Development and validation of a qPCR assay for the non-invasive determination of fetal sex in cattle and African Buffalo

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ABSTRACT

The expansive possibilities and research progress of cell-free DNA (cfDNA) and cell-free foetal DNA (cffDNA) have engendered numerous articles published on a regular basis in recent years. However, this has mainly been centred on early diagnostic protocols and detection of different types of cancer or non-invasive genetic testing of human fetuses. Various challenges and novel aspects still exist regarding cfDNA and cffDNA when it comes to investigating human samples. Work on the animal side of the spectrum has been scarce and most studies have focused on mouse models to be used for human testing at later stages. Knowledge regarding the presence of cfDNA and cffDNA in cattle and African buffalo biological fluids is substantially insufficient. This study attempted to be the first to confirm the presence of cffDNA in maternal plasma of African buffalo.

The study used cffDNA isolated from cattle and African buffalo plasma samples to determine the foetal sex of the animals non-invasively. The aim of the study was to develop a practical and robust method that could be used on a daily basis by veterinarians. cfDNA was isolated from cattle and African buffalo plasma samples, confirming the presence of cffDNA in the maternal plasma of these animals. We attempted to amplify the isolated cfDNA by means of real-time PCR (qPCR) to confirm the presence of the sex determining region y (SRY) gene only present in male animals. After qPCR of the maternal plasma samples the results were compared to the sex of the calves after birth. Because of the contamination of the nuclease-free water that occurred during the qPCR step of the cattle samples, 50% of samples presented inconclusive results in both of the duplicates, while 27% of the results were correct in both duplicates and 23% of the samples amplified incorrectly. The contamination was eliminated during the qPCR step of the African buffalo duplicates. A total of 44% of samples were correct in both replicates, but only one of the nine male samples amplified in both replicates. A further 19% of male samples amplified in at least one of the replicates with no false positives present. Based on the high number of false negatives considerable work remains to improve these methods for future studies. This might include alternate and more sensitive methods to qPCR and more specialised isolation procedures.

Keywords: cell-free DNA, cell-free foetal DNA, real-time PCR, sex determining region y, non-invasive prenatal testing.

LIST OF ABBREVIATIONS:

DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
ssDNA	single strand DNA
dsDNA	double strand DNA
fDNA	fetal DNA
SRY	sex determining region y
cfDNA	Cell-free DNA
cffDNA	Cell-free fetal DNA
gDNA	genomic DNA
HMG	High motility group
RhD	Rhesus D
NIPT	non-invasive prenatal testing
FDA	Food and Drug Administration
PCR	Polymerase chain reaction
qPCR	real-time PCR
NTC	non-template control
PC	Positive control
NC	Negative control
Cq	Quantification cycle
dNTPs	deoxyribonucleotide triphosphates
LOD	limit of detection
SD	Standard deviation
HS	High sensitivity
(PPT™)	Plasma preparation tube

SOP	Standard operating procedure

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CHAPTER 1 – INTRODUCTION TO THE SOUTH AFRICAN WILDLIFE INDUSTRY

1.1 The South African wildlife industry

South Africa is host to a unique breeding environment when it comes to the wildlife industry. According to a presentation by the Department of Environmental Affairs in 2018, South Africa has the largest allocated area for commercial/ private wildlife ranching in the world (Environmental Affairs RSA, 2018). This unique industry has played a crucial role in the conservation of certain species, but it also expanded into a lucrative breeding industry. In a study published in 2016, the number of large wildlife animals in South Africa increased from 575 000 in 1960 to more than 6 million in 2016 (Crowley, 2016). One of these industries involves disease free buffalo projects. It is estimated that there are roughly 25 000 – 30 000 disease free African buffalo (*Syncerus caffer*) in South Africa. All of these animals have to be put into quarantine and have to undergo certain required tests when the breeder wants to sell them. Most of these animals are female because only one bull is required in a breeding herd consisting of approximately 30 females. It is every breeder's goal to sell as many pregnant cows as possible because they fetch higher selling values. However, when the calves are born, their values differ significantly. Female calves are sometimes worth seven times more than bull calves. In 2016, the average heifer prices on *Vleissentraal* auctions were R 200 000.00 while the average price for a bull calf was R58 363,64 (Vleissentraal, 2016). Hence the need for a test that can accurately determine the sex of the unborn calf without putting additional stress on the calf or mother.

Non-invasive prenatal genetic diagnosis and sex prediction for foetuses during early pregnancy is now a very real possibility due to the discovery of foetal DNA (fDNA) in maternal circulation (Lo et al., 1997). Various studies (Payen and Cotinot, 1993, Bryja and Konecny, 2003) have shown that Y-chromosome DNA sequences can be used as probes for sex determination. Pomp et al. (1995) and Sánchez et al. (1996) first designed primer sequences to amplify the sex determining region y gene (SRY gene) in various species. The SRY gene is known as the sex determining region only found in males with a Y sex chromosome. Bryja et al. (2003) adapted and used these primers to successfully amplify the SRY gene in 40 different species for confirmation of sex. Extrapolating the findings made by Bryja and Konecny (2003) to accurately determine foetal sex, however, adds additional uncertainty as fDNA is fragmented in maternal circulation. Also, fDNA in maternal circulation (cffDNA) is only present in very low concentrations and does not provide stable measurements when using conventional sampling methods. Thus, alternate methods need to be investigated to sample and extract cffDNA to improve the accuracy of these tests.

In the case of the African Buffalo, it is not known whether fDNA is even present in maternal plasma or serum. fDNA has, however, been reported in cattle (Wang et al., 2010). Although the African Buffalo and cattle are genetically different (Buntjer et al., 2002) their anatomy and reproductive systems are similar, while the main difference involves smaller ovaries in buffalo (Ali, 1994). This might indicate that fDNA is also present in maternal plasma or serum in buffalo. A fast and reliable method for bovine embryo sexing has been developed by Lu et al. (2007) by employing the amplification of the bovine high motility (HMG) box of the sex-determining region of the SRY gene. However, the method was only used on transferred embryos. Using cells from an artificially inseminated embryo is a tedious process that requires highly skilled personnel and complicated equipment while, on top of this, it is time sensitive and poses various risks for the embryo, leaving many of them damaged and unusable. The method uses a micro-manipulator to extract cells from the nucleus or fluid from the cytoplasm. As indicated, this is time consuming and extremely specialised. Thus, a method to determine foetal sex non-invasively in cattle and buffalowould be of considerable value.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

In the relatively new field of cell-free foetal DNA (cffDNA) research in animals, a significant number of unknown elements and factors have remained present even in recent studies. Most of these concern the practical implications of working with these types of samples. For instance, a standard protocol for handling and processing cell-free DNA in humans indicates that the samples must be processed within six hours. Even in the case of domesticated animals such as cattle this can become quite difficult to achieve - all the more so when working with large quantities of animals. If, as is the case in wildlife contexts, a veterinarian has to collect the samples in the field this becomes nearly impossible. Establishing an optimised workflow that includes the most recent innovations might therefore prove critical. In the present study, we will try to elucidate some of the unknown aspects including proper collection, handling and quantification of cffDNA in maternal blood of cattle and buffalo, while simultaneously using the results to develop a commercial foetal sex determination test for farmers/ breeders.

2.2 Brief history of cell free DNA (cfDNA) and cell free foetal DNA (cffDNA)

Scientists have noted the transmission of DNA from external sources such as pathogens to the host as early as the 1940s (McCarty and Avery, 1946), but the method used to achieve this transmission has eluded researchers for years. Mandel (1948) discovered the presence of DNA outside the confinement of cells (cfDNA), which further intrigued researchers. However, these discoveries preceded the structural definitions of DNA and thus further exploration was strained. Emlen and Mannik (1982) were the first to notice the effects of systemic lupus erythematosus on clearance rates of “small fragmented” DNA in the blood of mice. This was later classified as cfDNA. This was the first among many investigations around the presence of cfDNA in animals (Lo et al., 2000).

All DNA fragments in the bloodstream that are not confined or encapsulated is referred to as cell-free DNA (or cfDNA) (Brune, 2017). These fragments have various points of origin that are still debated, but the widely accepted hypothesis is that it originates from apoptosis, necrosis or active release of cells (see Figure 1). The portion of cell-free DNA that originates from tumour cells is called circulating tumour DNA (or ctDNA). Normally, these fragments are cleaned up by macrophages but in cases of cancer it is believed that the overproduction of cells leaves more cfDNA behind (Bronkhorst et al., 2015). Still, this does not account for fDNA fragments found in maternal blood. It is speculated that the transmission of foetal cells to maternal blood through the placenta accounts for the presence of cffDNA fragments. In other

words, foetal cells enter the mother's bloodstream where they are viewed as foreign. The immune system reacts by destroying the cells whereby, as in the case of ctDNA, macrophages clean up the fragments (Bronkhorst et al., 2015). These ctDNA and cffDNA fragments average around 170 bases in length. ctDNA has a half-life of approximately two hours and is present in early- and late stage disease in many common tumours including non-small cell lung and breast cancer. The concentration of cffDNA increases throughout pregnancy as more cells enter the mother's bloodstream, but is cleared rapidly after birth (Phillippe, 2014). That said, normal cfDNA concentrations vary greatly, occurring at between 1 and 100,000 fragments per millilitre of plasma. Phillippe (2014) noticed a gradual increase of foetalDNA (fDNA) in maternal plasma as pregnancy progresses with a sudden 1.67 x spike in the last two months. Phillippe (2014) hypothesised that higher fDNA fragments in the maternal circulation might be a trigger for parturition or might be responsible for those hormonal changes that trigger the birthing process.

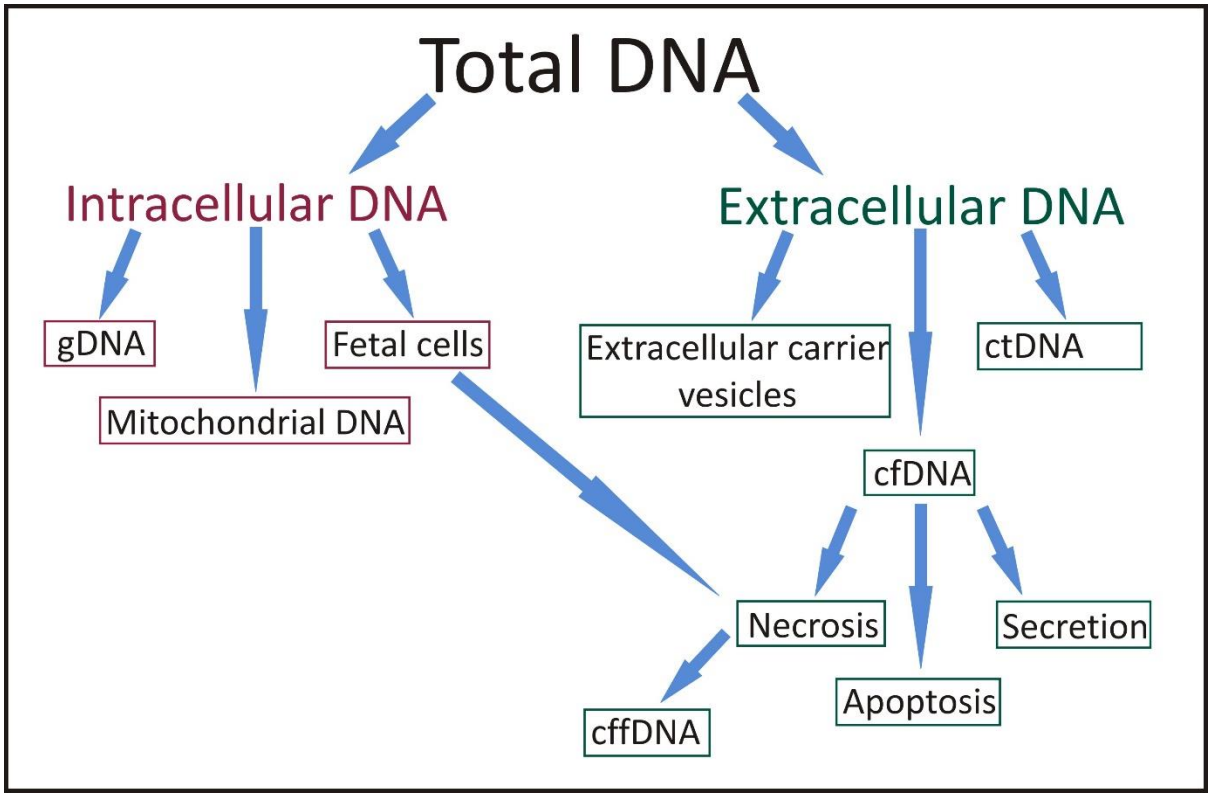

Figure 2.1: ***Breakdown of the total DNA present in vascular circulation of maternal blood.***

2.2.1 Foetal (fDNA)

Lo et al. (1998) note that the phenomenon of foetal nucleated cells in maternal plasma was discovered as early as 1969. Since then it has been a sought-after goal to use these cells for

non-invasive prenatal diagnosis but the research techniques and technologies continued to lag behind, remaining inadequate to achieving the goal. Schröder and de la Chapelle (1972) first noted foetal lymphocytes in human maternal blood and studies soon followed to use this discovery to study Y-linked foetal hereditary diseases from the mother's blood. Initial studies only focused on Y-linked diseases in humans as there was no way to distinguish between the mother's DNA and the DNA of a female foetus. Lo et al. (1998) describe methods developed by Bianchi et al. (1993) and Cheung et al. (1996) that made significant advances in the isolation and enrichment of foetal cells from maternal circulation. These techniques were crucial for the earlier studies of non-invasive molecular testing.

2.2.2 Cell-free foetal DNA (cffDNA)

The first notation of cell-free foetal DNA (cffDNA) was made in 1989 when Lo et al. used cffDNA to amplify a Y-linked gene from maternal plasma of humans, thus confirming the presence of foetal DNA in maternal plasma.

Because the methods to isolate and enrich foetal cells described by Bianchi et al. (1993) and Cheung et al. (1996) were time consuming and labour intensive, Lo et al. (1997) set out to investigate other sources of fDNA. They showed that plasma and serum were reliable sources of fDNA. This was one of the first confirmed sources of fDNA occurring outside the confinement of cells inside of maternal plasma and serum, that is, cffDNA. Lo et al. (1998) developed a method to quantify the fDNA for use in methods such as real-time PCR. At that time, this was considered to be a turning point that opened up the field for research to develop diagnostic tests that could predetermine certain genetic defects.

These advances increased the likelihood of the use of cffDNA for accurate prenatal diagnostic tests. Avent (2008) explored ways to conduct Rhesus D (RhD) blood group incompatibility testing between mother and foetus including RhD genotyping by non-invasive means instead of using invasive chorionic villus extractions. This became the first large-scale application of non-invasive prenatal testing (NIPT) for diagnostic purposes. The basis of the test was subject to a mother being RhD-negative, thus a positive RhD amplification in a PCR run indicated an RhD-positive offspring. Unfortunately, these initial tests were based on the belief that all negative phenotypes were engendered by the complete deletion of the RHD gene. This proved incorrect as certain ethnic groups showed only partial deletion or mutated RHD genes. More comprehensive tests are currently available to include these groups.

