

Development of DNA-based assays for the detection of *Batrachochytrium dendrobatidis* in environmental samples and amphibians

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Abstract

Chytridiomycosis is an emerging infectious disease affecting amphibians on a pandemic scale. The disease causing organism, *Batrachochytrium dendrobatidis*, has been recorded on a global scale to be the top causative driver behind the enigmatic declines in frog populations and species. Certain limitations do exist in the detection of chytridiomycosis, such as invasive sampling methods, low detection sensitivity and protocols being time intensive. The aim of this study was to develop a DNA-based assay, Loop-mediated Isothermal Amplification (LAMP) and conventional PCR, for the detection of *B. dendrobatidis*. *In vitro* tests involving live *B. dendrobatidis* cultures were undertaken to develop the LAMP assay. From given results the assay delivered promising data. Six LAMP primers were designed specific to *B. dendrobatidis*, labelled as Bd3F3, Bd3B3, Bd3FIP, Bd3BIP, Bd3LF and Bd3LB. A LAMP assay was developed which tested positive for both BdGPL and BdCAPE lineages from South Africa. The assay proved to be extremely sensitive for these isolates, yielding positive results up to a high DNA serial dilution of 5fg in 40 minutes and 0.1 zoospores in less than an hour which was only subjected to boiling. An additional PCR assay was also developed from the outer primers of the LAMP primer set. This additional PCR assay proved to be more sensitive than previously developed PCR assay for *B. dendrobatidis*, detecting positive DNA samples at 0.05pg and 0.1 zoospores per ml. *In vitro* tests were undertaken to test the viability and integrity of the developed LAMP assay. Mont Aux Sources was used as a field test since the population of *Amietia hymenopus* inhabiting its rivers are known to be infected with chytridiomycosis. LAMP successfully detected chytridiomycosis on both ventral skin swabs and toe clippings from several individuals across four rivers. Additional archived African samples of various frog species were tested for chytridiomycosis and revealed for the first time that Botswana frog populations are infected with chytridiomycosis in the Okavango Delta. The developed LAMP assay is a promising and powerful tool which can be implemented to overcome several short-comings that can be associated with histology, PCR and qPCR. Furthermore, LAMP has much higher reducibility with crude DNA samples.

Key words: amphibian disease, *Batrachochytrium dendrobatidis*, chytridiomycosis, loop-mediated isothermal amplification (LAMP), molecular diagnostics, polymerase chain reaction (PCR), primers

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Decleration

I, Nicolaas du Preez, declare herewith that this dissertation which I herewith submit to the North-West University, Potchefstroom Campus, is in compliance with the requirements set for the degree, Masters in Environmental Sciences, is my own work, has been text-edited in accordance with the requirements and has not already been submitted to any other university.

Signed: _____

On the _____ day of _____ of the year _____

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Chapter 1 Introduction and literature review

Introduction

1.1 Amphibian-chytridiomycosis relation

1.1.1 Evolutionary history regarding amphibians

Despite being known as the 'Age of Fish', the Devonian period - 360 million years ago (mya) to 420 mya - boasted an array of revolutionary expansions. These expansions regard marine evolution, soil advances and tetrapod metamorphosis, fabricating the primal and primitive embodiment of the world's wonders as they exist (Speer, 2011).

Devonian ocean

Before the two mighty land masses - Euramerica and Gondwana - were colonised, the ocean was teaming tirelessly with life. The Devonian period gave birth to most of today's fish found in the oceans and freshwater bodies. The three biggest groups belonging to Osteichthyes (bony fish), Chondrichthye (cartilaginous fish / 'sharks') and the extinct group of Placoderms (armoured fish) (Speer, 2011).

Devonian land mass

Known as the 'Devonian Explosion', this was the pinnacle in vegetation evolution in which plants fabricated the organic polymer, lignin. This allowed for the structural formation of cell walls, vascular tissue, seeds and tall growth – parting with the primitive pioneer vegetation and algal mats (Garwood & Dunlop, 2014). This diversified blossom led to the formation of new habitats and food sources from whence and when arthropods advanced and tetrapods arose, encouraging evolution of the first terrestrial vertebrates (Gess, 2013).

Devonian tetrapods

A class descending from mentioned Osteichthyes, termed Rhipidistia - clade group to the famous Sarcopterygii - gave rise to the first tetrapod, which were the amphibian class. The lungfish is an example of the last surviving Rhipidistia group and sister group to all extant land dwelling vertebrates. Two important amphibian subclasses include Labyrinthodontia and Lissamphibia. Labyrinthodontia is the ancestor to all extant land dwelling vertebrates while Lissamphibia includes all modern living amphibians – Salientia (Frogs), Caudata (Salamanders) and Gymnophiona (Caecilians) (Niedzwiedzki, *et al.*, 2010).

According to Amphibianweb (2017) the word 'Amphibian' is Greek for 'amphi' (of both / double kinds) and 'bios' (life), referring to life on both land and in water. Amphibians evolved an estimated 370 mya, being the efficacy behind land occupation (for vertebrates) for the first time in history.

Thus consisting of aquatic and terrestrial life stages, organs and / or bodily functions since the class' birth. Larvae are subjected to an aquatic lifestyle, post metamorphoses adults obtain more terrestrial-focused qualities. However still remain semi-aquatic depending on the order, species and breeding specificities (A Dictionary of Biology, 2008). This ancient entity of a class have adapted conscientiously to literally all eco-regions (with the polar regions as exceptions) ranging from dry deserts to wet rainforests due to having vast diversity among the three orders (Frost, *et al.*, 2006). Three attributes that play an important role behind the successful evolution, development and adaptation of the amphibians are: phyletic size change, spatial- and temporal patterning and paedomorphosis (Hanken, 1989).

The amphibian class boasts several biologically important properties such as: being 370 million years old, they are the split group between primordial aquatic swimmers and terrestrially evolved tetrapods having both aquatic and terrestrial attributes, and have intricate bodily functions and structures which prove to be of scientific value. This makes amphibians an extremely important group, regarding several planes – primarily research towards evolution and pharmaceuticals, and biodiversity since they are essential elements in the food chain and bio-indicators of a given ecosystems health, however amphibian numbers are dwindling (IUCN, 2017).

1.1.2 Enigmatic amphibian declines

According to the 2004 IUCN global species assessment, as seen in figure 1.1, amphibians count as the class with the second lowest species count among the 5 classes of vertebrates at 5 743 species, despite species richness increases by at least 80 new discoveries annually and is steadily rising (IUCN, 2017; Glaw, *et al.*, 1998). Despite this an estimated total of 1 856 (32%) are threatened and 427 (7.4%) are critically endangered – the highest respective percentages among the vertebrate, invertebrate and plant classes – for 2004. Since 2004 the threat level rose to a staggering 41%. Regardless of regular new species' discoveries, the extinction rate surpasses the species discovery rate at an alarming tempo (IUCN, 2017; Kohler, *et al.*, 2005). Up until more recent surveys (2015), the number of discovered and described amphibian species has risen to a total of 6300. However, amphibians remain at the top of the list as the vertebrate class with the most threatened species at an estimated 43% (Sabino-Pinto, *et al.*, 2015).

	Number of described species	Number of species evaluated	Number of threatened species in 2004	Number threatened as % of species described	Number threatened as % of species evaluated*
Vertebrates					
Mammals	5,416	4,853	1,101	20%	23%
Birds	9,917	9,917	1,213	12%	12%
Amphibians**	5,743	5,743	1,856	32%	32%
Reptiles	8,163	499	304	4%	61%
Fishes	28,500	1,721	800	3%	46%
Subtotal	57,739	22,733	5,274	9%	23%
Invertebrates					
Insects	950,000	771	559	0.06%	73%
Molluscs	70,000	2,163	974	1%	45%
Crustaceans	40,000	498	429	1%	86%
Others	130,200	55	30	0.02%	55%
Subtotal	1,190,200	3,487	1,992	0.17%	57%
Plants					
Mosses***	15,000	93	80	0.5%	86%
Ferns and allies***	13,025	210	140	1%	67%
Gymnosperms	980	907	305	31%	34%
Dicotyledons	199,350	9,473	7,025	4%	74%
Monocotyledons	59,300	1,141	771	1%	68%
Subtotal	287,655	11,824	8,321	2.89%	70%
Others					
Lichens	10,000	2	2	0.02%	100%
Subtotal	10,000	2	2	0.02%	100%
Total	1,545,594	38,046	15,589	1%	41%

Figure 1.1: Species assessment from 2004 (IUCN, 2017)

Exponential amphibian declines started from 1970. Since 1500 only an estimated 34 species have gone extinct, of which 9 (26%) since 1980. Possible extinction is a term given to formally critically endangered species when individuals of a population cannot be found in spite of intensive survey efforts. Currently between 9 and 122 species from 1980 have gained the status of 'possibly extinct'. Approximately 435 (7.5%) amphibian species are given the title of 'rapidly declining'. These species are divided into one of three categories grounded on the cause of decline: "overexploitation" (80 species), "reduced habitat" (183 species) and "enigmatic decline" (207 species). "Enigmatic decline" refers to reasons unknown for dwindling species (Stuart, *et al.*, 2004).

Batrachochytrium dendrobatidis (Bd) was identified in 1997 and found to be a chief driver among these enigmatic declines of frogs. This pathogen is found virtually globally on every continent except for Antarctica since no amphibian hosts inhabit the icy desert. Known to infect over 350

species, having a role in more than 200 extinctions and being the sole reason for individual species extinctions in the wild confirms this disease's virulence. Belonging to the fungal class Chytridiomycota, it is only one of two chytrids to parasitize invertebrates, with zoospores infecting amphibian skin cells, developing into sporangia within the keratinized epidermal cells causing cutaneous chytridiomycosis disease (Fisher, *et al.*, 2009). Similarly, *Batrachochytrium salamandrivorans* (Bsal / Bs), has recently been linked to the decline in salamanders. Representing a sister group to the species of Bd, Bsal seems to have a high affinity towards the Caudata (salamander-specific) clade. So far it seems that Bsal exhibits similar symptoms such as skin lesions although hosts experience higher levels of pathogenicity (Martel, *et al.*, 2013; Sabino-Pinto, *et al.*, 2015).

1.1.3 Chytridiomycosis

Amphibian skin is a vital organ necessary for the efficient operation of osmotic homeostasis, which is one of the most important biochemical traits that distinguish amphibians from other terrestrial tetrapods. The skin consists of multiple epithelial layers: *stratum corneum*, *stratum granulosum*, *stratum spinosum* and *stratum germinativum* (from superficial to deep). Bd sporangia are found mostly embedded within the *stratum granulosum* and *stratum corneum* since they are mostly found in keratinized cells. Frog skin is extremely rich in sustenance and moisture for the harmonious balance of osmoregulation. It is also an ideal substrate on which microbes can proliferate. Serous- and mucous glands are two secretory glands that help simultaneously maintain absolute skin function (osmoregulation) and defence (pathogenic microbes). Serous glands (poison-/granular glands) are responsible for secretion of the primary active chemicals against unwanted microbial invasion. Mucous glands are responsible for the facilitation of temperature control, prevention of desiccation and protection from injury or abrasive harm (Voyles, *et al.*, 2011)

Despite the skin being permeable to water it is also a harbour for regulated ion (electrolyte) transport and respiratory gas exchange. Amphibian osmotic balance is kept when the internal environment (amphibian) upholds a hyperosmotic concentration relative to the external environment. This is done by the active transport of several electrolytes (chloride, magnesium, sodium and potassium) across the skin. The sodium-potassium pump (AMP-regulated pathway) makes it possible for the steady continuous inflow of sodium against an active electrolyte gradient. Sodium ions from the external environment are exchanged with potassium ions from within the frog in which ion concentrations are regulated over both intracellular and extracellular regions. The establishment of this osmotic gradient by means of electrolytic currents (ion exchanges) allows for the regulation of water flow across the skin, keeping the frog moist. Electrolytic ion transport across skin is the primal determination factor of frogs' regulatory properties which is done in the flask-shaped mitochondrial cells found in the *stratum granulosum* layer. The epidermal cells and

mitochondrial cells within work together to ensure balanced and required water and electrolyte balance, hence the skin is a vital organ responsible for osmotic equilibrium – an essential process regarding frog health (Voyles, *et al.*, 2011).

Chytridiomycosis disrupts the keratinized epidermal cells leaving the amphibians susceptible to impaired osmoregulatory functions, desensitised immune responses and eventually the individuals' health diminishes until death pursues (Bai, *et al.*, 2012). The Americas, Europe, New Zealand, Australia and east-Africa have suffered direct and irregular-/indirect deaths leading to sharp population declines and the complete extinction of entire species due to the aggressive pathogenic nature of Bd (Bosch & Martinez-Solano, 2006, Weldon, *et al.*, 2018 in press).

This is a major problem since millions of frogs are used globally for several reasons: farming (food), bio-indicators of natural habitats, conservation of biodiversity, aesthetic reasons such as ornamenting ponds and gardens, biological control agents, personal pets, bait for certain pets of prey and research orientated grounds. Besides these 'uses' of frogs, they are also a proximate driver in ecosystem function and stability. Hence the dwindling numbers of frogs needs to be assessed with great haste and concerns since frogs play a vital role in many regards. The decline in frogs may result in ecosystem deterioration – recently the World Organisation of Animal Health (OIE) registered Bd as being a notifiable pathogen with emphasis on its deteriorating prospect towards biodiversity. During the 2005 Amphibian Conservation Action Plan (ACAP) Bd chytridiomycosis was described as *"the worst infectious disease ever recorded among vertebrates in terms of the number of species impacted and its propensity to drive them to extinction"* (ACAP, 2005). The Global Invasive Species Programme (GISP) also ranked Bd in the top 100 *"World's worst invasive alien species"* which annotates the importance and severity of this parasitic amphibian pathogen (GISD, 2017). In order to combat these dwindling amphibian numbers one needs to look at the current biggest threat and treat accordingly (Fisher & Garner, 2007; Olson, *et al.*, 2013; Voyles, *et al.*, 2010).

The origin of the disease, mode of spread and mechanism of death is largely unknown – due to this 100% mortality rate is evident in both transmission experiments and during disease outbreaks associated with susceptible amphibian species. Depending upon the amphibian species, incubation temperature and Bd strain, death pursues exposure after 18 to 70 days (Berger, *et al.*, 2005; Morgan, *et al.*, 2007).

Statement of the problem

Among the several vertebrate classes, amphibians top the list of being most threatened with an extinction crisis of slightly above 30% in regards to the entire group of species, surpassing both mammals (23% of species) and birds (12% of species) (Staurt, et al., 2004). Bd is a pathogenic amphibian fungus found almost globally - except on Antarctica - which has been linked to being a huge driving force behind this pandemic decline in amphibian communities. Over 350 amphibian species are model targets of Bd while more than 200 species are experiencing a forceful dwindle in numbers due to Bd (Fisher, et al., 2009). Bd infects the cutaneous epidermis of anuran larvae and post-metamorphic individuals including adult amphibians and juveniles (Longcore, et al., 1999 & Walker, et al., 2007). However, Bd only affects post-metamorphic amphibians, developing chytridiomycosis. When infected, anuran larvae develop deformed/eroded toes and mouthparts while affected adults only show symptoms when heavily infected and near death (Annis, et al., 2004).

From a biological and conservation viewpoint, chytridiomycosis is a universal problem since the majority of affected species experience a 100% mortality rate if left untreated (Berger, et al., 2005 & Cheng, et al., 2011). According to Weldon and Fisher (2011) Bd is listed among the world's top 100 invasive alien species, labelling them as a hardy pioneer- and competitive species which are well equipped to adjust to many/most regions. Furthermore, frogs are the primary vectors of Bd, and with the current travel and trade (as pets, cuisines or experimental studies) of amphibians Bd is spread over universal borders, leading to infections in natural and protected areas. Ultimately a loss of natural biodiversity occurs, endangered species die off and fewer individuals are present for environmental conservation studies (Weldon, & Fisher, 2011).

Detection of chytrid is crucial as a key element in the isolation and identification of Bd in/on infected individuals as well as an effective means of tracking nursing programs in which chytridiomycosis is treated (Boyle, et al., 2004). Traditional and conventional diagnoses include histological investigation of skin scrapings and toe clippings via H&E (haematoxylin and eosin) staining (Daszak, P.B.L., et al., 1999). However, some drawbacks regarding histological testing arise: an extent of expertise is required, live animals are needed, the protocol is time extensive, tests may exhibit low sensitivity, and environmental samples (soil and water) cannot be used (Boyle, D.G., et al., 2004). Though three of the biggest factors concerning the present day includes: non-invasive techniques, use of environmental samples, high tempo and specificity of identification (Kirshtein, J.D., et al., 2007; Longo, A.V., et al., 2013; Walker, S.F., et al., 2007). This led to the development and implementation of molecular methods such as PCR- and qPCR-based assays which proved to overcome most of these drawbacks with great success (Boyle, D.G., et al., 2004).

However, some limitations for PCR and qPCR exist as well. PCR and qPCR are not generally mobile – they cannot readily be applied on site - and the ITS1 gene from Bd varies in copy number

which affects qPCR quantification and identification – insufficient specificity (Longo, A.V., et al., 2013). Furthermore, several rapid thermal cycles are required along with low amplification efficiency. Loop-mediated isothermal amplification (LAMP) is a novel DNA-based method to overcome such disadvantages, operating under isothermal conditions (65°C) with the use of up to 6 primers. This allows for extremely fast (30-60min) detection with a high specificity and – amplification efficiency (Mori, Y., et al., 2001). LAMP can also be used as a mobile device for in-field detection of samples (Phalen, D., et al., 2011). This study will therefore attempt to develop a LAMP assay for detection of Bd in environmental samples and amphibian skin.

Aim

The aim of this study was to develop a DNA-based assay, Loop-Mediated Isothermal Amplification (LAMP), for the detection of *B. dendrobatidis*.

Objectives included:

- To design novel universal LAMP primers for detection of *B. dendrobatidis*
- To develop and optimise LAMP assay
- Using outer LAMP primers for the development and optimisation of a conventional PCR assay
- To evaluate the efficiency of newly developed LAMP and PCR assays in detection of wild *B. dendrobatidis* in nature

Dissertation outline:

Chapter 1 - Introduction and literature review

Chapter 2 - Design of universal LAMP primers

Chapter 3 - Development of LAMP assay

Chapter 4 - Development of PCR assay

Chapter 5 - Evaluation of developed LAMP and PCR assays with field samples

Chapter 6 - Conclusion and recommendations

Literature review

1.2 Characteristics of *Batrachochytrium dendrobatidis*

1.2.1 Life cycle

Bd consists of two life stages and portrays a simple asexual yeast life cycle: a short lived motile-waterborne infective zoospore responsible for dispersal and stationary thalli developing into zoosporangiums responsible for asexual reproduction (Berger, *et al.*, 2005; Longcore, *et al.*, 1999; Voyles, *et al.*, 2010).

Zoospores are the infective motile stage of Bd yeast cells which are amoeboid (mostly spherical) in form, unwallled and waterborne. Aquatic motility is due to a single posteriorly directed flagellum (19-20 μm). The diameter of the zoospores is 3-5 μm . When zoospores have dispersed through the water and found a suitable host the flagellum is reabsorbed after which the zoospores forms a cell wall – undergoing the formation of a hardened cyst. After encystment fine developing rhizoids divaricate from the germling (immature sporangium) (Berger, *et al.*, 2005; Longcore, *et al.*, 1999).

With the growth of the thallus the cytoplasm increases in both content and complexity. The most important and notable is multinucleation by means of mitotic divisions, cleaving and yielding fully functional zoospores. As the thallus becomes enlarged and swollen it receives the title of 'zoosporangium'. Colonial development is evident when fine septa divide the thalli so that every compartment develops its own sporangium accompanied with a single papillary discharge tube. With monocentric development no septa are present to divide the thalli, thus a single sporangium persists. The papillae may have a length of 0-10 μm , as soon as maturity is reached the discharge tube plug dissolves, allowing the release of the zoospores. Zoospores are only discharged when sufficient moisture is available in the surrounding environment; if the surrounding area is too dry the zoosporangium may retain the zoospores until preferable environmental conditions are met. After the release of zoospore culture the sporangium capsule is clear and only the chitin walls remain. Zoospores stuck within this empty capsule will remain there for the remainder of their growth while superficial frog bacteria may deposit and replicate within (Berger, *et al.*, 2005; Longcore, *et al.*, 1999).

The zoospores are the infective stage of Bd. Once a frog comes into contact with infected water bodies, the zoospores infect the amphibian epidermis and develop into sporangia. Several sporangia may infect a single selective epidermal cell. Frog epidermis consists of 5 layers of which the stratum corneum (horny outermost layer) and the stratum granulosum are infected with sporangium. The deeper growing cells house the infantile sporangia while the keratinized horny layer houses the mature sporangia from which the discharge papillae protrude to the surface.

When sporangia are expectant with zoospores they are released via these openings as soon as contact with water is made (Berger, *et al.*, 2005; Marantelli, *et al.*, 2004).

1.2.2 Nutrient utilisation

In vitro – Bd did not grow on dilute salts solution (Fuller and Jaworski, 1987), asparagine-glucose agar (Stevens, 1974) or yeast nitrogen base plus 1% glucose and thiamine (Sigma). Extremely low growth was observed in gelatin hydrolysate, yeast extract, malt extract, asparagine, peptone and a simple glucose media. More complex media with a strong nitrogen base are better suited for growth of Bd. The 1% peptonised milk and especially 1% tryptone broth (more so than peptonised milk) are the two best nutrient sources suited for Bd growth. There are some minor differences between these two nutrient sources. Tryptone contains 0.4µg thiamine per 1g tryptone whereas peptones contain only 0.1µg thiamine per 1g peptone (it is known that chytrids do require excess exogenous thiamine). Also peptonised milk contains less than half total nitrogen than tryptone does even though the growth is somewhat similar. Addition of different carbon sources to the 1% tryptone broth brought upon no increased growth rate of concentration. Growth was affected slightly by increasing the glucose and tryptone concentration to 2% and 1.5% respectively, any higher increase and the growth decreased. Furthermore, decent growth is evident in snakeskin liquid medium and 1% keratin agar. When grown on 1% skim milk and gelatin clear zones are evident with the conformation of azocasein- and gelatin degradation while growth in keratin azure gave no activity of keratin utilisation. This could indicate that Bd only grows (and doesn't utilise keratin as an energy source) within the keratinised epidermal layers because of easy access into the dead cells (Piotrowski, *et al.*, 2004).

Sporangia develops inside the deeper viable epidermal cells (stratum germanitivum and – spinosum) where the development coincides with the epidermal cell's growth as it slowly matures and moves outward to eventually keratinise (stratum corneum). These immature sporangia require prekeratin which are found in the deeper viable cells. Initial sporangium growth begins in the deeper viable cells but complete the final stages of growth in the empty keratinised cells from where zoospores are deposited – these keratinised cells are a key requirement for the mature sporangia when acting as parasite. Studies have yet to determine what specific nutrients are required and or utilised by sporangia within the frog skin (Berger, *et al.*, 2005).

1.2.3 Pathogenesis

The exact method regarding primary epidermal cell entry is still unclear. Longcore, *et al.*, (1999) proposed a hypothesis in which the motile zoospores encyst on the outer epidermal layers' exterior surface. From here a germ tube extends from the zoospore into the individual epidermal cells, transporting nuclear material into the cell matrix from which sporangia develop – the same mode of

transfer as with transduction or conjugation. The *in vitro* studies revealed that extracellular proteases are excreted which degrade gelatin and casein along with proteolytic enzyme activity. Furthermore, it was found that in Bd gene families with differential expression, Bd has the potential of fungalysin metallopeptidase- and serine protease expression – which are two enzymes involved during the process of infection during cell host penetration in other microbial pathogens (Voyles, *et al.*, 2010).

There is a major difference between infection intensity *in vitro* and in frog skin. In the *in vitro* conditions where the internal environment is even kept at a favourable constant with high-nutrients and optimal temperatures a typical growth curve can be observed. After an exponential growth peak follows a stationary- and eventual death phase in which zoospore production declines due to the accumulation of waste materials and/or inhibitory metabolic substances, resource exhaustion can also have a drastic declining effect regarding zoospore production and growth (Douglas, *et al.*, 2008). However, nutrient depletion on/in frog skin doesn't affect zoospore production and/or growth, this could lead to several re-infections which could develop acute infections and eventually mortality (Cheryl, *et al.*, 2009; Douglas, *et al.*, 2009).

The surrounding epidermis (stratum corneum region) of the thallus always undergoes hyperkeratosis which is an abnormal thickening of epidermal cells. Furthermore, the epidermis can undergo irregular hyperplasia where tissue or organs undergo abnormal growth due to increased reproduction rate of the surrounding cells as with a tumour. Spongiosis, also known as intercellular edema, is the anomalous accumulation of fluid within the infected skin representing fluid-vesicles. Individual cell vacuolation – not spongiosis - leads to epidermal lifting, shearing the skin apart. Skin erosions can occur where epithelial cells are partially eroded but can worsen up to where skin ulcers degrade entire layers of epithelial linings. Individual cells undergoing necrosis may encounter the event of pyknosis (chromatin condensation) followed by karyorrhexis (nucleus fragmentation) which leads to an anuclear necrotic cell (Berger *et al.*, 2005; Kroemer, *et al.*, 2009).

Voyles *et al.*, (2009) tested six variables for chytrid pathogenesis and found the following:

The plasma biochemistry sustained drastic decreases in both sodium and potassium concentrations which correlated directly with Bd zoospore infection load. In contrast to this decrease, total protein and globulin resulted in a slight increase which may represent an immune response. Dehydration was not a factor as one might suspect since a loss of water/moisture would be accompanied by this increase of total protein and albumin. However only since an insignificant increase in both total protein and albumin was noted, it only represented an immune response. From the findings, electrolyte changes were the greatest form of alteration among all variables and the most life-threatening. Urine biochemistry (glucose, ketones, pH, osmolality, etc.) remained unchanged and kept constant values around the normal range except for potassium and calcium

concentrations which decreased as Bd-load increased. Kidney health and functions remain unaffected since uric acid and plasma phosphorus levels stay put at the normal range. From skin biopsies, affected frogs suffered from spongiosis, hyperkeratosis, erosions and ultimately ulcerations. Cardiac biotelemetry indicates a slowed heart rate, decrease in the amplitude of ventricular depolarisation while an increase in the time of ventricular depolarisation occurs, all within the last couple of hours prior to when death settles in. Cause of death includes asystolic arrest and cardiac standstill due to these physiological cardiac changes (Voyles, *et al.*, 2009).

The pathogenic fungus still poses the power to potentially harm and even kill amphibians even though it is only limited to the epidermis. It has come down to two hypotheses how this is possible. Damage to the epidermal layer results in the functional disturbance of osmoregulation, this includes irregular fluctuation of essential electrolytes, water and oxygen. Active compounds such as proteolytic enzymes might be secreted by Bd which is easily assimilated through the highly permeable frog skin. Both these pathways are damaging to the frog skin and the animal itself to such an extent that death may pursue (Berger, *et al.*, 1998; Berger, *et al.*, 2005; Pessier, *et al.*, 1999).

It was found that isolates' genotypes may differ significantly even if the genomes contained a great degree of relatedness. Unpublished data by M.C. Fisher indicated an isolate (TF5a1) from Spain to represent 50% less virulence than an isolate (UKTvB) from U.K. in two different host species. Proteomic analysis of these two isolates revealed a significant variation in the degree of protein expression. The difference in these morphological traits suggests selective pressure to be at work here such as the local environmental conditions. With this in mind Bd has the potential to adapt to new host species and/or climates and an increase in its fitness may lead to the development of drug resistance (Fisher, *et al.*, 2009).

1.2.4 Genetics

Rosenblum and colleagues (2013) sequenced 49 isolates from intercontinental regions from which they managed a rooted Bd phylogeny based on nuclear single nucleotide polymorphisms (SNP's). Within the phylogenetic tree several unique clades (strains) were identified. BdGPL (Global Panzootic Lineage), BdCAPE (Southern African Cape Lineage), BdBrazil (Brazil Lineage) and BdCH (Swiss Lineage). Within each of these strains a difference in the degree of virulence expression was found (Berger, *et al.*, 2005; Farrer, *et al.*, 2011; Morehouse, *et al.*, 2003). However, BdGPL was found to be hyper-virulent with regard to the other lineages, being the chief driver behind the most catastrophic population declines and / or extinctions (Farrer, *et al.*, 2011). Recently a new and fifth Bd lineage was discovered, the hyper-diverse BdASIA lineage which was divided into two associated lineages. BdASIA-1 was grouped with BdCH while BdASIA-2 was

grouped with BdBrazil since these two BdASIA lineages were closely related to BdCH and BdBrazil respectively (O'Hanlon, *et al.*, 2018)

1.3 *Batrachochytrium dendrobatidis* emergence and the frog trade

The definition of “emerging infectious diseases” concurs when localised pathogens spread beyond their usual geographical region and/or host, affecting boundaries which are not native to them. This ‘emerging infectious disease’ should meet the following set of principles if it is to be regarded as an emerging disease. The original source/origin 1) should contain greater genetic variation than regions that were recently invaded, 2) would be the location of the earliest / first known phenomenon, 3) with the native hosts indicating minimal to no clinical symptoms, 4) with the date of this emergence would precede any host declines in immaculate neighbouring regions, 5) would provide sufficient global dissemination of the infectious disease, 6) would show no geographic spreading pattern over time and 7) with the occurring host(s) will deliver a stable prevalence within them over time (Weldon, *et al.*, 2004).

1.3.1 Out of Africa hypothesis

Weldon and colleagues (2004) hypothesised that *B. dendrobatidis* originated from Africa with epidemiological support and evidence – termed the ‘Out of Africa Hypothesis’. A historical survey was conducted on several *Xenopus sp.* from southern African institutions by means of typical histological examinations. Results indicated the earliest positive chytridiomycosis result to be from 1938 in a *X. laevis* individual from the Western Cape. Since 1940 no significant change in chytridiomycosis prevalence was detected and after 1973 chytridiomycosis geographic distribution showed no significant changes and was already found across all regions in southern Africa. This extended the earliest date by 23 years which came from Canada in 1961. In the wild *X. laevis* shows no clinical symptoms and have never experienced unsuspected die-offs. This, along with the geographic dissemination and number of frogs, makes *X. laevis* a perfect transmission host of chytridiomycosis through the international trade. When the pregnancy assay was discovered in 1934 for humans, extensive amounts of *X. laevis* were exported from southern Africa globally. In 1949 a total of 3 803 frogs were exported and in 1970 a total of 4 950 frogs were exported, all for use as means of biological pregnancy assays. With the introduction of non-biological pregnancy assays *X. laevis* remained an important scientific model organism especially in the fields of molecular biology, embryology and immunity. Since these exported individuals were wild-caught they had a high chance of carrying the disease. Escaped frogs from importing countries could have come into direct or indirect contact through water with other local native frogs, allowing transmission of Bd and subsequently the spread of chytridiomycosis globally (Weldon, *et al.*, 2004).

Although the study carried out by Weldon *et al* (2004) identified the earliest positive Bd host to be located in southern Africa (1938), there remains no indication whether southern Africa was the

origin of Bd. This is said with certainty due to other Africa regions having a wide dissemination regarding the disease since Bd has been found in eastern- and western Africa after exporting mass populations to the United States. African exports could act as a primary spread of the disease while the native frog population from the receiving country could act as a secondary spread of Bd. Another important host and vector of Bd is *Rana catesbeiana* which is the well-known American Bullfrog. The earliest record for *R. catesbeiana* infected with Bd is from 1978 which is 40 years after the first southern Africa record is 1938. The results from this study indicate by use of epidemiological evidence that Africa is the origin of Bd by supporting six of the seven criteria listed above. Only criteria 1 (genetic diversity) was not investigated (Weldon, *et al.*, 2004).

Morehouse *et al.*, (2003) undertook multi locus sequence typing (MLST) on the genomes of 35 strains (North America, Australia and Africa) to examine their relationship and genetic diversity. From the 10 loci (gene regions) that were selected to test for nucleotide variation, 6 loci (*tef1*, *rnap50*, *uorf48*, *bdc42*, *bdc33* and *bdc3*) indicated null nucleotide substitution among all strains while the other 4 loci contained at most 2 variable sites. Heterogeneity was also found in 4 loci (*r6046*, *lsu35*, *aprt13* and *ctsyn1*) in which more than 1 allele was present throughout all strains. These findings conclude a low genetic diversity level with intercontinental samples supporting the notion that Bd is a newly emerged pathogen. These low levels of polymorphism suggest four possible explanations: 1) Bd's global population size is small, 2) mutation rates are extremely low, 3) population size experienced a recent bottleneck, or 4) natural selection favoured a certain genotype(s) from which present day Bd arose. A small effective population is not possible due to the broad range of hosts and distribution globally, dismissing point 1. Point 2 can also be dismissed since no studies and evidence mentions/suggests extraordinarily low mutation rates in Bd. This leaves the consensus of low polymorphism to either the occurrence of a recent historical bottleneck effect or the emergence of the pathogen from a single strain from a specific selected source (Morehouse, *et al.*, 2003).

