NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Cellular and Molecular Biology

New Veterinary *in vitro* Diagnosis (IVD) using the Doctor Vida® Platform

MASTER IN BIOCHEMISTRY

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ABSTRACT

Point-of-Care Testing (POCT) is characterized as the diagnosis near the specimen. POCT devices in veterinary practice are getting more attention due to the increasing spread of zoonotic diseases, such as COVID-19. POCT based on isothermal Nucleic Acid Amplification Technologies (NAATs) are gaining popularity for consuming fewer resources and time compared to PCR. Doctor Vida® Pocket (DV Pocket) is a POCT device developed by STAB VIDA that allows to perform LAMP in real-time for diagnosis of COVID-19.

Herein we describe the development of two new *in vitro* veterinary POCT using the DV Pocket platform: Swine Dysentery (SD) and Bird sexing.

SD is a mucohaemorrhagic disease caused by *B. hyodysenteriae* affecting swine. The spread of SD through faeces and antibiotic resistance often leads to financial loss, thus the importance of developing a POCT. A LAMP-based assay targeting the *nox* gene from *B. hyodysenteriae* and *E. coli* as the sample control was developed, as well as two different stool collection kits. Ultimately, the assay presented 78.9% sensitivity and 45.5% specificity using kit 1 and a 68.4% sensitivity and 72.7% specificity for kit 2 allowing to detect *B. hyodysenteriae* within 40-minutes with only 2 minutes hands-on.

Bird sex determination is essential for conservation and evolutionism studies. STAB VIDA provides a Bird Sexing Service, so this project aimed to facilitate the process for its clients. It was attempted for birds from the Psittacidae family, *Columba livia* and finally *Psittacula krameri*. The latter had the best results, with a sensitivity of 80%, within 90 minutes and 30 minutes of sample incubation.

Optimizations on both assays are still required before progressing to clinical validation, ensuring higher sensitivity and specificity.

Keywords: Point-of-Care, Diagnosis, Isothermal Amplification, LAMP, Swine Dysentery, *B. hyodysenteriae*, Bird Sexing, Doctor Vida Pocket

RESUMO

Testes *"point-of-care"* (POCT) são caracterizados como o diagnóstico junto dos espécimes. Este tipo de diagnóstico tem adquirido cada vez mais atenção do ramo veterinário devido ao aumento de zoonoses, tal como a COVID-19. POCT baseados em tecnologias de amplificação de ácidos nucleicos isotérmicas têm ganho popularidade por consumirem menos recursos que a PCR. O Doctor Vida® Pocket (DV Pocket) é um dispositivo POCT desenvolvido pela STAB VIDA que faz uso de LAMP em tempo real para o diagnóstico de COVID-19.

Neste estudo desenvolveram-se 2 novos POCT, utilizando a plataforma DV Pocket, para diagnóstico da Disenteria Suína (DS) e Sexagem de Aves.

DS é uma doença muco-hemorrágica causada por *B. hyodysenteriae,* afetando suiniculturas. Devido à rápida infeção por fezes e resistência a antibióticos pode causar grandes perdas financeiras, daí a importância de um POCT para o seu diagnóstico. Um teste baseado em LAMP, usando o gene *nox* de *B. hyodysenteriae* como marcador e *E. coli* como controlo, bem como 2 kits de colheita de fezes, foram desenvolvidos. Este apresentou uma sensibilidade de 78.9% e especificidade de 45.5% para o kit 1, 68.4% e 72.7% para o kit 2, sendo detetado dentro de 40 minutos, com apenas 2 minutos de *hands-on.*

Sexagem de aves é essencial para estudos de conservação e evolução. A STAB VIDA possui serviços de sexagem de aves, desta forma este projeto visa facilitar o processo para os clientes. Testou-se em aves da família Psittacidae, *Columba livia* e *Psittacula krameri*. Neste último obtiveram-se os melhores resultados, com uma sensibilidade de 80%, dentro de 90 minutos de teste, com 30 minutos para incubação da amostra.

No entanto, otimizações são ainda necessárias para ambos os testes de modo a que possam prosseguir para validação clínica, e assegurando uma maior sensibilidade e especificidade.

Palavras chave: *Point-of-Care*, Diagnóstico, Amplificação Isotérmica, LAMP, Disenteria Suína, *B. hyodysenteriae*, Sexagem de pássaros, Doctor Vida Pocket

CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Cycle Threshold Number of cycles it takes the method to detect the target DNA.

Time-to-Result Time after which fluorescence exceeds a certain threshold, corresponding to the detection of target DNA.

ACRONYMS

B3	Loop-mediated Isothermal Amplification backward outer primer
BIP	Loop-mediated Isothermal Amplification backward inner primer
CHD	Chromo-Helicase Domain
COI	Cytochrome c Oxidase Subunit I
Ct	Cycle threshold
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-Linked Immunosorbent Assay
F3	Loop-mediated Isothermal Amplification forward outer primer
FIP	Loop-mediated Isothermal Amplification forward inner primer
HNB	Hydroxy Naphthol Blue Disodium Salt
HRM	High-resolution Melt analysis
LAMP	Loop-mediated Isothermal Amplification
LFA	Lateral Flow Assay
NAATs	Nucleic Acids Amplification Technologies
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
NTC	Non-template Control
PCR	Polymerase Chain Reaction
POCT	Point-of-Care Testing
qPCR	Quantitative PCR
RC	Reaction Condition
rRNA	Ribossomal RNA
RT-LAMP	Reverse Transcriptase Loop-Mediated Isothermal Amplification

SD	Swine Dysentery
SDS	Sodium Dodecyl Sulphate
TEC-LAMP	Tth Endonuclease Cleavage Loop-Mediated Isothermal Amplification
TtP	Time-to-Positive

1

INTRODUCTION

1.1 Point-of-Care Testing (POCT)

The term *Point-of-Care testing* (POCT) appeared in the 80s with the development of a biosensor for monitorization of the ionized calcium levels on whole blood of specimens on the operation room, when going through hepatic transplant[2]. Nowadays, POCT is characterized as the diagnosis near the specimens, decentralized from traditional laboratories and were firstly developed to decrease the therapeutic turn-around time in cases where this window is relatively small, aiding on a quicker therapeutic response from physicians [2,3].

POCT platforms are being currently used in hospitals, clinics, emergency rooms, ambulatory services and at home by the specimens. These platforms are an important component on chronic disease monitoring, such as diabetes mellitus, eliminating the need of translocation to hospitals or laboratories[4]. Some devices use smartphones sending the information directly to the physicians, which makes it more accessible for the specimen and the healthcare professionals[5]. Due to this growing necessity and investigation of alternatives to central laboratory testing, the POCT market is predicted to grow from 43.2 billion US dollars to 72 billion US dollars in 2027[6].

The main advantages of the use of POCT platforms are the decentralization of diagnosis from central laboratories to specimens and healthcare sites, the accessibility to the healthcare professionals, the decrease of the result-to-response window and the possibility of testing in sites with lack of resources thanks to the relatively low cost of the equipment, compared to the ones used in central laboratories. However, POCT platforms still face some limitations. Some of the techniques used in POCT lack the sensitivity and precision of results from central laboratories; the production cost since some of the materials used are high priced; errors during the sample collection or testing by the specimen or unqualified personnel performing the test; the results and specimen data management and coordination with laboratory databases and the specimen medical records still need improvements [3,4,7–9].

The mostly used techniques for POCT devices are Lateral Flow Assays/Immunoassays (LFA), such as at-home Pregnancy Tests, and Nucleic Acid Amplification Techniques (NAATs) such as qPCR and isothermal methods. LFA tests have a lower production cost and great reproducibility, but sometimes fail in terms of sensitivity and are only qualitative or semi-quantitative[7]. POCT tests based on

NAATs gained popularity during the COVID-19 pandemic for being more sensitive and specific, similarly to conventional PCR, and thanks to microfluidic techniques the costs of production are reduced due to the smaller volume of reagents needed. Nevertheless, POCT devices based on NAATs have some unwanted implications if using conventional PCR because of the need for special equipment capable of thermal cycling. This problem can be solved with the use of isothermal NAATs, such as Loop-mediated Isothermal Amplification (LAMP), that are also less sensitive to PCR inhibitors hence the possibility of eliminating extra steps for DNA/RNA extraction [7].

Although the advantages of the use of these platforms are important and solve some of the problems of current and centralized diagnostic methods it has some setbacks that cannot be ignored. Therefore, guidelines and international standards to evaluate POCT devices as marketable diagnostic tools were developed. The World Health Organization defined the ASSURED guidelines that POCT devices should follow: Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment free and Deliverable to end-users[9]. The International Organization for Standardization also defined the ISO 22870:2016 to be used with ISO 151189, for accreditation of the POCT diagnostic device. These standards are used for the assessment of quality management and control of the device, minimizing the risks for the specimen[3,10]. For this, accreditation instruction, control and calibration protocols need to be created and evaluated stating the type of sample used, details of the collection device and procedure, the technique used for the diagnosis and inhibitors, the reference values to be compared with the results, instructions for quality control, calibration and controls[3].

1.1.1 Point-of-Care Testing in Veterinary Practices

In the veterinary practice the need for low-cost diagnostic methods and a decrease on turnaround time for results is also on demand[11]. Most of the reference diagnostic techniques delay the diagnosis and treatment application which in certain cases can be fatal. Currently, veterinarians offer some in-clinic analysis, which gradually decentralizes the process[12]. Besides, veterinary physicians are free to make choices based on the results from in-clinic testing without the need of confirmation from reference methods[12]. Therefore, the increase need and demand for POCT testing in the veterinary setting could help decrease the cost on veterinary care, cut down the use of more invasive techniques and animal handling, and taking accurate and reliable diagnostic methods to more remote areas[5,11].

Nowadays, POCT testing in veterinary practice is especially important due to the growing net of animal and animal products trade, which could increase the wide and rapid spread of zoonotic and exotic diseases to non-endemic areas[5,13]. Thus, the rapid and accurate diagnosis with POCT devices and the identification of the pathogen in the early stages of spreading will reduce the costs of healthcare, disease management and decrease the degeneration of certain communities, as was observed with the recent COVID-19 pandemic[5]. Although the regulation for veterinary POCT tests is not as strict as for human POCT tests, and in some countries non-existent, the ASSURED criteria from the World Health Organization still applies. The American Society for Veterinary Clinical Pathology settled quality

assurance protocols for veterinary POCT tests to assure the safety of the animals and the elaboration of control and calibration procedures[14].

The first portable testing device available for animals, besides the dipstick test for urine analysis, was developed to analyse blood cell differentials[14].

Dipsticks and strip tests are simple, rapid and low-cost. The results are compared to a colour-coded chart, with interval levels for the target, which makes it a semi-quantitative test. The most used dipstick tests on veterinary practice were actually developed for human urine analysis. For companion pets it is also used for urine analysis as a way to diagnose chronic kidney disease and management of diabetes[11,15].

POCT tests based on LFAs are fast and simple, and gradually more sensitive due to developments in conjugation methods[14]. Due to its low cost compared to other methods, and relatively high sensitivity LFA tests are the most used in veterinary practice. Many LFA POCT tests are available for the diagnosis of heartworm, Lyme disease, canine parvovirus, *Giardia*, *Ehrlichia*, *Anaplasma* and feline leukaemia/immunodeficiency virus. SNAP tests from IDEXX benefit from a new technology based on lateral flow assays. These test cassettes use bidirectional flow generating two binding moments of the sample to the test pad – increases sensitivity. The activation of the cassette prompts a wash step – increases specificity. After the washing there is an amplification step which helps to detect low quantities of the target – increases sensitivity. This format is available for the diseases mentioned above, and studies demonstrated that it is more sensitive and specific than the LFA tests from a direct competitor, Zoetis[16].

Figure 1.1: Generic SNAP rapid LFA test from IDEXX. From https://serviveportugal.com/servive_pt/index.php?id_category=104&controller=category (consulted at 2022.12.18).

POCT based on NAATs has been gaining popularity as mentioned in the previous section, especially its application on lab-on-chip platforms reducing the equipment and volume of reagents and sample needed[15]. To date there are some lab-on-chip based on NAATs being commercialized, such as Vivalytic (Bosch), and some studies applying this technique with isothermal amplification have showed great results. For example, Jung *et al* (2015) developed a lab-on-chip RT-LAMP for the detection of Influenza A strains in a polycarbonate disk. Using a heat plate and a miniaturized fluorescence detector

they were able to obtain results within 47 minutes with an LOD of 10 copies of viral RNA[17]. With advancements on molecular methods and on the microfluidics field as well, in He *et al* (2020) applied the CRISPR-Cas12a and CRISPR RNA into a microfluidic cartridge for the detection of the African Swine Fever virus. This device was able to detect up to 100 fM of the target DNA[18].

1.2 Isothermal Amplification of Nucleic Acids

Methods for the amplification of nucleic acids have been used for years for diagnostic purposes. PCR, described by Mullis *et al* (1986)[19], is the most used method for clinical practices mostly due to its high efficiency. However, not all laboratories or hospitals can afford it thanks to the need for specialized equipment such as thermocyclers. Besides that, samples analysed by this technique have to go through nucleic acid extraction and purification, due to a high sensitivity to reaction inhibitors present on clinical samples[20]. Owing to these steps, PCR can be a rather time-consuming method, and the test itself takes hours because of the temperature cycles needed for the DNA polymerization and annealing of primers.

Since time and accuracy are so important for administering the right treatment at the right time, non-PCR based methods have been developed, claimed to be more efficient, sensitive, specific, rapid and simple, as it requires fewer sample treatments before analysis[20]. Most of these methods are isothermal, the test proceeds at the same temperature conditions, eliminating the need for thermocyclers, which in itself is a great step on the application of NAATs on the field. Such methods are strong candidates for application on POCT devices, decentralizing the diagnosis from hospital environments and making it more accessible and convenient for the specimens[21].

To this day a lot of isothermal amplification methods have been developed and applied to the detection of pathogens and diagnoses such as Human Immunodeficiency Virus, *Leishmania*, *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, etc[21]. The most used methods are the following[22]:

- Nucleic Acid Sequence-Based Amplification: amplification of RNA by 3 enzymes (avian myeloblastosis virus (AMV) reverse transcriptase, RNase H and T7 RNA polymerase) and 2 primers, at usually 41ºC;
- Helicase-Dependent Amplification: method analogous to PCR, but uses a helicase to dissociate the double-stranded DNA and single-stranded DNA-binding proteins, at 37ºC or between 60 to 65ºC. Only needs 2 primers as well;
- Exponential Amplification Reaction: this technique makes use of a probe with 2 of the same sequence, complementary to the target one, that is connected by a nickase recognition site, at 37ºC or between 55-60ºC;
- Strand-Displacement Amplification: method based on a cyclic reaction involving polymerization, cleavage and displacement, with 2 or 4 complex primers, at 37ºC;
- Recombinase Polymerase Amplification: involves the formation of a complex between a recombinase and the forward and reverse primers, at temperatures between 22-45ºC;

- ❖ Rolling Circle Amplification: uses a circular DNA probe, at temperatures between 30-37ºC;
- ❖ Loop-mediated Isothermal Amplification: makes use of 4 to 6 specific primers, recognizing up to 8 regions of the target sequence, and is performed at temperatures between 60 to 65ºC.

These isothermal amplification methods have a clear advantage over PCR, the fact that they are performed at a constant temperature, eliminating the need for expensive equipment. However, due to the exponential amplification and low amplification temperatures, false positives and primer dimers are common but can be mitigated by new primer designs or additives[21].

1.2.1 LAMP: Loop-Mediated Isothermal Amplification

LAMP is, out of the isothermal amplification methods described previously, one of the most used compiling more than 3700 publications in the last 22 years, and most importantly in our recent lives a rapid and simple tool for the detection of SARS-CoV-2[21].

This method was first described by Notomi *et al* (2000) as an amplification technique with a set of four specific primers and a DNA polymerase with high strand displacement activity derived from *Bacillus stearothermophilus* DNA polymerase I, both contributing to the specificity, sensibility and rapidity of the detection[23]. LAMP is proven to be more sensitive than PCR, about 10 to 100-times, with a limit of detection of about 1 copy of the target DNA/mL and more tolerant to possible PCR inhibitors, being a great candidate for clinical samples or in-field applications[21].

In a more in-depth view of the technique, it uses 4 core primers that are essential for amplification[23]:

- ❖ F3 or Forward Outer Primer: hybridizes with the F3c region;
- ❖ B3 or Backward Outer Primer: hybridizes with the B3c region;
- ❖ FIP or Forward Inner Primer: hybridizes with both the F2c and F1 region in different phases of the process;
- ❖ BIP or Backward Inner Primer: hybridizes with both the B2c and B1 regions in different phases of the process.

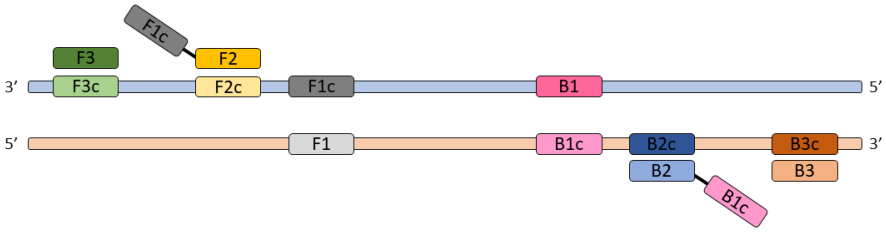

Figure 1.2: Representation of LAMP primers and the organization of its hybridization regions on the target sequence. The outer primers F3 and B3 are designed complementary to the F3c and B3c regions. The inner primers FIP and BIP include the F1 and F2, B2 and B1c regions, respectively. Designed on PowerPoint (Microsoft Office Professional Plus 2016).

The amplification process starts with the hybridization of the primer FIP with F2c and the synthesis of the complementary strand. Primer F3 then hybridizes with F3c, a step called strand invasion and

induces the synthesis of a new strand, which causes the displacement of the one generated by hybridization of FIP. The strand released serves now as a template for BIP-mediated complementary strand synthesis. Then, the hybridization of the B3 primer to B3c and synthesis causes the displacement of the strand generated by BIP. This last strand is the precursor for LAMP cycling. Due to the complementarity between the ends and inner regions of the sequence (F1c and B1), it forms a dumbbell or stem-loop like structure. With the formation of this structure, FIP binds to F2c, now located in the loop. The generation of the new strand complementary to the target causes strand displacement, which generates a stem-loop with 2 complementary copies of the target, and a loop on the opposite end of the original loop. The next loop amplification cycle generates a stem-loop complementary to the original. These structures serve then as templates for the last 2 steps of LAMP amplification: elongation and recycling.

LAMP amplification final products consist of stem-loop structures with stems of variable lengths, as well as structures with multiple loops.

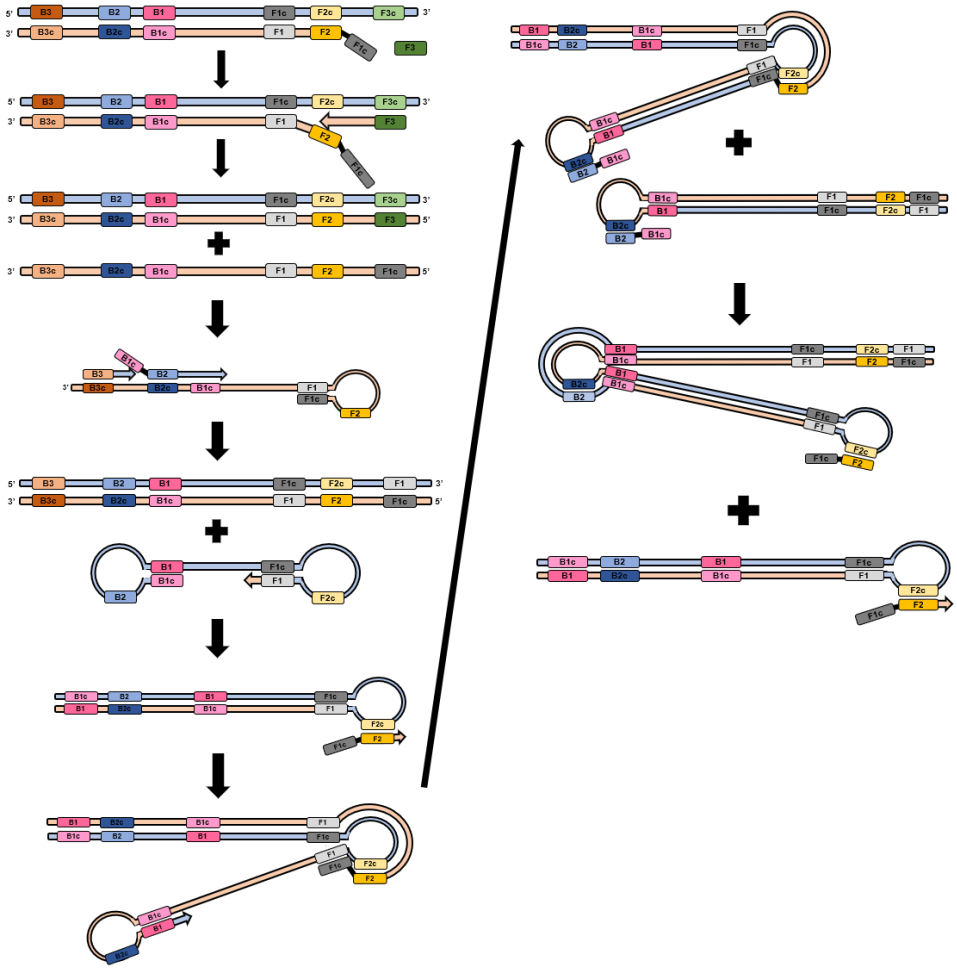

Figure 1.3: The first step of LAMP amplification is the FIP-mediated synthesis, then strand displacement by F3-mediated amplification. This process originates a strand with the F1c region at 5' end, which hybridizes with the F1 region creating a loop structure. BIP-mediated amplification of this sequence and further strand displacement by B3-mediated amplification generates a strand with F1c and B1c at the ends. F1c hybridizes again with F1 and B1c hybridizes with B1, generating a stem-loop like structure. This structure is the precursor to the cycling phase. This phase starts with FIP-mediated amplification and the formation of another loop, and so on, for the elongation and recycling phase. Designed on PowerPoint (Microsoft Office Professional Plus 2016).

1.2.2 Detection of LAMP Products

There are a variety of methods for the detection of LAMP amplicons, some more convenient than others. The most frequently used are turbidity, agarose gel electrophoresis, colorimetric detection under the naked eye or UV-light[24].

Turbidity consists on the detection of a white precipitate or changes in the opacity of the reaction mixture due to the formation of magnesium pyrophosphate ($Mg_2P_2O_7$), which is formed by the DNA polymerase activity[22,24–26]. These changes can be observed by the naked eye.

Figure 1.4: Endpoint detection of LAMP products by turbidity analysis. The tube on the left represents a negative result, and the tube on the right a positive. Adapted from Mori *et al* (2009)[27].

Agarose gel electrophoresis allows the visualization of LAMP amplification in the form of a ladder-like pattern since the amplification products generated by this method have various lengths[24,26]. However, due to the need to open the LAMP reaction tubes for loading the samples into the gel, it can increase the risk of crossover contamination between assays. As so, the space for reaction mixture preparation and agarose gel electrophoresis should be separate[24,25].

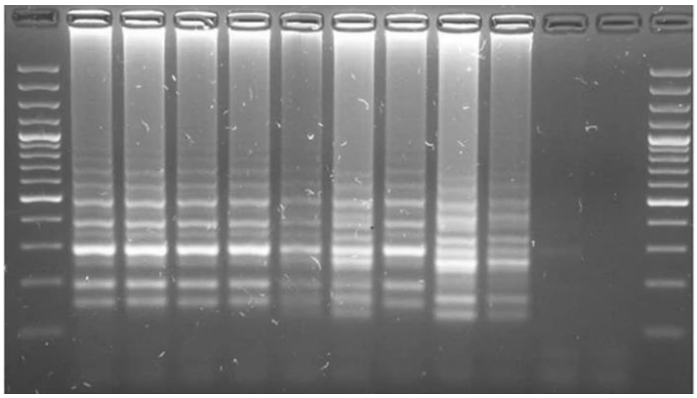

Figure 1.5: Agarose gel electrophoresis of LAMP products. Adapted from Mugambi *et al* (2015)[28].

Intercalating dyes are often used for colorimetric detection under the naked eye or under a UV-light. Calcein dye for example works in both ways[24,25].

- UV-light detection: calcein is a fluorophore with a high affinity for metal ions. When added to the reaction mixture, the manganese ion carried by calcein interacts with the pyrophosphate formed during amplification. The now free calcein interacts with the magnesium ions released, which

generates an increase in the fluorescence that can be visualized under a UV-light, at a wavelength of 365 nm;

Figure 1.6: Endpoint detection of LAMP products using intercalating dyes such as calcein, under the UV/light. The tube on the left represents a negative result and the tube on the right a positive. Adapted from Mori *et al* (2009)[27].

- Naked-eye visualization: the interaction between magnesium and calcein not only causes an increase in fluorescence but also noticeable colour changes on the reaction mixture, which changes from orange to green in case of amplification.

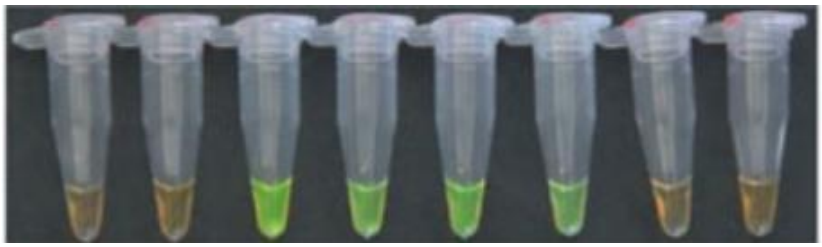

Figure 1.7: Colour change at the naked eye by the interaction of calcein with magnesium. Positive results present a green tone and negative results an orange tone. Adapted from Wong *et al* (2017) [24].

Adding intercalating dyes to the reaction such as SYBR green I and HNB is extremely advantageous since it gives the possibility for real-time fluorescence detection, aiding on the optimization of the assay as it gives information about the cycle threshold (Cq) for each sample and enables melt analysis of the LAMP products, which can be helpful to distinguish between specific and unspecific amplification, and on multiplex tests[24,25]. Besides those, pH-sensitive dyes are also an option due to the high generation of protons during amplification. LAMP amplification generates a decrease in pH equal to or higher than 2 units, so pH-sensitive dyes such as phenol red, cresol red or metacresol purple cause a strong colour change in positive samples that is visible by the naked eye[24,25].

Although the use of these dyes has numerous advantages, with or even without a melt analysis, sometimes it is still a bit complicated to discriminate a true positive from a false positive since the information it gives is the total DNA synthesis. For that, the use of a probe is advised, since it only gives a signal if connected to the specific target. For example, TEC-LAMP makes use of an endonuclease that cleaves abasic sites on double-stranded DNA and a probe. In this technology the probe consists on the abasic site flanked by a 5' fluorophore and a quencher, connected to the FIP primer. In short, if the TEC-LAMP modified FIP primer hybridizes with the specific target, the endonuclease cleaves the modified primer's abasic site, releasing the 5' fluorophore, which now free from the quencher produces an

increase in fluorescence[29]. This method was introduced by Higgins *et al* (2018), with a multiplex test for the detection of pathogens related to bacterial meningitis.

Some studies have also developed a lateral flow test consisting of a dipstick for the detection of LAMP products since it is low-cost, portable and simple[30].

1.2.3 Advantages and Limitations of LAMP

As mentioned before LAMP has a lot of advantages over PCR and other nucleic acid amplification methods. The greatest advantage considered is the rapidity of diagnosis, since a test usually lasts 30 to 40 minutes.

Sample preparation is also usually minimal or not required since this technique is less sensitive to inhibitors present in samples such as blood, urine and saliva than PCR. Given the amplification process, LAMP is able to amplify with high efficiency under isothermal conditions, which is a clear advantage over PCR since there is no need for expensive thermocyclers, and it can reach similar limits of detection[25,31]. The use of 4 to 6 highly specific primers makes a huge contribution to the reaction efficiency and nullifies the interference of non-target DNA/RNA if present in the reaction mixture[23].

Comparing LAMP to other isothermal nucleic acid amplification methods, such as Helicase-Dependent Amplification or Rolling Circle Amplification, requires fewer enzymes which is beneficial for the success and robustness of the reaction and does not need any pre-amplification reaction steps[31]. Besides direct visualization of the results is possible without agarose gel electrophoresis, with end-point detection methods as the use of fluorescent dyes that cause a change in the reaction mixture colour, and can be visualized by the naked eye or under a UV-light.

Although its advantages and developments, LAMP still has some limitations. For starters, primer design and target selection are complex since the primers need to recognize 6 to 8 different regions of the target, and those regions require to have a certain length. The target sequence together with the primer regions should not have more than 250/300 base pairs, since up that length the amplification is not as effective and does not yield as many target copies due to a low enzyme efficacy for longer strands[24,25].

The use of 4 to 6 primers contributes to the method's specificity. However, working with such a number of primers increases the possibility of primer-dimer formation, which in combination with the low temperatures of the assay causes most of the false-positives observed on isothermal amplification techniques[24,25]. That is why the use of a software for primer design such as PrimerExplorer V5 (https://primerexplorer.jp/e/) is so important since it gives information such as 5' and 3' ends stability, entropy energy and melting temperatures, parameters to keep in check while choosing and evaluating the primers on the possibility of primer-dimer formation and stability on its interaction with the target.

Carryover contamination is also very common on LAMP due to the amounts of copies generated and their high stability. That is why the use of an uracil DNA glycosylase and dUTPs or a separate place to analyse the results, in case of open-tube methods such as agarose gel electrophoresis, are important strategies to avoid such contaminations, which can also generate false-positive results[32].

Additionally, agarose gel electrophoresis is useful to confirm amplification as LAMP products leave a characteristic pattern on the gel, but since it is a ladder-like pattern it is not possible to identify if it is the desired one by band or target size[24].

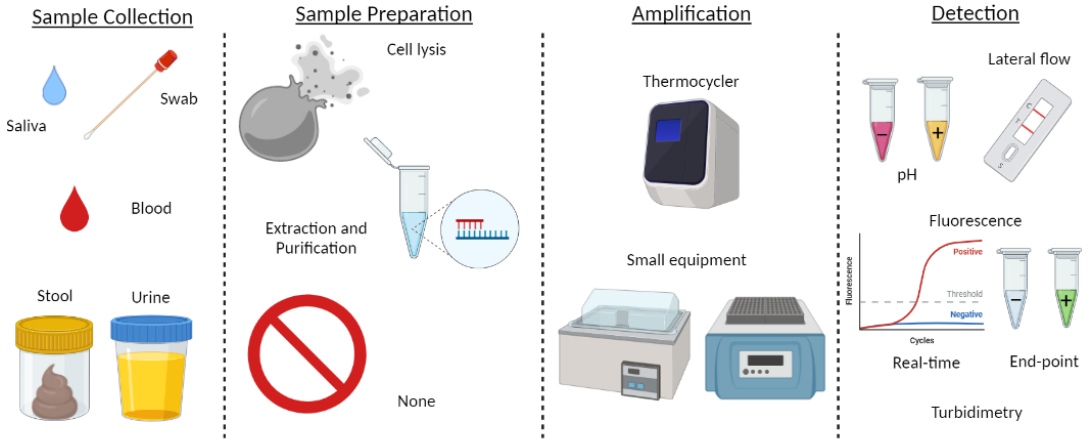

Figure 1.8: Graphic summary of the Loop-mediated Isothermal Amplification method, including samples collection, preparation, the possible equipment for the amplification process and methods of detection. Based on Moehling *et al* (2021)[25], designed on BioRender (https://biorender.com/).

1.2.4 Improvements and Future Perspectives

Over the years the LAMP method suffered optimizations and developments for the detection of various pathogens and diseases. For example, Reverse Transcription-LAMP (RT-LAMP) for the detection of RNA viruses such as SARS-CoV-2. Contrary to Reverse Transcription-PCR, RT-LAMP is a one-step method since the Bst 2.0 DNA polymerase was shown to have a high reverse transcription activity. However, it is advised to use a reverse transcriptase since it will still be a one-step reaction, but in this case with a higher yield[24].

Besides RT-LAMP some multiplex LAMP assays have been tested and developed. Multiplex LAMP was first described by Lau *et al* (2015) for the detection of the dengue virus and consists on the detection of multiple targets in the same reaction[33]. Due to the number of primers used for the detection of a single target, multiplex LAMP can be difficult to achieve since the mixture of multiple primers can easily generate false positives due to the formation of primer-dimers. The use of additives such as betaine, DMSO, crowding agents i.e. Polyethylene Glycol, proline, N-methylformamide and isobutyramide can help stabilize the enzyme, increase the reaction yield and reduce the background signals or false positives, for both single and multiplex LAMP[31,34–37].

