general principle behind a RT-PCR assay, is that the data is collected throughout the PCR process with the help of a specific fluorescence probe that is targeted against the DNA of interest. The amount of amplified DNA is measured by the amount of fluorescence detected for each cycle also known as the Ct value. The reactions are usually run for 50 cycles in total with different temperature shifts in each cycle in order to: separate the double stranded DNA, annealing of primers and elongation of new synthesizes DNA(45). The settings used for the RT-PCR machines are show in **table 2.3** for study two, three and four. The 7500 and Q5 machines are both able to detect either an absolute or relative quantity of DNA present in a sample.

Cycling conditions	Steps and cycles	
50°C for 2 mins.	Hold stage 1. step	1 cycle
95°C for 10 mins.	Hold stage 2. step	
95°C for 15 secs.	PCR stage 1. step	50 cycles
60°C for 1 min.	PCR stage 2. step	

Table 2.3. The program used for the RT-PCR reaction which is equivalent for study 1,2 and 3. This table shows the temperature, time and step for each cycle with 50 cycles in total.

Study 2.

The purpose of study two was to validate the sensitivity of the current RT-PCR assay. The sensitivity of the RT-PCR assay is defined by the assay's capability to detect different *Leptospira* species and in different specimens such as, urine and histological tissue. The samples utilized in this project, are urine samples from pigs and clinical kidney tissue samples from aborted pig fetuses and dogs. Moreover, pure *Leptospira* strains were also included and these were cultured, and DNA was extracted prior to this study. Initially, the study was executed by extracting DNA from urine and tissue samples by using Chelex. Eventually, the extracted DNA was added to a 96-well PCR plate as seen in **figure 2.2** and run on 7500 and Q5 RT-PCR machines.

Extraction of DNA from urine samples with Chelex method

The yielded DNA from urine samples were obtained by Chelex.

Chelex is a chelating ion exchange resin bead that is used for DNA purification. Usually a urine sample contains several contimants that can potentially inhibit the RT-PCR reaction by inhibiting the polymerase. For this reason, Chelex resin contains a functional group that acts as the chelating group which can bind to DNases or other potential contimants and isolate the DNA in a sample solution. Chelex has a high selectivity for different ions depending on the pH and therefore it's essential to suspend Chelex in a Tris-EDTA buffer. In this project, the urine samples obtained from pigs are centrifuged in (30.000 rpm for 15 minutes) to obtain a pellet. The suspended Chelex with Tris-EDTA buffer is added to the pellet and the samples are incubated on a heat block in 94º C for 10 min. Consequently, the cells in a sample will denature and *Leptospira* DNA will be released (if present) into the solution. The purified Leptospira DNA will be detected in each sample by running a RT-PCR reaction as described in previous section. As a note, Chelex resin beads can potentially also bind to

fluorescence which can lead to a false positive result if transferred into the RT-PCR reaction. (46)

Extraction of DNA from tissue samples with Chelex method

The DNA yielded from kidney tissue samples were also obtained by Chelex.
This was performed by transferring the kidney tissue to a 1.5ml Eppendorf tube with x µL Tris-EDTA buffer and Chelex resin beads. The solution was centrifuged for (30.000 rpm for 15 minutes) and furthermore transferred to a heat block in 94º C for 10 min. Since the tissue was coated in paraffin it was necessary to keep the solution warm to prevent the paraffin from solidifying.
The solution was transferred to a 96-well PCR plate and a master mix reaction was added to each well.

Study 3.

The purpose of study three is to validate the robustness of the current RT-PCR assay. The robustness of the RT-PCR assay is defined by the assay's capability to withstand variation when using different RT-PCR machines. In addition, this study was performed by three different technicians to also examine the robustness of the assay when applying human variation.

To assure the robustness of the RT-PCR assay and the compliance of the two RT-PCR machines, a comparison was performed by creating two identical PCR setups. This was done in a 96-well PCR plate where extracted Leptospira DNA was added. The DNA extracted correspond to study 2. Additionally, the setup was generated in triplicates performed by three different technicians. The two identical setups were run on the two different RT-PCR machines; 7500 and Q5 as seen in **figure 2.3**. The data yield from the RT-PCR assay were analyzed by statistical methods including Bland-Altmann plot and One-way ANOVA. This will be described in later section.

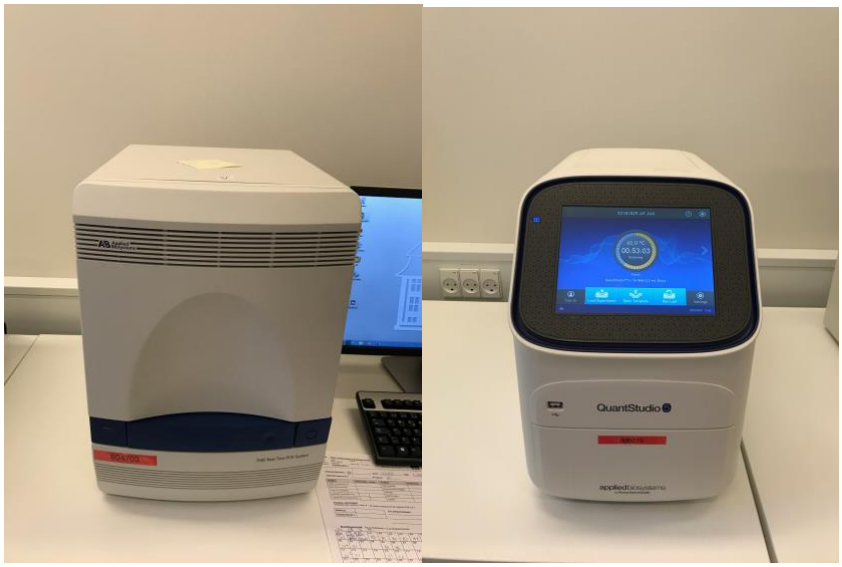

Figure 2.3. The two RT-PCR machines used in the diagnosis of leptospirosis at SSI and used in this project in the validation of the RT-PCR assay. A; Bioappliedsystems® 7500, B; Quantstudio™ 5.

Study 4.

Study four was carried out to determine the sensitivity or detection limit of the RT-PCR assay. This was achieved by diluting the positive Leptospira control 10 folds. A 10-fold serial dilution is obtained by diluting a stock solution 10x in serial. In this study, the stock solution was a positive Leptospira control and the initial concentration was 10^3. The Leptospira control was diluted to 10^8 by taking out 10µl of the 10^3 control and added to a new Eppendorf tube that contains 90µl DNA buffer (TE-buffer and calf thymus). This results in a 1:10 dilution ratio and for each tube the control sample is diluted 10x as shown in figure 2.4 Moreover, between each dilution the tube was well mixed to ensure homogeneity. Eventually, the serial diluted controls were added to a 96-well PCR plate as seen in figure 2.2 and run on the Q5 machine.

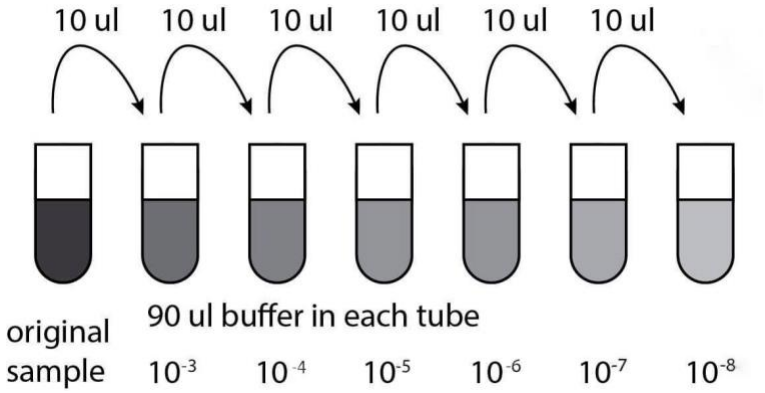

Figure 2.4. 10-fold serial dilution of the positive Leptospira control samples. The original solution (10^3) was diluted 10x by taking out 10µl and adding it to a new tube with 90µl DNA buffer. This was repeated until a dilution of 10^8 was achieved.

Quality assurance

Throughout the laboratory experiments, all the procedures were performed sterile with the use of latex gloves and in a laminar air-flow bench to prevent contamination. The master mixes for the RT-PCR reaction were generated in a separate sterile laboratory free from biological materials. Additionally, all the guidelines for the specific laboratory were followed.

To assure the quality of the collated data it was necessary to apply several controls to the RT-PCR reaction including positive controls, negative controls and IK internal control.

Initially, a positive control is added to the assay which is diluted in the range (10^3-10^6) to assure that the assay can detect DNA in that specific range. Furthermore, an internal control called IK control is added to the master mix to assure that the RT-PCR assay has run successfully and ensuring "true" negative if no target DNA is detected. The IK control contains an assay-specific sequence usually greater in size than the target DNA. The IK internal control is a synthetic lambda oligo sequence which can be used as a link between the forward and reverse primer. However, the IK control is not detectable without a fluorescence probe. For this reason, an IK lambda probe
is added to the master mix which targets the IK control and acts as a fluorescent signal during the RT-PCR reaction. As a note, in case of high concentrations of target DNA in a sample, the IK control can be inhibited and thus not be detected. In addition, a negative control is added to verify no contamination when preparing the master mix or when extracting DNA.

Statistical analysis

The statistical analysis used are only relevant for study three.

In order to validate the robustness of the RT-PCR assay and determine the compliance of the two RT-PCR machines, statistical analysis was performed such as, Bland Altman's plot and One-Way ANOVA.

Bland-Altman plot

Bland-Altman plot is an analysis to examine the agreement between two measuring methods. By studying the differences of the two methods it is possible to view any variance. The analysis is illustrated as a XY-plot where X equals the average between the two measurements and Y equals the difference between two measurements. Moreover, with a Bland-Altman plot it is possible to establish an interval where the data is predicted to lie within 95% confidence. This interval is called limit of agreement and it is suggested that data points must lie within 2±SD. However, this interval may not fit completely and therefore, accept limits must be defined beforehand based on clinical necessity, biological considerations or other aims. In addition, as seen in **figure 2.5** the Bland-Altman plot can reveal any bias meaning that one method is measuring more or less than the other. Nevertheless, to determine the significance of any observed variation statistical methods such as t-test or one-way ANOVA can be executed. Though to perform these statistical test's the data point must be normally distributed (47).

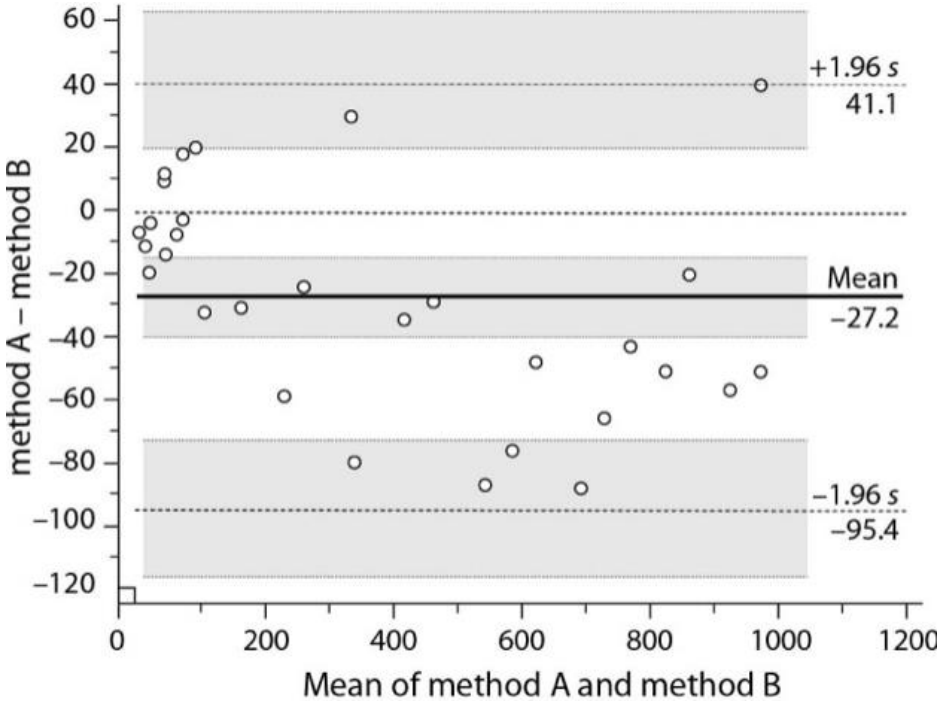

Figure 2.5 shows an illustration of a Bland-Altman plot where two measuring methods are compared. The X axis demonstrates the mean of measuring method A and B whereas the Y axis demonstrate the difference between method A and method B. Moreover, the upper and lower limit of agreement are illustrated as ±1,96SD. Furthermore, the black line marked as mean indicates the bias.

One-way ANOVA

To validate if potential variance between the RT-PCR machines are significant, a One-way ANOVA was performed. The statistical calculations were achieved on excel where a significant limit of 0.05% was set. Furthermore, to execute a one-way ANOVA it is required that yielded data are normally distributed. The data included in this study is expected to be normally distributed.

Result and analysis

In the following section a review and elaboration of the results conducted will be given for each study respectively. The results were obtained by performing RT-PCR assay on extracted Leptospira DNA from cultivated strains, urine and tissue samples. When performing RT-PCR two separate master mixes were generated for LipL32 and 16S, these will be referred to as LipL32 assay and 16S assay. Finally, a review of the performance of the current primers is also presented and were achieved by performing primer blast and sequence alignment. As a note, raw data is given in appendix 13-16 and a validation report of the obtained data is given in appendix 19.

Study 1.

In study one the location of the forward and reverse primers including their probes were identified and is given in **table 3.1** The primers have been checked for accurate annealing on both positive and negative strands on the template sequence. Moreover, the probes were localized in between the forward and reverse primers for both LipL32 and 16S. This applies for all four strains that have been checked on UGENE.

Strain	Accession number	Lipl32 Forward primer (bp)	Lipl32 Reverse primer (bp)	Lipl32 Probe (bp)	16S (F/R) Forward primer (bp)	16S (F/R) Reverse primer (bp)	Probe Lepto A-1 (16S rRNA) (bp)
Bratislava*	Whole genome: CP011410	1724883-1724913	1724827-1724847	1724849-1724870	(1):1922278-1922292 (2):3163283-3163297	(1):1922348-1922365 (2):3163210- 3163227	(1):1922312-1922335 (2):3163240- 3163263
Hardjo	Whole genome: CP012603	2672279-2672309	2672345-2672365	2672322-2672343	(1):2474617-2474631 (2):3090193-3090207	(1):2474544-2474561 (2):3090120-3090137	(1):2474574-2474597 (2):3090150-3090173
Hebdoma-dis	Lipl32: AY609328 16S: FJ154551	619-649	685-705	662-683	171-185	241-258	205-228
Pomona	Lipl32: EU871716 16S:NZ_AFLT02000042	619-649	685-705	662-683	185910-185924	185837-185854	185837-185890

Table 3.1. The four strains included in the primer blast with accession numbers and location of annealing for each forward- and reverse primers including their probe to the target sequence. The annealing locations are found by UGENE. Bratislava is marked with a star since the genome sequence was reversed when downloaded from NCBI.

Primer-blast

Primer blast provided different information about the primers such as Tm, GC content, PCR product length and possible unintended targets, and is given in **table 3.2** for LipL32 and 16S respectively. Overall for both LipL32 and 16S primers, the PCR product length of the intended target is around 87-88 bp Moreover, the Tm value varies between 60-62°C for LipL32 and between 53-58°C for 16S. The temperature between forward and reverse primer for both LipL32 and 16S does not vary more than 5°C. The GC content is generally higher for 16S compared to LipL32. Furthermore, several unintended targets were identified by performing primer blast however, no further investigation has been made to determine the significance of these unintended targets.

LipL32			
Strain	**Product length**	**Tm**	**GC%**
Bratislava **Forward** primer	87	~60°C	~48%
Bratislava **Reverse** primer		~62°C	~36%
Hardjo **Forward** primer	87	~62°C	~36%
Hardjo **Reverse** primer		~60°C	~48%
Hebdomadis **Forward** primer	87	~62°C	~36%
Hebdomadis **Reverse** primer		~60°C	~48%
Pomona **Forward** primer	87	~62°C	~35%
Pomona **Reverse** primer		~60°C	~48%

16S			
Strain	**Product length**	**Tm**	**GC%**
Bratislava **Forward** primer	88	~53°C	~67%
Bratislava **Reverse** primer		~58°C	~56%
Hardjo **Forward** primer	88	~58°C	~56%
Hardjo **Reverse** primer		~53°C	~67%
Hebdomadis **Forward** primer	88	~53°C	~67%
Hebdomadis **Reverse** primer		~58°C	~56%
Pomona **Forward** primer	88	~58°C	~56%
Pomona **Reverse** primer		~53°C	~67%

Table 3.2. Data achieved from primer blast about the performance of the primers (LipL32 above and 16S below) when amplifying the four target strains. The data contains information's such as PCR product length of the target, Tm and GC values.

Sequence Alignment

The results obtained by sequence alignment was only for the 16S primers, probe and *16S* gene found in several *Leptospira* strains as mentioned in **table 2.2**. As seen in **figure 3.1**, the results from the sequence alignment shows the exact annealing location of the probe and the primers to the target DNA sequence. Overall, the results from the alignment indicates that most of the strains are aligned with an exception of Patoc I which have several nucleotide differences compared to the other strains. The forward primer has five mismatches with Patoc I. Furthermore, the probe has possibly two mismatches with Patoc I. Additionally, the reverse primer has two mismatches near the 3'end, which may cause disturbance for the binding of the polymerase. This will be discussed later.