In 2012, Nicolaides et al. developed a routine cfDNA check that provided a risk score for trisomy 21 and 18. This test proved to have a 0.1% false-positive rate with less than 5% failure. Okun et al. (2014) determined that non-invasive prenatal testing increased Down syndrome

detection while also decreasing the number of amniocenteses performed. This is significant as it reduced the need for highly invasive amniocenteses. Currently, cffDNA tests are used mainly for screening purposes and not diagnostics. Companies that offer screening tests to assess the risks related to the foetus include Minipcr (https://www.minipcr.com) and Lab tests online (https://labtestsonline.org). These tests are mainly performed on mothers who present high risk factors such as high maternal age, pre-existing medical conditions, high blood pressure or familial history of genetic abnormalities. It is important to note that these tests are not Food and Drug Administration (FDA) approved yet and, thus, can only be used for screening purposes.

2.2.3 cfDNA and cffDNA research in animals:

Research done on foetal DNA found in maternal blood proved a rarity as most studies only focused on collecting foetal DNA directly from the placenta (Hooper et al., 1991, Bokar et al., 1989, Gerschenson and Poirier, 2000, Olson and Massaro, 1977). Research on cfDNA and cffDNA in animals have focused mainly on a small number of species that have included mice, bovids and horses. Below is a summary of these studies.

In 2004, Khosrotehrani *et al* investigated the relevance of cffDNA in mouse models to see whether they correlated with characteristics in humans. It was found that the clearance of the fDNA from maternal blood after delivery was similar to the process in humans. They also found that allogenic mating displayed higher fDNA concentrations when compared to congenic matings. Furthermore, they concluded that foetomaternal trafficking could be used in mouse models for testing. Foetomaternal trafficking is the movement or exchange of biological material between the mother and the foetus, normally through the placenta

Lemos et al. (2011) mention that the phenomenon of cffDNA in maternal circulation as found in humans is not well known in bovids. They ascribe the lack of information to structural differences of the placenta (Lemos et al., 2011). The synepitheliochorial placenta in bovids has no direct contact with the maternal blood circulation, which and discouraged researchers in investigating the possibility of cffDNA in the maternal plasma. However, Lemos et al. (2011) not only accurately confirmed the presence of cffDNA in maternal plasma of cattle, but simultaneously correctly determined the sex of the unborn foetuses.

As in humans, most cfDNA research topics have centred on tumour-derived circulating DNA studies in mouse models and how this translates to humans (Barták et al., 2018, {Emlen, 1982 #83, Rakhit et al., 2011), }. In animals, the development of non-invasive diagnostic tests have not been performed successfully and, as mentioned, only to a certain degree in humans. Most animal studies in cattle, goats, horses and others have focused on using cffDNA for foetal sex

determination (instead of genetic related abnormalities). All of these with varying degrees of success (see table 1) , (Bryja and Konecny, 2003, Lu et al., 2007, Lemos et al., 2011, Tavares et al., 2015, Davoodian and Kadivar, 2016). A NESTED-PCR approach was employed by Kadivar et al. (2016) and they achieved an 88% accuracy in equine samples. In their turn, Davoodian and Kadivar (2016) conducted various studies and developed separate tests to determine foetal sex in common farm animals.

Table 2.1: Summary of the studies that determined foetal sex in animals including the technique used, gene that was targeted and the accuracy of the test.

Species	Gestation (weeks)	PCR technique	Specificity	Gene	Reference
Horse	12	PCR	85%	SRY	de Leon et al. (2012)
Horse	12	2nd PCR & qPCR	95%	SRY	de Leon et al. (2012)
Horse	8-20	qPCR	88%	SRY & GAPDH	Kadivar et al. (2016)
Cattle	4-8	PCR	60%	SRY	Xi (2006)
Cattle	4-36	Nested-PCR	100%	SRY	Wang (2010)
Cattle	4-38.5	PCR	100%	TSPY	Lemos (2011)
Cattle	8-38	PCR	99.9%	Y-specific amplicons	da Cruz et al. (2012)
Goat	8-17	qPCR	100%	SRY	Kadivar et al. (2013)
Goat	8-18	qPCR	93.3%	Amelogenin	Kadivar et al. (2015)

2.3 Physiological characteristics of bovids

2.3.1 Bovids

Bovids, as the name implies belonging to the family Bovidae, are classified as cloven-hoofed animals and include antelopes, sheep, goats, cattle, buffalo and bison. Bovidae/ bovinae carry horns that consist of a sheath that covers a bony core that is capable of growing. This differs from other species like rhino or other ruminants such as deer that carry horns that consist only of a softer hairy layer (Estes, 1999).

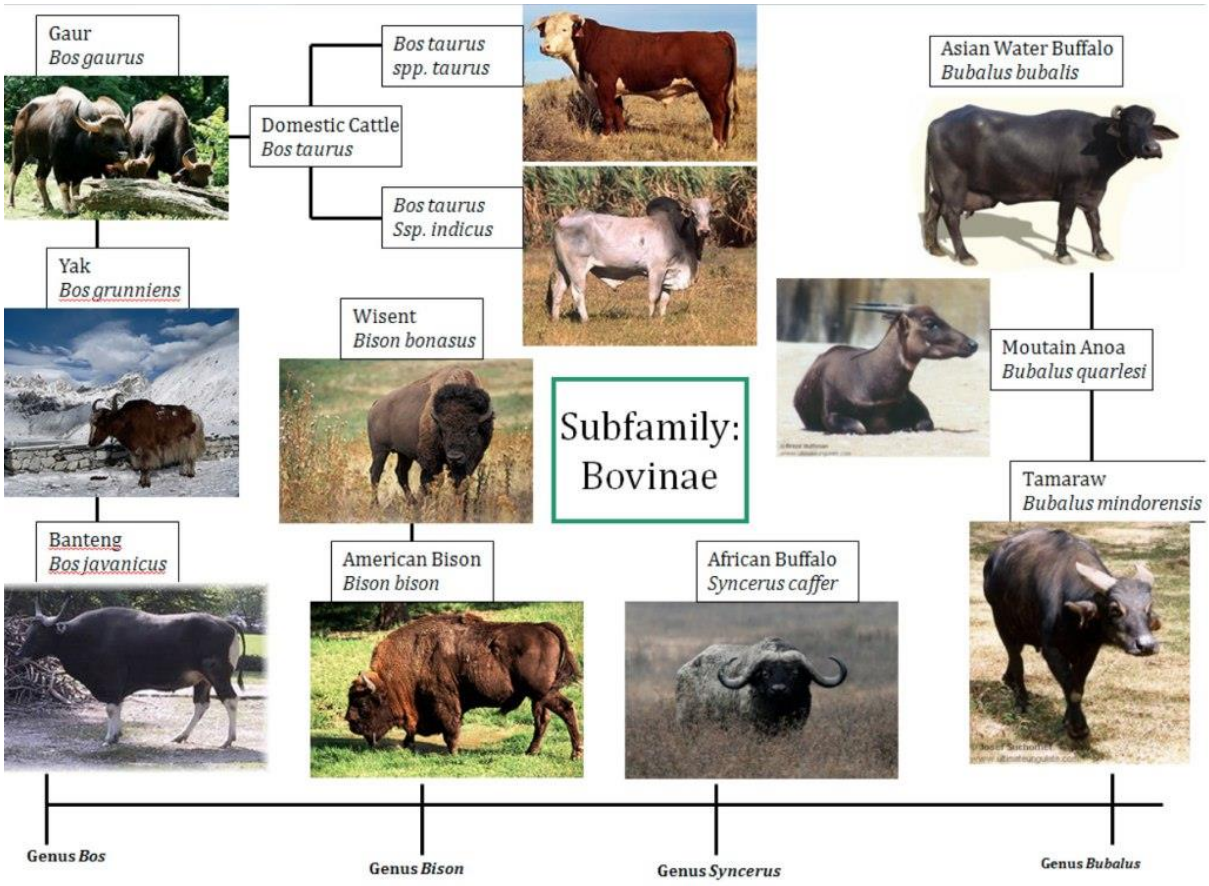

Figure 2.2: ***This Figure shows the differences between genuses and species within the Bovidae family. The Bos genus includes significantly more cattle species than what we used in this study. Only Bos taurus and Bos indicus are commercially used in South Africa.***

2.3.2 Cattle (*Bos taurus* and *Bos indicus*)

Cattle have been domesticated all over the world with a view to utilising their meat, milk and leather among others (Britannica, 1999). In general, all domesticated bovids are considered as cattle including, in some literature, the Asian water buffalo, yak and bison (see Figure 2). The present study will restrict the term to domestic cattle that are used for farming purposes in South Africa such as *Bos taurus*, *Bos indicus* and crossbreeds. Most breeds did not exist until very recently as certain breeds were selected for various purposes such as size, milk production and so forth.

2.3.3 African buffalo (*Syncerus caffer*):

The African buffalo is the largest of the African bovid species and weighs up to 900 kg (Estes, 1999). On average, bulls weigh 100 kg more than cows and they display thicker and wider

horns, which are quite predominant in mature bulls. They are widely adapted to various terrains and are interestingly immune to certain diseases that plague domestic cattle and other bovids such as bovine sleeping sickness which is transmitted through the tsetse fly. However, they are susceptible to cattle-borne diseases such as rinderpest which killed more than 90% of the African buffalo in the 1890s and other diseases such as foot-and-mouth and bovine tuberculosis continue to cause a reduction in their numbers after the subsequent recovery in recent years (Estes, 1999). African buffalo have a gestation period of 11 months and when they are in good health they can undergo oestrus three to four weeks after birth.

2.3.4 Diversity of mammalian placenta

Although all placentas have different morphologies, they all consist of foetal trophoblast cells and maternal uterine cells (Nakaya and Miyazawa, 2015). There are four basic morphologies as presented in Figure 3 below, and these differ among species. Humans and mice for instance possess a haemochorical (discoidal) placenta while bovids like cow and sheep mainly possess a synepitheliochorial (cotyledonary) placenta. The other two types of placentas are diffuse ones as found in horses and pigs and zonary ones present for instance in cats and dogs. The main difference is a retained endometrial epithelium layer in epitheliochorial and synepitheliochorial placentas, while it is degraded in endotheliochorial and hemochorial placentas (Nakaya and Miyazawa, 2015). This additional layer increases the regulation of substances that are interchanged between foetus and mother. Nakaya and Miyazawa (2015) speculate that this is the main reason for the lower concentration of fDNA in bovids when compared to humans.

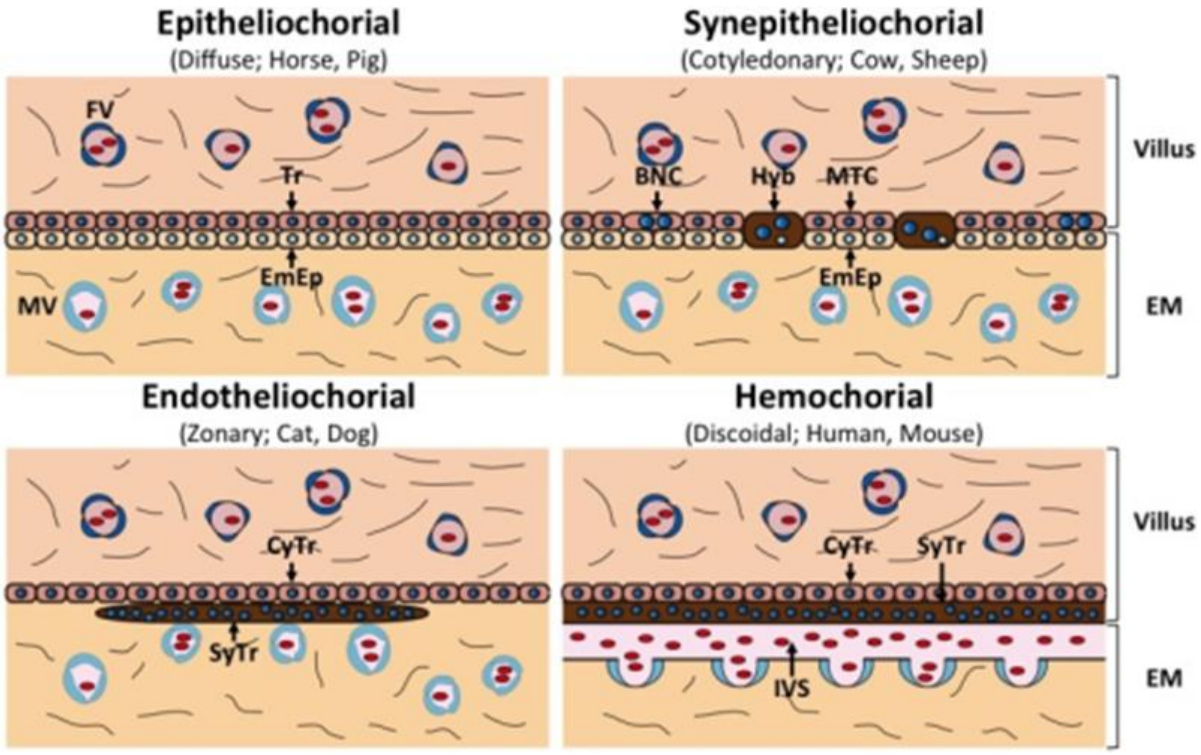

Figure 2.3: ***Structure of different types of placentas. The foetomaternal interfaces of the placentas are represented. The endometrial epithelium is retained in epitheliochorial and synepitheliochorial placentas, while it is degraded in endotheliochorial and hemochorial placentas. Abbreviations: FV; Foetal blood vessel, MV; Maternal blood vessel, Tr; Trophoblast, EmEp; Endometrial epithelium, BNC; Binucleate cell, Hyb; Hybrid cell, MTC; Mononucleate trophoblast cell, CyTr; Cytotrophoblast, SyTr; Syncytiotrophoblast, IVS; Intervillous space, EM; Endometrium.***

2.4 Gender determining genes in mammals

2.4.1 SRY gene

SRY is a gene that is male specific in most placental animals (Sinclair et al., 1990a). It is a single copy normally located on the pairing region of the Y-chromosome. However, some rodents presented exceptions to this. Some studies even revealed that they found multiple copies on the Y-chromosome (Bianchi et al., 1993, Bullejos et al., 1999), while other studies have even found copies on the X-chromosome (Bullejos et al., 1997). Just et al. (1995) and Vogel et al. (1998) found that the SRY gene was absent in two mole-vole species

and Soullier et al. (1998) also did not find a SRY gene in two sub-species of the spiny rat. Bryja and Konecny (2003) used the available SRY sequences or sequences determined by themselves to develop rapid sexing methods in various species that normally require more invasive methods. This was also effective in the cases of species that only showed differentiation in later phases of development. Building on studies such as those of Sánchez et al. (1996), Bullejos et al. (1999) and Pomp et al. (1995) they developed sexing methods and primer sets for more than 40 species including insects, rodents, mammals, primates and more.