The amplification process can also be accelerated by the use of loop primers (LF, loop forward and LB, loop backwards), usually added at a concentration of 0.4 µM. The hybridization regions of these primers are located between F1 and F2 for LF and B1 and B2 for LB. During the elongation and recycling phases, they hybridize to the stem-loops that were not hybridized by the inner primers, generating a structure that was not observed on the LAMP reactions with only the 4 core primers. Nagamine *et al* (2002) documented the use of loop primers for the first time and showed that despite not being essential for the process, they decreased the Time to Positive (TtP) by less than a half compared to the reaction

with only the core primers[38]. Adding a poly-T linker between the regions of the FIP and BIP primers can also accelerate the reaction by improving the formation of the loop structures[31].

Studies have been made to assess the stability and robustness of LAMP assays at different temperatures, pH values and different sample matrixes, conditions that the test could face in field diagnosis. Thekisoe *et al* (2009) tested LAMP reaction mixtures for the detection of *Trypanosoma brucei brucei* with Bst DNA polymerases stored at -20ºC, 25ºC and 37ºC, the last two temperatures mimicking the ambient of sub-tropical and tropical regions. The TtP for positive samples using the polymerases at different temperatures was similar, not affecting significantly the performance of the assay[39]. Francois *et al* (2011) tested the robustness of LAMP, compared to qPCR, at different pH values and samples matrixes, as well as different reaction mixture preparation times, for the detection of *Salmonella enterica* [40]. For the preparation time, some were prepared in a cold rack in under 5 minutes, and others at 22ºC and 37ºC, and up to 30 minutes. The reaction mixtures prepared at 37ºC for 30 minutes generated some false positives. However, the others prepared at 22ºC and at 37ºC, the latter for shorter periods of time, did not lose specificity contrary to qPCR. For the pH variations, only pH values inferior to 7.3 or superior to 9.5 generated alterations to the assay results. The use of urine, decanted stool and whole blood, although only 1 μL per reaction, did not inhibit the assay. For blood-culture medium, only a 20-fold dilution was necessary for the LAMP detection of *Salmonella*, as for qPCR a 2000-fold dilution was crucial. This data comes to show that LAMP as a method has a better capacity of adaptation than qPCR, maintaining its specificity and sensitivity through different conditions, which further proves that this method can be applied in field diagnosis in POCT devices.

However, this method still has a long way of improvements and adaptations, especially in the reaction mixture, since that for in-field applications a reaction mixture that can be stored at ambient temperature, in a non-control environment such as laboratories, without affecting its robustness would be the ideal. Nowadays lyophilized forms of LAMP reaction mixtures are being studied and tested, there are inclusively some lyophilized mixtures on the market, but there is still work to be done for its application on clinical diagnosis.

Nonetheless, LAMP offers a specific, sensitive and great alternative for other non-isothermal NAATs, that are more expensive due to the equipment required, more laborious thanks to the need of sample purification, and overall, more time consuming. LAMP is more resistant to some sample matrixes and inhibitors than PCR, giving it a great advantage to field applications or less qualified laboratories. The possibility of applying this technique on POCT devices helps on the decentralization of diagnosis from hospital environments and making the process more convenient, rapid and simple to the specimens and health professionals.

1.3 Doctor Vida® Pocket Platform

The Doctor Vida® Pocket platform was developed by STAB VIDA. This equipment is accredited for isothermal amplification and qualitative molecular detection of nucleic acids by fluorescence emission using an intercalating dye, in POCT.

This platform was first created and market approved for the POCT detection of the COVID-19 virus, using the RT-LAMP technology. This device is portable, user-friendly and controlled by and application available at both the Google Play Store and Apple Store, which delivers the result of the test within 40 minutes to the user's email address. Besides, the user is able to observe the evolution of the reaction in real-time through the app[41].

1.4 Part 1: Diagnosis of Swine Dysentery

1.4.1 Swine Dysentery

Swine dysentery (SD) is an enteric disease that mainly affects swine and is caused by bacteria from the *Brachyspira* genus[42]. This disease is characterized by the excretion of mucohaemorrhagic faeces, which lead to dehydration, decreased growth rate and, in more severe cases, death. It is most observed in pigs between 6-26 weeks of age, which has a great financial impact on pig farms[43,44]. The occurrence of SD was first described in 1921, but the causing agent was not found until the 70s[42].

1.4.2 Etiology

The causative agent of SD are bacteria from the genus *Brachyspira*. These anaerobic, oxygen tolerant, Gram-negative spirochetes evolved to occupy the large intestine of mammals, including swine and some rodents, and birds[42]. *Brachyspira hyodysenteriae* was found to be the origin of the disease in the 70s, however, nowadays it is known that *B. hampsonii* and *B. suanatina* can also cause SD symptoms in susceptible pigs due to their strongly beta-haemolytic activity[42]. Nonetheless, *B. hyodysenteriae* remains the most identified species on diseased pigs, and as so the most studied species, with many genetically diverse strains identified[45].

It is suggested that the success of the infection is mostly due to affinity to the mucins on the large intestine's epithelium, to its beta-haemolytic activity and nicotinamide adenine dinucleotide hydrogen oxidase (NADH) activity which protects *B. hyodysenteriae* against oxygen toxicity[42–44].

1.4.3 Epidemiology

B. hyodysenteriae is the most common cause of SD infecting swine as its main host. However, it infects some species of birds and rodents, which work as transmission vectors between and inside farms. The incidence of the disease is variable, but it still represents an endemic problem in many farms[42]. Disease can spread by direct contact or transmission vectors[46].

Evaluation of the survival of *B. hyodysenteriae* in pig faeces showed that it is more resistant in moist faeces, and that it could survive in that environment for 48 hours or 7 days on temperatures ranging from 0 to 10ºC or 25ºC, respectively[42]. Infected migratory ducks and lagoon water have been observed in North America and Europe, infecting susceptible pigs[42,44].

Pigs with SD are considered infectious for at least 90 days, and mice for 180 days. *B. hyodysenteriae* can also survive in soil for 18 days at 4ºC, in flies for 4 hours, and can be carried by cats and

dogs for 13 days[45]. That is why eradication and testing plans are so important for the elimination of the disease, as it will be discussed later.

1.4.4 Pathogenesis

The mechanisms for development of SD are still not fully characterized. In short, the mucus layer and goblet cells of the colon and large intestine are the primary targets, due to the source of nutrients and its anaerobic environment, which is ideal for the proliferation of *B. hyodysenteriae*[43,47]. Besides, mucus is a strong chemoattractant for spirochaetes and it is proven that the genes involved in the bacteria's motility target mucins, resulting in the association to the gut mucosa[44,47].

Once entering the large intestine, the infection of the goblet cells promotes an increase in the production of mucins and a state of inflammation, by virulence factors (hemolysins, lipopolysaccharide, etc.) [43,47]. The increasing production of mucins promotes morphological changes on the epithelium and *B. hyodysenteriae* binding [43]. *B. hyodysenteriae* then expresses NADPH oxidase to protect itself from reactive oxygen species and oxidative stress, encoded by the *nox* gene. This has an important role in the proliferation of the bacteria since mutants for this enzyme showed weak proliferation and consequently less clinical symptoms[42,44,47].

The progression of infection causes the lysis of goblet cells and the increase production of mucus with its subsequent release results in haemorrhage, producing the mucohaemorrhagic faeces characteristic of SD. The release of the mucus and vulnerability of the intestine's epithelium can give rise to further infections by other anaerobic bacteria, increasing morbidity as well as mortality[43,47].

Furthermore, studies have shown that the development of SD is not completely dependent on infection with *B. hyodysenteriae*, since it has been detected although in low numbers on apparently healthy pigs [48]. In another study, gnotobiotic pigs that were infected with *B. hyodysenteriae* did not develop SD symptoms, but pigs infected with intestinal contents from ones with SD showed clinical signs. This suggests the intestine's microenvironment has a role in the susceptibility of contracting the disease[42,49].

1.4.5 Clinical Signs

The first sign of an outbreak is usually the death of a low number of pigs before others show any clinical signs. Primary symptoms include soft yellow faeces and increased rectal temperatures, around 40ºC. With disease progression, the soft faeces turn into mucohaemorrhagic diarrhoea with staining of the perineum [42]. Diarrhoea then leads to dehydration, stagnant growth, hyperkalaemia and metabolic acidosis.

The incubation period of SD can range from 4 days to 3 months, but the clinical signs are usually present within 10 to 14 days after infection. However, *B. hyodysenteriae* can be detected in faeces 1 to 4 days before the observation of the clinical signs[42,44].

On endemically infected farms, outbreaks can be cyclic, and appear at every 3 to 4 weeks. Morbidity in a herd can reach 90% and if left untreated, mortality can be higher than 50% [42].

1.4.6 State of Art of Diagnosis

The traditional diagnosis of SD consists on the observation of the symptoms and characteristic lesions in the colon and large intestine's epithelium. Observation of spirochaetes by silver staining in a histological cut, faecal or mucosal smears can be performed, but always with the confirmation of diagnosis by isolation of *B. hyodysenteriae* from faeces. Nonetheless, these methods are time-consuming or need a second method for confirmation of the results[42].

Nowadays, PCR can be considered the gold standard, providing faster and more specific results than the aforementioned methods, being less invasive as well. These PCR tests use targets such as the *nox* gene (NADPH oxidase)[50], *tlyA* gene (hemolysin)[51,52] or genes that are conserved between strains such as the 23s rRNA[53] and the 16s rRNA[54]. More recently, new studies have emerged with possible new methods based on genotyping and NAATs for detection and differentiation of *B. hyodysenteriae* strains and other *Brachyspira* species. Gasparrini *et al* (2017) was able to characterize 180 isolates through multilocus sequence typing and multiple locus variable number tandem repeat analysis[55]. Rohde *et al* (2019) used the chaperonin *cpn60*UT to discriminate isolates more successfully than with the *nox* gene[56]. Other based on immunoblotting and immunosorbent methods. Lobova *et al* (2011) used immunoblotting with recombinant lipoprotein pig serum to detect asymptomatic carriers of *B. hyodysenteriae*[57] and Song *et al* (2015) applied ELISA to detect the pathogen at a herd level[58].

Commercial tests for the detection of *B. hyodysenteriae* consist only of qPCR test kits such as *Brachyspira hyodysenteriae* NADH Oxidase Gene genesig® Standard Kit (Primerdesign™ Ltd), VetMAX™ *B. hyodysenteriae* Kit (Applied Biosystems), BactoReal® Kit *Brachyspira hyodysenteriae* (ingenetix), EXOone *B. hyodysenteriae* qPCR kit (Exopol) and a kit with primers and probes for *B. hyodysenteriae* (cador *B. hyodysenteriae* Primers/Probes, Indical Bioscience). Most of these are marketed for research purposes only. At the moment, there are no available commercialized POCT diagnostic kits, either through molecular or immunologic detection. Considering the morbidity and mortality of this disease when left untreated, a rapid POCT test could be useful in reducing the consequences of this infection.

1.4.7 Risk Factors

The development of SD is usually correlated to the bad hygiene of the herd's environment, overstocking, the transportation of weaner or finishing pigs to the farm, as well as the conditions of transportation, and cold temperatures since *B. hyodysenteriae* survives longer at lower temperatures[44].

Wild rodents, cats and dogs are also a risk factor as carriers of the disease, and are considered important factors for inter and intrafarm spreading[44].

The diet has been shown to have a huge role on the development of clinical signs, since it influences the intestinal microbiota. Diets rich in fermentable feeds, resistant carbohydrates and lignins increases disease expression, since it provides energy and macromolecules needed for the colonization and replication of *B. hyodysenteriae*. On the contrary, highly digestible feeds and diets rich in inulin help reduce SD expression[42,44].

1.4.8 Treatment and Control

Direct treatment of SD with antibiotics is not always possible or successful since many strains of *B. hyodysenteriae* are multidrug resistant[42]. Treatment or symptom management is performed by the administration of antibiotics and electrolytes. When antibiotics fail to stop the infection and the deterioration of the pig's health, euthanasia should be considered, sometimes of the whole herd[42,45].

As for control measures, the infected population should be isolated from the apparently healthy one while on treatment. As a last resource, the herd should be repopulated with non-infected swine. A plan for control and elimination of rodents should be implemented[45]. All the materials that came into contact with the pigs and their pen should be thoroughly cleaned and disinfected [45].

All pigs, cats and dogs in the farm should be tested to prevent a new outbreak, and in case of positives the treatment prescribed should be applied[45].

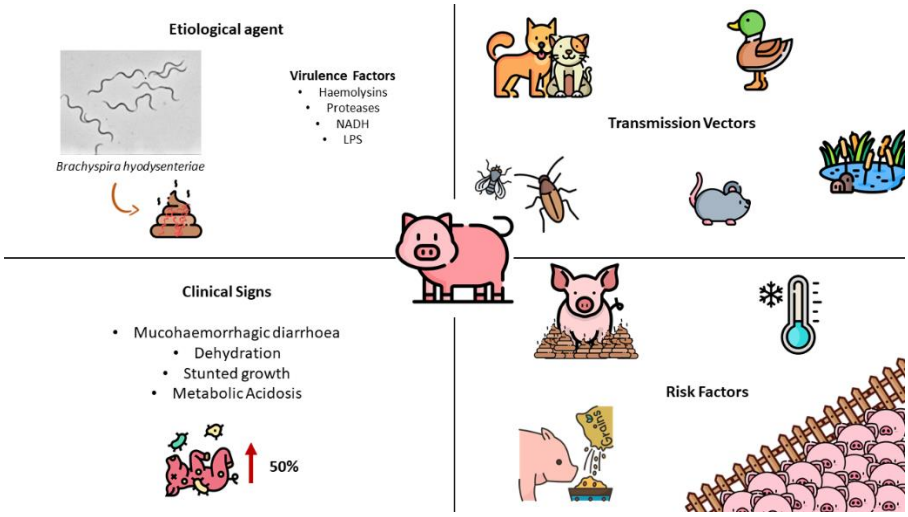

Figure 1.9: Graphic summary of the etiological agent, transmission vectors clinical signs and risk factors for the development and spreading of Swine Dysentery, caused by *B. hyodysenteriae*. Based on Alvarez-Ordóñez *et al* (2013)[46]. Designed on PowerPoint (Microsoft Office Professional Plus 2016), vector icons from Flaticon (https://www.flaticon.com/).

1.4.9 Goal of the Project

This Project aimed to develop a POCT diagnostic test to detect *B. hyodysenteriae*, the cause of SD, using LAMP. Once the achievement of the Proof-of-Concept, the test would be applied to Doctor Vida® Pocket Platform, and its feasibility for POCT applications would be evaluated and optimized. For a POCT testing using faecal matter as a sample, the formulation of steps and kits for sample collection, processing and purification will be necessary.

POCT tests that use stool as the sample are already commercialized, although none use the sample directly and are usually immunologic tests. For example, Buhlmann developed an immunoturbidimetry rapid test for the quantification of human faecal calprotectin (fCAL® turbo). The test kit uses a phone application (IB*Doc*®), which gives the information regarding the concentration of the molecule in the sample, distinguishing between inflammatory and irritable bowel disease. For sample collection, the

kit uses a device formulated by Bulhmann, that collects approximately 10 μg of stool thanks to the grooves on the tip, and the funnel on the tube that removes the excess sample from the dosing tip [59]. After collecting the sample, the tube is left to incubate for at least 2 hours on the extraction buffer, and the sample is viable to be analysed from 2 to 24 hours after the incubation. A few drops are transferred to the test cassette, and takes only about 12 minutes. A study comparing this test to the standard ELISA performed in laboratory practice found the same accuracy of results, with Quantum Blue fCal giving a better differentiation between inflammatory and non-inflammatory diseases[60].

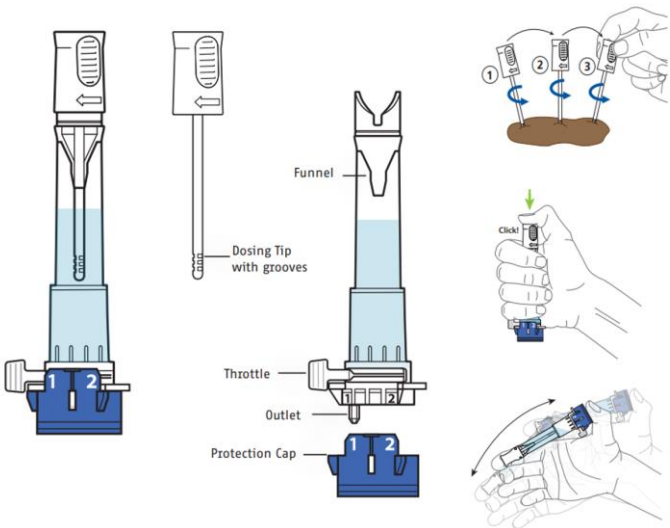

Figure 1.10: Stool collection device from Buhlmann fCAL® turbo test and IB*Doc*® (https://www.ibdoc.net/support/).

To detect *B. hyodysenteriae* the target chosen was the *nox* gene. The *nox* gene codifies for the enzyme NADPH oxidase that protects the bacteria from oxygen toxicity. For the sample control the *gad* gene from *E. coli*, a commensal bacterium to mammal's intestines and as so always present in faeces. The ultimate goal would be the development and clinical validation of a multiplex test.

1.5 Part 2: Bird Sex Determination

The determination and distinction of the sex in birds is an essential task for ecology, behavioural and genetic studies, breeding, conservatory and evolutionism studies[61,62]. From the Aves class, the order Psittaciformes is the one with the greater number of endangered species. According to The International Union for Conservation of Nature in 2019, approximately 400 out of the 3800 species in this order were considered endangered[63,64]. This is one of the practical examples of the importance of accurate bird sexing, aiding substantially on conservation and restauration programs.

Visual distinction of female and male characters in birds can be quite cumbersome, especially in monomorphic species that are almost indistinguishable. Dimorphic birds are easier to distinguish physically thanks to differences in the colour of the feathers and disparity of the sizes between male and female[64,65].

Bird sexing continues to be a challenge to perform with precision and efficiency, being the focal point for many researches. Especially due to the lack of sexual dimorphism in most bird species and the frustration of finding a universal marker valid across Aves class. Even through the capture of birds and observation of their external genitalia, composed by the cloaca, can be difficult to distinguish male and female, since that only in ducks and swans the cloaca is not identical in both sexes[66–68].

1.5.1 State of Art of Bird Sex Determination Methods

1.5.1.1 Non-molecular techniques

Non-molecular techniques for sex determination of birds include surgical methods, karyotyping, and measuring of steroid hormones.

Surgical sex identification can be performed in 2 ways: for both, an incision is made in the abdomen. For laparotomy, a metal probe is inserted to put the intestines away, so that the gonads can be visualized. For laparoscopy, in the same incision a fiber-optic cable is passed through, without the need to put the intestines away to visualize the gonads. Either way, these techniques are quite invasive, agitate the bird and put to rest any future attempts for breeding[66,67].

Karyotyping culture live cells and identification of the nucleus chromosomes is used to distinguish the W and Z chromosomes. Male birds are homozygous (ZZ) while the females are heterozygous (WZ). Besides, the W chromosome is smaller than the Z due to evolutionary loss of genes, which should be relatively easy to visualize. However, live culture cells are not quite simple to culture, and birds have a minimum of 40 chromosomes up to 126, in average 80. All these factors make this technique time-consuming and inconvenient[66,67].

Other alternative method for sex determination is the measurement of steroid hormones on the yolk of male and female eggs. However, this method needs further research to optimize the precision and specificity of the measurements[67].

Recent Advances

The methods described above are already being used for bird sexing. However, new non-molecular techniques are being studied to minimize animal handling, cost and the need for consumables. Raman spectroscopy has been described as a non-invasive, *in ovo*, consumables-free method for sex determination[69,70]. This technique is based on the Raman radiation interaction with embryonic blood components. It has been demonstrated that the embryonic blood composition of males and females is different, and as so could be distinguished using Raman spectroscopy. To summarize, Galli *et al* (2016) recognized that the Raman spectrum of males' embryonic blood was typically higher than for females. However, they also observed an overlap of the spectra of some male and female specimens, corresponding to the lowest male spectrum and highest female spectrum. After data refinement, the method was able to correctly identify the sex *in ovo* of 93% of the specimens, at the fourth day of incubation. The technique still needs some tunning due to data overlapping and some interferences of Raman signal from the eggshell membrane. Nonetheless, it is an alternative to the invasive methods described previously, and it is performed *in ovo* which means that the bird is sexed even before hatching.

1.5.1.2 Molecular techniques

Over the past few years, the study of molecular markers aided on the advancement of molecular techniques for bird sexing. These methods use the PCR technology, are non-invasive, low-cost and the genetic material can be obtained from simple samples such as feathers. The searching of universal markers for the last 25 years found multiple length polymorphisms in intronic sequences of the Chromo-Helicase DNA-binding domain (*CHD1*) between the homologous copies of the W and Z chromosome[63]. Some of these polymorphisms have already been used, targeted by primers such as P2/P8, 2550F/2718R and CHDF/CHDR for PCR, identifying the sex of birds among different orders with some thermal protocol changes[62,63,67]. The basic principle of this method is summarized on **Figure 1.11**. This technique has been applied to samples such as feathers, blood and eggshells, so it can be performed at any life stage of the bird[68].

The maker targeted by the P2/P8 primers was discovered by Griffiths *et al* (2000) and primarily used in 28 species of birds, correctly identifying the sex of 27[66]. Later applied to non-ratite species, it was able to correctly determine the sex of 80% of those[63]. These primers amplify intron 23 of *CHD1* of *Gallus gallus*, generating a fragment of approximately 362 bp for the W chromosome and 345 bp for the Z chromosome. This band pattern is relatively similar amongst parrot species but since the base pair differences between the fragment of the Z and W chromosome is small, resolving the 2 bands for female samples is almost impossible. Thus, sexing with these primers might require further procedures such as fragment analysis.

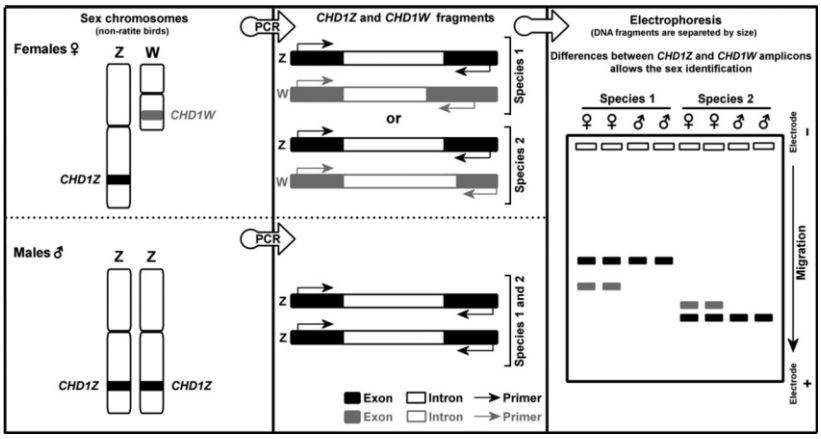

Figure 1.11: Graphic summary of the principle in which are based the molecular techniques used for bird sex determination, such as PCR with the primer sets P2/P8, 2550f/2718R and CHD1F/CHD1R. Taken from Morinha *et al* (2012)[68].

Another marker, CHD1iA, is present on the intron 17 of the *CHD1* gene of *Gallus gallus.* It is targeted by the primers 2550F/2718R and generates a band of 593 bp for the Z chromosome and 447 bp for the W chromosome in chickens[71]. When applied to 135 species from the Psittaciformes order, the primers determined correctly the sex of 113, with a band pattern of 2 bands for females (750 bp Z chromosome, 450 bp W chromosome) and 1 band for males (750 bp Z chromosome)[63].

On another study these 3 primer sets were applied across 10 bird orders (Anseriformes, Galliformes, Pelecaniformes, Ciconiiformes, Phoenicopteriformes, Aciipitriformes, Columbiformes, Psittaciformes, Piciformes and Passeriformes)[65].

	Success rate (%)									
	Anserif. (n = 38)	Gallif. (n = 6)	Pelecanif. (n = 14)	Ciconiif. (n = 12)	Phoenicopterif. (n = 10)	Accipitrif. (n = 29)	Columbif. (n = 2)	Psittacif. (n = 6)	Picif. (n = 2)	Passerif. (n = 55)
Agarose										
CHD1F/CHD1R	100	83.3	100	91.7	100	100	50	16.7	50	100
2550F/2718R	84.2	83.3	100	100	100	100	50	66.7	0	3.6
P2/P8	89.5	0	0	0	0	0	0	100	0	96.4
Capillary										
CHD1F/CHD1R	100	83.3	100	91.7	100	100	50	16.7	50	100
2550F/2718R	84.2	83.3	100	100	100	100	50	66.7	0	3.6
P2/P8	100	83.3	0	100	100	41.4	50	83.3	100	96.4

Figure 1.12: Success rates for each Primer sets across the 10 avian orders tested. The results were visualised through agarose gel and capillary electrophoresis. Taken from Çakmak *et al* (2017)[65].

The success rates for each primer set calculated for the agarose gel visualization were 70.2%, 57.9% and 64.9% for CHD1F/R, 2550F/2718R and P2/P8, respectively. Although, after performing fragment analysis on the PCR products, the success rate increased only for P2/P8 primers, from 64.9% to 98.4%. This happened since the P2/P8 primers produce bands for both the W and Z chromosomes that are very similar in length, and so they are difficult to resolve on agarose gel, as can be seen on **Figure 1.13**.

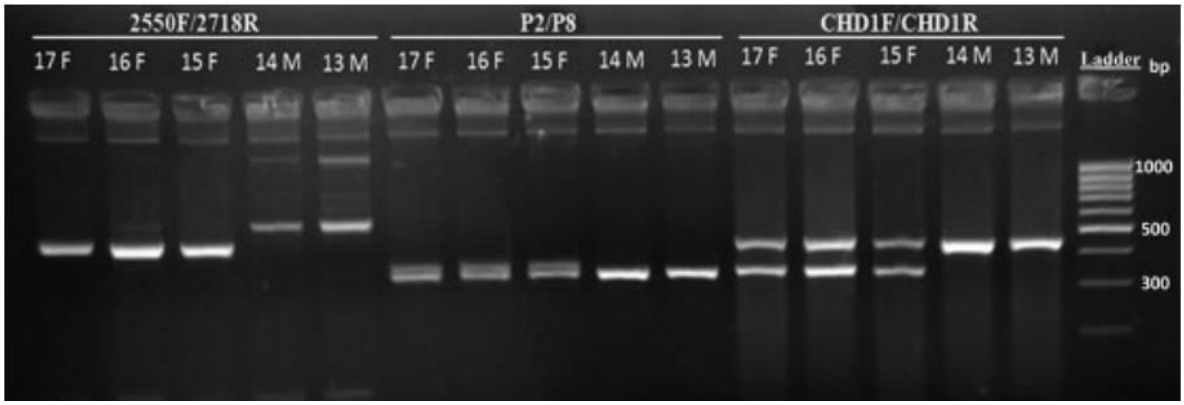

Figure 1.13: Typical agarose gel band pattern in *Cynus olor* blood for the 3 primer sets used for bird sexing. Taken from Çakmak *et al* (2017)[65].

Recent Advances

A real-time PCR with high resolution melt analysis (HRM) has been described using *CHD1* gene, to determine the sex of *Gallus gallus* individuals, using DNA extracted from feather tips[72]. With HRM the need to perform an agarose gel to visualize the results is eliminated, as well as further downstream methods such as fragment analysis. The method described by England *et al* (2021) uses an intercalating dye that binds to double stranded DNA and primers specific for the *CHD1* gene. When performing HRM, after amplification there is a gradual increase in temperature that will denature double stranded DNA and release dye molecules, causing a quenching effect or a decrease in fluorescence. This data is then collected and the temperatures at which the quenching was higher can be visualized. In this study they obtained a single melting peak for males, corresponding to its homozygous genotype (ZZ chromosomes), and 2 melting peaks for females, corresponding to its heterozygous genotype (ZW chromosomes). With this analysis, investigators were able to correctly identify the sex of 45 chickens.

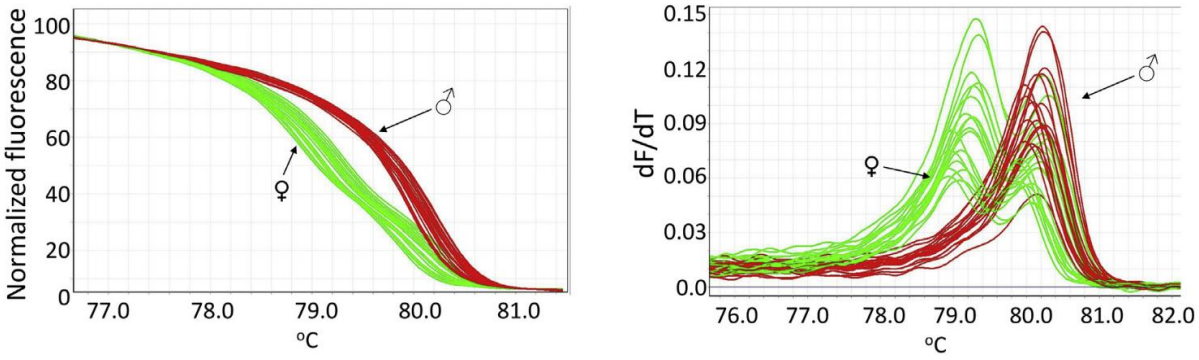

Figure 1.14: High-resolution melt analysis (HRM) of the 45 chickens. Females are characterized by 2 melt peaks because of the amplification of homologous *CHD1* on its W and Z chromosome, and males only have 1 peak since they are homozygous (ZZ chromosomes). Taken from England *et al* (2021)[72].

The recent rise of other PCR-based methods such as LAMP opened a new door for bird sex determination, with the possibility of shifting this test into POCT since LAMP technology does not require thermal cycling.

Centeno-Cuadros *et al* (2017) successfully described LAMP primers for sex determination of 3 raptor species using *CHD1* on both chromosomes as the target and DNA extracted from blood

samples[73]. Later in 2018 published what could be a universal marker for bird sexing, using the CHD-W sequences available on GenBank from 12 bird orders of the superorder Neognathae (NEO-W)[61]. These primers identified the sex of 21 species across 10 orders, so it cannot be deemed as universal. However, they designed CHD-W and CHD-Z specific primers for some of these families and orders (Ciconiidae, Estrildidae, Icteridae, Psittacidae and Falconiformes), and observed that the success level increased when the primers were designed for families rather than orders. Nonetheless, these primers were more successful at determining the sex than NEO-W primers. Elnomrosy *et al* (2022) later used the primers designed for the Psittacidae family (PSI-W) in 150 parrots from 21 species[64]. These primers identified correctly the sex of all individuals, with optimal reaction temperatures ranging from 57ºC and 63ºC. For example, for the species tested of the Cacatuidae family, the optimal reaction temperature was 57ºC, while for the Psittacidae family different species had different optimal reaction temperatures. When comparing the LAMP results to PCR, Elnomrosy observed that some samples that were female for LAMP did not amplify in PCR, which demonstrates the robustness and specificity of LAMP compared to PCR. Besides species from the Psittacidae family, Chan *et al* (2012) described LAMP primers for the sex determination of *Columba livia*[74]*.*

1.5.2 Goal of the Project

The goal of the Project consists on the development of a POCT test for the sex determination of bird species, either from Columbiformes or Psittaciformes order, and application of the test to DoctorVida® Pocket, performing then a clinical validation of the test and prepare for its commercial availability. The development and validation of a multiplex test with the target and internal control primers is the ultimate goal.

2

MATERIALS AND METHODS: PART 1

2.1 Targets and LAMP Primer Design for the Diagnosis of Swine Dysentery

The targets for the detection of *B. hyodysenteriae*, the etiologic agent of SD, and *E. coli* as the sample control were suggested by the collaborator of the project, Exopol S.L.

2.1.1 Targets and LAMP Primers for the Detection of *B. hyodysenteriae*

The primers for the diagnosis of SD target the *nox* gene from the strain ATCC 27164 of *B. hyodysenteriae* (GenBank Accession no.: KC984311.1), which is considered the reference strain of *B. hyodysenteriae spp.* All primer sets presented were designed using the PrimerExplorer V5 software (http://primerexplorer.jp/lampv5e/index.html). Primers LF and LB from primer set 2 were designed manually due to certain constraints on the abovementioned software. An alignment of the *nox* gene sequences of *B. hyodysenteriae* strains available on GenBank (https://www.ncbi.nlm.nih.gov/genbank/) was also performed to evaluate the conservation among strains and determine the best region for primer design. GenBank accession numbers of the strains used available at **Appendix 8.1.**

Table 2.1: Primer set 1, 2 and 3 for the detection of *B. hyodysenteriae*, targeting its *nox* gene. (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.).