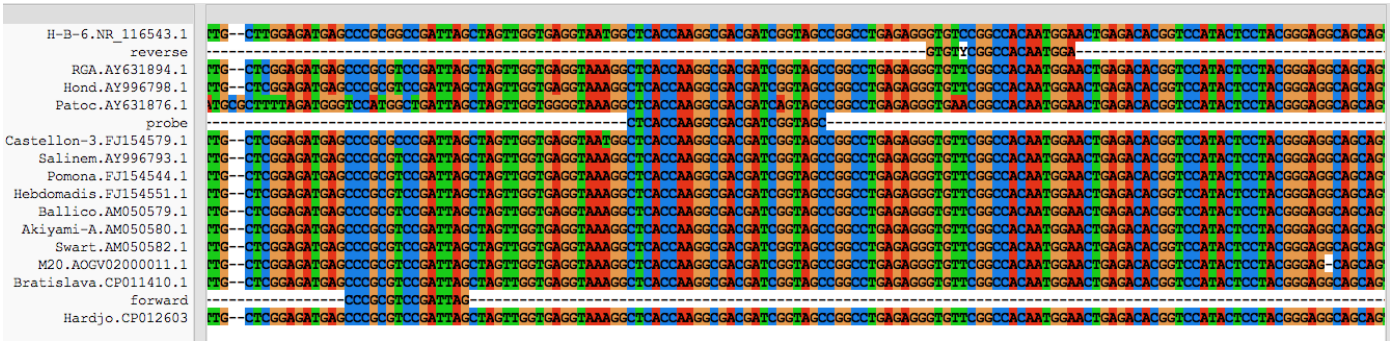

Figure 3.1 The results obtained of the sequence alignment performed on several *Leptospira* strains. This alignment includes 16S forward and reverse primer, the 16S probe and 16S gene found in each strain.

RT-PCR analysis

The results obtained from the RT-PCR reaction will be reviewed in this section. The results from the RT-PCR assay consist of several amplification curves of the controls and sample materials. All these amplification curves can be found in appendix 17 and 18. for all the studies, the RT-PCR reaction was achieved successfully since the positive controls were amplified as expected. In addition, the negative controls were also accepted as true negative and is found in appendix 17 and 18. In study two, the RT-PCR assay was repeated since the controls could not be accepted the first time. Moreover, **figure 3.2** illustrates the amplification curve of the controls achieved from study 2. Observed from figure 3.2 the positive control 10^3, 10^4, 10^5 and 10^6 are all amplified, whereas the negative control was not and therefore accepted as true negative. In addition, the samples were

generated in triplicates and no variation was observed between them, except the 10^6 control for LipL32 where one of the triplicates did not amplify. This will be considered later in the discussion.

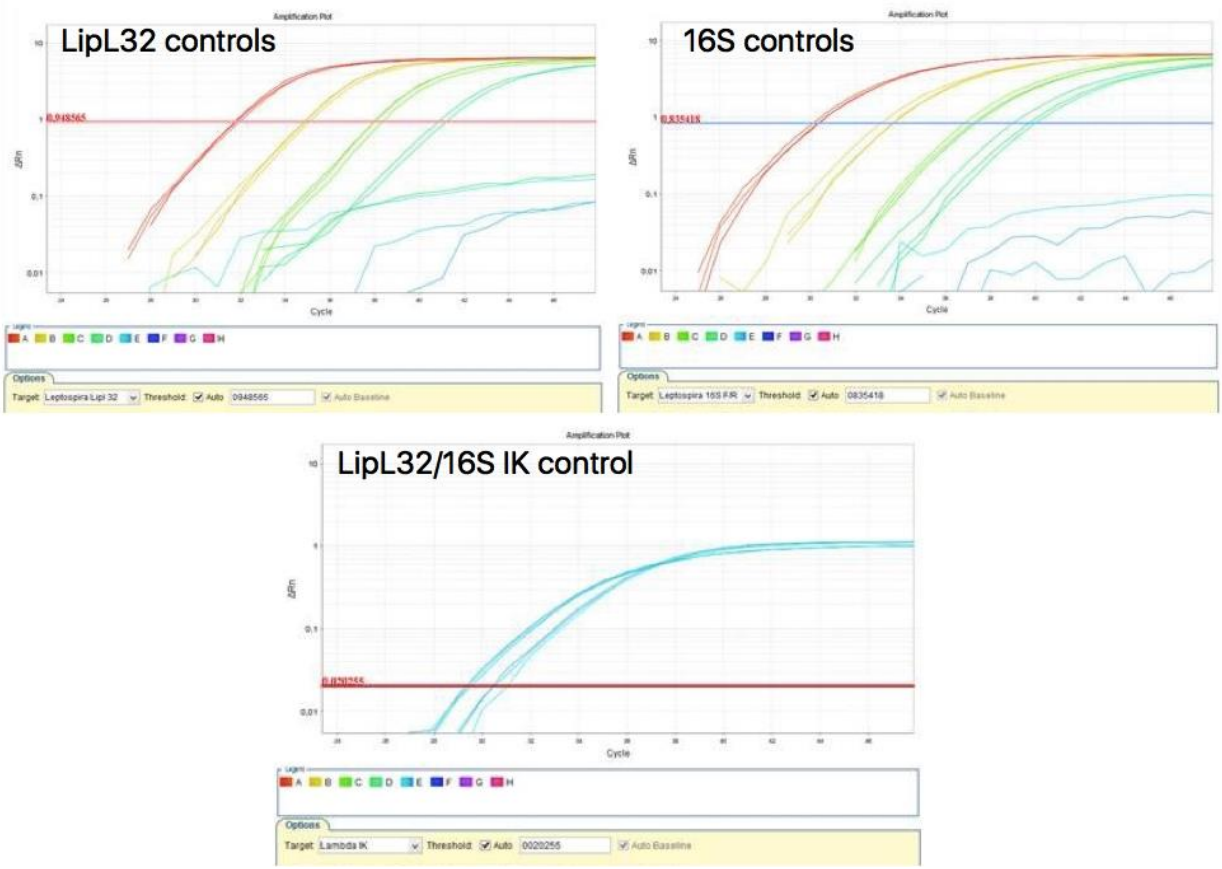

Figure 3.2 The figure illustrates the amplification curve for the controls achieved from study two. The above left figure illustrates the positive and negative control for LipL32 assay and the above right figure for 16S assay. In the above figures, the red curve indicates the 10^3 control, yellow 10^4, green 10^5, blue-green 10^6 and the blue is a negative control. The amplification curve below, is the IK control indicating true negative when it's been amplified.

Study 2.

In **table 3.3** an overview of the results obtained from study two is given. Overall, the obtained data shows that the current RT-PCR assay were able to detect several *Leptospira* strains except Patoc I, which also belongs to the saprophytic specie, this will be discussed later. Furthermore, the RT-PCR assay was also able to detect *Leptospira* spp. in kidney tissue from both pig fetuses and dogs. It was also observed that the RT-PCR assay was not able to detect *Leptospira* in urine from pigs when

using the LipL32 assay. However, when using the 16S assay it was possible to detect DNA in urine from the same pigs. This will be further discussed.

Sample material	Primary (LipL32)	Confirmatory (16S)
Akiyami A	Detected	Detected
Ballico	Detected	Detected
Bratislava	Detected	Detected
Castellon 3	Detected	Detected
Hardjo	Detected	Detected
Hebdomadis	Detected	Detected
Hond Utrecht IV	Detected	Detected
H.B.6	Detected	Detected
M20	Detected	Detected
Patoc I	Undetermined	Undetermined
Pomona	Detected	Detected
RGA	Detected	Detected
Salinem	Detected	Detected
Swarts	Detected	Detected
RGA	Detected	Detected
Urine from pig	Undetermined	Detected
Kidney tissue from pig fetus	Detected	Detected
Kidney tissue from dog	Detected	Detected

Table. 3.3. illustrates the results obtained from the RT-PCR assay performed on different sample material such, pure DNA from *Leptospira* strains, Urine from pigs, kidney tissue from pig fetus and dogs.

Study 3.

The results obtained from study three is represented in **figure 3.3** as a Bland-Altman's plot. The results illustrate a comparison between the two RT-PCR machines 7500 and Q5. Since the same setup where analyzed on both RT-PCR machines, the difference of them (x-axis) was plotted against the mean value (y-axis). The upper and lower limit of agreement is marked as a red line indicating ±2SD, whereas the bias marked as yellow illustrates the mean of difference. For both LipL32 and 16S assays there is observed a small bias indicating that one of the RT-PCR machines may measure generally higher or lower than the other. In addition, as observed in figure 3.3 a few data points fall out of the ±2SD interval for both LipL32 and 16S assays. An examination of the data revealed that the 10^6positive control was the one sample that exceeds the ±2SD interval for both LipL32 and 16S assays. As a result, for LipL32 it is 1/3 of the triplicates that exceeds the ±2SD interval, whereas it is 2/3 of the triplicates in 16S that exceed the interval. In addition, to determine the significance of the observed difference between 7500 and Q5 a one-way ANOVA was performed. The statistical method

revealed a P-value > 0,05 meaning that no significant difference was determined between 7500 and Q5 for both assays.

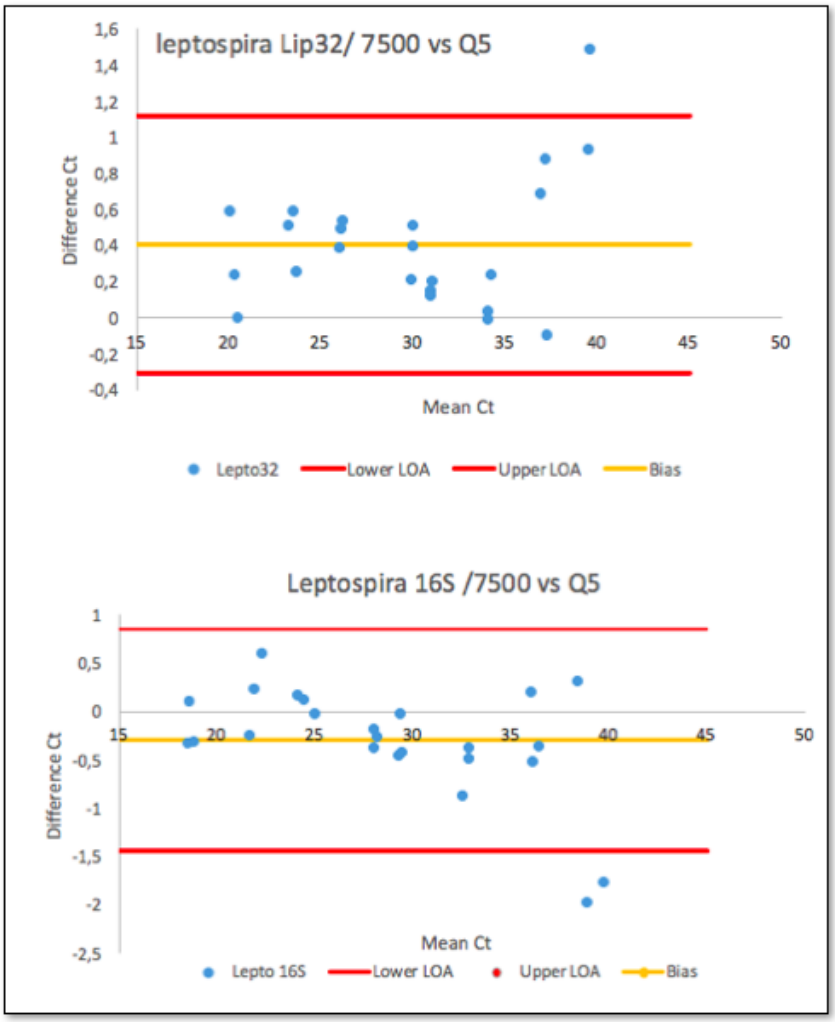

Figure 3.3 The results obtained from study three are demonstrated as a Bland-Altman's plot. The x axis indicates the difference in Ct value and the y axis indicates the mean Ct value. The blue data point illustrates the difference between the RT-PCR machines; 7500 and Q5. The red line illustrates the upper and lower limit of agreement (LOA) where the limit is determined as ±2SD. In addition, the yellow line presents the bias which is the mean of the differences.

Study 4.

The results obtained from study four are illustrated in **table 3.4** and demonstrates the detection limit of the RT-PCR assay, when detecting *Leptospira* DNA in a diluted sample. The yielded data presented is for LipL32 and 16S assays. For both assays the RT-PCR assay can detect *Leptospira*

DNA in a control that is diluted 10^5. However, the detection of Leptospira DNA in the 10^6diluted control becomes inconsistent for both LipL32 and 16S assays. This means that in diluted samples (around 10^6) the detection of *Leptospira* DNA may vary and in some cases become non-detectable. The detection inconsistency continues as the controls become more diluted and the DNA material becomes less concentrated. The relevancy of the detection limit for the RT-PCR assay will be further discussed.

Titer experiment for limit of detection		
Titer samples	LipL32	16S
10^3	Determined	Determined
10^4	Determined	Determined
10^5	Determined	Determined
10^6	Variable/inconsistent	Variable/inconsistent
10^7	Variable/inconsistent	undetermined
10^8	undetermined	undetermined

Table 3.4 The results obtained from study four is given where the limit of detection of the RT-PCR assay is determined. By diluting the 10^3control sample to 10^8 the table reveals the assays ability to detect Leptospira DNA in a 10^5diluted sample.

Discussion

In this section, an elaboration and discussion will be given on the obtained data for all four studies with the intention to validate the current RT-PCR assay used to detect DNA of *Leptospira* ssp. at SSI.

Reliability of the obtained data

The results obtained in this project were limited to the number of samples available and provided by SSI, which could potentially affect the optimal representation of the data. Moreover, not all *Leptospira* spp. where available to be analyzed by the RT-PCR assay, this will also be taken into consideration when discussing the results. Sample materials utilized were from pigs and dogs exclusively, therefore the obtained results will only provide evidence based on animals. Finally, all the controls used during the RT-PCR reactions were accepted and had shown no contamination that could potentially interfere with the data.

Study 1.

For study one, a primer blast was generated in order to assure the performance of the primers used to detect *Leptospira* DNA during a RT-PCR reaction. The primers were checked for Tm, GC content and product length along with specificity and homology with other prokaryotic and eukaryotic organisms. The primers were checked individually for each forward and reverse primer to assure their separate performance and quality when annealing to the template sequence.

How is the quality and performance of each primer when annealing to specific *Leptospira* sequences, in primer blast?

For LipL32 primers, the quality has shown to be optimal during annealing to the template sequences in primer blast for; Bratislava, Hardjo, Hebdomadis and Pomona. The Tm value was around 60-62°C, which is within the optimal temperature range for the RT-PCR assay performed. Furthermore, the GC content has shown to be 35-48% which is relatively lower than the desired 50-60%. Nevertheless, this did not have significant importance when performing RT-PCR in this project or in (48). In addition, the primers were tested and validated in study (49).

Moreover, the PCR product length was around 87 bp which is within the range of optimal PCR product length. When LipL32 was checked for specificity, several unintended targets were

discovered. However, the unintended targets were only discovered when Forward and reverse primer where checked separately. The reason for this, is due to primer blast program and a lack of similar genome sequences on the database (48).

For 16S primers, the quality has also shown to be optimal during annealing to the template sequences in primer blast for the same four strains mentioned above. The Tm value was around 53-58°C which is slightly less than the 60°C used in the RT-PCR settings. This may cause minor disruptions during the RT-PCR reaction. Moreover, the GC content 56-67% which was slightly higher than the optimal 50-60% GC content. Though this did not have any significant effect when testing the 16S primers during the RT-PCR reaction in this project and in (48,49). Moreover, the PCR product length was within the optimal length with a PCR product size of 88bp. Furthermore, study x has investigated the specificity of the 16S primers and has shown to be 91.5% specific and not 100%. This is due to cross reaction with other bacteria. However, in our study the specificity for the 16S primers could not be determined when blasting both forward and reverse primers collectively. Despite of that, the specificity of the 16S primers were determined by blasting the forward and reverse primers separately, equivalent to LipL32 primers.

How does the probe, forward and reverse primer for 16S align with several *Leptospira* strains?

The sequence alignment was shown to be successful since the 16S gene sequences of several *Leptospira* strains were aligned with the probe and the primers, excluding Patoc I. The alignment revealed several nucleotide differences between Patoc I and the other strains including mismatches with both forward and reverse primers along with the probe. Since there are several mismatches between Patoc I and the 16S primers, it could indicate that the sequence may not be amplified during the RT-PCR reaction. This has also shown to be true from study 2 performed in this project, where Patoc I could not be detected by using 16S assay. Furthermore, since Patoc I belong to the non-pathogenic *Biflexa* sp., some laboratories may intentionally exclude the amplification of this strain in the diagnosis of leptospirosis. This was also demonstrated in study (49). Furthermore, study (49), intentionally excluded the amplification of *Biflexa* sp. when using the 16S assay. Moreover, observed from the alignment, Patoc I had two mismatches at the 3' end of the reverse primer, which may cause interruption with the RT-PCR reaction by preventing binding of polymerase. If the purpose is to detect all spp. of Leptospira with the 16S assay, then a modification of the 16S reverse primer may be necessary, to achieve a non-specie specific primer. A suggestion could be to remove the first five

nucleotides from the 3’ end of the reverse primer and then extend with five new nucleotides from the 5’ end to obtain similar GC content. This may result in a greater annealing with other *Leptospira* spp. However, more *Leptospira* spp. must also be included in an alignment to check if the primers are able to anneal to all of them. Consequently, if the primers get modified, then they must be tested in different temperatures ± 60°C in order to assure their performance before being used in diagnostic settings.

Study 2.