Due to having acquired the title of 'emerging infectious disease' and being recognised as a globally widespread invasive and aggressive pathogen, two hypotheses was developed to report for the transpiring nature of Bd. The 'novel pathogen hypothesis' (NPH) declares that the disease spread quite recently onto new host species and geographic regions due to the amphibian trade and anthropogenic effects affecting the natural environment, spread and habitat preference of Bd. Also known as the 'spreading pathogen hypothesis' introduction of epidemics into the Americas and Australia has been identified along with infected vectors in the frog trade. The 'endemic pathogen hypothesis' (EPH) declares that chytridiomycosis is a widespread global endemic existence and may even be an amphibian symbiont. However, chytridiomycosis and a higher degree of virulence emerged due to environmental changes (global warming). This led to hosts becoming more susceptible to this disease due to pre-existing infections (Fisher, *et al.*, 2007; Fisher, *et al.*, 2009). Even though there are two hypotheses both contribute to the explanation of the current pandemic.

Support for the NPH comes from data illustrating epidemic introduction, globally-collected Bd isolates having very little genetic diversity which suggests a point-origin for the disease and that the amphibian trade contain detectable amounts of infected populations. Support for the EPH comes from data illustrating global Bd population presence from decades ago (South Africa 1938, Canada 1961, USA 1974 and Australia 1978), also having vast amounts of association between global warming and chytridiomycosis transmission and infections (Fisher, *et al.*, 2007).

1.3.2 Novel pathogen hypothesis (NPH)

NPH – exotic species being imported, human impingement of the wilderness and Bd host translocation all contribute to the NPH which give reason to the remote locations and high density of this highly virulent pathogen (Rachowicz, *et al.*, 2005). Laurance and colleagues (1996) presents several pieces of evidence supporting the NPH in which Bd acts as an epidemic disease. 1) Ecological similarities indicates amphibian species that assemble near or in lotic systems are affected by some type of waterborne pathogen however amphibians that assemble at lentic or terrestrial habitats indicate no apparent infection or declines. 2) Australia witnessed a mass frog decline in which a wave-like pattern commenced from southern Queensland to northern Queensland at a rate of 100 km per year for 15 years. Such a norm of declination is representative to that of an epidemic involving pathogens with a high degree of virulence infecting and affecting populations with little to no immunity. 3) An extremely rapid decline in affected species was witnessed in/around Brisbane, Eungella and Tableland in which the specific population numbers crashed within a couple of months. The fast tempo at which populations decline represents an infection with a highly virulent novel pathogen. 4) Experimental and pathological results indicated that frogs would showcase clinical signs when exposed to lotic systems of known pathogenic nature while they remained symptom free in aquariums filled with rainwater. The most prevalent clinical signs included lethargy, skin necrosis and motor dysfunction. 5) The simple absence of credible alternative factors is also one piece of additional evidence in regards to the NPH. Some of these alternatives which are rendered negative include: natural population fluctuations, environmental deterioration, habitat modifications, unbalanced physical- and chemical water parameters, UV radiation increase from the sun, unusual weather patterns. Over-predation and over-collection could only cause some local declines however doesn't have the potential to cause large-scale regional declines of populations (Laurance, *et al.*, 1996).

1.3.3 Endemic pathogen hypothesis (EPH)

EPH – the emanation of an infectious disease is the result of immunological-, behavioural- and/or ecological change in the normal parameter of the parasite or host tipping the scale from a non-virulent symbiotic relationship (possibly mutualism or commensalism to a relationship pathogenic in nature (parasitism or antibiosis amensalism). Amphibian immune systems may be affected by one of the following in combination or as a single stressor: changes in chemical concentrations, temperature, moisture levels, temperature and/or ultraviolet radiation – abiotic stressors. Host

density is one biotic stressor which may suppress individual immunity. These environmental changes may alone already increase pathogen virulence, but can also lead to immunosuppression stress which further increase host susceptibility. This may well be an explanation to this emerging infectious disease. Immunosuppression is thought of as the major role player in the EPH since infection of a host represents being immunosuppressed when infected (Rachowicz, *et al.*, 2005). However, Berger and colleagues (2000) found only 1 frog out of 147 Bd infected individuals to be immunosuppressed based on histological organ examination. Furthermore, no other pathogens of significance were found other than Bd based on bacterial and viral cultures, hematology and electron microscopy. Immunocompetent individuals exposed to low concentrations of Bd died off nonetheless. Despite this immunosuppression can't be ruled out since Bd might still be the predominant opportunistic pathogen in immunosuppressed individuals. Immunosuppression may well just be a catalyst in the infection rate/density in regards to Bd instead of a definite reason behind the emergence of chytridiomycosis. Environmental changes may alter a previously non-harmful parasite's growth rate, reproduction and/or transmission giving emergence to a disease causing pathogen, tipping the scale between possible mutualistic environments to a parasitic one and also causing an outbreak. These environmental changes include eutrophication, which increases the transmission and growth rate of Bd. Climate change leading to drier conditions in the natural habitats of amphibians force them to become overcrowded in the small areas left with humidity/water which in turn increases susceptibility of the host and increase transmission rate of Bd (Rachowicz, *et al.*, 2005). However, analysis done by Morehouse and colleagues (2003) found low geographic structuring and low pathogen-host specificity contradicting the EPH in which frog-fungus symbiosis could have been a possibility.

The export and import of amphibians range from the use in farming, conservation, ornamentation, bio-control and science. The amphibian trade may run annually into the millions covering hundreds of species. *X. laevis*, *R. catesbeiana* and *Bufo marinus* are the three species which are the most widely exported and imported as well as some of the top chytridiomycosis hosts/vectors who have established populations in Asia, Australia, Europe and America (Fisher, *et al.*, 2007).

1.3.4 Ancestral Bd origin

Using population genetics to get a genetic diversity pattern will help understand the phylogenetics behind Bd and in turn the origin and/or cause behind the emergence of this pathogen. Epidemiological studies involving virulence, phenotypic and genetics will help understand the factors leading to amphibian declines. Two isolates, JEL423 (isolated from *Phyllomedusa lemur*, Panama) and JAM81 (isolated from *Rana mucosa*, California) yielded some interesting molecular results done by the Joint Genome Institute and the Broad Institute. Results indicated Bd to be diploid, containing low genetic diversity (both in regards to the nuclear- and mitochondrial genomes) and being highly heterozygous. These genetic findings along with the historical data done by Weldon and colleagues (2004) support the NPH in which Bd has a single origin (Fisher, *et*

al., 2009). However, Rosenblum and colleagues (2013) acquired molecular data which indicated Bd to be both novel in some form of manifestation but endemic in others. It might be a combination of both, in which the NPH is the sole primary reason behind the emergence of this pathogen but the EPH did have an effect(s) in the rate of infection covering various factors (transmission, susceptibility, virulence, etc.). Similar results were found by Morgan and colleagues (2007) in which 14 of the 15 loci they studied for genetic divergence had no more than 2 alleles for every individual. Furthermore, it was found that these individuals were heterozygous and diploid. Geography and fungal genotype had little to none correlation with no pathogen-host specificity. A more recent survey conducted by James and colleagues (2009) found the same results than above mentioned by including the same loci used in both above studies however from 59 isolated strains from five continents. All of these molecular studies' outcomes are exactly the same, supporting the NPH.

According to Rosenblum, *et al.* (2013), genetic analysis suggests that either Brazil or Africa have the highest possibility of being the origin for the emergence of Bd due to containing ancestral variation and Bd-dynamics which is largely consistent with the NPH and endemism. According to Goka, *et al.* (2009), Bd may be endemic to Japan due to several reasons. The giant salamander, *Andrias japonicas*, was infected with different haplotypes from the ones found in imported alien amphibian species from Brazil. This giant salamander is endemic to certain habitats in Japan. The giant salamander also has higher toleration and resistance to infection and clinical symptoms. From unpublished data, traces of Bd were found in giant salamander individuals who were fixed in formalin and ethanol dating back to 1902. Thus far three centres for the emergence of ancestral Bd have been postulated: Africa, America or Asia. Since the detection of Bd in southern African specimens (1938), more hosts have been detected positive for Bd in Cameroon (1933) and Uganda (1934). However, from the 1902 Japanese sample and genotyping data suggesting higher genetic diversity in North America relative to Africa, this raises the question even further for the origin of emergence of Bd with the use of archived species (Bai, *et al.*, 2012). With recent development in genetic analysis and the emergence of BdASIA-1 and BdASIA-2, Asia and more so Korea is said to be the global hub for hyper-diverse ancestral Bd (O'Hanlon, *et al.*, 2018).

1.4 Diagnosis of *Batrachochytrium dendrobatidis*

Amphibians play major roles as ectotherms in the ecosystems and revolving food chains. However, the disease causing pathogen Bd is a serious threat to amphibian populations. Chytridiomycosis is extremely virulent with hosts experiencing extreme infection with death eventually following (Alemu, *et al.*, 2008). Heavy population declines have been noted in Europe, Australia and North- and Central America. The detection of this pathogen is of utmost importance with regards to the understanding of the biochemical pathways, transmission, reproduction, infection, virulence and also lastly to identify means of management. Diagnosis of Bd is done by means of histological-,

histochemical-, electron microscopy and/or molecular assays (Annis, *et al.*, 2004; Soto-Azat, *et al.*, 2009).

1.4.1 Histology

Amphibian tissue fixed in 10% neutral buffered formalin is embedded in paraffin after which the tissue (5-6 μ m sections) is stained with hematoxylin and eosin. Hereafter basic routine histology procedure is followed, and a trained eye is used to visually detect the zoosporangia structures which reside within the stratum corneum of which some zoosporangia may contain spores and/or discharge papilla (Berger, *et al.*, 1998; Pessier, *et al.*, 1999).

1.4.2 Electron microscopy

With scanning electron microscopy (SEM) amphibian tissue is fixed in 2.5% glutaraldehyde after which it is fixed in 1% osmium tetroxide. Hereafter the tissue is dehydrated, critical point-dried then sputter-coated with gold. A SEM can then be used to visualize the tissue at 5kV. For transmission electron microscopy (TEM) tissue is fixed in 10% neutral buffered formalin after which it is fixed in 2.5% glutaraldehyde, 1% osmium tetroxide and 2% uranyl acetate in that order. Hereafter the tissue is dehydrated and embedded in Araldite epoxy resin followed by staining with Reynold's lead citrate. A TEM can then be used to visualize the tissue (Berger, *et al.*, 1998; Berger, *et al.*, 2005; Pessier, *et al.*, 1999).

1.4.3 Molecular assays

According to Soto-Azat, *et al.* (2009), two molecular assays have been developed, namely a conventional polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR) for detection of Bd. Annis and colleagues (2004) developed the first PCR assay. ITS4 and ITS5 are universal fungal primers that anneal to the conserved regions of the 18S and 28S rRNA genes. These universal fungal primers were used to amplify the 5.8S rRNA gene and internal spacer regions, ITS1 and ITS2, which are predominantly found in fungi of the chytrid genera. The resulting sequences of Bd were aligned with neighbouring species to find conserved regions specific to Bd. From here Bd-specific primers were developed: forward primer Bd1a and reverse primer Bd2a. This primer set was specific to Bd with detection limits ranging from 10 ng down to 10 pg and 10 000 zoospores down to 1 zoospore in which positive amplification can be expected.

Boyle *et al.* (2004) developed the first qPCR assay. Generic primer set BOB5 and BOB6 which targets the 18S and 28S rDNA genes were used for sequencing Bd isolated. From acquired results the sequences were aligned to test for specificity and find conserved regions. Developed Bd-specific primers were: forward primer ITS1-3 Chytr, reverse primer 5.8S Chytr and the probe CHYTR MGB2.

1.5 Current challenges

For histology, a high degree of expertise and lengthy period of time is required if one is to visually detect and identify chytridiomycosis. Furthermore, the chance of acquiring positive results from only a couple of 6µm sized skin section is low due to the variability of infection levels among the limbs. Inhibitors such as formalin which is a basic chemical used to fix amphibians cause cross-linking in PCR and qPCR inhibiting the reaction. Formalin also causes DNA to degrade over time which proves troublesome when trying to extract DNA (Boyle, *et al.*, 2004; Soto-Azat, *et al.*, 2009).

PCR and qPCR can detect Bd both at earlier infection stages and at much lower infection concentrations in regards to histology. This delivers faster results at a higher sensitivity, reproducibility and repeatability. qPCR in its turn yield results at a more sensitive and faster level with the ability to quantify the sample (Soto-Azat, *et al.*, 2009).

However, for each of these assays invasive destructive techniques are exercised in order to examine zoosporangia for histology and extract DNA for molecular detection. These invasive techniques include swabbing the amphibian's skin, skin scraping, excising tissue, toe clippings, etc. (Soto-Azat, *et al.*, 2009).

According to Hyatt, *et al.* (2007), performing skin swabs are less invasive than excision but equally sensitive. However, DNA extraction from these swabs leading up to PCR sometimes yield inconsistent results. This could be due to inconsistency in swabbing the same regions of the frog's skin leading to inconsistent Bd prevalence among individuals. The depth in which Bd infects frog epidermis varies among species. Due to this the collection of zoospores may be inconsistent among species. Certain environmental factors may trigger the release of zoospores which complicates the accumulation of zoospores on the swab. Environmental inhibitors accumulating on the skin may cause PCR inhibition and yield to unreliable results (Shin, *et al.*, 2014).

Bd detection not only requires early detection but the use of non-invasive techniques as well. This is important to exercise control and management for the infection and spread of chytridiomycosis especially in the international pet, food and laboratory trade (Boyle, *et al.*, 2004).

1.6 Liquid-mediated Isothermal Amplification

Notomi and colleagues (2000) developed a novel amplification mechanism termed Loop-mediated Isothermal Amplification (LAMP). This method consists of the potential to amplify DNA within the hour or less using only a few DNA copies. Relying on auto-cycling strand displacement DNA synthesis, it incorporates a specifically designed DNA Polymerase termed Bst DNA polymerase and can use 4-6 primers (Notomi, *et al.*, 2000).

Steneth and Roe (1972) isolated *Bst* DNA Polymerase from the thermophilic bacterium *Geobacillus stearothermophilus*. *Bst* DNA Polymerase consist of 5'-3' exonuclease activity but not from 3'-5' thus no proofreading exists in the 3'-5' strand. Optimum temperature for the species and its respective polymerase is between 55°C and 65°C. *Bst* DNA Polymerase has a special helicase-

like activity allowing it to synthesize a new DNA strand while dissociating the double-stranded template (strand displacement DNA Polymerase) DNA's hydrogen bonds allowing synthesis at a single constant temperature since the double-strand requires no additional dissociation such as an increase in temperature with PCR and qPCR (Alliota, *et al.*, 1996; Nazina, *et al.*, 2001).

The primers include two outer primers termed F3 and B3, forward primer and backward primer respectively. Two inner primers termed FIP and BIP, Forward inner primer and Backward inner primer respectively. Both the two sets of outer and inner primers (four primers) are used in the initial steps while in the later stages of cycling the two inner primers are used – these four primers are crucial to the successful operation of LAMP. The F3 and B3 primers attach to the sense (5'-3') and anti-sense (3'-5') sequences respectively while the FIP and BIP each contain two apparent different sequences that commensurate to both the sense and anti-sense sequences one for priming (first stage) and the other for self-priming (later stages) (Notomi, *et al.*, 2000). Furthermore, two loop primers termed Loop forward (LF) and Loop backward (LB) can be developed if the sequence within the other 4 primers allows it. This increases the sensitivity and selectivity of LAMP (Nagamine, *et al.*, 2002).

LAMP amplifies specific target DNA regions with a high degree of efficiency, rapidity and specificity under constant isothermal conditions of 60°C-65 °C. The method consists of a single isothermal step during amplification delivering visual real-time detection of amplicons. Final results may be acquired in as little as 15mins but may take up to an hour depending on primer design. Using 4 primers that can recognise up to 6 regions leads to extreme target specificity. LAMP is quite cost effective since no special equipment and/or reagent are required. Large volumes of amplified products are expected – up to 10^{10} within specified time of 15-60 mins (Notomi, *et al.*, 2000; Thekisoe & Inoue, 2011).

Chapter 2 The design of novel universal LAMP primers for the detection of BdGPL and BdCAPE

2.1 Introduction

2.1.1 Significance of molecular diagnostics

End-point diagnosis and genetic analytic precedence of infectious diseases are of utmost importance. The analysis behind genetic information yields valuable data regarding the disease' antibiotic resistance, virulence and pathogenesis which could in turn be used to understand and combat the disease and/or disease-causing organism. Nucleic acid amplification has been a major breakthrough in the scenario of application-related fields for investigators specialising in molecular biology and clinical medicine. Genetic disorders and infectious diseases can be monitored by the use of one of several molecular diagnostics (Nagamine, *et al.*, 2002).

Traditional molecular diagnostics are a great improvement on the historical histological detection. However, LAMP carries even greater advantages with regards to both histology and traditional molecular diagnostics (PCR, SDA, LCR, etc.). LAMP runs at an isothermal temperature (reducing incubation time), incorporates auto-cycling strand displacement activity (increasing copy numbers in a reduced time) and only a few DNA copies (<10) are needed for amplification to yield a positive result within 1 hour. LAMP is a perfect molecular substitute for traditional molecular diagnostics and biochemical culture media which is still in practise today for certain pathogens such as tuberculosis (Nagamine, *et al.*, 2002 & Notomi, *et al.*, 2000). These advantages are due and thanks to the *Bst* DNA polymerase enzyme functions and traits as well as the number (up to 6 primers) and specific design (recognising up to 8 regions) of the primers. This allows for pin-point precision specificity, extremely high sensitivity and a ridiculously short amplification time resulting in high precision (high DNA product output) (Mori, *et al.*, 2004).

However, to achieve these high sensitivity and specificity activities, proper primer design needs to be taken into account, considering several factors such as GC content, base composition and secondary structures. A LAMP primer set consists of 6 primers – two outer primers, two inner primers and two loop primers (Parida, *et al.*, 2008).

2.1.2 LAMP primer characteristics

Designing LAMP primers hinge on the six target regions on the sequence of interest. These regions include F3, F2 and F1 on the template strand as well as B3, B2 and B1 on the complementary strand. The outer (F3 and B3) – and inner primers (FIP and BIP) are essential obligate oligonucleotides designed directly from these regions. Furthermore, loop primers (LF and LB) can be designed additionally if the sequence and developed obligate oligonucleotides allow it (Parida, *et al.*, 2008 & PrimerExplorer, 2005). Each primer represents the following region(s) on the strand in the 5'-3' ends as shown in figure 2.1.

Forward primer (F3) – F3

Backward primers (B3) – B3

Forward inner primer (FIP) – F2 + F1c

Backward inner primer (BIP) – B2 + B1c

Loop forward primers (LF) – LF

Loop backward primer (LB) – LB

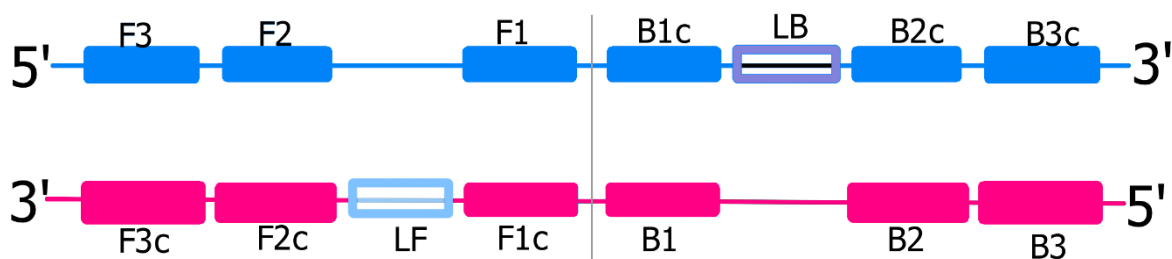


Figure 2.1: LAMP primer positions on the target gene

Five key factors in LAMP primer design include the melting temperature (T_m), primer stability, GC content, secondary structures and primer length / distances between primers. Optimum T_m for F3, F2, B3 and B2 range between 59°C - 61°C, for F1c and B1c between 64°C - 66°C and for LF and LB between 64°C - 66°C. The Nearest-Neighbour method used for estimating nucleic acid thermodynamic properties are the current tried and tested method to predict the most accurate measurement of a nucleotides melting temperature (Parida, *et al.*, 2008 & PrimerExplorer, 2005).

DNA synthesis starts at the end of the given primers; thus these ends should have a standard stability for efficient amplification. The 3' ends of F3/B3, F2/B2 and LF/LB as well as the 3' ends of F1c and B1c should be designed with a free energy quantity of -4kcal/mol or less. The stability at the end of F1c is important since it corresponds to the 3' end of F1 after amplification and creates a loop structure. A more negative ΔG (change in free energy) will allow higher chance and degree of annealing between primer and template. This ΔG is the difference between reactant (starting material) free energy and product free energy in which the reaction proceeds towards a negative ΔG (Parida, *et al.*, 2008 & PrimerExplorer, 2005).

Designing primers, it is preferable that they contain a GC content of between 40-65% while a GC content of 50-60% yields primers of relative quality when it comes to annealing and amplification. In order to prevent formation of secondary structures and primer dimers it is of importance to make certain the 3' ends are not complementary with each other. Taking the distance between primers and primer length in consideration. This could add to the quality of amplification and increase the chance of possible loop primer development since loop primer development is optional and depends of the distance and available number of nucleotides between region F2c and F1c. Optimum distances between regions for primer design is as follow: 120-160 base pairs (bp) between the 5' end of F2 and 5' end of B2. 40-60 bp between 5' end of F2 and 5' end of F1. 0-60 bp between 3' end of F3 and 5' end of F2 (Parida, *et al.*, 2008 & PrimerExplorer, 2005). Optimum primer length for F3/B3 is 17-21 nucleotides (nt), for F2/B2 its 23-24nt, for F1c/B1c its 12-21nt. FIP consisting of the F1c and F2 primers as well as the BIP consisting of the B1c and B2 primers are

both connected respectively with a TTTT spacer, giving a total nt length of 35-45. LF and BF consists of a nt length of approximately 17-21 (Nagamine, *et al.*, 2002; Notomi, *et al.*, 2000).

LAMP barely produce any non-specific DNA products. The first step when starting material is produced by F3/B3 and FIP/BIP, the synthesized strands contains sequences which FIP/BIP recognise immediately during the cycling and elongation step. The same goes for LF and LB in which the synthesized strand in the elongation step contains sequences which they recognise. This limits the potential production of non-specific products (Nagamine, *et al.*, 2002). Thus the objective entails the design of novel LAMP primers for the two lineages of *Batrachochytrium dendrobatidis*, BdGPL and BdCAPE, with scrutiny towards high specificity and sensitivity

To date 3 methods of LAMP primer development exist. Either through means of manual primer design, which could be tedious and time consuming. However, this works great when software doesn't or can't deliver loop primers. Additionally, through means of software, current software for primer design include the PrimerExplorer software by Eiken Chemical Co. Ltd. and PrimerDesign software by Premier Biosoft.

2.2 Materials and methods

2.2.1 Software

For the development of LAMP primers several molecular software and genetic datasets were needed such as the NCBI genbank database, MEGA software, PrimerExplorer software and AnnHyb software.

The National Centre for Biotechnology (NCBI) genbank is a massive all-inclusive genomic database with descriptive biological annotation, bibliographic and nucleotide sequences of 260 000 species (as of 2013). It was built and distributed by the U.S. National Institutes of Health's (NIH) NCBI division (Benson, *et al.*, 2013).

Molecular Evolutionary Genetics Analysis (MEGA) is a user-friendly but sophisticated software used for the wide range which befalls DNA and protein sequence analyses (sequence alignments, molecular clocks, phylogeny, etc.) and statistics (maximum parsimony, maximum likelihood, Bayesian, etc.). These analysis and statistics includes individual species up to large populations which is made possible with powerful visual tools (timetree wizard, tree explorer, alignment/trace editor, etc.) (Kumar, *et al.*, 2015).

PrimerExplorer is a software program which is readily and freely available to the public for the design of LAMP primers. A user-friendly basic design protocol option can be used or a manual-based operating design in which the user can change the various factors which are of interest to the primers such as melting temperature and length of each primer, 3'-end stability, elimination factors, etc. (Eiken Chemical Co., Ltd., 2017).

AnnHyb analyse nucleotide sequences in several ways. The sequences can be viewed, edited, format conversion is possible, multi-alignment viewer, align oligonucleotides to sequences, etc. (Friard, 2012).

2.2.2 Specifications on selection

The design and selection of Bd-lineage LAMP primers were subjected to the present indigenous Bd lineages (BdGPL and BdCAPE) within South Africa since *in vivo* tests could be performed on infected frog hosts (swabs vs toe clippings) after the primer assay was developed from *in vitro* experimentation on live Bd cultures.

2.2.3 Lineage data

Data for several Bd lineages from the GPL and CAPE lineage were acquired from both the NCBI Genbank and Imperial College, London.

NCBI Genbank strains:

1) Accession number: AY997031

Isolate: AFTOL-ID 21

```
001 tttgattttg ggtgggggag ttttattgat gtgtaaagt tgatggaatg acccattggt
061 tttttcaaaa aacacccttg atataatata gtgtgccata tgtcacgagt cgaacaaaat
121 ttattttattt tttcgacaaa ttaattggaa attgaataat ttaattgaaa aaaattgaaa
181 ataaatatta aaaacaactt ttgacaacgg atctcttggc tctcgcaacg atgaagaacg
241 cagcgaaatg cgatacgtaa tgtgaattgc aaacctttgt gaattattaa atctttgaac
301 gcacattgca ctcgtaaaag agtatacatg tttgagaatt ataaaaatac attgtccgaa
361 ttgactggac agatatgaac catgtcaaaa atatttgaca ggtttttaaaa gtagtagtaa
421 atgagtgata caaaaagtag tgggtctaaa caaccccgtc catcacacca acaaaa
```

Lineage: BdGPL

Strain: JEL197 (J.E. Longcore)

Host: Culture

Locality: United States of America

Gene: Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; internal transcribed spacer 2, partial sequence.

2) Accession number: JQ582938

Isolate: CW34

```
001 ttagttgttg ggtgggggtt ttttattgat gtgtaaagt tgatggaatg acccattggt
061 ttttttcaaa aacacccttg atataatata gtgtgccata tgtcacgagt cgaacaaaat
121 ttattttattt tttcgacaaa ttaattggaa attgaataat ttaattgaaa aaaattgaaa
181 ataaatatta aaaacaactt ttgacaacgg atctcttggc tctcgcaacg atgaagaacg
241 cagcgaaatg cgatacgtaa tgtgaattgc aaacctttgt gaattattaa atctttgaac
301 gcacattgca ctcgtaaaag agtatacatg tttgagaatt ataaaaatac attgtccgaa
361 ttgactggac agatatgaac catgtcaaaa atatttgaca ggtttttaaaa gtagtagtaa
421 aaaagagtga taaaaaagt agtgggtcta aacaaccccg tccatcacac cataca
```

Lineage: BdGPL

Strain: CW34 (C. Weldon)

Host: *Xenopus laevis*

Locality: United States of America

Gene: Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; internal transcribed spacer 2, partial sequence.

Imperial College strains:

1) Isolate: TF5a1

```
001 tattagagga agtaaaagtc gtaacaaggt aaccgtaggt gaacctgcgg
051 ttggatcatt aatatttggt ggggtggggt tattttattg acatttatgt
101 caataaatga cccattgttt tttcaaaaaa cacccttgat ataatacagt
151 gtgccatatg tcacgagtcg aacaaaatth atttatthtt tcgacaaatt
201 aattggaaat tgaataatth aattgaaaaa aaattgaaaa taaaaacaac
251 ttttgacaac ggatctcttg gctctcgcaa cgatgaagaa cgcagcgaaa
301 tgcgatacgt aatgtgaatt gcaaaccctt gtgaatcatt aaatctttga
351 acgcacattg cactcgtaaa agagtataca tgtttgagaa ttataaaaat
401 acattgtccg aattgactgg acagatatga accatgtcaa aaatatttga
451 caggthttta aagtagtagt aaatgagtga tacaaaaagt agtgggtcta
501 aacaaccccg tccatcacac catacaaata atctcaaata atgcaagatt
551 acccgctgaa cttagcata tcaataacgg g
```

Lineage: BdCAPE

Strain: TF5a1

Host: Unknown

Locality: Spain

Gene: Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; internal transcribed spacer 2, partial sequence.

2) Isolate: TRBOMB

```
001 trbmbtcatt tagaggaagt aaaagtcgta acaaggtaac cgtaggtgaa
051 cctgcggttg gatcattaat atttgtgggg ggtggatggg agttttattg
101 atgtgtaa atgtgatggaa tgaccattg tttttttcaa aaaacaccct
151 tgatataata cagtgtgcc a tatgtcacga gtcgaacaaa atttatttat
201 tttttcgaca aattaattgg aaattgaata atttaattga aaaaaaattg
251 aaaataaaaa caacttttga caacggatct cttggctctc gcaacgatga
301 agaacgcagc gaaatgcgat acgtaatgtg aattgcaaac ctttgtgaat
351 cattaatct ttgaacgcac attgcactcg taaaagagta tacatgtttg
401 agaattataa aaatacattg tccgaattga ctggacagat atgaaccatg
451 tcaaaaatat ttgacagggt ttaaaagtag tagtaaatga gtgatacaaa
501 aagtagtggg tctaaacaac cccgtccatc acaccataca aataatctca
551 aatcatgcaa gattaccgc tgaacttaag catatcaata agc
```

Lineage: BdCAPE

Strain: TRBOMB

Host: Unknown

Strain: Unknown

Gene: Internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; internal transcribed spacer 2, partial sequence.

2.2.4 Primer design

In order to develop universal primers that would be able to identify, attach and amplify the same genes between different species and or lineages, conserved regions within the gene(s) of interest between the species/lineages should be used to develop the primers. These developed primers which contain bases that are conserved within all the isolates (of different species and or lineages) can thus be used as universal primers for the broad scale detection of multiple species and/or lineages. Figure 2.2 illustrates the conserved regions between BdGPL and BdCAPE lineages which were used to develop LAMP primer sets Bd3, Bd4 and Bd5.

Each colour represents a respective nucleotide base. The asterisk (*) appears whenever conserved regions represent themselves over the course of the selected gene that was aligned from several individuals. As seen from figure BdGPL and BdCAPE was highly conserved across the entire gene of interest. This made lineage specific primer development unlikely for the given gene although a universal primer set for both BdGPL and BdCAPE was possible.

DNA Sequences		Translated Protein Sequences	
Species/Abbrev	Group Name	*****	
1. AFTOL-ID 21		GGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAACCTTTGTGAATCATTAAATCTTTGAACGCACAT	
2. CW34		GGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAACCTTTGTGAATTATTAAATCTTTGAACGCACAT	
3. TF5a1		GGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAACCTTTGTGAATCATTAAATCTTTGAACGCACAT	
4. TRBOMB		GGCTCTCGCAACGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAACCTTTGTGAATCATTAAATCTTTGAACGCACAT	

Figure 2.2: MEGA alignment indicating conserved regions between the BdGPL and BdCAPE lineage

2.2.5 NCBI BLAST

The outer primers of all three designed LAMP primer sets were tested for integrity and identification purposes. Only the outer primers were tested, reason being, once the outer primers anneal and amplify the strand of interest it delivers the starting material for the rest of the reaction. The inner- and loop primers are designed based on the sequence of the starting material. Thus, positive results from NCBI BLAST using designed outer primers delivers promising results which allows investigation and implementation of additional trials and testing systems. Figure 2.3 represents the NCBI BLAST results from LAMP primer set 4's outer primers. The primer set was specific to Bd alone across different clones and strains.

Primer pair 1

	Sequence (5'→3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGAAGAACGCAGCGAAAT	18	55.30	44.44	5.00	2.00
Reverse primer	CCACTACTTTTGTATCACTCAT	23	54.39	34.78	3.00	3.00

Products on target templates

>[JQ582941.1](#) Batrachochytrium dendrobatidis strain CW34 clone H internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

product length = 212

```
Forward primer 1 TGAAGAACGCAGCGAAAT 18
Template      231 ..... 248

Reverse primer 1 CCACTACTTTTGTATCACTCAT 23
Template      442 ..... 420
```

>[JQ582940.1](#) Batrachochytrium dendrobatidis strain CW34 clone M internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

product length = 220

```
Forward primer 1 TGAAGAACGCAGCGAAAT 18
Template      241 ..... 258

Reverse primer 1 CCACTACTTTTGTATCACTCAT 23
Template      460 ..... 438
```

>[JQ582935.1](#) Batrachochytrium dendrobatidis strain CW34 clone K internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence

product length = 212

```
Forward primer 1 TGAAGAACGCAGCGAAAT 18
Template      240 ..... 257

Reverse primer 1 CCACTACTTTTGTATCACTCAT 23
Template      451 ..... 429
```

Figure 2.3: NCBI BLAST result of LAMP primer set Bd4's outer primer

2.3 Results and discussion

Several LAMP primer development software were available for the development of LAMP primers. Primer sets Bd3, Bd4 and Bd5 were developed using PrimerExplorer V5 (Eiken Chemical Co. Ltd) which is an online webpage-development software. Given primer sets' individual results can be viewed from table 2.1.