2.4.2 HMG boxes

High motility group boxes (HMG-boxes) are high affinity domains involved in protein or DNA binding. Inside each HMG box a variety of HMG-related protein structures are found. They are involved in various areas of the regulation of DNA related processes such as translation, repair and replication. Binding of proteins or DNA to a specific HMG-protein causes conformational changes associated with the activation of the relevant process (Štros et al., 2007). The SRY-related DNA-binding domain called the SRY HMG-box is conserved across various species of placental mammals (Sinclair et al., 1990b, Gubbay et al., 1990). Among others, it contains the HMG1 and HMG2 proteins as well as the SRY gene binding domain that facilitates its replication. It has been around for approximately 1 000 million years and has been found in mammals, plants, yeast and insects. It is highly conserved among various species. It consists of approximately 79 amino acid residues.

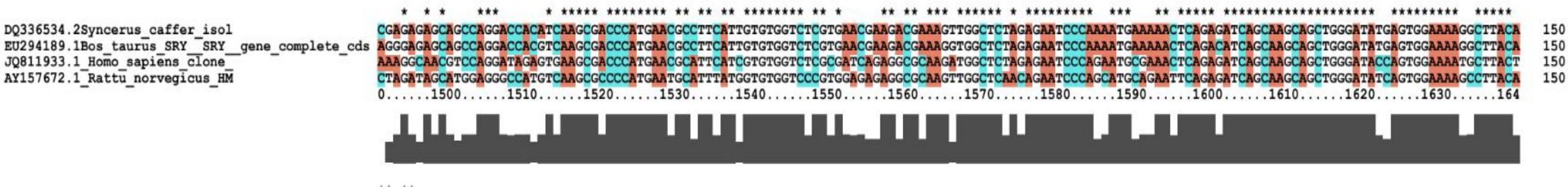

Figure 2.4: Shows the conservation of the SRY HMG-box in various species.

2.5 Methodological literature

2.5.1 Introduction

As demonstrated by Bronkhorst et al. (2015), cfDNA is an extremely difficult sample type to work with. Bustin et al. (2009) state that various technical deficiencies affect assay performance and include the following: a) inadequate sample storage, preparation and nucleic acid quality; b) poor choice of primers and probes for the PCR and c) inappropriate data and statistical analysis that lead to misleading results. This list covers faulty aspects that arise before, during and after the use of qPCR tests. Thus, when designing a protocol aimed at amplifying cfDNA, all aspects from collection to validation have to be carefully considered and selected. Quantification calibrators and controls are required for all qPCR reactions. It is therefore recommended that a positive control (PC), negative control (NC) and non-template control (NTC) be used in all qPCR runs (Bustin et al., 2009). PCs are ideallyextracted nucleic acid sequences that indubitably contain the sequence that will be amplified by employing the designed primers. Such PCs are essential to monitoring assay variation between runs, to ensure that the primers are still functional and that the reaction took place successfully (Bustin et al., 2009). A NC embodies a control of a similar biological sample as the positive control, but where the target sequence is definitely absent. A good example is a biological sample tested for specific viral RNA where a PC would be a sample that is contaminated with the virus while the NC would be a sample that definitely does not contain the virus. An NTC is a control reaction that contains all the reagents needed for the reaction but without template DNA/ RNA. NTCs detect PCR contamination when probes are used and they also distinguish unintended amplification products such as primer dimers (Bustin et al., 2009). NTCs should be included in every batch of samples and conditions should be established around where a quantification cycle (Cq) limit should be positioned for an assay, as no amplification is supposed to take place. The Cq value indicates the number of the cycle where the fluorescence of the target sequence's amplification exceeds the background fluorescence. For example, if the PC has a Cq of 32, a Cq value exceeding 40 of a NTC might show primer dimers and all subsequent amplification should be interpreted as such, while all amplification of the NTC below a Cq value of 35 might indicate contamination in a reagent. Below follows a brief discussion of the various considerations around each step as well as aspects that might influence results.

2.5.2 Choosing a sample matrix

Choosing a sample matrix that is reliable is one of the main considerations when designing a protocol. Various studies have proven the presence of cfDNA in urine, serum

and plasma. In bovids, however, attaining urine samples can prove difficult. Although some studies note that cfDNA was more abundant in serum than in plasma, the latter is more stable and less likely to be contaminated by cellular DNA (Lee et al., 2001; Van der Vaart and Pretorius, 2010; Board et al., 2008; Chan et al., 2005; Lui et al., 2002). Chiu et al. (2013) also attribute the higher concentrations of cfDNA in serum to contamination of cellular DNA rather than higher expulsion into serum when compared to plasma.

2.5.3 Collection and processing of samples

The *Minimum Information for Publication of Quantitative Real-Time PCR Experiments* (MIQE) mentions that sample collection and the methods that accompany the collection are among the most important aspects that influence experiments. This is also the first source where experimental variability may occur. Samples that are collected should include a brief description and report any incidences that might affect the sample, for instance when animals were stressed or if the sample was processed after the allotted time indicated in a protocol (Bustin et al., 2009)

Bronkhorst and co-workers note that various factors affect the concentration and measurement of cfDNA and propose certain requirements and variables when sampling cfDNA (Bronkhorst et al., 2015). These requirements were stipulated on comparing various other studies:

- Anticoagulants in blood collection tubes might affect isolated cfDNA if samples are not processed within 6 hours after collection (Lam et al., 2004). This should be an important consideration when developing a collection process.
- Storage conditions after collection, but before processing, greatly influence cfDNA yield. If samples are not stored at -80^{0}C within 6 hours after collection, there is an enormous spike in cfDNA yield in normal collection tubes although this is most probably due to gDNA contamination (El Messaoudi et al., 2013). Bronkhorst et al. mention that this time-limiting factor can be overcome by using Streck cell-free DNA™ blood collection tubes (Streck). When processing samples, it is important to remove material that might contaminate cfDNA or release gDNA (van Wijk et al., 2000). Minimising gDNA contamination is imperative to ensure less competition with low amounts of cffDNA during extraction and amplification.
- Protein digestion/ denaturation before extraction: Bronkhorst et al. conclude that protein digestion/ denaturation before extraction is an essential step to increase cfDNA yield. Exact reasons for this increase are not known, but it is speculated

that some of the cfDNA is bound to proteins and/ or enveloped in protein vesicles. Protein digestion then releases the cfDNA and an increase in their concentration after isolation results.

- Types of tubes used: using Vacutainer, S-Monovette, EDTA or Streck tubes carried no significant difference in yield when samples were processed promptly after collection (Gautschi et al., 2004). When collected samples can only be processed after longer periods cell-free DNA™ blood collection tubes performed best (Bronkhorst et al., 2015). However, these tubes are significantly more costly than other tubes: they can be up to 60 times more expensive as they are not readily available in South Africa and need to be imported.

2.5.4 Isolation of DNA

Extracting nucleic acids is the second critical step subsequent to collection and processing of samples. Efficiency depends on adequate homogenisation, sample type, target density, physiological status, genetic complexity and the amount of biomass processed. Providing adequate information around these steps is crucial for accurate reproducibility of the results. If used, information regarding the methods for concentration determination of the nucleic acids should also be included in detail. This is of particular importance when working with samples that are unstable after various thawing cycles (Bustin et al., 2009).

Isolation protocols and kits for cfDNA have become increasingly available over the previous couple of years and the most common include phenol-chloroform methods, salting-out, Guanidine/ Promega Wizard resin, magnetic bead based extractions and conventional column-based kits adapted for smaller fragments.

2.5.4.1 Magnetic bead based DNA extraction

Magnetic separation of nucleic acids has become a very accurate and efficient method over recent years. Particles that encompass a magnetic charge, such as nucleic acids, can be removed by using a magnet and applying a magnetic field (Tan and Yiap, 2009). Nucleic acids are generally considered to present a negative charge because of the negatively charged phosphate backbone in the DNA structure. Magnetic carriers are then designed that bind to the nucleic acids. Different types of carriers have already been designed and include synthetic polymers, biopolymers, porous glass or inorganic magnetic materials. Larger surface area materials such as beads are generally preferred for nucleic acids. The rounded structure of the beads also increases the strength of the bond as the nucleic acid wraps around the bead. The magnet is then activated and pulls

the bead-nucleic acid combination to itself and away from the rest of the sample (Tan and Yiap, 2009).

Commercial kits for separation have become available and are based on an alkaline lysis procedure followed by binding the nucleic acids. The contaminants are washed away by a wash buffer after which the nucleic acids are eluted with the elution buffer. Advantages of these kits are considerable and include the following: no organic solvents, less centrifugation steps, no vacuum filters and no column separation (Tan and Yiap, 2009).

Other methods include encapsulating the particle in polymers that are magnetised under specific conditions. These involve magnetisation of magnetisable cellulose in the presence of certain salt concentrations. This can be used when only certain sizes of nucleic acids are required. Higher salt concentrations bind smaller fragments and vice versa. Although suppliers for commercial cfDNA isolation kits do not stipulate concentrations or reagents in their kits, it can be assumed that these kits have a higher salt concentration to extract smaller fragments by preference.

Bronkhorst et al. (2015) found varying results obtained from different studies that tested commercial cfDNA isolation kits. Legler et al. (2007) tested various methods for achieving foetal DNA extraction from maternal blood and found that the QIAamp DSP Virus Kit performed best. However, an automated method as compared by Bratz et al. (2016) also showed reliable results. It is notable furthermore that automated methods yielded more DNA than conventional column extraction methods. Amplifying a gene that is not present in maternal gDNA such as SOX9 or SRY substantially reduces the risk of gDNA competition for dNTPs. Moreover, using an isolation method that favours shorter DNA fragments further reduces risk. This method will be discussed in the remainder of the present study.

2.5.5 KingFisher Duo prime (ThermoFisher, Cat.no. N16622)

KingFisher Duo prime is an automated system for the purification of nucleic acids and various protein and cell separation applications. The instrument uses magnetic rods to move particles through the various purification-, binding-, washing- and elution phases to yield high quality DNA, RNA or proteins. This automated method minimises contamination and impurities with a view to down-stream applications. The system includes pre-programmed protocols as well as software to customise protocols during optimisation. Up to 24 samples can be accommodated per run with up to 4mL of reagent per well, but the cfDNA purification protocol only accommodates six samples per run as the reagents need to be kept separate. This kit is optimised for fragmented DNA and

yields higher peaks for 100bp – 275bp fragments; however, up to 7000bp fragments have been extracted (Biosystems, 2017).

2.5.6 Proteinase-K treatment

Proteinase K is a serine-based protease that exhibits a very broad cleavage specificity. It is produced by the fungus *Tritirachium album* and works by cleaving specific peptide bonds that are adjacent to the carboxylic group of aliphatic and aromatic amino acids. It is therefore useful as a general digestive of proteins in biological samples. Proteinase K has also been purified further for RNase and DNase activity and can thus be used for preparation of chromosomal DNA in a variety of applications such as gel electrophoresis, protein fingerprinting and removal of nucleases from DNA and RNA (Promega, 2017). As mentioned, it is speculated that some of the cfDNA fragments are either protein-bound or encapsulated in protein based vesicles. Protein digestion is therefore essential to release these cfDNA fragments for isolation.

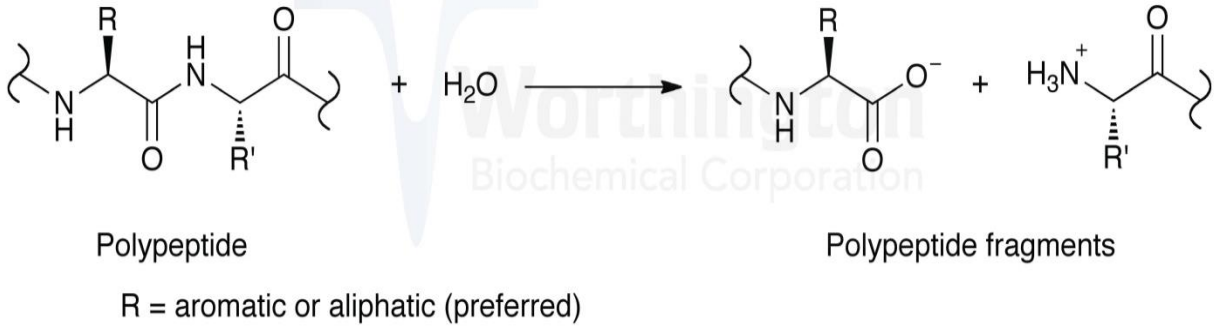

Figure 2.5: Enzymatic reaction of proteinase K (www.worthington-biochem.com)

2.5.7 Quantification of DNA and cffDNA

Several methods are well-known in the quantitation of nucleic acids in solution. Earlier methods employed included colour reactions of which the most frequently used were based on the reaction between deoxyribose and diphenylamine for the determination of DNA (Dische, 1930). Because of several interactions with other compounds such as sialic acid as well as the time constraints of the method, it has been adjusted a number of times over the past years by altering relative volumes of sample and reagent, increasing diphenylamine or replacing sulfuric acid with acetaldehyde in the recommended protocols (Burton, 1956). Fundamentally, the method was a colour reaction of DNA with diphenylamine in a mixture of acetic and sulfuric acid. The mixture

was then incubated and the fibrous tissue removed and washed with 80% ethanol. These were then compared with the same reaction mixture of known DNA concentrations to determine a relative DNA concentration (Dische, 1930).

In 1966, (Le Pecq and Paoletti) developed a more accurate method by staining double stranded DNA with ethidium bromide. A direct correlation was found between the fluorescence of the stained DNA and the DNA concentration. Furthermore, no other biological interference was found. The absorbance of the sample at 260nm was then measured to determine the concentration. However, further adjustments was found to be necessary to determine the exact concentration, such as subtracting turbidity (A320nm measurement) and adjusting for dilutions. This is still the most commonly used method. Most modern fluorometers adjust for these values automatically.

2.5.7.1 BioAnalyzer - DNA size determination

The Agilent BioAnalyzer is a lab-on-a-chip approach to gel electrophoresis and flow cytometry. It increases the speed of gel electrophoresis while drastically reducing sample volume. Available chips can be used for DNA/ RNA analysis, protein analysis and cell analysis. Separate glass wells and disposable chips reduce cross-contamination between samples (CMMT, 2003).

For the purpose of DNA assays the method is used to determine size and quantitation of the relevant sample.

Figure 4 presents an example of a BioAnalyzer run. Left displays gel electrophoresis results. The ladder (well 1) includes upper- and lower internal markers (50 bp and ~10 000 bp for this run) with a view to quantification purposes. Figures on the right show the fluorescence of differently sized DNA fragments compared to the duration of electrophoresis. Higher peaks indicate higher concentrations of the relevant size of a fragment. Consider in this context, though, that lack of consensus on the origin of cfDNA have stalled the formulation of a standardised definition and size description for cfDNA. It is however well known that cffDNA makes up less than 5% of the total DNA present in maternal circulation, as indicated by Jorgez and Bischoff (2009). These authors also used size separation methods to determine that more than 50% of cffDNA occurred between 100-300 bp and only 10% was maternal cfDNA in human maternal samples (Jorgez and Bischoff (2009).