***B. hyodysenteriae* Primer set 1**	
Name	**Sequence (5'-3')**
Bhyo_nox_F3	STAB VIDA's proprietary sequence #2022MV001
Bhyo_nox_B3	STAB VIDA's proprietary sequence #2022MV002
Bhyo_nox_FIP	STAB VIDA's proprietary sequence #2022MV003
Bhyo_nox_BIP	STAB VIDA's proprietary sequence #2022MV004
Bhyo_nox_LF	STAB VIDA's proprietary sequence #2022MV005
Bhyo_nox_LB	STAB VIDA's proprietary sequence #2022MV006
***B. hyodysenteriae* Primer set 2**	
Name	**Sequence (5'-3')**
Bhyo_F3	STAB VIDA's proprietary sequence #2022MV007
Bhyo_B3	STAB VIDA's proprietary sequence #2022MV008
Bhyo_FIP	STAB VIDA's proprietary sequence #2022MV009
Bhyo_BIP	STAB VIDA's proprietary sequence #2022MV010
Bhyo_nox_LF1	STAB VIDA's proprietary sequence #2022MV011
Bhyo_nox_LB1	STAB VIDA's proprietary sequence #2022MV012
***B. hyodysenteriae* Primer set 3**	
Name	**Sequence (5'-3')**
Bhyo_nox_F3-3	STAB VIDA's proprietary sequence #2022MV013
Bhyo_nox_B3-3	STAB VIDA's proprietary sequence #2022MV014
Bhyo_nox_FIP-3	STAB VIDA's proprietary sequence #2022MV015
Bhyo_nox_BIP-3	STAB VIDA's proprietary sequence #2022MV016
Bhyo_nox_LF-3	STAB VIDA's proprietary sequence #2022MV017
Bhyo_nox_LB-3	STAB VIDA's proprietary sequence #2022MV018

2.1.2 Targets and LAMP Primers for Sample Control: *E. coli*

The primers for the detection of *E. coli* for the sample control target the *gad* gene, that is conserved within the *E. coli spp*. The reference strain for this gene is NCTC86 (GenBank Accession no.: CP019778.1, nts 1835760 to 1837160). Similar to the primer sets for detection of *B. hyodysenteriae*, the primers for detection of *E. coli* were designed using the softwares PrimerExplorer V5.

Table 2.2: Sequences of the primers from primer set 1 and 2 for the detection of *E. coli*, targeting its *gad* gene. (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.)

Sample Control Primer set 1	
Name	**Sequence (5'-3')**
Ecoli_gad_F3	STAB VIDA's proprietary sequence #2022MV019
Ecoli_gad_B3	STAB VIDA's proprietary sequence #2022MV020
Ecoli_gad_FIP	STAB VIDA's proprietary sequence #2022MV021
Ecoli_gad _BIP	STAB VIDA's proprietary sequence #2022MV022
Ecoli_gad_LF	STAB VIDA's proprietary sequence #2022MV023
Ecoli_gad_LB	STAB VIDA's proprietary sequence #2022MV024
Sample Control Primer set 2	
Name	**Sequence (5'-3')**
Ecoli _F3	STAB VIDA's proprietary sequence #2022MV025
Ecoli _B3	STAB VIDA's proprietary sequence #2022MV026
Ecoli _FIP	STAB VIDA's proprietary sequence #2022MV027
Ecoli _BIP	STAB VIDA's proprietary sequence #2022MV028
Ecoli_LF	STAB VIDA's proprietary sequence #2022MV029
Ecoli_LB	STAB VIDA's proprietary sequence #2022MV030

2.2 Samples

The DNA and direct samples tested were provided by Exopol. The results and Cqs for all the samples, except for the ones in **bold** to which the DNA was extracted and tested in STAB VIDA with EXOone *B. hyodysenteriae* kit (Exopol, BHYO) provided by Exopol as well.

The names, IDs and results of each sample are represented on the next table.

Table 2.3: Samples provided by Exopol for testing and development of the test. The results and Cq present in the table were obtained by testing the samples with Exopol's kit EXOone *Brachyspira hyodysenteriae* (Exopol, BHYO).

	Sample ID	Sample Name	Sample Type	*B. hyodysenteriae*		*E. coli*	
				Result	Cq	Result	Cq
DNA SAMPLES	163998	Cepa Bhyo-3	DNA from Isolate	Positive	N/A	N/A	N/A
	164534	Cepa BHYO		Positive	N/A	N/A	N/A
	164835	Cepa Bhyo-1		Positive	N/A	N/A	N/A
	161378	Pool 5 His	DNA from Rectal Swabs	Positive	27,96	Positive	27,33
	164233	Pool 5 His		Positive	27,15	Positive	28,96
	164375	Pool 5 His		Negative	0	Positive	24,75
	164293	Pool 4 His		Positive	28,25	Positive	22,76
	164835	Pool 2 His		Positive	31,81	Positive	24,88
	164308	Pool 5 He	DNA from Faeces	Negative	0	Positive	23,36
	164278	Pool 2 He		Negative	0	Positive	19,25
	164188	He s-n		Positive	25,86	Positive	24,24
	164847	1# Pool He 1-5 N		Positive	24,94	Positive	N/A
	164959	Pool 3 He		Positive	29,08	Positive	23,95
DIRECT SAMPLES	163998	Cepa Bhyo-3	Isolate	Positive	N/A	N/A	N/A
	164534	Cepa BHYO		Positive	N/A	N/A	N/A
	164835	Cepa Bhyo-1		Positive	N/A	N/A	N/A
	164233	Pool 5 His	Rectal swabs in Exopol's Buffer	Positive	27,15	Positive	28,96
	164293	Pool 4 His		Positive	28,25	Positive	22,76
	164835	Pool 2 His		Positive	31,81	Positive	24,88
	161378	Pool 5 His		Positive	27,96	Positive	27,33
	164188	He s-n	Faeces in Exopol's Buffer	Positive	28,86	Positive	24,24
	164278	Pool 2 He		Negative	0	Positive	19,25
	164308	Pool 5 He		Negative	0	Positive	23,36
	164959	Pool 3 He		Positive	29,08	Positive	23,95
	164847	1# Pool He 1-5 N		Positive	24,94	Positive	N/A
	170610	**He s/n**	Faeces	Negative	0	Positive	31.60
	171695	**He**		Positive	30.82	Positive	35.07
	171652	**He MB7**		Positive	32.56	Positive	28.26
	170800	**He**		Positive	30.53	Positive	32.03
	170254	**Pool 2 He**		Positive	30.84	Positive	30.11
	171771	**1 # He**		Negative	0	Positive	32.89
	179162	**Pool 3 He**		Positive	24.11	Positive	25,37
	178707	**He diarrea c**		Positive	23.46	Positive	25.56
	177161	**2# Pool He 3+4+5**		Negative	0	Positive	31.98
	176894	**1 He**		Positive	22.86	Positive	25.75
	176058	**He**		Positive	28.25	Positive	29.66
	175568	**He**		Positive	27.90	Positive	29.94
	175499	**He**		Positive	31.03	Positive	29.33
	174575	**1# He 000479246**		Positive	26.94	Positive	29.43
	174485	**1# He 1**		Positive	32.13	Positive	38.76
	174485	**2# He 2**		Positive	32.34	Positive	38.76
	169254	**Pool 4 He**		Positive	28.16	Positive	30.31

(continuation) Table 2.3: Samples provided by Exopol for testing and development of the test. The results and Cq present in the table were obtained by testing the samples with Exopol's kit EXOone Brachyspira hyodysenteriae (Exopol, BHYO).

	Sample ID	Sample Name	Sample Type	*B. hyodysenteriae*		*E. coli*	
				Result	Cq	Result	Cq
	168935	**1 He**		Positive	27.71	Positive	29.85
	173629	**Pool 4 He**		Positive	25.1	Positive	25.64
	172429	**He s/n**		Positive	28.12	Positive	30.16
	172234	**He**		Positive	27.41	Positive	29.84
	179363	**He**		Positive	29.64	Positive	28.82
	179041	**He**		Positive	25.55	Positive	30.02
	179020	**He s/n**		Positive	31	Positive	29.01
	178989	**Pool 2 He**		Positive	25.82	Positive	29.78
	178570	**He**		Positive	30.09	Positive	33.33
	177161	**1# Pool He**		Negative	0	Positive	34.14
	178903	**1 He**		Negative	0	Positive	25.2
	178969	**1 He**		Negative	0	Positive	24.46
	178284	**He**		Negative	0	Positive	28.04
	178283	**He**		Negative	0	Positive	29.17
	178278	**He**		Negative	0	Positive	32.57
	177353	**He**		Negative	0	Positive	31.74
	176680	**1 He**		Negative	0	Positive	25.71
	176050	**1# He 20220505016**		Negative	0	Positive	28.39
	176050	**2# He 2022050017**		Negative	0	Positive	28.39

2.3 Reaction conditions and Optimizations

The samples tested were all provided by Exopol, and are identified by Sample ID and name on **Table 2.3**, as well as the respective sample type, and the Cq obtained with Exopol's EXOone *B. hyodysenteriae* qPCR kit (Exopol, BHYO) at their facilities. With the samples they provided a EXOone BHYO qPCR kit (100 reactions) for internal testing and result confirmation.

Initial tests for performance evaluation of the primer sets and reaction optimizations were performed in a Quantabio qPCR instrument (Quantabio, 95900-4C) using the respective reaction tubes (Quantabio, 95910-20). The amplification products were detected through the use of an intercalating dye, and the Green Channel (Excitation λ at 465 nm, Emission λ at 510 nm) of the device. The cycle protocol used on the Quantabio software was 60 cycles of 60 seconds at the same temperature, resulting in a 1-hour test.

Different reaction mastermixes were used, respectively A and B. The different buffers added to the reaction mastermix, temperatures tested and sample treatments are presented on the further sections, for both the detection of *B. hyodysenteriae* and the sample control.

Tests started with purified DNA from faeces and rectal swabs to assess the sensitivity and rapidity of the primer sets. Then with direct samples such as faeces, rectal swabs, homogenized in Exopol's buffer. Subsequently, faecal swabs from dry faeces were made in the laboratory with different collection kits and tested, evaluating the possibility and feasibility of this kind of test on the field performed by veterinary professionals, or even by farm owners.

2.3.1 Detection of *B. hyodysenteriae*: Reaction Conditions and Optimizations

The tests for the proof-of-concept of the detection of *B. hyodysenteriae* were conducted with 3 primer sets and different temperatures for optimization of the results. Different reaction additives were also tested since it has been demonstrated that some reagents could help increase important parameters, such as sensitivity, specificity, and decrease the TtP.

All the conditions tested can be consulted on the following Table, and will be referred through the document as the respective Reaction Condition (RC) number.

Table 2.4: Reaction Conditions (RC) tested for the detection of *Brachyspira hyodysenteriae*. The faecal swabs were made with a variety of swabs, such as Microbrush from Microbrush International (MRP400), Sterile Viscose swab from Deltalab (300284), Sterile Viscose swab from Citotest Scientific (2122-0201).

Reaction Mastermix: A						
RC (#)	**T (ºC)**	**Sample**	**Primer sets**	**Primer Conc. (mM)**	**Reaction Additives**	**Vol. of Reaction Mixture (µL)**
B1.1	65	DNA	1	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	N/A	15
B1.2	68	DNA		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B1.3	65	DNA	1 (w/o loop primers)	F3/B3: 0.2 FIP/BIP: 1.6		
B1.4		DNA	1	1×; 0.3×; 0.2×		
B2.1		DNA	2 (w/o loop primers)	F3/B3: 0.2 FIP/BIP: 1.6		
B2.2	67	DNA	2	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B2.3		Direct samples (in Exopol's buffer)		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B2.4	65	Direct samples boiled at 95ºC, 5'		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		10
B2.5	67	Faecal swabs		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B2.6		DNA from faecal swabs extracted w/ E.Z.N.A Tissue DNA Kit		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B3.1	65	DNA from faecal swabs extracted w/ E.Z.N.A Tissue DNA Kit	3	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B3.2		DNA from faecal swabs extracted w/ E.Z.N.A Tissue DNA Kit		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	30 mM Component A (STAB VIDA)	

(continuation) Table 2.5: Reaction Conditions (RC) tested for the detection of *Brachyspira hyodysenteriae*. The faecal swabs were made with a variety of swabs, such as Microbrush from Microbrush International (MRP400), Sterile Viscose swab from Deltalab (300284), Sterile Viscose swab from Citotest Scientific (2122-0201).

Reaction Mastermix: A						
RC (#)	**T (ºC)**	**Sample**	**Primer sets**	**Primer Conc. (mM)**	**Reaction Additives**	**Vol. of Reaction Mixture (µL)**
B3.3		DNA from faecal swabs extracted w/ E.Z.N.A Tissue DNA Kit		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.8	30 mM Component A (STAB VIDA)	
B3.4		DNA from faecal swab extracted w/ Column format A, Filter A		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B3.5		Faecal swab (1:1, 1:2, 1:5, 1:10, 1:50 dilutions)		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B3.6		Faecal swab treated w/ Chellex 100, 7.4% p/V		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		10
B3.7		DNA from Faecal swab w/ Column format B, Filter A		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	N/A	
B3.8		DNA from Faecal scoop extracted w/ Sterile 0.45 µm PVDF Filter Media		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
B3.9	63	DNA DNA extracted from faecal swabs w/ E.Z.N.A Tissue DNA Kit		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
Reaction Mastermix: B						
B3.10	65	DNA	3	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.8	N/A	25

2.3.2 Sample Control: Reaction Conditions and Optimizations

Diagnostic tests require a Sample Control to evaluate both the quality of the sample and enzyme activity.

The tests for the sample reaction control using *E. coli* were conducted with 2 primer sets and different temperatures. Reaction additives were also used.

All the conditions tested can be consulted on the following Table, and will be referred through the document as the respective Reaction Condition (RC) number.

Table 2.6: Reaction Conditions (RC) tested for the Sample Control. The faecal swabs were made with a variety of swabs, such as Microbrush from Microbrush International (MRP400), Sterile Viscose swab from Deltalab (300284), Sterile Viscose swab from Citotest Scientific (2122-0201).

Reaction Mastermix: A						
RC (#)	**T (ºC)**	**Sample**	**Primer sets**	**Primer Conc. (mM)**	**Reaction Additives**	**Vol. of Reaction Mixture (µL)**
E1.1	65	DNA	1	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	N/A	15
E1.2	68	DNA		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E1.3	65	DNA	1 (w/o loop primers)	F3/B3: 0.2 FIP/BIP: 1.6		
E1.4		DNA	1	1×; 0.3×; 0.2×		
E2.1		DNA	2	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.2		DNA	2 (w/o loop primers)	F3/B3: 0.2 FIP/BIP: 1.6		
E2.3	68	DNA	2	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.4	67	DNA		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.5		Direct samples (in Exopol's buffer)		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.6		Faecal swabs		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		10
E2.7	65	DNA from faecal swab extracted w/ Column format A, Filter A		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.8		Faecal swabs (1:1, 1:2, 1:5, 1:10, 1:50 dilutions)		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.9		Faecal swab treated w/ Chellex 100, 7.4% p/V		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.10		DNA from Faecal swab w/ Column format B, Filter A		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
E2.11		DNA from Faecal scoop extracted w/ Sterile 0.45 µm PVDF Filter Media		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4		
Reaction Mastermix: B						
E2.12	65	DNA	2	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.8	N/A	25

2.3.3 Multiplex Testing

Table 2.7: Reaction Conditions (RC) tested for the detection of *B. hyodysenteriae* and the sample control (*E. coli*) in multiplex.

Reaction Mastermix: A				
RC (#)	**Sample**	**T (ºC)**	**Primer sets**	**Primer Conc. (mM)**
1	DNA	67	2 (*B. hyo*) 2 (*E. coli*)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
2	Direct samples (in Exopol's buffer)	67	2 (*B. hyo*) 2 (*E. coli*)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
3	Direct samples (in Exopol's buffer)	67	2 (*B. hyo*) 2 (*E. coli*)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4 (w/o *E. coli* loop primers)
4	Spiked Samples	67	2 (*B. hyo*) 2 (*E. coli*)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4 0.3X F3/B3: 0.06 FIP/BIP: 0.48 LF/LB: 0.24

2.4 Reaction Conditions tested with Doctor Vida® Pocket

Testing with Doctor Vida® Pocket started after optimizing the reaction conditions. The primers chosen to continue to this evaluation were *B. hyodysenteriae* primer set 3 and *E. coli* primer set 2. The reaction mastermix used was **Reaction Mastermix A**.

For Doctor Vida® Pocket is used a total volume of reaction mixture of 50 μL, of which 10 μL is the sample. To the reaction tube is added 30 μL of a mineral oil, to avoid the evaporation of the reaction mixture during the test.

While testing with the device, some alterations were done to the sample treatment and collection, trying to find the best and most adaptable way to perform this test in POCT. The description of the collection kits created and its versions are described on the **subsection 2.4.1 Sample collection kits**, and on **Results and Discussion: Part 1, subsection 4.3.1 Sample collection kits**.

Table 2.8: Reaction Conditions (RC) tested for the detection of *B. hyodysenteriae* and the sample control on Doctor Vida® Pocket.

RC (#)	**T (ºC)**	**Collection buffer (μL)**	**Sample collection**	**Sample Weight (g)**	**Sample treatment**	**Primer sets**	**Primer Conc. (mM)**
DV1	63 (*B. hyo*) 65 (*E. coli*)	A 100 μL	Scoop spatula	~0.05 g	Column format B, Filter A	3 (B. hyo) 2 (E. coli)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

(continuation) Table 2.9: Reaction Conditions (RC) tested for the detection of *B. hyodysenteriae* and the sample control on Doctor Vida® Pocket.

RC (#)	T (ºC)	Collection buffer (µL)	Sample collection	Sample Weight (g)	Sample treatment	Primer sets	Primer Conc. (mM)
DV2	63 (*B. hyo*) 65 (*E. coli*)	A 100 µL Exopol's Buffer 100 µL	Scoop spatula	0.04 g 0.05g 0.1 g 0.2 g	Column format B, Filter A	3 (B. hyo) 2 (E. coli)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DV3	63 (*B. hyo*) 65 (*E. coli*)	A 100 µL	Scoop spatula	0.05 g 0.1 g 0.2 g	N/A	3 (B. hyo) 2 (E. coli)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DV4	63 (*B. hyo*) 65 (*E. coli*)	A 400 µL	Collection kit 1	~0.04 g	Filter A	3 (B. hyo) 2 (E. coli)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DV5	63 (*B. hyo*) 65 (*E. coli*)	A 400 µL	Collection kit 2	~0.05g	Filter A	3 (B. hyo) 2 (E. coli)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

2.4.1 Sample collection kits

Two alternative kits were assembled and tested.

The tube for kit 1 was provided by STAB VIDA, with the alterations to the tube being the incorporation of Filter A on the dropper piece.

For kit number 2, a 1 mL extraction tube was used attached to a piece designed and 3D printed internally at STAB VIDA, to which was attached to a tube for collection of the eluted sample. It is in this piece that Filter A is incorporated.

Both kits and redesigns are presented with photos on **Results and Discussion: Part 1, subsection 4.3.1 Sample collection kits**.

2.5 Sample Treatments

For the extraction and purification of DNA from the faecal swabs to test with the EXOone BHYO qPCR kit we used the E.Z.N.A Tissue DNA Kit (Omega Bio-Tek, D3396-01), following the manufacturer's instructions. In short, the sample was subjected to the lysis step for approximately 3 hours at 55ºC, and then centrifuged at maximum speed (≥10000×*g*) to pellet cellular debris. After adjusting binding conditions, the sample was transferred to the HiBind® DNA Mini Column, to which the DNA binds. The membrane is washed to eliminate salts and other contaminants. After completely drying the column to remove trace amounts of ethanol, the DNA is eluted.

For testing with LAMP reaction mixture, different sample treatments were tested, as will be discussed later on:

- Boiled at 95ºC for 5 minutes: faeces and rectal swabs in Exopol's buffer boiled at 95ºC for 5 minutes;
- Faecal swab homogenized in Sample collection buffer A: different swabs were used to make faecal swabs from dry faecal samples, such as a Microbrush from Microbrush

International (MRP400), a Sterile Viscose swab from Deltalab (300284), a Sterile Viscose swab from Citotest Scientific (2122-0201) and a swab from a tube available at STAB VIDA and were homogenized in 100 μL of Sample collection buffer A;

- Column format A, Filter A: filter by gravity flow. Faeces were collected with a scoop spatula and homogenized on 100 μL of Sample collection buffer A. The top and bottom cap of the column was removed and the preservative was allowed to flow through. Then the column was equilibrated with 10 mL of Sample collection buffer A. One hundred microliters of the sample were transferred to the column and allowed to enter the gel bed, and then 400 μL of Sample collection buffer A were transferred and allowed to enter the gel as well. For the elution step a 1.5 mL Eppendorf tube was placed under the column, 500 μL of Sample collection buffer A were transferred and the eluate was collected by gravity flow;
- Column format B, Filter A: filter in a spin-column format. In 100 μL of Sample collection buffer A and with a Microbrush or spoon spatula, the faeces collected were homogenized. The column was resuspended by vortex. The cap was loosened and the bottom closures were taken off, then placed into a collection tube and centrifuged for 1 minute at 735×*g*. The column was placed into a 1.5 mL Eppendorf tube, and 50 μL of the sample previously prepared was transferred to the column. The column was centrifuged for 2 minutes at 735×*g*, eluting the now purified sample;
- PVDF filter (GE HealthCare, 6900-2504): approximately 0.05 g of stool were collected with a scoop spatula and homogenized in 100 μL of Sample collection buffer A. The sample was transferred to a syringe, and filtrated through the PVDF filter;
- Chelex-100 (Bio-Rad, 1422822): 0.05 g of stool were collected with a scoop spatula and homogenized in 100 μL of Sample collection buffer A. 7.4 mg of Chelex-100 were weighted and transferred to the sample. The mixture was shaken and left at room temperature until the Chelex beads deposited on the bottom of the tube. The supernatant was collected for testing.

3

MATERIALS AND METHODS: PART 2

3.1 Sex Determination of Birds from Psittacidae Family

STAB VIDA provides a bird sexing service for its clients, giving results within 48 hours after receiving the samples in their laboratory. However, transportation of these samples from the breeder to the laboratory can take a few days. Then, it was on STAB VIDA's interest to create a simpler way for the company and for the breeder to correctly and rapidly determine the sex of their birds. Psittacidae is the largest family from Psittaciformes, and one of the families from which the company receives more samples.

3.1.1 Targets and LAMP Primers

The sequences of the first set of primers used for sex determination of species from Psittacidae family are described below, and target the *CHD1* gene from the bird female specific W chromosome. The primers for the sample control target a highly conserved fragment on the 6 chromosomes of birds.

Table 3.1: Primer sequences for Sex Determination of species from the Psittacidae family, and for sample control. (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.).

Psittacidae Family Sex Determination Primer set 1	
Name	**Sequence (5'-3')**
PSI-W_F3	STAB VIDA's proprietary sequence #2022MV031
PSI-W_B3	STAB VIDA's proprietary sequence #2022MV032
PSI-W_FIP	STAB VIDA's proprietary sequence #2022MV033
PSI-W_BIP	STAB VIDA's proprietary sequence #2022MV034
Sample Control Primer set 1	
Name	**Sequence (5'-3')**
UCE_F3	STAB VIDA's proprietary sequence #2022MV035
UCE_B3	STAB VIDA's proprietary sequence #2022MV036
UCE_FIP	STAB VIDA's proprietary sequence #2022MV037
UCE_BIP	STAB VIDA's proprietary sequence #2022MV038

3.1.2 Reaction Conditions with PSI-W and UCE

The samples used for these tests were feather tips. Feathers for this test were always provided by the Bird Sexing Service from STAB VIDA, after testing and delivering the results for those samples.

Tests with these primer sets were performed on Quantabio qPCR instrument (Quantabio, 95900-4C) using the respective reaction tubes (Quantabio, 95910-20). The amplification products were detected through the use an intercalating dye, and the Green Channel (Excitation λ at 465 nm, Emission λ at 510 nm) of the device. The cycle protocol used on the Quantabio software was 60 cycles of 60 seconds at the same temperature, resulting in a 1-hour test.

The reaction mixture used was **Reaction Mastermix A.**

Two different sample treatments were tested.

Table 3.2: Reaction Conditions (RC) tested for with primers PSI-W and UCE for bird sex determination of species from the family Psittacidae and control of the reaction, respectively.

RC (#)	T (ºC)	Sample	Sample treatment	Primer sets	Primer Conc. (mM)	Vol. of Reaction Mixture (µL)
P1.1	65	Feather Tips	Buffer E (STAB VIDA)	PSI-W	F3/B3: 0.2 FIP/BIP: 1.6	10
				UCE		
P1.2			STAB VIDA's Routine A (DNA extraction steps)	PSI-W		15
				UCE		

3.1.3 Polymerase Chain Reaction

A PCR was performed with primers forward and reverse of both sets. The PCR products were then sequenced.

DNA from feather tips was extracted and tested by PCR with the routine procedures used by STAB VIDA's Bird Sexing Services (Routine A).

The thermal PCR protocol used was the following.

Table 3.3: PCR thermal-cycle protocol used with forward and reverse primers from the LAMP primer sets PSI-W and UCE.

Phase	n Cycles	Time	Temperature
Initial denaturation	1	2'	95ºC
Denaturation	40	15''	95ºC
Annealing	40	15''	47ºC
Extension	40	15''	72ºC
Final extension	1	7'	72ºC

3.2 Sex Determination of *Columba livia*

Columba livia is a highly required species due to the participation in competitions and very specific breeding to maintain certain genetic characteristics.

After testing with these primers, 2 other set of primers for both bird sexing and for the sample control were designed.

3.2.1 Target and LAMP Primers for Sex Determination

The primers used for sex determination target a female-specific sequence of *Streptopelia orientalis*, a species from *Columba livia* family.

The second primer set was based on a CHD-W sequence from *Columba livia* available at GenBank (Accession number: AY517718.1).

Table 3.4: Primer sequences for Sex Determination of *Columba livia.* (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.).

***Columba livia* Sex Determination Primer set 1**	
Name	**Sequence (5'-3')**
CL_Sex_F3	STAB VIDA's proprietary sequence #2022MV039
CL_Sex_B3	STAB VIDA's proprietary sequence #2022MV040
CL_Sex_FIP	STAB VIDA's proprietary sequence #2022MV041
CL_Sex_BIP	STAB VIDA's proprietary sequence #2022MV042
***Columba livia* Sex Determination Primer set 2**	
Name	**Sequence (5'-3')**
CHDW_CLSEX_F3	STAB VIDA's proprietary sequence #2022MV043
CHDW_CLSEX_B3	STAB VIDA's proprietary sequence #2022MV044
CHDW_CLSEX_FIP	STAB VIDA's proprietary sequence #2022MV045
CHDW_CLSEX_BIP	STAB VIDA's proprietary sequence #2022MV046
CHDW_CLSEX_LF	STAB VIDA's proprietary sequence #2022MV047
CHDW_CLSEX_LB	STAB VIDA's proprietary sequence #2022MV048

3.2.2 Target and LAMP Primers for Sample Control

Both the primer sets target the gene for 18S ribossomal RNA of *Columba livia* (GenBank Accession number: AF173630.1).

Table 3.5: Primer sequences for Internal Control of the *Columba livia* Sex Determination. (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.).

Sample Control Primer set 1	
Name	**Sequence (5'-3')**
CL_18s_F3	STAB VIDA's proprietary sequence #2022MV049
CL_18s_B3	STAB VIDA's proprietary sequence #2022MV050
CL_18s_FIP	STAB VIDA's proprietary sequence #2022MV051
CL_18s_BIP	STAB VIDA's proprietary sequence #2022MV052
Sample Control Primer set 2	
Name	**Sequence (5'-3')**
Clivia_IRC_F3	STAB VIDA's proprietary sequence #2022MV053
CL_18s_B3	STAB VIDA's proprietary sequence #2022MV050
Clivia_IRC_FIP	STAB VIDA's proprietary sequence #2022MV054
Clivia_IRC_BIP	STAB VIDA's proprietary sequence #2022MV055

3.2.3 Polymerase Chain Reaction

An initial PCR reaction and purification of the products was made to obtain a positive control (target *Columba livia* sequence on the W-chromosome) for the following LAMP reactions.

Further PCR reactions were performed to assess if the primers forward and reverse were designed correctly, under STAB VIDA'S PCR reaction Routine A.

Table 3.6: PCR thermal-cycle protocol used with forward and reverse primers from the LAMP primer sets CL_Sex and CL_18s.

Phase	n Cycles	Time	Temperature
Initial denaturation	1	2'	95ºC
Denaturation	40	15''	95ºC
Annealing	40	15''	47ºC
Extension	40	15''	72ºC
Final extension	1	7'	72ºC

3.2.4 Reaction Conditions and Optimizations

The samples used for these tests were feather tips. Feathers for this test were always provided by the Bird Sexing Service from STAB VIDA, after testing and delivering the results for those samples.

Tests with these primer sets were performed on Quantabio qPCR instrument (Quantabio, 95900-4C) using the respective reaction tubes (Quantabio, 95910-20). The amplification products were detected through the use of an intercalating dye, and the Green Channel (Excitation λ at 465 nm, Emission λ at 510 nm) of the device.

The reaction mixture used was **Reaction Mastermix A**.

3.2.4.1 *Columba livia* Sex Determination

The samples used for these tests were feather tips. Feathers for this test were always provided by the Bird Sexing Service from STAB VIDA, after testing and delivering the results for those samples.

Table 3.7: Reaction Conditions (RC) tested for with primers CL_Sex and CHDW_CLSEX for sex determination of *Columba livia*.

Reaction time: 1 hour						
RC (#)	**T (ºC)**	**Sample**	**Reaction additives**	**Primer sets**	**Primer Conc. (mM)**	**Vol. of Reaction Mixture (µL)**
CL1.1	65	Feather Tips treated with Buffer E (STAB VIDA) or Routine A (DNA extraction step)	N/A	1	F3/B3: 0.2 FIP/BIP: 1.6	10
CL1.2	60	Feather Tips treated with Buffer E (STAB VIDA)			F3/B3: 0.2 FIP/BIP: 1.6	
CL1.3	65	Feather Tips boiled at 95ºC, 5’			F3/B3: 0.2 FIP/BIP: 1.6	
CL2.1				2 w/o loop primers	F3/B3: 0.2 FIP/BIP: 1.6	15
CL2.2			40 mM Component A (STAB VIDA)		F3/B3: 0.2 FIP/BIP: 1.6	
Reaction time: 2 hours						
CL2.3	65	Feather Tips boiled at 95ºC, 5’	N/A	2 w/o loop primers	F3/B3: 0.2 FIP/BIP: 1.6	15
CL2.4			N/A 7.5% (w/v) Component B (STAB VIDA)		F3/B3: 0.2 FIP/BIP: 1.6	
CL2.5			N/A 15, 20, 25, 30, 35 mM Component A (STAB VIDA)	2	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	10
CL2.6	67	DNA from feather tips extracted w/ E.Z.N.A Tissue DNA Kit	N/A 30 mM Component A (STAB VIDA)		F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4	
CL2.7	65				F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4/0.8	

3.2.4.2 Sample Control

The samples used for these tests were feather tips. Feathers for this test were always provided by the Bird Sexing Service from STAB VIDA, after testing and delivering the results for those samples.

The reaction mixture used was **Reaction Mastermix A**.

Table 3.8: Reaction Conditions (RC) tested for with primers CL_18S and Clivia_IRC for the sample control of sex determination of *Columba livia*.

Reaction time: 1 hour						
RC (#)	**T (ºC)**	**Sample**	**Reaction additives**	**Primer sets**	**Primer Conc. (mM)**	**Vol. of Reaction Mixture (μL)**
SC1.1	65	Feather Tips treated with Buffer E (STAB VIDA) or Routine A (DNA extraction step)	N/A	1	F3/B3: 0.2 FIP/BIP: 1.6	10
SC1.2		Feather Tips treated with Buffer E (STAB VIDA)			F3/B3: 0.2 FIP/BIP: 1.6	
SC1.3		Feather Tips boiled at 95ºC, 5’			F3/B3: 0.2 FIP/BIP: 1.6	
SC2.1		Feather Tips boiled at 95ºC, 5’		2	F3/B3: 0.2 FIP/BIP: 1.6	15
SC2.2		Feather Tips boiled at 95ºC, 5’	30 mM Component A (STAB VIDA)		F3/B3: 0.2 FIP/BIP: 1.6	
Reaction time: 2 hours						
SC2.3	65	Feather Tips boiled at 95ºC, 5’	N/A 7.5% (w/v) Component B (STAB VIDA)	2	F3/B3: 0.2 FIP/BIP: 1.6	15
SC1.4	67	DNA from feather tips extracted w/ E.Z.N.A Tissue DNA Kit	N/A 30 mM Component A (STAB VIDA)	1	F3/B3: 0.2 FIP/BIP: 1.6	10

3.3 Sex Determination of *Psittacula krameri*

On the company interest, sex determination testing proceeded to another species, *Psittacula krameri,* from Psittaciformes order. The plan was to design specific primers for this species that only target a fragment of the *CHD1* gene that is specific for the W-chromosome. Therefore, only identifying female

samples. If successful, the primer set would be then modified and tested for other species from this order.

First, a PCR was performed with primers 2550F/2718R and the product was sequenced. For *P. krameri* these primers only amplify a DNA fragment for the W-chromosome, and so they were used in hopes of creating LAMP specific primers for females only.

Later, aligning the PCR products from P2/P8 primers on male and female *P. krameri*, new LAMP primers were designed for both the female and male chromosome. P2/P8 primers generate 2 fragments of the same length for males (ZZ chromosomes), and 2 fragments of different lengths for females (ZW chromosomes) being the fragment from the W chromosome the smallest[65].

