Evaluation of ELISA and qPCR assays for detection of *Mycoplasma bovis* in milk from ruminants

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Abstract

Mycoplasma bovis (*M. bovis*) infections are difficult to diagnose and treat, causing severe economic losses to farms with infected cows. Eurofins Milk Testing Sweden AB screens cow's milk using qPCR (quantitative Polymerase Chain Reaction) to detect *M. bovis* infections. Five performance characteristics were chosen to evaluate if ELISA (Enzyme-Linked Immunosorbent Assay) could replace the current screening method; limit of detection (LOD), repeatability and intermediate precision, selectivity and matrix effects and PCR inhibition. The comparison between the two methods was complicated by the fact that qPCR test for the presence of *M. bovis* DNA (qPCR), while the ELISA test for the presence of anti-*M. bovis* antibodies. In addition, the analysis methods were compared regarding health and environmental aspects. Based on the two assays performance characteristics the qPCR outperformed the ELISA regarding the LOD and the matrix effects and PCR inhibition, while the ELISA surpassed the qPCR with respect to the repeatability and intermediate precision. Regarding the selectivity, both methods performed equally well. The qPCR includes extra steps compared to the ELISA and hence needs additional solutions, this contributes to that the qPCR is considered to be less health and environmentally friendly. The ELISA is also cheaper and more user friendly than the qPCR. Comparing of testing for antibodies and testing for DNA is complex, there is no guarantee that either antibodies or *M. bovis* cells are present in the milk at the time of testing. Based on the results in this degree project it is suitable to use the two methods in parallel; ELISA as the primary screening method and then to verify positive results with qPCR. Except for when a bacteriological culture is to be verified, then qPCR is suitable since the presence of *M. bovis* cells is to be tested.

Preface

The degree project resulting in this report was performed during a period of five moths between the 13th of January and 11th of June 2020. It was performed at Eurofins Milk Testing Sweden AB; Kabelvägen 2, 551 15 Jönköping, Sweden. The project was initiated by Eurofins Milk Testing Sweden AB to help them evaluate screening assays.

I am very grateful towards Eurofins Milk Testing Sweden AB for allowing me to do the master thesis at their company. I would also like to extend my warmest thanks to my examiner Jenny Schelin, my supervisor from LTH Johannes Hedman, my supervisor from Eurofins Milk Testing Sweden AB Louise Karlsson Budnik, Daniel Granstrand who helped me in the laboratory on site, and lastly Ronja Lillienau and Felix W. Holmin who gave me valuable feedback on the report.

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1 Introduction

1.1 Motivation for the degree project

Eurofins Scientific is a group of laboratories, that performs a large number of different analyses on products belonging to the food, pharmaceutical, and environmental sector. Eurofins Milk Testing Sweden AB (will further on be referred to as Eurofins) is a part of this group of laboratories, that performs accredited analysis of samples from dairies and pet associations. Among these are analysis of milk from the supplier, screening for unhealthy cows, and simplified water analysis for dairies. One of the pathogens that is screened for is *Mycoplasma bovis* (*M. bovis).*

Bulk tank milk (BTM) is continuously screened to see if *M. bovis* is present at farm level. Tests are also conducted on individual cows upon request, prompted by the display of symptoms or to confirm that it is *M. bovis* in a bacteriological culture. Today, Eurofins screens for *M. bovis* using quantitative polymerase chain reaction (qPCR). Previously, they have used the PathoProof™ Mastitis Complete-16 Kit from Thermo Fisher Scientific (Waltham, MA), to test for *M. bovis* in milk samples. They switched to using the PathoProof Mastitis Major-4.2 Kit (below called Major-4.2 Kit) from Thermo Fisher on the 27th of January 2020. However, they have the desire to switch detection method to Enzyme-Linked Immunosorbent Assay (ELISA), using another commercial kit. Both qPCR and ELISA methods are well established as detection methods for *M. bovis,* and competing companies use either of them. There are several reasons motivating the aspired change; the qPCR test is more expensive, it screens for additional pathogens that are not of interest, and an alternative screening method has also been requested from the customers. The National Veterinary Institute (SVA) offers screening both with qPCR and ELISA, and the prices differ from 630 kr for the qPCR, to 150 kr for the ELISA (Statens Veterinärmedicinska Anstalt, 2020). The Complete-16 Kit tested for 14 mastitis causing pathogens in addition to *M. bovis,* as well as for the Staphylococcal β-lactamase gene. The Mastitis Major-4.2 Kit tests for three pathogens in addition to the *M. bovis* (*Staphylococcus aureus*, *Streptococcus agalactiae,* and *Streptococcus uberis*) (Thermo Scientific, 2015; Thermo Scientific, 2015). However, the species of primary interest is *M. bovis*, making it unnecessary to test for the presence of the other species. This is a contributing factor to the customer request to change method; the multitude of results is experienced as somewhat confusing since only a few of the pathogens are of interest. This is the reason triggering the change to the Major-4.2 Kit.

1.2 Aim

The aim of the degree project was to investigate available ELISA kits for the detection of *M. bovis*, and to determine which of them that was of interest to test. Furthermore, the aim was to plan experiments, to evaluate the performance of the chosen ELISA kit, and to compare this with the performance of the qPCR detection that was currently used at the laboratory. In addition, it was to be decided if it was feasible to replace the qPCR method with the chosen ELISA kit.

2 Background

2.1 *Mycoplasma bovis:* Species, symptoms and treatment

M. bovis causes pneumonia in calves, spontaneous abortion of fetuses, mastitis, arthritis, and cornea inflammation. This entails substantial economic losses, due to calves dying, and grown cattle not being fit to serve their purpose. Carriers of *M. bovis* do not always display

symptoms of being infected, and this is one cause for the spreading of the pathogen. Another reason is that there are several laboratories that do not screen for *M. Bovis* at all. Due to the clinical symptoms being identical to symptoms caused by other pathogens and diseases, and the possibility of carrying *M. bovis* without displaying any symptoms, laboratory diagnosis is necessary (Nicholas & Ayling, 2003). Since the sample type is milk, the *M. bovis* infections that can be detected are limited to mastitis. The bacteria and the antibodies will only be present in the milk if the udder is infected, and not if *M. bovis* is only present in other tissues (Petersen, et al., 2018).

The *Mycoplasma mycoides* subspecies *mycoides* SC is even more pathogenic than *M. bovis*, but it is considered to be eradicated from Europe, making it unnecessary to screen for (Nicholas & Ayling, 2003). *M. bovis* was first detected in Sweden in 2011, and due to the considerable economic effects, and the difficulty of treating *M. bovis* infections, it is of substantial interest to avoid any larger outbreaks (Ericsson Unnerstad, et al., 2012; Nicholas & Ayling, 2003).

M. bovis is a small, pleomorphic bacterium that lacks a cell wall (Nicholas & Ayling, 2003). This makes it immune to ß-lactams, such as penicillin. In addition to being resistant to all antibiotics targeting the cell wall, *M. bovis* has also a natural resistance to several other antibiotics (polymyxins, sulfonamides, trimethoprim, nalidixic acid, and rifampin). Members from a few groups of antibiotics are used to treat *M. bovis;* amphenicols, tetracyclines, macrolides and some fluoroquinolones (Lysnyansky & Ayling, 2016; Klein, et al., 2019). The fluoroquinolones are the only ones able to kill the *M. bovis*, while the other antibiotics only inhibit growth, allowing the cows own immune defense to defeat the infection (Klein, et al., 2019). However, there seems to be an increasing resistance against these antibiotics as well. The increasing resistance is due to point-mutations, which means that there is a difference in resistance between different strains, making it difficult to have a general treatment plan (Lysnyansky & Ayling, 2016). In addition, *M. bovis* is present in many different tissues and can form biofilms in the body, making it even more difficult for the antibiotics to reach the bacteria (Nicholas, et al., 2016). Some autogenous vaccines (prepared using the *M. bovis* from the infected cow) are available in the USA and in Europe, but it is unclear how effective they really are (Klein, et al., 2019; Dudek, et al., 2016).

The reason for no general vaccine being available on the market, despite the severe consequences of *M. bovis* infections, is the variability of the surface proteins of *M. bovis*. It is believed that the expression of different surface antigens is affected by the presence of antibodies targeted against these antigens. Hence, *M. bovis* escapes the produced antibodies by decreasing the amount, or by altering the length, of targeted surface proteins. This variability hinders the immune system from beating the infection, which can develop into a chronic stage, and it also hinders the development of an effective vaccine (Le Grand, et al., 1996). Since pharmaceutical treatments have a limited effect, other recommendations are given concerning the procedure upon infection. Screening is an important means to rapidly detect *M. bovis* upon infection, allowing for the infected cows to be isolated to prevent spreading to healthy cows. The infected cow can either be allowed to self-heal, and possibly be re-introduced to the herd if it can be verified that it is healthy or be euthanized. Other factors also affect the risk of a herd being infected with *M.* bovis, and there are a set of preventative measures to take. One of them is to limit the herd size, since this decreases the number of susceptible members, and makes it more difficult for the pathogen to survive in the herd. It is also recommended to be vigilant, and to not introduce infected cows into the herd upon acquisition from other farms (Nicholas, et al., 2016).

2.2 Analytical methods

2.2.1 Available screening methods

In addition to the mentioned screening methods, qPCR and ELISA, there are a number of other analysis methods that can be used for *M. bovis* diagnosis. Traditionally, bacteriological culturing (BC) has been the method of choice. The culture has then been based on milk, joint fluids, eye swabs or nasal swabs (Petersen, et al., 2018). However, there are some disadvantages with BC, which have prompted the switch to other analytical methods. Enriched media is needed since *M. bovis* cannot synthesize amino acids or fatty acids, and antibiotics are required to prevent other bacteria to overgrow the *M. bovis.* In addition, the culture needs to be incubated for 7-10 days before any diagnosis can be made. The risk of *M. bovis* being overgrown poses a risk for false negative results. There is also a risk for false positive results due to the similarity between *M. bovis* colonies and colonies of other bacteria belonging to the same class. To be able to verify that the sample is indeed positive for *M. bovis*, another analysis needs to be employed e.g. qPCR or gel electrophoresis. Benefits with BC is that it is a cheap and simple method, and it facilitates further analysis of isolated colonies to see which clone is present in the sample. Since qPCR also tests for the presence of *M. bovis* in the sample, there are some similarities between these methods. The major one being the dependency of the animal shedding *M. bovis* at the time of sampling, which is not guaranteed. However, for BC there is also an additional demand on the presence of viable bacteria, which puts constraints on the handling of the milk. When comparing BC and qPCR, BC falls short regarding efficiency, specificity, and sensitivity. qPCR only takes about four hours to run, and the limit of detection (LOD) is 10 – 240 cfu (colony-forming unit)/ml for milk and the LOD for BC is 100 – 1000 cfu/ml in milk (Parker, et al., 2018). The LOD for the qPCR depends on the gene that is being amplified, and this is not stated for the Major-4.2 Kit.

Traditional PCR has mostly been replaced with qPCR, due to the additional identification and quantification step that is needed for PCR e.g. sequencing or gel electrophoresis. In addition, the LOD for traditional PCR is slightly higher than for qPCR; 400 cfu/ml in milk. The LOD for the qPCR is also dependent on the detection method, since the less specific methods have a higher background and hence a higher LOD (Parker, et al., 2018). There are some risks for false negative results due contaminations in the sample or primers that do not bind to the target sequence, to mention a couple of risks. In addition, there are risks for false positive results due to detection molecule of primer-dimers or amplification of the wrong sequence, caused by unspecific primers (Hedman, et al., 2018). However, if the used primers and protocol have been properly verified this should not pose a problem.

The third commonly used method for *M. bovis* screening is ELISA. Since ELISA detects the presence of anti-*M. bovis* antibodies, and not the presence of *M. bovis*, it is more difficult to compare this method with the others. However, there are some factors that could be considered to be drawbacks when comparing ELISA to qPCR and BC. Firstly, the development of antibodies is not immediate, making the status of a sample dependent on the time of sampling, as well as making it difficult to backtrack the time of infection. Secondly, it is difficult to assess the risk of false positive results based on in-field studies. However, this possible problem is mitigated by the use of databases, to find a unique antigen, as well as western blot being used to investigate cross-reactivity. Due to the differences between ELISA, PCR and BC it is sometimes recommended to use ELISA in combination with one of the former methods. This would eliminate any false positive results caused by healthy animals carrying anti-*M. bovis* antibodies (Parker, et al., 2018). Also, worth to mention is that ELISA is cheaper than both PCR and BC (Petersen, et al., 2016).