Table 2.1: Primer sets Bd3, Bd4 and Bd5 as designed with the use of PrimerExplorer V5

PRIMER SET	LABEL	LENGTH	TM	SEQUENCE
BD3-SET				
	F3	18	55.10	CTCGCAACGATGAAGAAC
	B3	23	55.94	CCACTACTTTTGTATCACTCTT
	FIP	43		GTGCAATGTGCGTTCAAAGATTTAA- CAGCGAAATGCGATACGT
	BIP	46		TACATTGTCCGAATTGACTGGAC- ACTACTACTTTTAAACCTGTCA
	F2	18	57.22	CAGCGAAATGCGATACGT
	F1c	25	61.30	GTGCAATGTGCGTTCAAAGATTTAA
	B2	23	55.00	ACTACTACTTTTAAACCTGTCA
	B1c	23	60.38	TACATTGTCCGAATTGACTGGAC
BD4-SET				
	F3	18	56.73	TGAAGAACGCAGCGAAAT
	B3	23	56.00	CCACTACTTTTGTATCACTCAT
	FIP	44		GAGTGCAATGTGCGTTCAAAG- ATACGTAATGTGAATTGCAAACC
	BIP	46		TACATTGTCCGAATTGACTGGAC- ACTACTACTTTTAAACCTGTCA
	F2	23	57.65	ATACGTAATGTGAATTGCAAACC
	F1c	21	60.25	GAGTGCAATGTGCGTTCAAAG
	B2	23	55.00	ACTACTACTTTTAAACCTGTCA
	B1c	23	60.38	TACATTGTCCGAATTGACTGGAC
BD5-SET				
	F3	18	55.42	TGACAACGGATCTCTTGG
	B3	21	56.51	CAAACAATTTTGGCATGGTTC
	FIP	44		GATTCACAAAGGTTTGAATTCACA- TCTCGCAACGATGAAGAAC

	BIP	43		AATCTTTGAACGCACATTGCACTC- GTCCAGTCAATTCGGACAA
	F2	19	57.01	TCTCGCAACGATGAAGAAC
	F1c	25	61.08	GATTCACAAAGGTTTGCAATTCACA
	B2	19	56.68	GTCCAGTCAATTCGGACAA
	B1c	24	62.60	AATCTTTGAACGCACATTGCACTC

As seen in table 2.1 PrimerExplorer developed only the necessary outer- (F3 and B3) and inner primers (FIP and BIP) but no loop primers, although the software does possess this feature. After obtaining designed primers it is preferable to test them on a theoretical basis first using NCBI BLAST (for both the primers and expected nucleotide product after amplification) in order to see whether or not the designed primers and expected nucleotide product yields a theoretically positive detection for the specified organism and gene of interest. It is preferable to BLAST the outer primers (F3 and B3) and the expected nucleotide product as seen in figures 2.3 and 2.4. For the sake of redundancy only primer set Bd4's BLAST results were given since primer set Bd3 and Bd5 also yielded almost similar positive results.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected: 0

Alignments  Download GenBank Graphics Distance tree of results								
	Description	Max score	Total score	Query cover	E value	Ident	Accession	
☐	Batrachochytrium dendrobatidis strain CW34 clone H internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	392	392	100%	2e-105	100%	JQ582941.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone K internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	392	392	100%	2e-105	100%	JQ582935.1	
☐	Batrachochytrium dendrobatidis JEL 197 ITS region; from reference material	392	392	100%	2e-105	100%	NR_119535.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone F internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	388	388	99%	3e-104	100%	JQ582927.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone D internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	388	388	99%	3e-104	100%	JQ582906.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone T internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	377	377	100%	6e-101	99%	JQ582937.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone B internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	377	377	99%	6e-101	99%	JQ582926.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone J internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	377	377	100%	6e-101	99%	JQ582903.1	
☐	Batrachochytrium dendrobatidis strain JEL648 clone A internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582931.1	
☐	Batrachochytrium dendrobatidis strain UM142 clone I internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582930.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone A internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582924.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone C internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582923.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone U internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582922.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone R internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582921.1	
☐	Batrachochytrium dendrobatidis strain JEL648 clone G internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582919.1	
☐	Batrachochytrium dendrobatidis strain JEL648 clone D internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582918.1	
☐	Batrachochytrium dendrobatidis strain JEL648 clone J internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582917.1	
☐	Batrachochytrium dendrobatidis strain CW34 clone P internal transcribed spacer 1, partial sequence; and 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582915.1	
☐	Batrachochytrium dendrobatidis strain UM142 clone N internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582909.1	
☐	Batrachochytrium dendrobatidis strain UM142 clone R internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, complete sequence	374	374	99%	8e-100	99%	JQ582908.1	

Figure 2.4: NCBI BLAST results of LAMP primer set Bd4's expected nucleotide product

As seen in figures 2.3 and 2.4 the Bd4 primer set and expected nucleotide product both tested positive for Bd. After designing and testing them through BLAST, these 3 primer sets were ordered from Inqaba Biotec (South Africa) to test the integrity and capability of each set.

No loop primers were designed from the used software and gene of interest yet. Thus loop primers were designed manually without the use of software. LF (forward loop primer) attaches to the gene of interest between F2 and F1 (in red, respectively) and represents the reverse complement of the template sequence while LB (backward loop primer) attaches to the gene of interest between B1 and B2 (in blue, respectively) and is identical to the template sequence.

```

001 ttgatttttg ggtgggggag ttttattgat gtgtaaatgt tgatggaatg acccattggt
061 tttttcaaaa aacacccttg atataataca gtgtgccata tgtcacgagt cgaacaaaat
121 ttattttattt tttcgacaaa ttaattggaa attgaataat ttaattgaaa aaaattgaaa
181 ataaatatta aaaacaactt ttgacaacgg atctcttggc tctcgcaacg atgaagaacg
241 cagcgaaatg cgatacgtaa tgtgaattgc aaacctttgt gaatcattaa atctttgaac
301 gcacattgca ctcgtaaaag agtatacatg tttgagaatt ataaaaatac attgtccgaa
361 ttgactggac agatatgaac catgtcaaaa atatttgaca ggttttaaaa gtagtagtaa
421 atgagtgata caaaaagtag tgggtctaaa caaccccgtc catcacacca acaaaa

```

Designed LF and LB loop primers:

LF: 5'-GATTTAATGATTCACAAAG-3' on Complementary strand

LB: 5'-AGATATGAACCATGTCAA-3' on Template strand

The LF and LB primers that were developed manually acquired a nt length of 19 and 18 base pairs respectively which befits the preferable range of 17-21 nt as prescribed by Nagamine, *et al.*, (2002) and Notomi, *et al.*, (2000).

composition, optimal melting temperature and preferable primer nt length are just some of the few requirements for a perfect LAMP reaction (Parida, *et al.*, 2008).

Table 2.1 indicates primer set Bd4's individual primer nt length to be in reasonable bounds for optimal reactions. The overall melting temperature of 57.66°C is rather low due to the low GC content of the gene. This simply means the annealing temperature of the reactions would have to be adjusted to the lower side in regards to the normal prescribed ranges of 63 to 65°C (Notomi, *et al.*, 2000). This would not have a significant drawback for the reactions, only a slight increased incubation time for LAMP. With the added loop primers, this increased reaction time would be insignificant.

Regardless of Bd4's perfect preliminary theoretical results, all three developed primer sets will be tested with *in vitro* systems to determine which primer set has a higher detection success rate. This particular primer set will then be sent for further integrity testing by means of *in vivo* systems.

Chapter 3 Development of a novel universal LAMP assay for the detection of BdGPL and BdCAPE

3.1 Introduction

3.1.1 A novel molecular diagnostic assay

Since the emergence of LAMP several conventional PCR assays, qPCR assays and culture media has been replaced with the former for simple point-of-care diagnostics. Probably the biggest attribute of LAMP lies within its auto cyclic strand displacement property which allows LAMP to have high rapidity, selectivity and sensitivity in regards to other molecular diagnostic assays and culture growth (Parida, *et al.*, 2008; Thai, *et al.*, 2004). Traditional point-of-care diagnostics for Bd which include PCR, qPCR and culture media may sometimes prove difficult in which unacceptable results are yielded for several reasons. PCR and qPCR are extremely sensitive to inhibitory substances and/or contaminants which may result from crude DNA and/or raw environmental samples influencing amplification in a multitude of arrays. Furthermore, culture media occasionally becomes contaminated and eventually fecund yeasts and/or bacteria proliferate, increasing difficulty with identification and for later use in genetic analysis (Parida, *et al.*, 2008; Wang, *et al.*, 2015).

LAMP has already replaced a profusion of assays in the identification of a multitude of human-, animal- plant- aquaculture- and zoonotic infectious diseases including several parasitic protozoans, parasitic helminths, pathogenic viruses, pathogenic bacteria and pathogenic fungi (Abd-Elsalam, *et al.*, 2011; Alhassan, *et al.*, 2007; Cai, *et al.*, 2013; Das, *et al.*, 2012; Endo, *et al.*, 2004; Iwamoto, *et al.*, 2003; Kong, *et al.*, 2012; Kuboki, *et al.*, 2003; Li, *et al.*, 2009; Niessen & Vogel, 2010; Nzelu, *et al.*, 2014; Parida, *et al.*, 2004; Perera, *et al.*, 2009; Promparon, *et al.*, 2011; Savan, *et al.*, 2005; Thai, *et al.*, 2004; Thekisoe, *et al.*, 2005; Wang, *et al.*, 2014; Wasserman, *et al.*, 2014; Wozniakowski, *et al.*, 2012). Furthermore, LAMP assays have also been developed in the detection of genetically modified organisms (GMOs), embryo sex identification and tumour detection (Fu, *et al.*, 2010).

Among all of these infectious diseases, pathogenic fungi have received the least amount of attention in regards to developed fungi LAMP assays with viruses and bacteria sharing the podium for first place, subsequently followed by parasites (protozoans/helminths) (Savan, *et al.*, 2005). To date only a handful of fungal LAMP assays have been developed and described, of which the fungi (yeasts and molds) include *Paracoccidioides brasiliensis*, *Ochroconis gallopava*, *Phytophthora ramorum*, *Brettanomyces/Dekkera* yeasts, arbuscular mycorrhizal fungi (such as *Glomus intraracices*), *Fusarium* species, *Candida* species, *Trichosporon* species and *Aspergillus* species (Kasahara, *et al.*, 2014; Luo, *et al.*, 2012;

Niessen, *et al.*, 2010). This yields a golden opportunity to broaden the fungal horizon with regards to end-point-diagnostics and genetic analysis and overcome limitations that exist within PCR and histology.

3.1.2 Potential beneficial advantages of LAMP

LAMP consist of several potential benefits in regards to many other gene amplification techniques. Some of the most notable and potential beneficial advantages include simplicity, rapidity, specificity, sensitivity, cost effectiveness, DNA and RNA amplification, high product output and tolerance towards inhibitors. Furthermore, the type of DNA polymerase used, *Bst* DNA polymerase, adds to the list of beneficial advantages since auto cycling strand displacement can take place which means no DNA denaturation step is required thus amplification can be carried out at a constant isothermal temperature (Aliotta, *et al.*, 1996; Nagamine, *et al.*, 2002; Thekiso & Inoue, 2011).

LAMP illustrates superior tolerance towards PCR when it comes to amplification inhibitors. *In vitro* cultures containing substances such as phosphate buffered saline (PBS), saline, vitreous and aqueous humour as well as Eagle's minimum essential medium (MEM) have been found to inhibit PCR reactions since Taq DNA polymerase has a lower tolerance while LAMP and the associated *Bst* DNA polymerase has a superior tolerance (Kaneko, *et al.*, 2007). When Boehme and colleagues (2007) used biological samples (sputum-filled blood) as template for the clinical diagnosis of pulmonary tuberculosis, LAMP reactions underwent no inhibition. EDTA, IgG, IgM, heparin and haemoglobin (typical blood components) has been identified as inhibitors to the Taq DNA polymerase enzyme and thus its associated conventional gene amplification methods while *Bst* DNA polymerase and the associated LAMP remains unaffected (Thekiso, *et al.*, 2009).

3.1.3 Principle behind LAMP reaction

The chemistry behind LAMP hinge on the essence of auto cyclic strand displacement reaction due and thanks to the unique nature that *Bst* DNA polymerase poses. The LAMP reaction can be divided into two major phases, a non-cyclic and a cyclic phase, each containing several intricate steps (Nagamine, *et al.*, 2002; Parida, *et al.*, 2008).

Non-cyclic phase

The non-cyclic phase incorporates the use of the inner- (FIP and BIP) and outer primers (F3 and B3). Double stranded target DNA does not have to undergo heat denaturation into single stranded DNA due to the unique function *Bst* DNA polymerase exhibits. The Isothermal temperature of LAMP ($\pm 65^{\circ}\text{C}$) creates an environment of dynamic equilibrium for double stranded DNA. In essence, FIP anneals to the double stranded target DNA, with the

aid of Bst DNA polymerase to simultaneously synthesise a FIP complementary strand to the target DNA it was attached to while displacing the original complementary strand. F3 anneals to the original target DNA strand, in turn displacing the newly formed complementary FIP strand which eventually forms a stem-loop structure due to 'self-priming' at the 5'-end. This stem-loop structure serves as template for BIP and B3 strand displacement DNA synthesis, repeating above mentioned order of FIP and F3, finally delivering a final product with two stem-loops at each end of the displaced BIP strand which is termed a dumbbell stem-loop DNA structure. This final dumbbell structure acts as starting material for the cyclic phase (Nagamine, *et al.*, 2002; Parida, *et al.*, 2008).

Cyclic phase

The cyclic phase incorporates the use of only the inner primers. The dumbbell structure initiates self-priming strand displacement from 3' to 5' delivering a single stem-loop structure which acts as the starting material for the cyclic phase. FIP anneals to this single stem-loop, initiating strand displacement synthesis delivering a complementary stem-loop strand towards the one acting as starting material for the cyclic phase as well as a cauliflower like structure containing alternating inverted repeats of the target stem-loop sequence. BIP can anneal to both these structures to either repeat the sequence of events that followed (BIP attachment to complementary stem-loop strand) or continue over into the elongation/recycling step (BIP attachment to cauliflower-like structure) reproducing more cauliflower-like structures of different sizes (Nagamine, *et al.*, 2002; Parida, *et al.*, 2008).

Addition of loop primers

The original dumbbell structures (final product of non-cyclic phase) and the final cauliflower-like structures (final product of cyclic phase) can act as starting materials for inner primers and loop primers. With the addition of loop primers, the final product output is significantly increased, selectivity and sensitivity is heightened exponentially while the incubation time is notably reduced. This is due to multiple strand displacement synthesis that can be carried out simultaneously on the same DNA template (Nagamine, *et al.*, 2002; Parida, *et al.*, 2008). The objective was to develop and optimise a novel LAMP assay for the detection of BdGPL and BdCAPE from subsequent designed primer sets with the use of *in vitro* models.

3.2 Materials and methods

3.2.1 LAMP kit

Common LAMP kits' assays can be followed step by step, delivering optimal results. However, some species / genes are a bit less user friendly, yielding non-desirable results requiring further optimisation beyond these universal assays. The norm of assays requires a pre-heat step of 95°C for 5mins, incubation step of 65°C for 60mins and lastly an annealing step of 80°C for 10mins, some may vary from this by a couple of degrees or minutes (Notomi, *et al.*, 2000). The kit of choice for this assay development was 'OptiGene Isothermal Master Mix' [OIMM] (Table 3.1). Lucigen OmniAmp DNA & RNA kit and Loopamp DNA Amplification kit were also notable contenders. However, the addition of each chemical separately increased prep time significantly and so contamination as well.

Table 3.1. LAMP reaction conditions

PRIMER SET	AMPLIFICATION TIME	ANNEAL DERIVATIVE	ASSAY
BD3	-	-	25μL total volume 15μL MM
BD4	52- & 54 mins	81.8- & 81.9°C	5μL template DNA 5μL primers mix
BD5	80 mins	83.5- & 83.4°C	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{4 \text{ parts}}$ No additional loop primers 65°C isothermal temperature 90 minutes' incubation time

Non-optimised OIMM instructions along with standardised LAMP conditions were followed for the first two optimisation experiments including, primer sets' integrity testing and temperature optimisation as set out on the leaflet of OIMM. The final reaction volume (25μL) consisted of 15μL master mix, 5μL template DNA and 5μL primer mix. The 5μL primer mix consisted of the following primer constituent relationship; $\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{4 \text{ parts}}$. The isothermal reaction was run at 65°C for 90mins. Reconstitution of lyophilized primer powder was done with the addition of double distilled water (ddH₂O) until a 100μM concentration was achieved.

After testing the primer sets' integrity and temperature optimisation with the non-optimised OIMM conditions, newly implemented optimised OIMM conditions were implemented to further optimise the LAMP assay. Newly optimised OIMM consisted of 15μL master mix, 5μL

template DNA and 2µL primer mix which was filled to a 25µL total with ddH₂O. Furthermore, the 2µL primer mix consisted of the following primer constituent relationship; $\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ part}}$. This reduction of 5µL primer mix to 2µL as well as the reduction of loop primers from 4 parts to 1 part seemed to help marginally with the reduction in non-specific amplification albeit the total reaction (positive controls included) time taking a toll of 5-10mins delay. Furthermore, the LAMP device used was the Genie 2 instrument which accompanied 2 wells that fitted 8-strip PCR reaction tubes respectively. LAMP reaction with LF and LB primers were also conducted with following reaction conditions shown in table 3.2.

Table 3.2. LAMP reaction including LF and Lb

PRIMER SET	AMPLIFICATION TIME	ANNEAL DERIVATIVE	ASSAY
BD4 W LOOPS	28- & 29 mins	81.5- & 81.6°C	25µL total volume 15µL MM 5µL template DNA 5µL primers mix
BD4 W/O LOOPS	57- & 58 mins	81.7- & 81.5°C	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{4 \text{ parts}}$ Primer set Bd4 used 65°C isothermal temperature 90 minutes' incubation time

3.2.2 *In vitro* systems

The two Bd lineages incorporated into this study was BdGPL (Global Panzootic Lineage) and BdCAPE (Cape lineage). Development of the LAMP assay was done with *in vitro* models of Bd which were grown in culture. The BdGPL strain was MG04 which was isolated in 2008 of off an *Amietia fuscigula* individual at the Silver Mine in the Western Cape province. The passarge used for the *in vitro* model testing was passarged on the 24th of April 2017 (passarge number 27) and the BdCAPE strain was SA4C which was isolated in 2010 of off an *Amietia angolensis* individual in Greytown, Kwazulu-Natal. The passarge used for the *in vitro* model testing was passarged on the 24th of April 2017 (passarge number 10). Both strains were kept in culture flasks at a steady 4°C and passarged every 3 months. Sub-cultures were passarged during the next passarging wave and incubated at 20°C for 3 days to ensure proliferated growth after which they were subjected to 4°C storage. Growth was examined every day during the 20°C incubation period by noting zoosporangium attachment

to substrate and increase in size, motile zoospore number increase, overall viability of the culture as well as contamination.

Several sub-cultures were used for DNA extraction containing both zoosporangiums and zoospores for total genomic DNA for LAMP assay optimisation. However, zoospores were also separately harvested to determine the sensitivity of serially diluted zoospores numbers in raw format, boiled and with DNA extracted. This is an especially useful approach since Bd's zoospores are motile waterborne cells.

Harvesting zoospores entails the growth of Bd in tryptone broth until sufficient growth was noted. Pipetting only 2mL of the culture media onto a petri dish with solidified agar as nutrient base, the petri dish was allowed to set in a Class II bioflow cabinet, drying out the fluid portion of the broth leaving the zoospores and zoosporangiums behind. After drying out over the time-span of roughly 1 hour, the petri dishes were closed and left in the incubator at 21°C for 3 days to allow the zoosporangiums to attach to the agar substrate and proliferate. After 3 days the petri dishes were flooded with sterile tryptone broth, this allows the zoosporangia to germinate, releasing all the zoospores into suspension which can be harvested via a pipet while the zoosporangia remain attached to the substrate. Typical cell counting was followed using a haemocytometer and compact light microscope.

These two strains acted as positive controls for *in vitro* development of the LAMP assay. Implemented blanks consisted of the same reagents except any template DNA, this was to detect any contamination and / or the formation of primer dimers. Negative controls included host DNA (*Xenopus laevis*), another closely related pathogenic yeast (*Candida albicans*) and an out-group which was the hard-bodied southern African dog tick (*Haemaphysalis elliptica*).

3.2.3 DNA extraction

Several DNA extraction methods were tested which included 4 kitchen methods (microwave method, Hoffman & Winston protocol, salting out and the phenol chloroform method) and 3 commercial DNA extraction kits (PrepMan, Quick g-DNA blood mini-prep and Zymo bacterial and fungal kit). From these several methods, one kitchen method and one DNA extraction kit proved to be equally effective in delivering sufficient quantity and quality genomic DNA.

At first the Zymo bacterial and fungal kit had great success in extracting genomic DNA. The protocol was followed out exactly as set out in the instruction manual however, an additional action was implemented between step 1 (addition of 200µL and 750µL into a bashing lysis tube) and step 2 (process said tube for 5 minutes at maximum speed in a bead beater) to further optimise DNA yield. This additional action included the heating of said tube with reagents at 70°C for 10 minutes before the bead beating processing. The additional heat

step works by weakening the robust cell wall consisting of complex polysaccharides and intricate chitin.

Secondly, the salting out method proved to deliver promising results after implementing purification of the final DNA product directly post extraction or after running an agarose gel.

3.2.4 Visual detection methods

Increased synthesized template DNA is accompanied by increased turbidity during LAMP amplification. After amplification this turbidity is seen as a white precipitate known as Magnesium Pyrophosphate. This precipitate can be represented by the following formula: $(DNA)_{n-1} + dNTP = (DNA)_n + P_2O_7^{4-}$ (pyrophosphate ions are released from nucleotides due to DNA polymerisation as by-products); $P_2O_7^{4-} + 2Mg^{2+} = Mg_2P_2O_7$ (these pyrophosphate ions react with the reaction buffer – magnesium ions – producing a white precipitate). Thus, precipitate turbidity increases in linear correlation with template DNA amplification. To analyse and detect whether DNA amplification was successful several methods include the use of magnesium pyrophosphate (Mori, *et al.*, 2001; Mori, *et al.*, 2004).

Naked eye detection can be done by judging the visual accumulation of turbidity between positive-, negative- and control groups. Furthermore, centrifuging (at 6000rpm) the LAMP reaction tube for a couple of seconds, the precipitate will accumulate as a white pellet in the bottom. Both these methods are only for the end-point confirmation of positive and/or negative amplicons. Real-time detection, which gives a descriptive result of DNA yield per time interval, can be interpreted by use of either a spectrophotometer or real-time turbidimeter (Mori, *et al.*, 2001; Mori, *et al.*, 2004).

Mori and colleagues (2004) designed a real-time turbidimeter which was used in this study for both the LAMP amplification reaction(s) and real-time analyses. Simultaneous real-time measurement of 8 samples' (in 0.2mL 8-strip tubes) turbidity are carried out at an isothermal temperature. Eight light emitting diodes (LED) blasts a light of 650nm wave-length through the 8-strip tubes (containing the amplified precipitate) illuminating the photodiodes (PD) while being heated at a constant isothermal temperature (60-65°C) by means of a heating block. A circuit board containing an 8-bit CPU, amplifier, 12-bit A/D converter and an analog multi-prexer. The PC receives both the LED luminescence intensity (I_{LED}) and PD light intensity (I_{PD}) from the CPU after A/D conversion. I_{PD} is the light intensity received by PD while I_{LED} is the light intensity emitted by LED. Using the calculation, $Turbidity = 1n(I_{PD} / I_{LED})$, turbidity was calculated by repeating the measurement every 30 seconds for the estimate LAMP reaction time (Mori, *et al.*, 2004).

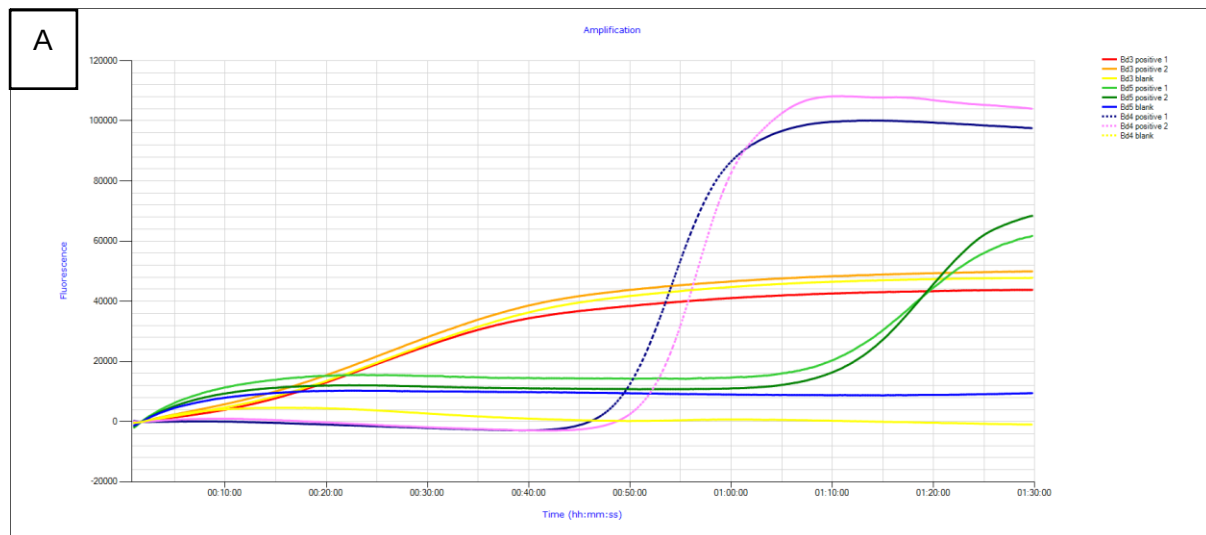
One of the oldest and most implemented visual detection method for DNA molecules was agarose gel electrophoresis. This simple method is highly effective in the separation, purification and identification of DNA products. First, a gel is to be prepared based upon the expected DNA product size, the product size will determine the agarose gel concentration. Second, the agarose gel liquid is poured into a tray containing wells in which the DNA samples are loaded respectively when cooled down. The agarose gel is then run at a specific voltage for a given time that will deliver most favourable separation of DNA fragments. Third, the gel has to be stained by a dye such as ethidium bromide, SYBR gold, SYBR green, SYBR safe and/or Eva green which can be visualised by UV light illumination (Johansson, 1972).

For this study three detection methods were implemented. Real-time visualisation using the turbidimeter, addition of ethidium bromide directly into the tubes post-incubation and running an agarose gel with the final DNA products.

3.3 Results and discussion

3.3.1 Primer sets Bd3, Bd4 and Bd5 testing

Primer sets Bd3, Bd4 and Bd5 were run in comparison with each other to illustrate amplification potential. From perceived results primer set Bd4 gave the best amplification curve yielding positive amplification at 52 and 54 mins respectively in regards to Bd3 that didn't amplify at all within the 90-minute mark and Bd5 that yielded a positive amplification curve at 80 minutes. From here on out primer set Bd4 was chosen as the primer set to be used in further optimisation. The anneal derivatives for Bd4 and Bd5 duplicates were 81.8/81.9°C and 83.5/83.4°C respectively. The function of the anneal derivative temperatures is to act as a control if by any chance a negative control or blank amplified. An amplified negative control or blank (without the DNA of interest) will deliver a different annealing temperature in comparison to a true positive control's annealing temperature. An amplified negative control or blank (with intentional target DNA contamination) will deliver the same annealing temperature as a true positive control's annealing temperature. Different primers used (Bd4 and Bd5) will deliver different annealing derivative temperatures. While annealing derivative temperatures remain relative constant within the same primer set whether loop primers are present or absent.



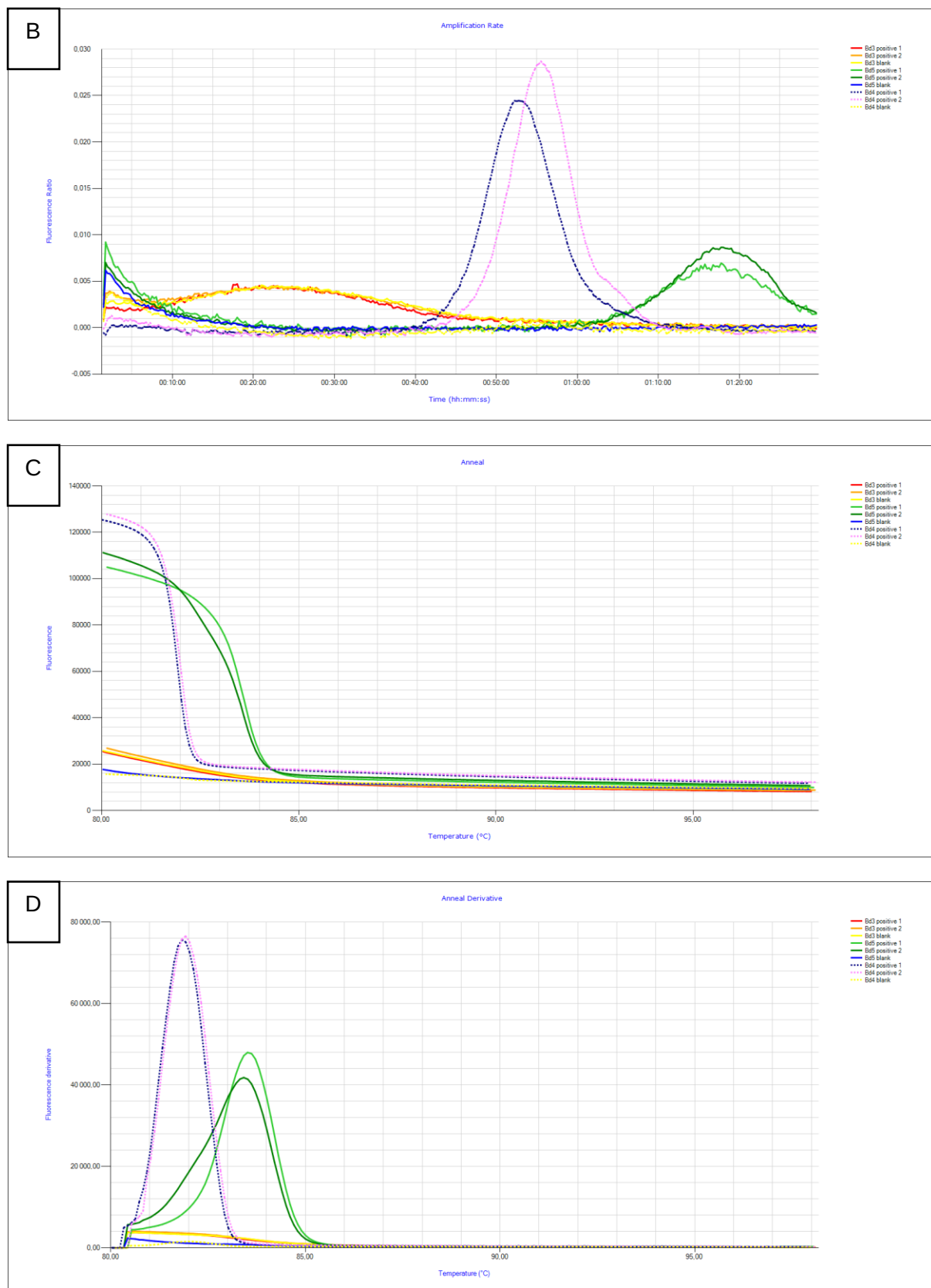


Figure 3.1: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers without added loop primers. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

As seen in figure 3.1 primer set Bd3 showed no real-time amplification from the turbidimeter but also had no positive result in the agarose gel (figure 3.2). While Bd5 indicated both a lagging and low amplification in real-time it also had a lower intensity band on the agarose gel. Bd4 showcased the fastest and highest amplification in real-time accompanied with a bright band on the agarose gel. Although ± 50 minutes was a lengthy time to detect a positive control, this was done without optimisation and more importantly without the additional loop primers which greatly increases the specificity and reduces incubation time needed to yield a result.