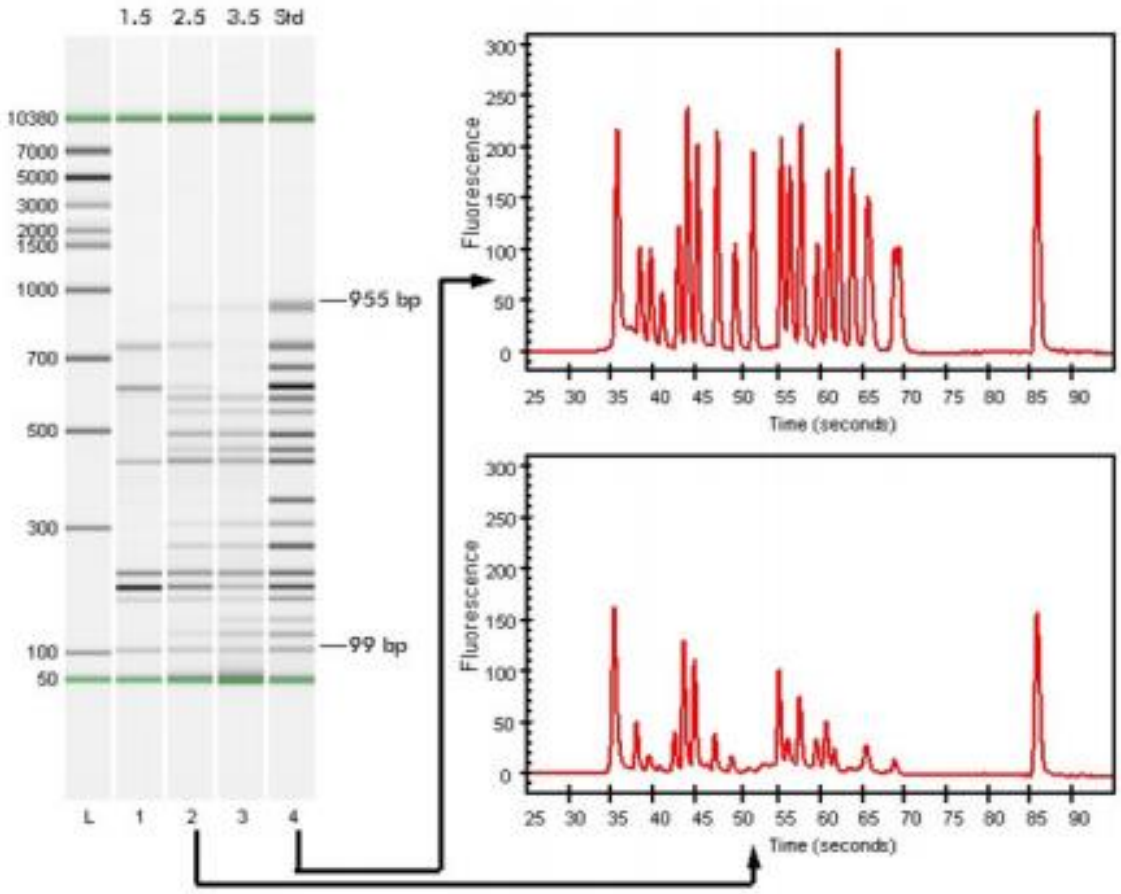

Figure 2.6: Example of BioAnalyzer results (Agilent Technologies)

2.5.8 PCR vs real-time PCR

Polymerase chain reaction (PCR) is a method that selectively and exponentially amplifies those nucleic acid molecules initially present in very small quantities (Singh and Kumar, 2001). PCR is an enzymatic reaction that employs well-understood mathematical principles, while efficacy nonetheless frequently relies on the primer design (Singh and Kumar, 2001). Below are the factors that influence this efficacy during a PCR reaction as stipulated by Biosoft (2018):

- A polymerase enzyme synthesizes a complementary sequence strand of bases onto any single strand DNA (ssDNA) provided the starting point was double stranded. Primers are designed to “prime” the starting point where the polymerase must begin extending ssDNA. At the start of the reaction a mix of primers and bases are added to the DNA. Temperature changes are used to control a PCR reaction by controlling the polymerase activity. A basic guideline for a reaction can be seen as the following:

- Temperature is raised to ensure that dsDNA is un-winded to form ssDNA.
- The temperature is then lowered for the primers to bind according to a target sequence. This temperature varies depending on the primers and needs to be optimised for every reaction. Now the polymerase has a double strand to bind to.
- The temperature is slightly raised again for the polymerase to work.
- The steps above are known as “one cycle.” One cycle (in theory) doubles the amount of copies of the target gene. Thus, after each cycle the number of copies are doubled and the number of copies increase exponentially. 40 cycles are normally used to ensure that billions of copies are present.

To test whether the reaction was successful, the DNA can be stained and run on an agarose gel. The higher the copy number the brighter the band will be.

Real-time PCR (q-PCR) uses the same principles of amplification as conventional PCR, but instead of looking at bands on a gel, the reaction is measured in real time. This is done by a camera or detector that monitors the reaction throughout. Various methods of monitoring exist but all are based on the principle of DNA amplification that is linked to fluorescence. Thus, more gene copies after each cycle equal a higher fluorescence. One of the most commonly used principles is Taq polymerase. The biggest advantages of qPCR compared to normal PCR are 1) that the efficiency of the reaction can be measured and 2) quantitative analysis of gene expression can be performed.

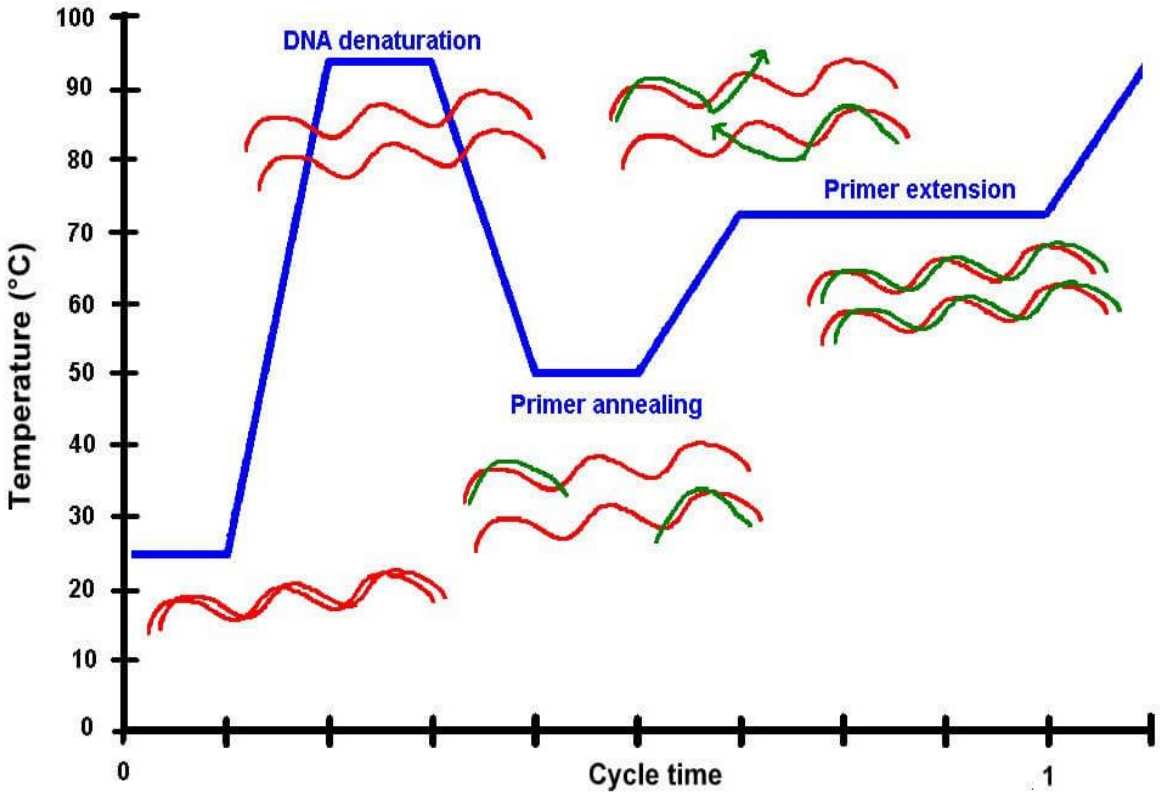

Figure 2.7: ***Graph showing the various steps of a PCR cycle. Each step corresponds to a certain temperature. Each phase also shows how the DNA responds to the change in temperature and how the DNA is replicated.***

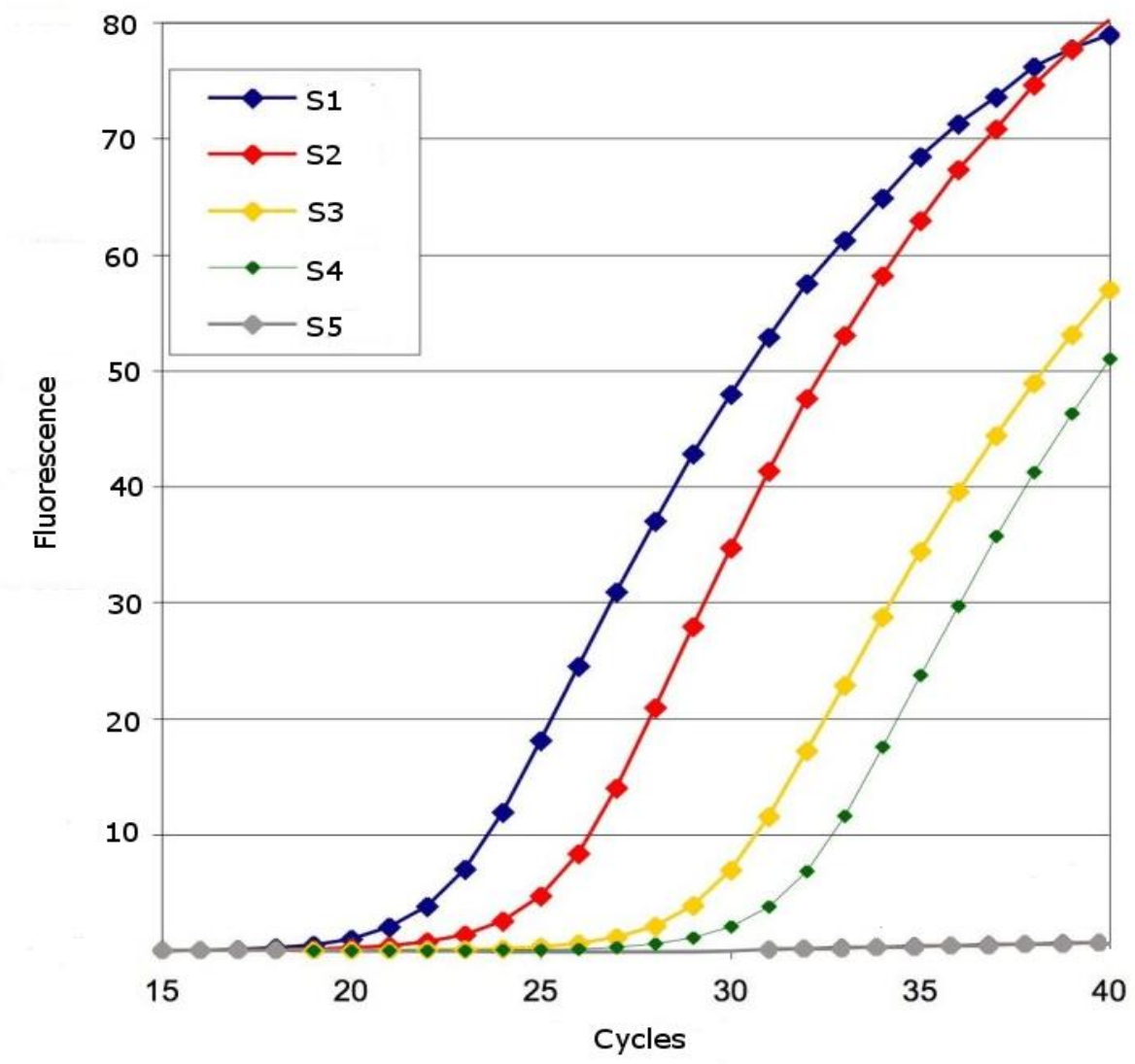

Figure 2.8: ***Graph showing an amplification profile of a qPCR run with serial dilutions of the same sample. The less copies present in the sample the later it starts to amplify.***

When it comes to development of qPCR primers it is important to test the effectiveness of the primers beforehand. The MIQE guidelines stipulate certain considerations during development such as testing the analytical sensitivity of the primers with a limit of detection (LOD) test. This involves determining the lowest point of detection (or lowest possible concentration of biological samples) ensuring 95% accuracy. Multiplexing is a very valuable utilisation of qPCR especially for simultaneous point mutations or polymorphisms detection. Multiplexing is essentially the amplification of more than one gene simultaneously by using multiple primer sets. In multiplexing, it is essential to demonstrate that the accuracy of detecting each target in individual assays is not

impaired when detecting the targets simultaneously. Thus, the LOD and assay efficiency should remain the same for uniplexing and multiplexing. This is particularly difficult to achieve when one target is more abundant than another.

2.5.9 Taq Polymerase qPCR

Escherichia coli was originally used when PCR was developed in the mid-1980s. Polymerases are used to synthesise DNA strands in a PCR reaction. Taq polymerase emanates from a bacterium that is found in hot springs and is thus more suitable for higher temperatures. In qPCR reactions a probe/ fluorophore is added. As soon as the polymerase synthesises the DNA strand past the point where the probe was bound, the fluorophore is released and a signal is generated. The fluorescence of the released probe is measured after each cycle and can then be translated into a concentration/ copy number (Dotson, 2018).

2.5.10 Analysis of generated data

The last step in a qPCR assay is data analysis. Various and vastly different techniques have been developed for data collection and processing and inevitably their performance also differs substantially. This has led to countless inaccurate and irreproducible articles and assays. Thus, detailed information regarding methods of data analysis as well as software used is essential. Specifying and identifying outliers in the process is also necessary. All statistical methods used must be documented and must include these specifications to ensure reproducibility and accuracy (Bustin et al., 2009).

Using qPCR to detect and not quantify is known as “qualitative PCR.” This is widely used for pathogen detection or methods where quantity is not necessary. In the case of this type of PCR, the low-end sensitivity of an assay is needed before a yes/ no answer can be generated. Thus, even when using a qualitative assay certain information will be necessary regarding performance of the assay, as mentioned.

When it comes to diagnostic assays, an independently verified calibrator that lies within a certain linear range should be included.

Assay performance should also be monitored by measuring or maintaining the following characteristics: a) PCR efficiency, b) linear dynamic range, c) limit of detection (LOD) and d) precision.

Cq values higher than 40 are usually suspect and indicate low PCR efficiency. However, employing predetermined cut-offs can sometimes eliminate valid results when they are too low or increase false positives when they are too high.

Linear dynamic range occurs where amplification is linear. This is needed for SYBR® green assays but is not essential for TaqMan assays. The highest to lowest copy number value must be established by means of a calibration curve of a known template. Ideally, three orders of magnitude should be covered and should normally extend five to six $\log^{10}$ concentrations. The generated calibration curve should include the interval for the target nucleic acids.