For study two, a validation of the sensitivity of the current RT-PCR assay was performed. The sensitivity of the assay was carried out by detecting different *Leptospira* spp., strains and in different specimens such as, urine and histological tissue. The investigations were performed by creating two different master mixes targeted for the *LipL32* and *16S genes*. The results were obtained by RT-PCR and will be discussed and evaluated in this section.

How does the RT-PCR assay perform when detecting *Leptospira* DNA from several strains, urine from pigs and kidney tissue from both pigs and dogs?

In study two, the RT-PCR assay were able to detect several *Leptospira* strains as demonstrated in table 3.3 except of Patoc I. Considering that Patoc I was shown to have mismatches with the primer sequences from the sequence alignment, may suggest that the detection deficiency is caused by this. Another possible reason for Patoc I not being detected, could be due to no *Leptospira* DNA present in the sample because of DNA degradation. However, the 16S and LipL32 assays showed great performance toward detecting pathogenic *Leptospira* strains. The two assays were also able to detect *Leptospira* DNA in kidney tissue from pigs and dogs. However, when analyzing pig urine, the RT-PCR assay had conflicted results. The LipL32 assay did not have any amplifications for all the 62 urine samples implying that the samples where “negative”, whereas the 16S assay did have amplification for all the 62 samples implying “positive” for *Leptospira*. This may suggest that either the samples contained *Leptospira* DNA that had lost the *LipL32* gene which could not be detected by the LipL32 assay or that other nonpathogenic *Leptospira* ssp. or microorganisms were present in the 16S assay, which resulted in positive samples. For this reason, it would be interesting to sequence the obtained PCR product to reveal which organisms that has been amplified and to assure true positive results of Leptospira. In addition, study (48) also observed same events as above mentioned.

Moreover, the study identified *Peptostreptococcus stomatitis* and *P. anaerobius* through sequencing, which are members of the normal gastrointestinal and vaginal flora (for human beings). This could potentially cause false positive results when using the 16S assay to detect *Leptospira* DNA in humans (48)(50).

Study 3.

Study three was performed by creating two identical RT-PCR setups and testing the assay on two different RT-PCR machines; 7500 and Q5.

How is the robustness of the RT-PCR assay that is used in the diagnosis of leptospirosis when testing the assay on two different RT-PCR machines?

The machine comparison in study three revealed compliance between 7500 and Q5, indicating that the results obtained from both machines are equivalent to each other. This implies that the robustness of the RT-PCR assay is solid when using different RT-PCR machines in the detection of *Leptospira* DNA. Furthermore, the reaction was performed in triplicates by different technicians, to study potential errors occurred by human variation. The assay also showed great robustness towards human variation. Moreover, by performing a Bland-Altman plot a small bias was observed for both LipL32 and 16S assay's, though a one-way ANOVA showed no significant variance. In addition, it was noticeable that for both LipL32 and 16S assay, the 10^6control exceeded the 2±SD. This means that the 10^6control was not within the established interval and the importance of this will be further discussed in study four.

Study 4.

The aim of study four was to examine the sensitivity or detection limit of the RT-PCR assays LipL32 and 16S when detecting *Leptospira* DNA. This was achieved by generating a serial dilution 10^3-10^8 of the positive *Leptospira* control.

Is the RT-PCR assay sensitive enough to detect *Leptospira* DNA in diluted samples and how can the sensitivity of the assay be optimized?

The RT-PCR assay for both LipL32 and 16S were able to detect the positive control in a 10^5 dilution. This means that the sensitivity of the assay is optimal enough to detect *Leptospira* DNA in diluted samples as low as 10^5. In addition, both assays were also able to be detect DNA in the 10^6positive control however, the detection was very inconsistent and sometimes the assay was not able to detect DNA in the 10^6control. This also confirms the results from study three, where the 10^6control exceeded the 2±SD for both LipL32 and 16S assay. Till this date, it has been difficult to detect *Leptospira* bacteria in the early stages of the disease, since some diagnostic assay requires larger quantities of the bacteria in order to determine a positive sample. This is also the reason why clinicians have chosen RT-PCR for diagnostic purposes to detect *Leptospira* in the early stages of the illness. However, when using RT-PCR it is not always possible to detect *Leptospira* DNA in very diluted samples or in samples with low amounts of DNA material. Therefore, to investigate the actual detection limit for the RT-PCR assay, the number of DNA copies of Leptospira could be determined in a sample. This can assure the detection of DNA in very diluted samples and with low amounts of DNA material in a sample when using RT-PCR. However, some factors can affect the determination of the detection limit since the amount of DNA in a sample may vary depending on factors such as the degree the sample has been diluted or the magnitude of infection with *Leptospira* cells. Hence, if the aim is to determine the specific amount of *Leptospira* DNA present in a sample, different measures should be considered. Initially, a ratio of DNA copies/*Leptospira* cells must be noted. Consequently, this has many challenges such as difficulties when cultivating *Leptospira* cells due to inconsistent cultures (some grows better than others), risk of laboratory staff getting infected and by the reason of that it is almost impractical to count live panels of *Leptospira* bacteria due to their high motility. Moreover, an alternative method to determine DNA copies/known amount of *Leptospira* cells could be to measure the DNA copies of the positive controls and thereupon be able to set a detection limit. However, some diagnostic laboratories are not interested in the absolute quantity of *Leptospira* DNA in a sample. Laboratories such as SSI are more aimed to assure the quality of detection of *Leptospira* DNA in a sample and not in the actual quantity since it's not relevant for diagnostic purposes. Moreover, other research-based laboratories could be more interested in knowing the quantity due to experimental motives.

The overall performance of the PCR assay

In this section, a review and discussion of the overall PCR method used in the diagnosis of leptospirosis will be given.

What are some of the advantages and disadvantages when using PCR for detection of *Leptospira*?

There are several advantages when using PCR to detect *Leptospira* some of which the ability to; detect several pathogenic *Leptospira* spp., discriminate between pathogenic and non-pathogenic spp., relatively fast and simple performance of procedure, uniformity in routine diagnostic settings, detection within a variety of sample materials such as blood, cerebrospinal fluid and urine and importantly, high sensitivity within early stage of illness (34). Nevertheless, PCR has also drawbacks and consequently, express disadvantages such as; costly and analysis are limiting to advanced equipment, low sensitivity in later stages of illness and consequently, low sensitivity after antibiotic treatment has been initiated. Moreover, there are several components that may potentially inhibit the PCR reaction such as, components within a test sample, from the extraction method or from plastics used during sample preparation (51). Furthermore, study (49) demonstrated different sensitivity in different samples, which should be taken into consideration when performing PCR. Moreover, PCR cannot identify the exact *Leptospira* spp. that has caused the infection (34)

Is PCR better than serological diagnostics such as MAT and ELISA?

When considering the complications of diagnosing leptospirosis, it is crucial not to exclude MAT and ELISA over PCR. Depending of the stage of diseases one method is superior to the other. However, it is favorable to use both methods simultaneously, since it would elevate the opportunities to detect Leptospira however, this is cost expensive. Additionally, the advantage of PCR over serology is mainly meaningful during early stages of leptospirosis (34).

How can the PCR assay be optimized?

The PCR assay as it is to date, exhibits high sensitivity and specificity towards pathogenic *Leptospira* DNA. Some of the measures that can be optimized includes the type of sample that is used to test

with PCR. Some studies have proposed that urine samples may be an optimal specimen for early diagnosis of leptospirosis by PCR. However, these studies were performed on a small population and needs to be verified with larger populations (6). Furthermore, it would be relevant to increase the sensitivity of the diagnostic setup using PCR assays, by increasing the concentration of target DNA in the DNA extraction. The increased target DNA would be useful when detecting *Leptospira* in diluted test samples were the amount of DNA would be undetectable otherwise.

Conclusion

An overall validation of the current RT-PCR assay has been completed. The RT-PCR assay consist of a primary (LipL32) and confirmatory (16S) assay which targets pathogenic *Leptospira* spp. The data obtained from all four studies collectively has demonstrated satisfying results. The performance of the RT-PCR has proven to detect several *Leptospira* strains and spp. which are relevant in the diagnosis of leptospirosis. Furthermore, the RT-PCR assay has exhibited abilities to distinguish between pathogenic and non-pathogenic *Leptospira* spp. When the RT-PCR assay were tested on different animal samples, the assay was able to detect *Leptospira* DNA in histological tissue such as kidney from pigs and dogs. However, the 16S assay seemed to have cross reaction with other bacteria's in urine samples from pigs. In addition, the RT-PCR were able to detect *Leptospira* DNA as low as 10^5-10^6test dilution nevertheless, the 10^6 dilution happens to be detected though inconsistently. Moreover, the RT-PCR assay demonstrated great robustness towards machine and human variation. Such findings of the RT-PCR assays are favorable in diagnostics settings, since it can provide reliable results during diagnosis. Therefore, this study can conclude that the current RT-PCR assay used to detect *Leptospira* in human samples at SSI can also be used in animal samples. Consequently, it is crucial to apply the right detecting method to the appropriate state of illness of leptospirosis. Considering, that the sensitivity of the detection methods varies significantly according to the state of illness. As an example, the first 1-2 weeks of illness it is favorable to use PCR to detect *Leptospira* since the DNA are present in blood, cerebrospinal fluid and urine. Hereafter antibodies can be detected through serological methods. To improve the diagnosis of leptospirosis it is first and foremost essential to increase the knowledge of *Leptospira* spp. Therefore, further investigation must be made in understanding virulence factors and other molecular structures of *Leptospira* spp.

Perspectivation

Based on this study, there are still many approaches that can be made in the diagnosis of leptospirosis. Moreover, since the taxonomy and classification of the bacteria has been delayed comprehensive knowledge about *Leptospira* and leptospirosis has also been delayed. As a result, the exact structure and virulence mechanisms of the pathogenic *Leptospira* spp. is still unclear. Therefore, there is an urgent need to further investigation and understanding of *Leptospira* and the mechanisms that leads to infection in human and animals. Nevertheless, the scientific research in whole genome sequences of several strains and spp. of *Leptospira* is still incomplete. Therefore, as a consideration, it would be beneficial to complete this domain in order to execute more novels in vivo as well as in vitro to gain greater understanding of *Leptospira* and leptospirosis and ultimately update the detection methods including PCR.

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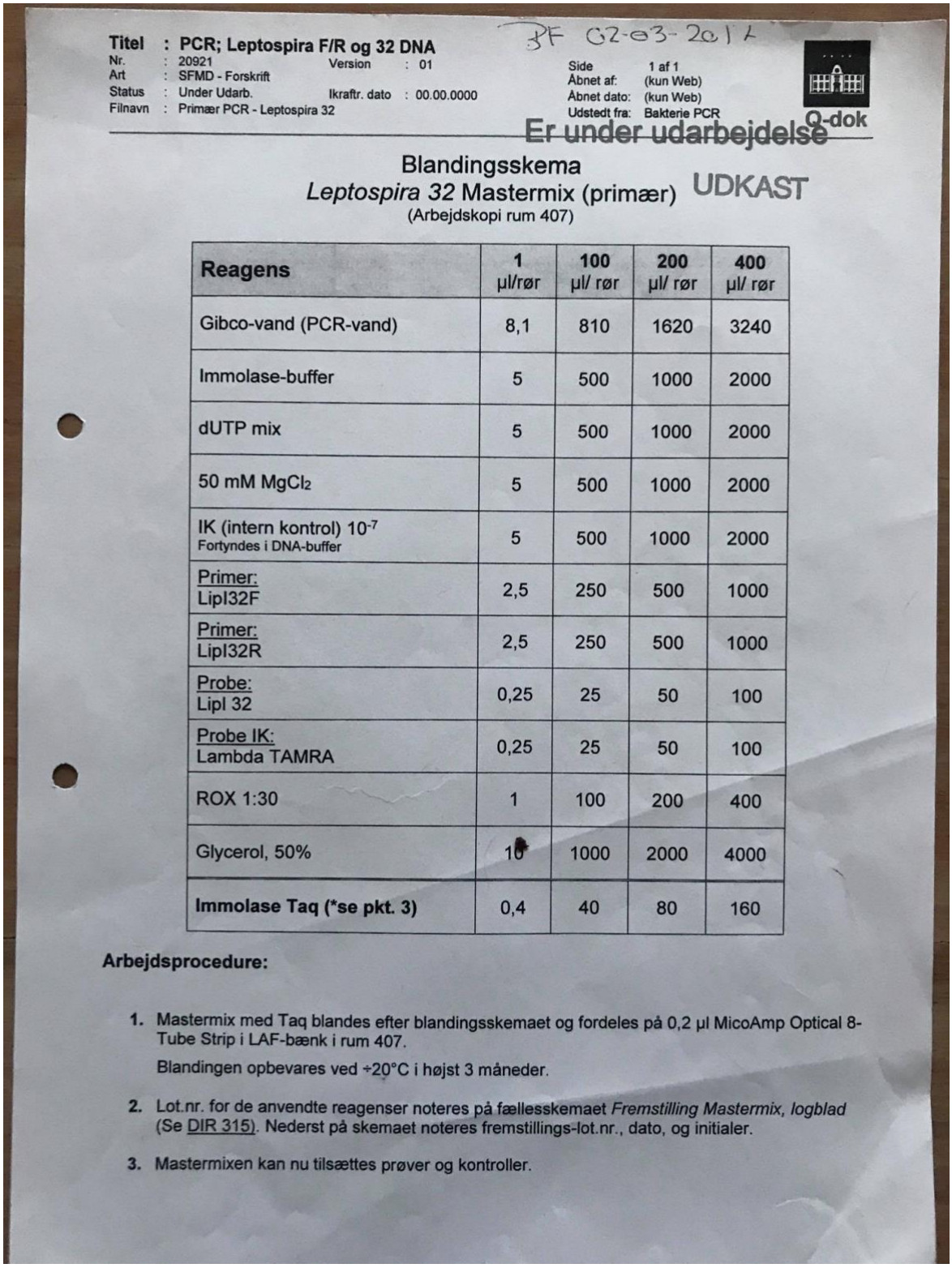
Appendix 1 Primary master mix LipL32



Titel : PCR; Leptospira F/R og 32 DNA
Nr. : 20921 Version : 01
Art : SFMD - Forskrift
Status : Under Udarb. Ikraftr. dato : 00.00.0000
Filnavn : Primær PCR - Leptospira 32

BF 02-03-2011

Side 1 af 1
Åbnet af: (kun Web)
Åbnet dato: (kun Web)
Udstedt fra: Bakterie PCR

Q-dok

Er under udarbejdelse

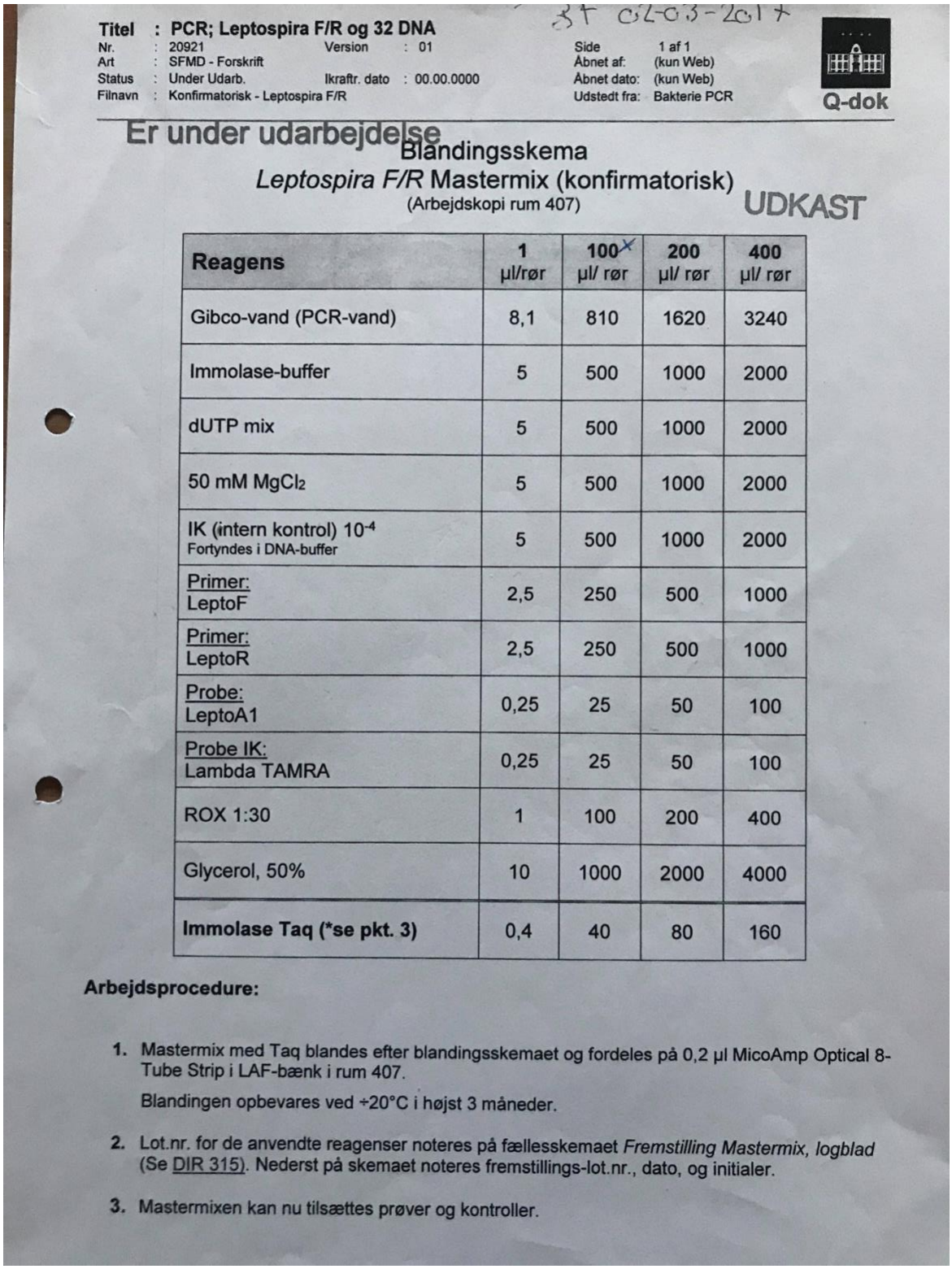
Blandingsskema
Leptospira 32 Mastermix (primær) UDKAST
(Arbejdskopi rum 407)

Reagens	1 µl/rør	100 µl/ rør	200 µl/ rør	400 µl/ rør
Gibco-vand (PCR-vand)	8,1	810	1620	3240
Immolase-buffer	5	500	1000	2000
dUTP mix	5	500	1000	2000
50 mM $MgCl_2$	5	500	1000	2000
IK (intern kontrol) 10^{-7} Fortyndes i DNA-buffer	5	500	1000	2000
Primer: LipI32F	2,5	250	500	1000
Primer: LipI32R	2,5	250	500	1000
Probe: LipI 32	0,25	25	50	100
Probe IK: Lambda TAMRA	0,25	25	50	100
ROX 1:30	1	100	200	400
Glycerol, 50%	10	1000	2000	4000
Immolase Taq (*se pkt. 3)	0,4	40	80	160

Arbejdsprocedure:

1. Mastermix med Taq blandes efter blandingsskemaet og fordeles på 0,2 µl MicoAmp Optical 8-Tube Strip i LAF-bænk i rum 407.
   Blandingen opbevares ved ÷20°C i højst 3 måneder.
2. Lot.nr. for de anvendte reagenser noteres på fællesskemaet *Fremstilling Mastermix, logblad* (Se DIR 315). Nederst på skemaet noteres fremstillings-lot.nr., dato, og initialer.
3. Mastermixen kan nu tilsættes prøver og kontroller.