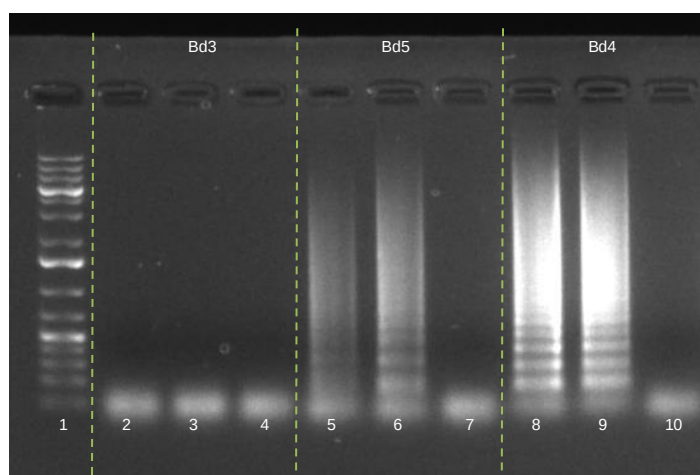
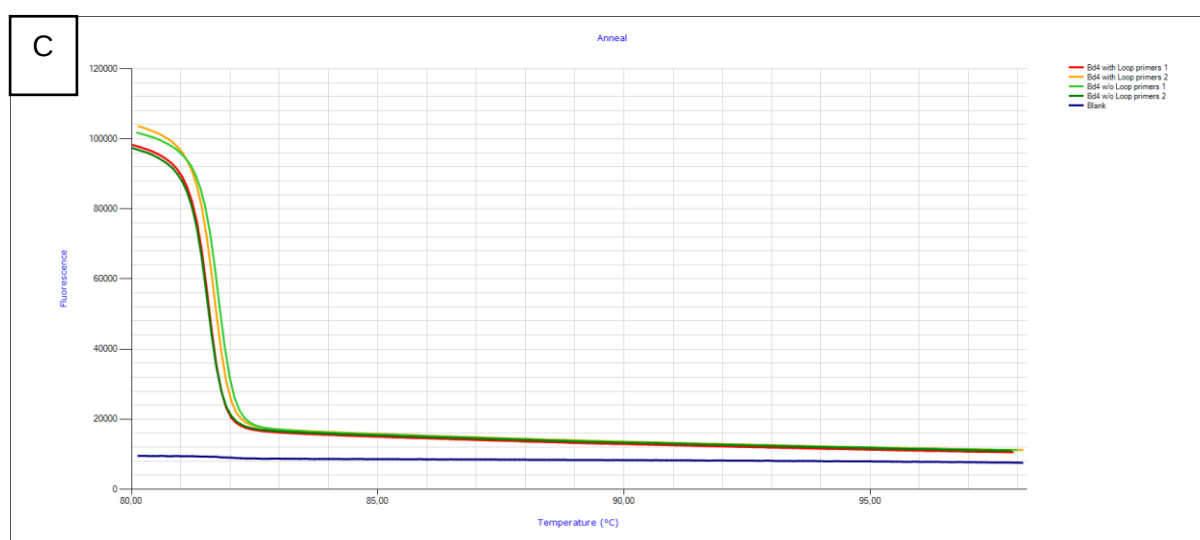
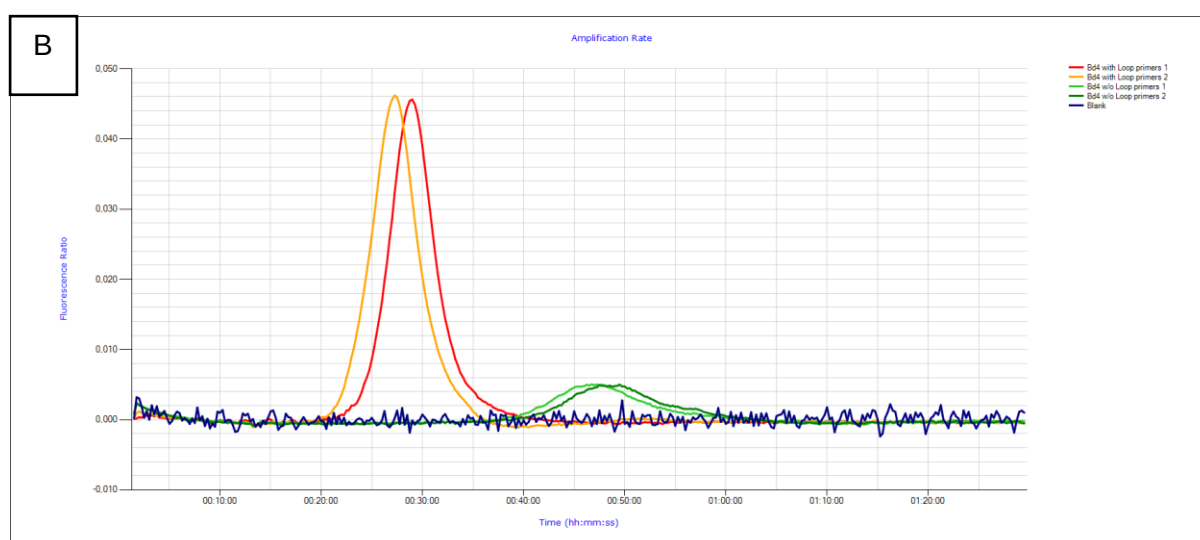
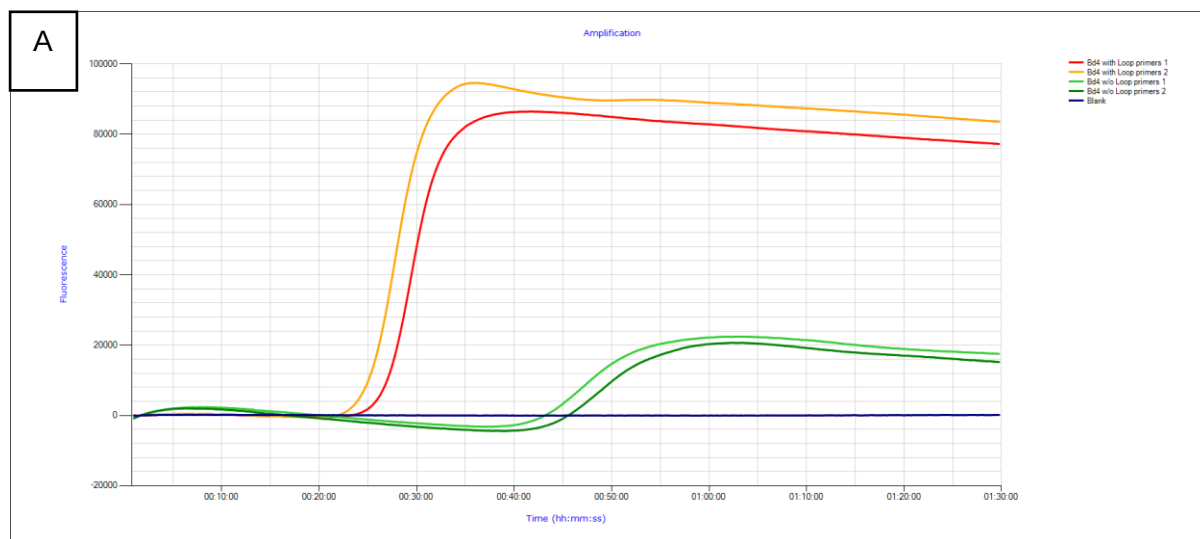


Figure 3.2: An agarose gel electrophoresis image of primer sets Bd3, Bd5 and Bd4

3.3.2 Bd4 amplification without loop primers vs with loop primers

Primer set Bd4 was run in conjunction with and without loop primers (LF and LB) to illustrate the difference in amplification potential when and whether loop primers were used. From perceived results primer set Bd4 containing no loop primers gave a positive amplification curve at approximately 57 and 58 mins respectively in regards to Bd4 that did contain the loop primers which had a massive reduction in amplification time, yielding positive results at approximately 28 and 29 minutes. The loop primers not only increases specificity and selectivity but also reduced amplification time by roughly 50%. Anneal derivatives for Bd4 with loop primers and without are 81.5/81.6°C and 81.7/81.5°C respectively, which as I mentioned would remain relatively constant whether or not loop primers are present in the same primer set as seen here. However, the addition of loop primers could also add to the possibility of non-specific amplification if pristine primer design was not taken into consideration. This non-specific amplification may arise by non-specific binding to the negative control or by primer dimer formation within the blank control. To avoid this, assay optimisation was needed to eliminate or at least reduce non-specific amplification.



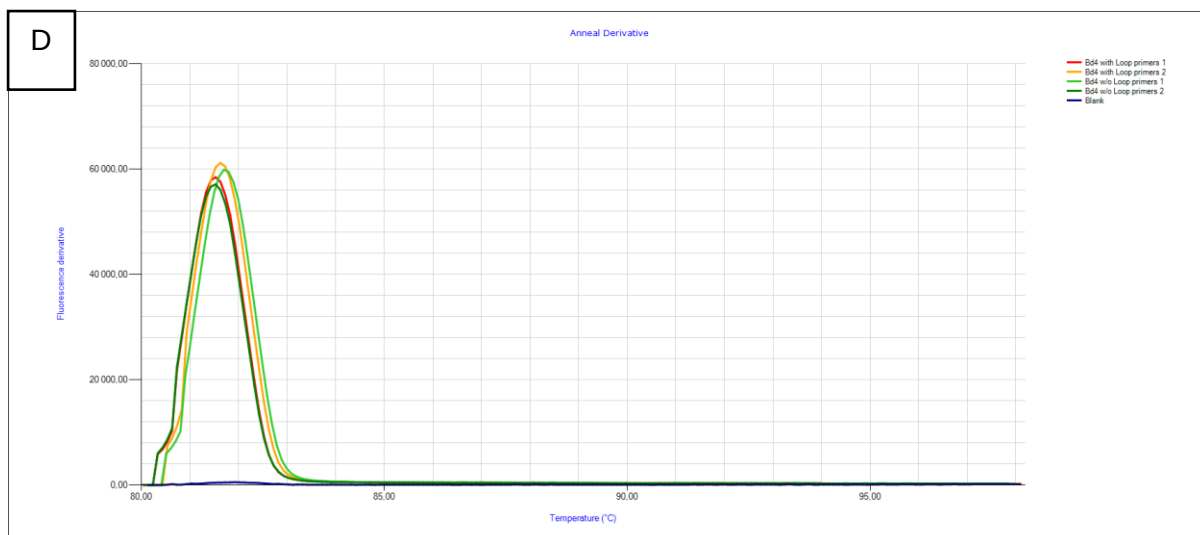
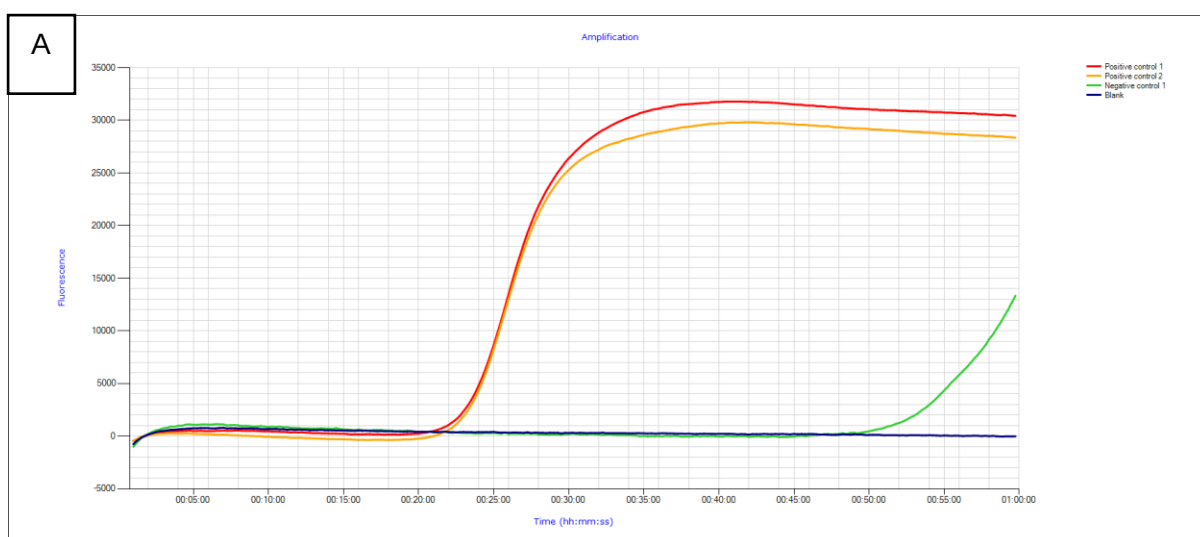


Figure 3.3: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers with added loop primers. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

3.3.3 Temperature optimisation

As illustrated by figure 3.3, positive controls 1 and 2 yielded a positive amplification curve at 26 and 25:30 minutes respectively. With anneal derivative temperatures reaching 81.9 and 81.8°C respectively. The start of an amplification curve for the negative control can be seen to arise at ± 50 mins while no set amplification time was captured by the Genie2 instrument since the negative amplification curve was not allowed to reach a maximum fluorescence and death phase. The anneal derivative for the negative control was recorded at 81.1°C.

55°C:



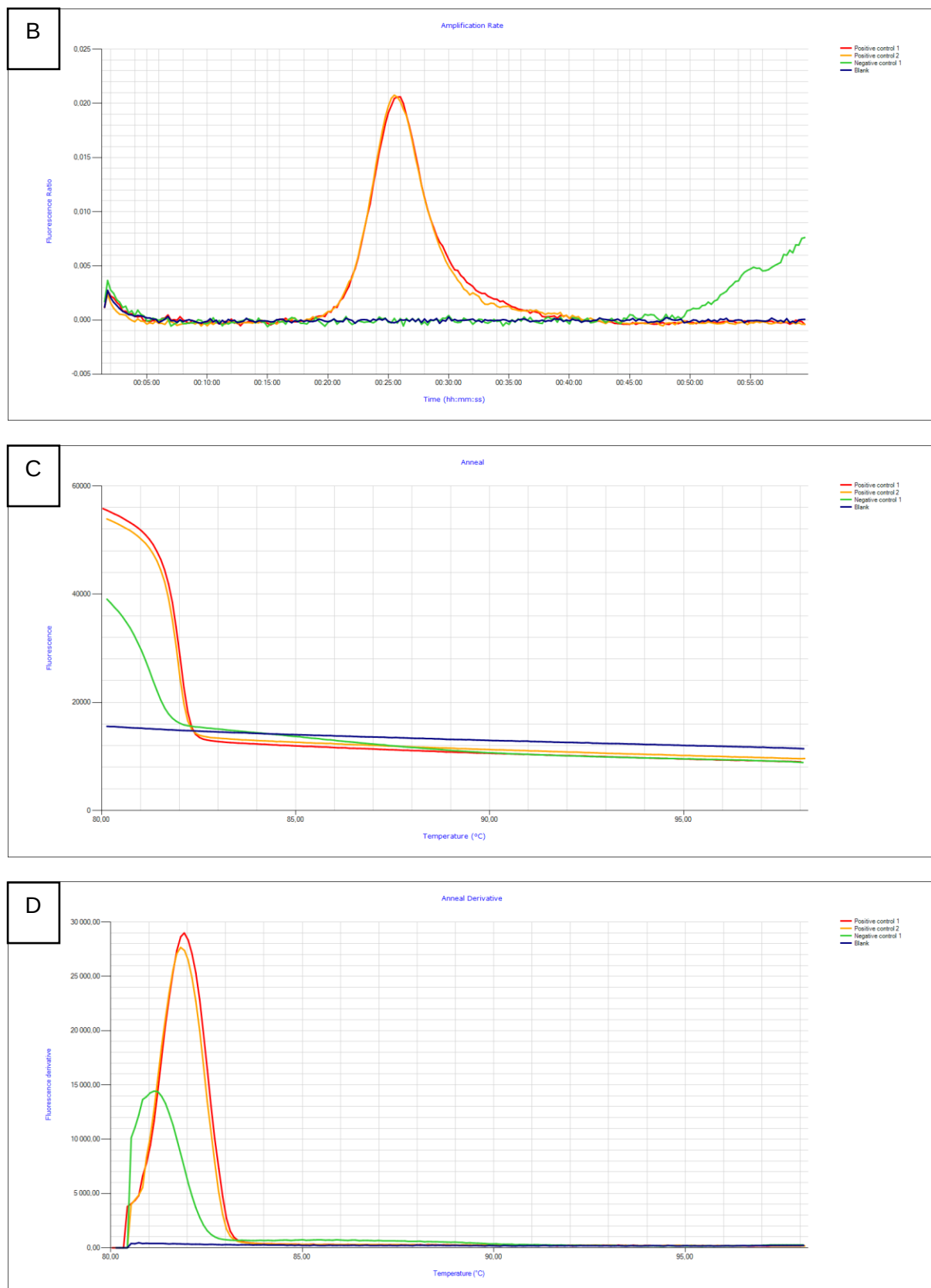
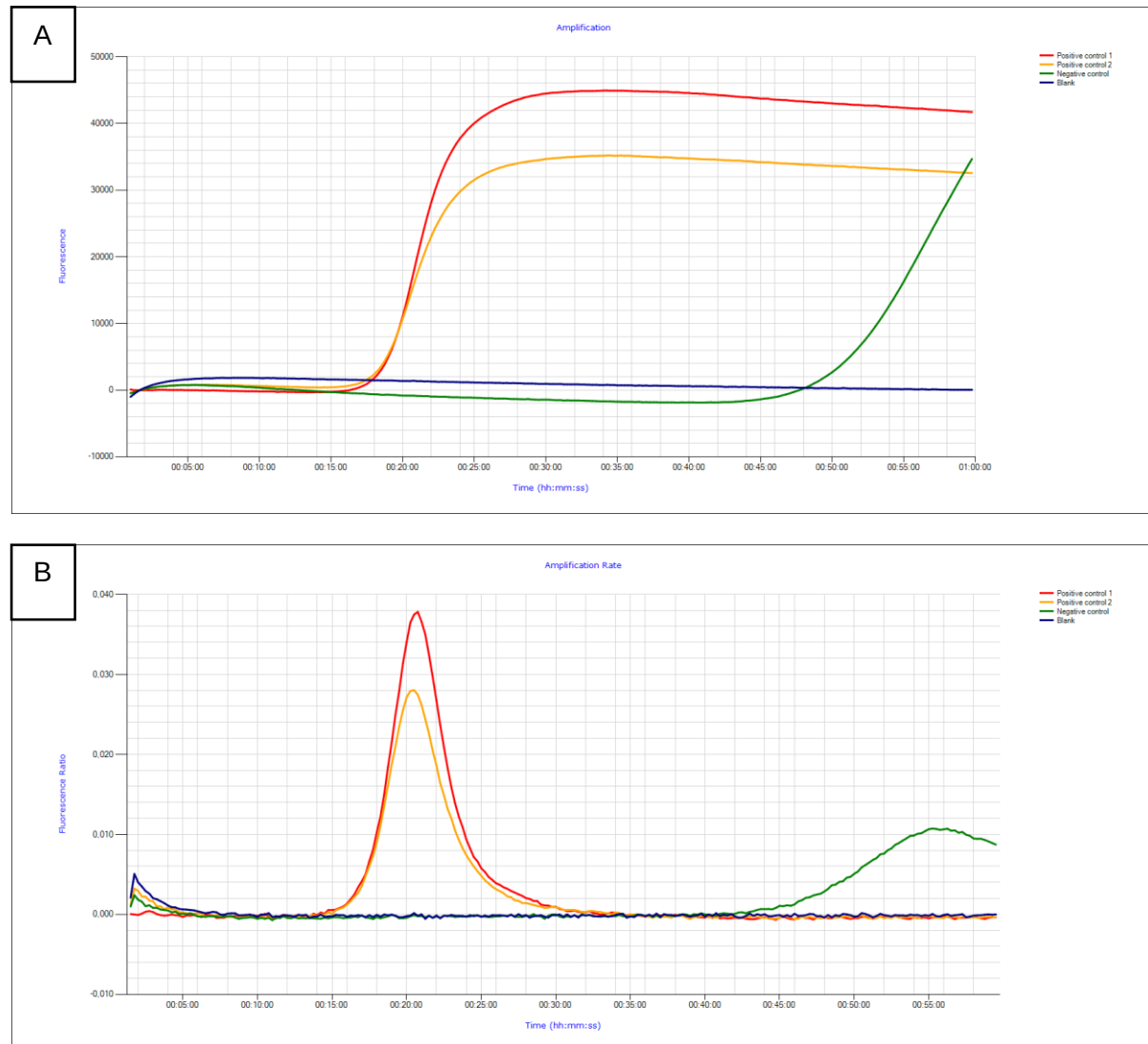


Figure 3.4: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers with added loop primers at 55°C. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

As illustrated by figure 3.4, positive controls 1 and 2 yielded a positive amplification curve at 20:45 and 20:30 minutes respectively. With anneal derivative temperatures reaching 82 and 81.9°C respectively. The start of an amplification curve for the negative control can be seen to arise at ± 48 mins while no set amplification time was captured by the Genie2 machine since the negative amplification curve was not allowed to reach a maximum fluorescence and death phase. The anneal derivative for the negative control was recorded at 81.3°C.

58°C:



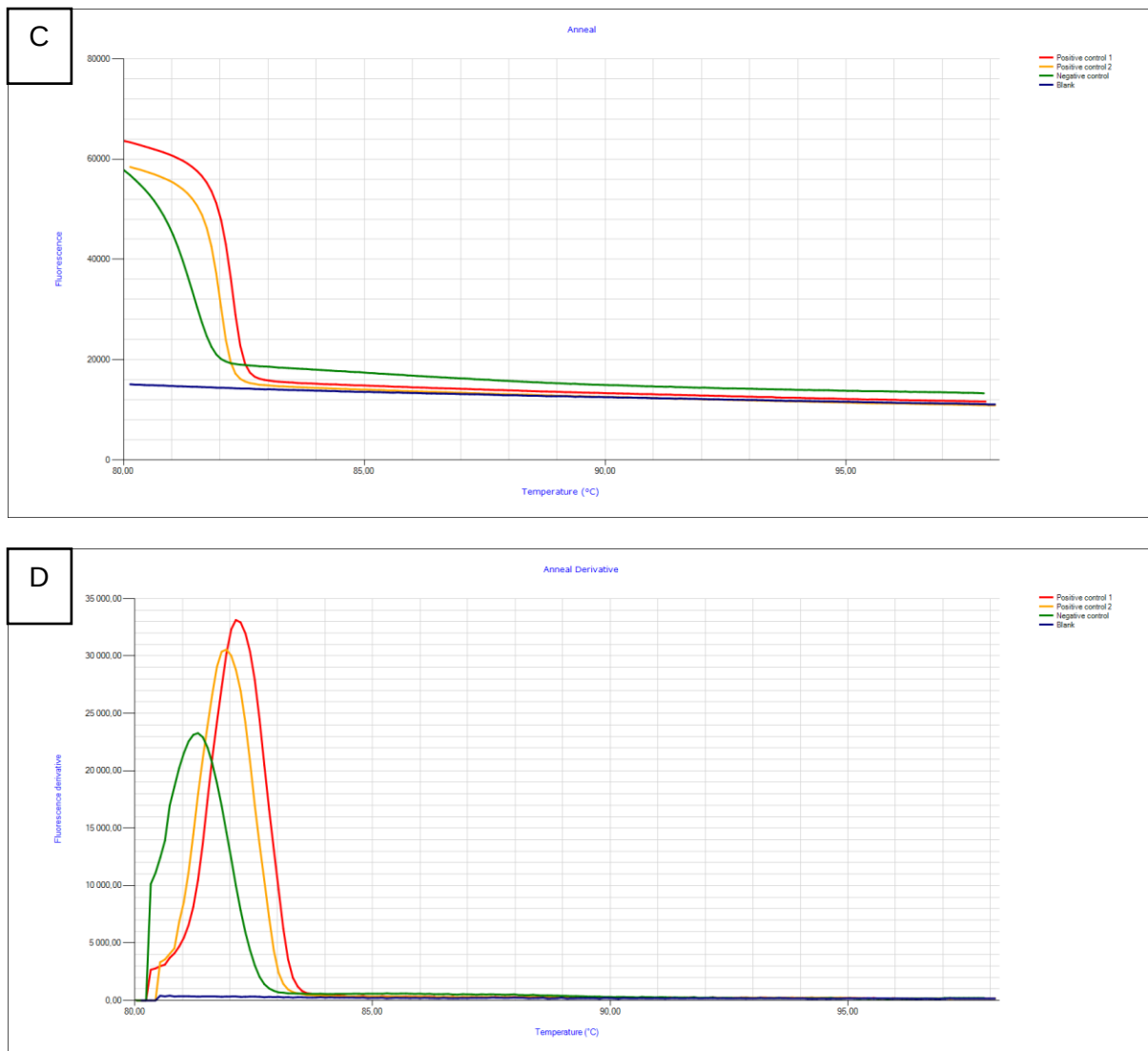
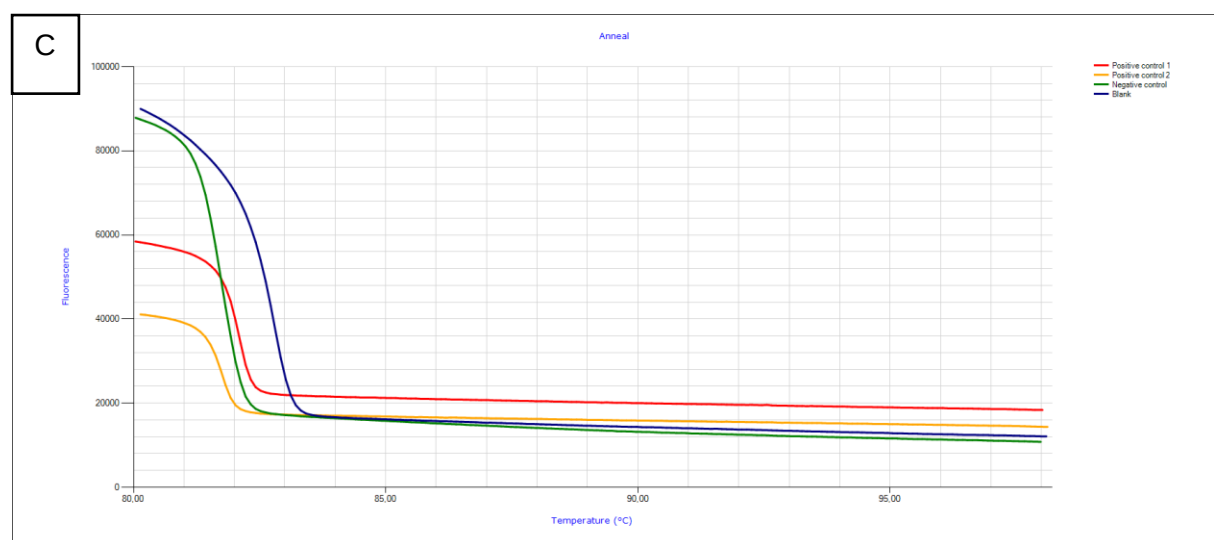
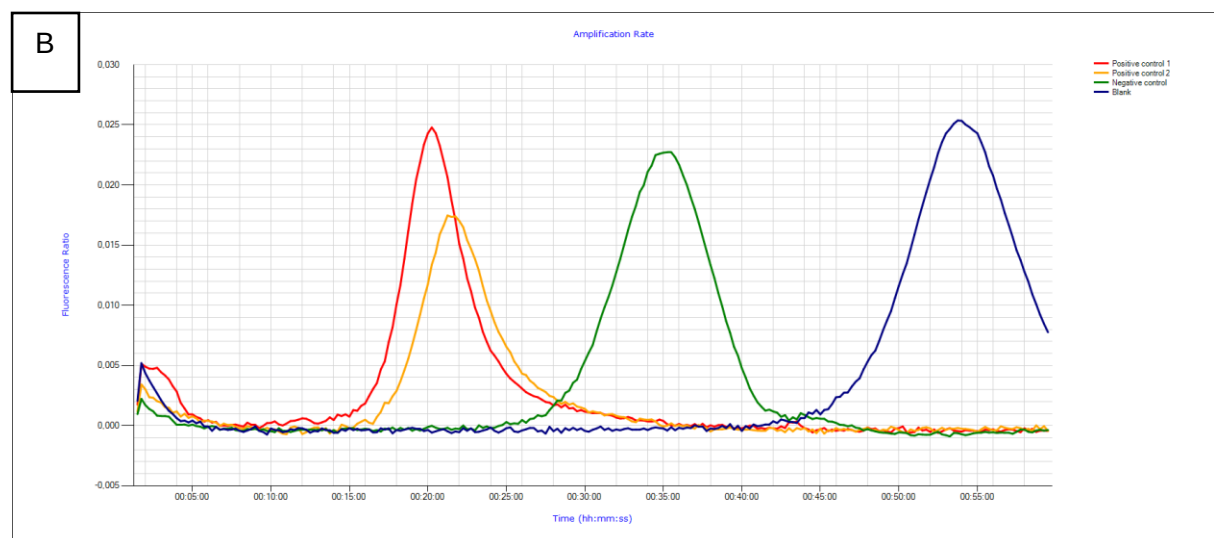
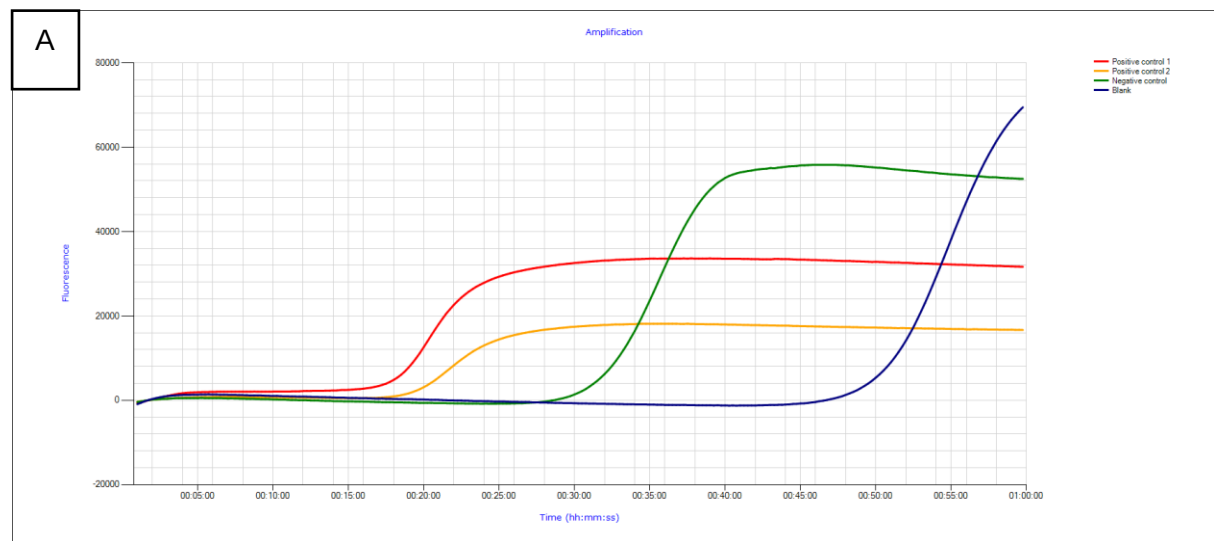


Figure 3.5: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers with added loop primers at 58°C. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

As illustrated by figure 3.5, positive controls 1 and 2 yielded a positive amplification curve at 20:15 and 21mins respectively. With anneal derivative temperatures reaching 82 and 81.9°C respectively. The start of an amplification curve for the negative control can be seen to arise at ±28 minutes with a set amplification time that was captured by the Genie2 machine of 35:15 minutes with an anneal derivative of 81.6°C. The blank control was seen to start amplification at ±47 minutess with a set amplification time of 53:45 minutes and anneal derivative of 82.6°C.