2.6 Study motivation and rationale

No standardised- or commercial method for non-invasive foetal sex determination in African buffalo or cattle in South Africa currently exists. To date, no studies have focused on fDNA in African buffalo. The similarities of the placenta in cattle and buffalo have led to the assumption that cffDNA might also be present in maternal buffalo plasma.

After confirming the presence of fDNA in the maternal plasma of buffalo, the present study will use the known sequence of the SRY gene located on the Y-chromosome to develop real-time PCR (qPCR) assays for cattle and buffalo. Subsequent to cell-free DNA isolation the engendered primers will be used to develop a test (qPCR assay) to determine the sex of a foetus. After successful development the assay will be validated for commercial purposes.

2.7 Aim of the study

To develop a standard operating procedure (SOP) with a view to determining the sex of buffalo and cattle at a foetal stage.

2.8 Objectives of the study:

i. Design a protocol for the correct collection of samples.
ii. Collect samples from cattle and buffalos.
iii. Determine whether cffDNA is present in maternal plasma of buffalo.
iv. Optimise a protocol to extract cell-free DNA in cattle and buffalo on the KingFisher Duo Prime.
v. Design primers to amplify the SRY gene in both species.
vi. Develop a sensitive method for the amplification of extracted cffDNA in both species.

vii. Test diagnostic accuracy.

CHAPTER 3: METHODOLOGY

3.1 Introduction:

The methodological design phase of the study made apparent current unknowns of stabilising, isolating, quantifying and amplifying cffDNA. Existing methods and literature do not provide a robust and practical non-invasive approach to determine foetal sex in humans and animals. Standardised methods for collecting and isolating cffDNA in animals have not been documented thoroughly and even finding articles that confirm the presence of cffDNA in maternal plasma proved difficult to achieve. Although standard protocols for the optimisation of qPCR protocols exist, the present study optimised multiple steps to compensate for low cffDNA concentrations. These steps included comparing collection tubes, following proteinase K treatment steps including volume of sample for isolation and designing and testing unique primer assays for African buffalo.

3.2 Ethical clearance

Ethical clearance was obtained from AnimCare, North-West University – ethics reference number: NWU-00279-17-S5.

Owners signed a consent form for collection of their samples (see Appendices 1, 2 and 3).

3.3 Collection and processing of samples

3.3.1 Collection of samples for genomic DNA isolation

Male cattle gDNA samples were collected in BD vacutainer® plasma preparation K2E tubes (PPT™) that were processed within eight hours subsequent to collection and these were sent to the laboratory for storage. Tubes were processed by centrifugation at 1 860 g for 5 minutes and plasma removed and stored at -80^{o}C.

Streck cell-free blood collection tubes vs BD vacutainer® plasma preparation K2E tubes (PPT™) were used for collection.

DNA concentrations were compared after isolation of human plasma samples. Human samples were only used to compare the stability of cfDNA in the different tubes. While comparing the collection tubes, the natural environmental conditions had to be simulated. Thus, the tubes were left in direct sunlight after collection as would be the case when collecting samples in the field. The (PPT™) tubes were compared by employing Streck cell-free blood collection tubes. A total of 12 blood tubes were collected (six of each

respective tube) to compare the stability of cfDNA in each tube. Samples were then left in direct sunlight for different time periods after which one of each tube type was processed. Tubes were processed by centrifugation at 1 860 g for 5 minutes and approximately 5mL plasma removed and stored at -80°C. The tubes were processed on day zero, one, two, four, six and eight. All plasma samples were used simultaneously for cfDNA isolation.

3.3.2 Sample collection from cattle

Pregnancy status and the stage of gestation of the animals were confirmed by rectal examination before blood collection was performed by a veterinarian employing venepuncture of the jugular vein of the 40 pregnant animals using an 18G vacutainer needle into a STT II Advance Vacutainer silver (PPT™) tube. Between the actual blood collection instances of the different animals, the collected blood was placed on a table in direct sunlight or in the back of an open vehicle until such time as when all the samples had been collected. To simulate the natural process that veterinarians use, no special care was taken to preserve samples from the time of collection to that of processing.

Blood was centrifuged within eight hours after collection at 1 860 g for 5 minutes and plasma was extracted from the sample for collection. Plasma was centrifuged at 3 380 g for 30 minutes to remove any remaining protein. Samples were frozen at -20^{0}C and transported on ice until they could be stored at -80^{0}C.

3.3.3 Sample collection from African buffalo

Immobilization was performed with a combination of etorphine, thiafentanil oxalate and azaperone. Individual doses were decided on according to the size of the animal to ensure optimal effectiveness. This dosage also ensured lower pain- and stress levels for the animals.

After immobilization and tranquilization, the pregnancy status and stage of gestation was confirmed by the veterinarian. Blood collection was undertaken by employing venepuncture of the jugular vein of 20 pregnant animals using an 18G vacutainer needle into a Streck tube. Incidentally, samples were initially taken in (PPT™) tubes as discussed above under heading 3.3.1 but after stability issues arose they had to be retaken. Once blood collection was complete, an antidote was administered where the veterinarian monitored each animal for an additional 15 minutes. To simulate the natural process that veterinarians use (as described in 3.3.1) no special care was taken, once more, to preserve samples from the time of collection to that of processing.

Processing of samples was performed in the manner described in section 3.3.1 above.

Two additional (PPT™) samples were taken from a male animal to be used as a positive control. These tubes were transported on ice and then frozen at -80°C until.

3.4 Isolation of gDNA and cfDNA

3.4.1 Proteinase K treatment

Samples were thawed to room temperature after which a proteinase K treatment was conducted with the following components in a 15mL falcon tube, presented in Table 3.1 below:

Table 3.1: Relevant reagents and volumes needed for Proteinase K treatment.

Reagents:	Volume:
Proteinase K, 20 mg/mL	60 µL
Plasma sample	4mL
20% SDS solution	200µL
Total volume	4.26mL

Samples were mixed by inverting them numerous times before they were incubated at 60^0C for 20 min in a thermal shaker at low revolutions. After incubation the samples were placed on ice for 5 minutes.

3.4.2 MagMax cfDNA isolation

Bronkhorst et al. (2015) demonstrated comparable results with other isolation methods by employing the KingFisher Duo automated magnetic bead system. Because there was one of these systems at our disposal, it was decided to use this isolation method. The MagMax cfDNA isolation kit (Thermo Fisher Scientific, USA) has two available methods where the amount of input material is varied. The 4mL protocol uses 4mL plasma as starting material while the 2mL protocol uses 2mL. Because of the low volumes at our disposal after elution, the two methods were compared to test yield efficacy. Because the MagMax cfDNA kit preferentially (but not exclusively) isolates shorter fragments of DNA, our male-only cattle gDNA samples were isolated by using this method. Since male-only cffDNA is however not freely available, this method was found to be the closest to simulating a positive control. Due to the difficulty of acquiring male buffalo samples and low volumes after isolation with the MagMax cfDNA kit, it was decided not to use

this method in their case. The method used for buffalo will therefore be described in 3.4.3 below. Male buffalo are not tranquilised as often as female buffalo and therefore the samples are more difficult to come by.

Samples used with a view to the 4mL isolation protocol were then set up on the KingFisher Duo Prime (Thermo Fisher Scientific, USA) instrument as described in the Table below:

Table 3.2: Below is the layout of the two plates and their reagents used for every 4mL cfDNA isolation on the KingFisher Duo Prime

Plate #	Row ID	Plate Row	Reagent	Volume per well
				4mL of plasma
1	Sample 1	A	MagMax cell free DNA lysis/binding solution	2.5mL
			MagMax cell free DNA magnetic beads	30µL
	Sample 1	B	MagMax cell free DNA lysis/binding solution	2.5mL
			MagMax cell free DNA magnetic beads	30µL
	Wash 1	C	MagMax cell free DNA wash solution	1mL
	Wash 2	D	MagMax cell free DNA wash solution	1mL
2	Elution	A	MagMax cell free DNA elution solution	60uL
	Low volume wash	B	80% ethanol	500uL
	High volume wash	C	80% ethanol	2mL
	Tip comb	D	Deep well tip comb	

After the plates were set up, 2mL of plasma sample was added to row A and B of plate 1. The KingFisher Duo Prime was set up for the “MagMax cfDNA-4mL-DUO” protocol around a deep-well magnetic head and the programme was run. Appendix 4 below presents the exact steps followed by the machine.

Protocol information

The machine protocol subsequently prompted insertion of plates.

Once isolation was complete, the extracted cfDNA (see plate 2, row A) was added to individual 1.5mL Eppendorf tubes and stored at 4^0C for up to 24 hours or at -20^0C for longer periods.

Table 3.3 shows the plate layout for the 2mL MagMax cfDNA isolation method on the KingFisher Duo Prime

Table 3.3: Plate layout for the 2mL MagMax cfDNA isolation method on the KingFisher Duo Prime

Plate #	Row ID	Plate Row	Reagent	Volume per well
				2mL of plasma
1	Sample 1	A	MagMax cell free DNA lysis/binding solution	2.5mL
			MagMax cell free DNA magnetic beads	30µL
	Sample 1	B	Empty	
	Wash 1	C	MagMax cell free DNA wash solution	1mL
	Wash 2	D	Empty	
2	Elution	A	MagMax cell free DNA elution solution	60uL
	Low volume wash	B	80% ethanol	500uL
	High volume wash	C	80% ethanol	2mL
	Tip comb	D	Deep well tip comb	

Once plates had been set up, 2mL of plasma sample was added only to row A of plate 1. The KingFisher Duo Prime was subsequently set up for using the “MagMax cfDNA-2mL-DUO” protocol with the deep-well magnetic head and the programme was run. Details are shown in Appendices 5.1 and 5.2 below.

As mentioned, once isolation was complete, the extracted cfDNA (see plate 2, row A) was added to individual 1.5mL Eppendorf tubes and stored at 4^0C for up to 24 hours or at -20^0C for longer periods.

3.4.3 gDNA isolation:

Because of the difficulty in acquiring samples from buffalo, it was decided to use gDNA isolated from whole blood to test the newly designed buffalo SRY primers (see 3.6.2). This approach provided more DNA to work with, although the samples had to be diluted significantly to simulate cfDNA concentrations. The gDNA was isolated from whole blood African buffalo samples by using the Zymo spin genomic DNA extraction kit (Zymo research, USA) following the manufacturer's instructions for whole-blood extractions.

3.5 Quantification of gDNA and cffDNA.

Due to low concentrations of cfDNA the Qubit 3.0 as well as the BioAnalyser 2100 were needed for quantification. The BioAnalyser detection limits are 500pg to 100ng of DNA, thus there was a need to verify on the Qubit that sufficient DNA had been added for the BioAnalyser. Thus, the Qubit was used for total dsDNA quantification while the BioAnalyser was used to confirm the presence of shorter fragments of DNA between 100-400bp.

3.5.1 Qubit 3.0

To quantify the isolated DNA a working solution was prepared in a plastic Eppendorf tube (or falcon tube for bigger batches) in the case of all samples. This was performed by diluting 1µL Qubit dsDNA HS Reagent in 200µL Qubit dsDNA HS buffer for every sample and the two standards. Standards were needed to create a calibration curve for the Qubit by providing a linear range whereby the samples concentrations could be determined.

The working solution was then added to 0.5mL thin-wall, clear PCR tubes in the following manner:

- 190µL working solution for standard 1 + 10µL of Qubit standard 1
- 190µL working solution for standard 2 + 10µL of Qubit standard 2
- 199µL working solution per tube for each sample + 1µL of each sample per tube.

Samples were vortexed for two to three seconds each and all bubbles removed. All samples were then incubated at room temperature for two minutes before they were analysed on the Qubit 3.0 in accordance with the dsDNA High Sensitivity assay protocol.

3.5.2 BioAnalyser 2100

The Agilent 2100 BioAnalyser follows a lab-on-a-chip approach which requires a chip priming station to “push” samples into the chip wells. A DNA 1000 HS (high sensitivity) chip was chosen since it is highly sensitive when it comes to short fragments of DNA.

The syringe was pressure-tested before each chip to ensure that the syringe was still usable: The syringe was pushed down to its lowest position and secured with a clip. The clip was released and the syringe shot to 0.1mL within two seconds. The base plate was set to position C and the screw retightened.

The DNA dye concentrate (blue cap) and DNA gel matrix (red cap) were allowed to equilibrate to room temperature for 30 minutes. The DNA dye concentrate was vortexed for ten seconds and spun down. 25µL of the blue capped concentrate was added to a DNA gel matrix vial and stored away from light at 4^0C for further use.

Before use, the gel-dye mixture was vortexed and transferred to a spin filter inside a microcentrifuge tube and spun for 15 minutes at room temperature at 3 380 g . The gel was then stored up to four weeks after preparation. When the mixture was used, it was equilibrated to room temperature for 30 minutes while it was protected from light.

A new chip was used for each new run and placed on the priming station. 9 µL of the gel-dye mix was added to the bottom well marked G (see right row, second well from the bottom in figure 3.1. The plunger was placed at 1mL and the priming station locked. The plunger was pushed down until it was held by the clip for exactly 60 seconds on which the clip was released. After five seconds the plunger was pulled back to the 1mL mark. The priming station was opened and 9uL of the gel-dye mix was added in the wells marked G (see right row, first and second wells figure 3.1

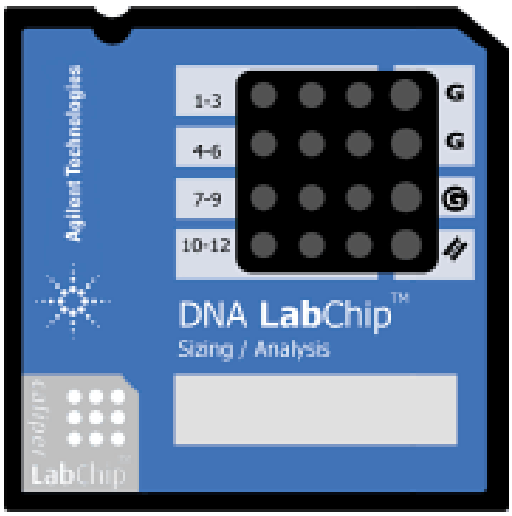

Figure 3.1: ***Example of a Agilent BioAnalyzer DNA 1000 HS chip.***

After priming the chip, 5µL of the DNA marker (green cap) was added to each of the 12 sample wells and the well with the ladder symbol. Another 1µL of the DNA 1000 ladder (yellow cap) was added to the well with the ladder symbol and 1µL of each sample was added to each of the 12 sample wells.

The prepared chip was vortexed for 60 seconds at 1000 g and inserted into the Agilent 2100 BioAnalyser for analysis.

3.6 qPCR assay design and primer assessment

3.6.1 Bovine qPCR assay

A validated TaqMan assay for the SRY gene was ordered from Thermo Fisher Scientific (Bt03286265_s1). The only available information on the kit was that the primers were designed by using the NCBI Reference Sequence: NM_001014385.1 and that it uses a FAM-MGB probe.

These primers were tested on low concentrations of male gDNA extracted with the Zymo spin genomic DNA extraction kit (Zymo research, USA), as described in section 3.3.4. A PCR mixture (20x TaqMan Gene expression assay and 2x TaqMan Gene expression Master Mix) was prepared for 20µL reactions and 0.1 – 20 ng DNA template was added to each reaction. Each sample was run in duplicate and each run included a PC, NC and NTC. The PC consisted of male-only gDNA (2.14ng/µl). The NC comprised female-only gDNA extracted with the MagMax cfDNA isolation kit. The NTC consisted of nuclease-free water as template.