3.3.1 Target and LAMP primers for Sex Identification

Both female primers designed target a different region from the CHD-W. The primers designed to identify males target the CHD-Z. All primers were designed with Primer Explorer V5.

Table 3.9: Primer sequences for the Sex Determination of female and male *Psittacula krameri.* (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.).

Female *Psittacula krameri* Sex Determination Primer set 1	
Name	**Sequence (5'-3')**
krameri_F3	STAB VIDA's proprietary sequence #2022MV056
krameri_B3	STAB VIDA's proprietary sequence #2022MV057
krameri_FIP	STAB VIDA's proprietary sequence #2022MV058
krameri_BIP	STAB VIDA's proprietary sequence #2022MV059
krameri_LF	STAB VIDA's proprietary sequence #2022MV060
krameri_LB	STAB VIDA's proprietary sequence #2022MV061
Female *Psittacula krameri* Sex Determination Primer set 2	
Name	**Sequence (5'-3')**
female_krameri_F3	STAB VIDA's proprietary sequence #2022MV062
female_krameri_B3	STAB VIDA's proprietary sequence #2022MV063
female_krameri_FIP	STAB VIDA's proprietary sequence #2022MV064
female_krameri_BIP	STAB VIDA's proprietary sequence #2022MV065
female_krameri_LF	STAB VIDA's proprietary sequence #2022MV066
female_krameri_LB	STAB VIDA's proprietary sequence #2022MV067
Male *Psittacula krameri* Sex Determination Primer set 1	
Name	**Sequence (5'-3')**
male_krameri_F3	STAB VIDA's proprietary sequence #2022MV068
male_krameri_B3	STAB VIDA's proprietary sequence #2022MV069
male_krameri_FIP	STAB VIDA's proprietary sequence #2022MV070
male_krameri_BIP	STAB VIDA's proprietary sequence #2022MV071
male_krameri_LF	STAB VIDA's proprietary sequence #2022MV072
male_krameri_LB	STAB VIDA's proprietary sequence #2022MV073

3.3.1.1 PCR protocol for 2550F/2718R and P2/P8 primers

These primers are from the bird sexing service, and both target different regions of the *CHD1* gene from W and Z chromosomes, that have length polymorphisms, thus can distinguish male and female since it generates products of different lengths for the W and Z chromosomes[65]. These were

used for sequencing of the products, observe the conservation of this region between individuals and subsequent primer design, under STAB VIDA's Bird Sexing Services Routine A.

Table 3.10: PCR thermal-cycle protocol used with PCR bird sexing primers 2550F/2718R and P2/P8.

Phase	n Cycles	Time	Temperature
Initial denaturation	1	3'	95ºC
Denaturation	40	15''	95ºC
Annealing	40	15''	51ºC
Extension	40	15''	72ºC
Final extension	1	7'	72ºC

3.3.2 Sample Collection Kit

Two alternative kits were considered for this test:

- Kit 1: tube from STAB VIDA, with 500 μL of Sample collection buffer A;
- Kit 2: 1 mL tube with an adaptor for a syringe tip and 500 μL of Sample collection buffer A.

3.3.3 Doctor Vida® Pocket Testing with Female *P. krameri* Primer set 1

Testing with Doctor Vida® Pocket started only with the primer set 1 for sex determination of *P. krameri*. The mastermix used was **Reaction Mastermix A.**

The samples for this and further tests with other primers were provided by the Bird Sexing Service. The detection of the products is made by the use of an intercalating dye.

For these tests a total volume of 50 and 63 μL was used for the reaction mixture, of which 10 and 30 μL was the sample volume, respectively. To the reaction tube we added mineral oil to avoid the evaporation of the reaction mixture during the test.

Different reaction additives, sample collection volume and number of feather tips were tested.

Table 3.11: Reaction Conditions (RC) tested for the sex determination of *P. krameri* with Female Sex Determination Primer set 1 on Doctor Vida® Pocket.

RC (#)	T (ºC)	Collection buffer (μL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
DVK1	61	200	Kit 1	1	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK2		300			N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK3		400			N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

(continuation) Table 3.12: Reaction Conditions (RC) tested for the sex determination of *P. krameri* with Female Sex Determination Primer set 1 on Doctor Vida® Pocket.

RC (#)	T (ºC)	Collection buffer (µL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
DVK4	61	500	Kit 1	1	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK5		700			N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK6		1000			N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK7		0	-	1 directly on reaction tube	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK8		1000	Kit 2	1	19.6% (v/v) Component E (STABVIDA)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK9		500			33.4 mM Component A (STABVIDA)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK10			Kit 1	3	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
DVK11			Kit 2			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

3.3.4 Reaction Conditions and Optimizations with Female *P. krameri* Primer set 2

This primer set was first tested with Doctor Vida®, but due to the results obtained further reaction were performed on the qPCR instrument.

Table 3.13: Reaction Conditions (RC) tested for the sex determination of *P. krameri* with Female Sex Determination primer set 2 on Doctor Vida® Pocket and on the real-time instrument.

RC (#)	T (ºC)	Collection buffer (µL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
DVFK1	61	500	Kit 2	3	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
FK1			- (2 µL)			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

(continuation) Table 3.14: Reaction Conditions (RC) tested for the sex determination of *P. krameri* with Female Sex Determination primer set 2 on Doctor Vida® Pocket and on the real-time instrument.

RC (#)	T (ºC)	Collection buffer (μL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
FK2	63	500	- (2 μL)	3	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
FK3	65		- (2 μL)			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
FK4	63		- (2 μL)		0.50×, 0.63×, 0.75×, 0.88× Component C (STABVIDA)	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

3.3.5 Reaction Conditions and Optimizations with Male *P. krameri* Primer set 1

The first test with these primers was performed with Doctor Vida® Pocket, but further testing was performed with the qPCR instrument.

Table 3.15: Reaction Conditions (RC) tested for the sex determination of *P. krameri* with Male Sex Determination primer set 1 on Doctor Vida® Pocket and on the real-time instrument.

RC (#)	T (ºC)	Collection buffer (μL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
DVMK1	61	500	Kit 2	3	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
MK1	63		- (2 μL)		N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
MK2	57		- (2 μL)		N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

3.3.6 Target and LAMP primers for Sample Control

Primers dgLCO1490/dgHCO2198 were used for females and males *P. krameri*. These primers target the mitochondrial COI gene, a universal biomarker used for species ID[75,76]. The primers used were provided by the Genomic Species Identification of STAB VIDA. From the alignment of the PCR products, internal control LAMP primers were designed, specific to the *COI* gene of *P. krameri*.

Table 3.16: Primer sequences for the Sample Control of Sex Determination of *Psittacula krameri.* (Note: Sequences are omitted to maintain confidentiality. To get access please contact STAB VIDA Lda.)

Sample control Primer set 1	
Name	**Sequence (5'-3')**
COI_krameri_F3	STAB VIDA's proprietary sequence #2022MV074
COI_krameri_B3	STAB VIDA's proprietary sequence #2022MV075
COI_krameri_FIP	STAB VIDA's proprietary sequence #2022MV076
COI_krameri_BIP	STAB VIDA's proprietary sequence #2022MV077
COI_krameri_LF	STAB VIDA's proprietary sequence #2022MV078
COI_krameri_LB	STAB VIDA's proprietary sequence #2022MV079

3.3.7 Reaction Conditions and Optimizations with Sample Control Primer set 1

Table 3.17: Reaction Conditions (RC) tested for the internal control *P. krameri* Female Sex Determination on Doctor Vida® Pocket and on the real-time instrument.

RC (#)	T (ºC)	Collection buffer (µL)	Sample collection	Num. feather tips	Reaction additives	Primer Conc. (mM)
DVCOI1	61	500	Kit 2	3	N/A	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
COI1			- (2 µL)			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
COI2	63		- (2 µL)			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
COI3	65		- (2 µL)			F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
COI4	63		- (2 µL)		0.50×, 0.63×, 0.75×, 0.88× Component C	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4

3.3.8 Multiplexing with Female *P. krameri* Sex Determination Primer set 1 and Sample Control Primer set 1

Table 3.18: Reaction Conditions (RC) tested for the Multiplex test with the sample control and *P. krameri* Female Sex Determination primer sets 1 on the real-time instrument.

RC (#)	T (ºC)	Collection buffer (µL)	Sample volume (µL)	Num. feather tips	Reaction additives	Primer Conc. (mM)

1	61	500	2 μL	3	0.50× Component C	F3/B3: 0.2 FIP/BIP: 1.6 LF/LB: 0.4
2	63					
3	65					

3.4 Sample treatment

Different sample treatments were tested, including E.Z.N.A Tissue kit for DNA extraction and purification from feather tips, following the manufacturer's instructions. Briefly, feather tips in a certain amount of Sample collection buffer A were subjected to the lysis step for approximately 3 hours at 55ºC, and then centrifuged at maximum speed (≥10000×*g*) to pellet cellular debris. After adjusting binding conditions, the sample was transferred to the HiBind® DNA Mini Column, to which the DNA binds. The membrane is washed to eliminate salts and other contaminants. After completely drying the column to remove trace amounts of ethanol, the DNA is eluted.

When using Buffer E, 20 μL of this solution was transferred to 3 feather tips, vortexed and incubated for approximately 30 minutes. When boiled, 2 to 3 feather tips were transferred to 20 μL of Sample collection buffer A, and incubated at 95ºC for 5 minutes.

4

RESULTS AND DISCUSSION: PART 1

4.1 Diagnosis of Swine Dysentery

4.1.1 Market Analysis

This Project began with a Market Analysis to determine what type of tests are more recurringly solicited in veterinary practices, as well as to what diseases have a higher incidence and which type of diagnosis are used. A research for rapid diagnostic tests currently in the market was also conducted, according to the diseases previously determined to have a high incidence.

After conducting an interview with Veterinary Doctor Tânia Freitas, from BioVETnatura (Santana, Madeira), was determined that diseases/pathogens such as Parvovirus, *Giardia duodenalis*, *Anaplasma* spp., *Leishmania infantum* and *Borrelia burgdorferi* greatly affect the pet's life and are the ones that are diagnosed more frequently in her clinic. However, the rapid diagnosis of these diseases is exclusively done with immunological tests. The molecular diagnosis is made by PCR in qualified laboratories, with an average 2 to 6 days' time window between sample transportation and delivery of results. The next table sums up this information.

Table 4.1: Summary of the initial market study, with the diseases and pathogens with higher prevalence and example rapid tests for its diagnosis, as well as the time that takes to receive the result when the sample is sent to a lab, in this case Cedivet.

	Company	**Method of detection**	**Sample**	**Time to result**	**PCR Time to result (Cedivet)**
Feline leukemia virus and Feline immunodeficiency virus	Kruuse	ELISA	Whole blood, plasma, serum	10 min	2-6 days
Borrelia burgdorferi	Zoetis	Lateral flow sandwich assay	Whole blood, plasma, serum	8-10 min	2-6 days

(continuation) Table 4.2: Summary of the initial market study, with the diseases and pathogens with higher prevalence and example rapid tests for its diagnosis, as well as the time that takes to receive the result when the sample is sent to a lab, in this case Cedivet.

	Company	Method of detection	Sample	Time to result	PCR Time to result (Cedivet)
Parvovirus	Uranovet	Immunochromato-graphy	Faeces	5-10 min	2-6 days
Giardia duodenalis	Uranovet	Immunochromato-graphy	Faeces	5-10 min	-
Anaplasma phago-cytophilum and *A. platys*	Uranovet	Immunochromato-graphy	Whole blood, plasma, serum	15 min	2-6 days
Ehrlichia canis	Zoetis	Double antigen sandwich assay	Whole blood, plasma, serum	10 min	2-6 days
Dirofilaria immitis	Uranovet	Immunochromato-graphy	Whole blood, plasma, serum	15 min	-
Leishmania infantum	Uranovet	Immunochromato-graphy	Whole blood, plasma, serum	15 min	2-6 days

To receive a result from a rapid molecular diagnostic test one can expect a waiting time ranging from twenty minutes up to one and a half hours. As so, rapid molecular diagnostic tests cannot compete with immunological rapid tests when it comes to the time to result. Furthermore, the cost of production of a rapid POCT molecular diagnostic test is considerably higher than that of an immunological test, and as so the selling price would need to be higher to achieve the same profit. Therefore, was agreed that the development of a POCT molecular diagnostic test, for these specific diseases, would not be of interest to the company since it wouldn't be able to compete with the prices of rapid immunological tests.

After a brief deliberation over the ramifications of the matter at hand, the possibility of a partnership with Exopol, a client of STAB VIDA, was put forth. An initial meeting was setup to discuss current statistics related to the most recurring solicited tests, and to which type of animals these tests were carried out on. It was concluded that the swine molecular diagnostic tests (qPCR) are the more solicited by veterinarians and farmers in Spain, more specifically the diagnoses of digestive tract related diseases. The following figure (**Figure 4.1**) shows the statistics related to the pathogens analysed by qPCR on the digestive tract diagnostic panel in the last five years by Exopol. Although in the last five years the highest percentage of positive cases per number of tests performed belongs to *Clostridium perfrigens*, the pathogen with the greatest number of diagnostic tests performed against is *Brachyspira hyodysenteriae*. Our Exopol contacts have mentioned that the incidence of *Brachyspira hyodysenteriae* is seasonal, being higher during the colder and humid seasons, and that a growing incidence of the pathogen behind Swine Dysentery in Spain has also been observed.

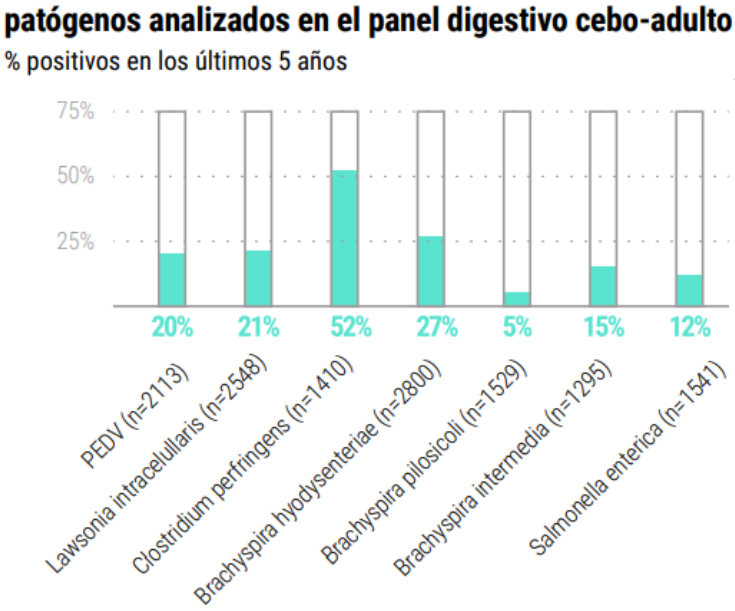

Figure 4.1: Pathogens analysed by Exopol's laboratory on the digestive tract diagnostic panel by qPCR. Taken from Exopol's "La etiología porcina en estadísticas"[77].

Exopol has recently published a diagram related to the incidence of infection of certain pathogens in swine in the last three years, related to the number of diagnostic tests made and its positive cases in certain independent provinces in Spain. The figure bellow (**Figure 4.2**) represents the incidence of *B. hyodysenteriae* in that period. The province with the higher incidence is Murcia, however, its corresponding study population is one of the lowest (n=13).

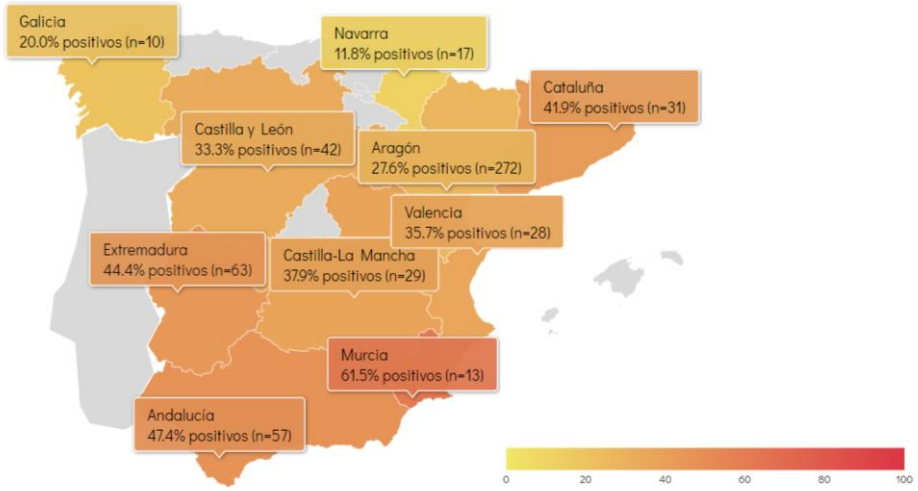

Figure 4.2: Incidence of *B. hyodysenteriae* in the last three years in Spain's independent provinces. The province with the greatest incidence is Murcia, with the highest number of positive results, although the total number of tests made is only 13. Aragón for example has a total of 272 tests made, and an incidence of 27.6%, which totals 75 positive cases, being the province with the higher number of positive cases. Diagram from https://www.exopol.com/php_fm/mapa_calor_ccaa.php (consulted at 2022-07-23).

Taking all of this into account, STAB VIDA and Exopol agreed on a partnership for the development of a diagnostic POCT test for the detection of *B. hyodysenteriae*, the causing agent of SD. Exopol

took responsibility in providing the targets for primer design alongside with samples for testing. STAB VIDA would then be in charge of developing the test and perform the clinical validation on Exopol's facility.

4.1.2 Proof-of-Concept for the Detection of *B. hyodysenteriae*, Bhyo-LAMP

Reaction Condition B1.1

After analysing the target sequences and designing the primer set for the detection of *B. hyodysenteriae*, the first test was performed under **RC B1.1** (**Table 2.4**). The results can be visualized on **Table 4.3**.

The primers were able to identify all positive samples, however with a false positive i.e., the amplification of the non-template control (NTC). The true positive samples had a TtP between 18 to 45 minutes. The TtP for the non-template control was 22 minutes, which could invalidate the true positive sample results which TtP was beyond 22 minutes.

Table 4.3: Bhyo-LAMP test results with DNA samples under **RC B1.1**, at 65ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit for the tested samples.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP RC B1.1 TtP (min)
Positive, 161378	27.96	44.98
Positive, 164188	25.86	35.23
Negative, 164278	0	0
Positive Control, 163998	-	18.36
NTC	-	22.30

Reaction Condition B1.2

One way of increasing the specificity of primer hybridization is using a higher reaction temperature. This increases the stringency of the primers to the template, theoretically increasing the specific hybridization and decreasing secondary structures between primers[78]. This method has been used in PCR, when one increases the annealing temperature.

As so, the primers were tested with a temperature of 68ºC (**RC B1.2**, **Table 2.4**).

Table 4.4: Bhyo-LAMP test results with DNA sample under **RC B1.2**, at 68ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP RC B1.2 TtP (min)
Positive, 161378	27.96	0
Negative, 164278	0	0
Positive Control, 163998	-	0
NTC	-	0

The temperature increase was not favourable for the primer's annealing to the template. The melting temperatures (50 nM NaCl) of primers F3 and B3 from primer set 1 are 47.21ºC and 43.25ºC (Multiple Primer Analyser from ThermoFisher, based on the method described by Breslauer *et al* (1986)[79]), respectively, which could explain the lack of amplification when performing the reaction at higher temperatures such as 68ºC. One could also argue that the decrease on the amount of sample is related to this, but as will be seen in the next conditions tested with the same sample volume, it is less probable to be the cause.

Reaction Condition B1.3

A new reaction condition was tested, **RC B1.3** (**Table 2.4**), without the loop primers due to the prevalence of false positives on the previous reaction conditions, and because the increase in temperature inhibited the reaction. This condition was tested to assess if primer dimers and secondary structures between the loop primers could be the cause of the false positive results. The results can be consulted on **Table 4.5**.

The reaction in the absence of loop primers did not amplify the positive sample, and amplified a negative sample. This could mean that the loop primers in this set are essential to amplification, and that the unspecific amplification could also be due to the core primers.

Table 4.5: Bhyo-LAMP test results with DNA samples under **RC B1.3**, at 65ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP RC B1.3 TtP (min)
Positive, 161378	27.96	0
Negative, 164278	0	50.05
Positive Control, 163998	-	0
NTC	-	0

Reaction Condition B1.4

Since the absence of the loop primers was not ideal, different concentrations were tested:

- 1× Primer Concentration (0.8 μM loop primers)
- 0.3× Primer Concentration
- 0.2× Primer Concentration

It has been noticed that the use of different primer concentrations could aid on increasing the TtP between true positives and false positives.

Table 4.6: Bhyo-LAMP test results with DNA samples under **RC B1.4**, at 65ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit. In this condition 3 different primer concentrations were tested.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP 1× TtP (min)	Bhyo LAMP 0.3× TtP (min)	Bhyo LAMP 0.2× TtP (min)
Positive, 161378	27.96	53.87	0	0
Negative, 164278	0	33.77	38.97	55.75
Positive Control, 163998	-	19.32	32.54	33.03
NTC	-	31.51	44.37	37.66
ΔTtP (NTC - PosC)	-	12.19	11.83	4.63
ΔTtP (Neg - PosC)	-	14.45	6.43	22.72

There was a delay on the amplification of all samples, although not quite as significative between the **RC B1.1** and **RC B1.4 1×** probably due to the fact that the only difference between these two conditions was the concentration of the loop primers.

None of these conditions can be considered ideal since in all of them both the NTC and Negative sample were amplified. The condition that produced greater differences on the TtPs between the NTC and the Positive Control was the primer concentration 0.2×, and between the Negative samples and Positive Control was the primer concentration 0.3×. However, only the condition with the primer concentration 1× amplified the positive sample and due to this, conclusions about these conditions are hard to draw, but it does not seem suitable for this set of primers.

Reaction Condition B2.1

A new primer set was designed since the previous was generating false positives. Primarily, the set did not contain loop primers since PrimerExplorer V5 was not able to design them with the core primers. So initially, the primer set was tested without the loop primers (**RC B2.1**, **Table 2.4**).

There is a clear difference between the Primer set 1 and 2, at the same temperature, the same volume of sample and without loop primers: the amplification of the negative sample was delayed for approximately 7 minutes and both the positive sample and positive control were amplified. Comparing

this reaction condition with **RC B1.1**, the TtP for the positive sample 161378 was lowered (44.98 vs 35.01 minutes) even in the absence of loop primers, and it did not amplify the non-template control, which demonstrates a higher sensitivity and specificity for this primer set relatively to the previous.

Table 4.7: Bhyo-LAMP test results with DNA samples under **RC B2.1,** without loop primers, at 65ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP RC B2.1 TtP (min)
Positive, 161378	27.96	35.01
Negative, 164278	0	57.31
Positive Control, 163998	-	27.01
NTC	-	0

Reaction condition B2.2

Loop Primers were manually designed and tested with **RC B2.2** (**Table 2.4**). For this reaction a higher temperature was also used since in the previous, at 65ºC and without these primers we obtained false positives.

In the absence of these primers the amplification of the positive sample was faster than with the Primer set 1 with its loop primers (**RC B1.1**), then it was expected that with the loop primers the amplification of the positive samples would have an even lower TtP. The results, in duplicates, of this reaction condition can be observed on **Table 4.8**.

Table 4.8: Bhyo-LAMP test results with DNA samples under **RC B2.2**, at 67ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit and **RC 1.3**, to compare the primer sets performances.

Samples DNA	EXOone BHYO Cq	Bhyo LAMP RC B2.2 TtP (min)	Average TtP (min)	ΔTtP (RC B2.1 – RC B2.2)
Positive, 161378	27.96	26.50 25.19	25.85	9.16
Negative, 164278	0	0 0	0	57.31
Positive Control, 163998	-	20.32 20.71	20.51	6.50
NTC	-	0 0	0	0

The results between duplicates were reproducible, with an average of 0.85 minutes difference between duplicates of each sample. The TtP of primer set 2 of all samples compared to primer set 1 although in a different temperature was lower. Besides the negative sample did not amplify. Owing to

this, primer set 2 seems to generate more specific and rapid results. As so, we started testing them with direct samples.

4.1.3 Optimizations

Reaction condition B2.3

The direct samples tested consisted of faeces and rectal swabs, homogenized on 50 μL of Exo-pol's buffer. The samples were transferred directly into the reaction mixture without any sample treatment or incubation time. This reaction was performed at 67ºC as the previous one. The results, in duplicates, can be consulted on the following table.

Table 4.9: Bhyo-LAMP test results with direct samples under **RC B2.3**, at 67ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit.

Direct Samples	EXOone BHYO Cq	Bhyo LAMP RC B2.3 TtP (min)
Positive, Faeces 164188	28.86	0 52.55
Positive, Rectal Swab 164233	27.15	36.93 30.53
Negative, Faeces 164308	0	0 0
NTC	-	0 0

The sample with the lowest TtP was the one from rectal swabs, with an average TtP of 33.73 minutes between duplicates. In addition, the difference between the TtP of the positive sample from rectal swabs and faeces is close to 22 minutes, and one of the duplicates of the faecal samples did not amplify. This could be due to the presence of inhibitors in this type of sample, although in some studies, when comparing rectal swabs and stool for molecular detection of pathogens, there was observed great concordance of results between the sample types, even when the number of target DNA/RNA copies was significantly lower in rectal swabs[80,81]. However, Gibory *et al* (2017) did experience false negatives in faecal samples.

When performing PCR with faecal samples the inhibition of the polymerase has been observed, and the possible inhibitors characterized[82–84]. From human stools Monteiro *et al* (1997) characterized PCR inhibitors in faeces as polysaccharides without sialic acid and negatively charged. Furthermore, it was hypothesized that these compounds were likely from a vegetable origin. Previously, Widjojoatmodjo *et al* (1992) had already characterized two other PCR inhibitors in faeces. Bilirubin, a product of haemoglobin degradation inhibited the reaction in such a low concentration as 10 μg/mL, and bile salts in concentrations of 50 μg/mL. More recently phytic acid has also been classified as a PCR inhibitor[85]. Phytic acid, known in its complex form as phytate phosphorus, is mostly present in plants, seeds and grains. This molecule has the task to store inorganic phosphate in its structure and chelates with divalent

and trivalent cations[85]. This last property is hypothesized to be the cause of PCR inhibition, since the polymerase is sensitive to fluctuating levels of magnesium (Mg^{2+})[85]. Pigs feed is mainly composed of grains and so it has high quantities of phytic acid, the same goes for fodder. Now, monogastric animals such as swine do not have the ability to digest this compound thus it is present in faeces in high quantities. However, recent articles described the advantage of introducing dietary phytase in pigs feed, in order to break down phytic acid and increase the bioavailability of inorganic phosphorous, which aids significantly the growing process in grower-finisher pigs[86,87]. Inclusively, Purina (Land O'Lakes, Inc.) sells a feed for Grower-Finisher that contains phytase[88]. Studies regarding the inhibition of faecal matter in LAMP and characterization of possible inhibitors are lacking. Although LAMP has been observed and proven to be more robust and less sensitive to some inhibitors than PCR, there could be some correlation between PCR inhibitors and LAMP inhibitors.

Taking the aforementioned into account, and considering a possible correlation, it is unlikely that phytic acid is to blame since phytase is now present in swine feed, breaking down the phytic acid. Some of the characterized inhibitors may be present, which could explain the results with the faecal samples, and the TtP differences.

Reaction condition B2.4

The question of whether or not the results from **RC B2.3** could also be the result of low availability of the target DNA was proposed and tested, especially since crude samples were tested without any previous lysis step.

As so, the direct samples were incubated at 95ºC for 5 min and then tested.

The results obtained from this experiment were not the expected, since most of the positive samples were not detected.

Since these samples were not purified DNases might be present, degrading the genetic material overtime. In addition, these samples were kept at -80ºC, and the freezing and thawing cycles could further DNA degradation[89].

Three out of seven positive samples were detected, and the sample 164233 had a TtP almost identical to the Cq from the qPCR kit, and was lower than the TtP without sample pre-treatment. Although in **RC 2.3** the TtP obtained for this sample was 30 and 36 min.

Table 4.10: Bhyo-LAMP test results with direct samples under **RC B2.4,** at 65ºC for 60 minutes and comparison with the Cq of the EXOone BHYO kit.

Direct Samples	EXOone BHYO Cq	Bhyo LAMP w/o pre-treatment TtP (min)	Bhyo LAMP RC B2.4 TtP (min)
Positive, Faeces 164188	28.86	0	40.48
Positive, Rectal Swab 164233	27.15	55.12	27.31
Negative, Faeces 164278	0	0	0
Positive, Rectal Swab 164293	28.25	0	0
Negative, Faeces 164308	0	0	0
Positive, Faeces 164959	29.08	0	0
Positive, Faeces 164847	24.94	0	57.61
Positive, Rectal Swab 164835	31.81	0	0
Positive, Rectal Swab 161378	27.96	0	0
Negative, Rectal Swab 164375	0	0	0
Pos Control, Isolate	-	19.42	-
NTC	-	0	0

Reaction condition B2.5

New faecal samples were received, dry faeces. These were useful to test different sample collection formats, discuss and test possible collection buffers for the final collection kit, and evaluate the possibility of inhibition of the reaction by the previous samples, since the samples were homogenized on Exopol's buffer.

Four different swabs were tested: Microbrush from Microbrush International (MRP400), a Sterile Viscose swab from Deltalab (300284), a Sterile Viscose swab from Citotest Scientific (2122-0201) and the swab from a collection tube from STAB VIDA. The material collected was homogenized in the collection buffer (100 µL of Sample collection buffer A).

Sample collection with Microbrush was performed better than expected, since this brush is used for dental restoration. Stool adhered well to the filaments of the brush, and the material was released easily on the collection buffer. However, the faecal samples received were not completely solid, and if this was not the case the sample would probably not adhere so well. The Viscose swab from Deltalab collected the sample fairly well, but when emerged in the collection buffer it absorbed approximately half of its volume. The swab from Citotest Scientific and the swab from STAB VIDA's behaved similarly to the Microbrush.

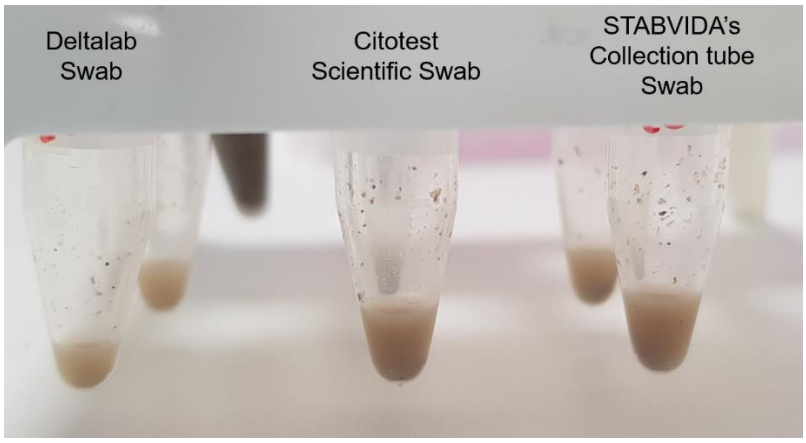

Figure 4.3: Volume comparison of Sample collection buffer after sample homogenization. As mentioned, the swab from Deltalab absorbed approximately half of the collection buffer during homogenization.

Table 4.11: Bhyo-LAMP under **RC B2.5**, in which were used 4 different swabs for collection of the faecal matter, and homogenization in 100 μL of Sample collection buffer A. Genetic material from the faecal swabs performed with the Microbrush was extracted and purified for testing with EXOone BHYO qPCR kit.

	EXOone BHYO Cq	**Bhyo LAMP RC B2.5 TtP (min)**			
Faecal Swabs	DNA Microbrush	Microbrush	Deltalab	Citotest	Collection tube
Negative, 170610	0	0	0	0	0
Positive, 171695	30.82	0	0	0	0
Positive, 171652	32.56	0	-	-	-
Positive, 170800	30.53	0	-	-	-
Positive, 170254	30.84	0	-	-	-
Negative, 171771	0	0	-	-	-
Pos Control, Isolate	-	37.12	26.94	29.31	30
NTC	-	0	0	0	0

The faecal swabs performed with the Microbrush underwent DNA extraction and purification to test on the EXOone BHYO qPCR kit. The lack of amplification suggested inhibition of the reaction due to the sample or that the swabs did not collect enough material for detection. It was later confirmed with Exopol which samples were positive and negative, and only the result for the sample 171771 was in discordance. Therefore, it was assumed the lack of amplification was caused by inhibitors in the samples, and started studying possible DNA extraction methods adaptable to POCT.

Reaction condition B2.6

The previous results could also be the consequence of lack of sensitivity from the primer set. To test this possibility, the extracted DNA from different faecal swabs was used, to compare with the results of the extracted DNA from faecal swabs with the Microbrush on the EXOone BHYO qPCR kit.

None of the positive samples were detected, which could indicate the lack of sensitivity of these primers, since extracted DNA of all positive samples was detected with the qPCR kit.

Table 4.12: Bhyo-LAMP under **RC B2.6,** at 67ºC for 60 minutes, using the DNA extracted from faecal swabs, and comparison of the LAMP results with Exopol's qPCR kit.