Besides theses established methods, there are several methods that are under development; latex agglutination, resonance devices, mass spectrometry (MS), and lateral flow sticks (Calcutt, et al., 2018). MALDI-TOF (matrix assisted laser desorption-time of flight) has been used as a MS-method, but it is not used in any larger extent. It is cheaper per run than PCR and suitable if it is of interest to investigate on clone level, however incubation of the sample for 24 hours is needed prior to running the sample and the investment cost for the equipment is expensive. In addition, there are some limitations regarding the available MS-libraries (Bokma, et al., 2019). Another method in the developing phase is the RPA-LFD (recombinase polymerase amplification-lateral flow dipstick) that is meant to be used for quick diagnostics at the site of infection. It is as sensitive as qPCR, and the analysis only takes 30 minutes at a temperature of 39 °C. The simplicity of this analysis method is supposed to help prevent major outbreaks of *M. bovis*. However, this method is currently more expensive than qPCR per test (Zhao, et al., 2018).

2.2.2 PCR

The polymerase chain reaction (PCR) is a method that is used to increase the amount of specific parts of DNA in a sample. This amplification is performed to enable detection, identification or quantification. To be able to perform PCR, parts of the sequence need to be known since primers (oligonucleotides), complementary to the sequence that is to be amplified, need to be added to the reaction. Two primers are needed, bordering the segment of interest, one is complementary to each DNA strand. Nucleotide triphosphates and the enzyme DNA polymerase also need to be added. One of DNA polymerases tasks *in vivo* is to copy the DNA prior to cell division, and this ability is taken advantage of *in vitro* during the course of PCR. Through thermocycling the complementary DNA strands are separated (higher temperature), and then the primers are allowed to anneal to the strands (lower temperature). Once the primers are annealed (intermediate temperature), the DNA polymerase uses the nucleotide triphosphates to prolong the 3´-end of the primer, forming a complementary strand. This process is then repeated, allowing the amount of the DNA segment to be copied and to increase exponentially. It is customary to use a thermostable enzyme, the *Taq* polymerase from *Thermus aquaticus* is often used, that is able to keep its activity despite the use of higher temperatures (Mullis, 1990).

Quantitative PCR (qPCR) is a method that allows for the DNA amplification to be monitored, and DNA to be quantified, in real-time during the course of the PCR. It is possible to correlate the number of cycles it takes to reach a certain signal strength with how much DNA is present in the original sample; i.e. the initial copy number (Higuchi, et al., 1993). qPCR is more reliable than end-point analysis to determine the initial copy number since the exponential phase will reach a plateau, making it impossible to extrapolate the curve to the starting point. Another parameter to consider is that the sample signal must be stronger than the background signal to be able get a reading. The number of rounds needed for the sample signal to become stronger than the background is called the cycle threshold (Ct), the Ct-value will be lower for a sample with a higher starting concentration of DNA since fewer rounds of amplification is needed to reach a certain signal stregth. The amount of generated DNA fragments can be measured in several ways, but the main principle is that the strength of the fluorescent signal increases when the amount of DNA increases. Three monitoring methods that can be used are; hydrolysis probe, molecular beacon or intercalating dyes. The signal from the hydrolysis probe is quenched when the probe is intact, but when DNA polymerase extends the primer it will reach the probe (which is bound to the DNA strand) and cleave off the quencher with its exonuclease activity. The signal from the molecular beacon is quenched when it is free, but

when it binds to a complementary strand of DNA the distance to the quencher increases and fluorescence will be emitted. Intercalating dyes emit fluorescence, which will be strongly intensified when they bind to double stranded DNA. However, the intercalating dye is less specific since it does not discriminate between different DNA sequences (Ginzinger, 2001). In Figure 1 the principle of qPCR is illustrated.

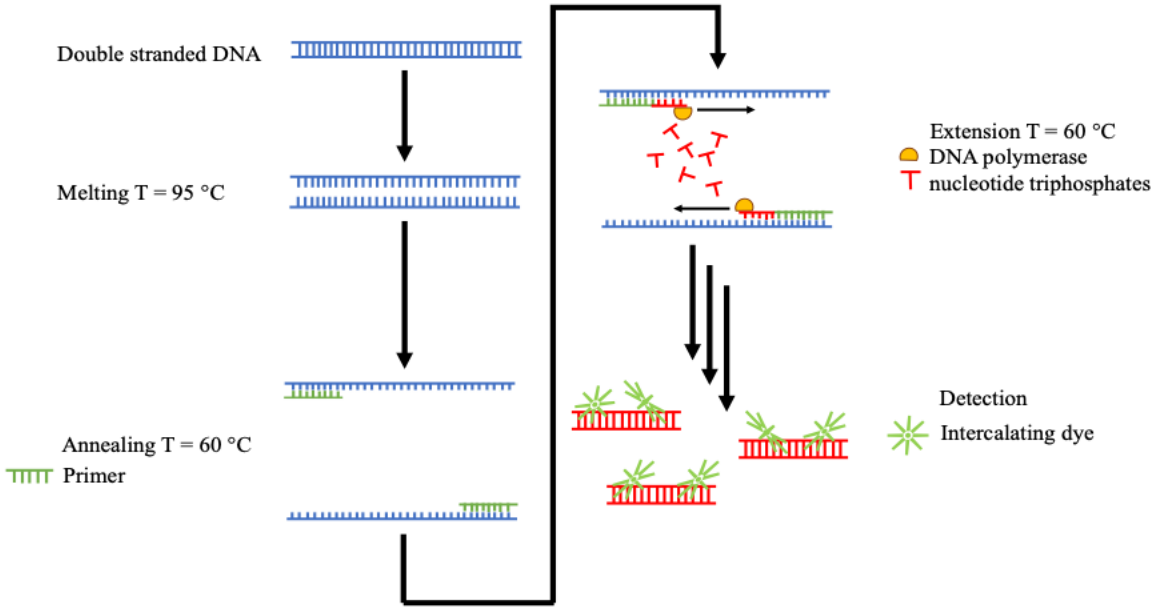

Figure 1. Illustration of the principle of qPCR. Double stranded DNA is denatured when the temperature is raised to 95 °C. Then the temperature is lowered to 60 °C, allowing the specific primers to anneal to the single stranded DNA. The DNA polymerases extend the primers by adding free nucleotide triphosphates. The cycle is repeated 40 times, resulting in an exponential build-up of PCR-product. The product is detected each cycle by intercalating dye, that fluorescence when bound to double stranded DNA. Stated parameters are based on the Major-4.2 Kit, except for the use of an intercalating dye as fluorescent reporter, probes are used in the Major-4.2 Kit (Thermo Scientific, 2016). Intercalating dye is used in this illustration to simplify the schematic process.

To be able to get the initial concentration of the template DNA a standard curve needs to be constructed. In the standard curve the Ct is plotted as a function of the logarithm of the known DNA-concentration (ng/µl) (Hedman, et al., 2018). A dilution series of 10-fold diluted samples can be used to create the curve, and if the efficiency of the PCR is 100% the slope will be -3.32 (Ginzinger, 2001). The Ct-value is affected by the pH and concentration of the reaction mixture, as well as by the instrument, making it impossible to use a general standard curve for all assays (Thermo Fisher Scientific, 2016).

2.2.3 ELISA

Enzyme-linked immunosorbent assay (ELISA) was first developed as an alternative to quantitative assays using radioactive labels, taking advantage of that enzyme coupled antibodies had previously been used to qualitatively detect antigens in tissue (Engvall & Perlmann, 1971). Due to the very specific binding between an antibody and its targeted antigen, ELISA is able to detect very low amounts of the analyte. The enzyme helps amplifying the signal by converting a substrate into product, thus making it readable by color development if the sample is positive. Today, there are several versions of ELSA, and it is possible to detect either antigens or antibodies as the analyte (Aydin, 2015).

In Direct ELISA the enzyme is conjugated directly to the antibody binding to the analyte (Engvall & Perlmann, 1971). In Indirect ELISA, used in this study, a secondary antibody is

labeled with the enzyme, which will bind to the primary antibody-analyte complex. In Sandwich ELISA two antibodies, one of which is immobilized at the capturing surface, bind the analyte at two different epitopes. The method can be either direct or indirect. The last ELISA method, Competitive ELISA, differs from the previous ones by that the signal decreases as the concentration of the analyte increases. Labeled analyte particles compete with the analyte particles in the sample, and the signal strength is reversed proportional to the amount of analyte in the sample. The Competitive ELISA can be either direct or indirect (Aydin, 2015). The principle of Indirect ELISA is demonstrated in Figure 2.

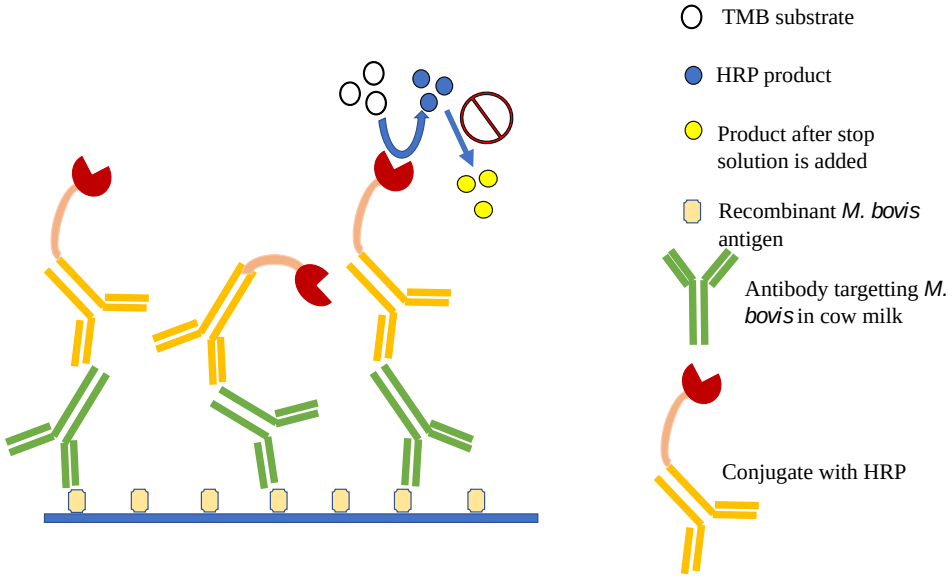

Figure 2. Illustration of the principle of indirect ELISA, used in this project. Other parameters in the illustration are also the same as in the kit. TMB substrate is 3,3',5,5'-Tetramethylbenzidine, and HRP is the enzyme horse radish peroxidase. If the target antibody is present in the sample, it will bind to the immobilized antigen. After a washing step the HRP-conjugate is allowed to form a complex with the target antibody. After an additional washing step, the HRP transforms the added TMB substrate to a colored product. The blue product will turn yellow when the stop solution is added.

To be able to connect the developed color with an analyte concentration, a calibration curve needs to be constructed. In the calibration curve, the signal is plotted as a function of the concentration and will yield a linear curve. For a noncompetitive ELISA, the calibration curve is constructed by measuring the color of at least three known concentrations. These dilutions should be located somewhere in the middle between the LOD and the maximum concentration that can be quantified (Sasaki & Mitchell, 2001).

2.3 Performance characteristics

There are a number of performance characteristics that can be tested and compared to deduce the aptitude of an analysis method. The comparison of the qPCR and the ELISA kits is somewhat complex since they only are qualitative, and since they have different analytes. However, five performance characteristics were chosen to be tested and used to compare the methods; limit of detection, repeatability and intermediate precision, selectivity, and matrix effects and PCR inhibition. These performance characteristics were chosen to give a well-rounded perception regarding the performance of the methods, and they are described in the following sections.

2.3.1 Limit of Detection

The limit of detection (LOD) is the lowest possible concentration that can be confidently detected with the method. It is included in the definition that this distinction can be made with a decided statistical certainty. The LOD for a method is highly dependent on the matrix in the sample to be tested, since the matrix affects the noise level that the analyte signal needs to be separated from. The LOD is determined differently depending on if the analysis method is quantitative or qualitative (Nilsson, et al., 2000).

LOD_{95} is the concentration where 95% of the positive samples are detected as positive (Hedman, et al., 2018). Consequently, if the number of samples is lower than 20 all of the samples need to be positive, otherwise the percentage of positive samples being detected as positive will be lower than 95%. The range of concentrations should be clustered around the expected LOD.

2.3.2 Repeatability and intermediate precision

Repeatability is a measure of how similar the results are from one run to another, when keeping every parameter constant (for example the operator, the day, the sample and so on). When measuring intermediate precision, one or several parameters are changed to see how much a certain parameter affects the result. These two parameters can be measured either as standard deviations, or as percentage false positives/negatives, depending on if the analysis is quantitative or qualitative (Nilsson, et al., 2000).