3.6.2 Buffalo qPCR assay

TaqMan primers were designed by Inqaba Biotech using the GenBank (DQ336534.2) sequence.

Table 3.4: shows the primers and probe sequences designed by Inqaba Biotech

Primer	Sequence (5' – 3')
TaqMan FWD	ggaggcacagagactactatccata
TaqMan RWD	ctgtggccttttggctttcc
FAM GSQ Probe	ccgggctataaatatc

Because the probes were designed by Inqaba, their Luna Mastermix was chosen for use in reactions. It employs universal cycling conditions optimised for Mastermix and primer sets. Primers were tested on male-only gDNA to see whether the designed primers and probes amplified male gDNA:

A PCR mixture for 20uL qPCR reactions was prepared: Luna Mastermix, forward primers (0.4µM), reverse primers (0.4µM), a probe (0.2µM)) and 2ng of DNA was added to each reaction. A PC consisting of male gDNA isolated with the MagMax cfDNA isolation kit as well as an NTC were included at each run, and 2ng DNA was added for the PC and water for the NTC.

The LOD was determined by making serial dilutions of two male-only gDNA samples isolated with the Zymo spin isolation kit as discussed in section 3.4.4. One male-only gDNA sample was diluted to 1.7ng/µl, 0.17ng/µl, 0.017ng/µl and 0.0017ng/µl while the other male-only dilutions were diluted to 2.4ng/µl, 0.24ng/µl, 0.024ng/µl 0.0024ng/µl. A total of 0.5µL of female gDNA (27.8ng/µl) was also mixed with aliquots of the dilutions to ensure that the maternal DNA present in our pregnant animals would not affect the test. None of the extant studies on cattle indicated any interference by maternal DNA (Davoodian and Kadivar, 2016, Lu et al., 2007) and thus this was only tested for buffalo

3.7 qPCR of cffDNA

3.7.1 Cattle qPCR

20µL reactions were prepared from the extracted cfDNA. A PCR mixture consisting of 20x TaqMan Gene expression assay and 2x TaqMan Gene expression Master Mix was prepared for 20µL reactions and 0.1 – 20 ng cffDNA template was added to each

reaction. The manufacturer protocol recommended at least 10ng template for good amplification, but due to lower cfDNA concentrations the present study attempted not to use this much. Each sample was run in duplicate and each run included a PC, NC, NTC. The PC consisted of diluted male-only gDNA (1.71ng/µl) isolated from plasma samples (see section 3.4.2). The NC was female-only gDNA extracted from plasma samples (3.4.2). The NTC consisted of nuclease-free water as template. The reactions were run on the Applied Biosystems QuantStudio 5 using the following reaction conditions: as instructed by the manufacturer's protocol, an initial two step holding phase of 50^{o}C for two minutes followed by 95^{o}C for then minutes to activate the Taq DNA polymerase. Totals of 20 and 50 cycles of amplification were performed by a 95^{o}C step for 15 seconds followed by 60^{o}C for one minute.

3.7.2 qPCR Buffalo

The following PCR mixture for 20uL or 50µL qPCR reactions was prepared: Luna Mastermix, forward primers (0.4µM), reverse primers (0.4µM), a probe (0.2µM) and 2ng of cffDNA were added to each reaction. A PC of male gDNA (see 3.4.2) and NTC were added to each run and 2ng DNA was added with a view to the PC while nuclease-free water with a view to the NTC. In addition, 50µL qPCR reactions were tested to allow amplification given that higher copies were thus present to compensate for low cffDNA concentrations. Thus, more copies would be present for amplification to occur.

Extracted cfDNA reactions were run on the Applied Biosystems QuantStudio 5 using the following reaction conditions: as instructed by the manufacturers protocol, an initial one step holding phase of 95^{o}C for ten minutes was performed to activate the Hot Start Taq DNA polymerase. Totals of 20 and 50 cycles of amplification were performed by a 95^{o}C step for 15 seconds followed by 60^{o}C for 30 seconds.

3.8 Statistical analysis

The qPCR data were visualised and analysed on QuantStudio Design and Analysis Software Version 1.4.3 (Applied Biosystems). Averages and standard deviations were determined on Microsoft Office Excel 2016. Positive amplification of the SRY gene was determined in samples where amplification occurred within five cycles of the PC. If no amplification occurred or amplification occurred within five cycles of the NC the sample was deemed to be negative.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Collection and processing of samples

After our initial collection of samples by means of BD vacutainer® plasma preparation K2E tubes (PPT™), which was sufficient according to literature, provided samples were processed within six hours after collection (Gautschi et al., 2004), we could not achieve reliable qPCR amplification (see section 4.5.2). Elbeik et al. (2005) however note that (PPT™) outperformed conventional EDTA tubes by physically separating cells from the plasma with a gel-like layer. This layer prevents contamination from cellular gDNA after collection. Elbeik et al. (2005) further found stability of various types of RNA and DNA in the plasma during this collection procedure. However, these authors did not address the matter of time constraints as the samples were handled in laboratory conditions and processed within four hours after collection. As presented by Table 4.1, the present study found that (PPT™) were not suitable if samples were not processed immediately, as the plasma total DNA concentrations increased with time. It was found further that adding a proteinase K treatment step before DNA isolation significantly improved the total DNA concentrations. Both sets of SRY primers (see sections 3.6.1, 3.6.2) were tested and worked well for the control male gDNA samples. When cfDNA from pregnant maternal samples was used, positive amplification was achieved in cattle as well as African buffalo, but not with great sensitivity. This positive amplification of the male SRY gene in buffalo also proved the presence of foetal DNA in maternal circulation, a new finding made by the present project.

4.1.1 Collection in Streck cell-free blood collection tubes vs BD vacutainer® plasma preparation K2E tubes (PPT™)

To determine the effect of different collection tubes on the stability and the concentration of cffDNA, Streck cell-free blood collection tubes were compared with BD vacutainer® plasma preparation K2E tubes (PPT™) over time. Initially samples were collected in (PPT™) tubes as described in extant literature (Bronkhorst et al., 2015) but consistent amplification could not be achieved once samples were processed. Thus, it was decided to compare (PPT™) tubes with Streck tubes, since the latter is known for ensuring better stability of cfDNA over time. These tubes were not initially considered because of their considerable cost while, moreover, they are not readily available for veterinarians. They have to be imported at approximately 100 times the cost of (PPT™) tubes.

Table 4.1: Table showing the cfDNA concentrations after isolation of human cfDNA from Streck and (PPT™) tubes. Samples were processed on the day of collection and 1,2,4,6 and 8 days after collection and DNA was isolated with the use of the MagMax cfDNA isolation kit.

Day	Streck tube DNA [ ] (ng/µl)	(PPT™) DNA [ ] (ng/µl)
0	0.158	0.542
1	0.376	0.340
2	0.168	1.51
4	0.382	3.48
6	0.312	4.9
8	0.232	16.3

Table 4.1 illustrates the stability of cfDNA in Streck tubes. The total DNA concentration in the plasma does not increase over time, which means that other cells that contain DNA are stabilised and do not undergo cell lysis. In contrast, the (PPT™) tubes' total DNA concentration increases significantly over time, which confirms that these tubes do not prevent cell damage as efficiently as the Streck tubes (Martignano, 2019). Minimising gDNA contamination in plasma samples is imperative when using the necessary template DNA for qPCR amplification. Minimising contamination improves efficiency of qPCR by reducing background DNA.

4.2 Proteinase K treatment as opposed to non-treatment

Initial cfDNA isolation of plasma samples from (PPT™) tubes results in observing substantially low yields. An additional Proteinase K step was added before isolation to compare yields. This was done to inhibit further nuclease activity and to degrade proteins present in the sample that might influence the isolation procedure. Below are the concentrations for the samples treated with Proteinase K contrasted with samples without the treatment.

Table 4.2: Comparison of the total DNA concentrations of (PPT™) samples pre-treated with proteinase K in contrast to samples that are not treated with proteinase K. Samples were isolated with the MagMAx cfDNA isolation method.

Sample #	Proteinase K treated sample DNA [ ] (ng/ul)	Non-treated sample DNA [ ] (ng/ul)
1.	16.8	0.304
2.	12.5	0.234
3.	11.4	0.818
Average	13.57	0.452

As stated, Bronkhorst et al. (2015) potentially attribute the increase of cfDNA concentrations after Proteinase K treatment to the release of encapsulated cfDNA in vesicles.

4.3 Isolation

Appendix 6 presents the results of the cfDNA isolation concentrations. Buffalo samples collected in Streck tubes showed a much lower total DNA concentration after cfDNA isolation when compared to the (PPT™) tubes used for cattle. This is most likely due to the instability of gDNA in the (PPT™) tubes. Higher cfDNA concentrations were not attributed to progression status of gestation as is the case in humans (Phillippe, 2014).

4.4 BioAnalyser

Because of the expensive nature of running samples on the BioAnalyser, samples had to be selected instead of running all the isolated cfDNA ones. Consequently, the focus was predominantly on buffalo samples (as presented in Table 4.3) while in the case of cattle samples ones showing the highest DNA concentration after isolation were selected, but it still proved difficult to amplify these during qPCR.

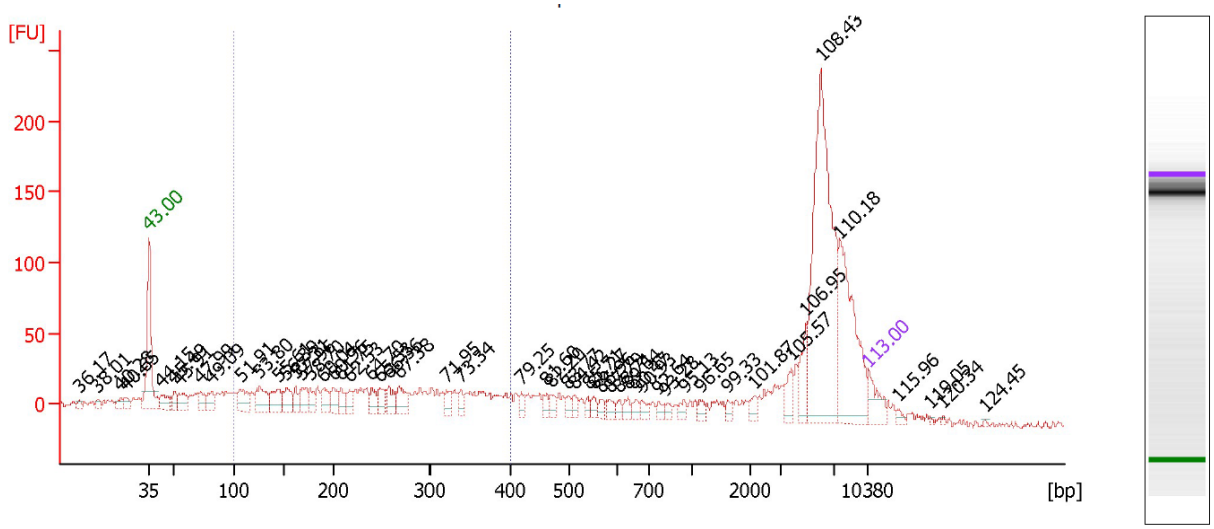

Figure 4.1: ***An example of the BioAnalyser results. The 35bp and 10380bp peaks are internal ladders added with a view to determining the concentrations of each peak.***

Table 4.3: Summary of the samples analysed on the BioAnalyser . Samples marked with ? are samples with inconclusive results as they amplified in-between the PC and NC.

Sample #)	Tube type	Gestation	Qubit total DNA Concentration (ng/µL)	Sex after birth	qPCR 1 sex	qPCR 2 sex	% of total DNA Between 100-400 bp
Cattle Samples							
FS-13	(PPT ™)	4 Weeks	16.7	M	M?	F	46
FS-23	(PPT ™)	6 Weeks	6.66	M	M?	F	1
FS-26	(PPT ™)	6 Weeks	1.71	M	M	M	4
FS-34	(PPT ™)	6 Weeks	5.1	M	F	M?	31
FS-32	(PPT ™)	10 Weeks	0.582	M	F	M?	2
FS-06	(PPT ™)	14 Weeks	8.52	M	M?	F	0
FS-09	(PPT ™)	32 Weeks	3.14	M	M?	M?	12
FS-11	(PPT ™)	32 Weeks	21.8	M	M?	M?	31
Average % Between 100-400 bp							16
Buffalo samples							
Red 6	Streck	3-4 Months	0.834	M	F	F	49
Red30	Streck	3-4 Months	0.136	M	F	F	8
Red 37	Streck	<2 Months	0.876	M	F	F	32
Blue 33	Streck	7+ Months	0.767	M	F	F	66
Red 49	Streck	<2 Months	2.54	Miscarriage/ resorption	F	F	32
Blue 37	Streck	Pregnant - mid to late	3.06	M	M	M	33

Sample #)	Tube type	Gestation	Qubit total DNA Concentration (ng/µL)	Sex after birth	qPCR 1 sex	qPCR 2 sex	% of total DNA Between 100-400 bp
Red 27	Streck	Pregnant - mid to late	3.8	F	F	F	54
Red 00	Streck	3-4 Months	1.1	M	F	F	30
Red 17	Streck	7+ Months	0.344	F	F	F	36
B - Red 34	Streck	3-4 Months	3.54	F			38
B - Blue 34	Streck	Pregnant - mid to late	2.16	M			46
G4	Streck	3-4 Months	2.42	F	F	F	38
P7	Streck	3-4 Months	5.46	M	M	F	46
G16	Streck	2 Months	1.65	F	F	F	42
G18	Streck	4 Months	1.03	M	M	F	20
G19	Streck	5 Months	3.2	M	F	M	50
G22	Streck	7+ Months	0.816	F	F	F	26
W25	Streck	7+ Months	3.02	F	F	F	34
Average % Between 100-400 bp							38

A summary of samples that were run on the BioAnalyser, as presented in Table 4.3, shows no significant correlation in the percentage of a sample between 100 – 400bp and the gestation period. And, contrary to current knowledge as found in human studies, the cffDNA percentage did not increase as the gestation period increased (Phillippe, 2014). One very significant difference was the average amount of DNA of a sample between 100 – 400bp with a view to samples collected in (PPT™) tubes as opposed to Streck tubes. The former showed an average of 16% while the average for Streck tubes were 38%. However, the (PPT™) tubes showed a much higher concentration of fragments bigger than 1000bp, which fall outside the ranges of the size for cffDNA in humans and might again point to gDNA contamination.

4.5 qPCR

As discussed in sections 3.4 - 3.7 above, each sample type was prepared to the specifications for qPCR amplification. Both primer sets were first tested on male-only gDNA and both showed appropriate amplification when diluted. The cfDNA samples of pregnant animals proved difficult to amplify consistently. Section 4.5 shows the results for testing both primer sets with male gDNA as well as results for amplification of cfDNA from maternal samples.