	EXOone BHYO Cq				**Bhyo LAMP RC B2.6 TtP (min)**			
Samples	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube
Negative, 170610	0	0	0	0	0	0	0	0
Positive, 171695	30.82	31.37	29.35	29.71	0	0	0	0
Positive, 171652	32.56	-	-	-	0	-	-	-
Positive, 170800	30.53	-	-	-	0	-	-	-
Positive, 170254	30.84	-	-	-	0	-	-	-
Negative, 171771	0	-	-	-	0	-	-	-
Pos Control, Isolate	-	-	-	-	0	32.70	34.80	34.74
NTC	-	-	-	-	0	0	0	

Reaction condition B3.1

New primers were designed with Primer Explorer V5 due to lack of sensitivity of the previous set. The new primer set was tested with DNA extracted from faecal swabs, at 65ºC.

This primer set appears to be more sensitive than the previous. It was able to detect all the positive DNA samples from the Microbrush swab, and only failed to detect the positive DNA sample from

Deltalab. These results are a clear improvement to the previous ones, so testing continued with these primers.

Table 4.13: Bhyo-LAMP under **RC B3.1,** at 65ºC for 60 minutes, using the DNA extracted from faecal swabs, and comparison of the LAMP results with Exopol's qPCR kit.

	EXOone BHYO Cq				**Bhyo LAMP RC B3.1 TtP (min)**			
Samples	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube
Negative, 170610	0	0	0	0	0	0	0	0
Positive, 171695	30.82	31.37	29.35	29.71	32.71	0	27.19	46.40
Positive, 171652	32.56	-	-	-	55.30	-	-	-
Positive, 170800	30.53	-	-	-	36.26	-	-	-
Positive, 170254	30.84	-	-	-	34.05	-	-	-
Negative, 171771	0	-	-	-	0	-	-	-
Pos Control, Isolate	-	-	-	-	20.81			
NTC	-	-	-	-	0			

Reaction condition B3.2

In the previous reaction condition six out of the seven positive samples tested were detected. However, two of them were detected only after 40 minutes. On an effort to try to increase the amplification speed, Component A (STAB VIDA) at 30 mM was added. This component A is supposed to increase the speed of amplification and sensitivity of LAMP.

Table 4.14: Bhyo-LAMP under **RC B3.2,** at 65ºC for 60 minutes, using the DNA extracted from faecal swabs, with 30 mM Component A (STAB VIDA).

	Bhyo LAMP RC B3.2 TtP (min)			
Samples	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube
Negative, 170610	0	0	0	0
Positive, 171695	56.52	56.22	44.38	0

(continuation) Table 4.15: Bhyo-LAMP under **RC B3.2,** at 65ºC for 60 minutes, using the DNA extracted from faecal swabs, with 30 mM Component A (STAB VIDA).

Bhyo LAMP RC B3.2
TtP (min)

Positive, 171652	0	-	-	-
Positive, 170800	57.65	-	-	-
Positive, 170254	37.35	-	-	-
Negative, 171771	0	-	-	-
Pos Control, Isolate	31.57			
NTC	0			

Five out of the 7 positive samples were detected, and with a higher TtP than on the previous reaction condition (**B3.1**). The TtP from the DNA of the Microbrush swab sample 170254 was closest to the previous reaction, with a difference of 3.3 minutes. Taking into account the lack of sensitivity and increase of the time-to-positives, this condition was considered not ideal for the test.

Reaction condition B3.3

The previous condition was carried out with a loop primer concentration of 0.4 μM. **RC B3.3** was tested with a loop primer concentration of 0.8 μM, since theoretically it could help increase the amplification speed. This condition was performed in the presence and absence of Component A (30 mM).

Table 4.16: Bhyo-LAMP under **RC B3.3,** at 65ºC for 60 minutes, using the DNA extracted from faecal swabs, with and without 30 mM of Component A (STAB VIDA), and loop primer concentration of 0.8 μM.

Bhyo LAMP RC B3.3
TtP (min)

Samples	W/o Component A				W/ Component A			
	DNA Microbrush	DNA Deltalb	DNA Citotest	DNA Collection tube	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube
Negative, 170610	50.14	0	0	56.93	0	0	0	0
Positive, 171695	43.41	55.49	43.16	42.71	41.96	0	33.81	0
Positive, 171652	0	-	-	-	0	-	-	-
Positive, 170800	43.97	-	-	-	0	-	-	-
Positive, 170254	37.02	-	-	-	0	-	-	-
Negative, 171771	0	-	-	-	0	-	-	-

(continuation) Table 4.17: Bhyo-LAMP under **RC B3.3,** at 65ºC for 60 minutes, using the DNA extracted from faecal swabs, with and without 30 mM of Component A (STAB VIDA), and loop primer concentration of 0.8 μM.

Bhyo LAMP RC B3.3
TtP (min)

Samples	W/o Component A	W/ Component A
Pos Control, Isolate	18.36	24.46
NTC	0	0

In the reaction without Component A six out of seven positive samples were detected, but two negative samples were identified as positive. In the presence of Component A, only 2 out of the 7 positive samples were identified, so this reaction condition had a worse performance compared to the previous (**RC B3.2**).

Reaction condition B3.4

As observed in previous experiences, even the faecal swabs without further sample treatment still had an inhibitory effect over the reaction. As so, different methods of DNA purification were tested. The one used for this **RC** was Column format A, Filter A, based on gravity flow gel filtration.

The faecal sample was collected with Microbrush and homogenized in 100 μL of Sample collection buffer A, and the column was used according to the procedure described on **Material and Methods: Part 1, subsection 2.5 Sample Treatments.** From the same sample, 2 eluted fractions were collected for testing at 65ºC.

Table 4.18: Bhyo-LAMP under **RC B3.4,** at 65ºC for 60 minutes, testing the 1st and 2nd fraction of the eluted sample using Column format A, Filter A.

Bhyo LAMP RC B3.4
TtP (min)

Sample	1st eluted	2nd eluted
Positive, 171695	42.51	51.77
NTC	0	

Both elution fractions came out as positive, thus both contained the target DNA. The first fraction had a lower TtP, a difference of approximately 9 minutes to the second fraction, which could mean this fraction carried the higher amount of target DNA or was the purest fraction. Though the results of this experiment were positive, the TtPs obtained were higher compared to the ones with **RC 3.1.** Besides, a gravity flow-based method would not be ideal for POCT due to the pipetting steps and waiting time for the collection of the sample for testing.

Reaction condition B3.5

To understand the inhibitory effect of faeces collected with a swab such as Microbrush and homogenized in Sample collection buffer A, different dilutions of the sample spiked with the Positive control were tested. Taking into account that the observed TtP for the positive control remains relatively constant when the reaction conditions are the same, the real sample's inhibition degree should therefore be observable.

For that, a Microbrush was used to collect the sample and homogenized in 50 μL of Collection Buffer instead of 100 μL to concentrate the sample. Then 1:2, 1:5, 1:10 and 1:50 dilutions of this sample were made. To each reaction tube, a mixture of 1 μL of the positive control and 2.3 μL of the correspondent dilution was added.

Table 4.19: Bhyo-LAMP under **RC B3.5,** at 65ºC for 60 minutes, testing dilutions from faecal swab with a Microbrush, to assess the inhibition of the reaction by this type of sample.

Bhyo LAMP RC B3.5

TtP (min)

Samples Faecal swab	W/o Sample dilutions	W/o Pos Control (3.3 μL sample)	W/ Pos Control (1 μL)			
			1:2 dilution	1:5 dilution	1:10 dilution	1:50 dilution
Positive, 171695	-	0	0	34.82	25.17	22.09
Pos Control, Exopol	24.38	-	-	-	-	-
NTC	0					

From this experience it was confirmed the hypothesis of this type of sample inhibiting the reaction. The positive control alone had a TtP of 24.38 min and the direct faecal swab did not amplify. The mixture of the positive control with the 1:2 dilution of the faecal swab had a TtP of 34.82 min, with the 1:5 dilution a TtP of 25.17 min, and with 1:50 dilution a TtP of 22.09 min. Notice that the TtP for the higher dilution factor was lower than that of the positive control alone because the inhibitors were more diluted, and also because this mixture theoretically has higher amounts of the target DNA. As for the other dilutions, the TtPs were higher than that of the positive control probably because the inhibitors were still in concentrations that were not tolerated by the enzyme. Therefore, the goal was to find a DNA extraction or sample purification method adaptable to POCT.

Reaction condition B3.6, B3.7 and B3.8

With the results obtained with the Column format A, Filter A method and the knowledge acquired with the previous experience, three other purification methods were tested. For these Reaction conditions, a scoop spatula was used to collect the faecal matter. This resulted in approximately 0.05 g of faeces homogenized in 100 μL of Sample collection buffer A.

The first method tested was Chelex-100 for extraction of DNA and/or removal of impurities that could inhibit the reaction. Chelex-100 is a resin that binds to polyvalent metallic ions such as copper and iron. This resin is usually used in suspension and mixed with the sample. Then incubated at 95ºC

for 2 to 10 minutes. Then the mixture is centrifuged, filtrated or left for the beads to deposit, and the supernatant is collected [90]. Walsh *et al* (1991) described the use of Chelex-100 for DNA extraction of forensic samples, using Chelex-100 5% and incubating with the sample at 95ºC[91]. It was observed that the samples incubated at 95ºC without Chelex did on amplify on PCR. On the other hand, the samples incubated with Chelex-100 did. There is then the hypothesis that Chelex-100 has a protective role over DNA when heated to 95ºC because it sequesters metallic ions that could cause damage to nucleic acids. Besides, it was verified that the sample extracted with Chelex had a lower probability of containing PCR inhibitors comparing to the other methods tested. Later, Yang *et al* (2008) used a suspension of Chellex-100 5% with 0.2 mg of protease K for bacterial DNA extraction from stool [92]. Chelex 5% and the sample collected were incubated at 56ºC for 30 minutes. Comparing this method with the other tested in this study, which included a DNA extraction kit for faeces, the Chelex-100 method was the one that produced the purest DNA, was the simplest and the most unexpensive.

Chelex-100 was then tested in **RC B3.6.** For **RC B3.7** Column format B, Filter A was used. For **RC B3.8** a syringe filter of PVDF (Polyvinylidene Fluoride) with pores of 0.45 μm in diameter. The procedures for all these methods can be consulted on **Materials and Methods: Part 1, subsection 2.5 Sample Treatments**.

For each sample collected of the methods used, a 1:2 dilution of that sample was tested to observe if the dilution of the inhibitors could help increase the TtPs, although the target DNA would also get diluted. The positive control was also tested, and a mixture of 1:2 positive control to the collected sample to assess the inhibitory effect.

Table 4.20: Bhyo-LAMP under **RC B3.6, B3.6** and **B3.7,** at 65ºC for 60 minutes. Chelex-100, Column format B, Filter A and a PVDF syringe filter were used, respectively.

	Bhyo LAMP RC B3.6 TtP (min)		**Bhyo LAMP RC B3.7 TtP (min)**		**Bhyo LAMP RC B3.8 TtP (min)**	
Samples Scoop spatula	No dilution	1:2 dilution	No dilution	1:2 dilution	No dilution	1:2 dilution
Positive, 171695	0	0	56.22	0	0	0
Pos Control, Exopol	25.82	0	25.82	22.59	25.82	24.08
NTC	0					

The only successful method of DNA extraction on this experiment was the Column format B, Filter A (**RC B3.7**) since it was the only method of which the sample tested positive. Besides, the mixture of the positive control with the eluted from the column resulted on a lower TtP than that of the positive control alone, which shows that the method successfully eliminates possible inhibitors. As for the 1:2 sample dilution tested from Column format B, Filter A, it was not detected. It was hypothesized that with the dilution the target DNA concentration was also diluted to levels below the ones detected by the reaction.

As for the Chelex, there are two possible reasons for its lack of success. Either due to the mixture of Chelex and the sample not having been heated as recommended, or more likely due to the fact that the available Chelex-100 was already past its expiration date which could have altered the beads' properties.

The PDVF filter used was somewhat unsuited for the process being carried out. In addition to its material being slightly hydrophobic, the suggested usage volume for a filter with 25 mm of diameter is 10 mL, and the volume sample available for testing was below this measurement. As so, of a filtered volume of 2300 μL with only 0.05 g of faeces, it was only possible to filter a total volume of 100 μL. Keeping in mind that the initial sample had been diluted, and that the volume filtered was far below that of the recommended amount, can be concluded that a great portion of the target DNA could have been retained by the filter itself and that the filtered portion held DNA concentrations below the detectable concentration level.

Reaction condition B3.9

After testing what were considered the most viable methods for POCT DNA extraction, testing with a lower temperature (63ºC) was suggested with DNA samples. Decreasing the reaction temperature decreases the stringency, which could result in unspecific binding and amplification. On the other hand, lower temperatures can facilitate the binding of the primers to the target and increase the speed of the reaction (lower TtP)[78].

As so, this primer set was tested with DNA samples, and the DNA extracted with E.Z.N.A tissue kit from faecal swabs.

Table 4.21: Bhyo-LAMP under **RC B3.9** at 63ºC for 60 minutes, using DNA samples and DNA extracted from faecal swabs.

DNA Samples	Bhyo LAMP RC B3.9 TtP (min)
Positive, 164188	13.54
Positive, 164233	15.06
Negative, 164308	40.06
Positive, Microbrush 171695	0
Positive, Column format B, Filter A 171695	56.75
Pos Control, Exopol	16.08
NTC	0

The times-to-positive of the DNA samples (164188 and 164233) are the lowest obtained so far for this type of sample amongst the 3 primer sets tested. Thus, and due to the previous results, this temperature and primer set 3 were chosen to further tests on Doctor Vida® Pocket.

Reaction Condition B3.10

Reaction Mastermix B has been observed to be faster than Reaction Mastermix A. As so, this mastermix was tested with primer set 3 at 65ºC.

Table 4.22: Test with *B. hyodysenteriae* primers for comparison of Reaction Mastermix A and B, at 65ºC for 60 minutes, with DNA samples.

DNA Samples	Reaction Mastermix B TtP (min)	Reaction Mastermix A TtP (min)
Pos Control, Exopol	24.49	12.17
NTC	0	0

Although the claims of this mix being faster than the previously used, this experience showed the exact opposite. Thus, further tests were conducted only with Reaction Mastermix A.

4.1.4 Proof-of-Concept for the Sample Control, *E. coli*

Reaction condition E1.1

Testing with the first primer set designed for the detection of *E. coli* started with DNA samples and at 65ºC (**RC E1.1**).

Table 4.23: Results from the LAMP test for the sample control in nucleic acid samples under **RC E1.1**, for 60 minutes at 65ºC and comparison between the Cq of samples tested on EXOone kit as the endogenous control and the results obtained with LAMP.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E1.1 TtP (min)
Positive, 161378	27.33	10.23
Positive, 164188	24.24	10.07
Positive, 164278	19.25	9.11
Negative Control, 163998	-	39.75
NTC	-	31.16

The TtP of the positive samples was between 9 to 10 minutes. The false-positives had TtPs of 31 and 40 minutes. So, there is a great time distinction between the true positives and false positives. It is

important to note that the amplification times for the *E. coli* primer set were significantly shorter (~20 minutes) than that of *B. hyodysenteriae* primers in the same condition, and the TtP of the true positive samples in LAMP were also lower than the Cq's from Exopol's kit.

Reaction condition E1.2

This reaction condition was performed at 68ºC to try to mitigate the false positives [78].

Table 4.24: Results for the LAMP test for the sample control in nucleic acid samples under **RC E1.2** for 60 minutes at 68ºC, and comparison between the Cq values of the samples tested with EXOone kit as the endogenous control and the TtP obtained with our test.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E1.2 TtP (min)
Positive, 161378	27.33	10.52
Negative Control, 163988	-	37.63
NTC	-	35.58

There were no significant differences between this reaction condition and **RC E1.1**, the complete opposite of the observed with **RC B1.2**. This was expected because as explained previously, the melting temperatures of the primers F3 and B3 of *E. coli* primer set 1 are significantly higher than those of *B. hyodysenteriae* primer set 1.

Reaction condition E1.3

Since the increase in temperature was not enough to eliminate false positives, a test was performed in the absence of the loop primers. These are not essential, used mainly to accelerate the reaction, and could possibly be the cause of the false positives due to the formation of primer dimers or other secondary structures and further amplification of these structures.

The absence of loop primers generated a delay on the amplification of the positive sample compared to **RC E1.1** (10.23 vs 31.18 minutes), and false positives were still present with a difference of 6 to 8 minutes comparing to **RC E1.1**. Examining the results so far, the false positives could be due to secondary structure between the core primers and the loop primers as well, or unspecific amplification.

Table 4.25: LAMP test for the sample control under the **RC E1.3** for 60 minutes at 65ºC, and comparison with the Cq values of the samples tested on the kit EXOone.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E1.3 TtP (min)
Positive, 161378	27.33	31.18

Negative Control, 163998	-	31.81
NTC	-	37.54

Reaction condition E1.4

In this condition, different primer concentrations were tested. Decreasing primer concentrations can help on increasing the specific amplification due to lower availability of primers[93]. As so, they would theoretically bind more to the target.

- 1× Primer Concentration (0.8 μM loop primers);
- 0.3× Primer Concentration;
- 0.2× Primer Concentration.

Table 4.26: LAMP test for the Sample control under **RC E1.4,** at 65ºC, which comprises 3 different primer concentrations.

DNA Samples	EXOone Endogenous Control Cq	LAMP 1× TtP (min)	LAMP 0.3× TtP (min)	LAMP 0.2× TtP (min)
Positive, 161378	27.33	11.40	13.66	17.34
Negative Control, 163998	-	44.36	25.05	57.61
NTC	-	39.05	37.43	47.80
ΔTtP (NTC - Pos)	-	27.65	23.77	30.46
ΔTtP (NegC - Pos)	-	32.96	11.39	40.27

The amplification of the positive sample was delayed with the decreasing primer concentrations although the TtP was never above 20 minutes.

As for the negative control, with the decreasing primer concentrations can be observed a significant delay, especially in 0.2× primer concentration where was calculated a delay of 17.86 minutes comparing to **RC E1.1**. The test with 0.2× primer concentration was also the one with a better separation between the true positive sample and false positives, 30.46 minutes between the true positive and the NTC, and 40.27 minutes between the true positive and negative control.

Although was obtained a better separation between true positives and false positives compared to previous reaction conditions, these are still not ideal because false positives are still being detected under a 60-minute reaction.

Reaction condition E2.1

A new primer set was design since the previous generated false positives in all conditions tested, either due to primer-dimer formation or unspecific binding and amplification.

Table 4.27: LAMP test for the Sample control under **RC E2.1**, at 65ºC for 60 minutes with DNA samples.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E2.1 TtP (min)
Positive, 161378	27.33	12.08
Negative Control, 163988	-	0
NTC	-	27.35

Comparing the behaviour of this new primer set with the previous in the same reaction conditions, the amplification of the positive sample was delayed for about 1 minute, and the NTC was amplified 8 minutes earlier. However, the negative control did not amplify. These results could be a sign of a more specific behaviour from these primers.

Reaction condition E2.2

Following what was done with the previous primer set, these were tested in the absence of loop primers at 65ºC.

Table 4.28: LAMP test for the Sample control under **RC E2.1**, at 65ºC for 60 minutes with DNA samples, in the absence of loop primers.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E2.2 TtP (min)	LAMP RC E2.1 TtP (min)
Positive, 161378	27.33	29.91	12.08
Negative Control, 163988	-	37.95	0
NTC	-	49.17	27.35

Comparing this reaction condition to the one in the presence of loop primers, the amplification of the NTC was delayed for 21.82 minutes, but the amplification of the positive sample was also delayed for 17.83 minutes. The TtP difference between the positive sample and the NTC was higher in this condition than in the previous. However, the negative control amplified at 37.95 minutes. Although this primer set generated false positives as well, those were detected with a higher TtP than that of Primer set 1. Besides, in the presence of loop primers the negative control sample was not detected.

Reaction condition E2.3 and E2.4

The experiments followed to the same line of action as with the previous primer set. Primer set 2 was then tested at 68ºC (**RC E2.3**) and 67ºC (**RC E2.4**), with loop primers, to once again evaluated if it was enough to mitigate the false positives [78].

Table 4.29: LAMP test for the Sample control under **RC E2.3**, at 68ºC for 60 minutes with DNA samples.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E2.3 TtP (min)
Positive, 161378	27.33	11.40
Negative Control, 163988	-	47.24
NTC	-	45.88

The amplification of the positive control at 65ºC and 68ºC did not suffer any alterations. The TtP of the NTC was delayed 18.53 minutes. The amplification of the negative control and NTC was not expected at this temperature from what was observed so far of this primer set. Therefore, it needs further optimizations.

Table 4.30: LAMP test for the Sample control under **RC E2.4**, at 67ºC for 60 minutes with DNA samples.

DNA Samples	EXOone Endogenous Control Cq	LAMP RC E2.3 TtP (min)
Positive, 161378	27.33	9.69 9.07
Negative Control, 163988	-	37.63 0
NTC	-	0 0

At 67ºC the reaction was carried out with duplicates to evaluate the reproducibility as well. The duplicates of the positive sample were detected under 10 minutes, earlier than at 68 and 65ºC. Only one of the duplicates of the negative control was detected (10 minutes earlier than at 68ºC). None of the duplicates of the NTC were detected. This outcome was not expected since a decrease in temperature decreases the stringency, which increases the possibility of false positives. It is possible that the amplification of the negative control and the NTC at 68ºC were caused by contamination.

4.1.5 Optimizations

Reaction condition E2.5

The condition that produced the best results so far was **RC E2.4** (67ºC). Thus, the next step was testing this condition with direct samples (faeces and rectal swabs in Exopol's buffer).

Table 4.31: LAMP test for the detection of *E. coli* under **RC E2.5**, at 67ºC for 60 minutes with direct samples.

Direct Samples	EXOone Endogenous Control Cq	LAMP RC E2.5 TtP (min)

Positive, Faeces 161188	24.24	14.08 15.11
Positive, Rectal Swab 164233	28.96	13.14 12.22
NTC	-	18.06 38.86

Although the duplicates of the positive samples amplified with similar TtPs, the NTC duplicates amplified as well. Further optimizations were made.

Reaction condition E2.6

The use of direct samples proved to have some inhibitory effect on the detection of *B. hyodysenteriae*. This was not the case for the Sample control but the since the sample used for both reactions will be the same, this primer set was tested with faecal swabs and other sample treatments applied previously on the detection of *B. hyodysenteriae*. This reaction was performed at 67ºC.

Table 4.32: LAMP test for the detection of *E. coli* under **RC E2.6**, at 67ºC for 60 minutes with faecal swabs.

	EXOone Endogenous Control Cq				**LAMP RC E2.6 TtP (min)**			
Faecal Swabs	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube	Microbrush	Deltalab	Citotest	Collection tube
170610	31.60	31.01	29.91	30.04	22.55	0	0	0
171695	35.07	34.47	31.91	31.60	48.50	43.72	0	32.58
171652	28.26	-	-	-	15.62	-	-	-
170800	32.03	-	-	-	19.04	-	-	-

(continuation) Table 4.33: LAMP test for the detection of *E. coli* under **RC E2.6**, at 67ºC for 60 minutes with faecal swabs.

	EXOone Endogenous Control Cq				**LAMP RC E2.6 TtP (min)**			
Faecal Samples	DNA Microbrush	DNA Deltalab	DNA Citotest	DNA Collection tube	Microbrush	Deltalab	Citotest	Collection tube
170254	30.11	-	-	-	16.69	-	-	-
171771	32.89	-	-	-	12.13	-	-	-
NTC	0				48.89			

Contrary to what was observed with the detection of *B. hyodysenteriae* in the same conditions, *E. coli* was detected in almost all the samples tested, which was expected since this bacterium is present in faeces. The samples prepared with Citotest swab tested negative for *E. coli*. As for Deltalab and the swab from STAB VIDA, only 1 of the 2 samples tested were identified as positive. Since all the samples prepared with the Microbrush were amplified, and after DNA extraction and purification the samples from all the swabs were amplified with Exopol's kit, it can be hypothesized that some component of the Deltalab, Citotest and collection tube swabs could cause some inhibition of the reaction. Accordingly, it was observed before that certain types of swab material absorb DNA differently[94]. Deltalab and Citotest's swabs are made of viscose (a type of rayon). Zasada *et al* (2020) demonstrated that this kind of swab material, out of 4 total materials tested, was the one that was able to absorb the least amount of DNA[95].

Reaction condition E2.7

As for the detection of *B. hyodysenteriae*, for *E. coli* the sample eluted from the Column format A, Filter A was tested.

Table 4.34: LAMP test for the detection of *E. coli* under **RC E2.7**, at 65ºC for 60 minutes testing the 1st and 2nd fraction of the eluted sample using Column format A, Filter A.

	LAMP RC E2.7 TtP (min)	
Sample	1st eluted	2nd eluted
171695	0	46.52
NTC	0	

In the case of *B. hyodysenteriae* both fractions collected tested positive, but the second fraction had a higher TtP. For *E. coli*, only the second fraction came positive, which would rule out the result of the first fraction as invalid, since the internal control was not detected. Besides, a gravity flow method is not ideal for POCT for the reasons presented before.

Reaction condition E2.8

An identical experiment was carried out with *B. hyodysenteriae* primers to assess the inhibitory effect of stool in the reaction.

Similarly, a Microbrush was used to collect the sample which was homogenized in 50 μL of Sample collection buffer A instead of 100 μL to concentrate the sample. Then 1:2, 1:5, 1:10 and 1:50 dilutions of the sample were made. To each reaction tube, a mixture of 1 μL of the positive control and 2.3 μL of the correspondent dilution was added.

Table 4.35: LAMP test for the detection of *E. coli* under **RC E2.8,** at 65ºC for 60 minutes, testing dilutions from faecal swab with a Microbrush, to assess the inhibition of the reaction by this type of sample

LAMP RC B2.8

TtP (min)

Samples Faecal swab	W/o Sample dilutions	W/o Pos Control (3.3 μL sample)	W/ Pos Control (1 μL)			
			1:2 dilution	1:5 dilution	1:10 dilution	1:50 dilution
171695	-	0	34.39	18.36	13.91	12.22
Pos Control, Cells	11.02	-	-	-	-	-

The effect of this dilutions on *E. coli*'s positive control was similar to the effect observed for *B. hyodysenteriae*, though the mixture of *E. coli*'s positive control and 1:2 sample dilution was detected, opposite to the observed in *B. hyodysenteriae* test. This could be one of the indicators that *E. coli*'s reaction is more robust than *B. hyodysenteriae*'s.

Reaction condition E2.9, E2.10 and E2.11

Three other methods of DNA extraction and sample purification were tested in parallel with the Sample control:

- Chelex-100: **RC E2.9**
- Column format B, Filter A: **RC E2.10**
- PVDF (Polyvinylidene Fluoride) syringe filter: **RC E2.10**

For each sample collected of the methods used, a 1:2 dilution of that sample was tested. Also, the positive control and a mixture of 1:2 positive control to the collected sample to assess the inhibitory effect.

Table 4.36: LAMP test for the detection of *E. coli* under **RC E2.9, E2.10** and **E2.11,** at 65ºC for 60 minutes. Chelex-100, Column format and a PVDF syringe filter were used, respectively.

	LAMP RC E2.9 TtP (min)		**LAMP RC E2.10 TtP (min)**		**LAMP RC E2.11 TtP (min)**	
Samples Scoop spatula	No dilution	1:2 dilution	No dilution	1:2 dilution	No dilution	1:2 dilution
171695	0	0	16.24	37.19	46.49	45.40
Pos Control, Cells	12.98	33.37	12.98	11.61	12.98	14.02

The only extraction method that was not successful was the Chelex-100. However, as mentioned the mixture of Chelex with the sample was not heated, and the Chelex had also expired. This result is similar to the one observed with *B. hyodysenteriae*.

With the other methods in all the dilutions tested, *E. coli* was detected. An overall comparison of the *E. coli* and *B. hyodysenteriae* primers show a higher sensitivity of the *E. coli* primers, and on the other hand a higher specificity of the *B. hyodysenteriae* primers.

Reaction condition E2.12

Reaction Mastermix B has been observed to be faster than Reaction Mastermix A, and was tested with primer set 2 at 65ºC.

Table 4.37: Test with the Sample control primer set 2 for comparison of Reaction Mastermix A and B, at 65ºC for 60 minutes, with DNA samples.

Samples	**Reaction Mastermix B TtP (min)**	**Reaction Mastermix A TtP (min)**
Pos Control, Cells	4.25	8.20
NTC	12.59	0

For *E. coli* Reaction Mastermix B was indeed faster, but also less specific that Reaction Mastermix A in this test.

4.2 Multiplex Testing

Before attempting a multiplex test, the melting temperatures of the LAMP products of primer sets 2 for detection of *B. hyodysenteriae* and for the internal reaction control were verified, to assess if distinction through HRM would be possible. This was performed by a melt step on Quantabio qPCR instrument, and the derivative of the melt curve was calculated by the instrument's software. **Figures 4.4** and **4.5** show the derivative of the melting curves for the LAMP products of primer sets 2 for the detection of *B. hyodysenteriae* and *E. coli* on DNA samples.

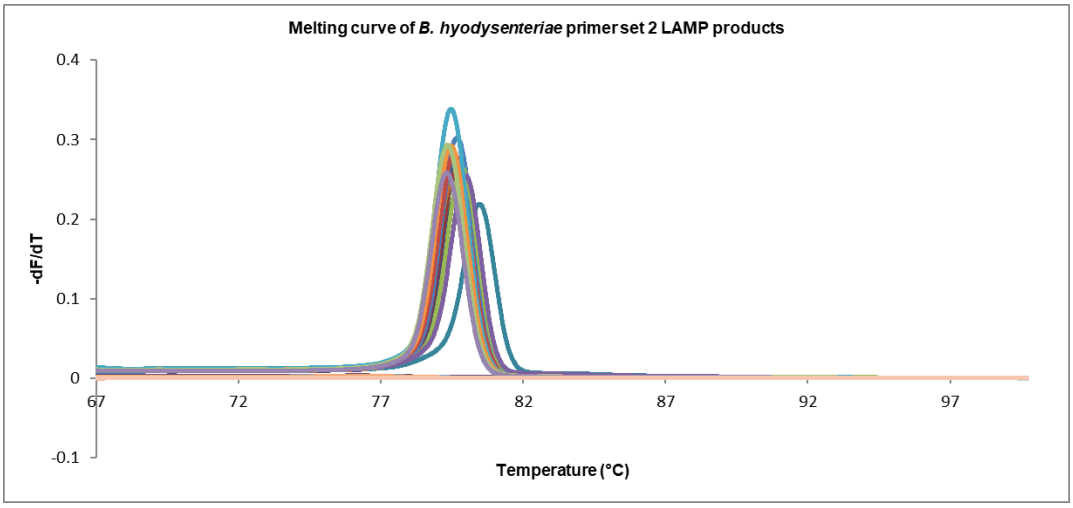

Figure 4.4: Melting curve of *B. hyodysenteriae* primer set 2 LAMP products, with DNA samples. The average melting temperature is 79.66ºC.

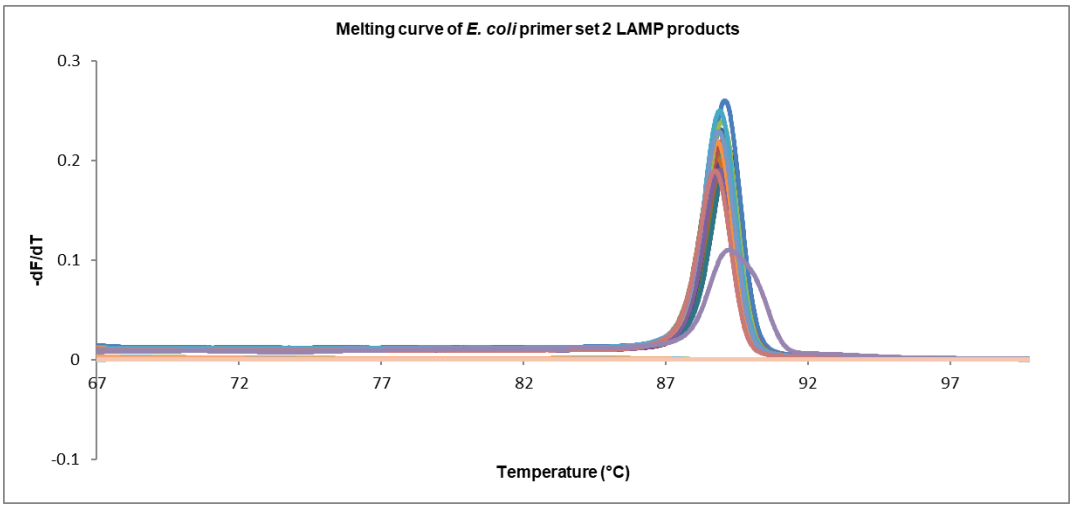

Figure 4.5: Melting curve of Sample control set 2 LAMP products, with DNA samples. The average melting temperature is 88.92ºC.