2.3.3 Selectivity

Selectivity means the ability of the analysis to distinguish the desired analyte from other species in a complex sample. The measured signal should reflect the presence of this analyte regardless of which other species, and the concentrations of these, that are present in the sample (Nilsson, et al., 2000).

2.3.4 Matrix effects and PCR inhibition

As mentioned above, the matrix affects the noise, and hence the LOD. However, it can also affect the signal of the analyte. Since milk is a liquid matrix, it is usually less problematic to work with compared to a solid matrix. Although unspecific binding to the wells in the ELISA might decrease the detected signal, blocking of unspecific binding will mitigate this problem (Brandon & Adams, 2015). Sample preparation is also a means to try and reduce the effects of the sample matrix (Hedman, et al., 2018).

To avoid false negatives in the qPCR, caused by matrix effects, inhibitors, faulty thermo cycling, Internal Amplification Control (IAC) is included in the test tube. The IAC is an additional DNA segment that should be amplified regardless of if the target sequence is present in the sample (Hoorfar, et al., 2003). If the sample is stated to have “IAC failure”, there is no guarantee that negative result is not due to a faulty PCR run, and the sample should be re-run.

3 Literature review

3.1 Differences when testing for DNA and antibodies

The qPCR tests for the presence of *M. bovis* DNA in milk, with no distinction between live and dead bacteria, while the ELISA gives a positive result if antibodies targeting *M. bovis* are present. This difference in target analyte raises the question if these two screening methods

indeed are interchangeable. As mentioned earlier, only mastitis can be detected when milk is tested. However, if this is the case bacteria and antibodies will appear in the milk at some point after the infection, but there is no guarantee that they will be present at the same time (Kantsø Nielsen, et al., 2015). In addition, the antibody response is highly individual and time dependent. It depends both on the cow and the expression pattern of surface antigens of the *M. bovis* clone (which can differ within a strain) (Petersen, et al., 2018). It has also been suggested that treatment with antibiotics, prior to proper diagnosis, can decrease the number of *M. bovis* cells that are present in the milk (Nicholas & Ayling, 2003).

As a result of the dynamic nature of the antibody production and the shedding (the presence of bacteria in milk), sampling can occur at a stage where neither bacteria nor antibodies are present in the milk (Kantsø Nielsen, et al., 2015). There is a lag period between infection and shedding of about three days, making this the earliest qPCR can be used for detection (Kauf, et al., 2007). However, it takes up to two weeks for the body to select and produce antibodies with the desired specificity, making the ELISA unable to detect *M. bovis* infection prior to this. On the other hand, the antibodies remain present in the milk for several months after the first infection, which enables the detection of chronic *M. bovis* infections (Akan, et al., 2014). In contrast, subclinical or chronical infections seldom result in any shedding (Parker, et al., 2018).

Due to the dynamic character of the antibody production, it has been suggested that the ELISA testing for antibodies should only be used on herd level. This to not be dependent on the antibody production of a single individual, but rather on the collective antibody production of the herd. This would also circumvent the issue of uninfected cows producing antibodies due to the presence of *M. bovis* on the farm, since these cows would test positive if they were tested as individuals (Petersen, et al., 2018). To try and avoid these false-positives, one suggestion is to increase the cut-off value for the ELISA, with the motivation that the concentration of antibodies is higher if the infection is ongoing. This suggestion was made based on data from diagnosis on herd-level (Petersen, et al., 2016). In addition, the clinical symptoms appear at roughly the same time as the antibody level in the milk is increased, making ELISA a viable option if analysis is promoted by the identification of symptoms (Petersen, et al., 2018).

3.2 Choice of commercial ELISA kit

Two companies were identified as providers of ELISA-kits screening for *M. bovis* in Europe; Bio-X and ID.vet. One of Eurofins Danish laboratories is currently using Mycoplasma bovis ELISA kit BIO K 302/2 (below called BIO K 302/2 kit) from Bio-X (Rochefort, Belgium) as screening method, but they are conducting tests to see if they can switch to the ID Screen® Mycoplasma bovis Indirect ELISA (below called ID Screen Kit) from ID.vet (Grabels, France). Hence, both of the relevant kits were currently used and investigated, respectively, within Eurofins Scientific. Since the Danish laboratory was interested in replacing the BIO K 302/2 kit with the ID Screen kit, the latter was the most relevant kit to investigate. In addition to these two kits being the only available kits in Europe, there are several advantages in using the same kit as the Danish laboratory; regarding purchase of the kit and assistance in working with the kit, to mention a couple. Both milk and serum samples can be used when testing for *M. bovis* with these kits.

An external interlaboratory study was performed to evaluate the ID screen Kit, the Bio K 302/2 Kit and PCR. According to this study the ID screen Kit performed equally well as PCR assays, while the Bio K 302/2 Kit was inferior both concerning diagnostic sensitivity and

specificity (Aspán, et al., 2018). This gives an indication that the ID screen Kit is superior to the Bio K 302/2 Kit, making the ID screen Kit the best choice to use as the ELISA assay.

3.3 Comparison of environmental and health aspects of PCR and ELISA

It is a fair assumption to make that there might be differences regarding the environmental and health aspects of running PCR and ELISA assays. The Major-4.2 Kit and the ID Screen Kit will be compared regarding these parameters, and the BIO K 302/2 Kit will also be mentioned. The Major-4.2 Kit involves more steps, due to the DNA extraction being performed prior to the PCR and includes more reagents. However, Lysis Solution 2, Master Mix, Primer Mix, Buffer AE, and Buffer AW2 are stated to be non-hazardous in the Safety Data Sheets provided from Thermo Fisher upon request (Thermo Fisher Scientific, 2012; Thermo Fisher Scientific, 2012; Thermo Fisher Scientific, 2012; QIAGEN, 2017; QIAGEN, 2017). Lysis Solution 1 is stated to be irritating for the eyes, and have a negative impact on aquatic organisms and contains the hazardous compound Trition X-100 (Thermo Fisher Scientific, 2012). Proteinase K is stated to possibly sensitize the respiratory tract and cause allergies or asthma, and also irritate eyes and skin. It contains the hazardous reagent protinase, tritirachium album serine (Thermo Fisher Scienific, 2012). Buffer AW1 is hazardous if swallowed or inhaled, and also irritating for skin and eyes, and contains the hazardous compound guanidine hydrochloride (QIAGEN, 2019). Buffer AL also contains maleic acid, in addition to guanidine hydrochloride, and is irritating skin, eyes and if inhaled (QIAGEN, 2017). The use of protective clothing, gloves and eye protection is recommended regarding the use of all of these reagents.

The Material Safety Data Sheet for the ID screen Kit was also obtained by request from ID.Vet and contained information regarding all of the solutions included in the kit. The concentrated Conjugate and the concentrated Wash Solution in the ID screen Kit are stated to be non-hazardous (ID.vet, 2018). The Positive and Negative Control contain sodium azide and are stated to be irritating to eyes and skin, and harmful to aquatic organisms. Dilution Buffers 13 and 3, contain Proclin 300, and pose the same hazards. The Substrate Solution which contains 3,3’,5,5’-tetramethylbenzidine, is also irritating for eyes and skin. The Stop Solution is also irritating to the eyes and the skin, in addition to being harmful if swallowed. This is due to it containing 3% organic acid. However, these compounds are only present in less than 0.1%, except for the organic acid. As for the Major-4.2 Kit the use of protective clothing, gloves and eye protection is recommended regarding the use of all of the reagents. In addition, all biological material present in the reagents are non-hazardous due to inactivation.

There are a lot of similarities between the ID screen Kit and the BIO K 302/2 Kit, but there are also a couple of differences; both the Washing solution and the Dilution Buffer contains Proclin 300, and the Stop solution contains 7% phosphoric acid. Besides the previously mentioned hazards with Proclin 300, the phosphoric acid can cause acute oral toxicity (Bio-X Diagnostics, 2015). The same protective garments are recommended as for the other two assays.

Considering the stated health and environmental aspects of the different ELISA assays, they are almost equally hazardous. However, the stronger acid used in the Stop Solution in the Bio K 302/2 Kit can be considered as a disadvantage when comparing it to the ID screen Kit. When comparing the Major-4.2 Kit with the ID screen Kit the most common hazards are irritation of eyes and skin, followed by respiratory sensation and oral toxicity. In addition, both assays employ reagents that are harmful to aquatic organisms, prompting waste not being

disposed of in the sink. Both assays utilize small volumes. With these similarities in mind, the major advantage of the ELISA over the PCR is that fewer solutions need to be handled.

4 Materials and methods

4.1 Sample information

Neither the concentration of *M. bovis* in the positive control included in the Major-4.2 Kit, nor the antibody concentration in the positive control included in the ID Screen Kit are known, since the tests are not quantitative. In addition, standard samples with known concentrations of these species were not available. Hence, the LOD was determined semi-qualitatively; as a dilution of a positive sample. Two positive bulk tank milk (BTM) samples, from two different farms, were detected during a qPCR screening and were used to investigate the performance characteristics of the two analysis methods. The samples will hereon forward be denoted Sample A and Sample B.

The samples were frozen after the qPCR screening. To avoid repeated thawing and freezing of the samples, they were divided into smaller tubes with approximatively 5 ml in each. When more sample was needed, a new tube was thawed, a drop of the conservative bronopol was added to keep the sample from degrading if kept in the fridge.

Milk samples containing other *Mycoplasma* species (M. spp.) (hereon after denoted Sample C and D), negative milk samples (denoted as Sample E and F), as well as the positive controls included in the kits, were also used in some experiments, see individual sections for details. Sample C and D had tested positive for M. spp. when the Complete-16 Kit was still in use.

4.2 qPCR with the Major-4.2 Kit

All qPCR reactions were run according to *Thermo Scientific PathoProof Major-4.2 kit Instructions for Use*, the protocol provided by the kit manufacturer (Thermo Scientific, 2015). The software was programmed according to *Software manual - Thermo Scientific PathoProof Norden Lab Mastitis Studio Software Instructions for use* (Thermo Scientific, 2016).

A positive extraction control ($PC_{extraction}$), consisting of saved milk from infected cows, was used to verify that the extraction was successful, see Table A - 1 in Appendix A 2 for required values. In addition, a positive PCR control (PC_{PCR}) containing DNA from *M. bovis* was run in a separate PCR-tube to verify that the PCR reaction worked.

In the experiments where positive PCR control (PC_{PCR}) was added to milk samples, this was done after the extraction had been carried out. This approach was chosen to avoid the free DNA in the PC_{PCR} to be degraded during the extraction process.

The measured cycle threshold value can be translated into a status regarding the level of contamination of *M. Bovis*, the translation should be done in accordance with Table 1.

Table 1. Interpretation guide for the measured cycle threshold (Ct) values, stating which status of M. Bovis *infection a certain Ct-value corresponds to. The guide is adapted from Appendix A 1.*

Ct-value:	**Status:**
No Ct	-
37.1 – 40	+/-
32.1 – 37	+
22 – 32	++
< 22	+++

4.2.1 Confirmation of positive samples

Two BTM samples, Sample A and B, were submitted to Eurofins as a part of a screening project and had tested positive for *M. bovis* when screened with qPCR, and it was these samples that were further used in the experiments. The analyses were performed by a Eurofins employee. The result was confirmed by rerunning the samples.

4.2.2 Limit of detection

The qPCR kit can be considered to be qualitative since it does not employ a standard curve to translate the result into a concentration. Hence, the LOD will be determined by testing samples with different dilutions and observing at which dilution all of the replicates are detected as positive. According to Appendix A 1 the theoretical LOD is Ct < 40. The theoretical LOD was used to calculate the corresponding dilution for Sample A and B, to determine which dilutions would be most appropriate to use. This was done using the correlation that a ten-fold dilution causes a decrease of 3.32 in Ct-value, and the mean Ct-value for the undiluted sample A was 32.1 and 27.3 for Sample B. Four dilutions were chosen and are stated in Table 2.

Table 2. The chosen dilutions for Sample A and Sample B are stated in the table, as well as their theoretical Ct-values.

Sample A:	**Theoretical Ct-value:**	**Sample B:**	**Theoretical Ct-value:**
x1	32	x1	27
x10	35.3	x100	33.6
x100	38.6	x2400	38.3
x1000	42.0	x12000	40.7

A dilution series was created by dilution of Sample A and B with distilled water, and triplicates of each dilution were run.