Appendix 2 Confirmatory master mix 16S (F/R)



Titel : PCR; Leptospira F/R og 32 DNA
Nr. : 20921 Version : 01
Art : SFMD - Forskrift
Status : Under Udarb. Ikraftr. dato : 00.00.0000
Filnavn : Konfirmatorisk - Leptospira F/R

3F 02-03-2017

Side 1 af 1
Åbnet af: (kun Web)
Åbnet dato: (kun Web)
Udstedt fra: Bakterie PCR

Q-dok

Er under udarbejdelse

Blandingsskema

Leptospira F/R Mastermix (konfirmatorisk)

(Arbejdskopi rum 407)

UDKAST

Reagens	1 µl/rør	100 µl/ rør	200 µl/ rør	400 µl/ rør
Gibco-vand (PCR-vand)	8,1	810	1620	3240
Immolase-buffer	5	500	1000	2000
dUTP mix	5	500	1000	2000
50 mM $MgCl_2$	5	500	1000	2000
IK (intern kontrol) 10^{-4} Fortyndes i DNA-buffer	5	500	1000	2000
Primer: LeptoF	2,5	250	500	1000
Primer: LeptoR	2,5	250	500	1000
Probe: LeptoA1	0,25	25	50	100
Probe IK: Lambda TAMRA	0,25	25	50	100
ROX 1:30	1	100	200	400
Glycerol, 50%	10	1000	2000	4000
Immolase Taq (*se pkt. 3)	0,4	40	80	160

Arbejdsprocedure:

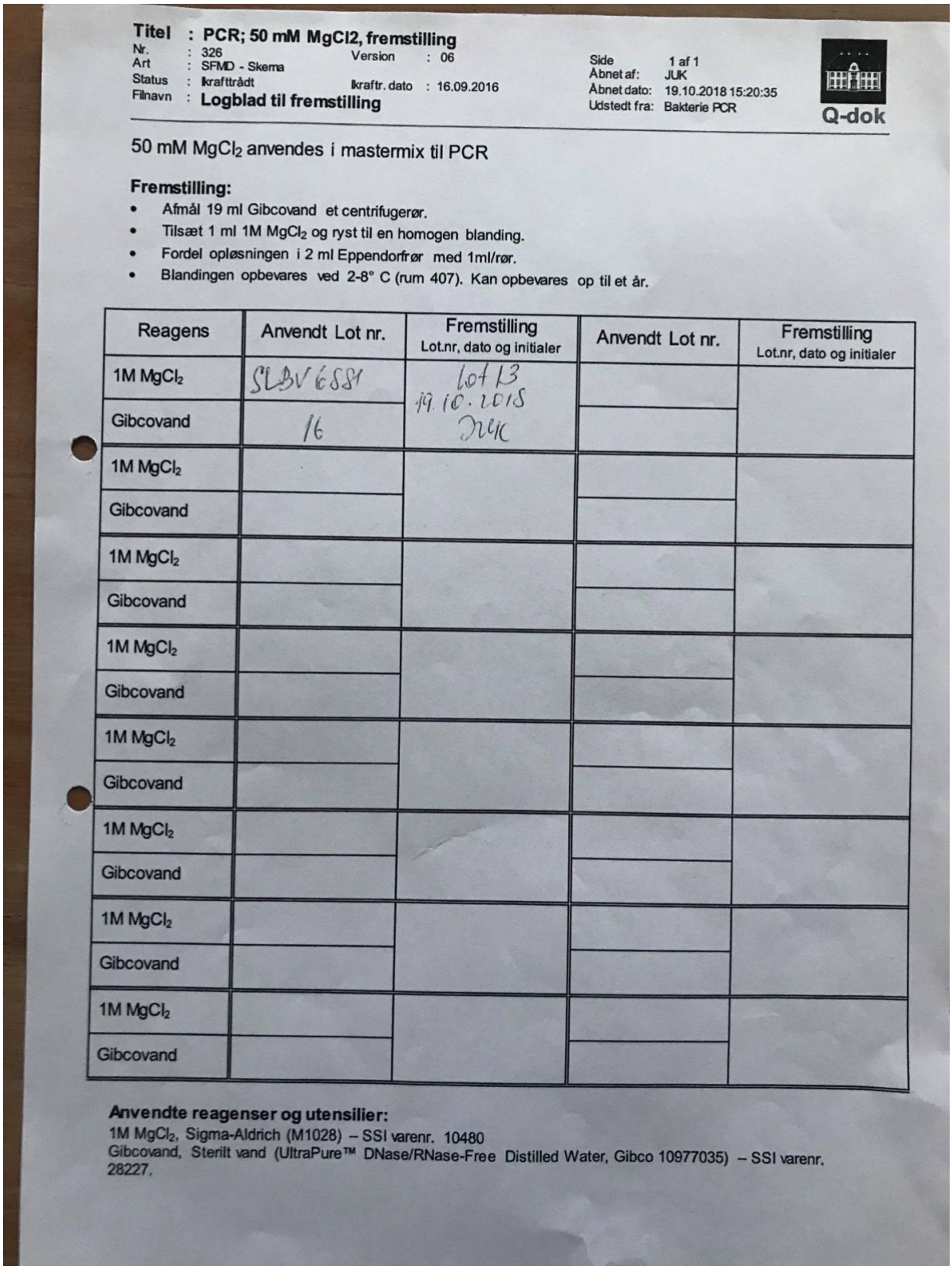
1. Mastermix med Taq blandes efter blandingsskemaet og fordeles på 0,2 µl MicoAmp Optical 8-Tube Strip i LAF-bænk i rum 407.

   Blandingen opbevares ved ÷20°C i højst 3 måneder.

2. Lot.nr. for de anvendte reagenser noteres på fællesskemaet *Fremstilling Mastermix, logblad* (Se DIR 315). Nederst på skemaet noteres fremstillings-lot.nr., dato, og initialer.

3. Mastermixen kan nu tilsættes prøver og kontroller.

Appendix 3 Creating 50mM MgCl2



Titel : PCR; 50 mM MgCl2, fremstilling
Nr. : 326 Version : 06
Art : SFMD - Skema
Status : Ikrafttrådt Ikraftr. dato : 16.09.2016
Filnavn : **Logblad til fremstilling**

Side 1 af 1
Åbnet af: JUK
Åbnet dato: 19.10.2018 15:20:35
Udstedt fra: Bakterie PCR

Q-dok

50 mM $MgCl_2$ anvendes i mastermix til PCR

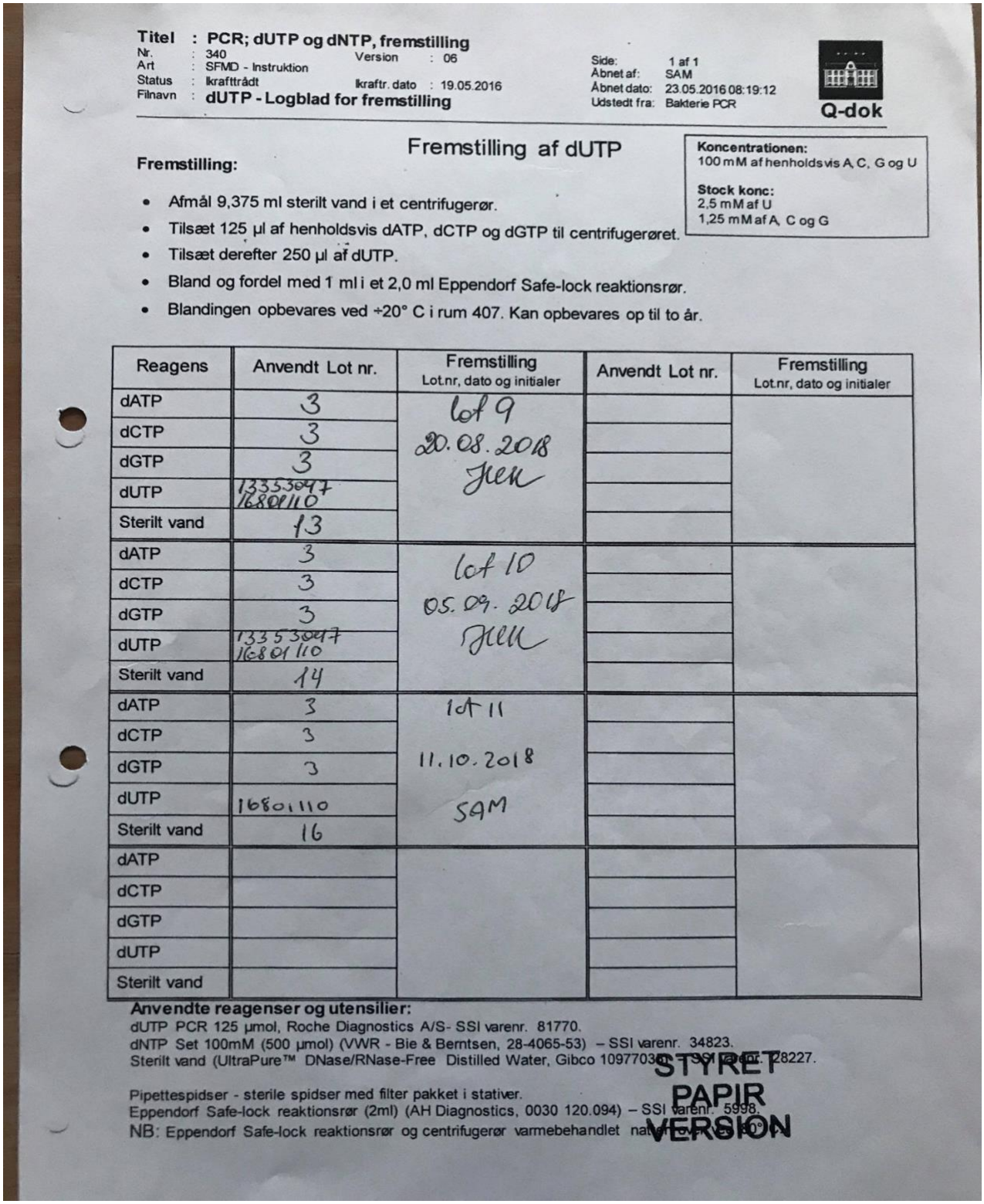
Fremstilling:

- Afmål 19 ml Gibcovand et centrifugerør.
- Tilsæt 1 ml 1M $MgCl_2$ og ryst til en homogen blanding.
- Fordel opløsningen i 2 ml Eppendorfrør med 1ml/rør.
- Blandingen opbevares ved 2-8° C (rum 407). Kan opbevares op til et år.

Reagens	Anvendt Lot nr.	Fremstilling Lot.nr, dato og initialer	Anvendt Lot nr.	Fremstilling Lot.nr, dato og initialer
1M $MgCl_2$	SLBV6881	lot L3 19.10.2015 JUK		
Gibcovand	16			
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				
1M $MgCl_2$				
Gibcovand				

Anvendte reagenser og utensilier:
1M $MgCl_2$, Sigma-Aldrich (M1028) – SSI varenr. 10480
Gibcovand, Sterilt vand (UltraPure™ DNase/RNase-Free Distilled Water, Gibco 10977035) – SSI varenr. 28227.

Appendix 4 Creating dNTP and dUTP



Titel : **PCR; dUTP og dNTP, fremstilling**
Nr. : 340 Version : 06
Art : SFMD - Instruktion
Status : Ikrafttrådt Ikraftr. dato : 19.05.2016
Filnavn : **dUTP - Logblad for fremstilling**

Side: 1 af 1
Åbnet af: SAM
Åbnet dato: 23.05.2016 08:19:12
Udstedt fra: Bakterie PCR

Q-dok

Fremstilling af dUTP

Fremstilling:

Koncentrationen:
100 mM af henholdsvis A, C, G og U

Stock konc:
2,5 mM af U
1,25 mM af A, C og G

- Afmål 9,375 ml sterilt vand i et centrifugerør.
- Tilsæt 125 µl af henholdsvis dATP, dCTP og dGTP til centrifugerøret.
- Tilsæt derefter 250 µl af dUTP.
- Bland og fordel med 1 ml i et 2,0 ml Eppendorf Safe-lock reaktionsrør.
- Blandingen opbevares ved ÷20° C i rum 407. Kan opbevares op til to år.

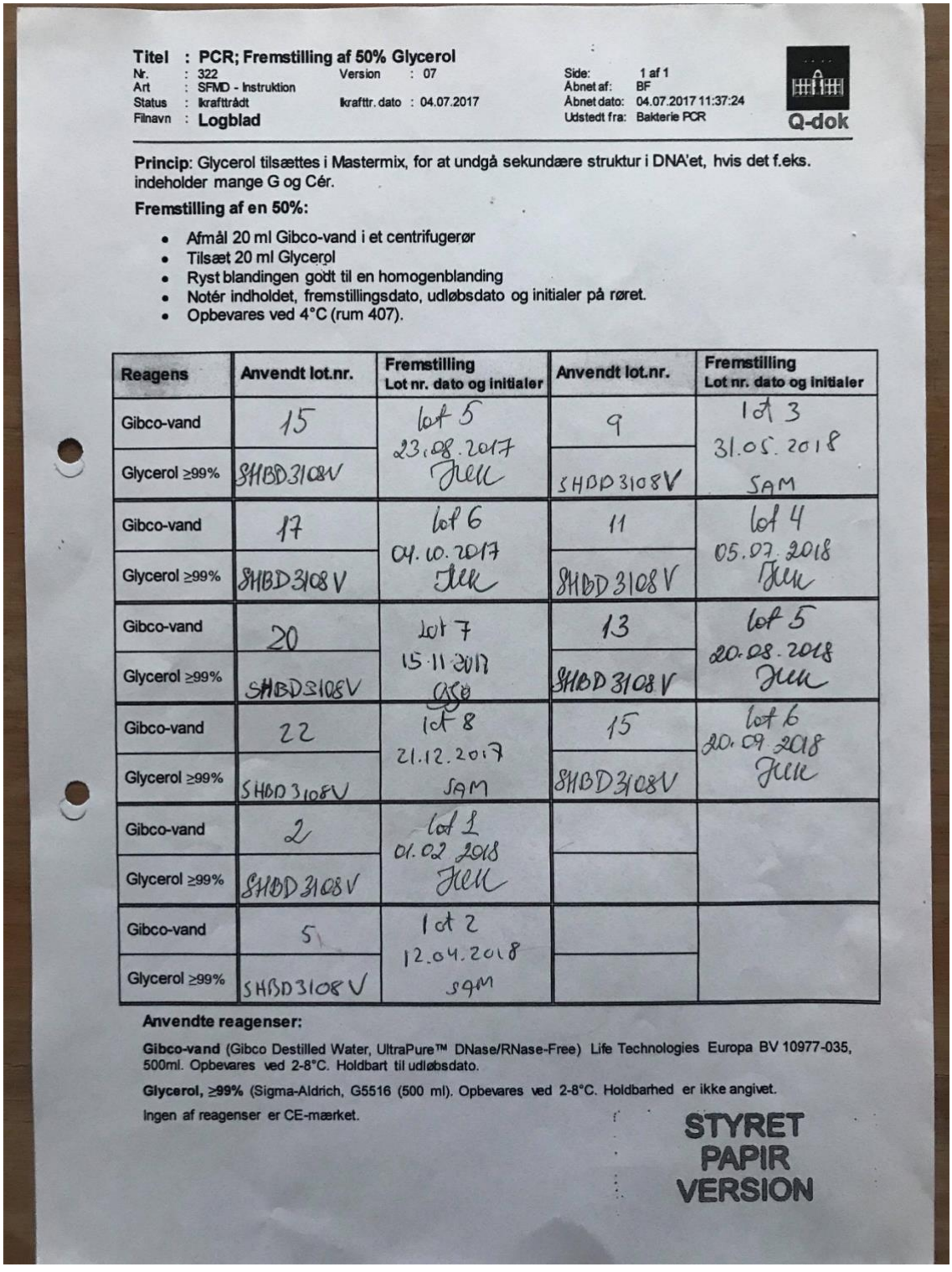
Reagens	Anvendt Lot nr.	Fremstilling Lot.nr, dato og initialer	Anvendt Lot nr.	Fremstilling Lot.nr, dato og initialer
dATP	3	lot 9 20.08.2018 JLK		
dCTP	3			
dGTP	3			
dUTP	13353047 16801110			
Sterilt vand	13			
dATP	3	lot 10 05.09.2018 JLK		
dCTP	3			
dGTP	3			
dUTP	13353047 16801110			
Sterilt vand	14			
dATP	3	lot 11 11.10.2018 SAM		
dCTP	3			
dGTP	3			
dUTP	16801110			
Sterilt vand	16			
dATP				
dCTP				
dGTP				
dUTP				
Sterilt vand				

Anvendte reagenser og utensilier:
dUTP PCR 125 µmol, Roche Diagnostics A/S- SSI varenr. 81770.
dNTP Set 100mM (500 µmol) (VWR - Bie & Berntsen, 28-4065-53) – SSI varenr. 34823.
Sterilt vand (UltraPure™ DNase/RNase-Free Distilled Water, Gibco 10977035) – SSI varenr. 28227.