The average melting temperature of *B. hyodysenteriae* LAMP products is 79.66ºC, and for *E. coli* is 88.92ºC, which is a difference of 9.26ºC. As so, it would be theoretically possible to distinguish both products by HRM in a multiplex test[72,96].

4.2.1 Multiplex test 1

A first attempt of a multiplex test was performed with primer sets 2 for *B. hyodysenteriae* and *E. coli* with DNA samples at 67ºC. The sample 161378 is positive for both, and 164308 is only positive for *E. coli*. The reaction was performed with duplicates.

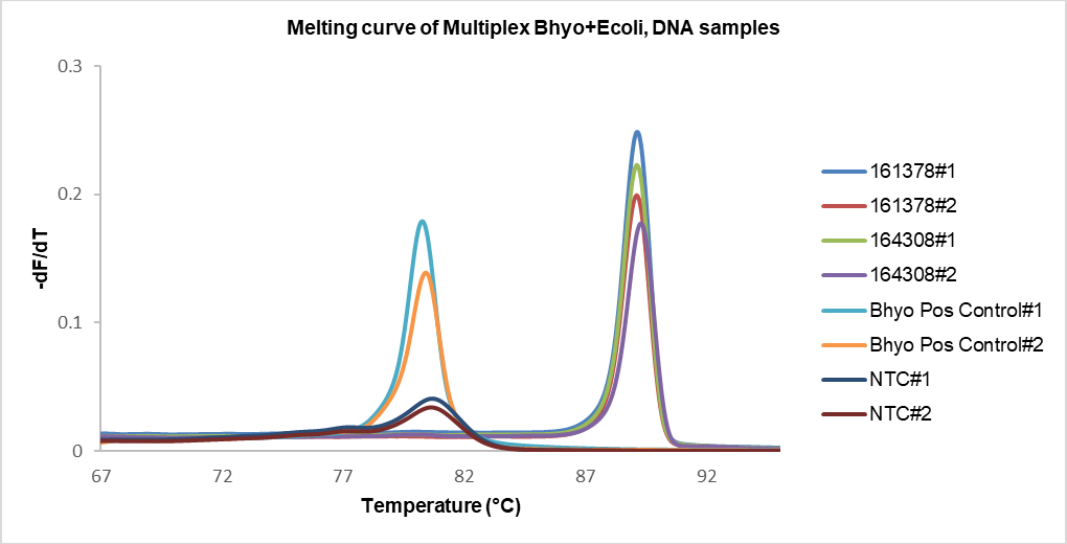

Figure 4.6: Melting curve of multiplex test 1 with primer sets 2 for *B. hyodysenteriae* and *E. coli*, with DNA samples at 67ºC.

The melting curves of 161378 only has one peak at approximately 89ºC, which indicates that this simple was only amplified with *E. coli* primers. The positive control for *B. hyodysenteriae* only has one peak as well at about 80ºC but this was the excepted.

4.2.2 Multiplex test 2

A second attempt was performed with direct samples, at 67ºC.

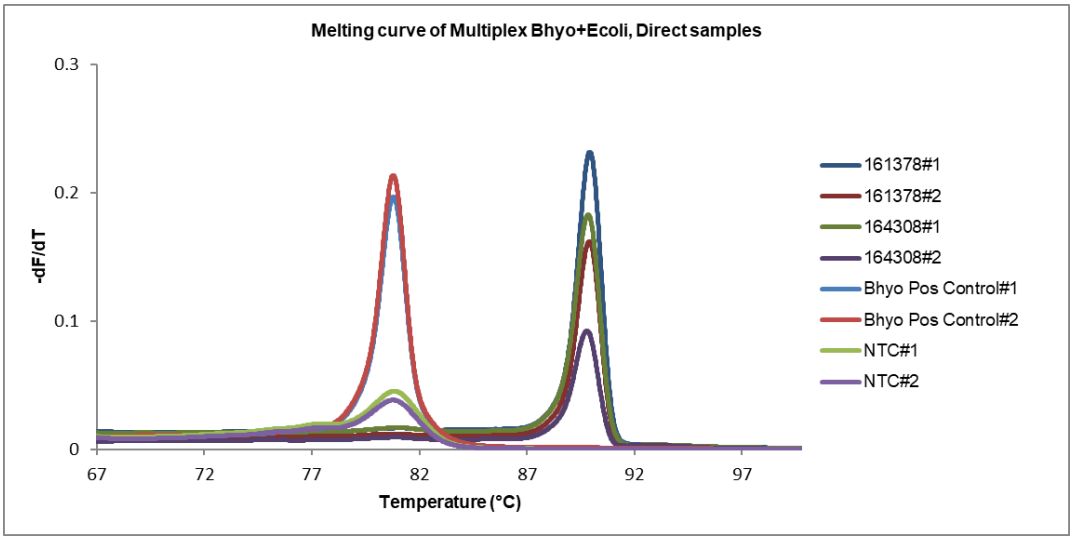

Figure 4.7: Melting curve of multiplex test 2 with primer sets 2 for *B hyodysenteriae* and *E. coli* with direct samples at 67ºC.

This test has the exact outcome as the previous test.

4.2.3 Multiplex test 3

Next, multiplex test 3 was performed in the same conditions as the previous, in the absence of *E. coli* loop primers. Sample 164188 and 164293 are positive for both, 164278 and 164308 are only positive for *E. coli.*

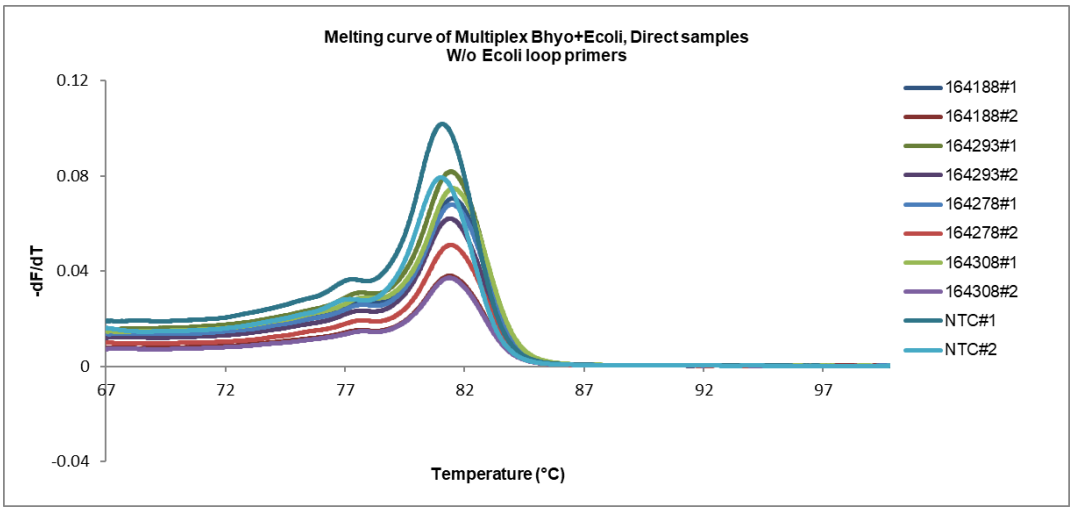

Figure 4.8: Melting curve of multiplex test 3 with primer sets 2 for *B. hyodysenteriae* and E. coli (without loop primers) with direct samples at 67ºC.

The melting curve deviated to a lower temperature interval, and the *E. coli* product characteristic peak at about 89ºC is not present. It was thought that the peak with the highest intensity (~81ºC) could be from *B. hyodysenteriae* specific detection. However, all the samples, including the one negative for *B. hyodysenteriae* and the NTC have this peak on their melting curves. Thus, there is a high possibility that these products are not specific.

Reviews and some studies have described the difficulty of creating a multiplex LAMP test with high specificity due to the number of primers present in one reaction tube[20,97]. The tendency observed in these multiplex tests so far is either the favouritism of the enzyme towards *E. coli* primers, or a higher content of *E. coli* DNA over *B. hyodysenteriae* DNA in the samples.

4.2.4 Multiplex test 4

To test the influence of the amount of each bacterial DNA, a direct sample (164375) only positive for *E. coli* was spiked with *B. hyodysenteriae* positive control (DNA). Then, from the total sample volume:

- 50% 164375 + 50% *B. hyodysenteriae* Positive Control;
- 25% 164375 + 75% *B. hyodysenteriae* Positive Control;
- 75% 164375 + 25% *B. hyodysenteriae* Positive Control.

Observing the melting curves of each spike, there is a clear pattern. All samples have two distinct peaks, one that corresponds to *B. hyodysenteriae* detection products (~81ºC) and other to the detection of *E. coli* (~90ºC). Furthermore, with the increasing percentage of *B. hyodysenteriae* in the total sample, the higher the intensity of the melt peak characteristic of its product.

Other conditions were tested (data not shown), but the best results obtained were with the spiked samples.

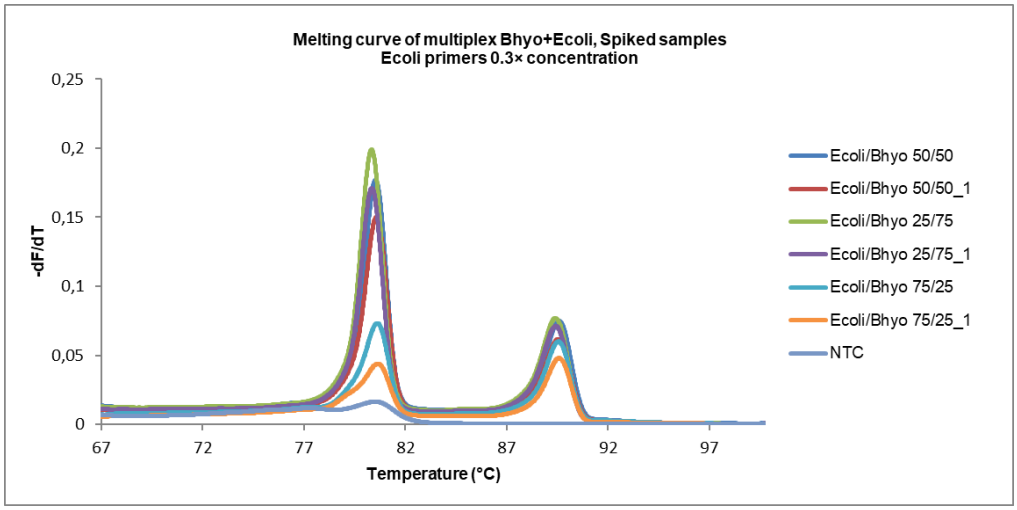

Figure 4.9: Melting curve of multiplex test 4 with primer sets 2 for *B. hyodysenteriae* and *E. coli* (0.3×) with a positive *E. coli* sample spiked with *B. hyodysenteriae* DNA, 3 different ratios, at 67ºC.

The ratio of *B. hyodysenteriae* DNA to *E. coli* DNA is a parameter that is impossible to control in real samples. Thus, it was decided to proceed testing with Doctor Vida® Pocket, but as 2 separate reactions.

4.3 Doctor Vida® Pocket Tests

DV test 1

After the optimizations made on the reaction for the detection of the target and the Sample control, the next step was testing on Doctor Vida® Pocket.

In the first test the samples used were 171695, 170254 and 170800, which are all positive for the target and Sample control. A scoop spatula was used for the collection of approximately 0.05g of each sample which were then homogenized in 100 μL of Sample collection buffer A. The Column format B, Filter A method was used as specified on **Materials and Methods: Part 1, subsection 2.5 Sample Treatments** for the extraction of DNA and removal of inhibitors.

Table 4.38: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the sample control (65ºC). All samples used are positive for both, and its DNA was extracted from faecal swabs with Column format B, Filter A.

Samples	*B. hyodysenteriae* DVPocket TtP (min)	Sample control DVPocket TtP (min)
171695	38.5	0
170254	25.5	18.5

(continuation) Table 4.39: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the sample control (65ºC). All samples used are positive for both, and its DNA was extracted from faecal swabs with Column format B, Filter A.

Samples	*B. hyodysenteriae* DVPocket TtP (min)	Sample control DVPocket TtP (min)
170800	30	19.5
Bhyo Pos Control	19.5	-
Ecoli Pos Control	-	17.5
NTC	0	35

All positive *B. hyodysenteriae* samples were detected under 40 minutes, with no false positives. As for the sample control, sample 171695 failed to amplify, which would make the positive result for *B. hyodysenteriae* invalid. The remaining samples were detected under 20 minutes. The primers for the sample control generated a false positive, detected at 35 minutes.

DV test 2

In the following tests, different sample concentrations were tested. The samples were once again collected with a scoop spatula and weighted, followed by a homogenization in 100 μL of Sample collection buffer A or Exopol's buffer, to evaluate the influence, if any, of their buffer comparing to STAB VIDA's on the reaction. The values weighted therein were 0.04g, 0.05g, 0.1g and 0.2g. The remaining quantity of sample was not enough to carry out tests with the same sample concentration as with Sample collection buffer A.

The samples were then again extracted by Column format B, Filter A method. The sample used for this experiment was 170254, positive for both *B. hyodysenteriae* and *E. coli*.

In the tested sample weights in both Sample collection buffer A and Exopol's buffer the discrepancy between the inhibition effect of both buffers is noticeable. As so, the decision to keep Sample collection buffer A as the collection buffer was made.

Table 4.40: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC). In this test both Sample collection buffer A and Exopol's buffer were used as the sample collection buffer, and different dilutions of the sample in its collection buffer were tested.

	B. hyodysenteriae **DVPocket TtP (min)**		**Control** **DVPocket TtP (min)**	
Samples	Sample collection buffer A	Exopol's buffer	Sample collection buffer A	Exopol's buffer
0.4 mg/mL	- -	0 50	- -	29.5 28.5
0.5 mg/mL	29.5 22.5	0 47	23 22	30.5 31
1 mg/mL	18.5 18	- -	29 26	- -
2 mg/mL	21 31.5	- -	35 29	- -
Bhyo Pos Control	24.5 21.5	25.5 18	- -	- -
Ecoli Pos Control	- -	- -	18 18.5	42 33
NTC	55.5 0	0 0	0 0	38 37.5

DV test 3

The following test was carried out the same way as the previous tests, with the sample homogenized in Sample collection buffer A and the usage of Column format B, Filter A. However, for this test the sample used is negative for *B. hyodysenteriae* to test the specificity of the results obtained until now.

Table 4.41: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC). In this test only Collection buffer A was used, and a sample negative for *B. hyodysenteriae* was tested to assess the specificity of the results obtained so far.

Samples	***B. hyodysenteriae*** **DVPocket TtP (min)**	**Control** **DVPocket TtP (min)**
0.5 mg/mL	0 0	0 0
1 mg/mL	0 0	0 39
2 mg/mL	47 0	0 23

(continuation) Table 4.42: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC). In this test only Collection buffer A was used, and a sample negative for *B. hyodysenteriae* was tested to assess the specificity of the results obtained so far.

Samples	*B. hyodysenteriae* DVPocket TtP (min)	Control DVPocket TtP (min)
Bhyo Pos Control	23 25.5	- -
Ecoli Pos Control	- -	19.5 20
NTC	47 55	31.5 0

4.3.1 Sample collection kits

Sample collection kit 1

STAB VIDA'S Sample collection kit 1, with Filter A incorporated in the dropper piece and 400 µL of Sample collection buffer A.

When testing with this device there was an attempt to collect the sample with the tube on **Figure 3.11**, but since the samples were quite liquid, the collection was not successful. The swab from this tube was the one of the swabs used during reaction optimizations, and so it was chosen as the collection swab for this kit.

Figure 4.10: Sample collection kit 1**.**

Figure 4.11: First attempt of the sample collector.

Sample collection kit 2

STAB VIDA's Sample collection kit 2.

On **Figure 3.12** is first version of this collection kit. Filter A is incorporated in the piece with the red oval, and 400 μL of Sample collection buffer A.

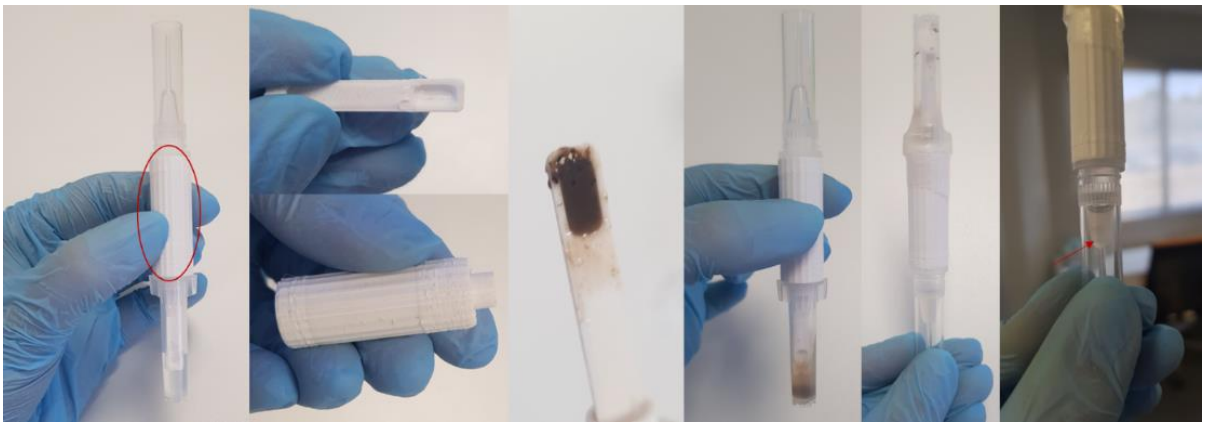

Figure 4.12: Sample collection kit 2 before optimizations.

The problem with this design was the format of the scoop, that sometimes made it a hard task to squeeze the collection tube to elute the sample. The scoop was then redesigned to have a thinner handle to facilitate the process (**Figure 3.13**).

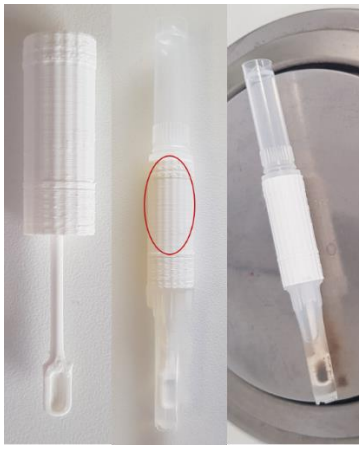

Figure 4.13: Sample collection kit 2 after optimizations.

DV test 4

This test was carried out with collection kit 1. This device possesses a pompet to which 400 μL of Sample collection buffer A are transferred, as well as a swab for the collection of the sample. The collection buffer is released through the pompet and travels through the interior of the swab, freeing the collected material and homogenizing the sample in the collection buffer. Following the homogenization process the sample is filtered through Filter A.

The 170800 sample was used, positive for both *B. hyodysenteriae* and *E. coli*. Two elution fractions were collected. The first collection was comprised of the first 3 drops of the eluted sample, while the second collection was comprised of the following 4th, 5th and 6th drops. Both collections appeared to be translucent, which implies a proper filtration of the sample by the column even when subjected to the pressure of the pompet. From each elution fraction 10 μL were transferred to the correspondent reaction tube.

B. hyodysenteriae was detected solely in the first collection even though *E. coli* was detected in both, which would lead to believe that the second collection was a false-negative. Thus, with the usage of this collection device of the collected from the first 3 drops of the eluted samples should be used for the test.

Table 4.43: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC), with sample collection kit 1.

	B. hyodysenteriae **DVPocket TtP (min)**		**Control** **DVPocket TtP (min)**	
Samples	1st collection	2nd collection	1st collection	2nd collection
170800	49.5	0	22.5	24.5
Bhyo Pos Control	21	23.5	-	-
Ecoli Pos Control	-	-	15	15
NTC	0	0	41	0

DV test 5

For this test the collection kit 2 was used before its optimizations to improve the Filter A separation resolution were implemented.

Table 4.44: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC), with the first version of sample collection kit 2.

Samples	*B. hyodysenteriae* DVPocket TtP (min)	Control DVPocket TtP (min)
170800	33.5	23
170254	41	21.5
NTC	0	0

After the optimizations were implemented to the device, the only sample available for testing was 170800, but later Exopol provided new samples for testing.

Table 4.45: Doctor Vida® Pocket test for the detection of *B. hyodysenteriae* (63ºC) and the control (65ºC), with the second version of sample collection kit 2.

Samples	*B. hyodysenteriae* DVPocket TtP (min)	Control DVPocket TtP (min)
170800	32	23
179163	20	16
NTC	0	0

Good results were achieved with this collection device. However, there were some leakage problems, which is not at all ideal to a diagnostic test.

Further optimizations to the conditions of Filter A are needed to prevent leakages and assure the proper filtration and separation of DNA from other components of the samples, since through experiments conducted during the dissertation work, it was observed the inhibitory effect of stool samples to the LAMP reaction.

After solving the leakage problems, a small validation was made with 30 samples for the 2 collection kits, to compare their performances and the overall viability of the test developed so far. The results can be consulted on **Appendix 8.2**. These last tests for the performance evaluation of the kits were performed by my supervisor Gonçalo Doria.

Table 4.46: Analysis of the number of concordant, non-concordant and invalid tests from the performances of Sample collection kit 1 and 2, with the results obtained with the EXOone *B. hyodysenteriae* qPCR kit.

Kits	Concordant results w/EXOone qPCR kit	Non-concordant results w/EXOone qPCR kit	Invalid results	Total tests (n)
Kit 1 (Swab Kit)	19 (63.33%)	6 (20.00%)	5 (16.67%)	30
Kit 2 (Scoop Kit)	20 (66.67%)	7 (23.33%)	3 (10.00%)	30

Both kits behaved similarly when it comes to the concordance results, with the Scoop kit detecting only 1 more sample correctly than the Swab kit (66.67% vs 63.33%), and non-concordant results (23.33% vs 20.00%). As for the invalid results, the Swab kit had 2 more invalid results than the Scoop kit. The test duration was 1 hour. In some of the negative results there could be observed a slight increase of the fluorescence signal at the end of the test. This could be a sign that the time of the reaction needs to be increased and tested on further experiments. For kit 1 a sensitivity of 78.9% and specificity of 45.5%, and for kit 2 a sensitivity of 68.4% and specificity of 72.7% were obtained with the final versions of the kits.

Besides the performance results, the user-friendliness of each kit was evaluated. In this case, the samples were stored in 1.5 mL Eppendorfs, and were quite liquid. Due to this it was easier to collect the sample with the swab kit rather than with the scoop kit.

All these parameters are to be evaluated again during clinical validation.

5

RESULTS AND DISCUSSION: PART 2

5.1 Sex Determination of Birds from Psittacidae Family

5.1.1 Proof-of-Concept for Sex Determination and Control

Reaction Condition P1.1

The samples for this test were incubated in Buffer E (STAB VIDA) and tested at 65ºC.

Table 5.1: LAMP test for female sex determination of species from the Psittacidae family under **RC P1.1**, at 65ºC for 60 minutes, for both sex determination primer set 1 and sample control primer set 1 in singleplex. Feather tips were treated with Buffer E (STAB VIDA).

Samples	Psittacidae Sex Determination TtP (min)	Sample Control TtP (min)
M. monachus Male	0 0	0 0
A. militaris Female	0 0	0 0
P. elegans Female	0 0	0 0
P. elegans Male	0 0	0 0
C. carduelis Female	0 50	0 0
C. carduelis Male	0 0	0 0
NTC	0 0	0 0

A female and male sample from *C. carduelis* (Passeriformes order) was used to analyse the specificity of these primers to the Psittacidae family. However, the only sample that was identified as female with the sex determination primers was one of the duplicates of the *C. carduelis* female. Besides, none of the samples were amplified with the sample control primers, which would rule out that result as

invalid. Since the control primers are supposed to target an ultraconserved sequence amongst Neoaves, and so it would theoretically detect *C. carduelis* as well. Therefore, the next test was performed at the same temperature, but with a different sample treatment.

Reaction condition P1.2

The samples for this test were treated with STAB VIDA's Bird Sexing Service Routine A (DNA extraction steps). The reaction proceeded at 65ºC like the previous.

Table 5.2: LAMP test for female sex determination of species from the Psittacidae family under **RC P1.2**, at 65ºC for 60 minutes, for both sex determination primer set 1 and internal control primer set 1 in singleplex. Feather tips were treated with STAB VIDA's Bird Sexing Service Routine A (DNA extraction steps).

Samples	Psittacidae Sex Determination TtP (min)	Control TtP (min)
P. krameri	0	0
Female	58	0
P. krameri	0	0
Male	0	0
P. molinae	0	0
Female	0	0
A. ochrocephala	0	0
Male	0	0
M. monachus	0	0
Female	0	0
M. monachus	0	0
Male	0	0
NTC	0	0
	0	0

As observed in the previous reaction, only one duplicate of a positive sample started to get detected almost at the end of the reaction, and the sample control primers did not detect any sample, ruling the test as invalid.

Due to the negative results obtained so far, a PCR with both the sex determination and sample control primers, only with the forward and reverse primers was performed. If the PCR was successful, the products could be sequenced and analysed.

5.1.1.1 PCR results

The samples were extracted and tested on PCR following STAB VIDA's Bird Sexing Service Routine A.

The gel on the left corresponds to the PCR products of PSI-W, and the gel on the right to the UCE products.

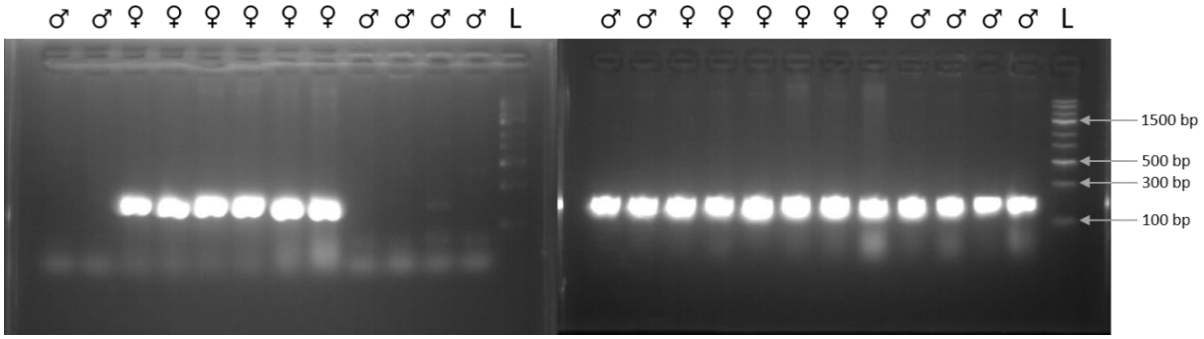

Figure 5.1: Agarose gel 2% with TAE 1×, 130 V for 30 minutes. The gel one the left are the results from the PCR with PSI-W primers, and the gel on the right with UCE primers. PSI-W primer set generated a ~200 bp band only on female samples. As for UCE Primers, it generated a ~200 bp band but for both male and female sample, as intended. L: GeneRuler Express DNA Ladder (ThermoScientific, SM1552).

All female samples were amplified with the forward and reverse primers from the PSI-W set, and all female and male samples were amplified with the forward and reverse primers from the UCE set. Since the sample treatment was the same as in **RC P1.2**, the lack of amplification might be due to the absence of the internal FIP and BIP primers on the sequence.

Therefore, these products were sequenced by Sanger and aligned to make a consensus sequence. In that sequence, a correspondence of the primer sequences was made.

The alignment of the sequences was made with CLC Genomics Workbench (Qiagen). When aligning the sequences, CLC generates an automatic Consensus sequence based on the alignment.

Between these sequences there were deletions, and various mismatches, although all the samples belong to the same family (Psittacidae). When trying to match the primers sequences on these products, only the F2 region and BIP primer could be found (with at least 4 mismatches on both B1 and B2). The variation of this region between species of the same family could be the reason for the negative results obtained so far.

The region targeted by the UCE primers is clearly more conserved between species of the same family than the regions targeted by the PSI-W primers. Both FIP and BIP primers are present in these products. Therefore, the negative results obtained so far with UCE primer set could have been due to not so ideal reaction conditions.

Due to these variations between the sequences, the fact that some of the primers were not found on those and the finding of a study with LAMP primers for the sex determination of female *Columba livia*, a bird species that is also of high interest for STAB VIDA, these tests were left at this stage.

5.2 Sex Determination of *Columba livia*

5.2.1 Proof-of-Concept for Sex Determination

Before testing this primer set with LAMP, a PCR was performed to assess the conservation of this region between individuals.

DNA was extracted from feather tips and tested in PCR with STAB VIDA's Bird Sexing Routine A.

All female *Columba livia* samples were detected correctly, with a strong band of about 200 bp. The male sample was not amplified. The bands delineated in red were thought to be primer dimers or unspecific amplification.

The products from this PCR were purified with magnetic beads and used in the LAMP reactions as the positive control.

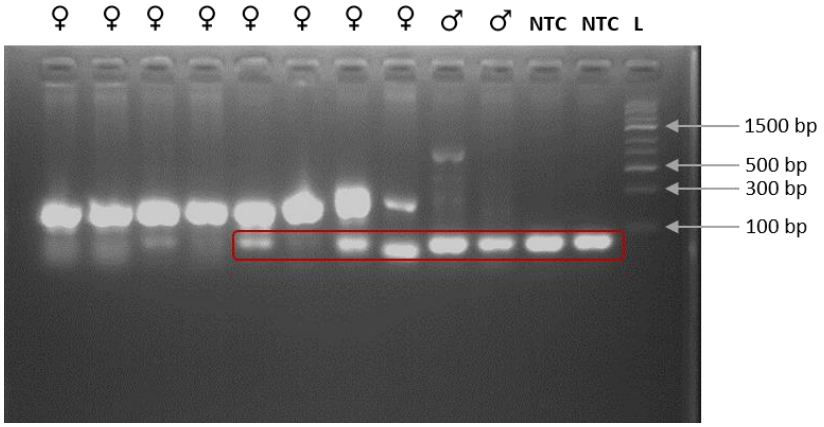

Figure 5.2: Agarose gel 2% with TAE 1×, 130 V for 45 minutes, of the PCR of primers CL_Sex, with *Columba livia* male and female samples. These primers generated a ~200 bp bands for females and no specific bands for the male sample, as expected. The bands delineated in red are thought to be unspecific amplification and primer dimers. L: GeneRuler Express DNA Ladder (ThermoScientific, SM1552), NTC: non-template control.

The PCR amplicons were sequenced by Sanger and analysed. The region aligned shows high conservation. The BIP primer is present in these sequences, but only F1 could be found. Nevertheless, this primer set was tested with LAMP.

Reaction condition CL1.1

The feather tips were treated with STAB VIDA's Buffer E or STAB VIDA's Bird Sexing Routine A (DNA extraction steps). The test was performed at 65ºC for 1 hour.

Table 5.3: LAMP test for the sex determination of *C. livia* females under **RC CL1.1**, with *Columba livia* Sex Determination primer set 1, at 65ºC for 60 minutes. The samples were treated with STAB VIDA's Bird Sexing Routine A (DNA extraction steps) and Buffer E.

Samples	*C. livia* Sex Determination TtP (min)
Female	0
Buffer E	0
Male	0
Buffer E	0
Female	0
Routine A	0
Male	0
Routine A	0
Pos Control	17.83
	23.50
NTC	0
	0

Only the positive control was detected, which could mean that all primers are present in the target sequence. Since the positive control used consists of the purified target sequence, the problem may be with the sample treatment, or the reaction temperature.

An agarose gel was run with the LAMP products. It was thought the real-time device could not be detecting the fluorescence increase correctly.

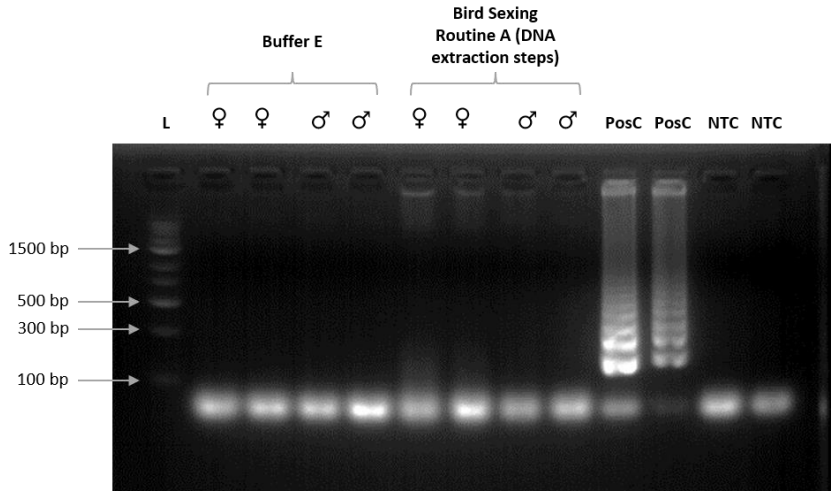

Figure 5.3: Agarose gel 2% with TAE 1×, 130 V for 45 minutes, of the LAMP products with *Columba livia* Sex Determination primer set 1. The typical LAMP band pattern is present only on PosC has verified with the real-time instrument results. L: GeneRuler Express DNA Ladder (ThermoScientific, SM1552), PosC: Positive Control, NTC: non-template control.

Only the positive control duplicates have the typical LAMP band pattern, thus only this sample was amplified.