4.2.3 Repeatability and intermediate precision

The repeatability and the intermediate precision were measured as the percentage of false positives/negatives since the qPCR gave qualitative results. The percentage of false positives (1) and the percentage of false negatives (2) were calculated as follow.

$$\%false\ positives = \frac{number\ of\ false\ positives \times 100}{total\ amount\ of\ true\ negatives} \quad (1)$$

$$\%false\ negatives = \frac{number\ of\ false\ negatives \times 100}{total\ amount\ of\ true\ positives} \quad (2)$$

To evaluate the repeatability, the dilutions of Sample A in Table 2 were tested in four replicates each, all of them run on the same plate. This was not the same plate that was used for the LOD measurements.

To evaluate the intermediate precision the results from the LOD measurement and from the repeatability measurement were used. This was done to assess the impact of the plate and the day on the results.

4.2.4 Selectivity

It was important that the method was selective enough to distinguish between *M. bovis* and other M. spp., and only gave a positive result if *M. bovis* was present in the sample. The Major-4.2 Kit did not test for other M. spp., which the Complete-16 Kit did (Thermo Scientific, 2015; Thermo Scientific, 2016).

Sample C and D were tested to see that other M. spp. did not give a positive reading. In addition, positive control was added to eluates from the DNA extraction of Sample C and D to test the selectivity for *M. bovis* when other M. spp. were present. Triplicates were tested for Sample C and D without any added *M. bovis* DNA, with a x2 dilution of added PC_{PCR} and with a x4 dilution of added PC_{PCR}.

4.2.5 Matrix effects and PCR inhibition

To test the matrix effects and PCR inhibition *M. bovis* DNA was added (in the form of PC_{PCR}) to the DNA extraction eluates of Sample E and F, that was negative for *M. bovis*. The PC_{PCR} was also added to water, meaning that no matrix was present. Since the Major-4.2 Kit does not test for the presence of M. spp., there was no guarantee that these samles were negative in that regard. The PC_{PCR} was added as a x2 dilution and as a x4 dilution to Sample E and F, and to distilled water respectively. The samples were then run in triplicates.

4.3 ELISA with the ID Screen Kit

All samples were run according to the *Short incubation protocol* in Appendix A 3. A centrifugation step (15 minutes at 600g) was performed for all samples, after dilution when this was performed, but prior to pipetting the sample into the wells, as was recommended when testing whole milk samples (IDvet, n.d.). Distilled water was used when diluting the Wash Concentrate (20X). After ocular inspection of the wells, soaking was deemed unnecessary, and the washing steps were repeated three times.

The plate reader was programmed as recommended in the instructions in Appendix A 3. The wavelength was set to 450 nm, and the test was deemed valid if (3) and (4) were fulfilled:

$$OD_{PC} > 0.350 \quad (3)$$

$$\frac{OD_{PC}}{OD_{NC}} > 3 \quad (4)$$

Where OD is the optical density, PC is the positive control and NC is the negative control, and the OD values are the mean values of the duplicates. The reading program was also programmed to calculate the ratio between the sample and the positive control based on the measured OD values according to (5).

$$\frac{S}{P}\% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100 \quad (5)$$

To determine the status of the sample, the calculated S/P% was interpreted according to Table 3.

Table 3. Interpretation guide for the calculated S/P% value, stating which status of M. Bovis *infection a certain SP % translates to. The guide is adapted from Appendix A 3.*

Result (S/P%):	**Status:**
S/P% <20%	-
20%≤ S/P% <40%	+
40%≤ S/P% <60%	++
60%≤ S/P% <80%	+++
S/P% ≥80%	++++

4.3.1 Confirmation of positive samples

The two milk samples that were positive according to the qPCR (Sample A and B) were tested with the ELISA-kit. The samples were tested as duplicates in the same run.

4.3.2 Limit of detection

The ELISA kit can be considered to be qualitative since it does not employ a standard curve to translate the result into a concentration. Hence, the LOD will be determined by testing samples with different dilutions and observing at which dilution all of the replicates are detected as positive. To determine which dilutions would be most appropriate to use when determining the LOD, the theoretical LOD was calculated using (5). The values that were used to calculate this were S/P% 20 (lowest S/P% for a positive sample), OD_{PC} was 1.276 and OD_{NC} was 0.045. The OD_{PC} and OD_{NC} were the mean values from the two replicates of each control when the positive samples were confirmed.

The theoretical LOD was calculated to be roughly OD 0.29. These values were then used to estimate which dilutions would yield an OD of 0.29, using (5), provided that the OD is linearly dependent on the antibody concentration. This was done for both samples. Six dilutions were chosen and are stated in Table 4.

Table 4. The chosen dilutions for Sample A and Sample B, as well as their theoretical S/P%, are stated in the table. The dilutions labeled with † were run in four replicates instead of three.

Sample A:	**Theoretical S/P%:**	**Sample B:**	**Theoretical S/P%:**
x1†	83	x1	27
x2†	39	x1.2†	22
x3	25	x1.3	20
x4	18	x1.4	18
x6†	10	x1.6†	15
x8†	7	x1.8†	13

Two dilution series for each sample, denoted α and β, were created by dilution of Sample A and B with distilled water, and triplicates of each dilution were run with the exception of the dilutions labeled † with in Table 4 where four replicates were run for one of the dilution series.

4.3.3 Repeatability and intermediate precision

Since the ELISA gave qualitative results, the repeatability and the intermediate precision were measured as the percentage of false positives/negatives. The percentage of false positives and the percentage of false negatives were calculated according to equation (1) and (2).

The repeatability was calculated using the results from the LOD measurements. The dilutions labeled with † in Table 4 were tested in four replicates each, when measuring the LOD, to evaluate the repeatability of the assay.

The dilutions labeled with † in Table 4, in dilution series β for Sample A, were then tested in triplicates the following day on the other plate in the package to test the intermediate precision, regarding the plate and the day.

4.3.4 Selectivity

It was important that the method was selective enough to distinguish between *M. bovis* and other M. spp., and only gave a positive result if *M. bovis* was present in the sample.

Sample C and D, that were known to be PCR-positive for M. spp., were tested to see if the ELISA detected these samples as negative for *M. bovis*. Triplicates were tested for Sample C and D without any anti-*M. Bovis* antibodies added, with a x50 dilution of added antibodies and with a x150 dilution of added antibodies. The added antibodies were in the form of the positive control (PC).

4.3.5 Matrix effects and PCR inhibition

To test the matrix effects anti-*M. bovis* antibodies were added (in the form of the positive control) to Sample E and F, that had been qPCR-negative for *M. bovis* when tested with the Major-4.2 Kit, and to Buffer 13 that did not contain any matrix. Since the Major-4.2 Kit did not test for the presence of M. spp., there was no guarantee that these samples were negative in that regard. The antibodies were added with a x50 dilution and with a x150 dilution to Sample E and F, and to Buffer 13 respectively. The samples were then run in triplicates.

5 Results

5.1 Confirmation of positive results

Two replicates each of Sample A and B were tested with qPCR to confirm that they were positive for *M. bovis* DNA. The calculated mean Ct-values were 32.1 for Sample A and 27.3 for Sample B (see Appendix B 1 for raw data). Both samples were graded as having the status ++.

Duplicates of Sample A and B were also tested with ELISA to confirm that they were positive for anti-*M. bovis* antibodies. The calculated mean S/P% values were 83.0 for Sample A and 27.1 for Sample B (see raw data in Appendix C 1). Sample A was graded as ++++ and Sample B was graded as +.

5.2 Limit of detection

To determine the LOD for the qPCR, dilution series β was run for four dilutions of Sample A and Sample B. The Samples were run as triplicates, and a sample is positive if it has a Ct-value of < 40. The measured Ct-values can be found in Table 5. All replicates need to be positive for a specific dilution to be considered as the LOD for a given sample. The LOD for Sample A was found to be x10-x100 and x2400-x12000 for Sample B.

Table 5. Dilutions of Sample A and B were run in the qPCR to determine the LOD. The measured cycle threshold (Ct) values for the stated dilutions for Sample A and B of dilution series β. Triplicates were run for each dilution.

		Sample A:				Sample B:			
	Dilution series:	**Undiluted (x1)**	**x10**	**x100**	**x1000**	**x1**	**x100**	**x2400**	**x12000**
Ct-value:	β	32.9	37.8	-	-	26.7	33.6	37.1	-
		33.3	37.0	-	-	27.1	33.6	37.0	-
		33.1	36.5	-	-	27.6	33.2	36.8	-

To determine the LOD for the ELISA dilution series α and β were run for six dilutions for Sample A and B. The undiluted Sample B samples were run on another day than the rest of the samples. The samples in dilution series α and the two intermediate dilutions for dilution series β were run in triplicates, while the remaining dilutions in dilution series β were run in four replicates. A sample is considered positive if the S/P% is ≥ 20%, and all replicates need to be positive for a specific dilution to be considered as the LOD. The calculated S/P% are found in Table 6 for Sample A and in Table 7 for Sample B (raw data are found in Appendix C 2). The LOD for Sample A was found to be x3-x4 and x1.3-x1.4 for Sample B.

Table 6. Dilutions of Sample A were run in the ELISA to determine the LOD. The rounded down S/P% for the chosen dilutions of Sample A are presented in the table for dilution series α and β.

		Dilutions Sample A:					
	Dilution series:	**x1**	**x2**	**x3**	**x4**	**x6**	**x8**
S/P%:	α	55	33	26	19	13	9
		64	36	24	18	13	12
		64	38	25	18	20	10
	β	64	38	24	19	13	9
		68	36	24	20	13	10
		71	35	24	18	15	16
		73	36			13	10

Table 7. Dilutions of Sample B were run in the ELISA to determine the LOD. The rounded down S/P% for the analysed dilutions of Sample B are presented in the table for dilution series α and β. The samples labeled with ‡ were run on a different day. N/A: not analysed.

		Dilutions Sample B:					
	Dilution series:	**x1**	**x1.2**	**x1.3**	**x1.4**	**x1.6**	**x1.8**
S/P%:	α	20‡	15	16	13	12	11
		22‡	16	14	14	13	10
		22‡	18	14	14	13	11
	β	N/A	15	11	12	11	8
		N/A	13	12	12	10	9
		N/A	15	13	12	11	9
		N/A	14			10	11

5.3 Repeatability and Intermediate precision

The repeatability and intermediate precision for the qPCR were calculated as the percentage of false negative and the percentage of false positive using equations (1) and (2). The

repeatability was calculated as 38% false negatives and 0% false positives, and the intermediate precision was calculated as 27% false negatives and 0% false positives. The calculations were performed using dilution series β for Sample A, and the used numbers are found in Table 8. The measured values for the dilutions were considered to be either true or false based on their theoretical Ct-value (Table 2).

Table 8. The repeatability and intermediate precision were calculated as the percentage false positive and the percentage false negative using the numbers that are stated in the table. The samples used to calculate the repeatability are dilution series β in Table B - 3, in Appendix B 2. The intermediate precision was calculated using the dilution series β in Table B - 3 and Table B - 6, in Appendix B 2 and B 3. Samples with Ct > 40 were considered negative, see Table 2.

	Repeatability:	**Intermediate precision:**
Number of True positives:	8	11
Number of True negatives	8	11
Number of False positives:	0	0
Number of False negatives	3	3

The repeatability and intermediate precision for the ELISA were calculated as the percentage false negatives and the percentage false positives using equations (1) and (2). The repeatability was calculated as 0% false negatives and 9% false positives, and the intermediate precision was calculated as 0% false negatives and 7% false positives. The calculations were performed using dilution series β for Sample A, and the used numbers are found in Table 9. The measured values for the dilutions were considered to be either true or false based on their theoretical S/P% (Table 4).

Table 9. The repeatability and intermediate precision were calculated as the percentage false positive and the percentage false negative using the numbers that are stated in the table. The samples used are the ones in the dilution series β for Sample A in Table C - 4 and Table C - 10 in Appendix C 2 and C 3. Samples with S/P% <20% were considered as negative, see Table 4.

	Repeatability:	**Intermediate precision:**
Number of True positives:	11	14
Number of True negatives	11	14
Number of False positives:	1	1
Number of False negatives	0	0

5.4 Selectivity

The qPCRs ability to distinguish between *M. bovis* and other M. spp. were tested using M. spp. positive samples (Sample C and D). Triplicates of Sample C and D were tested without any added *M. bovis* DNA (in the form of positive PCR control), when a x2 dilution of DNA had been added and when a x10 dilution of DNA had been added. The calculated mean Ct-values for these samples are displayed in Table 10 (raw data are found in Table B - 8 in Appendix B 4). The qPCR was able to detect M. spp. positive samples as negative, and both the *M. bovis* spiked samples as positive.