Pipettespidser - sterile spidser med filter pakket i stativer.
Eppendorf Safe-lock reaktionsrør (2ml) (AH Diagnostics, 0030 120.094) – SSI varenr. 5998.
NB: Eppendorf Safe-lock reaktionsrør og centrifugerør varmebehandlet natten over ved 180°

STYRET PAPIR VERSION

Appendix 5 Creating 50% glycerol



Titel : PCR; Fremstilling af 50% Glycerol
Nr. : 322 Version : 07
Art : SFMD - Instruktion
Status : Ikrafttrådt Ikrafttr. dato : 04.07.2017
Filnavn : **Logblad**

Side: 1 af 1
Åbnet af: BF
Åbnet dato: 04.07.2017 11:37:24
Udstedt fra: Bakterie PCR

Q-dok

Princip: Glycerol tilsættes i Mastermix, for at undgå sekundære struktur i DNA'et, hvis det f.eks. indeholder mange G og Cér.

Fremstilling af en 50%:

- Afmål 20 ml Gibco-vand i et centrifugerør
- Tilsæt 20 ml Glycerol
- Ryst blandingen godt til en homogenblanding
- Notér indholdet, fremstillingsdato, udløbsdato og initialer på røret.
- Opbevares ved 4°C (rum 407).

Reagens	Anvendt lot.nr.	Fremstilling Lot nr. dato og initialer	Anvendt lot.nr.	Fremstilling Lot nr. dato og initialer
Gibco-vand	15	lot 5 23.08.2017 JLK	9	lot 3 31.05.2018 SAM
Glycerol ≥99%	SHBD3108V		SHBD3108V	
Gibco-vand	17	lot 6 04.10.2017 JLK	11	lot 4 05.07.2018 JLK
Glycerol ≥99%	SHBD3108V		SHBD3108V	
Gibco-vand	20	lot 7 15.11.2017 CSO	13	lot 5 20.08.2018 JLK
Glycerol ≥99%	SHBD3108V		SHBD3108V	
Gibco-vand	22	lot 8 21.12.2017 SAM	15	lot 6 20.09.2018 JLK
Glycerol ≥99%	SHBD3108V		SHBD3108V	
Gibco-vand	2	lot 1 01.02.2018 JLK		
Glycerol ≥99%	SHBD3108V			
Gibco-vand	5	lot 2 12.04.2018 SAM		
Glycerol ≥99%	SHBD3108V			

Anvendte reagenser:

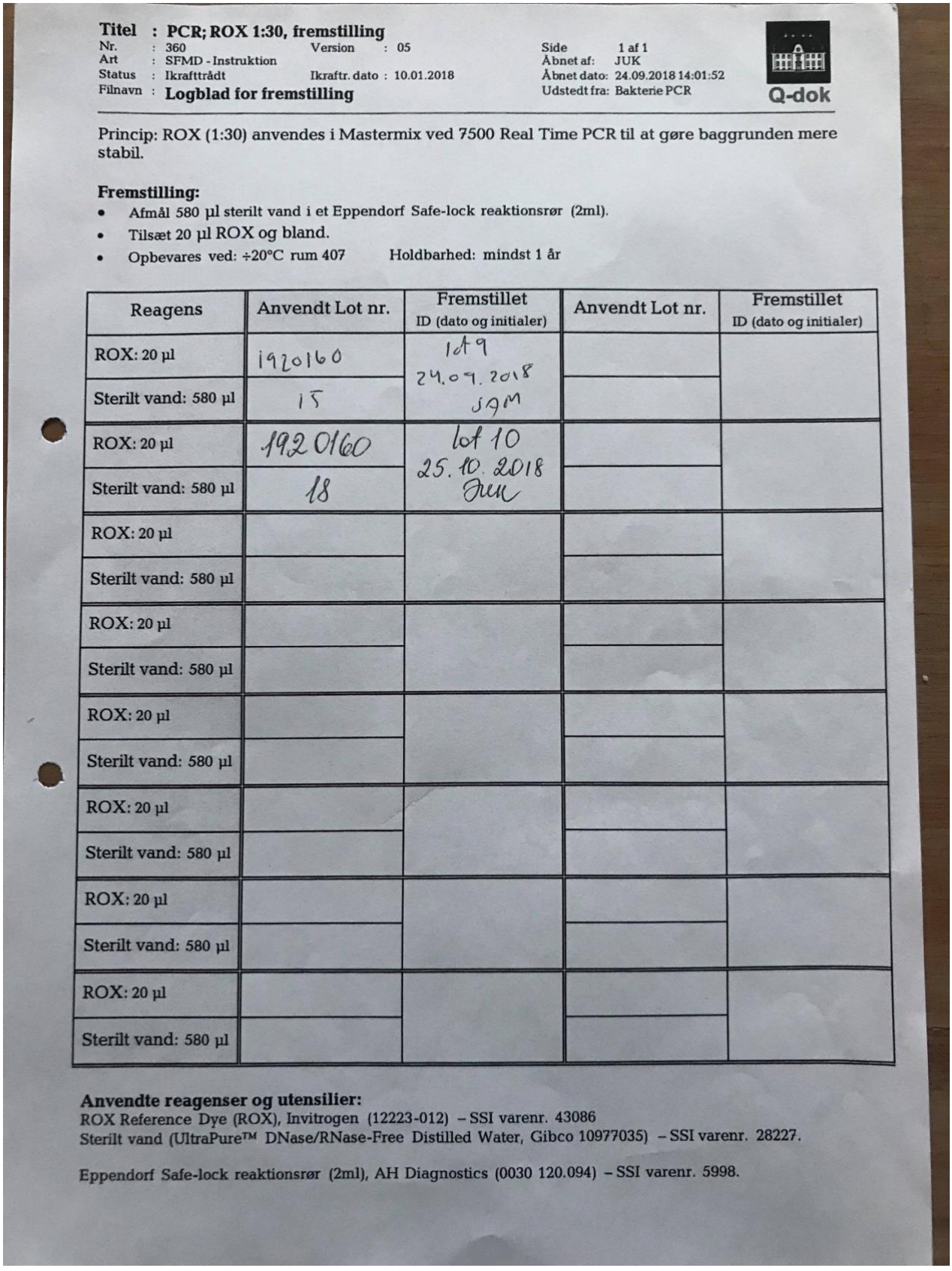
Gibco-vand (Gibco Destilled Water, UltraPure™ DNase/RNase-Free) Life Technologies Europa BV 10977-035, 500ml. Opbevares ved 2-8°C. Holdbart til udløbsdato.

Glycerol, ≥99% (Sigma-Aldrich, G5516 (500 ml). Opbevares ved 2-8°C. Holdbarhed er ikke angivet.

Ingen af reagenser er CE-mærket.

STYRET PAPIR VERSION

Appendix 6 Creating 1:30 ROX



Titel : PCR; ROX 1:30, fremstilling
Nr. : 360 Version : 05
Art : SFMD - Instruktion
Status : Ikrafttrådt Ikraftr. dato : 10.01.2018
Filnavn : **Logblad for fremstilling**

Side 1 af 1
Åbnet af: JUK
Åbnet dato: 24.09.2018 14:01:52
Udstedt fra: Bakterie PCR

Q-dok

Princip: ROX (1:30) anvendes i Mastermix ved 7500 Real Time PCR til at gøre baggrunden mere stabil.

Fremstilling:

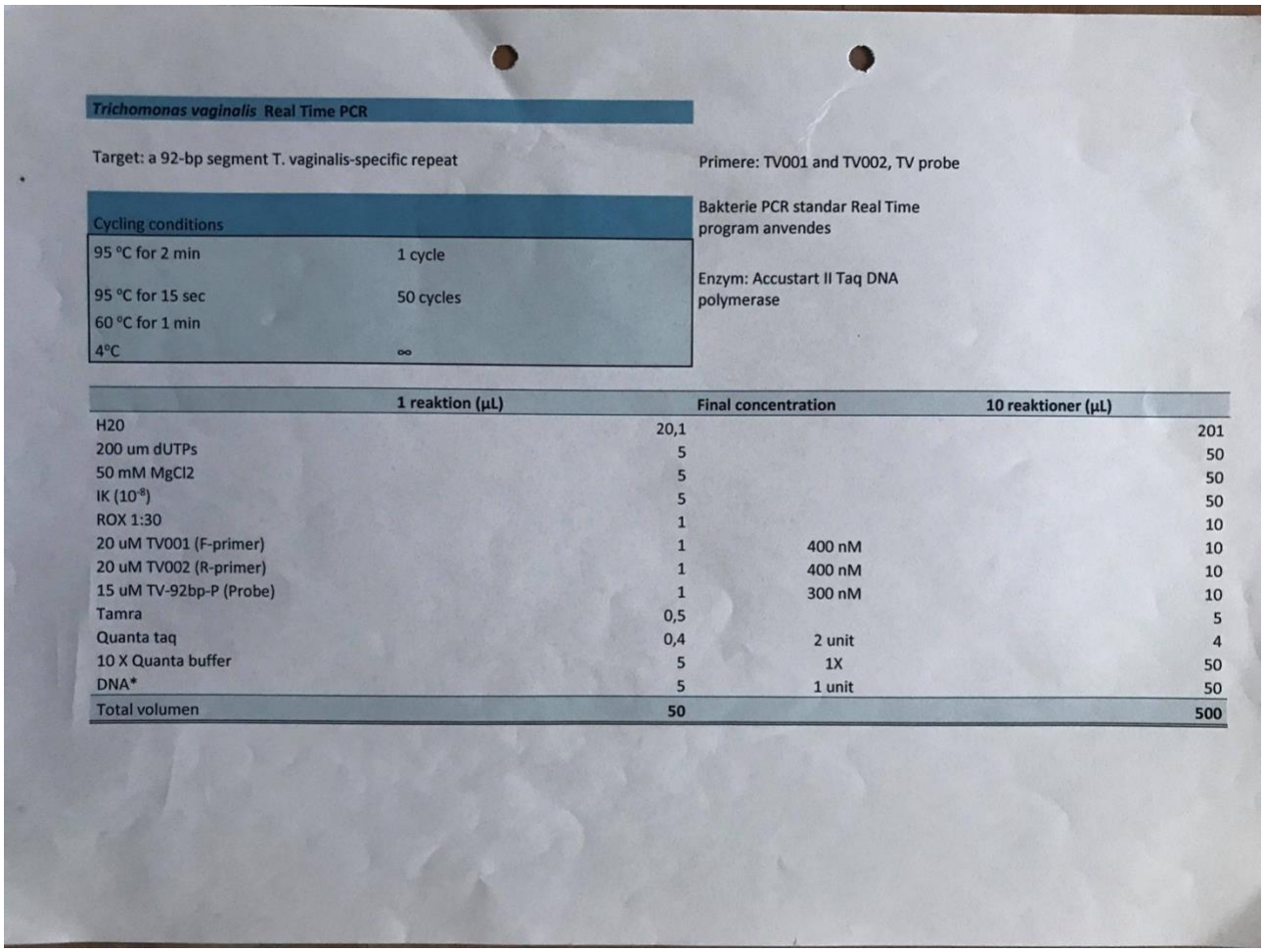
- Afmål 580 µl sterilt vand i et Eppendorf Safe-lock reaktionsrør (2ml).
- Tilsæt 20 µl ROX og bland.
- Opbevares ved: ÷20°C rum 407 Holdbarhed: mindst 1 år

Reagens	Anvendt Lot nr.	Fremstillet ID (dato og initialer)	Anvendt Lot nr.	Fremstillet ID (dato og initialer)
ROX: 20 µl	1920160	lot 9 24.09.2018 JAM		
Sterilt vand: 580 µl	15			
ROX: 20 µl	1920160	lot 10 25.10.2018 JUK		
Sterilt vand: 580 µl	18			
ROX: 20 µl				
Sterilt vand: 580 µl				
ROX: 20 µl				
Sterilt vand: 580 µl				
ROX: 20 µl				
Sterilt vand: 580 µl				
ROX: 20 µl				
Sterilt vand: 580 µl				
ROX: 20 µl				
Sterilt vand: 580 µl				
ROX: 20 µl				
Sterilt vand: 580 µl				

Anvendte reagenser og utensilier:
ROX Reference Dye (ROX), Invitrogen (12223-012) – SSI varenr. 43086
Sterilt vand (UltraPure™ DNase/RNase-Free Distilled Water, Gibco 10977035) – SSI varenr. 28227.

Eppendorf Safe-lock reaktionsrør (2ml), AH Diagnostics (0030 120.094) – SSI varenr. 5998.

Appendix 7 PCR setting for Real time PCR



***Trichomonas vaginalis* Real Time PCR**

Target: a 92-bp segment T. vaginalis-specific repeat

Primere: TV001 and TV002, TV probe

Cycling conditions	
95 °C for 2 min	1 cycle
95 °C for 15 sec 60 °C for 1 min	50 cycles
4°C	∞

Bakterie PCR standar Real Time program anvendes

Enzym: Accustart II Taq DNA polymerase

	1 reaktion (µL)	Final concentration	10 reaktioner (µL)
H20	20,1		201
200 um dUTPs	5		50
50 mM MgCl2	5		50
IK (10^{-8})	5		50
ROX 1:30	1		10
20 uM TV001 (F-primer)	1	400 nM	10
20 uM TV002 (R-primer)	1	400 nM	10
15 uM TV-92bp-P (Probe)	1	300 nM	10
Tamra	0,5		5
Quanta taq	0,4	2 unit	4
10 X Quanta buffer	5	1X	50
DNA*	5	1 unit	50
Total volumen	**50**		**500**

Note: these settings are also used for *Leptospira* RT-PCR assay (LipL32) and (16S)

Appendix 8 Creating IK control

Fremstilling af intern kontrol (IK)

1. Formål

At beskrive fremstilling af intern kontrol IK, dels til gelbaseret og dels til realtime PCR.

1.1 Princip

For alle akkrediterede diagnostiske PCR analyser gælder det, at der i den første PCR der udføres, er indbygget en intern amplifikationskontrol (IK), som skal afsløre om der har været komponenter i patientprøven som har hæmmet PCR, eller om den manglende opformering skyldes en fejl ved opsætningen. Disse fejl kan enten være menneskelige fejl, men kan også være tilfældigt forekommende "reaktions udfald".

PCR produktet vil indeholde et stykke af lambda-sekvensen flankeret af de relevante primere for analysen. Den samlede længe af den interne kontrol er således længden af lambda (ca. mellem 150-620 bp) plus analyseprimerne. Længden af lambda afhænger af den enkelte analyse, idet IK altid skal være længere end target. Target i konventionel PCR er generelt længere end i realtime PCR.

Gelbaseret PCR:

Lambda IPC-F	TGA CGG TTT CTA AC	Position 13663
Lambda IPC-R	GAC ATA CGG AAA TAG	Position 14280. Produkt størrelse 616 bp (med primere ca. 668 bp)

Eksempel på design af primere til fremstilling af IK kan ses herunder:

a) IK primere til PanDermatofyt PCR:

b) IK-PD-ITS-F: ACG GAT CTC TTG GTT CCG GCC TGA CGG TTT CTA AC

IK-PD-ITS-R: *GGA ATC GCG GCC TGG CCA GAC ATA CGG AAA TAG*

Realtime baseret PCR:

Lambda IPC-F	CCG GGA CGT ATC ATG CT	Position 13915
Lambda IPC-R	ACC GCT CAG GCA TTT GCT	Position 14061. Produkt størrelse 146 bp

Eksempel på design af primere til fremstilling af IK kan ses herunder:

c) IK primere til plyA PCR (meningitis) (Real Time PCR).

Ply-894-ipc-f *TGC AGA GCG TCC TTT GGT CTA T CCG GGA CGT ATC ATG CT*

Ply-974-ipc-r *CTC TTA CTC GTG GTT TCC AAC TTG A ACC GCT CAG GCA TTT GCT*

2. Procedure

Intern kontrol fremstilles for de fleste analyser ved, at et sæt specieldesignede primere med en lambda sekvens i 3'enden amplificerer et stykke lambda DNA. Fragmentet oprenses ved gelelektroforese og fortyndes således, at det giver en klar reaktion i PCR uden at forrykke detektionsgrænsen af den specifikke PCR test.

Selve fremstilling er ens for begge PCR typer. Forskellen er sekvenserne, amplificering samt test i den specifikke metode.