Reaction condition CL1.2

This reaction was performed at 60ºC and the samples were treated with Buffer E (STAB VIDA).

Table 5.4: LAMP test for the sex determination of *C. livia* females under **RC CL1.2**, with *Columba livia* Sex Determination primer set 1, at 60ºC for 60 minutes. The samples were treated with Buffer E (STAB VIDA).

	C. livia
Samples	**Sex Determination TtP (min)**
Female	0
Female	0
Female	0
Female	0
Female	0
Male	0
Male	0
Male	0
Pos Control	20.90
NTC	0

Lowering the temperature of the test did not interfere with the amplification of the positive control, nor did it generate false positives. However, it still could not detect the female samples. It was also hypothesized that this region could be highly condensed, and since LAMP does not use thermal cycling, the strand separation capacity of the polymerase could not be enough to separate the strands of a highly condensed, histone-rich region. This hypothesis could be supported by the fact that PCR amplification was possible.

Reaction condition CL1.3

Next, the sample treatment was changed. Two to three feather tips were boiled in 20 μL of Sample collection buffer A at 95ºC for 10 minutes.

Table 5.5: LAMP test for the sex determination of *C. livia* females under **RC CL1.3**, with *Columba livia* Sex Determination primer set 1, at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC.

Samples	*C. livia* Sex Determination TtP (min)
Female	0 0
Female	0 0
Male	0 0
Male	0 0
Pos Control	19.58
NTC	0

As observed before, only the positive control was detected. The other plausible cause discussed for these results was faulty primer design, because although the positive control was detected in every reaction so far, this positive control consists of the purified target region and all the other sample treatments used do not purify the DNA, only make it theoretically more available. Thus, other components of this type of sample could be decreasing amplification efficiency[62,98], or they are not specific enough to hybridize in such conditions.

Reaction condition CL2.1

A new primer set was designed based on a CHD-W sequence of *C. livia* on GenBank. The feather tips were boiled at 95ºC for 10 minutes.

Table 5.6: LAMP test for the sex determination of *C. livia* females under **RC CL2.1**, with *Columba livia* Sex Determination primer set 2 (without loop primers), at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC.

Samples	*C. livia* Sex Determination TtP (min)
Female	0
Male	0
NTC	0

The female sample was not detected, nor did it generate false positives.

Reaction condition CL2.2

Component A (STAB VIDA) was used at 40 mM. This component is supposed to enhance the sensitivity of LAMP reactions, as well as the speed of amplification.

Female genomic DNA was purified from feather tips boiled in Sample collection buffer A with E.Z.N.A Tissue Kit, to be used as a positive control.

Table 5.7: LAMP test for the sex determination of *C. livia* females under **RC CL2.2**, with *Columba livia* Sex Determination primer set 2 (without loop primers), at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC, and 40 mM Component A (STAB VIDA) was added to the reaction mixture.

Samples	***C. livia* Sex Determination TtP (min)**
Female	0
Male	0
Female Genome	0
NTC	0

While expecting at least the detection of the female genomic DNA it did not happen. The time of the reaction was then increased to 2 hours, to assess if the amplification of females can be observed with specificity after 1 hour.

Reaction condition CL2.3

This reaction was run for 2 hours, with feather tips boiled in Sample collection buffer A at 95ºC for 10 minutes, and the female genomic DNA sample.

Table 5.8: LAMP test for the sex determination of *C. livia* females under **RC CL2.3**, with *Columba livia* Sex Determination primer set 2 (without loop primers), at 65ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC.

Samples	***C. livia* Sex Determination TtP (min)**
Female	72.28
Male	0
Female Genome	73.68
NTC	108.44

The female sample was detected at 72.28 minutes and the female genomic DNA at 73.68, with the NTC amplifying only at 108.44 minutes. However, it still generated a false negative.

Reaction condition CL2.4

To this reaction 7.5% of Component B (STAB VIDA) was added to help increase the stringency of LAMP reactions. This percentage was used because a gradient of Component B was tested already and this percentage was the only one that produced good results (data not shown).

Table 5.9: LAMP test for the sex determination of *C. livia* females under **RC CL2.4**, with *Columba livia* Sex Determination primer set 2 (without loop primers), at 65ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC and 7.5% of Component B (STAB VIDA) was added to the reaction mixture.

	***C. livia* Sex Determination**	
	TtP (min)	
Samples	0% Component B	7.5% Component B
Female	100.22	0
Male	0	0
Female Genome	107.16	0
NTC	77.30	0

The use of Component B in these conditions produced a negative effect on the reaction. Without this buffer both the female and female genome sample were amplified, although the NTC was amplified before those. With Component B no samples were amplified.

Reaction condition CL2.5

Loop primers were designed for this primer set, and tested with a gradient of Component A (STAB VIDA).

Table 5.10: LAMP test for the sex determination of *C. livia* females under **RC CL2.5**, with *Columba livia* Sex Determination primer set 2 and newly designed loop primers, at 65ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC. A gradient concentration of Component A was tested.

***C. livia* Sex Determination**

TtP (min)

Samples	0 mM Component A	15 mM Component A	20 mM Component A	25 mM Component A	30 mM Component A	35 mM Component A
Female	63	92.04	94.3	0	52.3	81.28
Male	98.32	0	70.96	0	54.3	62.5
Female Genome	94.22	72.08	60.32	47.46	30.04	25.02
NTC	92.44	0	74.06	66.98	80.16	68.04

The condition with 15 mM of Component A was the only one that did not generate false positives. The lowest TtP obtained for positive sample was with 30 mM of Component A. However, in this condition the male sample amplified shortly after the female and the NTC also amplified at 80 minutes. This is something that could potentially be optimized, so the remaining tests were performed with 30 mM of Component A. Furthermore, taking into account that the male's TtP was so similar to the female's, it could have been due to contamination.

Reaction condition CL2.6

This condition was performed with 30 mM of Component A (STAB VIDA) and at 67ºC in order to try to eliminate or at least delay the appearance of false positives. The samples used were genomic DNA, extracted and purified from male and female feather tips with the E.Z.N.A Tissue kit.

Table 5.11: LAMP test for the sex determination of *C. livia* females under **RC CL2.6**, with *Columba livia* Sex Determination primer set 2 (with loop primers), at 67ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC, with 30 mM of Component A.

***C. livia* Sex Determination TtP (min)**

Samples	0 mM Component A	30 mM Component A
Female Genome	0	42.44
Male Genome	0	62.44
NTC	0	52.82

The increase in temperature caused a delay of approximately 12 minutes for the amplification of the female genomic DNA sample relative to the previous condition (65°C, 30 mM Component A). The

NTC amplification was reduced from 80.16 to 52.82 minutes. The increase in temperature, in this case, was not favorable for the specificity of the reaction.

Reaction condition CL2.7

The primers were tested again at 65ºC, with and without 30 mM of Component A (STAB VIDA), and with loop primers concentrations of 0.4 μM or 0.8 μM.

Table 5.12: LAMP test for the sex determination of *C. livia* females under **RC CL2.6**, with *Columba livia* Sex Determination primer set 2 (with loop primers), at 65ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes at 95ºC, with 30 mM of Component A, and different concentrations of loop primers.

***C. livia* Sex Determination TtP (min)**

	0 mM Component A		30 mM Component A	
Samples	0.4 μM	0.8 μM	0.4 μM	0.8 μM
Female Genome	0	47.88	22.58	23.62
Male Genome	82.26	0	75.36	71.38
NTC	100.92	92.2	39.7	51.02

Despite continuing to generate false positives, the condition that had the lowest TtP for the female genomic DNA sample was with 30 mM Component A and 0.4 μM loop primers. If the test time was 30 minutes there would be no false positives during that time. However, further testing with real samples (feather tips in the collection/lysis buffer) and optimizations will be needed to ensure that female samples amplify before this time, and that male samples would not amplify within those 30 minutes.

5.2.2 Proof-of-Concept for Sample Control

Before testing this primer set with LAMP, a PCR was performed to assess the conservation of this region between individuals.

DNA was extracted from feather tips and tested in PCR with STAB VIDA's Bird Sexing Routine A. All samples, both males and females were detected as was expected. The bands have all approximately 150-200 bp.

The products from this PCR were also purified with magnetic beads and used in the LAMP reactions as the positive control.

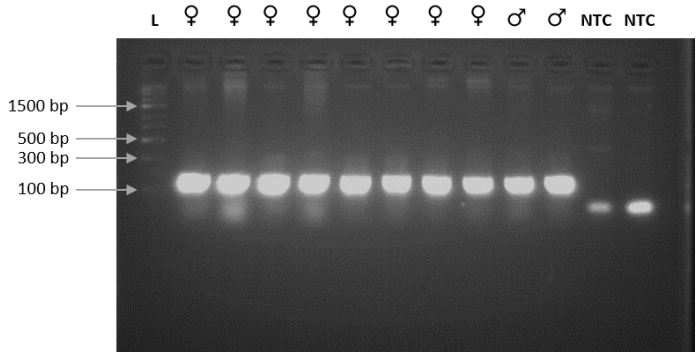

Figure 5.4: Agarose gel 2% with TAE 1×, 130 V for 45 minutes, with the PCR products of forward and reverse primers from the Sample Control primer set 1, with *Columba livia* male and female samples. These primers generated a ~150 bp bands for female and male samples, as expected. L: GeneRuler Express DNA Ladder (ThermoScientific, SM1552), NTC: non-template control.

The PCR products were sequenced by Sanger, and the alignments analysed. While trying to make correspondence of FIP and BIP primers to these sequences, F1 and BIP were found, although with some mismatches.

Reaction condition SC1.1

The feather tips were treated with STAB VIDA's Buffer E or STABVIDA's Bird Sexing Routine A (DNA extraction steps). The test was performed at 65ºC for 1 hour.

Table 5.13: LAMP test for the sample control of sex determination *C. livia* females under **RC SC1.1**, Sample control primer set 1, at 65ºC for 60 minutes. The feather tips were treated with STAB VIDA's Bird Sexing Routine A (DNA extraction steps) and Buffer E.

Samples	Sample Control TtP (min)
Female	11.03
Buffer E	11.15
Male	11.27
Buffer E	16.34
Female	0
Routine A	0
Male	0
Routine A	0
Pos Control	5.30
	8.45
NTC	30.10
	39.86

The only samples to amplify were the female and male samples to which the Buffer E treatment was applied. Keeping in mind the previous results obtained, there is a possibility that some component pertaining to the STAB VIDA's Bird Sexing Routine A (DNA extraction steps) could lead to inhibition of the reaction. Common components to lysis buffer are detergents such as sodium dodecyl sulphate (SDS)[99]. SDS and other ionic detergents have been known to inhibit PCR, by breaking down the polymerase protein structure[100,101]. One can deduce that it would have the same effect in LAMP. This could

be the reason for the lack of amplification on LAMP with samples treated with STAB VIDA's Bird Sexing Routine A (DNA extraction steps). Since the lysis and DNA extraction steps used from the Bird Sexing services are for samples that will be tested with PCR, the PCR mastermix used probably contains non-ionic agents such as Triton X-100 or Tween 20 that can attenuate the effect of SDS over the PCR reaction[100].

An agarose gel was run with the samples to verify the results.

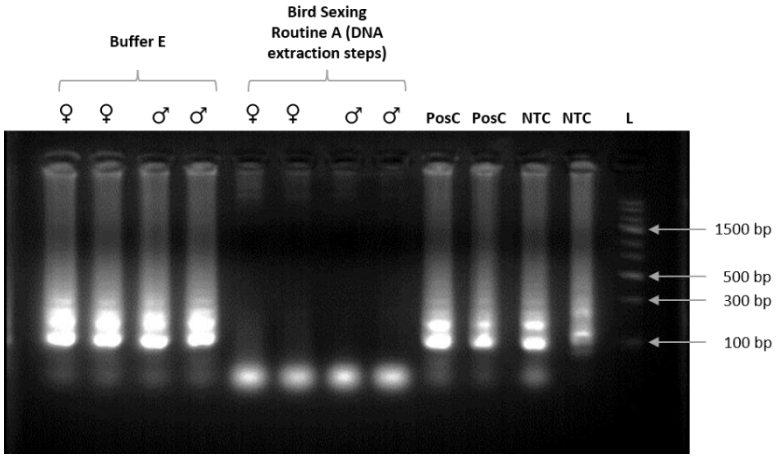

Figure 5.5: Agarose gel 2% with TAE 1×, 130 V for 45 minutes, of the LAMP products with Sample Control primer set 1. The typical LAMP band pattern is present in both female and male samples treated with Buffer E, PosC and NTC, as verified through the real-time instrument. L: GeneRuler Express DNA Ladder (ThermoScientific, SM1552), PosC: Positive Control, NTC: non-template control.

The results presented by the real-time PCR instrument are confirmed by the visualization of the agarose gel, including the amplification of the NTC.

Reaction condition SC1.2

More samples were treated with Buffer E and tested again at 65ºC to confirm the previous results.

Table 5.14: LAMP test for sample control of sex determination *C. livia* females under **RC SC1.2**, Sample control primer set 1, at 65ºC for 60 minutes. The feather tips were treated with Buffer E (STAB VIDA).

Samples	Sample Control TtP (min)
Female	11.20
Female	11.45
Female	12
Female	12.32
Female	12.35

(continuation) Table 5.15: LAMP test for sample control of sex determination *C. livia* females under **RC SC1.2**, Sample control primer set 1, at 65ºC for 60 minutes. The feather tips were treated with Buffer E (STAB VIDA).

Samples	Sample Control TtP (min)
Male	12.32
Male	12.40
Male	13
Male	12.24
Male	12.20
Pos Control	7.40
NTC	41.90

Both male and female samples were detected, same as the positive control. The NTC was also amplified, but with great separation from the true-positives.

Reaction condition SC1.3

Other sample treatment was tested since the method that was being used with Buffer E was not the most suitable for POCT. Thus, the feather tips, 2 or 3, were transferred to 20 μL of Sample collection buffer A and incubated at 95ºC for 10 minutes.

Table 5.16: LAMP test for sample control of sex determination *C. livia* females under **RC SC1.3**, Sample control primer set 1, at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes.

Samples	Sample Control TtP (min)
Female	11.40 12.30
Female	10.20 11.32
Male	10 11.15
Male	12.34 13
Pos Control	8.13 8.08
NTC	30.50 52.17

Both female and male samples were detected before 15 minutes, with the NTC amplifying after 30 minutes, although the other duplicate only amplified at 52 minutes.

Reaction condition SC2.1

New primers (sample control primer set 2) were designed and tested due to the false positives of the previous, targeting the same region. Feather tips were boiled at 95ºC for 10 minutes.

Table 5.17: LAMP test for sample control of sex determination *C. livia* females under **RC IC2.1**, Sample control primer set 2, at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes.

Samples	Sample Control TtP (min)
Female	40
Male	37.43
Female Genome	33.56
NTC	0

Although the TtPs increased for both the female, male and genomic female DNA samples, the NTC did not amplify. Next, optimizations for these primers were tested.

5.2.3 Optimizations for Sample Control

Reaction condition SC2.2

The feather tips were boiled at 95ºC for 10 minutes.

Table 5.18: LAMP test for the sample control of sex determination *C. livia* females under **RC SC2.2**, Sample control primer set 2, at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes.

Samples	Sample Control TtP (min)
Female	41.59
Male	46.04
NTC	54.36

This condition generated a false positive, though with a significant TtP increase. The increase of the female sample TtP is not significant, but the male TtP increase was of almost 10 minutes.

Reaction condition SC2.3

Component A was used at 40 mM, since it has been observed to improve both sensitivity and specificity.

Female genomic DNA was purified from feather tips boiled in Sample collection buffer A with E.Z.N.A Tissue Kit, to be used as a positive control.

Table 5.19: LAMP test for sample control of sex determination *C. livia* females under **RC SC2.3**, Sample control primer set 2, at 65ºC for 60 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes, and 40 mM of Component A was added to the reaction mixture.

Samples	Sample Control TtP (min)
Female	47.42
Male	51.35
Female Genome	41.89
NTC	31.38

The use of Component A at this concentration was not favourable for the reaction, since the TtPs of true samples increased compared to the previous reaction. The TtP of the NTC was the only one that decreased.

Reaction condition SC2.4

From this result on, the reaction is 2 hours.

To this condition with the sample control primer set 2, 7.5% Component B (STAB VIDA) was added in order to try and increase the stringency. As mentioned in the results presented for the bird sex determination reactions, a gradient of Component B was performed, and this percentage was the calculated for obtaining the best results (data not shown).

Table 5.20: LAMP test for sample control of sex determination *C. livia* females under **RC SC2.4**, Sample control primer set 2, at 65ºC for 120 minutes. The feather tips were boiled in 20 μL of Sample collection buffer A for 10 minutes, and 7.5% Component B was added to the reaction mixture.

Sample Control

TtP (min)

Samples	0% Component B	7.5% Component B
Female	16.7	21.06
Male	20.28	45.86
Female Genome	16.3	28.76
NTC	44.8	51.44

Without Component B, the samples' TtP are lower than that of the samples with the addition of the buffer. Despite the possible presence of inhibitors in this type of sample[62,98], the TtP pertaining to both the male and female sample display a similar TtP to that of the female genomic DNA sample in a great portion of the reactions.

Reaction condition SC1.4

Primer set 1 was tested again, in a 2-hour reaction, at 67ºC and with 30 mM of Component A, with genomic DNA for both male and female extracted from feather tips with E.Z.N.A Tissue kit.

Table 5.21: LAMP test for sample control of sex determination *C. livia* females under **RC SC1.4**, Sample control primer set 1, at 67ºC for 120 minutes. The samples tested were female and male genomic DNA extracted from feather tips with E.Z.N.A Tissue Kit, and 30 mM of Component A (STAB VIDA) was added to the reaction mixture.

Sample Control

TtP (min)

Samples	0 mM Component A	30 mM Component A
Female Genome	12.98	19.14
Male Genome	13.10	20.58
NTC	105.24	56.70

In the absence of 30 mM of Component A, the difference between the true positives and NTC TtPs was greater. At this reaction temperature, not using Component A is then more favourable to the specificity and sensibility of the primers.

5.3 Sex Determination of *Psittacula krameri*

5.3.1 PCR with *P. krameri* female samples with 2550F/2718R primers

According to the STAB VIDA's Bird Sexing Service, the primers 2550F/2718R only detect females on *Psittacula krameri.* Therefore, these primers were used, the PCR products were sequenced and aligned, and primers were designed based on those sequences.

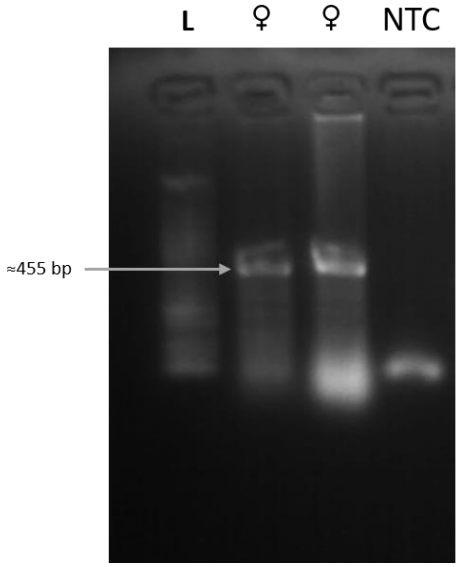

Figure 5.6: Agarose gel 2% with TAE 1×, 130 V for 60 minutes, with the products of PCR primers 2550F/2718R, with *Psittacula krameri* female samples. These primers generated a ~455 bp bands for the female samples, as expected. L: Quick-Load Purple 50 bp DNA Ladder (NEB, N0556S), NTC: non-template control.

This consensus sequence was uploaded to Primer Explorer V5, and LAMP primers for the identification of *P. krameri* females were designed.

5.3.2 Sample collection device

Sample collection kit 1

Sample collection kit 1 contains 500 μL Sample collection buffer A.

Figure 5.7: Collection kit 1 (STAB VIDA) for the collection and incubation of feather tips in 500 μL of Sample collection buffer A.

Drop assays were performed to estimate the drop volume of this tube. Three assays of 5 drops were made, the average value and standard deviation were calculated.

Table 5.22: Drop volume assay to measure the drop volume released by collection kit 1. The values are represented in mg, approximately equivalent to μL.

	Sample collection buffer volume: 500 μL		
	1st assay	2nd assay	3rd assay
Assay average	32.10	30.60	31.38
Standard Deviation	2.925	3.452	1.958
Global Average	31.36		
Global Standard Deviation	2.71		

The average volume of the drop from this kit is 30 μL.

Sample collection kit 2

Sample kit 1 with 500 μL of Sample collection Buffer A. The average drop volume from this kit is 10 μL.

Figure 5.8: Collection kit 2 (STAB VIDA) for the collection and incubation of feather tips in 500 μL of Sample collection Buffer A.

5.3.3 Proof-of-Concept: Doctor Vida® Pocket Testing

5.3.3.1 Female *Psittacula krameri* Sex Determination Primer set 1

The samples used were tested blind, i.e., without knowing the sex. The results obtained were later compared with the results from the bird sexing service.

Reaction condition DVK1, 2, 3, 4, 5 and 6

With a Sample collection buffer A volume of 200, 300, 400, 500, 700 and 1000 μL and 1 feather tip, and Kit 1 (30 μL sample/reaction tube). The tips were incubated by 30 minutes before testing.

Table 5.23: Doctor Vida® Pocket test **RC DVK1, 2, 3, 4, 5** and **6** for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was incubated in 200, 300, 400, 500, 700 and 1000 μL Sample collection buffer A, respectively, for 30 minutes. Then 30 μL (collection kit 1) were transferred to the reaction tube.

Sample Collection Buffer A Volume (μL)

DV Pocket TtP (min)

Bird Ring Num.	200	300	400	500	700	1000	Bird Sexing STAB VIDA
BIRD508788	0 Male	0 Male	0 Male	73 Female	0 Male	0 Male	Female
BIRD508729	35.5 Female	0 Male	35.5 Female	36.5 Female	37 Female	28 Female	Female
BIRD128701	29 Female	26.5 Female	22.5 Female	32 Female	72 Female	40.5 Female	Female

(continuation) Table 5.24: Doctor Vida® Pocket test **RC DVK1, 2, 3, 4, 5** and **6** for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was incubated in 200, 300, 400, 500, 700 and 1000 μL Sample collection buffer A, respectively, for 30 minutes. Then 30 μL (collection kit 1) were transferred to the reaction tube.

Sample Collection Buffer A Volume (μL)

DV Pocket TtP (min)

Bird Ring Num.	200	300	400	500	700	1000	Bird Sexing STAB VIDA
BIRD505586	N/A	0 Male	N/A	0 Male	N/A	N/A	Male
BIRD505585	N/A	0 Male	N/A	0 Male	N/A	N/A	Male
BIRD505583	N/A	0 Male	N/A	0 Male	N/A	N/A	Female
BIRD505584	N/A	0 Male	N/A	N/A	N/A	N/A	Male
BIRD508731	N/A	36 Female	N/A	23 Female	N/A	N/A	Female
BIRD508730	N/A	0 Male	N/A	N/A	N/A	N/A	Male
BIRD508733	N/A	75 Female	N/A	79 Female	N/A	N/A	Male
NTC	0						-

The condition that produced the best results was the one where 500 μL of sample collection buffer, although one of the female samples was detected only after 73 minutes, and the intention was to cut the time of the reaction to 60 minutes which in that case would make this sample a male in this test.

Reaction condition DVK7

Before optimizing some of the conditions already tested, a test was made with 10 samples, with the tip of 1 feather directly on the reaction tube with no incubation time.

Table 5.25: Doctor Vida® Pocket test **RC DVK7** for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was transferred directly to the reaction tube, and tested without any incubation time.

Bird Ring Num.	**DV Pocket TtP (min)**	**Bird Sexing STAB VIDA**
BIRD192135	0 Male	Female
BIRD126800	33 Female	Female
BIRD126817	0 Male	Male

(continuation) Table 5.26: Doctor Vida® Pocket test **RC DVK7** for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was transferred directly to the reaction tube, and tested without any incubation time.

Bird Ring Num.	DV Pocket TtP (min)	Bird Sexing STAB VIDA
BIRD126803	25 Female	Female
BIRD126804	78 Female	Male
BIRD126805	26.5 Female	Female
BIRD508814	0 Male	Female
BIRD129486	42.5 Female	Male
BIRD208307	34.5 Female	Female
BIRD208302	0 Male	Male
NTC	0	-

These results were expected. Some samples were detected, even before 40 minutes, while others were not detected and even generated 2 false-positives. This was probably caused by possible inhibitors present in this type of sample[62,98].

Reaction condition DVK8 and 9

The reaction conditions using 500 and 1000 μL of Sample collection buffer A were tested with different additives, with collection kit 1 (10 μL sample/reaction tube).

Table 5.27: Doctor Vida® Pocket test RC DVK8 and 9 for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was incubated for 30 minutes in 1000 μL or 500 μL of Sample collection buffer A, respectively. From that, 30 μL of sample (collection kit 1) were transferred to the reaction tube. Buffer E (19.6% v/v) and 33.4 mM of Component A (STAB VIDA) were added to the reaction mixture.

	DV Pocket TtP (min) 1000 μL			DV Pocket TtP (min) 500 μL			
Bird Ring Num.	No additives	Buffer E	33.4 mM Component A	No additives	Buffer E	33.4 mM Component A	STAB VIDA Results
BIRD508788	0 Male	0 Male	0 Male	0 Male	0 Male	0 Male	Female
BIRD508731	19.5 Female	24.5 Female	18.5 Female	N/A	N/A	N/A	Female
BIRD128701	0 Male	0 Male	0 Male	0 Male	0 Male	0 Male	Female

(continuation) Table 5.28: Doctor Vida® Pocket test RC DVK8 and 9 for sex determination of *P. krameri* at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 1 feather tip was incubated for 30 minutes in 1000 μL or 500 μL of Sample collection buffer A, respectively. From that, 30 μL of sample (collection kit 1) were transferred to the reaction tube. Buffer E (19.6% v/v) and 33.4 mM of Component A (STAB VIDA) were added to the reaction mixture.

	DV Pocket TtP (min) 1000 μL			**DV Pocket TtP (min) 500 μL**			
Bird Ring Num.	No additives	Buffer E	33.4 mM Component A	No additives	Buffer E	33.4 mM Component A	**STAB VIDA Results**
BIRD252715	N/A	N/A	0 Male	N/A	N/A	N/A	Male
BIRD252707	N/A	N/A	0 Male	N/A	N/A	N/A	Female
BIRD252710	N/A	N/A	66 Female	N/A	N/A	N/A	Female
BIRD127876	N/A	N/A	0 Male	N/A	N/A	N/A	Male
BIRD127875	N/A	N/A	0 Male	N/A	N/A	N/A	Male
BIRD127874	N/A	N/A	0 Male	61 Female	58 Female	60 Female	Female
BIRD126816	N/A	N/A	0 Male	N/A	N/A	N/A	Male
BIRD126812	N/A	N/A	52 Female	N/A	N/A	N/A	Female
BIRD127873	N/A	N/A	22 Female	N/A	N/A	N/A	Female
BIRD127872	N/A	N/A	71 Female	N/A	N/A	N/A	Male
NTC	0						-

It was observed that usually the samples tested that were incubated in 500 μL has better amplification times than the others, and even amplified samples that were not detection with other conditions. Thus, further tests were conducted with a sample collection buffer volume of 500 μL.

Reaction condition DVK10 and 11

It was discussed that the high TtPs obtained could be due to the use of only 1 feather tip. Purwaningrum *et al* (2019) also observed a visual difference of PCR products on agarose gel when using 1 or more feather tips, the band being clearer and more intense when using more feather tips[67]. The use of 3 feather tips per sample, incubated for 30 minutes in 500 μL of Sample collection buffer A, with kit 1 and 2 were tested.

Table 5.29: Doctor Vida® Pocket test RC DVK10 for sex determination of P. krameri at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 30 μL (collection kit 1) were transferred to the reaction tube. The failed sample (BIRD208307) was re-tested with both collection kit 1 and 2.

Bird Ring Num.	Kit 1 (30 μL/reaction tube) TtP (min)	Test repetition (30 μL/reaction tube) TtP (min)	Test repetition (10 μL/reaction tube) TtP (min)	Bird Sexing STAB VIDA
BIRD192135	29.5 Female	N/A	N/A	Female
BIRD126800	50 Female	N/A	N/A	Female
BIRD126817	0 Male	N/A	N/A	Male
BIRD126803	26.5 Female	N/A	N/A	Female
BIRD126804	0 Male	N/A	N/A	Male
BIRD126805	26 Female	N/A	N/A	Female
BIRD508814	18 Female	N/A	N/A	Female
BIRD129486	0 Male	N/A	N/A	Male
BIRD208307	0 Male	0 Male	26.5 Female	Female
BIRD208302	0 Male	N/A	N/A	Male
NTC	0			-

The sample that failed with kit 2 was repeated with both kit 1 and 2, and was only detected with kit 2. It was then hypothesized that using a smaller sample volume on the reaction mixture, although diluting the target also dilutes the possible inhibitors on the sample, which causes a decrease on its inhibitory effect.

Table 5.30: Doctor Vida® Pocket test RC DVK11 for sex determination of P. krameri at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 10 μL (collection kit 2) were transferred to the reaction tube. The failed sam-ples (BIRD506520 and BIRD129626) were re-tested with collection kit 1.

Bird Ring Num.	Kit 2 (10 μL/reaction tube) TtP (min)	Test repetition 10 μL/reaction tube TtP (min)	Bird Sexing STAB VIDA
BIRD123515	0 Male	N/A	Male
BIRD123517	23 Female	N/A	Female
BIRD123518	0 Male	N/A	Male
BIRD506516	25 Female	N/A	Female
BIRD506517	0 Male	N/A	Male
BIRD506519	0 Male	N/A	Male
BIRD506520	0 Male	62 Female	Female
BIRD129792	0 Male	N/A	Male
BIRD129626	0 Male	29 Female	Female
BIRD129795	0 Male	N/A	Male
NTC	0	N/A	-

With the repetition of the samples that were not detected in the first test, and since that in the repetition the same kit was used (10 μL of sample/reaction tube) and were detected, this test was considered to have a success rate of 100%.

Further improvements can be made in the incubation time, reaction temperature or sample treatment. Sample collection buffer A as the sample collection buffer seems to work on lysing the cells and releasing the DNA, but it does not eliminate or inactivate possible reaction inhibitors. Besides, it was already hypothesized that bird sex determination through NAATs could sometimes be unsuccessful due to intron variations between individuals and polymorphisms on the W chromosome[62,102,103].

5.3.4 PCR with *P. krameri* samples and P2/P8 primers

Female and male P. krameri samples were amplified by PCR with primers P2/P8. These primers are supposed to generate 2 different PCR products for females and 1 for males. Two separate PCR reactions were performed and it was only possible to visualize 1 band for females on a 2% agarose gel.

Therefore, the products were sequenced and aligned. A new primer set for detection of females and one for the detection of males (as a possible sample control for the reaction).

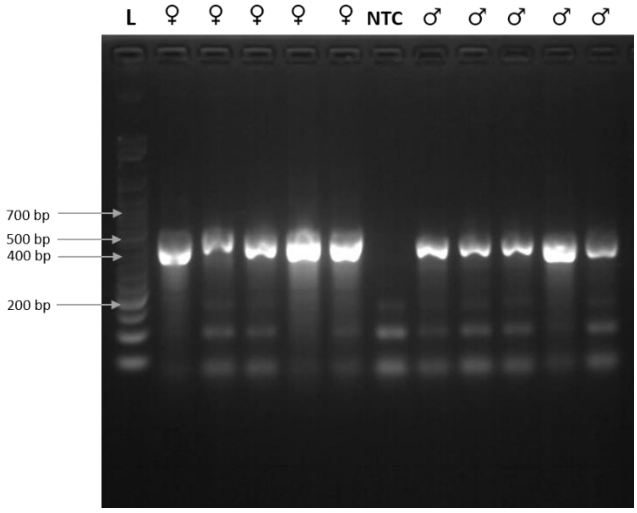

Figure 5.9: Agarose gel 2% with TAE 1×, 130 V for 80 minutes, with the products of PCR primers P2/P8, with *Psittacula krameri* female and male samples. These primers were supposed to generate 2 bands for females and 1 band for male samples, but in both sexes, it is only possible to visualize 1 band of approximately 350 bp. L: Quick-Load Purple 50 bp DNA Ladder (NEB, N0556S), NTC: non-template control.

The consensus sequences taken from the alignment of both female and male products with P2/P8 primers were uploaded to PrimerExplorer V5 and primers for female and male sex determination were designed.

5.3.5 Proof-of-concept with Female *P. krameri* Sex Determination Primer set 2

Reaction condition DVFK1

This first test was performed on Doctor Vida® Pocket test, at 61ºC, for one hour and a half.

Table 5.31: Doctor Vida® Pocket test **RC DVFK1** for sex determination of *P. krameri* females at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 10 μL (collection kit 2) were transferred to the reaction tube.

Samples	*P. krameri* Sex Determination TtP (min)
Female	0
Female	0
Male	0

(continuation) Table 5.32: Doctor Vida® Pocket test **RC DVFK1** for sex determination of *P. krameri* females at 61ºC for 90 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 10 μL (collection kit 2) were transferred to the reaction tube.