4.5.1 Cattle serial dilutions

As mentioned in section 3.6.1, the cattle qPCR assay was tested by performing serial dilutions for qPCR. Figure 4.2 below represents the results of these dilutions.

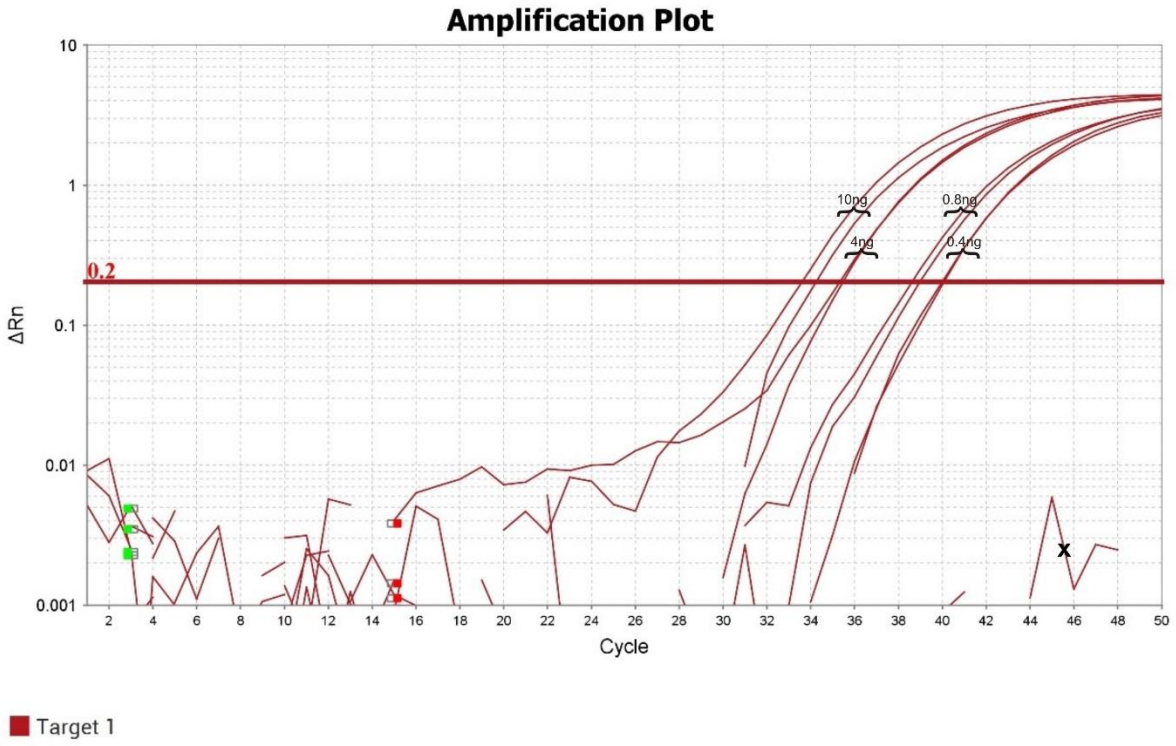

Figure 4.2: ***0.4ng, 0.8ng, 4ng and 10ng dilutions of male gDNA cattle samples run in duplicate. Target is the SRY gene with a FAM probe. NTC showed no amplification. X indicates the NTC.***

Figure 4.2 reflects the clear effect of dilutions around gDNA cattle samples on the target SRY gene: 10ng, 4ng, 0.8ng and 0.4ng of total DNA amplifying at 34, 35, 39 and 40 cycles respectively. Higher amounts of DNA amplified first and lower concentrations

later. The %CV values between the duplicates of these samples were 1.23, 0.3, 0.67 and 0.12, which affirms significant consistency between replicates. No amplification occurred in the case of the SRY target of the NTC.

4.5.2 qPCR of cattle samples

After confirming that the qPCR assay worked, cfDNA samples were analysed in duplicate, as described in section 3.7.1 above.

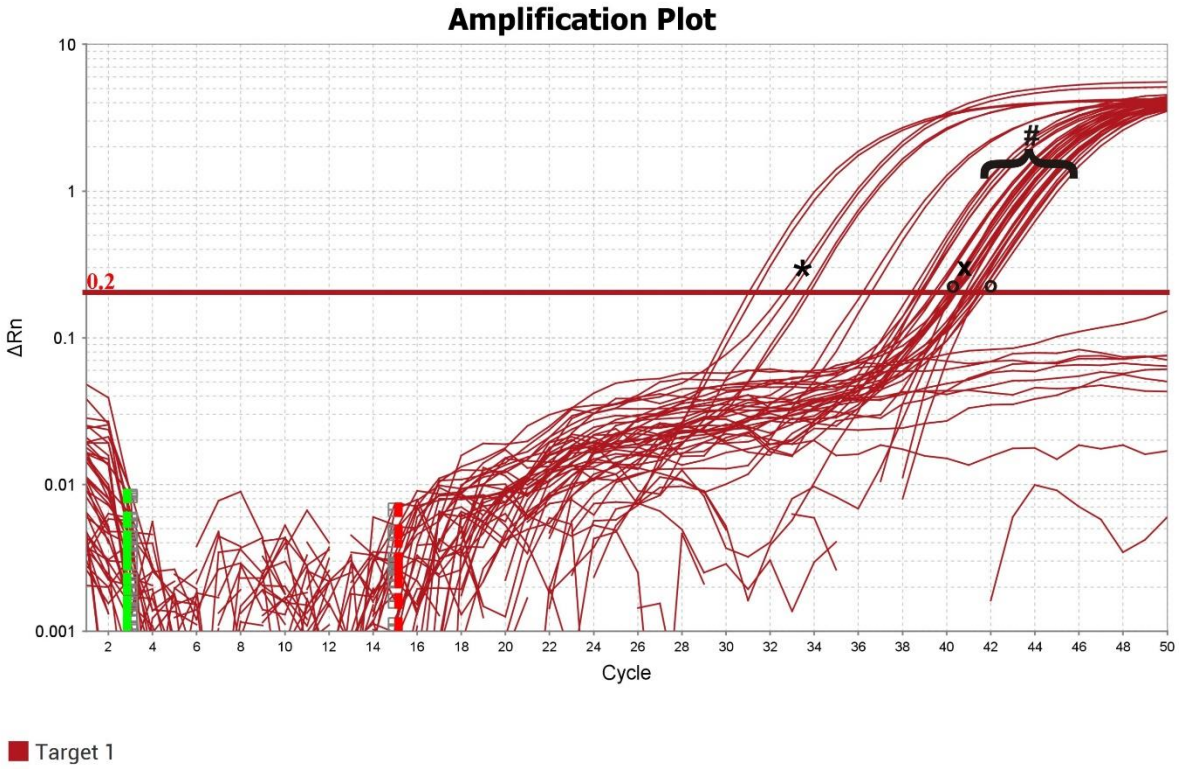

Figure 4.3: ***shows an example of a test of cattle samples run in duplicate with 50µl qPCR reactions. The SRY gene was amplified with the * (star) showing the PC. Late amplification was prominent with the NTC (x) also amplifying at approximately 42 cycles. The X indicates the NTC, O the NC and # a cluster of SRY positive and negative samples.***

This figure shows good amplification of the PC(*) at ~32 cycles. Approximately 10ng was used for the PC. The NC and NTC showed amplification at around 40 cycles. Normally, this would be held as primer dimer amplification and the sample would be viewed as negative, but in this case too many of the samples showed amplification in this region. The symbol # indicates a cluster of maternal samples of male and female offspring. This

is concerning, as there is no proper separation of male and female amplification. Unfortunately, the volumes needed for the quantification around BioAnalyser and duplicate 50µL qPCR reactions used most of the isolated cfDNA and the qPCR for cattle could not be repeated with new DNase free water

4.5.3 African Buffalo LOD test:

In contrast to the cattle, a custom assay was designed for the African buffalo samples. Instead of running normal serial dilutions an LOD test was performed to assess the assay (see section 2.5.8). The samples were set up as described in section 3.6.2 and the results are represented below in figure 4.4.

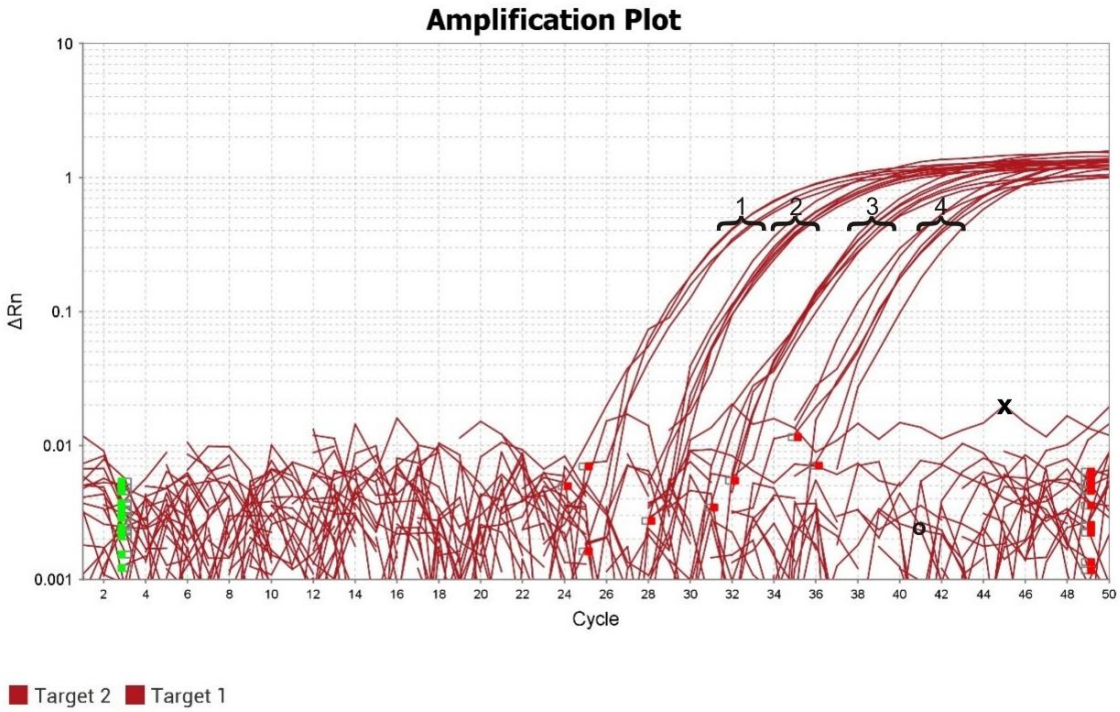

Figure 4.4: ***The figure demonstrates the effectiveness of our buffalo SRY primers. Samples were run in duplicate, but duplicates of a 1:1 mixture of male and female DNA were also run to test whether the maternal DNA had an effect on amplification. 10x, 100x, 1000x and 10000x dilutions were used, indicated by 1,2,3 and 4 respectively. X indicates the NTC and O the NC***

Figure 4.4 shows amplification of the duplicates to be very efficient. Two different male-only samples were used in duplicate. Dilutions were amplified in close proximity to their duplicates while the mixture of male + female DNA also amplified close to their male-

only counterparts. A measurement of ~20ng of male DNA was used as starting material before the dilutions were made. The 10x dilutions showed %CV of 0.33, 1.27 and 1.42 between replicates while there was 0.598 %CV difference between the male + female dilution mixture on the one hand and the male-only dilution on the other. The 100x solutions had a %CV of 0.589, 0.78 and 1.15 between replicates and a %CV of 0.182 between the male + female dilution mixture when compared to the male-only dilution. The 1000x dilutions had a %CV of 0.04, 1.04 and 0.17 respectively between replicates and a %CV of 0.272 between the male + female dilution mixture when compared to the male-only dilution. The 10 000x dilutions showed the highest %CV between replicates of 1.14, 1.78 and 2.24 and a %CV of 0.92 between the male + female dilution mixture when compared to the male-only dilution. The mixture of male and female DNA in the African buffalo LOD test reaffirmed that maternal DNA does not have any impact on amplification, given that the amplification occurred within one (or less) cycles of the male-only samples at lower dilutions. At 10 000x dilution, however, the finding was that the amplifications of the duplicates were moving further apart and four cycles separated the different samples and their duplicates. The amount of DNA added to these two different male samples ranged between 0.0016ng and 0.0025ng.

4.5.4 qPCR of African buffalo samples

Subsequent to the LOD test on the custom assay for African buffalo isolated cfDNA samples were used in duplicate as described in section 3.7.2.

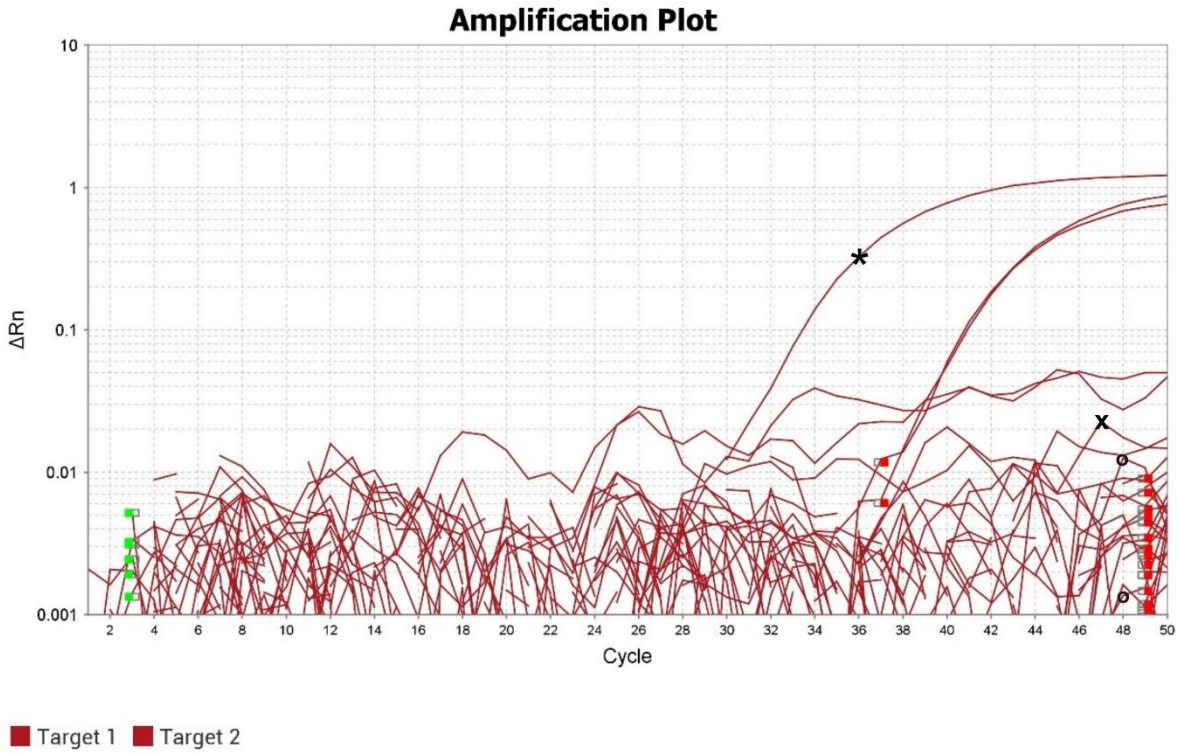

Figure 4.5: ***20uL Buffalo qPCR reactions run in duplicate. * indicates the PC, X the NTC and O the NC.***

Figure 4.5 shows the 20µL reactions of pregnant African buffalo cows. Only one sample amplified at ~38 cycles in both replicates – Blue 37. The PC amplified at ~33 cycles (~1ng of gDNA added). The NTC and the NC remained unamplified.