60°C:



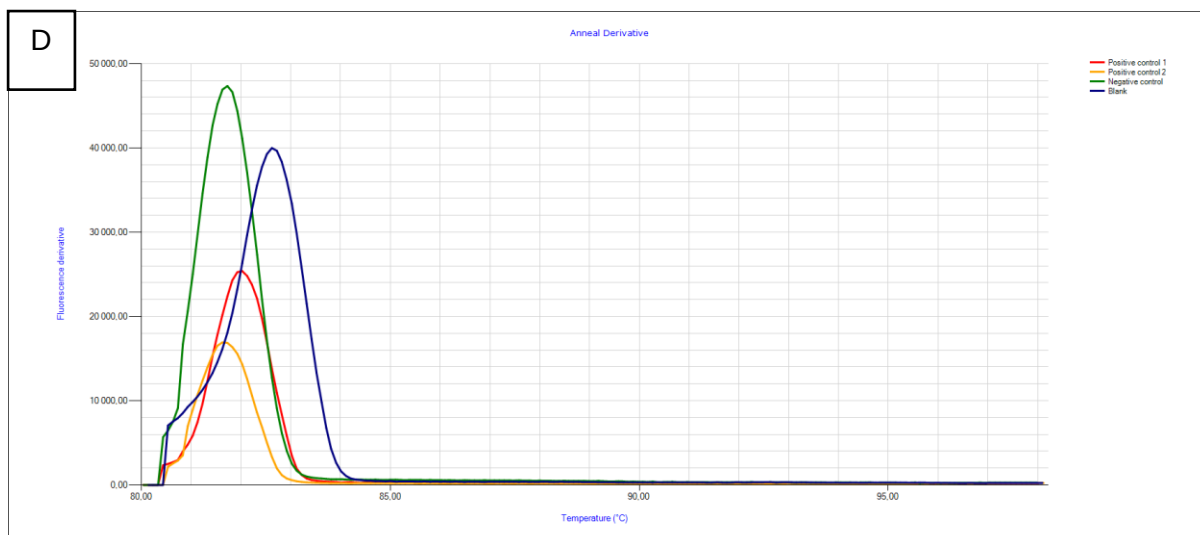
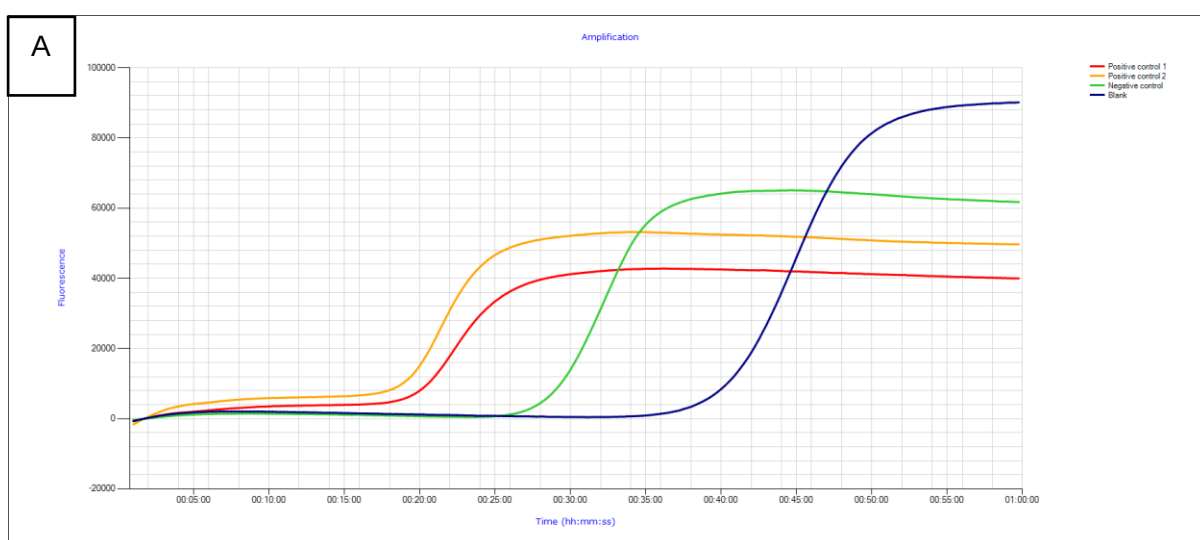


Figure 3.6: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers with added loop primers at 60°C. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

As illustrated by figure 3.6, positive controls 1 and 2 yielded a positive amplification curve at 22 and 21:15 minutes respectively. With anneal derivative temperatures reaching 81.9 and 82°C respectively. The start of an amplification curve for the negative control can be seen to arise at ± 26 minutes with a set amplification time that was captured by the Genie2 instrument of 31:30 minutes with an anneal derivative of 81.7°C. The blank control was seen to start amplification at ± 35 minutes with a set amplification time of 44:15 minutes and anneal derivative of 82.9°C.

63°C:



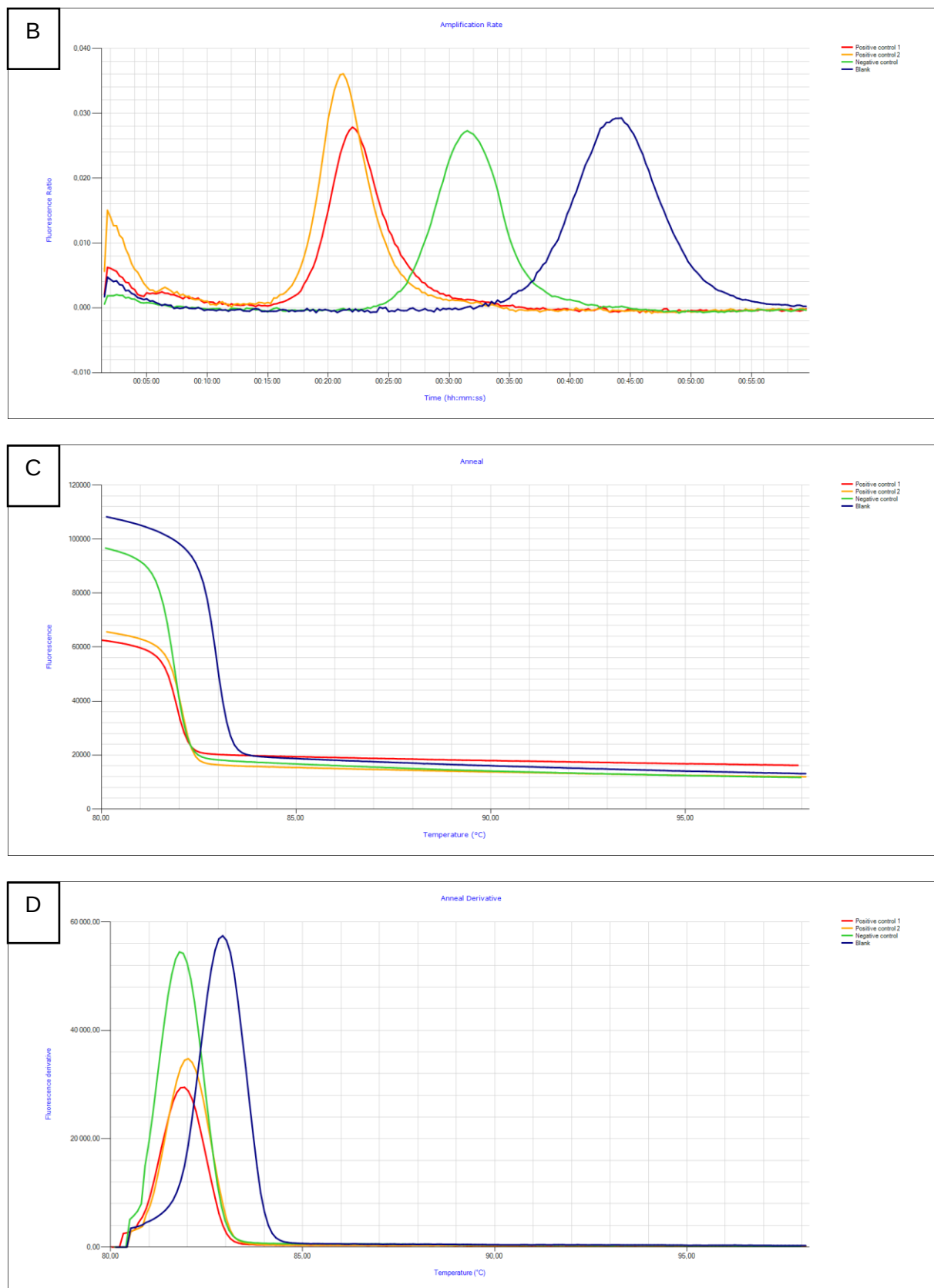


Figure 3.7: Real-time LAMP reaction results for Bd3, Bd4 and Bd5 primers with added loop primers at 63°C. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

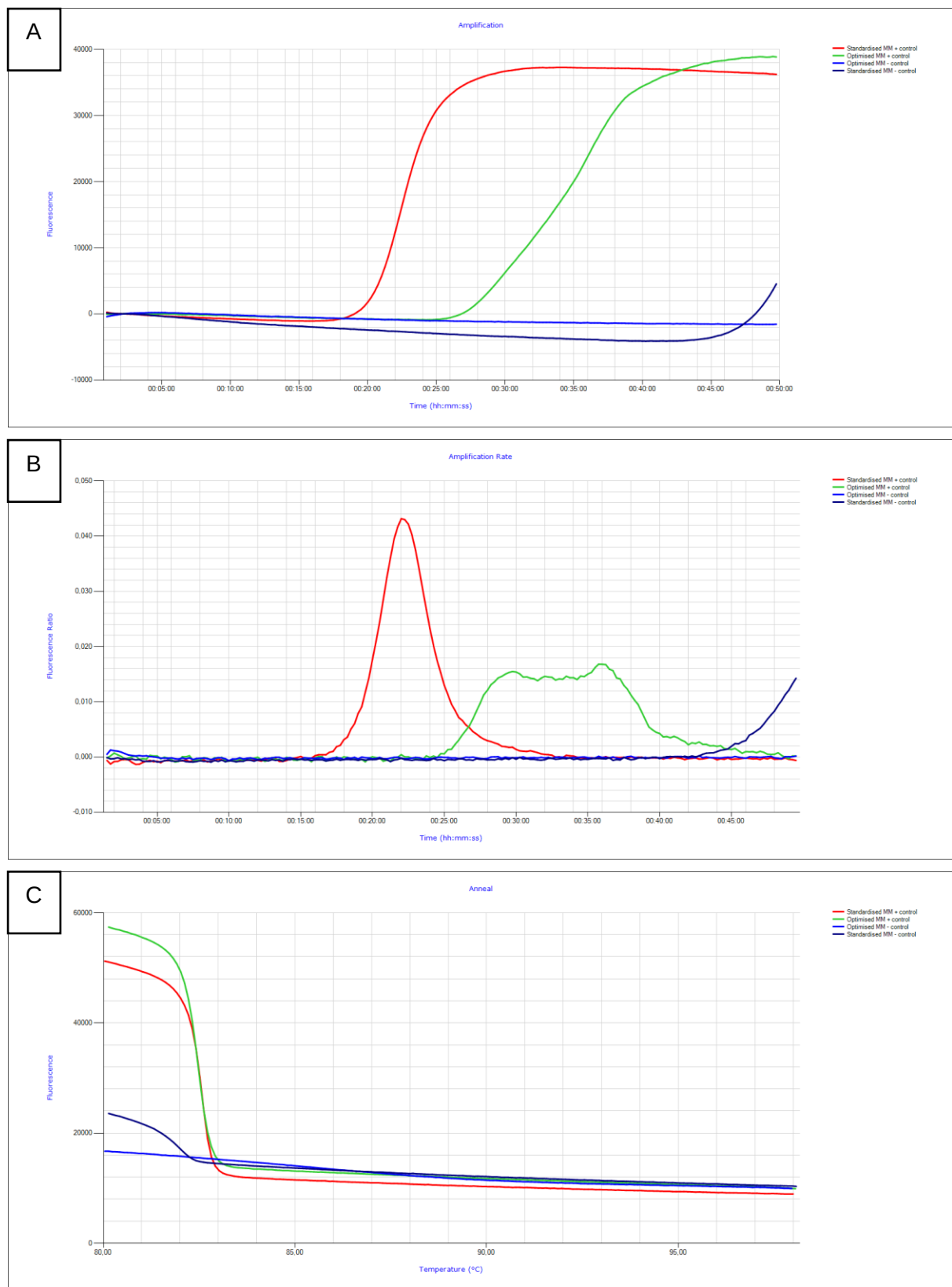
Temperature optimisation can greatly influence any amplification system as seen in table 3.3 and figure 3.7. From the removal or at least reduction of non-specific binding to negative controls (non-specific amplification) or primer dimer formation, reducing amplification incubation time, increasing amplification product output, etc. From this temperature optimisation it was clearly visible that with the temperature increase, differences were evident. The 63 and 65°C temperatures were eliminated, although short amplification times (20:15-22 minutes) were received, the higher temperatures increased non-specific binding to non-target DNA and primer-dimer formation. The 58°C temperature set was selected to carry out further assay optimisation due to having the shortest incubation times (20 minutes) without the presence or difficulty of non-specific binding up until a later stage – the negative control can be seen to start with amplification at ±48 minutes although never reaches true amplification since the incubation time was cut off before it could have completed the reaction. This simply indicates that the total reaction time should be set up for ±50 minutes maximum since positive results can be expected within that time frame.

Table 3.3: LAMP reactions at different temperatures

TEMPERATURE	AMPLIFICATION TIME	ANNEAL DERIVATIVE	ASSAY
55°C POSITIVE	26- & 25:30 mins	81.9- & 81.8°C	25µL total volume
NEGATIVE	50 mins	81.1	15µL MM
BLANK	-	-	5µL template DNA
58°C POSITIVE	20:45- & 20:30 mins	82- & 81.9°C	5µL primers mix
NEGATIVE	48 mins	81.3°C	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{4 \text{ parts}}$
BLANK	-	-	Primer set Bd4 used
60°C POSITIVE	20:15- & 21 mins	82- & 81.9°C	Added additional loop primers
NEGATIVE	35:15 mins	81.6°C	90 minutes' incubation time
BLANK	53:45 mins	82.6°C	
63°C POSITIVE	22- & 21:50 mins	81.9- & 82°C	
NEGATIVE	31:30 mins	81.7°C	
BLANK	44:15 mins	82.9°C	

3.3.4 Optigene Isothermal Master Mix optimisation

As seen in figure 3.8 and table 3.4 the non-specific amplification of the standardised OIMM negative control was completely eliminated when the optimised OIMM was implemented. Although at the cost of the positive control having amplified and yielded a positive result 7 minutes later (22 mins vs 29 mins). The anneal derivate temperatures for both standardised and optimised OIMM positive controls remained 82.4°C.



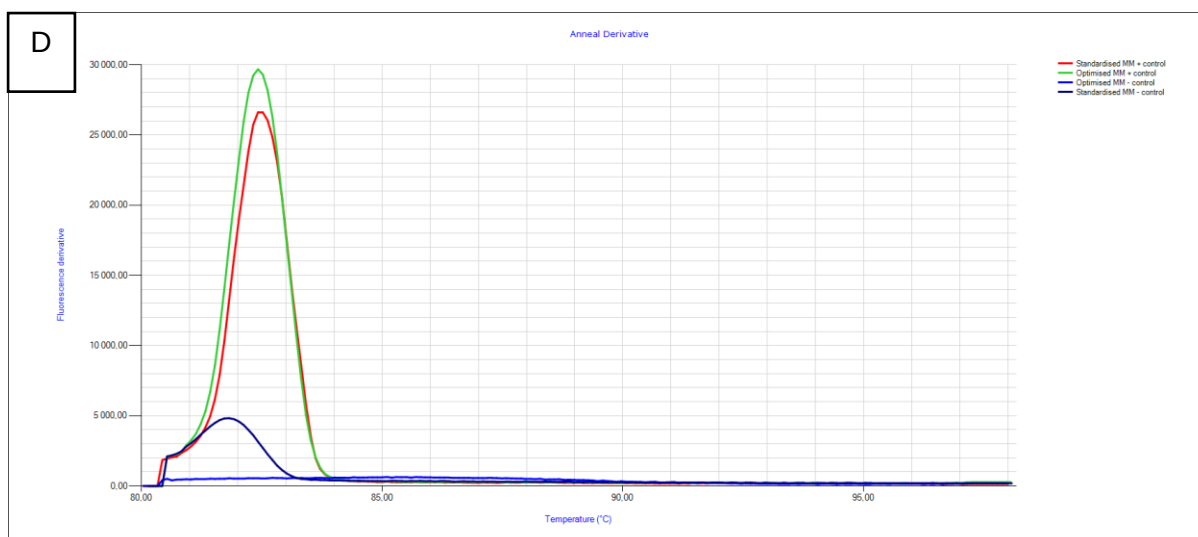


Figure 3.8: Real-time LAMP reaction results for Bd4 with non-optimised and optimised OIMM. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

Table 3.4: LAMP reactions with non-optimised and optimised OIMM

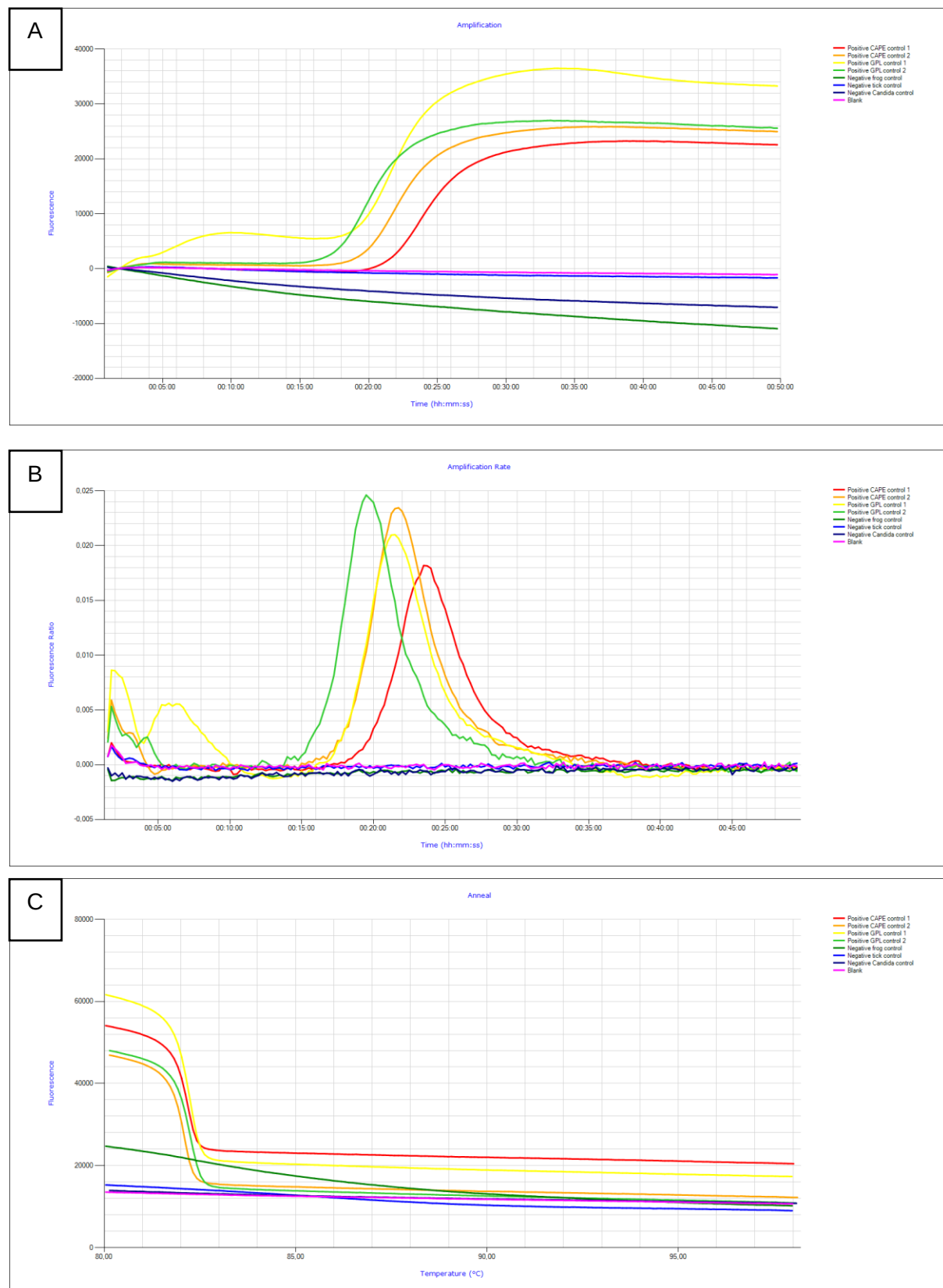
MASTER MIX	AMPLIFICATION TIME	ANNEAL DERIVATIVE	ASSAY [NO OPTIMISATION (N/O)]	ASSAY [OPTIMISATION (O)]
OIMM (N/O)			25μL total volume	25μL total volume
POSITIVE C	22 mins	82.4°C	15μL MM	15μL MM
NEGATIVE C	Starts at ±46 mins		5μL template DNA	5μL template DNA
			5μL primers mix	2μL primers mix
			$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{4 \text{ parts}}$	3μL ddH ₂ O
OIMM (O)			Primer set Bd4 used	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ parts}}$
POSITIVE C	27 mins	82.4°C	Added additional loop primers	Primer set Bd4 used
NEGATIVE C	-		58°C isothermal temperature	Added additional loop primers
			50 minutes' incubation time	58°C isothermal temperature
				50 minutes' incubation time

3.3.5 Specificity tests

Up until now the following variables were accepted into the assay: using primer set Bd4 as the set of choice, including the loop primers into the reactions, setting the incubation time to 58°C for optimal amplification and using optimised OIMM conditions.

Specificity tests revealed primer set Bd4 to be quite specific to *Batrachochytrium dendrobatidis* after some optimisation as seen in figure 3.9 and table 3.5. The two South African Bd lineages, BdGPL and BdCAPE, were included into the specificity tests. From results it seemed that both Bd strains tested positive with the use of primer set Bd4 with

BdGPL, usually being detected at an earlier time (21:30 and 19:30 minutes) with anneal derivatives of 82.1°C for both controls, while BdCAPE followed shortly after (23:30 and 21:45 minutes) with anneal derivatives of 82 and 81.9°C respectively.



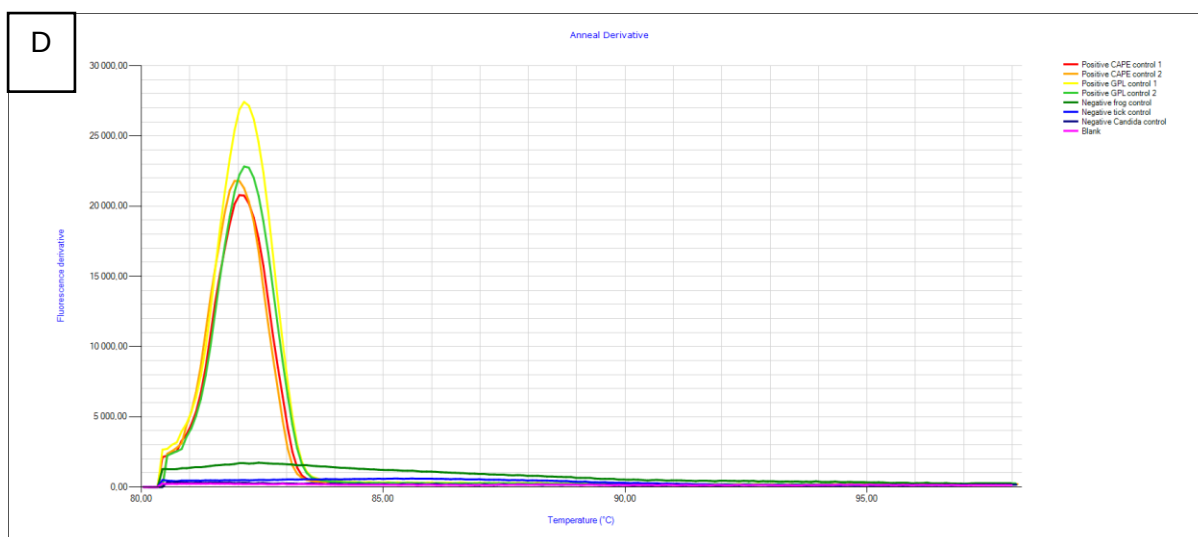


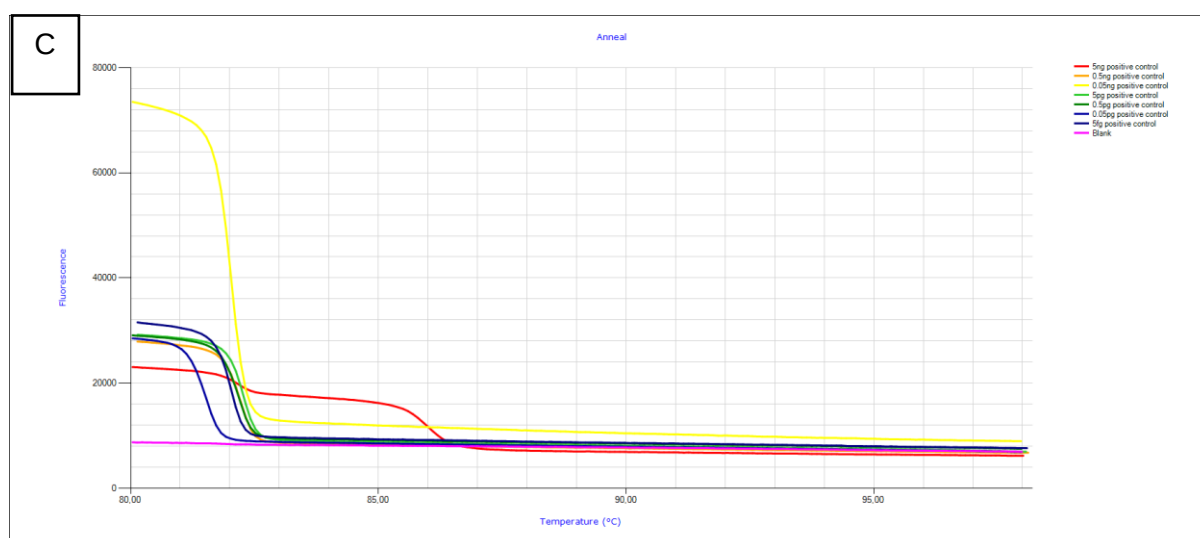
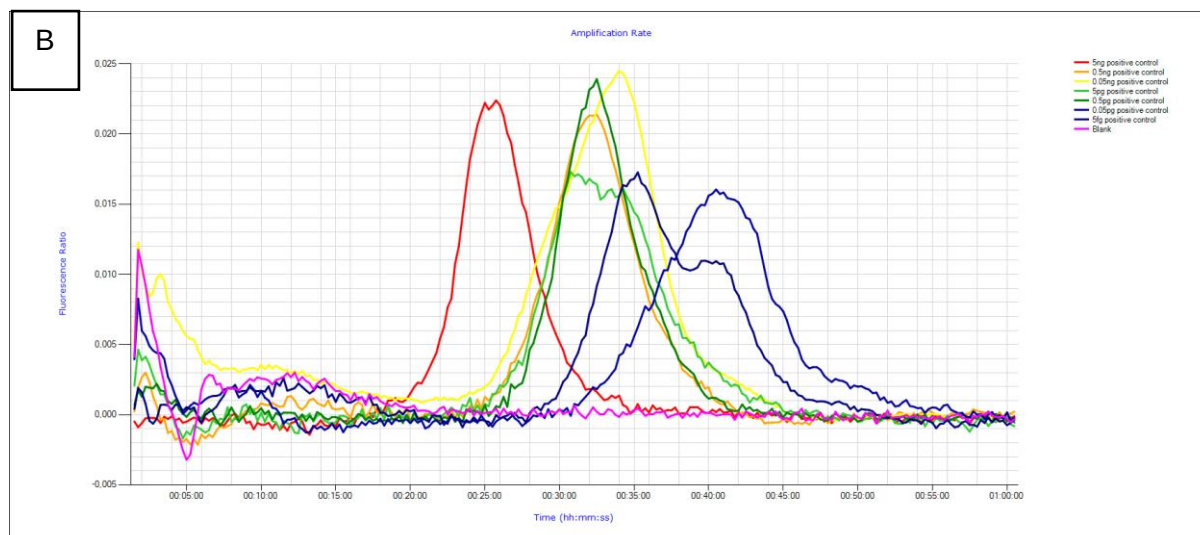
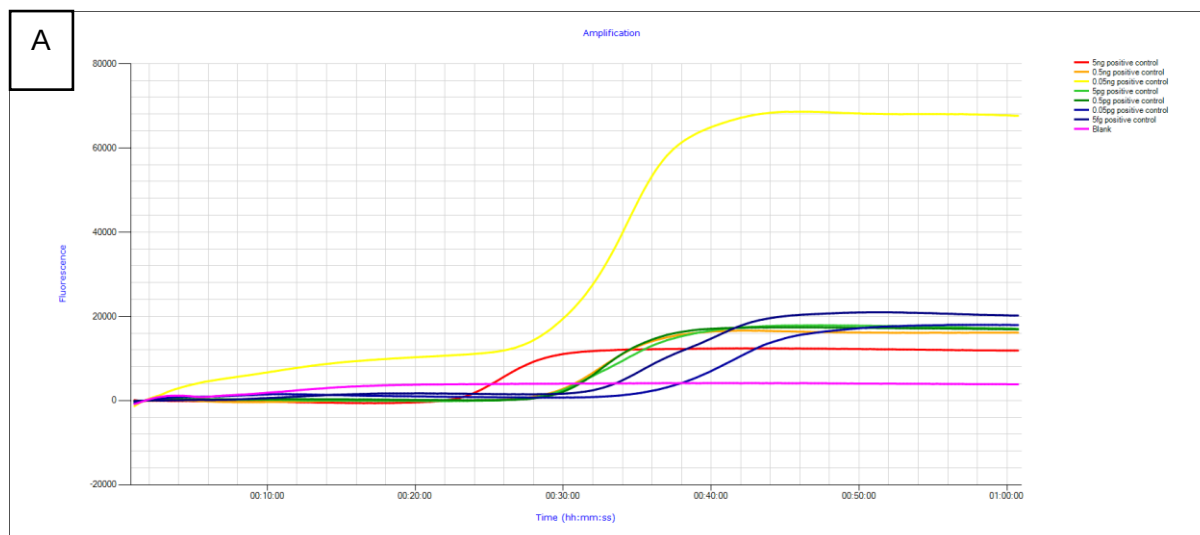
Figure 3.9: Real-time LAMP reaction results for specificity tests. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

Table 3.5: LAMP specificity reaction tests

CONTROL	AMPLIFICATION TIME	ANNEAL DERIVATIVE	ASSAY
<u>POSITIVE</u>			25μL total volume
BDGPL	21:30- & 19:30 mins	82.1°C	15μL MM
BDCAPE	23:30- & 21:45 mins	82- & 81.9°C	5μL template DNA
<u>NEGATIVE</u>			2μL primers mix
FROG	-	-	3μL ddH ₂ O
TICK	-	-	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ parts}}$
CANDIDA	-	-	
<u>BLANK</u>	-	-	Primer set Bd4 used
			Added additional loop primers
			58°C isothermal temperature
			50 minutes' incubation time

3.3.6 Sensitivity test (genomic DNA serial dilutions)

Primer sensitivity was tested using a serial dilution of extracted genomic Bd DNA. This serial dilution was constructed from 5ng up to 5fg, a 6-fold serial dilution series. The 5ng amplified the fastest (25 minutes) while the 5fg control amplified lastly as expected (40 minutes) as seen in figure 3.10.



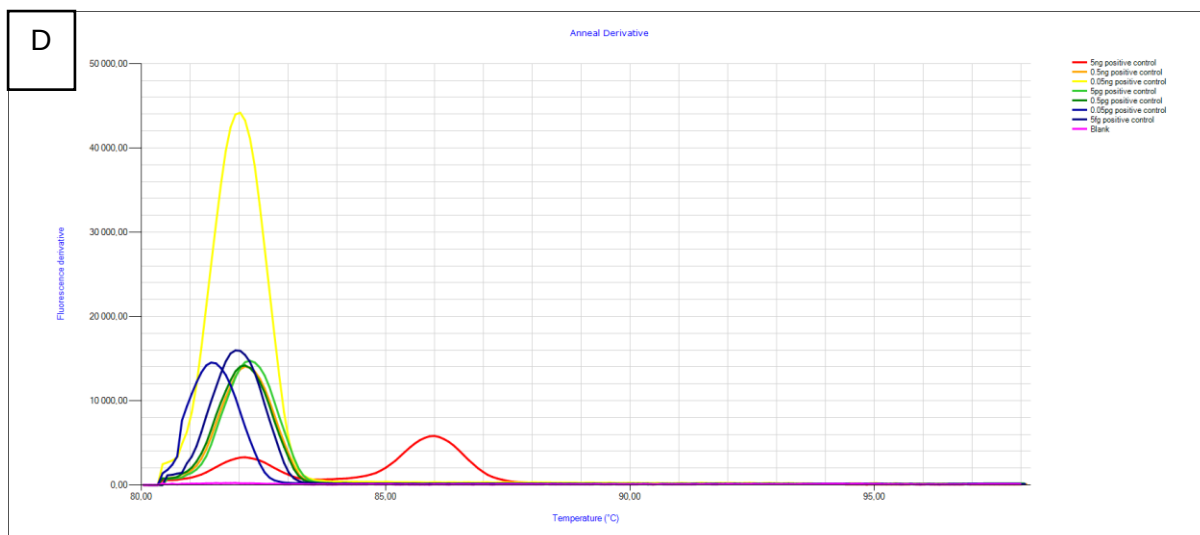
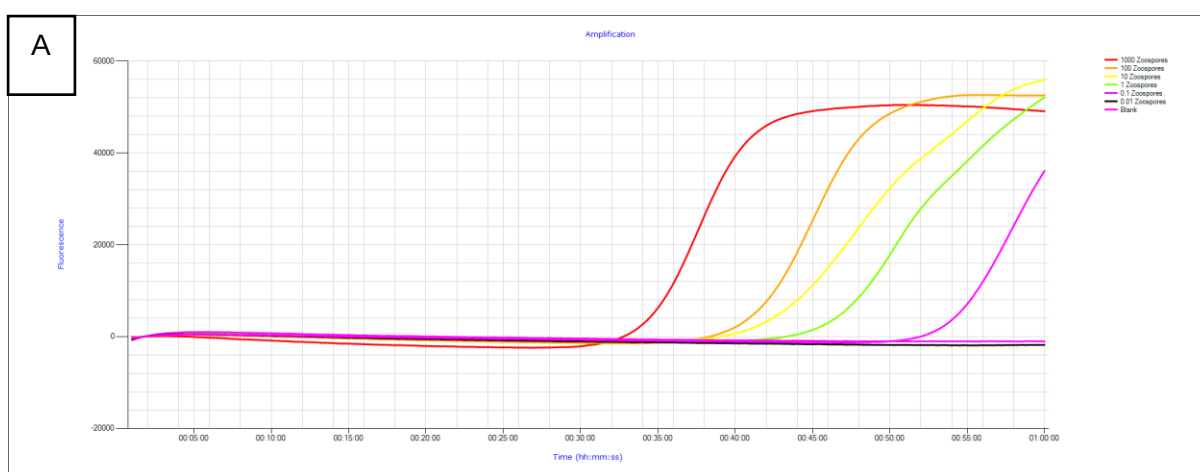


Figure 3.10: Real-time LAMP reaction results for sensitivity tests (genomic DNA serial dilution). A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

3.3.7 Sensitivity test (zoospore serial dilutions)

Primer sensitivity was again tested, this time using a serial dilution of Bd zoospores. This serial dilution was constructed from 1000 zoospores to 0.01 zoospores, a 5-time serial dilution series. The 1000 zoospores amplified the fastest (37 minutes) while the 0.1 zoospore control amplified lastly (57 minutes) as seen in figure 3.11. 0.01 zoospores were not amplified within the time given (60 minutes) however if a longer incubation time was allowed the 0.01 zoospores would most probably have amplified as well. Another thing to take into consideration is that these dilutions were not subjected to DNA extraction by means of either a kitchen method or commercial kit but were allowed to boil at 100°C for 5 minutes only.