Samples	*P. krameri* Sex Determination TtP (min)
Male	0
NTC	0

None of the male samples were amplified as expected. However, the female samples were not detected. This test was repeated on the real-time PCR instrument, as well as the following tests and optimizations.

Reaction condition FK1

With the repetition of the previous reaction temperature on the real-time, the instrument was able to detect the amplification of a female and 2 male samples, as well as the NTC.

Table 5.33: LAMP test for sex determination of *P. krameri* females under **RC FK1** for sex determination of *P. krameri* at 61ºC for 60 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A.

Samples	*P. krameri* Sex Determination TtP (min)
Female	44.64
Female	0
Male	36.95
Male	41.43
NTC	58.04

A smaller reaction and sample volume were used and so a direct comparison with the reaction on the DV Pocket cannot be made. However, the amplification of these samples at the same temperature and using the same samples could imply different fluorescence detection sensitivities between the real-time instrument and DV Pocket.

Reaction condition FK2 and FK3

Next, the temperature was increased to 63ºC and then 65ºC to try eliminating the false-positives.

Table 5.34: LAMP test for sex determination of *P. krameri* females under **RC FK2** and **3** for sex determination of *P. krameri* at 63 and 65ºC, respectively, for 60 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A.

Samples	***P. krameri*** **Sex Determination FK2 TtP (min)**	***P. krameri*** **Sex Determination FK3 TtP (min)**
Female	36.93	54.68
Female	42.46	0
Male	54.74	37.36
Male	33.81	53.87
NTC	34.55	41.25

The increase in temperature increased the TtP of female samples and did not eliminate false positives.

Reaction condition FK4

A Component C (STAB VIDA) was used in different concentrations to help increasing the stringency of the reaction, hence the increasing the specificity.

All Component C concentrations tested generated false-positives, either by the amplification of male samples or the NTC, at similar TtPs as the female samples.

Table 5.35: LAMP test for sex determination of *P. krameri* females under **RC FK4** for sex determination of *P. krameri* at 63ºC for 60 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. To the reaction mixture 0.5, 0.63, 0.75 and 0.88× of Component C were added.

	P. krameri **Sex Determination TtP (min)**				
Samples	0× Component C	0.50× Component C	0.63× Component C	0.75× Component C	0.88× Component C
Female	31.34	22.30	23.53	57.72	0
Female	38.15	35.86	30.73	0	0
Male	38.54	50.9	29.71	49.4	0

(continuation) Table 5.36: LAMP test for sex determination of *P. krameri* females under **RC FK4** for sex determination of *P. krameri* at 63ºC for 60 minutes, with Female *P. krameri* Sex Determination primer set 2. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. To the reaction mixture 0.5, 0.63, 0.75 and 0.88× of Component C were added.

Samples	***P. krameri* Sex Determination TtP (min)**				
	0× Component C	0.50× Component C	0.63× Component C	0.75× Component C	0.88× Component C
Male	0	44.51	30.09	0	0
NTC	39.21	54.05	39.13	0	0

The lack of specificity of these primers can possibly be linked to the fact that the P2/P8 primers generate 2 PCR fragments for females, 1 for the W chromosome and one for the Z. The fact that only 1 band could be observed on the agarose gel could be due to the small differences in length between those 2 fragments, which is about 30 bp. Therefore, the products sequenced were hypothetically a mixture of the fragment from the female and male chromosome. This resulted in primers that amplify both males and females in most conditions.

5.3.6 Proof-of-Concept with Male *P. krameri* Sex Determination Primer set 1

Reaction condition DVMK1

This first test was performed on Doctor Vida® Pocket test, at 61ºC, for one hour and a half.

Table 5.37: Doctor Vida® Pocket test **RC DVMK1** for sex determination of *P. krameri* at 61ºC for 90 minutes, with Male *P. krameri* Sex Determination primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 10 μL (collection kit 2) were transferred to the reaction tube.

Samples	***P. krameri* Sex Determination TtP (min)**
Female	0
Female	0
Male	0
Male	0
NTC	0

As observed with the female primer set 2, none of the samples were amplified, so the test was repeated with the real-time instrument.

Reaction condition MK1 and MK2

Even with the real-time instrument at 61ºC, no amplification was detected. Thinking the problem was the reaction temperature, and since that in some studies bird sexing with LAMP primers was optimized to 57ºC, the primers were tested at that temperature. At 57ºC there was only a false-positive.

Table 5.38: LAMP test for sex determination of *P. krameri* males under **RC MK1** and **2** for sex determination of *P. krameri* at 63 and 57ºC, respectively, for 60 minutes, with Male *P. krameri* Sex Determination primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A.

Samples	***P. krameri*** **Sex Determination MK1 TtP (min)**	***P. krameri*** **Sex Determination MK2 TtP (min)**
Female	0	0
Female	0	0
Male	0	0
Male	0	0
NTC	0	54.25

Although the observed conservation of the region used to design these primers was high, the primers have been unsuccessful for the sex determination of male *P. krameri* male samples.

5.3.7 PCR with *P. krameri* samples and dgLCO1490/dgHCO2198 primers

Female and male *P. krameri* sample were amplified by PCR with primers dgLCO1490/dgHCO2198. These primers target the *COI* gene, generating a 708-bp product, and are used for species-ID since it is a highly conserved region between related species.

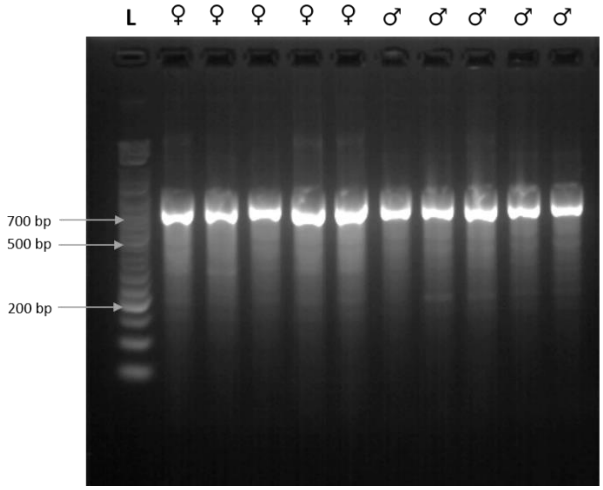

Figure 5.10: Agarose gel 2% with TAE 1×, 130 V for 80 minutes, with the products of PCR primers dgLCO1490/dgHCO2198, with *Psittacula krameri* female and male samples. These primers generated a ~700 bp band in both sexes, as expected. L: Quick-Load Purple 50 bp DNA Ladder (NEB, N0556S).

The alignment showed high conservation of this sequence between males and females. The consensus was uploaded to Primer Explorer V5 and LAMP primers were designed, targeting *P. krameri* COI gene.

5.3.8 Proof-of-Concept with Sample Control Primer set 1

Reaction condition DVCOI1

The primers targeting the COI gene were tested at 61ºC with Doctor Vida® Pocket.

Table 5.39: Doctor Vida® Pocket test **RC DVCOI1** for sample control of *P. krameri* female sex determination at 61ºC for 90 minutes, with Internal control primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 μL of Sample collection buffer A. From that, 10 μL (collection kit 2) were transferred to the reaction tube.

Samples	Sample Control TtP (min)
Female	35.50
Female	36
Male	80
Male	49
NTC	0

All samples were detected, although one of the male samples was amplified only after 80 minutes.

5.3.8.1 Optimizations

Reaction condition COI1

The previous condition (61ºC) was re-tested on the real-time instrument. Only one male was detected.

Table 5.40: LAMP test under **RC COI1** for sample control of *P. krameri* female sex determination at 61ºC for 60 minutes, with Sample control primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 µL of Sample collection buffer A.

Samples	Sample Control TtP (min)
Female	0
Female	0
Male	0
Male	34.24
NTC	0

Contrary to what was observed with the first test on the DV Pocket and then with the real-time instrument with primer set 2 for sex determination of Female *P. krameri*, more samples were detected with Doctor Vida® Pocket than with the real-time instrument. Other reaction conditions were tested for optimization.

Reaction condition COI2 and 3

The primers were tested at 63 and 65ºC to assess the influence of temperature in this test.

Table 5.41: LAMP test under **RC COI2** and **3** for sample control of *P. krameri* female sex determination at 63 and 65ºC for 60 minutes, with Sample control primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 µL of Sample collection buffer A.

Samples	Sample Control COI2 TtP (min)	Sample Control COI3 TtP (min)
Female	20.62	13.92
Female	21.60	0
Male	0	0
Male	0	0
NTC	0	0

None of the temperatures were ideal for the amplification of the male and female samples. However, the primers behaved better at 63ºC, amplifying the 2 female samples under 25 minutes.

Reaction condition COI4

This reaction was performed in the presence of gradient concentration of Component C (STAB VIDA), to help increase the specificity of the reaction, by increasing the stringency conditions.

Table 5.42: LAMP test under **RC COI4** for sample control of *P. krameri* female sex determination at 63ºC for 60 minutes, with Sample control primer set 1. For each sample 3 feather tips were incubated for 30 minutes in 500 µL of Sample collection buffer A. A concentration gradient of Component C (STAB VIDA) was tested.

	Sample Control				
	TtP (min)				
Samples	0× Component C	0.50× Component C	0.63× Component C	0.75× Component C	0.88× Component C
Female	16.16	10.11	11.31	37.08	21.08
Female	0	11.44	0	12.03	22.65
Male	14.82	9.11	7.79	11.31	25.78
Male	16.66	9.36	8.64	12.31	20.97
NTC	0	0	0	0	0

None of the concentrations of Buffer C generated false positives. The optimal concentration was 0.50× since all samples amplified around the same time, between 9 and 11 minutes, approximately, and had the lowest TtP from all the conditions tested so far.

This condition was then used for an attempt at multiplexing these primers were the Female *P. krameri* sex determination primer set 1, distinguishing the results from each reaction with high-resolution melt.

5.3.9 Multiplexing

Before testing these primers in a multiplex format, the melt curves of their products were obtained on the real-time instrument.

Figures 5.11 and 5.12 show the melting curves of Female *P. krameri* Sex Determination primer set 1 and Sample Control primer set 1, respectively.

The average melting temperature for the products of the sex determination primer set is 79.78ºC, and for in products of the sample control primers are 81.61ºC. These products could then be distinguished by high-resolution melting.

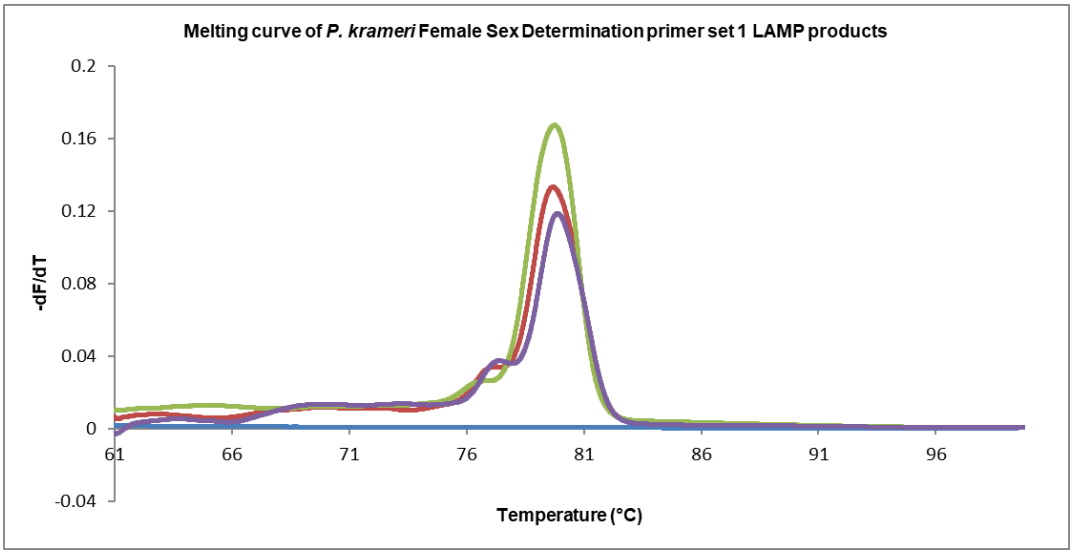

Figure 5.11: Melting curve of *P. krameri* Female Sex Determination primer set 1 LAMP products, with DNA samples. The average melting temperature is 79.78ºC.

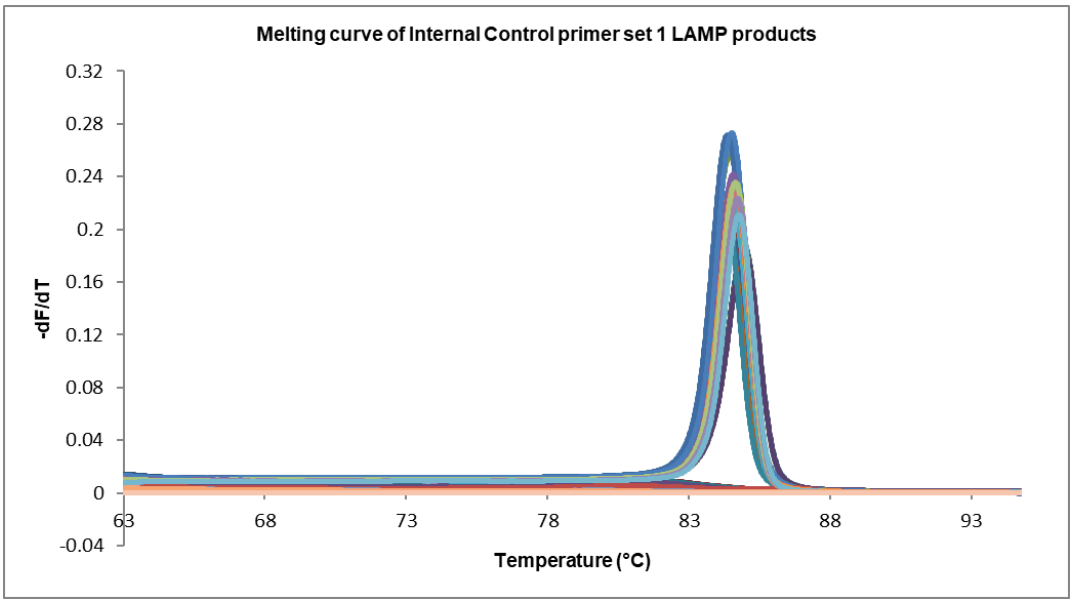

Figure 5.12: Melting curve of Sample Control primer set 1 LAMP products, with DNA samples. The average melting temperature is 84.61ºC.

Multiplex reaction condition 1

The first attempt was made at 63ºC, since it was the optimal temperature for the sample control primers with 0.5× of Component C (STAB VIDA).

Without Component C only one male and one female were detected, with an average melting temperature of 85.05ºC, which is closer to the melting temperature of the LAMP products of the internal control primers.

With 0.5× of Component C all male and female samples were detected, with an average melting temperature of 84.58ºC, that is also close to the melting temperature of the products for the in-ternal control primers. Therefore, this multiplex test was not successful because either the internal control

primers are favoured, as was observed with the attempts of multiplexing the test for the detection of *B. hyodysenteriae* and its internal control; or the products cannot be distinguished by HRM.

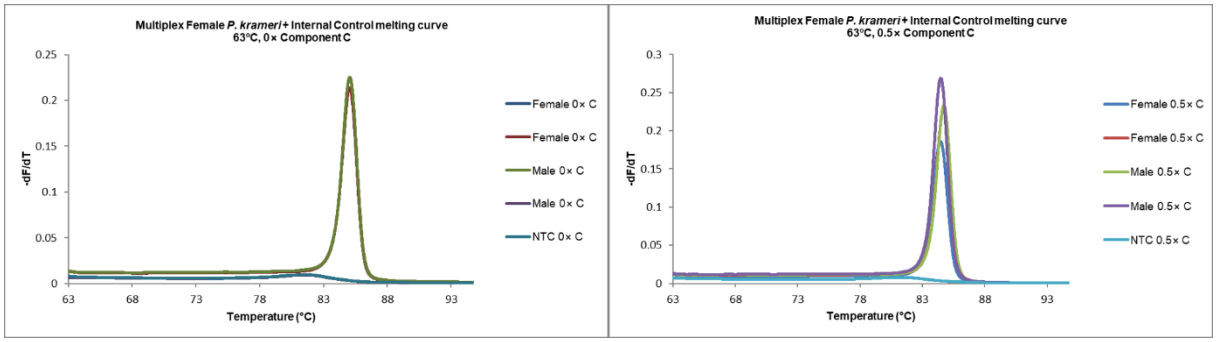

Figure 5.13: Melting curve of multiplex test 1 with primer sets 1 for Female *P. krameri* Sex Determination and Sample Control primer set 1, with feather tips incubated in 500 µL of Sample collection buffer A for 30 minutes, at 63ºC, in the presence and absence of 0.5× Component C (STAB VIDA).

Multiplex reaction condition 2

The reaction temperature was decreased to 61ºC, the reaction temperature of the Female *P. krameri* sex determination primer set 1.

The LAMP products with both female and male samples generated a melting curve with only 1 peak. Without Component C only 1 female and male sample was detected, with an average melt of 84.88ºC, which corresponds to melting of the internal control primers LAMP products.

As for the multiplex with 0.5× of Component C, all samples were detected, with an average melt temperature of 84.28ºC which is also close to the melt temperature of the products of the internal control primers. The non-template control melting curve had 3 peaks (79.81, 82.88 and 86.17ºC) which could be due to unspecific amplification.

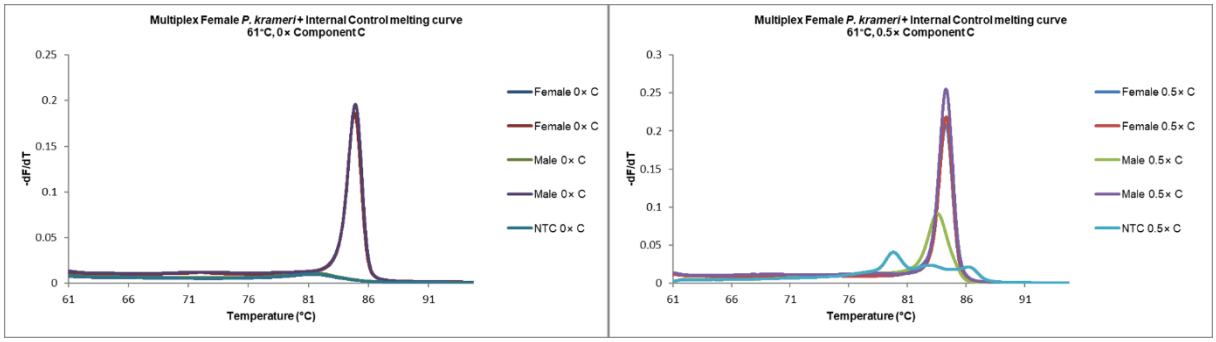

Figure 5.14: Melting curve of multiplex test 2 with primer sets 1 for Female *P. krameri* Sex Determination and Sample Control primer set 1, with feather tips incubated in 500 µL of Sample collection buffer A for 30 minutes, at 61ºC, in the presence and absence of 0.5× Component C (STAB VIDA).

Multiplex reaction condition 3

The reaction temperature was increased to assess if it was possible to slightly undermine the reaction for the internal control and observe amplification of the female-sex specific primers. The out-come of this multiplex was similar to the previous attempts, with the condition with 0.5× Component C

detecting all samples, and the condition without Component C detecting only 1 female and 1 male sample. The melting show equally showcase only 1 melt peak, corresponding to the melting peak of the products from the sample control primers.

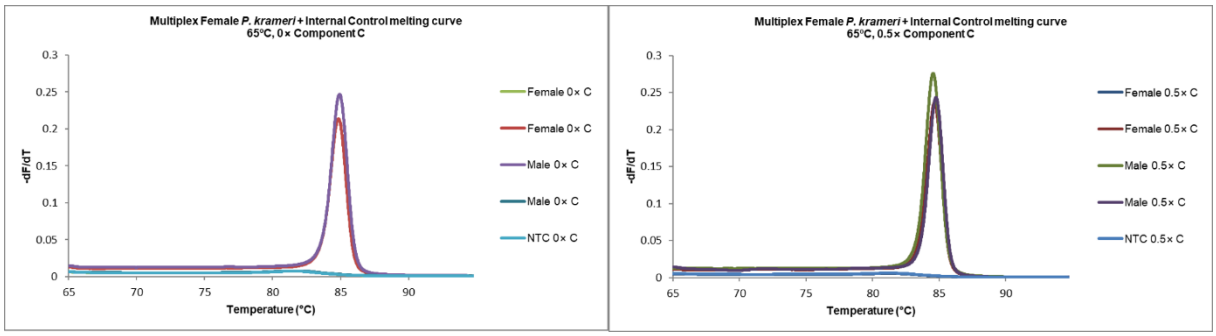

Figure 5.15: Melting curve of multiplex test 3 with primer sets 1 for Female *P. krameri* Sex Determination and Sample Control primer set 1, with feather tips incubated in 500 μL of Sample collection buffer A for 30 minutes, at 65ºC, in the presence and absence of 0.5× Component C (STAB VIDA).

Turning this reaction into a multiplex still needs further adjustments and optimizations, and probably new primer sets that have similar performances, so that both products can be observed by high-resolution melting.

6

CONCLUSIONS AND FUTURE PERSPECTIVES

The goals were partially completed since the proof-of-concept was obtained for both the target detection and sample control on both Projects, and the application of those on Doctor Vida® Pocket. For both projects a clinical validation was not attained.

For the detection of swine dysentery, the target was the *nox* gene from *Brachyspira hyodysenteriae*, and the proof-of-concept was obtained only for the third set of primers. After optimizations the optimal temperature for this reaction was considered at 63ºC without any reaction additives. Although the sample control primers generated a higher number of false positives, these were considered more sensitive since, contrary to what was observed with the primers for the detection of *B. hyodysenteriae*, *E. coli* was detected in all real samples tested (stool and rectal swabs in Exopol's Buffer). An attempt on multiplex testing development was made, in which the LAMP products of the target pathogen and the sample control would be distinguished by HRM. These tests were not successful, and was also observed, using spiked samples, that the ratio of *E. coli* to *B. hyodysenteriae* is an important factor for multiplexing with these primers. However, this parameter is impossible to control in real samples, and as so the multiplex attempts were terminated. Furthermore, during the application of the test to Doctor Vida® Pocket, two sample collection kits were designed and tested, one with a swab and the other with a scoop to collect stool, both with Sample collection buffer A. In these kits was also incorporated Filter A for sample purification, since there was a high probability that certain components of stool could be inhibiting the reaction in some way. In 30 samples tested, with the final version of both kits, was obtained 78.9% sensitivity and 45.5% specificity for kit 1, and 68.4% sensitivity and 72.7% specificity for kit 2, detecting *B. hyodysenteriae* within 40-minutes and 2 minutes hands-on. The clinical validation of the test was not possible during the dissertation's work.

As for the bird sexing, three targets were tested, being that only with the third target there were relevant results for a proof-of-concept. For the first target, CHD-W gene of Psittacidae and an ultraconserved sequence among Neoaves (UCE), a LAMP primer set for sexing and sample control respectively were tested. After sequencing the PCR products of the forward and reverse primers from both sets, a great genetic variability was observed between individuals in these regions and the further correspondence of the FIP and BIP primers in the respective sequences did not succeed.

For the second target, LAMP primers for the bird sexing of *Columba livia* females, using a female specific sequence of *S. orientalis*, and the sample control using the 18s rRNA gene were tested. A new primer design was required for both the bird sexing and the sample control. A different target was employed for the *C. livia* sex determination (CHD1-W) while for the control the same target was used. With both new primer sets it was possible to obtain the proof-of-concept for a 2-hour test. However, even with the tested optimizations the occasional appearance of false positives was inevitable, both for the sexing and sample control.

Finally, the sex determination of birds from the *Psittacula krameri* species was attempted. For the LAMP primer design, 2550F/2718R PCR primers commonly used for the sex determination of species along various orders were used since these only detect the female for *P. krameri*. Then, the products of the PCR were sequenced. LAMP primers were then specifically designed for *P. krameri* females, based on the alignment of the 2550F/2718R's products. In the best condition tested (Sample collection buffer A volume of 500 μL and collection kit 2), the success rate obtained was 80%, which for a commercialized diagnostic test is not ideal. Additionally, there was still no sample control for this reaction to confirm the results. As so, from the aligned products of the PCR P2/P8 primers, the LAMP primers for the detection of male *P. krameri* were designed. The *COI* gene was also targeted, which is primarily used for species ID, as a possible sample control. The male primers were tested at various temperatures, but none were capable to detect the male samples. The *COI* gene primers were tested and optimized for a reaction at 63ºC with 0.5× of Component C. New primers for the sexing of *P. krameri* females were also designed based on the products of the P2/P8 primers. These primers generate 2 different products for the female, from the male (Z) and female (W) chromosomes. Nevertheless, on the agarose gel only one band was perceptible in the female samples, and as so it was sequenced without the extraction of gel bands. Keeping in mind the results observed with these primers, which detected both males and females inconsistently, it is plausible that the sequenced product was a combination of fragments from CHD regions of W and Z chromosomes. Due to these circumstances, a multiplex involving the first primers designed for females and the sample control primers targeting the *COI* gene was attempted, in which the distinction of the target products and of the sample control would be made through HRM. However, the reaction gave preference to the sample control primers since in all tested conditions only the sample control's product melt peak was present.

As so, future work for the diagnosis of Swine Dysentery project would be further optimizations to the collection kits and the reaction, in a way to increase sensitivity. User-friendliness is also something very important to take into account, and could be better studied in further experiments, especially with potential users.

As for the bird sexing tests, more tests with the sample control primers are necessary to guarantee that the success rate observed in the first tests is reliable. The optimization of the primers used for the bird sexing of the *P. krameri* species will then be necessary to guarantee a higher success rate. Measures such as other reaction temperatures, use of additives or new primer designs for the target regions should also be taken.

7

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8

APPENDIX

8.1 Target for primer design for the detection of *B. hyodysenteriae*

Table 8.1: *B. hyodysenteriae* strains and the GenBank accession number of the respective *nox* gene sequences.

B. hyodysenteriae strain	**GenBank Accesion Number**
ATCC 27164	KC984311.1
AN 1409:2/01	DQ487115.1
AN 174/92	DQ487117.1
AN 2420/97	DQ487118.1
AN 383:2/00	DQ487116.1
B169	AF060801.1
B78	AF060800.1
R1	AF060802.1
3140	JX428806.1
49	KU215621.1

8.2 Validation of 30 samples tested with *B. hyodysenteriae* Sample collection kit 1 and 2

Table 8.2: Results from the tests with 30 real samples for the detection of *B. hyodysenteriae* and sample control on Doctor Vida® Pocket, with Sample collection kits 1 (Swab kit) and 2 (Scoop kit), and comparison with the results obtained with the EXOone qPCR kit, to compare the performance of both kits, and validate the previous results obtained. Results from DV Pocket coloured in green means they are in concordance with the ones obtained with the qPCR kit, in red they are not in concordance. The cells coloured yellow represent invalid results, where the sample control (*E. coli*) was not detected.

		Results DV Pocket (TtP)				qPCR Bhyo		qPCR Ecoli	
#	Sample ID	Swab kit		Scoop kit		Cq	Result	Cq	Result
1	179162	POS	Bhyo: 28.5	POS	Bhyo: 31.5	24.11	POS	27.37	POS
			Ecoli: 18.5		Ecoli: 20			POS	
2	178707	POS	Bhyo: 28	POS	Bhyo: 32	23.46	POS	25.56	POS
			Ecoli: 21		Ecoli: 27			POS	
3	177161	Invalid	Bhyo: 0	NEG	Bhyo: 0	-	NEG	31.98	POS
			Ecoli: 0		Ecoli: 26.5			NEG	
4	176894	POS	Bhyo: 25	POS	Bhyo: 28.5	22.86	POS	25.75	POS
			Ecoli: 19.5		Ecoli: 25			POS	
5	176058	POS	Bhyo: 35.5	POS	Bhyo: 38.5	28.25	POS	29.66	POS
			Ecoli: 25		Ecoli: 29.5			POS	
6	175568	POS	Bhyo: 33.5	POS	Bhyo: 27	27.9	POS	29.94	POS
			Ecoli: 23		Ecoli: 19.5			POS	
7	175499	NEG	Bhyo: 0	NEG	Bhyo: 0	31.03	POS	29.33	POS
			Ecoli: 22		Ecoli: 26.5			POS	
8	174575	POS	Bhyo: 28.5	POS	Bhyo: 31.5	26.94	POS	29.43	POS
			Ecoli: 19.5		Ecoli: 20			POS	
9	174485	POS	Bhyo: 25.5	Invalid	Bhyo: 0	32.13	POS	38.76	POS
			Ecoli: 41.5		Ecoli: 0			POS	
10	174485	Invalid	Bhyo: 0	Invalid	Bhyo: 0	32.34	POS	36.6	POS
			Ecoli: 0		Ecoli: 0			POS	
11	169254	POS	Bhyo: 49.5	POS	Bhyo: 53	28.16	POS	30.31	POS
			Ecoli: 35.5		Ecoli: 30			POS	
12	168935	POS	Bhyo: 45.5	NEG	Bhyo: 0	27.71	POS	29.85	POS
			Ecoli: 25.5		Ecoli: 33.5			POS	
13	173629	POS	Bhyo: 32	POS	Bhyo: 32.5	25.1	POS	25.64	POS
			Ecoli: 20		Ecoli: 27			POS	
14	172429	POS	Bhyo: 41.5	POS	Bhyo: 46	28.12	POS	30.16	POS
			Ecoli: 22.5		Ecoli: 26			POS	
15	172234	POS	Bhyo: 36.5	POS	Bhyo:39	27.41	POS	29.84	POS
			Ecoli: 21		Ecoli: 20.5			POS	

(continuation) Table 8.3: Results from the tests with 30 real samples for the detection of *B. hyodysenteriae* and sample control on Doctor Vida® Pocket, with Sample collection kits 1 (Swab kit) and 2 (Scoop kit), and comparison with the results obtained with the EXOone qPCR kit, to compare the performance of both kits, and validate the previous results obtained. Results from DV Pocket coloured in green means they are in concordance with the ones obtained with the qPCR kit, in red they are not in concordance. The cells coloured yellow represent invalid results, where the sample control (*E. coli*) was not detected.

		Results DV Pocket (TtP)				qPCR Bhyo		qPCR Ecoli	
#	Sample ID	Swab kit		Scoop kit		Cq	Result	Cq	Result
16	179363	Invalid	Bhyo: 0	NEG	Bhyo: 0	29.64	POS	28.82	POS
			Ecoli: 0		Ecoli: 29			POS	
17	179041	POS	Bhyo: 30.5	POS	Bhyo: 34	25.55	POS	30.02	POS
			Ecoli: 30.5		Ecoli: 37			POS	
18	179020	NEG	Bhyo: 0	Invalid	Bhyo: 0	31	POS	29.01	POS
			Ecoli: 27		Ecoli: 0			POS	
19	178989	POS	Bhyo: 49	POS	Bhyo: 33.5	25.82	POS	29.78	POS
			Ecoli: 20.5		Ecoli: 20.5			POS	
20	178570	POS	Bhyo: 37	POS	Bhyo: 32.5	30.09	POS	33.33	POS
			Ecoli: 19		Ecoli: 17.5			POS	
21	177161	Invalid	Bhyo: 0	NEG	Bhyo: 0	-	NEG	34.14	POS
			Ecoli: 0		Ecoli: 30.5			NEG	
22	178903	Invalid	Bhyo: 0	NEG	Bhyo: 0	-	NEG	25.2	POS
			Ecoli: 0		Ecoli: 29.5			NEG	
23	178696	NEG	Bhyo: 0	NEG	Bhyo: 0	-	NEG	24.46	POS
			Ecoli: 19		Ecoli: 16.5			NEG	
24	178284	POS	Bhyo: 34.5	NEG	Bhyo:0	-	NEG	28.04	POS
			Ecoli: 18		Ecoli: 18.5			NEG	
25	178283	NEG	Bhyo: 0	NEG	Bhyo: 0	-	NEG	29.17	POS
			Ecoli: 24.5		Ecoli: 17.5			NEG	
26	178278	Invalid	Bhyo: 0	NEG	Bhyo: 0	-	NEG	32.57	POS
			Ecoli: 0		Ecoli: 22			NEG	
27	177353	NEG	Bhyo: 0	POS	Bhyo: 27	-	NEG	31.74	POS
			Ecoli: 23		Ecoli: 33			NEG	
28	176680	NEG	Bhyo: 0	NEG	Bhyo:0	-	NEG	25.71	POS
			Ecoli: 14		Ecoli: 18			NEG	
29	176050	POS	Bhyo: 45	POS	Bhyo: 40	-	NEG	28.39	POS
			Ecoli: 19.5		Ecoli: 23.5			NEG	
30	176050	NEG	Bhyo: 0	POS	Bhyo: 42	-	NEG	31.17	POS
			Ecoli: 21.5		Ecoli: 23			NEG	