Table 10. The selectivity was tested by running samples with M. spp., and also these samples spiked with M. bovis. *The table displays the calculated mean Ct-values (n=3) for the M. spp. positive samples, the used raw data can be found in Appendix B 4. Positive PCR control (PC_{PCR}) was added in two concentrations. The status of the samples is also included in the table, see the interpretation guide in Table 1.*

PC$_{PCR}$ dilution:	Sample:	Mean Ct-value:	Status:
	C	-	-
	D	-	-
	C	24.0	++
x10	D	24.6	++
	C	22.0	++
x2	D	21.7	+++

The selectivity of the ELISA was tested in the same manner as the qPCR. Triplicates of M. spp. positive Sample C and D were tested with no added anti-M. bovis antibodies (in the form of the positive control), with a x50 dilution of antibodies added and with a x150 dilution of antibodies added. The calculated mean S/P% are found in Table 11 (raw data are found in Appendix C 4). The ELISA was able to detect M. spp. positive samples as negative, and both the *M. bovis* spiked samples were detected as positive.

Table 11. The selectivity was tested by running samples with M. spp., and also these samples spiked with M. bovis. *The table displays the calculated mean S/P% (n=3) for the M. spp. positive samples, the used raw data can be found in Appendix C 4. Positive control (PC) was added in two concentrations. The status of the samples is also included in the table, see the interpretation guide in Table 3.*

PC dilution:	Sample:	Mean S/P%:	Status:
	C	2	-
	D	5	-
	C	36	+
x150	D	42	++
	C	81	++++
x50	D	75	+++

5.5 Matrix effects and PCR inhibition

To assess the possible PCR inhibition caused by the milk matrix for the qPCR, M. bovis DNA (in the form of positive PCR control) was added to water and to M. bovis negative Sample E and F. Triplicates of the water, Sample E and Sample F were run when a x2 dilution of DNA had been added and when a x10 dilution of DNA had been added. If no PCR inhibition took place the Ct-value for the water sample and both the milk samples should be the same when the same amount of DNA was added to them. The calculated mean Ct-values for qPCR negative milk samples and water with added PC$_{PCR}$, are displayed in Table 12, the raw data is found in Appendix B 4. No PCR inhibition was observed.

Table 12. The PCR inhibition was investigated by running spiked water and negative milk samples (Sample E and F). The table displays the calculated mean Ct-values (n=3) for the negative samples, the used raw data can be found in Appendix B 4. Positive PCR control (PC$_{PCR}$) was added in two concentrations. The status of the samples is also included in the table, see the interpretation guide in Table 1.

PC$_{PCR}$ dilution:	Sample:	Mean Ct-value:	Status:

x10	H_2O	24.3	++
	E	24.7	++
	F	24.9	++
x2	H_2O	22.4	++
	E	21.4	+++
	F	21.5	+++

The matrix effects caused by sample background for the ELISA were investigated by comparing the S/P% for Buffer 13 compared with the S/P% for the M. bovis negative Sample E and F. Triplicates of the Buffer 13, Sample E and Sample F were run were run when a x50 dilution of antibodies had been added and when a x150 dilution of antibodies had been added. If no matrix effects were present the same antibody dilution should yield the same S/P% in the Buffer 13 and in Sample E and F. The calculated mean S/P% for qPCR negative samples and Buffer 13 with added positive control, are displayed in Table 13, the raw data is found in Appendix C 4. It was observed that the milk matrix enhanced the signal of the sample, the enhancement increased as the *M. bovis* concentration in the sample increased.

Table 13. The matrix effects were investigated by running spiked Buffer 13 and negative milk samples (Sample E and F). The table displays the calculated mean S/P% (n=3) for the spiked samples, the used raw data can be found in Appendix C 4. Positive control (PC) was added in two concentrations. The status of the samples is also included in the table, see the interpretation guide in Table 3. The Buffer 13 samples were run at another day than the rest of the samples.

PC dilution:	**Sample:**	**Mean S/P%:**	**Status:**
x150	Buffer 13	28	+
	E	34	+
	F	70	+++
x50	Buffer 13	59	++
	E	73	+++
	F	93	++++

5.6 Performance comparison

A summary of the outcome of the tested performance characteristics can be seen in Tabell 14. The qPCR performed better than the ELISA regarding the LOD and the matrix effects and the PCR inhibition, the ELISA performed better than the qPCR regarding the repeatability and the intermediate precision and both the methods performed equally well regarding the selectivity.

Tabell 14. The table displays a summary of the outcome of the tested performance characteristics for the qPCR and the ELISA. The method that performed the best in regards to a certain performance characteristic is labeled with √.

Performance characteristic:	**qPCR:**	**ELISA:**
LOD	√	
Repeatability and intermediate precision		√
Selectivity	√	√
Matrix effects and PCR inhibition	√	

6 Discussion

The purpose of this degree project was to investigate available commercial ELISA kits that screen cow's milk for *M. bovis* infection, and then to evaluate and compare the chosen ELISA kit (ID screen Kit) with the at Eurofins currently used qPCR assay (Major-4.2 Kit). This has been executed by the planning and performance of lab work, as well as a substantial literary study. Due to the lack of available samples with known *M. bovis* concentration, and the semi-quantitative nature of the assays, the experimental evaluation has been performed in regard to two *M. bovis* positive milk samples; Sample A and B. The following performance characteristics were evaluated for the two assays; limit of detection (LOD), repeatability, intermediate precision, selectivity and matrix effects and PCR inhibition.

Regarding the LOD for the Major-4.2 Kit and the ID screen Kit it is evident that the qPCR method has a substantially lower LOD. The LOD for Sample A is in the range x3-x4 dilution for the ELISA and in the range x10-x100 dilution for the qPCR. For Sample B the LOD is in the range x1-x1.2 dilution for the ELISA and in the range x2400-12000 dilution for the qPCR. These measured LODs differ somewhat from the theoretical LODs. For the ELISA the theoretical LOD for Sample B was calculated to be in the range x1.3-x1.4 dilution. However, the dilutions of Sample B were very close to each other due to the low starting concentration of antibodies in Sample B, making the difference from the theoretical LOD quite small. For the qPCR the theoretical ranges, x20-x40 dilution for Sample A and x600-x1200 for Sample B, fall within the measured ranges. The x2400 dilution for qPCR of Sample B should theoretically be negative, but two out of three samples were detected as positive. This can possibly be explained by the fact that the theoretical LOD is based on experimental values, or by unprecise dilution. These factors can also be the reason for the single false negative x4 dilution of Sample A, tested by ELISA. It is also worth taking into account that the DNA content in the original sample is decreased by as much as 85% by the DNA extraction step, depending on the extraction method (Norén, et al., 2013). This means that the amount of DNA in the original sample must be large enough to withstand the depletion during the extraction step. It is possible to optimize the used extraction method, however that is not within the scope of this project.

The developmental validations of these kits are limited due to the semi-quantitative nature of the analysis methods and they do not state a determined LOD. For the Major-4.2 Kit the only validation that was performed was a comparison with the Complete-16 Kit. For the ID screen Kit and the Bio K 302/2 Kit serum was used as sample, which makes it difficult to compare the results.

The calculated repeatability for the qPCR is 38% false negatives and 0% false positives and the calculated intermediate precision is 27% false negatives and 0% false positives. For the ELISA the calculated repeatability is 0% false negatives and 9% false positives and intermediate precision is 0% false negatives and 7% false positives. Hence, the ELISA kit is superior both regarding the repeatability and the intermediate precision compared to the qPCR kit. The high percentages for the qPCR could possibly be contributed to the use of more diluted qPCR samples, compared to the dilutions of the ELISA samples and compared to the LOD of the method.

When comparing the percentage of false positives with the percentage of false negatives it is necessary to keep the consequences of misdiagnosis in mind. A false positive result could

lead to the culling of a healthy cow. This can however be counteracted by a policy to retest all positive *M. bovis* samples. When it comes to false negative results the consequence is that the infected cow is kept together with the healthy cows, allowing the infection to spread. This could lead to a large number of cows being culled. These consequences have been taken into account when the cut-off value was set for the kit. Nonetheless, the percentage false negatives are high for the qPCR. The IAC is a means to detect false negative samples and implore that these samples are retested. This was the case for the dilution series β for Sample B, all of the false samples were stated to have IAC failure. However, for the dilution series β for Sample A none of the false samples were stated to have IAC failure, meaning that they would have been reported as negative.

The developmental validation for the ID screen Kit claimed that the assay had uniform results, consistent with no false negative results (ID.vet, 2019). However, the validation did not assess the repeatability for negative samples, which seems to be the weakness of this assay. The developmental validation for the Bio K 302/2 Kit stated that the repeatability and intermediate precision were up to par, that a negative dilution was tested for the intermediate precision and that there were no problems with false positives reported amongst these (Bio-X Diagnostics, 2020).

Both the qPCR and the ELISA tested negative for the M. spp. positive samples (Sample C and D). The spiked samples were tested positive for both assays. This proves that both analysis methods have adequate selectivity for the intended screening of *M. bovis* infection. The spiked qPCR samples did not differ much between Sample C and D with the same dilution, while there was a slightly bigger difference between the spiked ELISA samples with the same dilution.

These results are consistent with the developmental validation for the ID screen Kit, where no negative sample was detected as positive (ID.vet, 2019).

When comparing the Ct-values for the x10 dilution of the water sample with the Ct-values for the x10 dilution of Sample E and Sample F there is no difference. For the samples with x2 dilution there is a difference of roughly 1 Ct-unit between the water sample, which has the higher Ct-value, and Sample E and Sample F. This indicates that the milk matrix contributes to an enhancement of the DNA amplification, or that there is a small amount of DNA present in Sample E and F from the beginning. For the x150 diluted ELISA samples the mean S/P% are 28 for the water sample, 34 for Sample E and 70 for Sample F. The mean S/P% for the x50 dilutions are 59 for the water sample, 73 for Sample E and 93 for Sample F. These results exhibit a positive enhancement of the signal that increases when the antibody concentration increases.

Comparing the impact of the matrix on the analysis results it is evident that the ELISA assay is more affected. The ELISA samples are more diluted than the PCR samples, which means that the enhancement effect from the matrix should have been even more pronounced in the PCR results than in the ELISA results if the matrix effect would have been equally intensified by higher analyte concentration. Hence, it appears that there is not a problem with PCR inhibition due to the matrix, if any impact the DNA is further amplified in the spiked samples. The DNA extraction prior to the qPCR could be an explanation to the low impact of matrix on the qPCR results. Concerning the matrix effect on the ELISA samples, there is a large difference between Sample E and F and it would have been interesting to further investigate the heterogeneity of the matrix in different samples. This indicates that milk matrix is

heterogenous and that different milk samples cause differently severe matrix effects. These differences make it more difficult to compensate for the matrix effect. The fact that the S/P% is larger when more antibodies are present in the sample indicates that the Conjugate does not bind to the anti-*M. bovis* antibodies in a 1:1 ratio. This could cause a sample to be graded as more positive than it actually is, depending on the matrix in that particular sample.

In a European inter-laboratory study six laboratories compared the performance of the Bio K 302/2 Kit and the ID screen Kit to the results of western blot by testing serum samples from high and low prevalence areas. According to this study the ID screen Kit identified 87% of the samples from the high prevalence areas as positive and 0.4% of the samples from the low prevalence areas as positive. However, the Bio K 302/2 Kit only identified 46% of the samples from high prevalence areas as positive and 12% of the samples from the low prevalence areas as positive. In comparison, the western blot identified 86% of the samples from high prevalence areas as positive and none of the samples from the low prevalence area as positive. These results indicate that the ID screen Kit performs very well, while the Bio K 302/2 Kit probably needs to be optimized further. The increase of the cut-off value would decrease the number of the samples from low prevalence areas that were detected as positive, but that will also decrease the number of samples that were detected as positive from the high prevalence area (Andersson, et al., 2019). Another study compared the performance of the Bio K 302/2 Kit and the PathoProof Mastitis Major-3 kit from Thermo Fischer by testing BTM samples from Danish herds. They concluded that the Bio K 302/2 Kit had better selectivity and specificity than the qPCR kit if the cut-off for the ELISA kit had been increased with 26% compared to the cut-off stated by the manufacturer (Kantsø Nielsen, et al., 2015). This study had a different experimental set-up than the one in this project, which makes the results a bit difficult to compare. However, it seems reasonable to believe that the ID screen Kit is a better ELISA kit to use than the Bio K 302/2 Kit and that the ELISA assay can be considered to be a reasonable alternative to qPCR assay concerning BTM screenings.