2.1 Fremstilling

PCR-laboratoriet:

Primer fortynding og fremstilling af mix foregår i ren bænk.

a) Primerne fortyndes til 20 µM i TE-buffer (1x konc – se fremstilling af TE-buffer i DIR 325).

b) Mastermix fremstilles efter *Blandingsskemaet* (Bilag 1a).

c) Blandingen amplificeres på 2720-maskine ved program IK, rum 216 – se instruktionen DIR 27062

Program: IK				
1.	94°C/2 min.	-		1 cyklus
2.	94°C/30 sek.	40°C/30 sek.	72°C/1 min	40 cykler
4.	72°C/5 min.			1 cyklus
5.	4°C			→ ∞

DNA-laboratoriet:

Efter amplificering separeres DNA-fragmenterne efter størrelse og form ved hjælp af gelelektroforese.

For kørsel - se DIR 25302.

a) 20 µl amplificat blandes med 5 µl loading buffer

b) Køres i et rent kar med 0,5 X TBE på en 2% agarosegel i 1 time ved 150 V.

c) 100 bp markør loades i begge sider af gelen.

d) IPC-båndet skæres med en ren skalpel, og overføres til et Eppendorfrør med 1000 µl TE-buffer (1x konc.).

e) IPC-båndet smeltes på en forvarmet varmeblok i 3 min ved 90°C = stockopløsningen.

f) Stock-opløsningen fortyndes til 10^{-1} (100 µl stockopløsning + 900 TE (1x konc.).

g) Den oprensede IK påføres dato og initialer for fortyndingen, dette påføres alle rør hvis stamopløsningen af IK er blevet udportioneret.

h) Fremstillingen noteres på *Logblad for fremstilling* (Bilag 1c).

i) Stock-opløsningen opbevares ved ÷20°C i DNA-laboratoriet, og fortyndingen 10^{-1} afleveres til PCR-laboratoriet.

2.2 Test af den interne kontrol

Den optimale koncentration af IK-kontrol skal nu findes i den specifikke metode.

Den fortyndede kontrol (10^{-1}) titreres ud i en 10-foldsfortynding til f.eks. 10^{-8}.

Tre fortyndinger vælges ud fra hvor stærk båndet var ved stock-opløsningen og disse testes i variable koncentrationer alt efter metoden – se eksempel.

Eksempel:

Den anvendte Mastermix er variabel alt efter metoden. Det er dog vigtigt af mixen er homogen og anvendes til alle 15 rør.

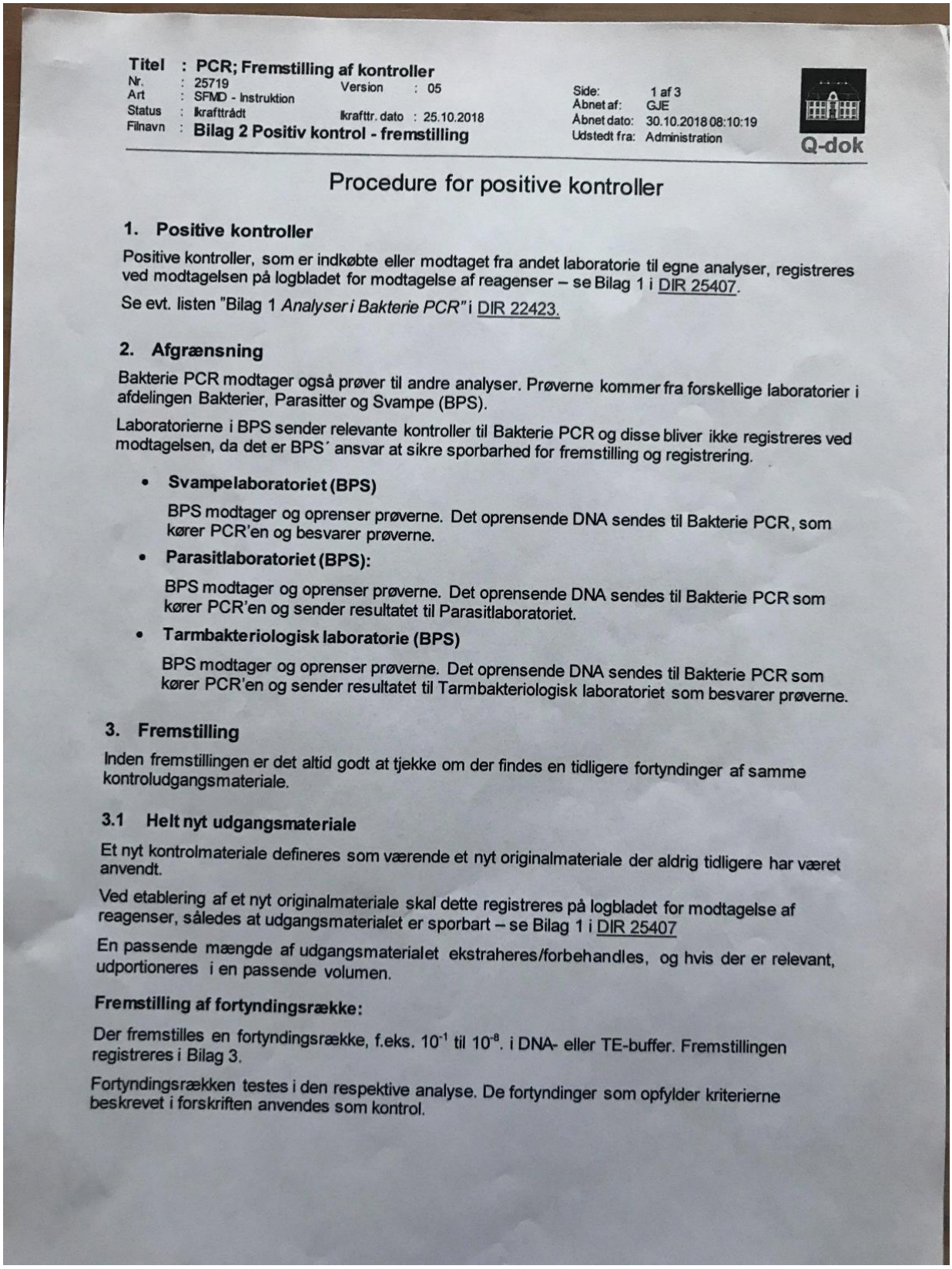
RØR	Pos kontrol		IK
1.	10-3	+	10-6
2.	10-3	+	10-7
3.	10-3	+	10-8
4.	10-3	+	DNA-buffer
5.	10-3	+	PCR-vand
6.	10-4	+	10-6
7.	10-4	+	10-7
8.	10-4	+	10-8
9.	10-4	+	DNA-buffer
10.	10-4	+	PCR-vand
11.	10-5	+	10-6
12.	10-5	+	10-7
13.	10-5	+	10-8
14.	10-5	+	DNA-buffer
15.	10-5	+	PCR-vand

a) Der tilsættes x µl af hver fortynding til relevante Mastermixen, og amplificeres.

b) Efter amplificering separeres DNA-fragmenterne efter størrelse og form ved hjælp af gelelektroforese. For kørsel – se instruktionen DIR 25302.

c) Den optimale IK vælges ud fra at den interne kontrol skal være tydelig, men må ikke hæmme den positive kontrol. Resultatet noteres på *Logblad for fremstilling* (Bilag 1c).

Appendix 9 Procedure of positive controls



Titel : PCR; Fremstilling af kontroller
Nr. : 25719 Version : 05
Art : SFMD - Instruktion
Status : Ikrafttrådt Ikrafttr. dato : 25.10.2018
Filnavn : Bilag 2 Positiv kontrol - fremstilling

Side: 1 af 3
Åbnet af: GJE
Åbnet dato: 30.10.2018 08:10:19
Udstedt fra: Administration

Q-dok

Procedure for positive kontroller

1. Positive kontroller

Positive kontroller, som er indkøbte eller modtaget fra andet laboratorie til egne analyser, registreres ved modtagelsen på logbladet for modtagelse af reagenser – se Bilag 1 i DIR 25407.

Se evt. listen "Bilag 1 *Analyser i Bakterie PCR"* i DIR 22423.

2. Afgrænsning

Bakterie PCR modtager også prøver til andre analyser. Prøverne kommer fra forskellige laboratorier i afdelingen Bakterier, Parasitter og Svampe (BPS).

Laboratorierne i BPS sender relevante kontroller til Bakterie PCR og disse bliver ikke registreres ved modtagelsen, da det er BPS´ ansvar at sikre sporbarhed for fremstilling og registrering.

- **Svampelaboratoriet (BPS)**

  BPS modtager og oprenser prøverne. Det oprensende DNA sendes til Bakterie PCR, som kører PCR'en og besvarer prøverne.
- **Parasitlaboratoriet (BPS):**

  BPS modtager og oprenser prøverne. Det oprensende DNA sendes til Bakterie PCR som kører PCR'en og sender resultatet til Parasitlaboratoriet.
- **Tarmbakteriologisk laboratorie (BPS)**

  BPS modtager og oprenser prøverne. Det oprensende DNA sendes til Bakterie PCR som kører PCR'en og sender resultatet til Tarmbakteriologisk laboratoriet som besvarer prøverne.

3. Fremstilling

Inden fremstillingen er det altid godt at tjekke om der findes en tidligere fortyndinger af samme kontroludgangsmateriale.

3.1 Helt nyt udgangsmateriale

Et nyt kontrolmateriale defineres som værende et nyt originalmateriale der aldrig tidligere har været anvendt.

Ved etablering af et nyt originalmateriale skal dette registreres på logbladet for modtagelse af reagenser, således at udgangsmaterialet er sporbart – se Bilag 1 i DIR 25407

En passende mængde af udgangsmaterialet ekstraheres/forbehandles, og hvis der er relevant, udportioneres i en passende volumen.

Fremstilling af fortyndingsrække:

Der fremstilles en fortyndingsrække, f.eks. 10^{-1} til 10^{-8}. i DNA- eller TE-buffer. Fremstillingen registreres i Bilag 3.

Fortyndingsrækken testes i den respektive analyse. De fortyndinger som opfylder kriterierne beskrevet i forskriften anvendes som kontrol.

3.2 Kendt kontrolmateriale

Der fremstilles en fortyndingsrække i DNA- eller TE-buffer, ud fra vurderingen af den tidligere fremstillede fortyndingsrække. Se eksempel på side 3.

Fremstillingen registreres på logbladet Bilag 3.

4. Kriterier for godkendelse

Kriterierne for godkendelse er de samme som står beskrevet i de respektive forskrifter.

Den godkendte kontrol stilles i kassen for anvendte kontroller.

5. Specielle fremstillinger

5.1 Rickettsia

Positiv kontrol materiale er indkøbt fra LCG Standards (www.lgcstandards.com).

Cellerne dyrkes og DNA oprenses fra cellekulturen ved QIAgen oprensning.
100 µl til 100 µl CM til QIAgen elueres i 100 µl fra renkultur og PCR udføres med primerne.
Fortyndinger af PCR produktet anvendes i de respektive analyser.

Fortyndingerne 10-3, 10-4 og 10-5 anvendes.

Appendix 10 Additionally, reading material

Titel : Leptospira antistof (Weil)

Nr.	: 816	Version	: 06	Side	1 af 1
Art	: SFMD - Forskrift			Åbnet af:	ISPD
Status	: Ikrafttrådt	Ikraftr. dato	: 04.04.2016	Åbnet dato:	07.11.2018 13:58:19
Filnavn	: Arbejdsgang - Screening og udtitrering			Udstedt fra:	MDV Serologi

Q-dok

Hvis en prøve er tidligere positiv eller de kliniske oplysninger gør at prøven formodes positiv, kan screeningen undlades i en eller flere serotyper, og udtitrering kan foretages med det samme.

Mht. dokumentation for anvendte antisera: Der skal skrives dato/initialer for ibrugtagning af antisera hver gang et nyt batch af brugsopløsningerne tages i brug, se DIR 697, *Leptospira* (Weil), diverse.

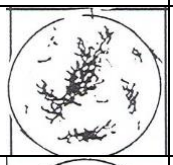
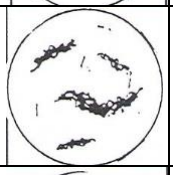
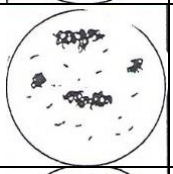
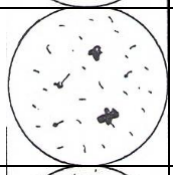
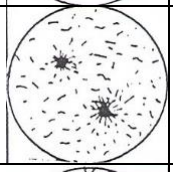
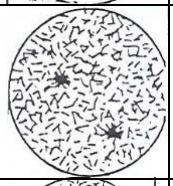
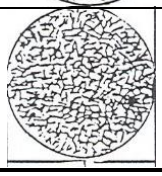
Kulturerne kontrolleres for god vækst og renhed mikroskopisk i et mørkefeltsmikroskop (foretrækkes) og/eller visuelt op mod en lyskilde med mørk baggrund. Der udvælges kulturer med god vækst og så vidt muligt ingen forurening, se DIR 697, *Leptospira* (Weil), diverse.
Mht. dokumentation fra anvendte kulturer: Så vidt muligt anvendes samme glas, som der har været anvendt til sidste omsåning, dvs. dokumentationen fremgår af logblad for omsåning af *Leptospira* kulturer. Såfremt der anvendes et andet batch, udfyldes et nyt eksemplar af samme logblad, hvor der afkrydses i "Andet" og under "Bemærkninger" skrives at batchet anvendes til agglutinationstest.

Screening:

- Udfyldelse af aflæsningsskema til screeninger opstartes (kan gøres løbende efterhånden som prøverne modtages).
- Widalglas opstilles som det fremgår af filen "Screening for *Leptospira*".
- På første glas i hver række skrives navnet på kulturen der skal tilsættes.
- Der tilsættes 1960 µl milli Q-vand til fortyndingsglasset, som det fremgår af filen "Screening for *Leptospira*".
- Der afpipetteres 40 µl prøvemateriale til fortyndingsglasset som det fremgår af filen "Screening for *Leptospira*".
- Der afpipetteres 100 µl fra fortyndingsglasset til hver af de 15 screeningsglas, svarende til de 15 stammer.
- Der tilsættes 1 dråbe af hver af de positive kontroller til de respektive tilhørende glas.
- Med sterile plastpipetter tilsættes 2 dråber kulturer til alle glas i de respektive rækker.
- Efter tilsætning af kulturer rystes stativet, dækkes løst med sort papir/pap og inkuberes ved stuetemperatur i 1½-2 timer inden aflæsningen påbegyndes.
- Prøverne aflæses for agglutination ved mørkefeltsmikroskopi (se anden fil mht. aflæsning og tolkning af det aflæste). Resultaterne skrives ind på aflæsningskema til *Leptospira* screeninger efterhånden som der aflæses.
- Hvis der er agglutination i en eller flere af stammerne, udtitreres i den/de pågældende stammer.

Udtitrering:

- Udfyldelse af aflæsningsskema til udtitrering opstartes.
- Widalglas opstilles som det fremgår af filen "Titrering for *Leptospira*".
- På første glas i hver række skrives navnet på kulturen der skal tilsættes.
- Med pipette tilsættes milli Q-vand, som det fremgår af filen "Titrering for *Leptospira*".
- Der foretages udtitrering som det fremgår af filen "Titrering for *Leptospira*".
- Som det fremgår af filen "Titrering for *Leptospira*" overføres 100 µl fortynding fra hvert fortyndingsglas til tomme widalglas i en række under fortyndingsrækken. Denne række kaldes udtitreringsrækken, og denne gentages for hver serotype, der skal udtitreres i.
- Hvis der ikke er nok fortyndet prøvemateriale i en fortyndingsrække, kan den gentages det nødvendige antal gange.
- Kontrollerne laves som ved screeningen.
- Med sterile plast transfer-pipetter tilsættes 2 dråber kulturer til alle glas i de respektive udtitreringsrækker.
- Efter tilsætning af kulturer rystes stativet, dækkes med sort papir/pap og inkuberes ved stuetemperatur i 1½-2 timer inden aflæsningen påbegyndes.
- Prøverne aflæses for agglutination ved mørkefeltsmikroskopi (se anden fil mht. aflæsning og tolkning af det aflæste). Resultaterne skrives ind på aflæsningsskema til udtitrering af *Leptospira* efterhånden som der aflæses.