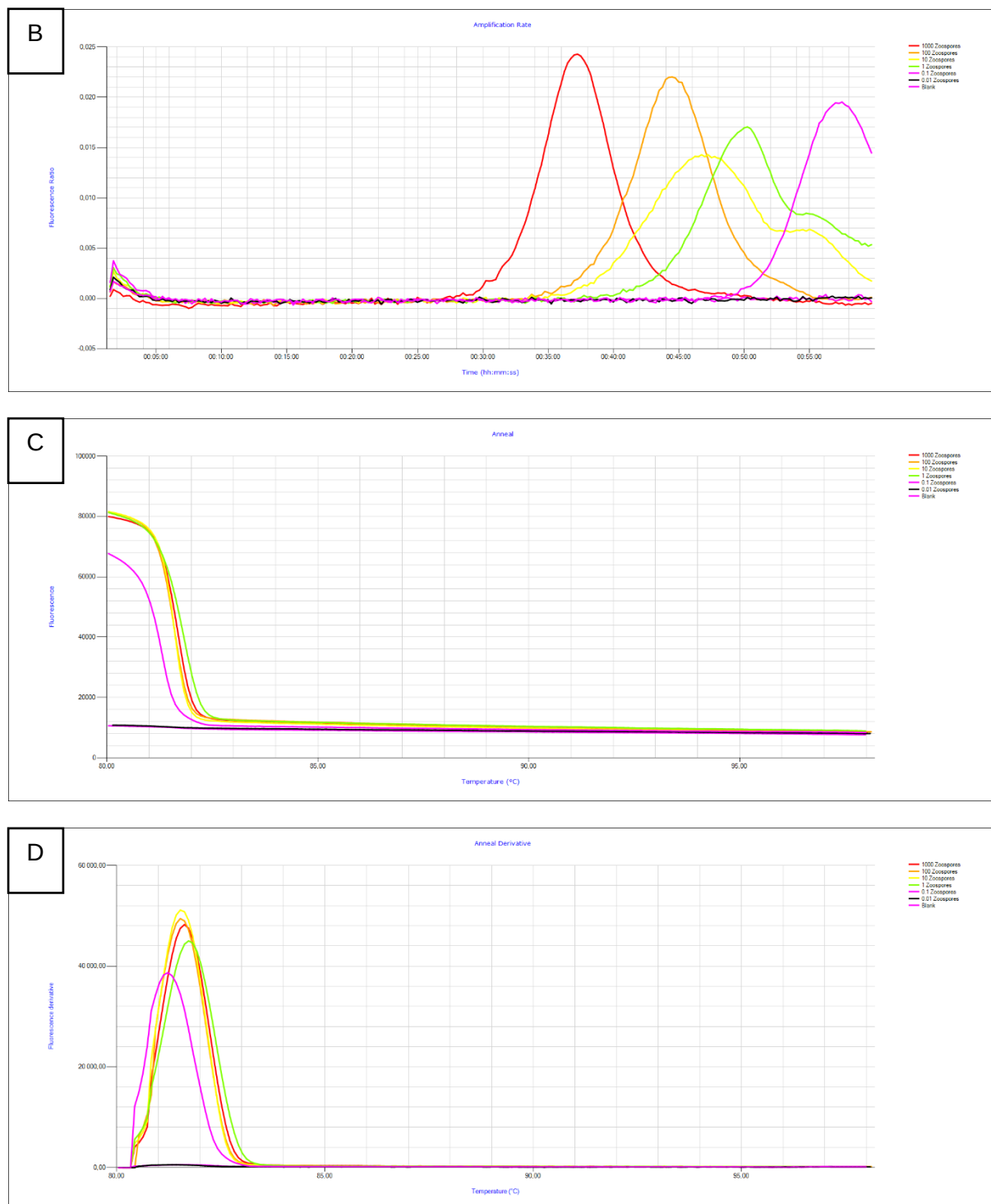
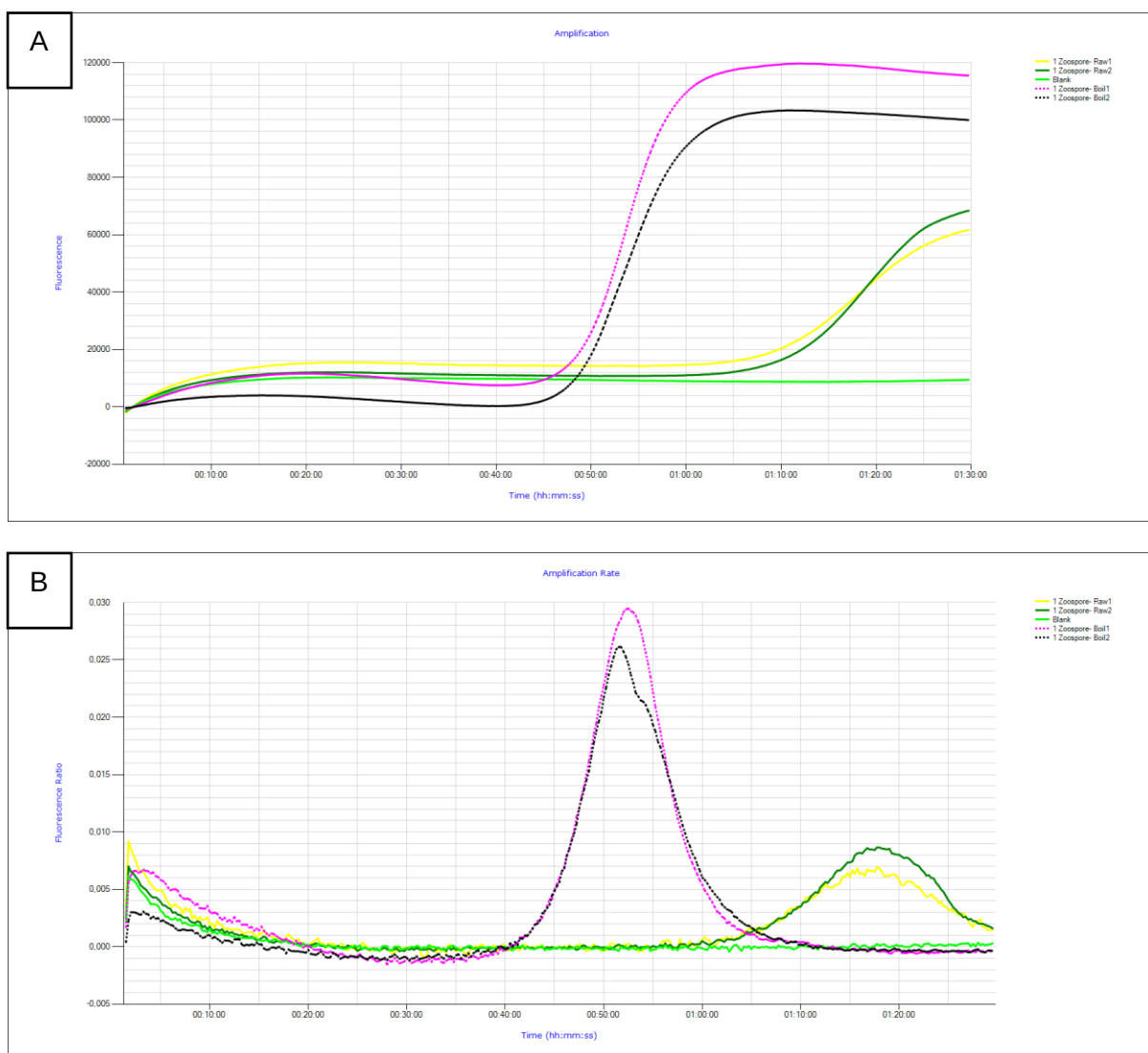


Figure 3.11: Real-time LAMP reaction results for sensitivity tests (zoospore serial dilution). A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

3.3.8 Sensitivity test (boiled zoospores vs raw zoospores)

As expected and indicated in figure 3.12, the boiled zoospores achieved a much faster amplification time of 51:45 and 52:30 minutes respectively whereas the raw zoospores were lagging a bit behind with amplification time of 77 minutes for both amplification curves. However, this is all done with only a single (1) zoospore per millilitre of culture. The temperature at which the reaction ran was 58°C after which a certain time the raw zoospore's cell walls became permeable in which DNA had the opportunity to differentiate outwards. Even just the slightest amounts of DNA (as low as pictograms) would be picked up by the primers.



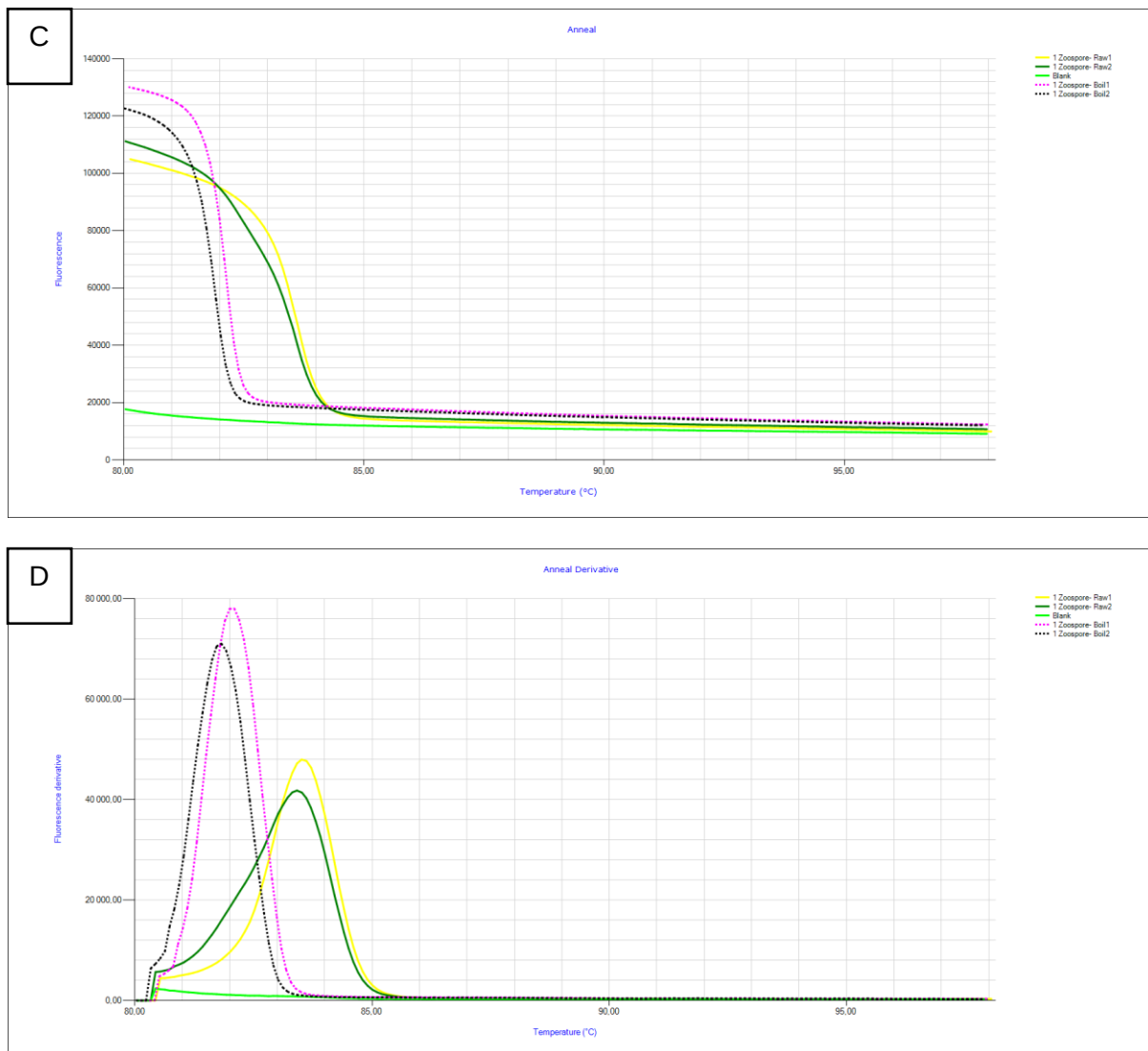
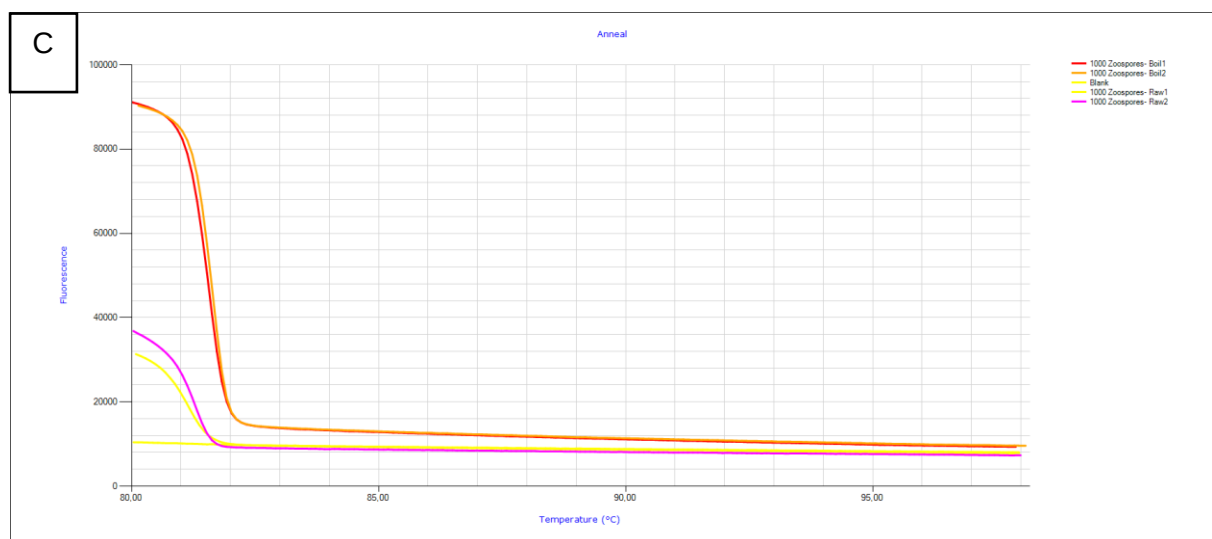
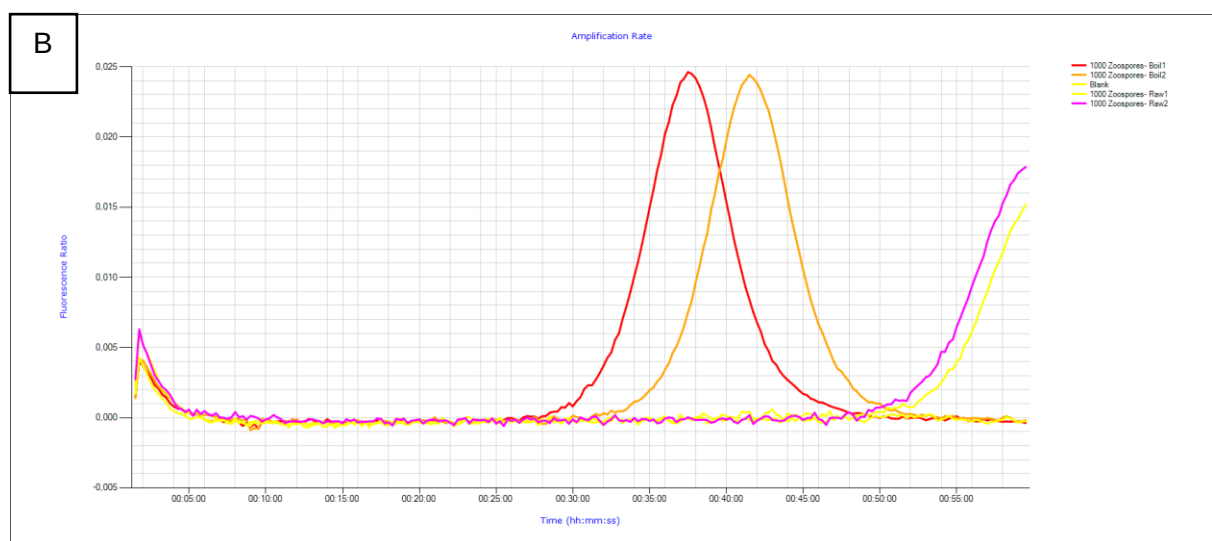
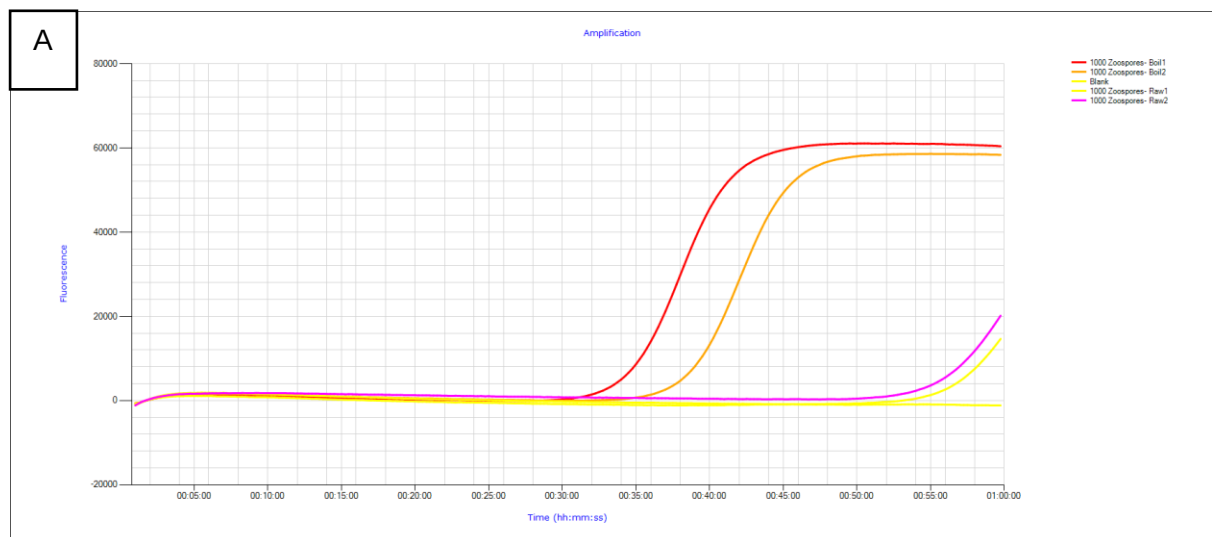


Figure 3.12: Real-time LAMP reaction results for sensitivity tests (1 boiled zoospore vs 1 raw zoospore). A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

As expected and indicated in figure 3.13, the boiled zoospores achieved a much faster amplification time of 37:30 and 41:30 minutes respectively whereas the raw zoospores were lagging a bit behind with an amplification time of roughly 60mins for both amplification curves. However, this is all done with 1000 zoospores per ml of culture.



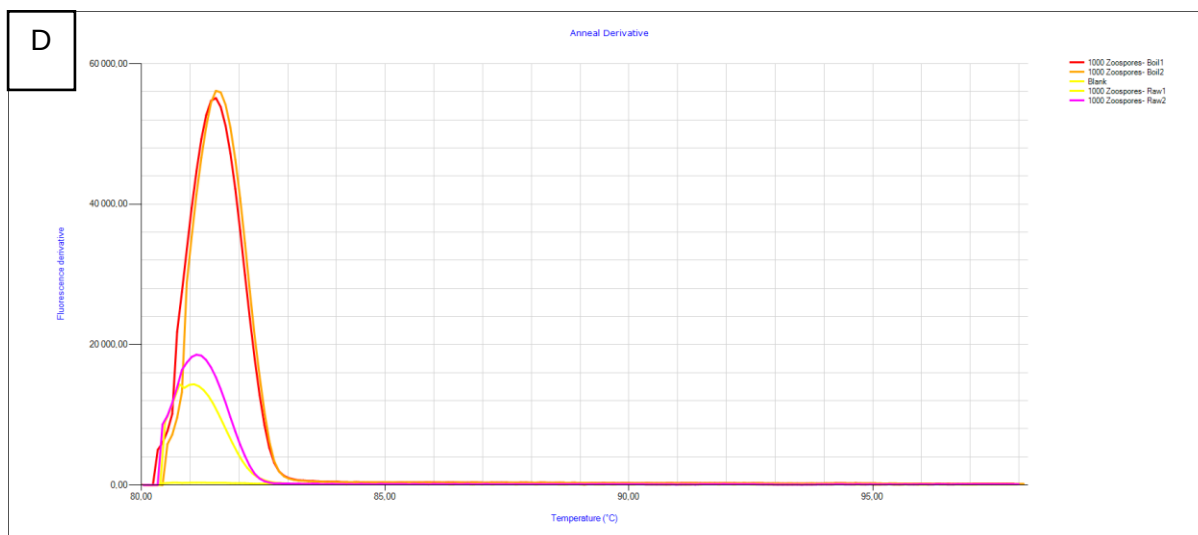


Figure 3.13: Real-time LAMP reaction results for sensitivity tests (1000 boiled zoospores vs 1000 raw zoospores). A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

The amplification of 1 zoospore (boiled) had an average positive amplification time of 52.13 minutes while the positive amplification time of 1 zoospore (raw) was 77mins. The amplification of 1000 zoospores (boiled) had an average positive amplification time of 39.50 minutes while the positive amplification time of 1000 zoospores (raw) was 60mins. Thus an additional amplification time of roughly 12mins was added to the total reaction time to test the for the presence of Bd (boiled) when the zoospore count was increased from 1 zoospore per ml to 1000 zoospores per ml. An additional amplification time of 11mins was added to the total reaction time to test the for the presence of Bd (raw) when the zoospore count was increased from 1 zoospore per ml to 1000 zoospores per ml. Thus the assay was developed in such a manner to detect positive samples of Bd by using extracted genomic DNA, boiling a culture/aliquot containing the Bd zoospores or using a culture/aliquot with raw (no extraction and no boiling) Bd zoospores. Table 3.6 yielding a summarised happenstance of above experiment.

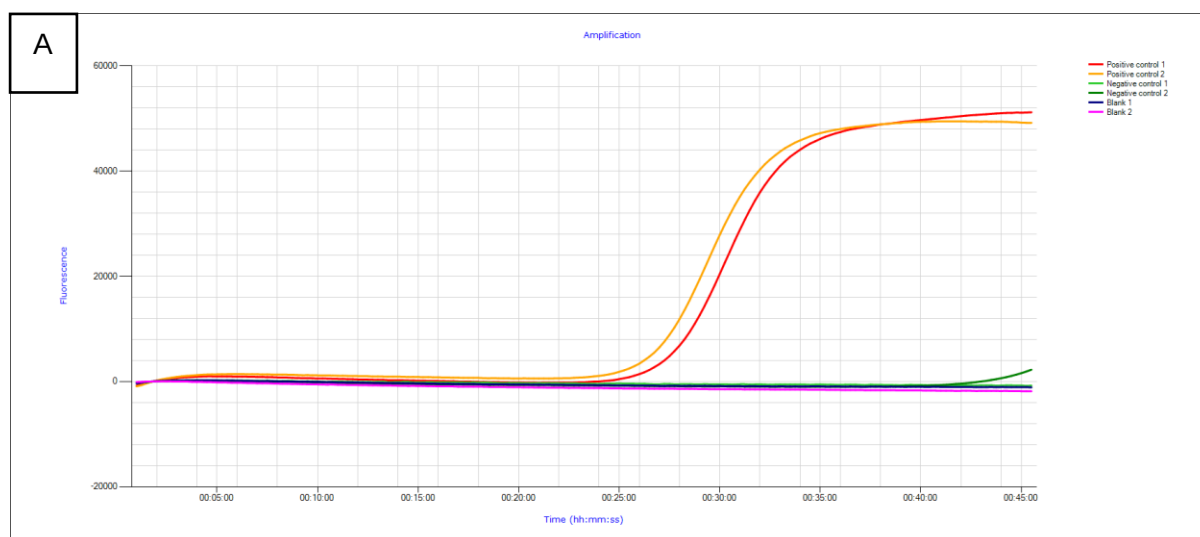
Table 3.6: LAMP serial dilution results

DILUTION	AMPLIFICATION TIME	ASSAY
DNA EXTRACTION		
5NG DNA	25 mins	25µL total volume 15µL MM
5FG DNA	40 mins	5µL template DNA
BOILED ZS		
1000 ZS	37- & 41:30 mins	2µL primers mix 3µL ddH ₂ O
1 ZS	51:45- & 52:30 mins	$\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ parts}}$
0.1 ZS	57 mins	Primer set Bd4 used
RAW ZS		
1000 ZS	60 mins	Added additional loop primers
1 ZS	77 mins	58°C isothermal temperature 60-90 minutes' incubation time

3.3.9 Detection methods

Real-time detection can be viewed in the present (as amplification occurs at the moment) with the Genie2's built-in touch screen. This real-time viewer allows the user to switch between several real-time screens which includes; amplification, amplification rate, anneal and anneal derivative. This yields the fastest possible means of perceiving results, as you can literally observe the end-result in real-time as seen in figure 3.14.

Real-time detection



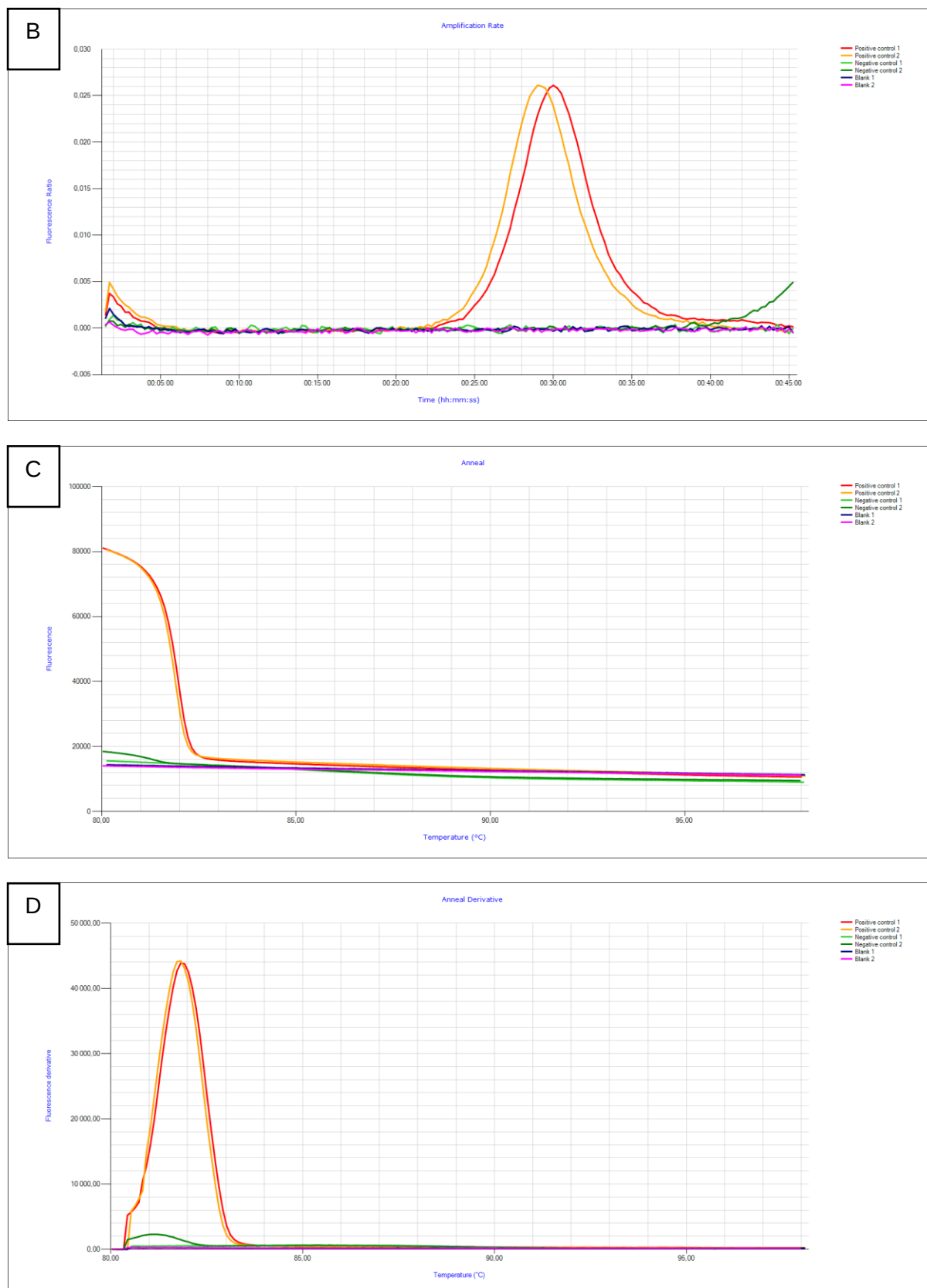


Figure 3.14: Real-time LAMP reaction results. A – Amplification. B – Amplification rate. C – Anneal. D – Anneal derivative.

Ethidium bromide

As seen in figure 3.15, tube strips 1 and 2 (positive control) illuminated under UV light which is the signal for a positive amplification while tube strips 3 and 4 (negative control) along with tube strips 5 and 6 (blanks) showed no signs of illumination.

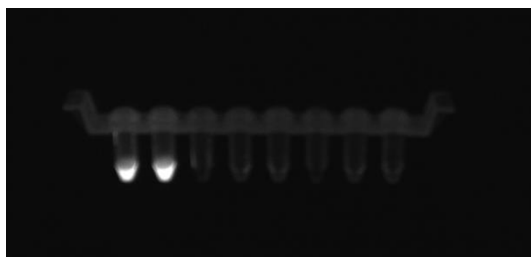


Figure 3.15: Ethidium bromide detection

Agarose gel electrophoresis

Agarose gel electrophoresis is the forefather of separating and viewing DNA molecules after amplification. Lane 1 represented the DNA ladder, lanes 3 and 4 represented the two positive controls, lanes 5 and 6 represented the two negative controls while lanes 7 and 8 represented two blank controls (Figure 3.16).

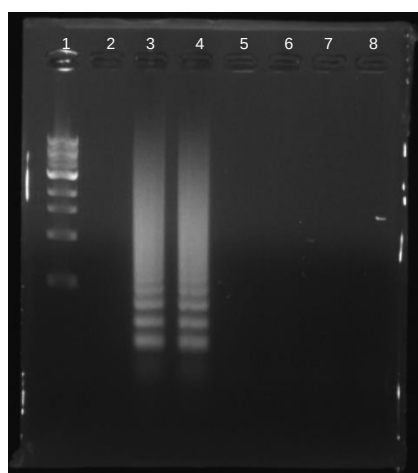


Figure 3.16: Agarose gel electrophoresis image

3.3.10 Viability of LAMP practicability

From perceived results LAMP proved to be of substantial feasibility for end-point-diagnostics in the application of detecting the pathogenic fungus *Batrachochytrium dendrobatidis*. LAMP and the developed assay in conjunction furthermore has high applicability towards field work. LAMP has been described as a simple amplification method acting as a rapid diagnostic, delivering fast and sensitive detection of microbes (Abd-Alsalam, *et al.*, 2011). From perceived *in vitro* system studies, this was witnessed first-hand. Positive controls (5ng) were amplified in less than 30 minutes and extremely low concentrations of DNA (5fg) were detected in less than 50 minutes. Furthermore, crude zoospore samples (saturated in

tryptone broth) that did not undergo DNA extraction were also positively amplified. Not only is this LAMP assay extremely rapid and sensitive but can also handle extremely crude DNA samples as well. This is promising in regards to further test the assay with *in vivo* (field and archived samples) systems.

Detection methods included a real-time turbidimeter, ethidium bromide and typical agarose gel electrophoresis. The real-time turbidimeter yielded results in present as the reaction is running. This proves to be the swiftest detection method among the three. In some cases, a turbidimeter may not be present for use, this is where ethidium bromide and typical agarose gel electrophoresis comes in handy. However, in the case of agarose gel electrophoresis another 30 to 60 mins waiting time is added to the entire experimental window. The total run time of the agarose gel electrophoresis depends on a couple of factors (voltage used, gel length, well sizes, etc.). This is a really economic and trusted approach towards DNA molecule separation. Some drawbacks include extra waiting time, chance of contamination and working with a carcinogen (ethidium bromide).