7 Future work

If this project was to be extended it would have been interesting to test the Bio K 302/2 Kit to evaluate its performance compared to the ID screen Kit and the Major-4.2 Kit and see if it would have been advisable to use this kit. It would also be of interest to investigate the robustness of the methods, regarding the incubation times and temperatures. When performing screenings different operators will perform the assay and this might affect the incubation times and temperatures, since the protocols allow for some variation. Only two *M. bovis* positive samples were available when this project was performed, and it would have been interesting to see if both the ELISA and the qPCR would have been able to detect all samples from infected animals as positive. The results of this more extensive testing could have led to other recommendations being made. It would also be interesting to talk to the Danish laboratory and see what they concluded based on their validations of the two ELISA kits.

It was not within the scope of this project to investigate how the matrix differ between different milk samples, but based on the results for the matrix effects and PCR inhibition measurements for the ELISA it appears that the matrix effects differs depending on the milk sample that was used as the matrix. Hence, it would have been interesting to see how much the matrix effects can differ between specific sample.

8 Conclusion

Comparing qPCR and ELISA as screening assays for *M. bovis* infections proved to be complex, and it is impossible to recommend a single assay to use exclusively. However, based on the experimental results and the knowledge gained from literature it is possible to make recommendations regarding when one of the methods is more suitable than the other.

Running experiments with Sample A and Sample B (positive for *M. bovis*) it was seen that the relationship between *M. bovis* DNA and anti-*M. bovis* antibodies is not fixed. Sample A had the highest antibody concentration (highest S/P%) according to the ELISA, while Sample B had the highest DNA concentration (lowest Ct-value) according to the qPCR. The heterogeneity of the milk samples where further displayed by the difference in matrix effects between Sample E and F when run in the ELISA.

According to the results of the performed experiments regarding the LOD the qPCR has a substantially lower LOD than the ELISA. Still, the ELISA detected both of the samples as positive. However, the ELISA was superior when it came to the repeatability and the intermediate precision. Despite the fail-safe of the IAC, several samples were undetected as false negatives. Both assays passed the selectivity test. The matrix effects were greater for the ELISA assay than for the qPCR assay and it is only slightly mitigated by the circumstance that the positive result is more amplified the higher the analyte concentration, preventing false results. Based on the performance characteristics of the assays, the qPCR performed better than the ELISA assay.

Considering other factors than the experimental results, it is not obvious that the qPCR is the best choice. The ELISA assay is cheaper than the PCR, making it an attractive choice for the farmers. Another advantage of the ELISA is that it is a simple assay with fewer steps than the qPCR. This would allow for more flexibility regarding which operator is able to run the assay, since the qPCR protocol is more complex and requires a seasoned operator. Alternatively, the automation of the DNA extraction would make it more user friendly. This is also a contributing factor to why the ELISA can be considered to be more environmentally friendly. In addition, there are advantages both with testing for DNA and for antibodies. When screening BTM there is a greater chance that there are antibodies and DNA present, than when screening individuals.

In conclusion, based on the experimental results and the information retrieved from the literature it depends on the origin of the sample if qPCR or ELISA is more suitable to use. The ELISA is a good choice when verifying a diagnosis made based on displayed symptoms since cows with symptoms have had time to develop antibodies against *M. bovis* and should test positive with the ELISA assay if they are infected. The qPCR assay could then be used to confirm that there is an ongoing infection by verifying the result by retesting positive samples. The qPCR is a better choice when confirming a bacteriological culture of milk since the positive culture proves that the pathogen is being shedded, so if the cow is infected by *M. bovis* the qPCR result will be positive. When it comes to screening BTM both assays have advantages and disadvantages but based on the results in this project ELISA could be used when screening bulk tank milk; it is the cheaper and more health and environmentally friendly assay, and it detected the qPCR-positive samples as positive.

References

Akan, M. et al., 2014. Diagnosis of *Mycopiasma bovis* Infection in Cattle by ELISA and PCR. *Kafkas Universitesi Veteriner Fakultesi Dergisi,* 20(2), pp. 249-252.

Andersson, A.-M.et al., 2019. A European inter-laboratory trial to evaluate the performance of three serological methods for diagnosis of *Mycoplasma bovis* infection in cattle using latent class analysis. Volume 15, pp. 1-10.

Aspán, A. et al., 2018. *An European interlaboratory evaluation of PCR and ELISA methods for* Mycoplasma bovis *diagnostics.* Prato, 5th Prato Conference on Animal Bacterial Pathogens.

Aydin, S., 2015. A short history, principles, and types of ELISA, and our laboratory experience with peptide/protein analyses using ELISA. *Peptides,* 10, Volume 72, pp. 4-15.

Bio-X Diagnostics, 2015. *BIO K 302* -. [Online]
Available at: https://www.biox.com/en/bio-k-302-monoscreen-abelisa-mycoplasma-bovis-indirect-monowell-p-250/
[Accessed 19 03 2020].

Bio-X Diagnostics, 2020. *Bio K 432 validation file.* Rochefort: Bio-X.

Bokma, J. et al., 2019. Optimizing identification of *Mycoplasma bovis* by MALDI-TOF MS. *Research in Veterinary Science,* 08, Volume 125, pp. 185-188.

Brandon, D. L. & Adams, L. M., 2015. Milk Matrix Effects on Antibody Binding Analyzed by Enzyme-Linked Immunosorbent Assay and Biolayer Interferometry. *Journal of Agricultural and Food Chemistry,* Volume 63, p. 3593−3598.

Calcutt, M. J. et al., 2018. Gap analysis of *Mycoplasma bovis* disease, diagnosis and control: An aid to identify future development requirements. *Transboundary & Emerging Diseases,* Volume 65, pp. 91-109.

Dudek, K. et al., 2016. An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. *Vaccine,* 08 06, 34(27), pp. 3051-3058.

Engvall, E. & Perlmann, P., 1971. Enzyme-linked immunosorbent assay (ELISA) Quantitative assay of immunoglobulin G. *Immunochemistry,* 09 09, 8(9), pp. 871-874.

Ericsson Unnerstad, H., Fungbrant, K., Persson Waller, K. & Persson, Y., 2012. *Mycoplasma bovis* hos kor och kalvar i Sverige. *Svensk Veterinärtidning,* Issue 13, pp. 17-20.

Ginzinger, D. G., 2001. Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology,* 05 03, Volume 30, p. 503–512.

Hedman, J. et al., 2018. Validation guidelines for PCR workflows in bioterrorism preparedness, food safety and forensics. *Accreditation and Quality Assurance,* 05 06, Volume 23, p. 133–144.

Higuchi, R., Fockler, C., Dollinger, G. & Watson, R., 1993. Kinetic PCR Analysis: Real-time Monitoring of DNA Amplification Reactions. *Nature Biotechnology,* 01 09, Volume 11, p. 1026–1030.

Hoorfar, J. et al., 2003. Making Internal Amplification Control Mandatory for Diagnostic PCR. *Journal of Clinical Microbiology,* 12, 41(12), p. 5835.

ID.vet, 2018. *Material Safety Data Sheet ID Screen® Mycoplasma bovis Indirect.* Grabels: ID.vet.

ID.vet, 2019. *Internal validation report ID Screen® Mycoplasma bovis Indirect.* Grabels: ID.vet.

IDvet, n.d. *Recommendations for Milk Testing.* [Online]
Available at: https://www.id-vet.com/wp-content/uploads/2015/09/Recommendations-for-Milk-Testing_DOC2629.pdf
[Accessed 02 03 2020].

Kantsø Nielsen, P. et al., 2015. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*. *Preventive Veterinary Medicine,* 10, 121(3-4), pp. 338-342.

Kauf, A. C. W., Rosenbusch, R. F., Paape, M. J. & Bannerman, D. D., 2007. Innate Immune Response to Intramammary *Mycoplasma bovis* Infection. *Journal of Dairy Science,* 07, 90(7), p. 3336–3348.

Klein, U. et al., 2019. New antimicrobial susceptibility data from monitoring of *Mycoplasma bovis* isolated in Europe. *Veterinary Microbiology,* 27 09, Volume 238, pp. 1-6.
Le Grand, D., Solsona, M., Rosengarten, R. & Poumarat, F., 1996. Adaptive surface antigen variation in *Mycoplasma bovis* to the host immune response. *FEMS Microbiology Letters,* 11, 144(2-3), p. 267–275.
Lysnyansky, I. & Ayling, R. D., 2016. *Mycoplasma bovis*: Mechanisms of Resistance and Trends in Antimicrobial Susceptibility. *frontiers in Microbiology,* 17 04, Volume 7, pp. 595-601.
Mullis, K. B., 1990. The Unusual Origin of the Polymerase Chain Reaction. *Scientific American,* 04, 262(4), pp. 56-65.
Nicholas, R. A. J. & Ayling, R. D., 2003. *Mycoplasma bovis*: disease, diagnosis, and control. *Research in Veterinary Science,* 74(2), pp. 105-112.
Nicholas, R. A. J., Fox, L. K. & Lysnyansky, I., 2016. *Mycoplasma mastitis* in cattle: To cull or not to cull. *The Veterinary Journal,* Volume 216, pp. 142-147.
Nilsson, A., Stensiö, K.-E. & Lundgren, B., 2000. *Validering av kemiska analysmetoder,* Borås: Just Nu-Tryck.
Norén, L., Hedell, R., Ansell, R. & Hedman, J., 2013. Purification of crime scene DNA extracts using centrifugal filter devices. *Investigative Genetics,* 4(8), pp. 1-8.
Parker, A. M. et al., 2018. A review of *mycoplasma* diagnostics in cattle. *Journal of Veterinary Internal Medicine,* 32(3), pp. 1241-1252.
Petersen, M. B., Krogh, K. & Nielsen, L. R., 2016. Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds. *Journal of Dairy Science,* 01 01, Volume 99, p. 3815–3823.
Petersen, M. B. et al., 2018. A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows. *Journal of Dairy Science,* 08, 101(8), pp. 7383-7396.
QIAGEN, 2017. *Buffer AE_00000068.* Manchester: QIAGEN.
QIAGEN, 2017. *Buffer AL_00000089.* Manchester: QIAGEN.
QIAGEN, 2017. *Buffer AW2_00000071.* Manchester: QIAGEN.
QIAGEN, 2019. *Buffer AW1_00000070.* Manchester: QIAGEN.
Sasaki, D. & Mitchell, R. A., 2001. *How to Obtain Reproducible Quantitative ELISA Results.* [Online] Available at: https://www.oxfordbiomed.com/sites/default/files/2017-02/How%20to%20Obtain%20Reproducible%20Quantitative%20ELISA%20results.pdf [Accessed 30 01 2020].
Statens Veterinärmedicinska Anstalt, 2020. *Prislista analyser och produkter från SVA.* [Online] Available at: https://www.sva.se/analyser-och-produkter/prislista-start/sok-i-prislistan?quicksearchquery=mycoplasma+bovis [Accessed 21 01 2020].
Thermo Fisher Scienific, 2012. *F-873 (Proteinase K).* Vantaa: Thermo Fisher Scienific.
Thermo Fisher Scientific, 2012. *F-871 (Lysis Solution 1).* Vantaa: Thermo Fisher Scienific.
Thermo Fisher Scientific, 2012. *F-872 (Lysis Solution 2).* Vantaa: Thermo Fisher Scienific.
Thermo Fisher Scientific, 2012. *F-882 (Master Mix).* Vantaa: Thermo Fisher Scienific.
Thermo Fisher Scientific, 2012. *F-928 (Primer Mix).* Vantaa: Thermo Fisher Scienific.
Thermo Fisher Scientific, 2016. *https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1503-PJ9169-CO019879-Re-brand-Real-Time-PCR-Understanding-Ct-Value-Americas-FHR.pdf.* [Online] Available at: Real-time PCR: understanding Ct [Accessed 29 01 2020].
Thermo Scientific, 2015. [Online] Available at: http://tools.thermofisher.com/content/sfs/manuals/D14909-02-

PathoProof%20Major-42%20IFU.pdf
[Accessed 15 06 2020].
Thermo Scientific, 2015. *Protocol Book Thermo Scientific PathoProof Complete-16 kit Instructions for Use.* [Online]
Available at: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMBD%2FInstructions%2FD14910-02-PathoProof%2520Complete-16%2520IFU.pdf&title=UGF0aG9wcm9vZiBDb21wbGV0ZS0xNiBLaXQgSUZV
[Accessed 21 01 2020].
Thermo Scientific, 2015. *Protocol Book Thermo Scientific PathoProof Major-4.2 kit Instructions for Use.* [Online]
Available at: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMBD%2FInstructions%2FD14909-02-PathoProof%2520Major-42%2520IFU.pdf&title=UGF0aG9Qcm9vZiBNYWpvci00LjIgS2l0IElGVQ==
[Accessed 28 01 2020].
Thermo Scientific, 2016. *PF0888A PathoProof Software Norden Lab Mastitis Studio.* [Online]
Available at: https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FMBD%2FInstructions%2FD14911-02-PathoProof%2520Norden%2520Lab%2520Studio%2520IFU.pdf&title=UGF0aFByb29mIE5vcmRlbiBMYWIgU3R1ZGlvIElG
[Accessed 23 01 2020].
Zhao, G. et al., 2018. Development of a recombinase polymerase amplification combined with a lateral flow dipstick assay for rapid detection of the *Mycoplasma bovis*. *BMC Veterinary Research,* 14(1), pp. 1-10.