Forklaring	Billedet i mikroskopet	Agglu-tinerede *Leptospirer* - positiv	Løse, svagt positive	Frie *Leptospirer* – negativ	Tolkning
Ved fuld agglutination bør billedet se nogenlunde således ud:		++++	0	0	+4 / +
Ved næsten fuld agglutination, men med få svagt positive, bør billedet se nogenlunde således ud:		+++	+	0	+3 / +
Ved mange agglutinerede, men lige så mange svagt positive, bør billedet se nogenlunde således ud:		++	++	0	+3 / +
Ved mange svagt agglutinerede men ingen frie, bør billedet se nogenlunde således ud:		+	+++	0	+2 / +
Ved mange svagt agglutinerede og få frie, bør billedet se nogenlunde således ud:		0	+++	+	+2 / +
Ved mange frie og få svagt agglutinerede *Leptospira*, bør billedet se nogenlunde således ud:		0	+	+++	+1 / (+)
Ved en negativ kontrol og ved negativ prøve bør billedet se nogenlunde således så:		0	0	++++	0 / ÷

Appendix 11 Excel calculation of Bland-Altman plot

lepto1/32	lepto2/32	diff	mean		lepto1F/R	lepto2/F/R	diff	mean
1	1,000				2	2		
30,980694	30,834	0,147	30,907216	Gitta	29,278442	29,309	-0,030	29,293519
34,094994	34,058	0,037	34,076368		32,584599	33,066	-0,482	32,825354
37,195404	36,506	0,690	36,850538		35,763962	36,285	-0,521	36,024672
Undetermine	38,237		38,23719		38,523026	38,216	0,307	38,369715
Undetermine	Undetermined				Undetermine	Undetermined		
23,777407	23,190	0,588	23,483478		22,003361	21,765	0,238	21,884413
26,305342	25,813	0,492	26,05939		24,457937	24,330	0,128	24,393865
30,186459	29,786	0,401	29,986094		27,927515	28,109	-0,182	28,01827
3	3				4	4		
20,3449	19,756	0,589	20,050613		18,584143	18,472	0,112	18,527922
5	5,000				6	6		
30,983294	30,861	0,122	30,922094	Mahdi	29,000576	29,450	-0,449	29,225121
34,293137	34,052	0,242	34,172371		32,029804	32,904	-0,875	32,467121
37,573807	36,695	0,879	37,134323		36,047424	35,851	0,197	35,949036
39,964466	39,033	0,931	39,498966		37,831825	39,799	-1,967	38,815334
Undetermine	Undetermined				Undetermine	Undetermined		
23,51486	23,005	0,510	23,259877		21,530087	21,774	-0,244	21,65202
26,169121	25,780	0,389	25,974443		24,174175	24,001	0,174	24,087377
30,027662	29,813	0,214	29,920543		27,768198	28,149	-0,380	27,95835
7	7				8	8		
20,463007	20,463007	0,000	20,463007		18,333075	18,660	-0,327	18,496461
9	9				10	10		
31,126806	30,920	0,206	31,02357	Rand	29,224459	29,653	-0,429	29,4389
34,04985	34,059	-0,009	34,054443		32,631798	33,012	-0,381	32,822063
37,189259	37,286	-0,097	37,237627		36,205811	36,561	-0,355	36,383549
40,303444	38,814	1,489	39,558773		38,813145	40,572	-1,759	39,692678
Undetermine	Undetermined				Undetermine	Undetermined		
23,752268	23,495	0,258	23,623399		22,605204	22,001	0,604	22,303204
26,457037	25,923	0,534	26,189803		24,976063	24,994	-0,018	24,985048
30,262526	29,750	0,513	30,00612		28,002174	28,268	-0,266	28,134945
11	11							
20,393387	20,157	0,236	20,275154		18,65764	18,968	-0,310	18,812715
	bias	0,407						
	std	0,3626963				bias	*-0,3006008*	0
	lower loa	-0,3038485	45			bias	-0,301	45
	lower loa	-0,3038485	0			std	0,5840428	
	upper loa	1,117921	45			lower loa	-1,4453247	0
	upper loa	1,117921	0			lower loa	-1,4453247	45
	bias	0,4070362	45			upper loa	0,8441232	0
	bias	0,4070362	0			upper loa	0,8441232	45

Appendix 12 Excel calculation of ONE WAY AVONA

lip32	
30,9806938	30,834
34,0949936	34,058
37,1954041	36,506
	38,237
23,7774067	23,190
26,3053417	25,813
30,1864586	29,786
20,3449001	19,756
30,9832935	30,861
34,2931366	34,052
37,5738068	36,695
39,9644661	39,033
23,5148602	23,005
26,1691208	25,780
30,0276623	29,813
20,463007	20,463007
31,1268063	30,920
34,0498505	34,059
37,1892586	37,286
40,3034439	38,814
23,7522678	23,495
26,457037	25,923
30,2625256	29,750
20,3933868	20,157

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	23	689,409128	29,9743099	39,0143197
Column 2	24	718,284485	29,9285202	39,1915416

ANOVA

Source of Variati	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Grou	0,02462505	1	0,02462505	0,00062972	0,9800908	4,05661246
Within Groups	1759,72049	45	39,1048998			
Total	1759,74512	46				

F/R	
29,2784424	29,309
32,5845985	33,066
35,7639618	36,285
38,5230255	38,216
22,0033607	21,765
24,4579372	24,330
27,927515	28,109
18,5841427	18,472
29,000576	29,450
32,0298042	32,904
36,0474243	35,851
37,8318253	39,799
21,5300865	21,774
24,1741753	24,001
27,768198	28,149
18,3330746	18,660
29,2244587	29,653
32,6317978	33,012
36,2058105	36,561
38,8131447	40,572
22,6052036	22,001
24,9760628	24,994
28,0021744	28,268
18,6576405	18,968

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	24	686,954441	28,6231017	42,7358124
Column 2	24	694,168859	28,9237025	46,8682656

ANOVA

Source of Variati	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Grou	1,08432985	1	1,08432985	0,02420269	0,87705098	4,05174869
Within Groups	2060,89379	46	44,802039			
Total	2061,97812	47				

Appendix 13 Raw data of the setup of figure 2.2 II analyzed on 7500 RT-PCR machine

Well	Reporter	Ct	Well	Reporter	Ct
A1	TAMRA	30,7079449	D6	TAMRA	29,5698261
A1	FAM	30,9806938	D6	FAM	37,8318253
A2	TAMRA	29,4806099	D9	TAMRA	30,5932865
A2	FAM	29,2784424	D9	FAM	40,3034439
A3	TAMRA	Undetermined	D10	TAMRA	29,4420853
A3	FAM	20,3449001	D10	FAM	38,8131447
A4	TAMRA	Undetermined	E1	TAMRA	30,9327602
A4	FAM	18,5841427	E1	FAM	Undetermined
A5	TAMRA	30,7531509	E2	TAMRA	29,4432869
A5	FAM	30,9832935	E2	FAM	Undetermined
A6	TAMRA	29,7352734	E5	TAMRA	30,5827904
A6	FAM	29,000576	E5	FAM	Undetermined
A7	TAMRA	Undetermined	E6	TAMRA	29,5771313
A7	FAM	20,463007	E6	FAM	Undetermined
A8	TAMRA	Undetermined	E9	TAMRA	30,4633999
A8	FAM	18,3330746	E9	FAM	Undetermined
A9	TAMRA	30,6679668	E10	TAMRA	29,3564739
A9	FAM	31,1268063	E10	FAM	Undetermined
A10	TAMRA	29,4828968	F1	TAMRA	Undetermined
A10	FAM	29,2244587	F1	FAM	23,7774067
A11	TAMRA	Undetermined	F2	TAMRA	Undetermined
A11	FAM	20,3933868	F2	FAM	22,0033607
A12	TAMRA	Undetermined	F5	TAMRA	Undetermined
A12	FAM	18,6576405	F5	FAM	23,5148602
B1	TAMRA	31,0682735	F6	TAMRA	34,6077194
B1	FAM	34,0949936	F6	FAM	21,5300865
B2	TAMRA	29,6404762	F9	TAMRA	27,6669903
B2	FAM	32,5845985	F9	FAM	23,7522678
B5	TAMRA	30,6630192	F10	TAMRA	39,7248764
B5	FAM	34,2931366	F10	FAM	22,6052036
B6	TAMRA	29,7105865	G1	TAMRA	29,3619385
B6	FAM	32,0298042	G1	FAM	26,3053417
B9	TAMRA	30,4744816	G2	TAMRA	29,2785873
B9	FAM	34,0498505	G2	FAM	24,4579372
B10	TAMRA	29,376482	G5	TAMRA	28,9054146
B10	FAM	32,6317978	G5	FAM	26,1691208
C1	TAMRA	30,6207275	G6	TAMRA	28,9726791
C1	FAM	37,1954041	G6	FAM	24,1741753
C2	TAMRA	29,5423794	G9	TAMRA	28,9236717
C2	FAM	35,7639618	G9	FAM	26,457037
C5	TAMRA	30,4627686	G10	TAMRA	29,0867329
C5	FAM	37,5738068	G10	FAM	24,9760628
C6	TAMRA	29,5056744	H1	TAMRA	30,5675354
C6	FAM	36,0474243	H1	FAM	30,1864586
C9	TAMRA	30,7053089	H2	TAMRA	29,5705643
C9	FAM	37,1892586	H2	FAM	27,927515
C10	TAMRA	29,243988	H5	TAMRA	30,5162029
C10	FAM	36,2058105	H5	FAM	30,0276623
D1	TAMRA	30,8730984	H6	TAMRA	29,3756447
D1	FAM	Undetermined	H6	FAM	27,768198
D2	TAMRA	29,6638546	H9	TAMRA	30,5210533
D2	FAM	38,5230255	H9	FAM	30,2625256
D5	TAMRA	30,4236774	H10	TAMRA	29,1506824
D5	FAM	39,9644661	H10	FAM	28,0021744

Appendix 14 Raw Data of the set up in figure 2.2 II analyzed on Q5 RT-PCR machine

Well	Well Position	Target Name	CT		Well	Well Position	Target Name	CT
1	A1	Leptospira 32	30,834		45	D9	Leptospira 32	38,814
1	A1	TAMRA	31,439		45	D9	TAMRA	30,922
2	A2	Leptospira F/I	29,309		46	D10	Leptospira F/I	40,572
2	A2	TAMRA	30,520		46	D10	TAMRA	30,038
3	A3	Leptospira 32	19,756		49	E1	Leptospira 32	Undetermined
3	A3	TAMRA	28,508		49	E1	TAMRA	30,965
4	A4	Leptospira F/I	18,472		50	E2	Leptospira F/I	Undetermined
4	A4	TAMRA	Undetermined		50	E2	TAMRA	30,193
5	A5	Leptospira 32	30,861		53	E5	Leptospira 32	Undetermined
5	A5	TAMRA	31,550		53	E5	TAMRA	31,528
6	A6	Leptospira F/I	29,450		54	E6	Leptospira F/I	Undetermined
6	A6	TAMRA	30,522		54	E6	TAMRA	30,029
7	A7	Leptospira 32	19,895		57	E9	Leptospira 32	Undetermined
7	A7	TAMRA	Undetermined		57	E9	TAMRA	31,503
8	A8	Leptospira F/I	18,660		58	E10	Leptospira F/I	Undetermined
8	A8	TAMRA	Undetermined		58	E10	TAMRA	30,370
9	A9	Leptospira 32	30,920		61	F1	Leptospira 32	23,190
9	A9	TAMRA	31,098		61	F1	TAMRA	27,150
10	A10	Leptospira F/I	29,653		62	F2	Leptospira F/I	21,765
10	A10	TAMRA	30,559		62	F2	TAMRA	40,252
11	A11	Leptospira 32	20,157		65	F5	Leptospira 32	23,005
11	A11	TAMRA	Undetermined		65	F5	TAMRA	32,150
12	A12	Leptospira F/I	18,968		66	F6	Leptospira F/I	21,774
12	A12	TAMRA	Undetermined		66	F6	TAMRA	37,768
13	B1	Leptospira 32	34,058		69	F9	Leptospira 32	23,495
13	B1	TAMRA	31,461		69	F9	TAMRA	Undetermined
14	B2	Leptospira F/I	33,066		70	F10	Leptospira F/I	22,001
14	B2	TAMRA	30,655		70	F10	TAMRA	37,557
17	B5	Leptospira 32	34,052		73	G1	Leptospira 32	25,813
17	B5	TAMRA	31,584		73	G1	TAMRA	30,427
18	B6	Leptospira F/I	32,904		74	G2	Leptospira F/I	24,330
18	B6	TAMRA	30,554		74	G2	TAMRA	31,000
21	B9	Leptospira 32	34,059		77	G5	Leptospira 32	25,780
21	B9	TAMRA	31,373		77	G5	TAMRA	30,523
22	B10	Leptospira F/I	33,012		78	G6	Leptospira F/I	24,001
22	B10	TAMRA	30,448		78	G6	TAMRA	30,584
25	C1	Leptospira 32	36,506		81	G9	Leptospira 32	25,923
25	C1	TAMRA	31,625		81	G9	TAMRA	30,590
26	C2	Leptospira F/I	36,285		82	G10	Leptospira F/I	24,994
26	C2	TAMRA	30,125		82	G10	TAMRA	30,628
29	C5	Leptospira 32	36,695		85	H1	Leptospira 32	29,786
29	C5	TAMRA	30,878		85	H1	TAMRA	30,707
30	C6	Leptospira F/I	35,851		86	H2	Leptospira F/I	28,109
30	C6	TAMRA	30,126		86	H2	TAMRA	30,403
33	C9	Leptospira 32	37,286		89	H5	Leptospira 32	29,813
33	C9	TAMRA	31,271		89	H5	TAMRA	30,866
34	C10	Leptospira F/I	36,561		90	H6	Leptospira F/I	28,149
34	C10	TAMRA	30,162		90	H6	TAMRA	30,177
37	D1	Leptospira 32	38,237		93	H9	Leptospira 32	29,750
37	D1	TAMRA	30,865		93	H9	TAMRA	30,749
38	D2	Leptospira F/I	38,216		94	H10	Leptospira F/I	28,268
38	D2	TAMRA	30,233		94	H10	TAMRA	30,507
41	D5	Leptospira 32	39,033					
41	D5	TAMRA	31,799					
42	D6	Leptospira F/I	39,799					
42	D6	TAMRA	30,200					

Appendix 15 Raw data of the set up in figure 2.2 III analyzed on Q5 RT-PCR machine

Well	Well Position	Target Name	CT	Well	Well Position	Target Name	CT
1	A1	Leptospira 32	31,303	49	E1	Leptospira 32	Undetermined
1	A1	TAMRA	30,966	49	E1	TAMRA	31,943
2	A2	Leptospira F/F	32,501	50	E2	Leptospira F/F	Undetermined
2	A2	TAMRA	30,700	50	E2	TAMRA	29,995
3	A3	Leptospira 32	26,987	51	E3	Leptospira 32	25,427
3	A3	TAMRA	35,478	51	E3	TAMRA	33,569
4	A4	Leptospira F/F	27,396	52	E4	Leptospira F/F	26,422
4	A4	TAMRA	30,625	52	E4	TAMRA	30,976
5	A5	Leptospira 32	27,098	53	E5	Leptospira 32	39,641
5	A5	TAMRA	31,276	53	E5	TAMRA	30,833
6	A6	Leptospira F/F	28,016	54	E6	Leptospira F/F	44,698
6	A6	TAMRA	29,454	54	E6	TAMRA	30,356
13	B1	Leptospira 32	34,467	61	F1	Leptospira 32	26,086
13	B1	TAMRA	31,679	61	F1	TAMRA	32,084
14	B2	Leptospira F/F	35,321	62	F2	Leptospira F/F	27,050
14	B2	TAMRA	30,179	62	F2	TAMRA	30,358
15	B3	Leptospira 32	28,085	63	F3	Leptospira 32	26,008
15	B3	TAMRA	30,563	63	F3	TAMRA	32,394
16	B4	Leptospira F/F	29,123	64	F4	Leptospira F/F	26,949
16	B4	TAMRA	30,686	64	F4	TAMRA	30,911
17	B5	Leptospira 32	33,393	65	F5	Leptospira 32	39,647
17	B5	TAMRA	31,058	65	F5	TAMRA	31,242
18	B6	Leptospira F/F	34,363	66	F6	Leptospira F/F	41,848
18	B6	TAMRA	31,173	66	F6	TAMRA	30,582
25	C1	Leptospira 32	37,833	73	G1	Leptospira 32	27,332
25	C1	TAMRA	30,974	73	G1	TAMRA	30,553
26	C2	Leptospira F/F	38,588	74	G2	Leptospira F/F	28,368
26	C2	TAMRA	30,033	74	G2	TAMRA	30,546
27	C3	Leptospira 32	30,631	75	G3	Leptospira 32	25,154
27	C3	TAMRA	30,900	75	G3	TAMRA	43,234
28	C4	Leptospira F/F	31,489	76	G4	Leptospira F/F	26,240
28	C4	TAMRA	30,096	76	G4	TAMRA	31,265
29	C5	Leptospira 32	33,491	77	G5	Leptospira 32	33,057
29	C5	TAMRA	31,724	77	G5	TAMRA	31,005
30	C6	Leptospira F/F	35,300	78	G6	Leptospira F/F	35,051
30	C6	TAMRA	30,730	78	G6	TAMRA	31,716
37	D1	Leptospira 32	Undetermined	85	H1	Leptospira 32	24,839
37	D1	TAMRA	31,419	85	H1	TAMRA	42,976
38	D2	Leptospira F/F	41,273	86	H2	Leptospira F/F	24,544
38	D2	TAMRA	29,416	86	H2	TAMRA	32,812
39	D3	Leptospira 32	Undetermined	87	H3	Leptospira 32	25,569
39	D3	TAMRA	31,413	87	H3	TAMRA	41,079
40	D4	Leptospira F/F	Undetermined	88	H4	Leptospira F/F	25,688
40	D4	TAMRA	30,070	88	H4	TAMRA	31,980
41	D5	Leptospira 32	32,360	89	H5	Leptospira 32	Undetermined
41	D5	TAMRA	31,207	89	H5	TAMRA	32,396
42	D6	Leptospira F/F	34,116	90	H6	Leptospira F/F	Undetermined
42	D6	TAMRA	30,357	90	H6	TAMRA	29,972

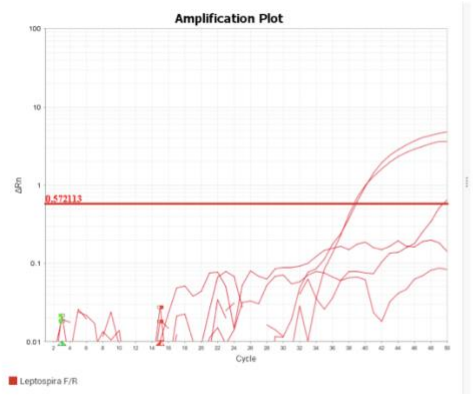
Appendix 16 Raw data of the set up in figure 2.2 IV analyzed on Q5 RT-PCR machine