Thus far LAMP shows high credibility for *in vitro* studies. The potential to substitute the other two molecular assays for Bd is evident. Some of the possible limitations may include having the expertise behind LAMP and primer design. These so called limitations could easily be overcome through some classes. The largest of concern for limitations may include primer stability and contamination. Certain *in vivo* tests had to be redone several times due to the problem of contamination since LAMP is ridiculously sensitive in regards to other molecular techniques. Primer stability also proved to be a challenge, losing primer stability within 3 days, contributing heavily to self-priming and resulting in false-positives.

Chapter 4 Development of a universal PCR assay for the detection of BdGPL and BdCAPE

4.1 Introduction

Annis and colleagues (2004) have developed a molecular PCR assay for the detection of Bd. This PCR assay delivers several advantages above traditional histology and histochemical analysis. The biggest attributing factors being the extreme sensitivity, the significantly reduced time in acquiring results and non-invasive applications of PCR in regards to histology. ITS4 and ITS5 are universal fungal primers that anneal to the specific fungal sequence encompassing the 18S to 28S rRNA genes. These universal fungal primers were used to attach to the 5.8S rRNA gene and internal spacer regions, ITS1 and ITS2, which are predominantly found in fungi of the chytrid genera (*Batrachochytrium dendrobatidis*, *B. salamandrivorans*, etc.), amplifying them and delivering 5.8S-ITS DNA sequences of Bd origin. The resulting sequences of Bd were aligned with neighbouring species to find conserved regions specific to Bd. From these conserved regions, Bd-specific primers were developed: forward primer Bd1a and reverse primer Bd2a (Soto-Azat, *et al.* 2009). The acquired PCR primers specific to Bd were designed from the ITS1 and ITS2 regions, respectively:

Bd1a: 5'-CAGTGTGCCATATGTCACG-3'

Bd2a: 5'-CATGGTTCATATCTGTCCAG-3'

Detection limits (sensitivity) was carried out using serial dilutions of both extracted DNA and zoospores. The zoospores were frozen at -20°C after which they were heated at 90°C for 5 minutes to release crude DNA from zoospore. The primers detected 10ng of extracted DNA up to a serial dilution of 10pg (0.01ng) for two isolates (JEL 197 and JEL 274) and 1pg for one isolate (JEL 215). For the zoospore serial dilution range positive detection ranged from 10 000 zoospores up to a dilution of 1 zoospore. The detection method used was simple agarose gel electrophoresis in which a 300bp band resulted from PCR amplification of designed primers (Annis, *et al.*, 2004).

Boyle and colleagues (2004) developed a qPCR assay for the detection of Bd with a high degree of specificity and sensitivity, with a non-invasive sampling method as preferable means and mode of action. Generic primer set BOB5 and BOB6 which targets the 18S and 28S rDNA genes were used for sequencing Bd isolated. From acquired results the sequences were aligned to test for specificity and find conserved regions. Developed Bd-specific primers were: forward primer ITS1-3 Chytr, reverse primer 5.8S Chytr and the probe CHYTR MGB2.

ITS1-3 Chytr: 5'-CCTTGATATAATACAGTGTGCCATATGTC-3'

5.8S Chytr: 5'-AGCCAAGAGATCCGTTGTCAAA-3'

CHYTR MGB2: 6FAM CGAGTCGAACAAAAT MGBNFQ-3'

qPCR detection limits were found to be effective up to 1 zoospore per infected tissue. The assay was also able to detect chytridiomycosis infection 1 to 2 weeks before it was detectable by histology after exposure where day 1 was the first day of frog exposure to Bd zoospores (Boyle, *et al.* 2004).

We developed an additional and new PCR assay for the detection of Bd. The outer primers (F3 and B3) of any LAMP primer set can be used with great success in a PCR reaction for the same gene of interest as well since they mimic the forward and reverse primers' characteristics and functions when used during PCR. A PCR assay was chosen to be developed predominantly due to conventional PCR being more economic, especially if a large number of individuals are to be tested for the presence of chytridiomycosis – seeking only end-point diagnostics.

The objective was to create an additional and new PCR assay that would prove more efficient in detecting Bd, more so specifically detecting low(er) chytridiomycosis infection concentrations which would aid in the help of effectively detecting this pathogen's presence in frogs.

4.2 Materials and methods

4.2.1 PCR kit

Three LAMP primer sets were designed, Bd3, Bd4 and Bd5. Bd4 was used as the chosen LAMP primer set for development of the LAMP assay. However, all three sets' outer primers (F3 and B3) were also tested for use in PCR. When ordering LAMP primers – F3, B3, FIP, BIP, FL and BL – each individualised primer is prepared in lyophilised (freeze dried) fashion and stored in a 1.5mL Eppendorf tube. Thus for PCR use the outer primers were easily separated. The F3 and B3 lyophilised primers were suspended in ddH₂O with a slight alkalinity (pH 7.5–8). It was of paramount importance to make several aliquots from stock solution, storing them separately at -20°C. Reason being to not consistently thaw and freeze the stock solution when running a new reaction. This reduced the possibility of contamination and lowered the chances of disrupting primer stability. The colder the temperature for storage, the longer DNA molecules (primers specifically) will stay viable and absolute for use in molecular studies instead of becoming obsolete with time. Non-optimised standardised PCR kit conditions were followed out (Table 4.1 and 4.2). The Phusion High-Fidelity DNA Polymerase protocol of New England BioLabs was used with great success.

Table 4.1: PCR reaction components

COMPONENT	25µL REACTION	50µL REACTION	FINAL CONCENTRATION
5X PHUSION HF OR GC BUFFER	5	10	1X
10MM DNTPS	0.5	0.1	200µM
10µM FORWARD PRIMER	1.25	2.5	0.5µM
10µM REVERSE PRIMER	1.25	2.5	0.5µM
PHUSION DNA POLYMERASE	0.25	0.5	1.0 units / PCR run
TEMPLATE DNA	Variable	Variable	-
NUCLEASE FREE WATER	Add to fill 25µL	Add to fill 25µL	-
TOTAL REACTION VOLUME	25µl	50µl	-

Table 4.2: PCR reaction conditions

STEP	TEMPERATURE	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C	5-10 seconds
	45-72°C	10-30 seconds
	72°C	15-30 seconds/kb
	72°C	5-10 seconds
Final Extensions	72°C	5-10 seconds
Hold	4°C	∞

The template DNA volume used for each 25µL PCR reaction was a mere 1µL. After the reaction was done, the samples were loaded into a typical 1% agarose gel and left to run for 45 minutes at 100 V. The make-up of the gel consisted of 1g agarose powder mixed into 100mL Tris-acetate-EDTA buffer which was mixed and heated until the agarose powder completely dissolved. A concentration of 2µL ethidium bromide (EtBr) per 100mL gel was added to the liquid agarose gel mixture and homogenised before allowing to set until a solidified jelly consistency was achieved. When DNA samples were loaded into the gel, the EtBr intercalated with the DNA double helix as the current pulled the DNA molecules from the anode (negative pole) to the cathode (positive pole) since the DNA self is negatively loaded delivering clear illuminated bands when viewed under UV light.

From preliminary elimination rounds, primer set Bd5 revealed to be a more stable and viable set in the “more” successful identification of Bd. Figure represents the fully kitted Bd5 LAMP primer set (excluding loop primers) containing the outer primers (F3 and B3) and inner primers (FIP and BIP). Bd5 outer primers represented the following sequences of interest:

F3: 5'-TGACAACGGATCTCTTGG-3'

B3: 5'-CTTGGTACGGTTTAAACAAAC-3'

For the *In vitro* system dynamics, DNA extraction and visual detection method refer to pages 40, 41 and 42 under sections 3.2.2, 3.2.3 and 3.2.4 respectively.

4.3 Results and discussion

Results stated in theory that Bd5F3 and Bd5B3 are specific to Bd alone. F3 carried a sequence length of 18nt accompanied by a melting temperature of 54°C and a Guanine/Cytosine (GC) percentage of 50%. B3 carried a sequence length of 21nt accompanied by a melting temperature of 54°C and a GC percentage of 38%. The NCBI BLAST results of primer set Bd5 matched with expected Bd reference in NCBI database. Once again high specificity theory results indicated high query coverage and high identification percentage for Bd5F3 and Bd5B3 towards Bd (Figure 4.1

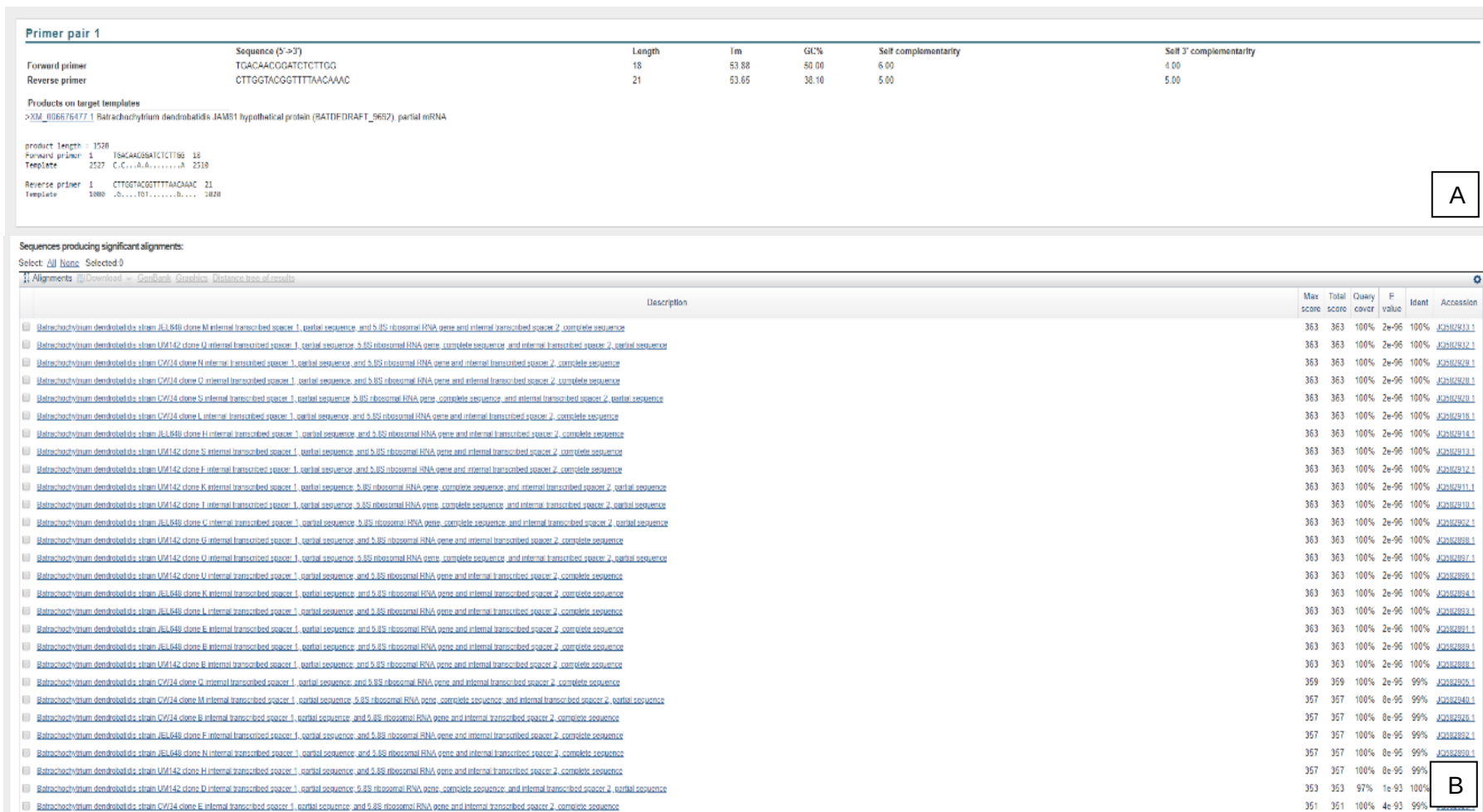


Figure 4.1: NCBI BLAST results. A – primer blast results. B – expected nucleotide product results

4.3.1 Specificity test

For all PCR results basic agarose gel electrophoresis were used for end-point diagnosis detection using ethidium bromide.

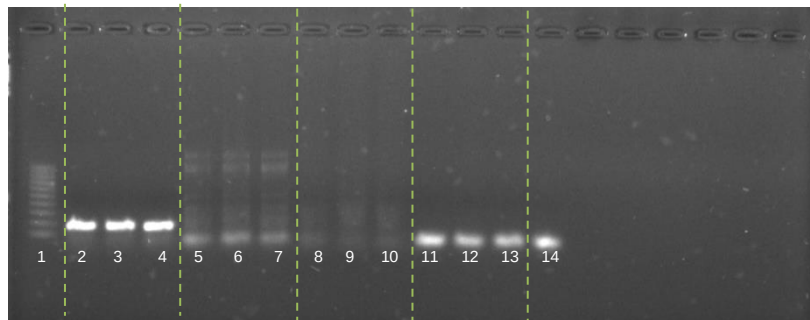


Figure 4.2: PCR specificity results run with Bd5F3 and Bd5B3 primers with DNA marker (lane 1), positive controls (lanes 2 to 4), negative controls (lanes 5 to 13) and a blank (lane 14).

Lane 1 represents a 1kb DNA ladder. Lanes 2 to 4 represented the positive controls containing Bd DNA. Lanes 5 to 7 represented negative controls of the African clawed frog, *Xenopus laevis*. Lanes 8 to 10 represented a secondary set of negative controls of the pathogenic yeast *Candida albicans*. Lanes 11 to 13 consisted of a tertiary set of negative controls of the hard-bodied southern African dog tick *Haemaphysalis elliptica*. Lane 14 consisted of the blank with no added DNA template (Double distilled water).

PCR with primer set Bd5 (F3 and B3) successfully amplified the positive controls (lanes 2 to 4) with high intensity bands (200bp) under UV illumination (Figure 4.2). When creating an assay for a pathogen that affects/infects host animals it is always recommended to run the host DNA as a negative control as well. Reason being, if one was wanting to detect a certain pathogen on or within a host, it would prove foolish if the designed primers can attach to and amplify the host DNA – every sample originating from the host (infected or not) would test positive. From figure it was noted that Bd5 did attach to amplify host DNA, resulting in vague light bands however, the delivered bands were not of the correct nucleotide length which should be 200 base pairs (bp). Thus this is of no concern since these uninfected hosts' DNA did not yield the desirable band size for a positive result. With the secondary set of negatives (*Candida albicans*), it was necessary to add in a close related yeast genus which can also be found in lotic freshwater systems. This eliminates the possibility of false positives from free living yeast cells that may make their way into a reaction by chance. The tertiary set of negatives are an outgroup for the sake of random amplification. The blank in lane 14 was used with great importance since it could indicate any presence of contamination. Since the primers had no template target DNA to anneal to in lanes 5 to 14 primer dimers were formed. This was simply the doing of primers annealing to each other, producing secondary non-specific products of extremely small size (± 20 bp).

4.3.2 Sensitivity test (DNA serial dilutions)

A clear visual reduction in band intensity indication could be seen as the serial dilution ran from 10^{-1} to 10^{-4} , with 10^{-4} (0.5pg) being the lowest detection range for primer set Bd5, representing vague dull bands (Figure 4.3)

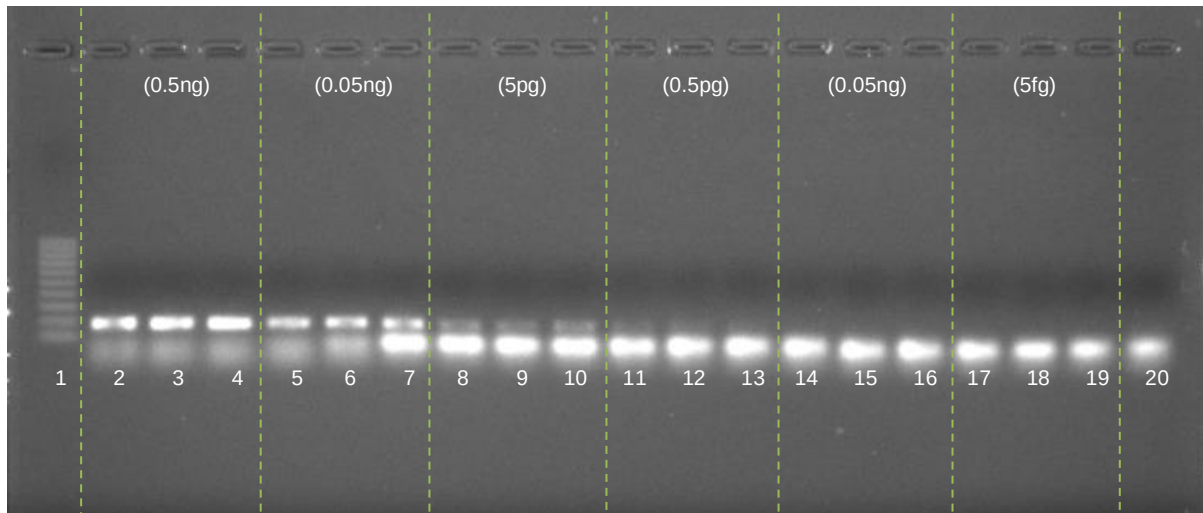


Figure 4.3: PCR sensitivity run with Bd5F3 and Bd5B3 primers with serially diluted Bd DNA from 5ng down to 5fg. With DNA markers (lane 1), serially diluted DNA (lanes 2 to 19) and a blank (lane 20).

Reactions were done in triplicate as seen in figure 4.3. Lane 1 represented a 1kb DNA ladder. Lanes 2 to 4 represented a serial dilution of 10^{-1} (0.5ng). Lanes 5 to 7 represented a serial dilution of 10^{-2} (0.05ng). Lanes 8 to 10 represented a serial dilution of 10^{-3} (5pg). Lanes 11 to 13 represented a serial dilution of 10^{-4} (0.5pg). Lanes 14 to 16 represented a serial dilution of 10^{-5} (0.05pg). Lanes 17 to 19 represented a serial dilution of 10^{-6} (5fg). Lane 20 represented the blank with no added DNA template.

4.3.3 Sensitivity test (Zoospores serial dilution)

A clear visual reduction in band intensity indication could be seen as the serial dilution ran from 10^{-1} to 10^{-5} , with 10^{-4} (1 zoospore) being the lowest clear visible detection range for primer set Bd5. However, the dilution 10^{-5} (0.1 zoospores) also contained very faint but non the less present bands of dull intensity (Figure 4.4).

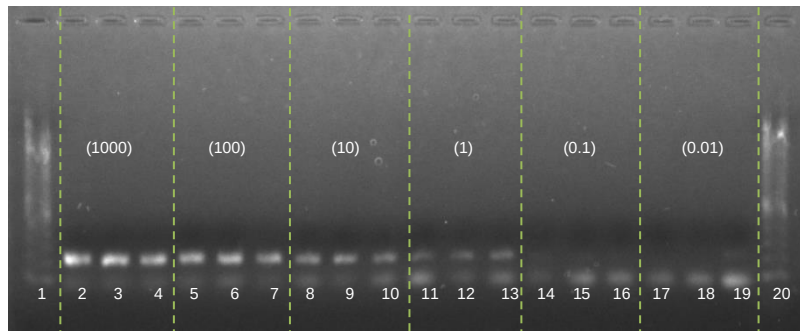


Figure 4.4: PCR sensitivity run for Bd5F3 and Bd5B3 in which the zoospores were serially diluted from 10 000 down to 0.01 with DNA markers (lanes 1 and 20) and serially diluted zoospores (lanes 2 to 19).

Reaction were done in triplicate as seen in figure 4.4. Lane 1 represented a 1kb DNA ladder. Lanes 2 to 4 represented a serial dilution of 10^{-1} (1000 zoospores). Lanes 5 to 7 represented a serial dilution of 10^{-2} (100 zoospores). Lanes 8 to 10 represented a serial dilution of 10^{-3} (10 zoospores). Lanes 11 to 13 represented a serial dilution of 10^{-4} (1 zoospore). Lanes 14 to 16 represented a serial dilution of 10^{-5} (0.1 zoospores). Lanes 17 to 19 represented a serial dilution of 10^{-6} (0.01 zoospores). Lane 20 represented another 1kb DNA ladder.

4.3.4 Purified samples (low DNA and Zoospore band intensity clean-up)

From figure 4.3 it was noted that the lowest DNA dilution was 10^{-4} (0.5pg) however, the band intensity was faint and hardly visible. From figure 4.4 it was noted that the lowest zoospore dilution was 10^{-5} (0.1 zoospores) but the bands were even more so difficult to see without a trained eye than the lowest DNA dilution.

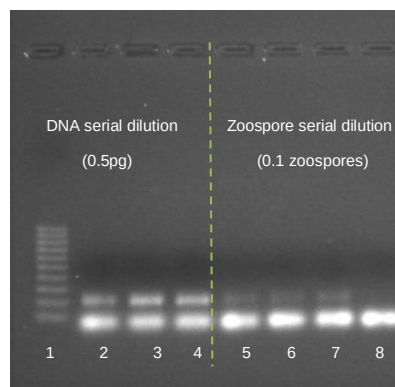


Figure 4.5: Agarose gel with purified PCR products. With DNA (lanes 2 to 4) and zoospore serial dilutions (lanes 5 to 7). Lane 1 represented the 1kb DNA ladder while lane 8 represented a blank.

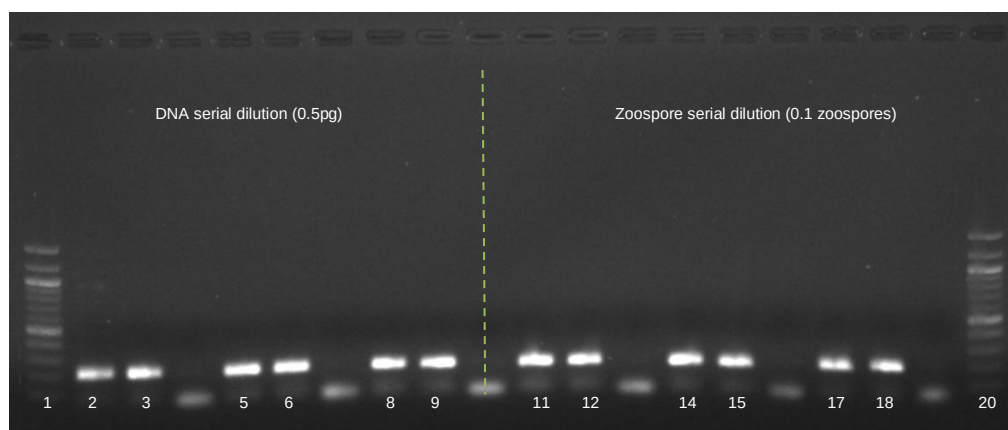


Figure 4.6: Agarose gel with purified agarose gel products (after PCR purification) with DNA markers (lanes 1 and 20), purified DNA samples (lanes 2,3,5,6,8 and 9) and purified zoospore samples (lanes 11,12,14,15,17,18).

After these serial dilution runs, the lowest positive dilution series as mentioned above were subjected to gel purification. These purified samples were loaded again in to a gel in order to perceive the effectiveness of the gel purification protocol as seen in figure 4.5. After purification the subsequent bands (serially diluted samples) were visible to a greater degree, still sitting at 300bp which was the expected band size after PCR. Following the clean-up step, purified samples were again subjected to PCR, yielding clear and highly vibrant bands as seen in figure 4.6. Lanes 2, 3 and 4 from figure 4.5 purified DNA serial dilution samples were each run in duplicates as seen in figure 4.6. Lanes 5, 6 and 7 from figure 4.5 purified zoospore serial dilution samples were each run in duplicates as seen in figure 4.6 with lanes 1 and 20 that represented a 1kb DNA ladder. All of the wells with left-out numbers represented blanks.

The assay developed by Annis and colleagues (2004) yielded a sensitivity range fit for molecular end-point diagnosis of Bd. Sensitivity was limited to 10pg for two strains (JEL 197 and JEL 274) and with one other strain (JEL 215) being detected at 1pg for DNA extractions. For zoospore dilutions, the assay was capable of detecting as low as a single zoospore.

The assay we developed proved to be even more sensitive in both regards of extracted DNA and zoospore dilutions. The assay detected a low DNA dilution of 0.5pg while a zoospore dilution of up to 0.1 zoospores were detected although a gel purification clean-up was required since the bands' intensity was a bit dull.

If one wishes to simply undertake end-point diagnostics of a given population such as to quantify infection status and/or prevalence of the population any one of the above PCR assays would prove fruitful due to the low costs (for large populations), ease of use (PCR being quite user friendly) and fast reaction time for positives in regards to histology. PCR is a great economic alternative to qPCR and better in the sense of practical application than histology. However, if a population is

known to carry low infection load and a slightly more sensitive diagnostic is preferred (once again or end-point diagnostics only), the PCR assay we developed would fit perfectly. When absolute sensitivity and real-time detection is required no other diagnostic assay would prove better than qPCR.

In comparison to the LAMP assay, PCR would most definitely come second in several planes – sensitivity, selectivity, rapidness and robustness. However, PCR may exhibit some other beneficial advantages. PCR's are able to house much more samples per selective run (up to 30 or 60 individual tubes) while LAMP is limited to 16. For simple end-point diagnostics, especially with a large number of specimens, PCR would prove to be much more economic. If multiplex was a consideration, PCR would again prove better suited since the chance of non-specific binding within LAMP (due to the many and large primers) increases tenfold during multiplex.

Chapter 5 Evaluation of newly developed LAMP and PCR assays in the detection of Bd in archived and wild frogs

5.1 Introduction

Out of the top 100 worst invasive alien species, only 8 consist of micro-organisms which include 1 protozoon (avian malaria), 2 viruses (banana bunchy virus and rinderpest virus) and 5 fungi (chestnut blight, crayfish plague, Dutch elm disease, phytophthora root rot and amphibian chytrid disease) (Lowe, *et al.*, 2000). From one hypothesis based on archived specimens, Bd was most probably native to Southern Africa after which it spread globally in the early 1900's as part of massive *Xenopus laevis* exports as research models and pregnancy tests (Weldon, *et al.*, 2004). A more recent study accompanied by genetic analysis indicated that Bd-Asia pre-dates Bd-GPL based on ITS DNA haplotyping, thus hypothesising that Bd more likely originated from Southeast Asia (Bai, *et al.*, 2012). Never the less, this emerging infectious and invasive alien species is a great threat to frogs globally as well as to the biotic and abiotic surroundings in which frogs play a significant role (Alemu, *et al.*, 2008). Bd has been known to infect and affect a variety of frog species around the world in several countries such as the West Indies, China, Japan, Bolivia, Argentina, Hawaii, New Zealand, Gabon, Spain, Ethiopia, USA, Italy and Southern Africa, just to name a few (Alemu, *et al.*, 2008; Bai, *et al.*, 2012; Barrionuevo and Ponssa, 2008, Barrionuevo, *et al.*, 2008; Beard, *et al.*, 2005; Bell, *et al.*, 2004, 2011; Bosch, *et al.*, 2006; Gower, *et al.*, 2012; Rachowicz, *et al.*, 2007; Simoncelli, *et al.*, 2005; Tarrant, *et al.*, 2013). Bd has been detected in 56 countries thus far globally, infecting a total of 516 frog species as of from 2013 (Olson, *et al.*, 2013).

The amphibian class is in the lead when it comes to being threatened among all known vertebrate classes. Exceeding 30% of total species labelled as having an extinction crisis surpassing both birds (12% extinction crisis) and mammals (23% extinction crisis) (Stuart, S.N., *et al.*, 2004). The enigmatic pandemic decline of these amphibians were directly linked to this pathogenic chytrid. The driving force of Bd is known to negatively affect more than 200 species, of which some experience mass die-offs and other experiencing terrible pathogenic symptoms (Fisher, M.C., *et al.*, 2009). Bd was listed as one of the top 100 invasive alien species for good reason, being robust pioneer species they are well equipped when it comes to competition and besetting new habitats. This along with the current affair in amphibian travel and trade makes Bd a force to be reckoned with since the pathogen has access to worldwide transport, both in natural and protected areas where Bd has never been before. Ultimately a loss of natural biodiversity occurs, endangered species die off, ecosystems and food webs are negatively affected and manipulated, also fewer individuals are present for environmental conservation studies. All these outcomes have severe consequences which may even indirectly or directly affect the well-being of the world and humans (Weldon, C. & Fisher, M.C., 2011).

The sampling site for *in vivo* modelling to test the LAMP and PCR assay integrity was in and around the proximity of Mont Aux Sources which is located on top of the Drakensberg. Bragging with several natural phenomena such as the majestic Tugela waterfall which is the highest fall in South Africa running at 946m. However, the defining and unique quality of Mont Aux Sources is the monstrous amphitheatre. Running a widespread 4km across the Drakensberg makes it a perfect strip of land to carry rivers, harbour soil and vegetation, all at an altitude of 1000+m. At this altitude a unique biome is borne which is cut off from the rest of the world (Souchon, 2005).

Despite the unforgiving and unpredictable weather (extreme temperatures, precipitate, fires, snow and lightning) some hardy animals still manage to survive and even proliferate in these conditions (Bristow, 2003; Nel & Summer, 2008). The sampling area on the amphitheatre was occupied predominantly by grass fauna, with the red oat grass (*Themeda trindra*) and tussock grass (*Festuca costata*) acting as a mat along with random growing protea bushes and several types of bulbous plants. Some of the animal inhabiting these extreme habitats include jackals, baboons and certain antelope (eland, klipspringer and bushbuck) (Bristow, 2003).

A limited number of frogs are found on this high altitude biome such as the clicking stream frog (*Stronylopus grayii*), Common river frog (*Amietia delalandii*), Maluti river frog (*Amietia umbraculata*) and finally the Phofung river frog (*Amietia hymenopus*) (Du Preez & Carruthers, 2009). The Phofung river frog was chosen as species of interest due to known records of this species being infected with chytrid. In 1998 a mass die-off of this species was witnessed, presumably due to a combination of dry weather reducing all the rivers to separate pools, and infection with Bd. Bd was found in archived specimens from that year and location, and was given as a possible cause for the mass mortality of 1998 (O'Grady, 1998; Weldon & du Preez, 2006). Chytridiomycosis epidemic has been known to follow after an intense drought (Berger et al. 2000). Newly developed LAMP and PCR assays were to be tested and evaluated in terms of their efficiency and efficacy by the detection of Bd in both wild (Mont Aux Sources, Drakensberg) and archived frog specimens (African Amphibian Conservation Research Group (AACRG) research collection).

5.2 Materials and methods

After optimisation of newly developed PCR and LAMP assays, the assays were implemented in the evaluation of their efficacy on frog populations of both known and unknown infection status. Amplification assessment on frogs with a known infection status were carried out first to establish the performance and precision of each assay. After amplification assessment was established, frog populations of unknown infection status were put through PCR and LAMP runs to detect whether or not frogs of these regions were affected by chytridiomycosis. The perceived results were compared with that of histology results from the same frogs done by Miss Celia Cloete, a B.Sc. Honours Zoology student of the North-West University.

Developed and optimised PCR and LAMP assays were undertaken for performance assessment against frogs of known and unknown chytridiomycosis infection status. Histology was already done on frogs of unknown chytridiomycosis infection status and were used as reference for the PCR and LAMP assay.

Ethics approval

The samples were collected with Permit: OP 4218/2016. The study was approved by the animal ethics committee (AnimCare) of North-West University, reference numbers: NWU-00015-16-A

5.2.1 Known chytridiomycosis infection site (Mont Aux Sources)

Tissue sampling for the frogs (Figure 5.1) of known chytridiomycosis infection status was done in and around Mont-Aux-Sources (28° 46' 21.07" S, 28° 52' 21.72" E) which can be found in southern Africa on the Drakensberg. Mont-Aux-Sources is roughly divided between South-Africa and Lesotho. These Phofung river frogs (*Amietia hymenopus*) that reside within the four rivulets (Tugela, Vemvane, Bilanjil and Ribbon falls) of Mont-Aux-sources were known to be infected with chytridiomycosis with the BdCAPE strain. Frogs were caught per river using a small dip net or by hand. As per collection the grouping of frogs were placed in plastic bags containing some river water. Using sterile nitrile laboratory gloves, 1 toe clipping was taken of each individual along with a skin swab of the ventral side of the body using sterile surgical scissors and sterile cheek swabs respectively. The individuals were marked in accordance to which river they were caught, followed by the species name and finally individual number. After collection and sampling was done, the frogs were released back to the specific river-location from which they were found.