Appendix A – Protocols

A 1. Interpretation guide for the qPCR (Provided from Thermo Fisher Scientific)

ThermoFisher SCIENTIFIC

The world leader in serving science

PathoProof Software v1.8.4
Approximate Ct ranges for each bacterial target and for each quantity class

Target	Ct-value range for +++	Ct-value range for ++	Ct-value range for +	Ct-value range for +/-	Negative result
Beta-lactamase gene	< 25	25 - 30	30.1 - 36	36.1 - 36.5	36.6 - 40
C. bovis	< 22	22 - 28	28.1 - 37	37.1 - 40	No Ct
E. coli	< 24	24 - 34	34.1 - 37	37.1 - 40	No Ct
Enterococcus spp.	< 24	24 - 28	28.1 - 37	37.1 - 40	No Ct
Klebsiella spp.	< 24	24 - 32	32.1 - 37	37.1 - 40	No Ct
M. alkalescens	< 21	21 - 32	32.1 - 37	37.1 - 40	No Ct
M. bovigenitalium	< 23	23 - 34	34.1 - 37	37.1 - 40	No Ct
M. bovis	< 22	22 - 32	32.1 - 37	37.1 - 40	No Ct
M. californicum	< 24	24 - 34	34.1 - 37	37.1 - 40	No Ct
M. canadense	< 23	23 - 33	33.1 - 37	37.1 - 40	No Ct
Mycoplasma spp.	< 19	19 - 29	29.1 - 37	37.1 - 40	No Ct
Prototheca spp.	< 21	21 - 31	31.1 - 37	37.1 - 40	No Ct
Staph. aureus	< 24	24 - 30	30.1 - 37	37.1 - 40	No Ct
Staph. spp.	< 24	24 - 31	31.1 - 34	34.1 - 36	36.1 - 40
Str. agalactiae	< 24	24 - 32	32.1 - 37	37.1 - 40	No Ct
Str. dysgalactiae	< 22	22 - 30	30.1 - 37	37.1 - 40	No Ct
Str. uberis	< 17	17 - 28	28.1 - 37	37.1 - 40	No Ct
S. marcescens	< 23	23 - 33	33.1 - 37	37.1 - 40	No Ct
T. pyogenes and P. indolicus	< 24	24 - 30	30.1 - 37	37.1 - 40	No Ct
Yeast	< 15	15 - 25	25.1 - 37	37.1 - 40	No Ct

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A 2. Required Ct-values for the positive extraction control

The positive extraction control ($PC_{extraction}$) consist of saved milk from infected cows, since *M. bovis* is still a rare pathogen on Swedish farms it is not included in the $PC_{extraction}$, see Table A - 1.

Table A - 1. The table states the ranges within which the cycle threshold (Ct) values need to be for the positive extraction control for the extraction to be considered successful.

Species:	**Ct-value:**
M. bovis	–
S. agalactie	30 – 40
S. uberis	29 – 34
S. auereus	30 – 39

A 3. Protocol ELISA (Provided from ID.vet)

General Information

This diagnostic kit is designed to detect antibodies against *Mycoplasma bovis*.

It can be used with bovine serum, plasma or milk (individual, pooled, or bulk milk samples).

Description and Principle

Wells are coated with *M. bovis* recombinant antigen.

Samples to be tested and controls are added to the microwells. Anti-*M. bovis* antibodies, if present, form an antigen- antibody complex.

An anti-ruminant horseradish peroxidase (HRP) conjugate is added to the microwells. It fixes to the anti-*M. bovis* antibodies, forming an antigen-antibody-conjugate-HRP complex.

After washing in order to eliminate the excess conjugate, the Substrate Solution (TMB) is added.

The resulting coloration depends on the quantity of specific antibodies present in the specimen to be tested:

- in the presence of antibodies, a blue solution appears which becomes yellow after addition of the Stop Solution.
- in the absence of antibodies, no coloration appears.

The microplate is read at 450 nm.

Kit Components

Reagents*
Microplates coated with *M. bovis* recombinant antigen
Concentrated Conjugate (10X)
Positive Control
Negative Control
Dilution Buffer 13
Dilution Buffer 3
Wash Concentrate (20X)
Substrate Solution
Stop Solution (0.5 M)

* *Quantities supplied are indicated on the kit label.*

1. The conjugate, controls and substrate solution must be stored at 5°C (± 3°C).
2. The other reagents can be stored between +2°C and +26°C.
3. Wash, substrate and stop solutions can be used for the entire IDvet product range. Dilution buffers with same batch numbers are interchangeable.

Materials required but not provided

1. Mono or multi-channel pipettes capable of delivering volumes of 5 µl, 50µl, 100 µl, 200µl and 500 µl.
2. Disposable tips.
3. Distilled or deionized water.
4. Manual or automatic wash system.
5. 96-well microplate reader.

Precautions

1. Do not pipette by mouth.
2. Contains components that can be harmful to the skin and eyes and may cause sensitisation by skin contact. Avoid contact with skin and eyes. Use protective lab coat, one-way gloves and safety glasses. The stop solution (0.5 M acid) may be harmful if swallowed.
3. Do not expose the substrate solution to bright light nor to oxidizing agents.
4. All waste should be properly decontaminated prior to disposal. Dispose in accordance with local regulations.
5. Please refer to the Material Safety Data Sheet, available upon request at info@id-vet.com , for more detailed information.

Sample Preparation

All samples types

In order to avoid differences in incubation times between specimens, it is possible to prepare a 96-well plate containing the test and control specimens, before transferring them into an ELISA microplate using a multichannel pipette.

Milk samples

This test can be performed on skimmed or whole milk samples, with or without preservatives.
When analysing whole milk samples, special washing precautions should be taken (please refer to "Recommendations for milk testing" available upon request).
Centrifuge each whole milk sample, or just let the samples sit, so that the cream separates from the lactoserum (cream on the top, lactoserum on the bottom): Pipette under the cream so that only the lactoserum enters the cone (antibodies are found in the lactoserum).

Wash Solution Preparation

If necessary, bring the Wash Concentrate **(20X)** to room temperature and mix thoroughly to ensure that the Wash Concentrate is completely solubilized.

Prepare the Wash Solution **(1X)** by diluting the Wash Concentrate **(20X)** in distilled/deionized water.

The quality of the wash step may influence results. Ensure that wells are completely empty between washes. If using an automatic washer, it is extremely important to correctly parameter the machine (mode, type of aspiration, aspiration height). For more information, please consult the "IDvet Washing Guide", available upon request.

Testing Procedure

Allow the reagents to come to room temperature (21°C ± 5°C) before use. Homogenize all reagents by inversion or vortexing.

It is possible to test in the same run individual serum/plasma and milk samples using the **short incubation protocol.**

However, to increase sensitivity, IDvet recommends using the **overnight incubation protocol** dedicated to milk samples described hereafter. The milk protocol allows for an excellent correlation between paired serum/milk samples.

■ Short incubation protocol (for serum, plasma, and milk samples in the same run):

1. Add controls:
   - 195 µl of **Dilution Buffer 13** to wells A1, B1, C1, D1 (for controls).
   - 5 µl of the **Negative Control** to wells A1 and B1.
   - 5 µl of the **Positive Control** to wells C1 and D1.

a) For serum or plasma samples add:
   - 195 µl of **Dilution Buffer 13** to each microwell for serum or plasma testing
   - 5 µl of each serum/plasma sample to be tested.

b) For milk (individual, pooled, or bulk milk) samples: *Refer to section "Milk sample preparation".* Add:
   - 50 µL of **Dilution buffer 13** to each microwell for milk testing
   - 100 µL of milk samples to be tested.

2. Cover the plate and incubate **45 min ± 4 min** at 21°C (± 5°C).

■ Overnight incubation protocol for milk only (individual, pooled or bulk milk samples)

1. Samples are added to the ELISA microplate undiluted and controls at a final dilution of 1:101 as follows:

a) Pre-dilute the **Negative Control** and the **Positive Control** to 1:101 in **Dilution Buffer 13** to generate the **NC milk** and **PC milk** controls using for each control one microtube with 1000µl of buffer and 10µl of control.

b) In the ELISA microplate, add:
   - 50 µl of **Dilution Buffer 13** to each microwell (for controls and samples)
   - 100 µl of the **NC milk** prepared as described hereabove to wells A1 and B1.
   - 100 µl of the **PC milk** prepared as described here above to wells C1 and D1.
   - 100 µl of each milk sample to be tested to the remaining wells.

2. Cover the plate and incubate **overnight (16-20 hours)** at 21°C (± 5°C).

■ For all protocols

3. Empty the wells. Wash each well at least* 3 times with at least 300 µl of the **Wash Solution**. Avoid drying of the wells between washes.

   Note: If testing milk samples, be careful that there is no fatty ring left in the well after washing. To avoid fat residues, it is possible to include a soaking time of 2–5 minutes between washes. Additional washing steps (up to 6) can also be added. These additional washes can also be done when testing serum/plasma samples together with milk samples without affecting test performance on these sample types.

4. Prepare the **Conjugate 1X** by diluting the **Concentrated Conjugate 10X** to 1:10 in **Dilution Buffer 3.**
5. Add 100 µl of the **Conjugate 1X** to each well.
6. Cover the plate and incubate **30 min ± 3 min** at 21°C (± 5°C).
7. Empty the wells. Wash each well 3 times with at least 300 µl of the **Wash Solution**. Avoid drying of the wells between washes.
8. Add 100 µl of the **Substrate Solution** to each well.
9. Cover the plate and incubate **15 min ± 2 min** at 21°C (± 5°C) in the dark.
10. Add 100 µl of the **Stop Solution** to each well, in the same order as in step No. 8, to stop the reaction.
11. Read and record the O.D. at 450 nm.

Validation

The test is validated if:

✓ the mean value of the Positive Control O.D. (OD_{PC}) is greater than 0.350.

$$OD_{PC} > 0.350$$

✓ the ratio of the mean values of the Positive Control O.D. (OD_{PC}) to the Negative Control O.D. (OD_{NC}) is greater than 3.

$$OD_{PC}/OD_{NC} > 3$$

Interpretation

■ Short incubation protocol

For each sample, calculate the S/P percentage (S/P %):

$$S/P\ \% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$$

Result	Status
SERUM or PLASMA	
S/P % < 60%	NEGATIVE
S/P % ≥ 60%	POSITIVE
INDIVIDUAL, POOLED or BULK MILK	
S/P % < 20%	NEGATIVE
S/P % ≥ 20%	POSITIVE

If requested, positive results can be classified as follows:

Result		Status
SERUM or PLASMA	***MILK***	
60%≤S/P %<80%	*20%≤S/P %<40%*	+
80%≤S/P %<110%	*40%≤S/P %<60%*	++
110%≤S/P %<140%	*60%≤S/P %<80%*	+++
S/P % ≥ 140 %	*S/P % ≥ 80 %*	++++

■ Overnight incubation protocol (milk only)

For each milk sample, calculate the S/P percentage (S/P_{milk} %)

$$S/P\ milk\ \% = \frac{OD_{sample} - OD_{NCmilk}}{OD_{PC\ milk} - OD_{NCmilk}} \times 100$$

INDIVIDUAL, POOLED or BULK MILK	
Result	Status
S/P_{milk} % < 30%	NEGATIVE
S/P_{milk} % ≥ 30%	POSITIVE

If requested, positive results can be classified as follows:

Result	*Status*
30%≤S/P %<50%	+
50%≤S/P %<100%	++
100%≤S/P %<150%	+++
S/P % ≥ 150 %	++++

Note: The IDSoft™ data analysis program is available free-of-charge. Please contact support.software@id-vet.com for more information. This software program can calculate many parameters (validation criteria, S/P or S/N values, titers, vaccination age, groups) and offers a graphic representation of the serological profiles of the animals tested).