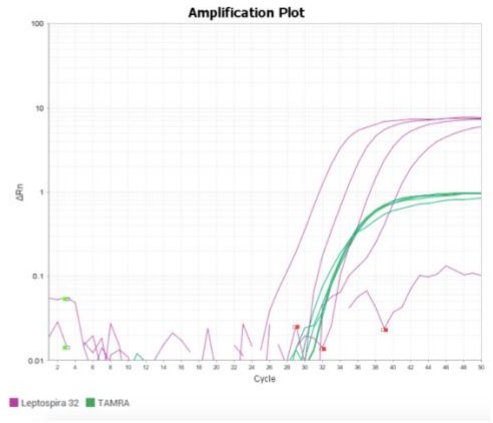
Well	Well Position	Target Name	CT
1	A1	Leptospira 32	30,657
1	A1	TAMRA	31,702
2	A2	Leptospira F/F	29,210
2	A2	TAMRA	31,344
3	A3	Leptospira 32	30,695
3	A3	TAMRA	31,274
4	A4	Leptospira F/F	29,188
4	A4	TAMRA	31,439
5	A5	Leptospira 32	30,647
5	A5	TAMRA	31,357
6	A6	Leptospira F/F	Undetermined
6	A6	TAMRA	31,706
13	B1	Leptospira 32	33,953
13	B1	TAMRA	31,447
15	B3	Leptospira 32	33,808
15	B3	TAMRA	31,889
16	B4	Leptospira F/F	32,338
16	B4	TAMRA	30,979
17	B5	Leptospira 32	34,136
17	B5	TAMRA	31,967
18	B6	Leptospira F/F	32,497
18	B6	TAMRA	31,218
25	C1	Leptospira 32	37,472
25	C1	TAMRA	32,009
26	C2	Leptospira F/F	35,527
26	C2	TAMRA	30,655
27	C3	Leptospira 32	36,505
27	C3	TAMRA	32,214
28	C4	Leptospira F/F	35,777
28	C4	TAMRA	30,407
29	C5	Leptospira 32	37,714
29	C5	TAMRA	31,907
30	C6	Leptospira F/F	35,517
30	C6	TAMRA	30,902
37	D1	Leptospira 32	Undetermined
37	D1	TAMRA	32,086
38	D2	Leptospira F/F	Undetermined
38	D2	TAMRA	30,823
39	D3	Leptospira 32	38,176
39	D3	TAMRA	32,026

Well	Well Position	Target Name	CT
40	D4	Leptospira F/F	37,934
40	D4	TAMRA	30,861
41	D5	Leptospira 32	Undetermined
41	D5	TAMRA	31,858
42	D6	Leptospira F/F	37,299
42	D6	TAMRA	31,126
49	E1	Leptospira 32	38,639
49	E1	TAMRA	31,779
50	E2	Leptospira F/F	Undetermined
50	E2	TAMRA	30,843
51	E3	Leptospira 32	Undetermined
51	E3	TAMRA	31,951
52	E4	Leptospira F/F	Undetermined
52	E4	TAMRA	30,603
53	E5	Leptospira 32	Undetermined
53	E5	TAMRA	31,758
54	E6	Leptospira F/F	Undetermined
54	E6	TAMRA	31,032
61	F1	Leptospira 32	Undetermined
61	F1	TAMRA	31,938
62	F2	Leptospira F/F	Undetermined
62	F2	TAMRA	31,154
63	F3	Leptospira 32	Undetermined
63	F3	TAMRA	31,945
64	F4	Leptospira F/F	Undetermined
64	F4	TAMRA	30,912
65	F5	Leptospira 32	Undetermined
65	F5	TAMRA	31,744
66	F6	Leptospira F/F	Undetermined
66	F6	TAMRA	30,559
73	G1	Leptospira 32	Undetermined
73	G1	TAMRA	32,030
74	G2	Leptospira F/F	Undetermined
74	G2	TAMRA	30,562
75	G3	Leptospira 32	Undetermined
75	G3	TAMRA	32,535
76	G4	Leptospira F/F	Undetermined
76	G4	TAMRA	30,698
77	G5	Leptospira 32	Undetermined
77	G5	TAMRA	31,697
78	G6	Leptospira F/F	Undetermined
78	G6	TAMRA	31,015

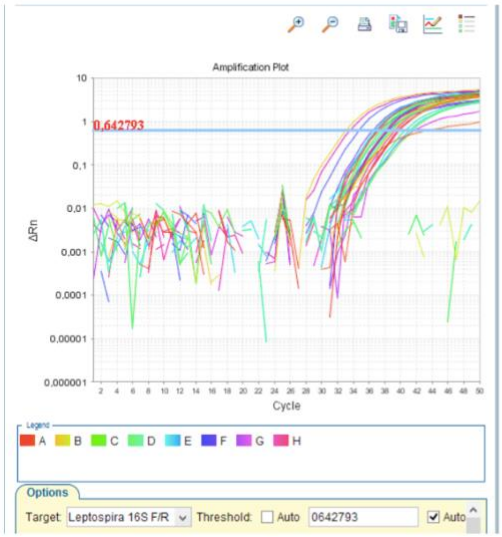
Appendix 17 Amplification plot for the control samples for Study 2

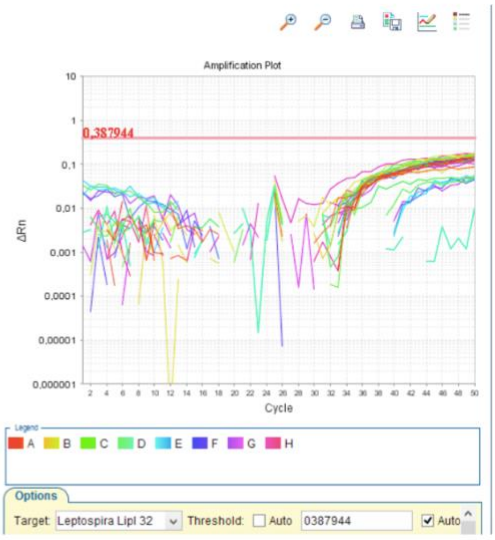
Amplification plots of strains first attempt



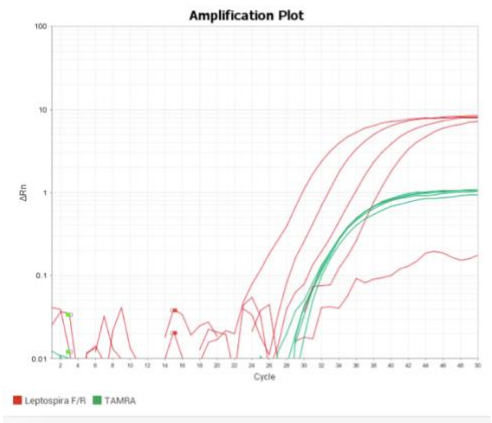


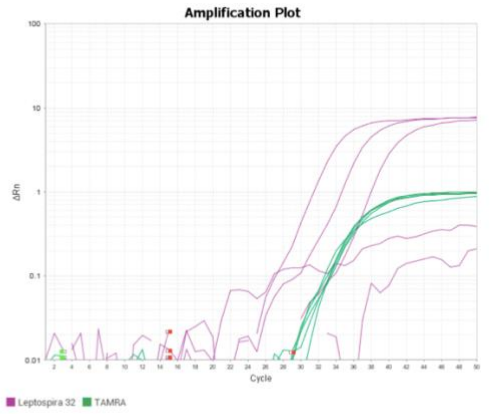
Amplification plots for animal sample: urine from pigs



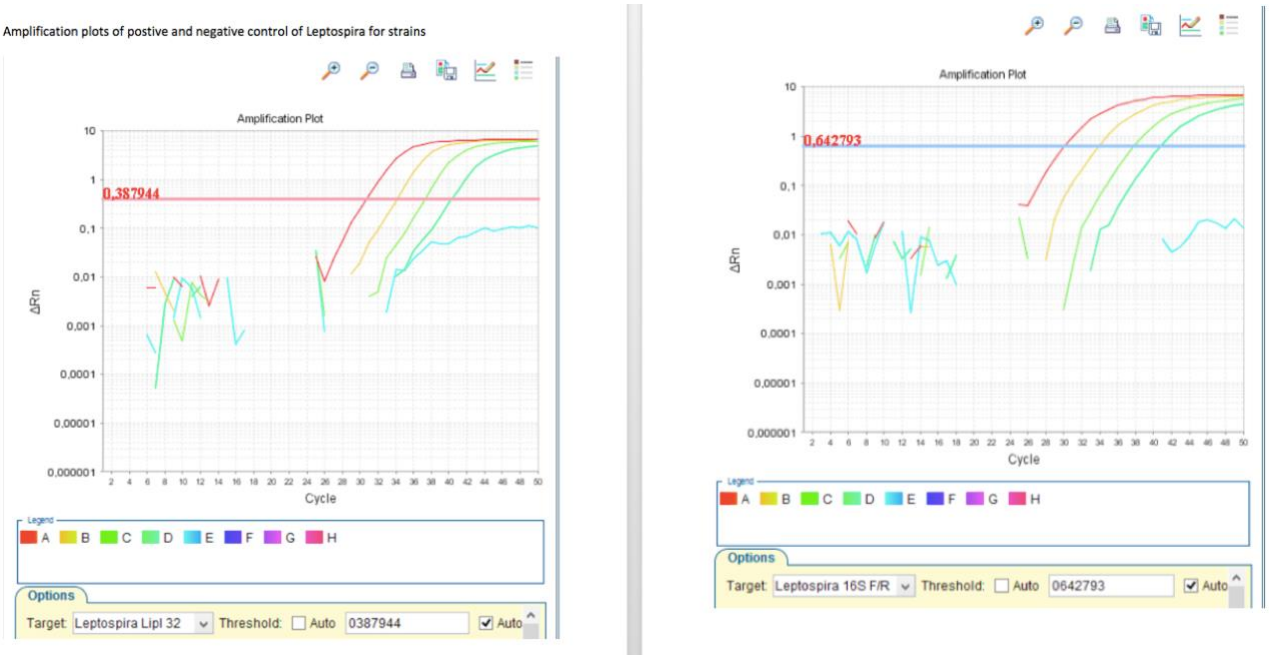


Amplification plots for possive and negative leptospira control in tissue and all strains





Appendix 18 Amplification plot for the control samples for study 3



Appendix 19 Validations report of the RT-PCR assay for SSI

Valideringsrapport

<table>
<tr><th></th><th>Analyse:</th><th></th></tr>
<tr><td>1</td><td>Formålet (kvalitetsmål):</td><td>At validere nuværende real time PCR assay LipL32 og 16S (F/R) at detektere Leptospira DNA i veterinære prøver</td></tr>
<tr><td>2</td><td>Udviklingsgruppen:</td><td>Randi Føns Petersen, Rand Hasan Khalil, Mahdi Adan</td></tr>
<tr><td>3</td><td>Metode (princip):</td><td>Real-time PCR (Taqman probe)</td></tr>
<tr><td>4</td><td>Reference:</td><td></td></tr>
<tr><td>5</td><td>Analyt:</td><td>Leptospira DNA</td></tr>
<tr><td>6</td><td>Prøvemateriale:</td><td>Urin, histologiskvæv</td></tr>
<tr><td>7</td><td>Apparatur/udstyr:</td><td>Bioappliedsystems 7500 (real time PCR)
Quantstudio Q5 (real time PCR)</td></tr>
<tr><td>8</td><td>Måleenhed:</td><td>Ct-værdier</td></tr>
<tr><td>9</td><td>Reagenser:</td><td>Mastermix - Immolase
<table>
<tr><th>Leptospira primer/probes</th><th>Sequence</th></tr>
<tr><td>LeptoF</td><td>CCC GCG TCC GAT TAG</td></tr>
<tr><td>LeptoR</td><td>TCC ATT GTG GCC GYA CAC</td></tr>
<tr><td>Probe Lepto A-1 (16S rRNA)</td><td>Fam-CTC ACC AAG GCG ACG ATC GGT AGC-BHQ-1</td></tr>
<tr><td>Lipl32 P</td><td>Fam-AAG TGA AAG GAT CTT TCG TTG C - MGB</td></tr>
<tr><td>Lipl 32F</td><td>AGA GGT CTT TAC AGA ATT TCT TTC ACT ACC T</td></tr>
<tr><td>Lipl 32R</td><td>TGG YAA AAG CAG ACC AAC AGA</td></tr>
</table></td></tr>
<tr><td>10</td><td>Kontroller:</td><td>IK (intern kontrol) 10^7, samt positiv Leptospira kontrol i fortyndingsrækken 10^3-10^6</td></tr>
<tr><td>11</td><td>Præstationsprøvning:</td><td>Ikke testet</td></tr>
</table>

<table>
<tr><th></th><th>Analyse:</th><th></th></tr>
<tr><td>12</td><td>Sensitivitet:</td><td>Evne til at detecktere alle nedenstående strains og spp. Af Leptospira pånær Patoc I. Som tilhøre Saprofytiske bakterier (jordbakterier)
<table><tr><th>Genomspecies</th><th>Serovar</th><th>Serogroup</th><th>Strain</th></tr><tr><td>L. Biflexa</td><td>Patoc</td><td>Semaranga</td><td>Patoc I</td></tr><tr><td>L. Borgpetersenii</td><td>Ballum</td><td>Castellonis</td><td>Castellon 3</td></tr><tr><td>L. Fainei</td><td>Hurtsbridge</td><td>Hurtsbridge</td><td>H.B.6</td></tr><tr><td>L. Interrogans</td><td>Autumnalis</td><td>Autumnalis</td><td>Akiyami A</td></tr><tr><td>L. Interrogans</td><td>Bataviae</td><td>Bataviae</td><td>Swart</td></tr><tr><td>L. Interrogans</td><td>Bratislava</td><td>Australis</td><td>Bratislava</td></tr><tr><td>L. Interrogans</td><td>Canicola</td><td>Canicola</td><td>Hond Utrecht IV</td></tr><tr><td>L. Interrogans</td><td>Copenhageni</td><td>Copenhageni</td><td>M20</td></tr><tr><td>L. Interrogans</td><td>Hardjo</td><td>Serjoe</td><td>Hardjo prajitno</td></tr><tr><td>L. Interrogans</td><td>Hebdomadis</td><td>Hebdomadis</td><td>Hebdomadis</td></tr><tr><td>L. Interrogans</td><td>Icterohaemorrhagiae</td><td>Icterohaemorrhagiae</td><td>RGA</td></tr><tr><td>L. Interrogans</td><td>Iustralis</td><td>Australis</td><td>Ballico</td></tr><tr><td>L. Interrogans</td><td>Pomona</td><td>Pomona</td><td>Pomona</td></tr><tr><td>L. Interrogans</td><td>Pyrogenes</td><td>Pyrogenes</td><td>Salinem</td></tr></table></td></tr>
<tr><td>13</td><td>Detektionsgrænse:</td><td>Detektion af positiv Leptospira kontrol i 10^5-10^6 fortynding</td></tr>
<tr><td>14</td><td>Specificitet:</td><td>Evne til at diskriminere mellem patogene og ikke patogene ssp. Af Leptopsira
<table><tr><th>Genomspecies</th><th>Serovar</th><th>Serogroup</th><th>Strain</th></tr><tr><td>L. Biflexa</td><td>Patoc</td><td>Semaranga</td><td>Patoc I</td></tr><tr><td>L. Borgpetersenii</td><td>Ballum</td><td>Castellonis</td><td>Castellon 3</td></tr><tr><td>L. Fainei</td><td>Hurtsbridge</td><td>Hurtsbridge</td><td>H.B.6</td></tr><tr><td>L. Interrogans</td><td>Autumnalis</td><td>Autumnalis</td><td>Akiyami A</td></tr><tr><td>L. Interrogans</td><td>Bataviae</td><td>Bataviae</td><td>Swart</td></tr><tr><td>L. Interrogans</td><td>Bratislava</td><td>Australis</td><td>Bratislava</td></tr><tr><td>L. Interrogans</td><td>Canicola</td><td>Canicola</td><td>Hond Utrecht IV</td></tr><tr><td>L. Interrogans</td><td>Copenhageni</td><td>Copenhageni</td><td>M20</td></tr><tr><td>L. Interrogans</td><td>Hardjo</td><td>Serjoe</td><td>Hardjo prajitno</td></tr><tr><td>L. Interrogans</td><td>Hebdomadis</td><td>Hebdomadis</td><td>Hebdomadis</td></tr><tr><td>L. Interrogans</td><td>Icterohaemorrhagiae</td><td>Icterohaemorrhagiae</td><td>RGA</td></tr><tr><td>L. Interrogans</td><td>Iustralis</td><td>Australis</td><td>Ballico</td></tr><tr><td>L. Interrogans</td><td>Pomona</td><td>Pomona</td><td>Pomona</td></tr><tr><td>L. Interrogans</td><td>Pyrogenes</td><td>Pyrogenes</td><td>Salinem</td></tr></table></td></tr>
<tr><td>15</td><td>Måleinterval (kvantitativ test):</td><td>Ikke testet</td></tr>
<tr><td>16</td><td>Usikkerhed for kvantitet i måleområde:</td><td>Ikke testet</td></tr>
<tr><td>17</td><td>Ydre begrænsninger (temperatur, luftfugtighed m.m.):</td><td>Ikke testet</td></tr>
<tr><td>18</td><td>Svarmuligheder:</td><td>Påvist, ikke påvist</td></tr>
<tr><td>19</td><td>Bilag (resultater):</td><td></td></tr>
</table>

Konklusion:	Blev kvalitetsmål/kravspecifikationer opnået?
	Det er muligt at detektere Leptospira DNA i veterinærprøver med LipL32 og 16S (F/R) primerne. Dog har 16S primerne udvist mindre specifitet i urinprøver fra svin.