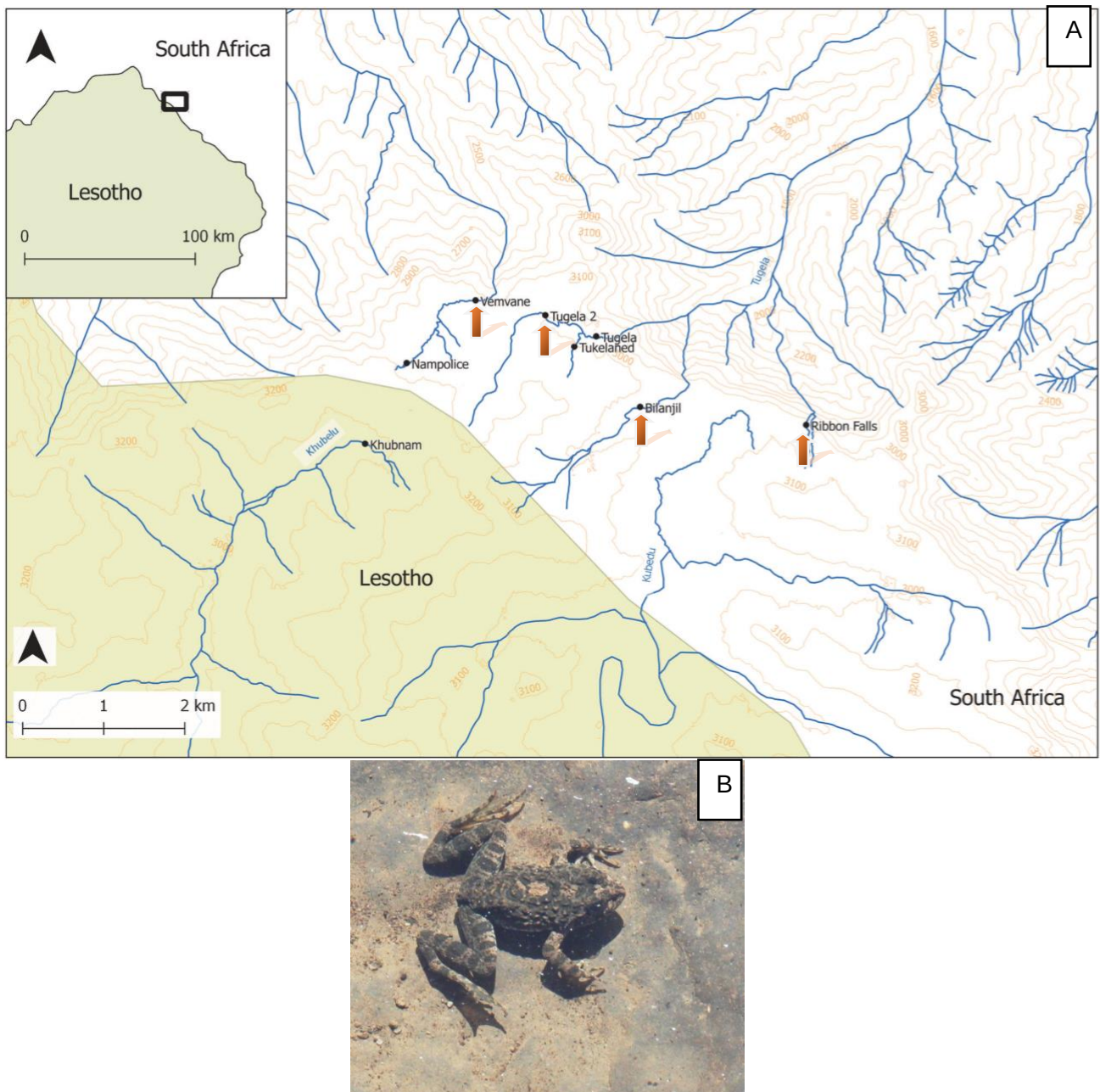


Figure 5.1: A – Mont Aux Sources [(sampling sites indicated with orange arrows) Griffiths, *et al.*, 2018]. B – Sampled frog specimens

5.2.2 Unknown chytridiomycosis infection (AACRG archived samples)

Frogs of unknown infection status were stored as historic specimens in 70% alcohol in the AACRG research collection of North-West University. Chosen archived specimens are listed in table. These frogs were sampled from a variety of different dates (from as early as 1999) and locations (Botswana, Zambia, Tanzania and Mozambique). Collected specimens were each assigned an AACRG field number following storage in 90% alcohol in a cool and dark place.

5.2.3 Assays used for Bd detection

Histology assay: histology was carried out by Celia Cloete (Hons. B.Sc) which included three major steps. The first is a tissue processing step for the material which includes several ethanol washes and finally being embedded in paraffin wax. Secondly the tissues were sectioned at 8µm with a Reickert-Jung 2050 automated microtome. Lastly, the slides containing the sections were stained with Ehrlich's haematoxylin and eosin, and coverslips mounted with Entellan® (Merck) mounting media.

LAMP assay: the LAMP assay consisted of three pairs of primers, the outer primers (Bd4F3 and Bd4B3), inner primers (Bd4FIP and Bd4BIP) and the loop primers (Bd4LF and Bd4LB).

Bd4F3	5'-TGAAGAACGCAGCGAAAT-3'
Bd4B3	5'-CCACTACTTTTTGTATCACTCAT-3'
Bd4FIP	5'-GAGTGCAATGTGCGTTCAAAG-ATACGTAATGTGAATTGCAAACC-3'
Bd4BIP	5'-TACATTGTCCGAATTGACTGGAC-ACTACTACTTTTAAACCTGTCA-3'
Bd4LF	5'-GATTTAATGATTCACAAAG-3'
Bd4LB	5'-AGATATGAACCATGTCAA-3'

This primer set was designed to be specific to the BdGPL and BdCAPE strains of *Batrachochytrium dendrobatidis*. The Optigene Isothermal Master Mix was used for the field samples' evaluation since it delivered better/optimal results in regards to the Lucigen OmniAmp DNA & RNA LAMP Kit and Loopamp DNA Amplification Kit. The Optigene Isothermal Master Mix was optimised to the following specification: 15µL MM, 5µL template DNA and 2µL primer mix which was filled to a 25µL total with ddH₂O. Furthermore, the 2µL primer mix consisted of the following primer constituent relationship; $\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ part}}$. This reduction of 5µL primer mix to 2µL as well as the reduction of loop primers from 4 parts to 1 part seemed to help marginally with the reduction in non-specific amplification. An isothermal reaction temperature of 58°C was used for all reactions since it delivered fast results (<30mins) at a total reaction time of 50mins.

PCR assay: the PCR assay consisted of the two outer primers (Bd5F3 and Bd5B3) of the Bd5 primer set.

Bd5F3	5'-TGACAACGGATCTCTTGG-3'
Bd5B3	5'-CTTGGTACGGTTTAAACAAAC-3'

PCR runs were non-optimised standardised conditions as laid out by the Phusion High-Fidelity DNA Polymerase kit's leaflet. The annealing temperature was set at 54°C for a total reaction time of 70mins.

5.2.4 DNA extraction

All samples were subjected to the well-known kitchen method of DNA extraction, the Salting out method, with great success.

5.3 Results and discussion

5.3.1 Known chytridiomycosis infection site (Mont Aux Sources)

Table 5.1 represents sampled buccal swabs and toe clippings of *Amietia hymenopus* individuals which has undergone both PCR and LAMP.

Table 5.1: Detection of Bd using PCR and LAMP

		VENTRAL SKIN SWABS		TOE CLIPPINGS	
RIVER – INDIVIDUAL		PCR	LAMP	PCR	LAMP
VEMVANE	1	Pos	Pos	Pos	Pos
	2	Neg	Pos	Neg	Neg
	3	Pos	Pos	Pos	Pos
TUGELA	1	Neg	Pos	Neg	Pos
	2	Pos	Pos	Pos	Pos
	3	Pos	Pos	Pos	Pos
BILANJIL	1	Pos	Pos	Pos	Pos
	2	Pos	Pos	Pos	Pos
	3	Neg	Neg	Neg	Pos
RIBBON-FALLS	1	Neg	Neg	Neg	Neg

As seen in table the only site with no positive indication for chytridiomycosis for both ventral skin swabs and toe clippings was Ribbon Falls. Also only a single individual was caught for this rivulet. This simply implies that no Bd was present (on host tissue at least) at the time of sampling since there was low host population numbers as with previous annual surveys for this specific site. However, to fully determine the Bd presence and prevalence abiotic samples would have to be analysed as well which would include water and riverbed soil. The other three rivulets all had a high incidence of positive samples.

Vemvane had only three negative results, all of which originated from the same specimen, individual 2 was negative for a buccal swab done by PCR and a toe clipping done by both PCR and LAMP. The negative swab done by PCR could be due to a low zoospore presence on the specimen's ventral side of the body. The negative toe clippings done by PCR and LAMP could be due to selecting a toe that was not infected with chytridiomycosis, since even a low infection load of 1 zoosporangium houses up to 10 zoospores which is well above the detection limits of both these amplification assays. Since the swab tested positive for only LAMP, this simply indicated that the sample chosen for analysis was under a low infection load. Despite these three negatives on individual 2 the LAMP buccal swab still tested positive. The rest of the individuals' tests were positive which was expected since high Bd prevalence was found in the Vevane during surveys from previous years.

The Tugela river delivered two negative tests from individual 1 from a swab done by PCR and a toe clipping done by PCR as well. The swab again could have been due to low zoospore prevalence on the skin. The negative toe clipping was probably due to the specific toe not being infected since PCR revealed it to be of low zoospore infection load. Despite these two negatives for individual 1, the LAMP buccal swab and LAMP toe clipping still tested positive. The rest of the individuals' tests were positive which was expected since high Bd prevalence was found in the Tugela with previous years' surveys (Griffiths, *et al.*, 2018).

Bilanjil had positive results for the first two individuals. The third individual only managed a positive result with the toe clipping and LAMP diagnostic. Thus extremely low infection load was suspected on individual 3.

5.3.2 Unknown chytridiomycosis infection (AACRG archived samples)

Only a few individual specimens were tested for chytridiomycosis which include numbers 1108, 0561, 0562, 0563, 0567, 0569, 0570, 2335, 2336, 2337, 2338, 0930 and 0859. Specimen 0563 and 2336 were specifically chosen since histological results yielded positive identification of the presence of chytridiomycosis (Table 5.2). Positive results by means of histology concludes that those specimens are most definitely infected with chytridiomycosis – since they are individually scrutinised with expert eyes-, making them excellent positive controls. The rest were simply chosen at random based on groupings around the histological positive specimens and some outgroups outside of the positive individuals. Several sister specimens (same species found in the same location) around the positive individuals were chosen as well since chytridiomycosis may sometimes not be visible with the naked eye whereas trace amounts (nanograms or less) of the DNA can be picked up by molecular diagnostics. Furthermore, three outgroups were also chosen for amplification.

Table 5.2: Detection of Bd by histology, PCR and LAMP

AACRG FIELD NO	LOCATION	LATITUDE	LONGITUDE	GENUS	SPECIFIC EPITHETON	HISTOLOGY	PCR	LAMP
1125	Hippo Lodge, Zambia	- 14,6520833	26,4054167	<i>Ptychadena</i>	<i>anchietae</i>	Ne	-	-
1129	Lefusa Camp, Zambia	- 14,6772222	26,1902777	<i>Ptychadena</i>	<i>anchietae</i>	Ne	-	-
2334	Botswana	- 19,1973333	23,4211389	<i>Ptychadena</i>	<i>anchietae</i>	Ne	-	-
0710	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
0711	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
0712	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
0716	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
0717	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
0718	Okavango	- 18,7616667	21,894444	<i>Hyperolius</i>	<i>angolensis</i>	Ne	-	-
1108	Upper Sani (2008)	- 29,5531667	29,2516666	<i>Amietia</i>	<i>umbraculata</i>	Ne	Ne	Ne
0943	Botswana	- 19,3915556	22,75375	<i>Ptychadena</i>	<i>guibei</i>	Ne	-	-
0944	Botswana	- 19,3915556	22,75375	<i>Ptychadena</i>	<i>guibei</i>	Ne	-	-
0945	Botswana	-19,348669	22,713629	<i>Ptychadena</i>	<i>guibei</i>	Ne	-	-
0557	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0558	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0559	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0560	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0561	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	Ne	Po
0562	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	Po	Po
0563	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Po	Po	Po
0564	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	NO DATA	-	-
0567	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	Ne	Po
0569	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	Po	Po
0570	Nxaraga Camp (2008)	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	Ne	Po
0571	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0572	Nxaraga Camp	- 19,5480556	23,177777	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0573	Shakawe	- 18,4355555	21,9055555	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0575	Shakawe	- 18,4355555	21,9055555	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
0576	Shakawe	- 18,4355555	21,9055555	<i>Xenopus</i>	<i>laevis</i>	Ne	-	-
1122	Hippo Lodge, Zambia	- 14,6772222	26,3769444	<i>Ptychadena</i>	<i>mapacha</i>	Ne	-	-
1123	Hippo Lodge, Zambia	- 14,6772222	26,3769444	<i>Ptychadena</i>	<i>mapacha</i>	Ne	-	-

1127	Hippo Lodge, Zambia	- 14,6520833	26,4054167	<i>Ptychadena</i>	<i>mapacha</i>	Ne	-	-
0574	Shakawe	- 18,4355555	21,9055555	<i>Ptychadena</i>	<i>mapacha</i>	Ne	-	-
0947	Botswana	- 19,2212778	22,7688611	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
1562	Tete, Mozambique	- 16,2938889	33,7561111	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
1563	Tete, Mozambique	- 16,2938889	33,7561111	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
0928	Botswana	- 19,3645556	22,7091944	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
0929	Botswana	- 19,3645556	22,7091944	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
0937	Botswana	- 19,3834722	22,7449722	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
0941	Botswana	- 19,3834722	22,7449722	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	-	-
2335	Okavango (1999)	- 18,9736111	22,5738888	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	Po	Po
2336	Okavango (1999)	- 18,9736111	22,5738888	<i>Ptychadena</i>	<i>mascariensis</i>	Po	Po	Po
2337	Okavango (1999)	- 18,9736111	22,5738888	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	Ne	Po
2338	Okavango (1999)	- 18,9736111	22,5738888	<i>Ptychadena</i>	<i>mascariensis</i>	Ne	Ne	Ne
0935	Botswana	-19,3925	22,7587777	<i>Hyperolius</i>	<i>parallelus</i>	Ne	-	-
0938	Botswana	- 19,3817778	22,7290555	<i>Hyperolius</i>	<i>parallelus</i>	Ne	-	-
1136	Lefusa Camp, Zambia-14.6166111	- 14,6772222	26,1902777	<i>Hyperolius</i>	<i>pyrrhodictyon</i>	Ne	-	-
1138	Lefusa Camp, Zambia-14.6166111	- 14,6772222	26,1902777	<i>Hyperolius</i>	<i>pyrrhodictyon</i>	Ne	-	-
0925	Botswana	- 19,3915556	22,7623889	<i>Ptychadena</i>	<i>subpunctata</i>	Ne	-	-
0930	Botswana (2009)	- 19,3645556	22,7091944	<i>Ptychadena</i>	<i>subpunctata</i>	Ne	Ne	Ne
0936	Botswana	- 19,3834722	22,7449722	<i>Ptychadena</i>	<i>subpunctata</i>	Ne	-	-
2330	Mopane Woodland, Xakanaxa Botswana	-19,19599	22,43793	<i>Ptychadena</i>	<i>subpunctata</i>	Ne	-	-
2331	Mopane Woodland, Xakanaxa Botswana	-19,19599	22,43793	<i>Ptychadena</i>	<i>subpunctata</i>	Ne	-	-
1124	Hippo Lodge, Zambia	- 14,6772222	26,3769444	<i>Ptychadena</i>	<i>taenioscelis</i>	Ne	-	-
1130	Lefusa Camp, Zambia-14.6166111	- 14,6772222	26,1902777	<i>Ptychadena</i>	<i>taenioscelis</i>	Ne	-	-
1104	Upper Sani	- 29,5531667	29,2516666	<i>Amietia</i>	<i>umbraculata</i>	Ne	-	-
1105	Upper Sani	- 29,5531667	29,2516666	<i>Amietia</i>	<i>umbraculata</i>	NO DATA	-	-
1108	Upper Sani	- 29,5812778	29,2886111	<i>Amietia</i>	<i>umbraculata</i>	Ne	-	-
0859	Tanzania (2008)	- 3,35822222	36,8375833	<i>Hyperolius</i>	<i>veridiflaves</i>	Ne	Ne	Ne

(Ne) ~ Negative

(Po) ~ Positive

(-) ~ No test on individual

As seen in table 5.2 only two specimens tested positive for chytridiomycosis by means of histology. An African clawed frog (AACRG field number 0563), *Xenopus laevis*, and a grass frog (AACRG field number 2336), *Ptychadena mascariensis*, both tested positive for chytridiomycosis by means

of histology. Following amplification by PCR and LAMP, both these specimens yielded positive for chytridiomycosis as well. Several sister specimens (same species found in the same location) around the positive individuals were chosen as well since chytridiomycosis may sometimes not be visible with the naked eye whereas trace amounts (nanograms or less) of the zoospores' DNA can be picked up by molecular diagnostics (Boyle, *et al.* 2004).

Five additional sister specimens of the African clawed frog (AACRG field number 0563) was chosen for examination by PCR and LAMP. These five specimens were found to be negative based on histological survey although some seemed to test positive by PCR and/or LAMP. Specimen 0561 tested positive for chytridiomycosis by LAMP only. Specimen 0562 tested positive for chytridiomycosis by PCR and LAMP. Specimen 0567 and 0570 tested positive for chytridiomycosis by LAMP only. Specimen 0569 tested positive by PCR and LAMP.

Three additional sister specimens of the grass frogs (AACRG field number 2336) were chosen for examination by PCR and LAMP. These three specimens were found to be negative based on histological survey although some seemed to test positive by PCR and/or LAMP. Specimen 2335 tested positive for chytridiomycosis by PCR and LAMP. Specimen 2337 tested positive for chytridiomycosis by LAMP only. Specimen 2338 remained negative for chytridiomycosis for both molecular diagnostics.

Reason being that several specimens tested positive for chytridiomycosis by either PCR and/or LAMP while not by histology was simply due to a probable low infection count. Histology depends on physical symptoms and the presence of zoosporangia which formed in the keratinised skin surface. Having a low infection (thus minimal symptoms or minimal presence of zoosporangia) will definitely affect histological examination precision while just the slightest load of infection (as low as 10 zoospores per infection) will prove adequate for molecular diagnostic tools.

Eukaryotes contain multi-copy rDNA genes with fungi seating 100 to 400 copies per individual haploid genome. Highly conserved regions (5.8-, 18- and 28S DNA) are separated by ITS regions and intergenic spacer units of which both evolve at a fast rate. These high copy numbers and variability entitles molecular diagnostics to a high degree of sensitivity and specificity when used to design an amplification assay. Thus, the high copy numbers allowed a low detection level of Bd as seen in Chapter 2 where LAMP was able to detect up to 0.1 zoospores or mere pictograms in a given sample. Histology was dependent on the presence of at least 1 zoosporangium (also the lowest possibility to detect chytridiomycosis) per serially sectioned toe for the detection of chytridiomycosis. Note that 1 zoosporangium houses at least up to 10 zoospores, this allowed for a 100 times greater detection potential than the lowest number of zoospores (0.1 zoospores) for LAMP (Boyle, *et al.* 2004). LAMP thus allowed a reliable means of infection detection at much lower concentrations of infection that is possible with histology. All thirteen samples were also tested and delivered optimal results in 60 minutes for LAMP and 90 minutes for PCR whereas the thirteen samples took several days for histological analysis. Molecular diagnostics also have the

ability to potentially detect Bd before infection settles, which can be 1 to 2 weeks in advance before histological examination delivers positive results, holding a major advantage over histology (Boyle, *et al.* 2004).

Chapter 6 Conclusions and recommendations

Among the several vertebrate classes, amphibians top the list of being most threatened with a high extinction crisis. Bd is a pathogenic amphibian fungus responsible for these enigmatic declines and huge pandemic die-offs. Chytridiomycosis is a universal problem since the majority of affected species experience a 100% mortality rate if left untreated or detected at a late stage of infection. Conventional means of diagnosis include histology, PCR and qPCR however these means of detection do have some limitations. The LAMP assay was developed to improve on these shortcomings.

The four basic primers (inner- and outer primers) were designed using PrimerExplorerV5 with exception towards the loop primers. The loop primers were designed manually without the use of software. Designing LAMP primers without any expertise behind the chemistry won't prove fruitful. A recommendation for another primer designing software would be PrimerDesign by New England Biolabs since they have a higher chance of including loop primers along with the four basic primers.

In vitro testing models revealed the LAMP assay to be superior towards histology, PCR and qPCR in regards to several disciplines. The sensitivity and total reaction time was particularly more desirable. However, the high sensitivity proved several times to be a minor drawback for the LAMP assay. Contamination was the biggest attributing factor to these drawbacks since even the most inappreciable minute possibility of contamination would yield false positives. Primers' stability was just as a big factor for consistent results. Newly designed primers would become unstable after 3 days' use and add to these drawbacks. Recommendations could include making / using LAMP specific laboratories. This simply means that the laboratory is in use for LAMP only and the organism / gene of interest only, this will greatly reduce the risk and problem of contamination. Furthermore, ordering highly purified LAMP primers and re-suspending them in a much more stable liquid such as TE buffer instead of double distilled water. Also making several smaller aliquots from the master solution could combat primer de-stabilisation since the same master solution doesn't get thawed and frozen repeatedly.

In vivo testing models indicated the Mont Aux Sources frog populations to still be infected with chytridiomycosis as suspected. The testing of archived African frog species allowed for the first positive results of the presence of chytridiomycosis in Botswana. These LAMP results were reproduced by both PCR and histology.

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Appendices

Appendix 1: Principle behind LAMP reaction

The conventional LAMP mechanism (without loop primers) and biochemical reaction steps are illustrated in figure 1A. The first step of LAMP is to get the starting material produced. Structure 1: either one of the inner primers (FIP in this case) hybridizes to the target DNA at region F2c initiating synthesis of the complementary strand. Structure 2: the outer primer F3 is a few nt bases shorter and lower in concentration than FIP, this allows slow hybridizing to the target DNA at region F3c initiating strand displacement synthesis releasing the FIP-linked complementary strand forming a stem-loop structure as illustrated in figure structure 4. This specific single DNA-strand acts as template for the BIP primer to initiate DNA strand synthesis followed by F3-primed displacement DNA synthesis once again. This displaced synthesized strand in structure 4 leads to the formation of a dumbbell DNA structure (structure 6) which undergoes self-primed DNA synthesis, quickly converting to a stem-loop DNA (structure 7) (Notomi, *et al.*, 2000).

The stem-loop structure serves as starting strand for the second step of LAMP, termed cycling amplification. The cycling amplification step is initiated when FIP hybridizes to the stem-looped DNA's loop (structure 7), priming strand displacement and DNA synthesis to generate an inverted complementary copy of the target stem-loop DNA (structure 7) along with a BIP loop (structure 8). From here self-primed strand displacement synthesis delivers a complementary strand (structure 10) of the target stem-loop DNA originally used (structure 7) as well as a gap repaired stem-loop DNA containing double copies of this target stem-loop DNA (structure 9). Products 9 and 10 serve as template for BIP-primed strand displacement synthesis in which structure 10 is designated to a recycling step (part of cycling amplification) yielding structure 20 which can be recycled in the cycling amplification step following self-priming to deliver a similar product to structure 7. Structure 10 following BIP-primed strand displacement synthesis also yields structure 11 and its by-product, structure 7 (recycled in cycling amplification step) (Notomi, *et al.*, 2000)

The delivered structures 9 and 11 serve as template for BIP-primed strand displacement synthesis in the elongation step. During the LAMP reaction the sequence of interest is amplified 3-fold during every half cycle. Final amplified DNA products are a combination of stem-loop- and cauliflower-like structures of various stem lengths and loops due to alternately inverted repeats annealing to the target sequence. All products in the elongation step derives from structure 12 which in turn derives from structures 9 and 11 (Notomi, *et al.*, 2000).

Figure 1B represents the LAMP mechanism when loop primers LF and BF are added to the reaction process. When no loop primers are added only the stem-loops containing F2c and B2c regions can be amplified with the FIP and BIP primers respectively. When loop primers are added into the reaction an additional two different stem-loops – F2/F1 and B2/B1 – can be amplified. The stem-loop regions between F1 - F2 and B1 - B2 is hybridized and subsequent amplification follows with the LF and BF primers respectively with the reaction proceeding from F1 to F2 or B1 to B2 - or

their respective compliments (F1c to F2c or B1c to B2c) depending from which strand the loop primers were developed. The use of loop primers reduces reaction time, increases reaction rate and increases specificity and sensitivity to a great degree (Eiken Chemical Co. Ltd., 2005; Nagamine, *et al.*, 2002; PrimerExplorer, 2005; Thekiso, *et al.*, 2011).

From figure 1B the loop primers begin working in throughout the elongation step by using the original LAMP products (figure) as templates. On structure 12 (one end product of the original LAMP process) one stem-loop DNA with a B1c-B2c region undergoes strand displacement DNA synthesis with LB (starting at B1c and going in the 5'-3' direction), when the 3' end is reached on the strand the synthesized DNA structure is displaced, yielding structure 15. Again on structure 12 another stem-loop DNA with a F2c region is hybridized and primed BY FIP for strand displacement DNA synthesis delivering structure 14 which in return yields structure 10 and its associated 'by-product' when primed for strand displacement DNA synthesis by FIP. This structure 10 and its associate get recycled back as structure 12. Structure 12 is one such example (from the many products of the elongation step in figure) which can be used by the loop primers for strand displacement DNA synthesis and figure represents all of the different products delivered from structure 12 alone (Nagamine, *et al.*, 2002).

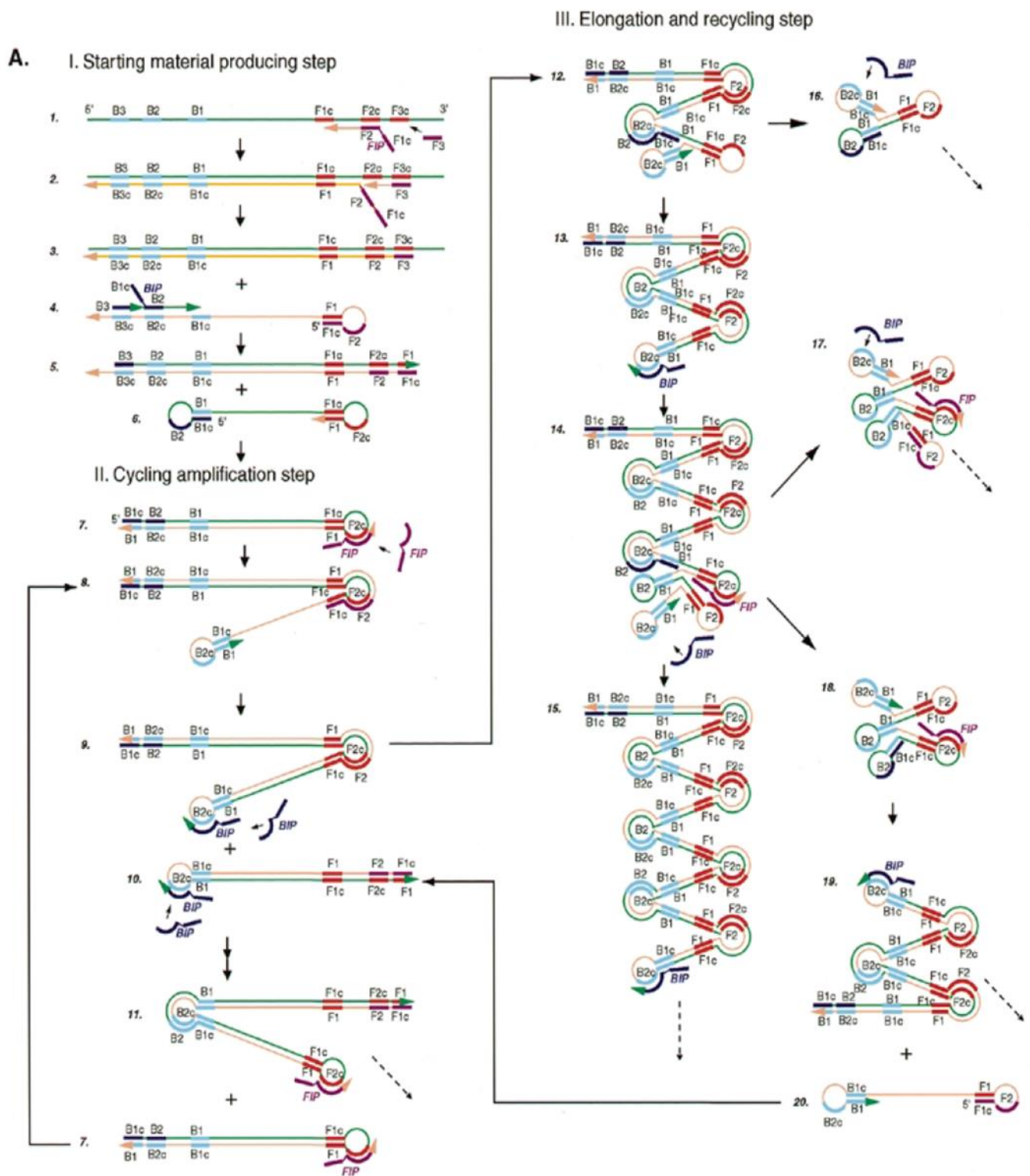


Figure 1A: LAMP reaction without loop primers

Appendix 2: Instructions pamphlet for final LAMP assay

The LAMP assay consisted of three pairs of primers, the outer primers (Bd4F3 and Bd4B3), inner primers (Bd4FIP and Bd4BIP) and the loop primers (Bd4LF and Bd4LB). Sequences are as follows:

Bd4F3	5'-TGAAGAACGCAGCGAAAT-3'
Bd4B3	5'-CCACTACTTTTTGTATCACTCAT-3'
Bd4FIP	5'-GAGTGCAATGTGCGTTCAAAG-ATACGTAATGTGAATTGCAAACC-3'
Bd4BIP	5'-TACATTGTCCGAATTGACTGGAC-ACTACTACTTTTAAAACCTGTCA-3'
Bd4LF	5'-GATTTAATGATTCACAAAG-3'
Bd4LB	5'-AGATATGAACCATGTCAA-3'

The kit used to develop the Bd LAMP assay was the OIMM. The protocol was optimised to deliver optimal results. The final reaction volume was 25µL which consisted of 15µL master mix, 5µL template DNA, 2µL primer mix and 3µL ddH₂O. Furthermore, the 2µL primer mix consisted of the following primer constituent relationship; $\frac{\text{outer primers}}{1 \text{ part}} : \frac{\text{inner primers}}{2 \text{ parts}} : \frac{\text{loop primers}}{1 \text{ part}}$. An isothermal reaction temperature of 58°C was used for a total reaction time of 50 minutes.

Appendix 3: Histology method

Fifty-nine specimens (from Zambia, Botswana, Mozambique and Tanzania) were taken from the African Amphibian Conservation Research Group (AACRG) collection. Toe clippings were taken from the specimens and stored in 70% EtOH. The clippings were subsequently subjected to various concentrations of alcohol and xylene as outlined in Figure 1 (as adapted from Weldon (2005)).

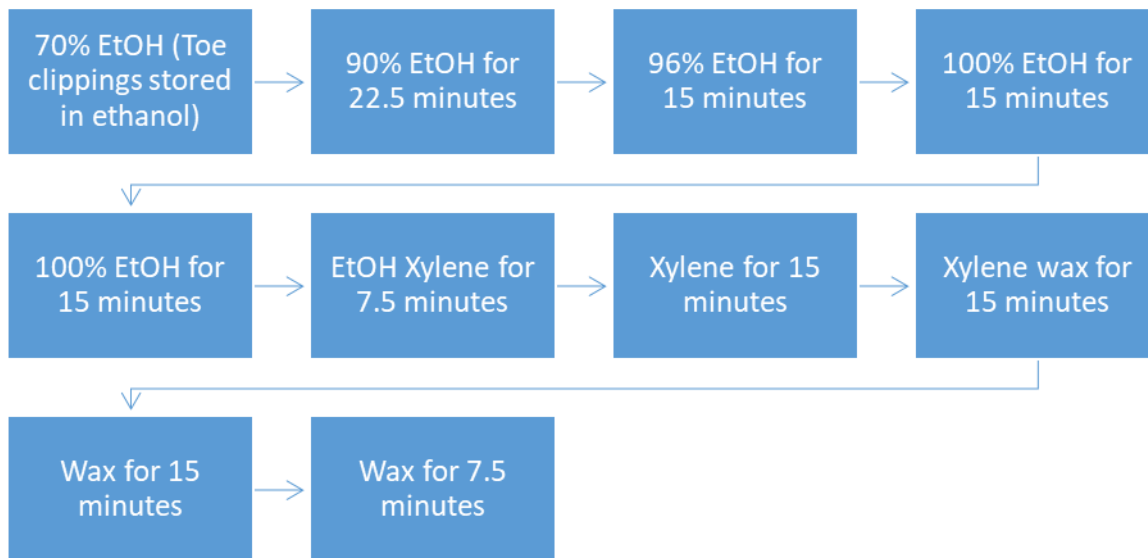


Figure 3A: The washing process of histology material.

The tissue was then embedded in wax blocks and cut in 0.6 μ m segments. The segments were then fixed on microscopic slides and left at 36°C for 24 hours. The slides were then stained as illustrated by figure 2 (as adapted from Weldon (2005)).



Figure 3B: The staining process of histology material.

After staining the slides, cover slides were fixed onto the slides using resin. The slides were then examined for signs of chytrid.