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Appendix B – Raw data qPCR

B 1. Raw data for the Confirmation of positive samples by qPCR

Table B - 1. The measured cycle threshold (Ct) values are displayed in the table for the two positive samples.

Sample:	**Ct-value:**
A	32.0
	32.2
B	28.8
	26.0

B 2. Raw data for the determination of the LOD and repeatability by qPCR

Table B - 2. The measured cycle threshold (Ct) values for the positive controls (PC) and the negative controls (NC). The positive extraction control was made up of milk, and the Ct-values for the other species were in the correct ranges. Samples labeled with $_{IAC}$ were reported as Internal Amplification Control (IAC) failure.

Sample:	**Ct-value:**
$NC_{extraction}$	-
$PC_{extraction}$	- (expected for *M.bovis*)
NC_{PCR}	35.6
PC_{PCR}	21.5_{IAC}

Table B - 3. The measured cycle threshold (Ct) values for the dilutions of Sample A. False results are labeled with an *, and samples marked with IAC were reported as Internal Amplification Control (IAC) failure.

		Dilutions Sample A:					
	Dilution series:	**x1**	**x10**	**x20**	**x40**	**x100**	**x1000**
Ct-value:	α	32.7	-*	-*	-	-	-
		31.9	-*	-*	-	-	-IAC
		31.6	-*	-*	-	-	- IAC
	β	32.2	36.7	-*	38.2	39.3	-
		32.5	38.2*	-*	-	-	- IAC
		32.2	-*	-*	-	-	-
		32.8	-*			-	-

*Table B - 4. The samples that were stated to have Internal Amplification Control (IAC) failure in Table B - 3 were re-tested the following day. The eluates from the extraction had been frozen in -20°C overnight. The measured cycle threshold (Ct) values for the dilutions of these samples are displayed in the table. The controls run in the same run as these samples are displayed in **Fel! Hittar inte referenskälla.**. The remaining samples the had IAC failure were re-tested as part of the intermediate precision run. False results are labeled with *, and samples marked with $_{IAC}$ were reported as Internal Amplification Control (IAC) failure.*

	Sample A:	**Dilutions Sample B:**		
	x1000	**x600**	**x1200**	**x12000**
Dilution series:	α	β	β	α
Ct-value:	-*	-* IAC	- IAC	-
	-*	-* IAC	- IAC	34.4
	-* IAC	-* IAC	- IAC	37.1

B 3. Raw data for the test of intermediate precision by qPCR

Table B - 5. The measured cycle threshold (Ct) values for the positive controls (PC) and the negative controls (NC). The $PC_{extraction}$ was made up of milk, and the Ct-values for the other species were in the correct ranges, see Appendix A 2. The positive extraction control ($PC_{extraction}$) consists of milk samples from infected cows, since M. bovis *is a rare pathogen on Swedish farms it is not included in the $PC_{extraction}$.*

Sample:	**Ct-value:**
$NC_{extraction}$	-
$PC_{extraction}$	- (expected for *M.bovis*)
NC_{PCR}	35.6
PC_{PCR}	20.5

*Table B - 6. The measured cycle threshold (Ct) values for the A and B samples. False results are labeled with *, and samples marked with $_{IAC}$ were reported as Internal Amplification Control (IAC) failure.*

		Sample A:				**Sample B:**			
	Dilution series:	**x1**	**x10**	**x100**	**x1000**	**x1**	**x100**	**x2400**	**x12000**
Ct-value:	β	32.9	37.8*	-	-	26.7	33.6	37.1	-
		33.3	37.0	-	-	27.1	33.6	37.0*	-
		33.1	36.5	-	-	27.6	33.2	36.8*	-

B 4. Raw data for the test of selectivity and matrix effects and PCR inhibition by qPCR

Table B - 7. The measured cycle threshold (Ct) values are presented in the table for the Sample C, Sample D, Sample E, Sample F, and water. Sample C and D are M. spp. qPCR *positive samples, and Sample E and F are qPCR negative samples when the Major-4.2 Kit was used. PC has been added in the stated dilutions. The controls that were run together with these samples are found in* ***Fel! Hittar inte referenskälla.****. False results are labeled with* **, and samples marked with* $_{IAC}$ *were reported as Internal Amplification Control (IAC) failure.*

Sample:	**PC dilution:**	**Ct-value:**
C		-
		-
		-
D		-
		-$_{IAC}$
		-
C	x10	24.1
		24.1
		23.7
D	x10	23.9
		-*
		25.2 $_{IAC}$
C	x2	22.1
		22.1
		21.8
D	x2	22.1
		21.5
		21.6
H_2O	x10	24.5
		24.7
		23.7
E	x10	24.7
		-* $_{IAC}$
		24.7
F	x10	24.5 $_{IAC}$
		24.9 $_{IAC}$
		-*
H_2O	x2	23.1 $_{IAC}$
		21.8
		22.4
E	x2	21.0
		21.7
		21.5
F	x2	21.5
		21.5
		21.6

Appendix C – Raw data ELISA

C 1. Raw data for the Confirmation of positive samples by ELISA

Table C - 1. The measured OD-values at 450 nm, as well as the calculated S/P% are presented in the table for the Negative Control (NC), the Positive Control (PC), Sample A and B.

Sample:	**OD 450:**	**S/P%:**
NC	0.043	-0.122
	0.046	0.122
PC	1.176	91.917
	1.375	108.083
A	1.066	82.981
	1.067	83.063
B	0.374	26.767
	0.384	27.579

C 2. Raw data for the determination of the LOD and repeatability by ELISA

Four replicates instead of three were used for four dilutions for Sample A and B, respectively, to test the repeatability.

Table C - 2. The measured OD-values at 450 nm, as well as the calculated S/P% are presented in the table for the Negative Control (NC) and the Positive Control (PC).

Sample:	**OD 450:**	**S/P%:**
NC	0.043	-0.292
	0.051	0.292
PC	1.338	94.337
	1.493	105.663

Table C - 3. The measured OD at 450 nm for the dilutions of Sample A.

		Dilutions Sample A:					
	Dilution series:	**x1**	**x2**	**x3**	**x4**	**x6**	**x8**
OD 450:	α	0.803	0.503	0.416	0.309	0.231	0.182
		0.927	0.547	0.379	0.301	0.231	0.214
		0.923	0.578	0.396	0.305	0.328	0.197
	β	0.928	0.569	0.378	0.316	0.227	0.183
		0.981	0.541	0.38	0.331	0.238	0.192
		1.027	0.526	0.381	0.304	0.256	0.266
		1.053	0.545			0.227	0.187

*Table C - 4. The calculated S/P% for the dilutions of Sample A. False results are labeled with *.*

		Dilutions Sample A:					
	Dilution series:	**x1**	**x2**	**x3**	**x4**	**x6**	**x8**
S/P%:	α	55.243	33.321	26.964	19.145	13.445	9.865
		64.304	36.536	24.26	18.56	13.445	12.203
		64.012	38.802	25.502	18.853	20.533*	10.961
	β	64.377	38.144	24.187	19.657	13.153	9.938
		68.25	36.098	24.333	20.753*	13.957	10.596
		71.611	35.002	24.406	18.78	15.272	16.003
		73.511	36.39			13.153	10.23

Table C - 5. The measured OD at 450 nm for the dilutions of Sample A.

		Dilutions Sample B:					
	Dilution series:	**x1**	**x1.2**	**x1.3**	**x1.4**	**x1.6**	**x1.8**
OD 450:	α	0.266	0.263	0.266	0.232	0.213	0.205
		0.28	0.277	0.251	0.239	0.226	0.195
		0.286	0.297	0.243	0.243	0.226	0.198
	β	0.242	0.258	0.218	0.218	0.205	0.165
		0.276	0.238	0.224	0.222	0.188	0.176
		0.279	0.254	0.228	0.212	0.201	0.183
		0.286	0.239			0.186	0.206

*Table C - 6. The calculated S/P% for the dilutions of Sample A. False results are labeled with *.*

		Dilutions Sample B:					
	Dilution series:	**x1**	**x1.2**	**x1.3**	**x1.4**	**x1.6**	**x1.8**
S/P%:	α	16.003*	15.784*	16.003*	13.518	12.13	11.545
		17.026*	16.807*	14.907*	14.03	13.08	10.815
		17.464*	18.268*	14.322*	14.322	13.08	11.034
	β	14.249*	15.418*	11.911*	12.495	11.545	8.623
		16.734*	13.957*	12.934*	12.788	10.303	9.426
		16.953*	15.126*	13.226*	12.057	11.253	9.938
		17.464*	14.03*			10.157	11.619

Table C - 7. Raw data for the additional measurement of the x1 and x1.2 B samples, including the measured OD at 450 nm and the calculated S/P%.

Sample:	Dilution:	OD 450:	S/P%:
NC		0.036	-0.072
		0.038	0.072
PC		1.477	104.046
		1.365	95.954
Sample B	x1	0.315	20.087
		0.35	22.616
		0.355	22.977
	x1.2	0.259	16.04
		0.282	17.702
		0.263	16.329

C 3. Raw data for the test of intermediate precision by ELISA

Table C - 8. The measured OD-values at 450 nm, as well as the calculated S/P% are presented in the table for the Negative Control (NC) and the Positive Control (PC).

Sample:	OD 450:	S/P%:
NC	0.046	-0.26
	0.052	0.26
PC	1.258	104.585
	1.152	95.415

Table C - 9. The measured OD at 450 nm for the dilutions of Sample A and B.

		Sample A:				Sample B:			
	Dilution series:	x1	x2	x6	x8	x1	x1.2	x1.6	x1.8
OD 450:	β	1.01	0.526	0.161	0.118	0.312	0.254	0.164	0.153
		1.018	0.495	0.155	0.162	0.318	0.26	0.186	0.148
		1.022	0.465	0.183	0.163	0.295	0.26	0.163	0.151

*Table C - 10. The calculated S/P% for the dilutions of Sample A and B. False results are labeled with *. The values used for the calculations are found in Table C - 8.*

		Sample A:				Sample B:			
	Dilution series:	x1	x2	x6	x8	x1	x1.2	x1.6	x1.8
S/P%:	β	83.131	41.263	9.689	5.969	22.751	17.734*	9.948	8.997
		83.824	38.581	9.17	9.775	23.27	18.253*	11.851	8.564
		84.17	35.986	11.592	9.862	21.28	18.253*	9.862	8.824

C 4. Raw data for the test of selectivity and matrix effects and PCR inhibition by ELISA

Table C - 11. The measured OD at 450 nm, as well as the calculated S/P% are presented in the table for the Negative Control (NC), the Positive Control (PC), Sample C, Sample D, Sample E, Sample F, and water. Sample C and D are M. spp. qPCR *positive samples, and Sample E and F are qPCR negative samples when the Major-4.2 Kit was used. PC has been added in the stated dilutions.*

Sample:	**PC dilution:**	**OD 450:**	**S/P%:**
NC		0.037	0
		0.037	0
PC	x40	1.477	96.807
		1.572	103.193
C		0.075	2.555
		0.075	2.555
		0.072	2.353
D		0.109	4.84
		0.113	5.109
		0.117	5.378
C	x150	0.557	34.958
		0.573	36.034
		0.615	38.857
D	x150	0.71	45.244
		0.644	40.807
		0.645	40.874
C	x50	1.303	85.109
		1.265	82.555
		1.169	76.101
D	x50	1.186	77.244
		1.146	74.555
		1.142	74.286
H_2O	x150	0.148	7.462
		0.232	13.109
		0.25	14.319
E	x150	0.556	34.891
		0.546	34.218
		0.539	33.748
F	x150	1.092	70.924
		1.034	67.025
		1.123	73.008
H_2O	x50	0.434	26.689
		0.458	28.303
		0.5	31.126
E	x50	1.098	71.328
		1.069	69.378
		1.215	79.193
F	x50	1.462	95.798
		1.423	93.176
		1.379	90.218

Table C - 12. Due to the poor results using water as the dilution background for the samples "without matrix" Buffer 13 was used instead in an additional run. Due to a limited number of available wells only one each of the controls were run. PC stands for Positive Control and NC stands for Negative Control.

Sample:	**PC dilution:**	**OD 450:**	**S/P%:**
NC		0.043	0
PC	x40	1.423	100
Buffer 13	x150	0.467	30.725
		0.418	27.174
		0.427	27.826
Buffer 13	x50	0.882	60.797
		0.814	55.87
		0.883	60.87