Table 4.4: **gives a summary of the qPCR results of the cattle and African buffalo samples including the stage of gestation, DNA concentration and the tube type the sample was collected in.**

Sample #	Tube type	Gestation	Qubit DNA Concentration (ng/µL)	Sex at birth	qPCR 1 sex	qPCR 2 sex
Cattle Samples						
FS-13	(PPT™)	4 weeks	16.7	M	M?	F
FS-02	(PPT™)	6 weeks	5.36	M	F	F
FS-23	(PPT™)	6 weeks	6.66	M	M?	F
FS-26	(PPT™)	6 weeks	1.71	M	M	M
FS-27	(PPT™)	6 weeks	5.42	F	M	M?
FS-29	(PPT™)	6 weeks	36.4	F	F	F
FS-34	(PPT™)	6 weeks	5.1	M	F	M?
FS-37	(PPT™)	6 weeks	3.24	F	F	M?
FS-01	(PPT™)	8 weeks	9.4	F	F	F
FS-04	(PPT™)	8 weeks	3	F	M?	F
FS-07	(PPT™)	10 weeks	7.02	M	F	F
FS-30	(PPT™)	10 weeks	31.6	M	M?	M?
FS-32	(PPT™)	10 weeks	0.582	M	F	M?
FS-36	(PPT™)	10 weeks	3.44	F	M?	F
FS-05	(PPT™)	12 weeks	2.36	F	M?	M?
FS-14	(PPT™)	12 weeks	9.42	F	M?	F
FS-24	(PPT™)	12 weeks	2.68	F	M?	M?
FS-33	(PPT™)	12 weeks	2.74	F	M?	M?
FS-35	(PPT™)	12 weeks	14.1	F	M?	F
FS-06	(PPT™)	14 weeks	8.52	M	M?	F
FS-09	(PPT™)	32 weeks	3.14	M	M?	M?
FS-11	(PPT™)	32 weeks	21.8	M	M?	M?
Buffalo samples						
Red 6	Streck	3-4 Months	0.834	M	F	F
Red30	Streck	3-4 Months	0.136	M	F	F
Red 37	Streck	<2 Months	0.876	M	F	F
Blue 33	Streck	7+ Months	0.767	M	F	F
Red 49	Streck	<2 Months	2.54	Miscarriage/ resorption	F	F

Sample #	Tube type	Gestation	Qubit DNA Concentration (ng/µL)	Sex at birth	qPCR 1 sex	qPCR 2 sex
Blue 37	Streck	Pregnant - mid to late	3.06	M	M	M
Red 27	Streck	Pregnant - mid to late	3.8	F	F	F
Red 00	Streck	3-4 Months	1.1	M	F	F
Red 17	Streck	7+ Months	0.344	F	F	F
B - Red 34	Streck	3-4 Months	3.54	F		
B - Blue 34	Streck	Pregnant - mid to late	2.16	M		
G4	Streck	3-4 Months	2.42	F	F	F
P7	Streck	3-4 Months	5.46	M	M	F
G16	Streck	2 Months	1.65	F	F	F
G18	Streck	4 Months	1.03	M	M	F
G19	Streck	5 Months	3.2	M	F	M
G22	Streck	7+ Months	0.816	F	F	F
W25	Streck	7+ Months	3.02	F	F	F

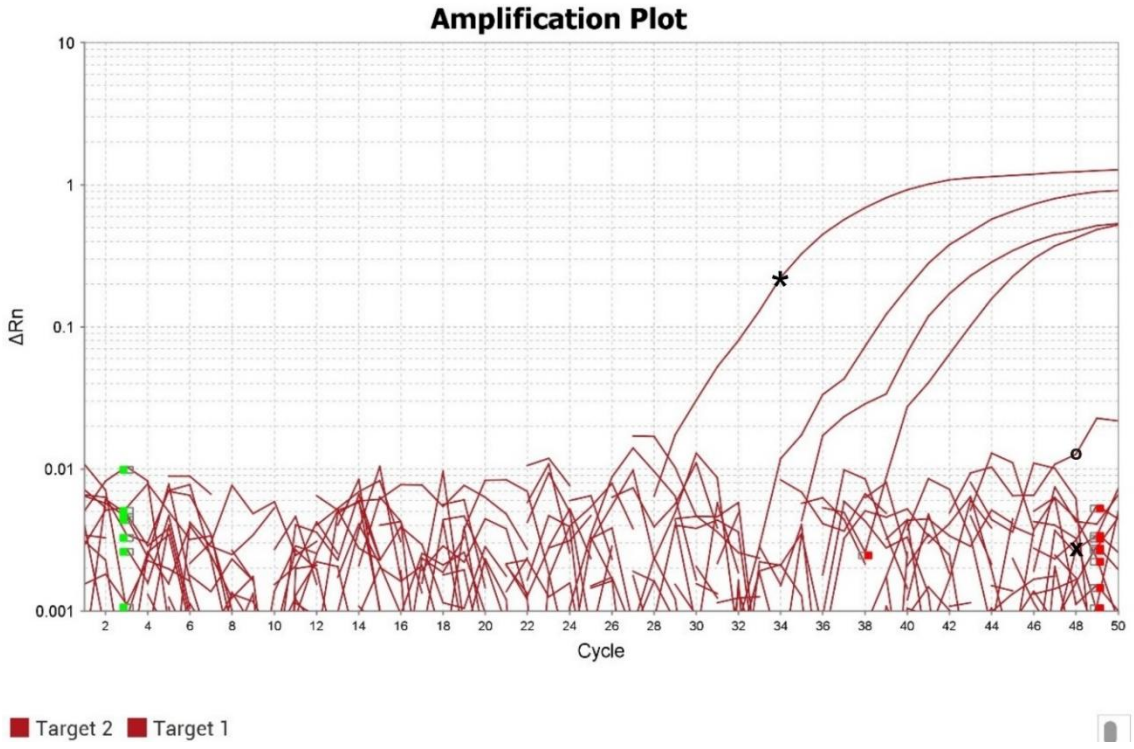

Figure 4.6: ***50uL Buffalo qPCR reactions run in duplicate. * indicates the PC, X the NTC and O the NC.***

The reaction of the African buffalo qPCR reactions was repeated by employing 50µL reaction volumes. This was performed to test whether a higher copy number in the sample might improve the results. Only three samples showed amplification and it was only in one of the replicates of each. This amplification took place between 39 and 41 cycles. The PC showed good amplification at ~31 cycles (approximately 1ng of gDNA had been added). The NC and NTC did not amplify.

CHAPTER 5: CONCLUSION AND FUTURE PROSPECTS

In the relatively new field of cffDNA, all the unknown factors associated with it compounded a considerable number of uncertainties around the design of the present study. Even when the factors collection-, processing-, and isolation procedures are considered with a view to various review articles, the process that runs from collection to results continue to present numerous limitations. Given the nature and extremely low concentrations of cffDNA even the chosen qPCR method is questionable. However, the goal of the study was to design a robust and practical method with the technology at our disposal.

That said, the results obtained in this study provided valuable insights with a view to future studies of this nature.

5.1 DNA Isolation (Objective I, II and IV)

Significant improvement occurred in terms of the collection- and isolation process, included comparisons of collection tubes and adding a proteinase K step before isolation. A higher yield after isolation was achieved with improved stability of cfDNA when Streck tubes were used (Table 4.2 and 4.1). However, these tubes are not as optimal as we thought they would be and it has recently come to light that cffDNA collection tubes are available from Qiagen. Future studies will explore this development.

5.2 qPCR (Objectives III, V, VI and VII)

The qPCR methods can be improved considerably. The LOD tests (Figure 4.2 and 4.4) showed good amplification at low gDNA concentrations isolated by way of an cfDNA isolation method, entailing the primers that were designed worked well with a view to reaction conditions in order to amplify shorter gDNA fragments. This isolation method was chosen because it worked on shorter fragments and this led to improved stimulation of cfDNA. At the time of performing this, cffDNA of which the calf sex was known was unavailable and testing the LOD on a PC of cffDNA was not possible. Even if the sex was known, however, no known methods existed (or exists) to determine the exact concentrations of cffDNA mixed with maternal cfDNA. Thus, the only way to test the primers was to isolate the short gDNA fragments by executing the cfDNA method. This however gave rise to new challenges as the level of degradation of cffDNA of our maternal samples when compared to the shorter gDNA samples were not anticipated. When substantially low accuracy was achieved by means of qPCR amplification it was speculated that the following two-fold problem had occurred due to degradation: 1) highly

degraded cffDNA denoted that there might be a decrease of the amount of fragments of sufficient size available for amplification and 2) an excessive percentage of the DNA was cut in areas where amplification should have taken place (see Figure 5.1). As has been stated, this is why a conventional qPCR method is questionable. When compared to previous studies of this nature, all samples were processed immediately to prevent the degradation as much as possible and in this study, we could not prevent this factor effectively (Davoodian and Kadivar, 2016, Bryja and Konecny, 2003, Lu et al., 2007). This halted further degradation of the cffDNA more efficiently than was the case in the present study's collection- and processing procedures.

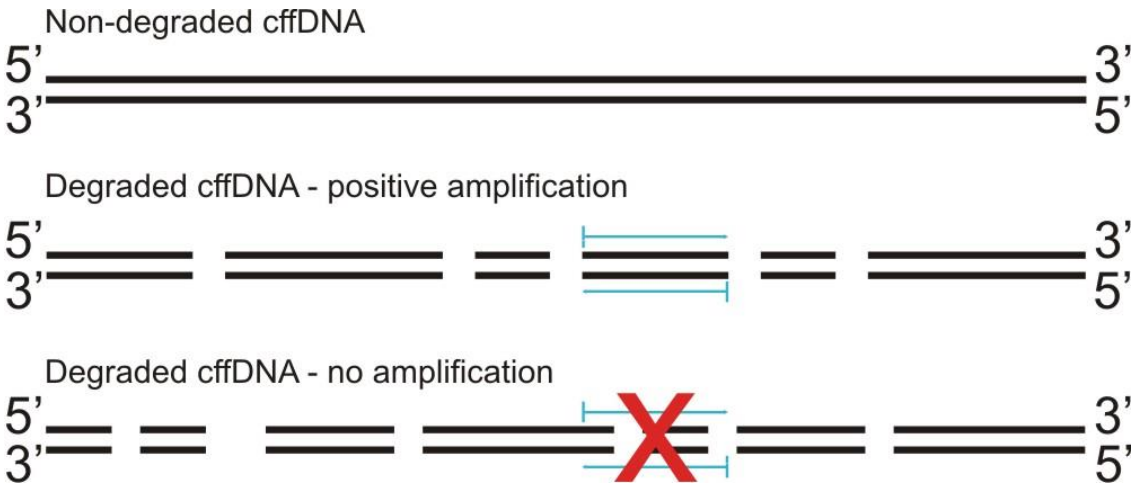

Figure 5.1: ***Shows the impact of degradation on qPCR. If the cffDNA is degraded in the wrong area amplification cannot occur.***

When cattle samples were amplified challenges arose with regard to NTC (see Figure 4.3). Ideally, no amplification should take place, an excessive number of samples amplified too closely to the NTC. The NTC amplified at ~40 cycles while various positive and negative samples amplified between 39 and 43 cycles. After the animals were born and the results were compared, it was found that many of the samples were male. 50% of samples presented inconclusive results in both of the duplicates, while 27% of the results were correct in both duplicates and 23% of the samples amplified incorrectly (figure 4.3). The false negatives that amplified close to the NTC might suggest contamination of the reagents in the present study – most likely the DNase-free water as all other reagents were unopened before experimentation started. The contamination was not found in the serial dilution test and thus could have occurred between testing sessions as the water in the laboratory was shared between other students. This was

corrected in the African buffalo LOD test and the African Buffalo qPCR tests (Figure 4.2 and 4.4). As mentioned in section 4.5.2 of the present text, the high volumes needed for the qPCR analyses usurped most of the isolated cfDNA and the reactions could not be repeated while using new nuclease-free water.

5.3 BioAnalyser (Objective III)

Due to the considerable cost of running the BioAnalyser HS chips, it was decided to select certain samples that engendered challenges in the qPCR amplification process, but in combination with controls that worked well. However, the consequent results were even more conflicting. Before running the samples it was suspected that the difficulty around amplification arose due to a low percentage of the sample actually containing cffDNA. Because there no documented method exists that would quantify the cffDNA fraction in the maternal plasma exactly, the study used the documented average size of cffDNA in humans (Phillippe, 2014). Thus, taking into account the average size of 170bp, BioAnalyser software was used to provide a percentage of DNA fragments between 100-400bp. The results showed that some of the samples where the cows had progressed only six weeks into their gestation period, entailing a low cfDNA concentration after isolation (1.71 ng/µL) and low percentage (~4%) of the sample between 100-400bp, still amplified satisfactorily in the course of the qPCR run (see Figure 4.5). In contrast to this, samples collected in (PPT™) tubes where the cows were at least 30 weeks into gestation presented high cfDNA concentrations after isolation and showed a high percentage between 100-400bp (~30%), but still underwent no amplification of the SRY gene during qPCR. This led to an assumption that the specific region of the SRY gene that needed to be amplified was not present in this fraction of cfDNA.

One positive finding engendered by BioAnalyser results were the averages of the percentage of the sample, which was found to be between 100bp – 400bp. For samples that were collected in (PPT™) tubes the average was only 16% compared to 38% in Streck tubes. This at least demonstrated that the stabilizing agents used in these tubes work better for shorter fragments of DNA.

5.4 Recommendations for future research

Future studies might consider different genes to amplify. For instance, SOX9 holds promise as it is a sex-determining gene. Apart from location, SOX9 differs from SRY in one crucial aspect: it is a multi-copy gene (Kent et al., 1996). If we were not limited to the low volume acquired from cfDNA extraction (~60µl), this gene would therefore also

have been tested in the present study. A multiplexed reaction around hypermethylated RASSF1A might be considered since it acts as a positive marker for foetal DNA in human maternal samples (Chan et al., 2006). It therefore serves to confirm the presence of fDNA and also determines whether the PCR was successful or not, which removes the need for the BioAnalyser step. However, the presence of hypermethylated RASSF1A has not been confirmed in cattle or African buffalo. Consider again that the reason for including the BioAnalyser step was to confirm the presence of short fragments of DNA that normally correlate to cfDNA and also cffDNA. When RASSF1A was added to the qPCR step the the presence of cffDNA could be confirmed and the required sex determining gene could simultaneously be confirmed. Another possible alteration might be the use of MassARRAY system (Agena Bioscience) instead of qPCR. The system is more sensitive to low concentrations and this will allow us to design multiple shorter primer assays and test them at once. Thus, even if degradation occured in one area, amplification will still occur at other designated areas for a positive result.

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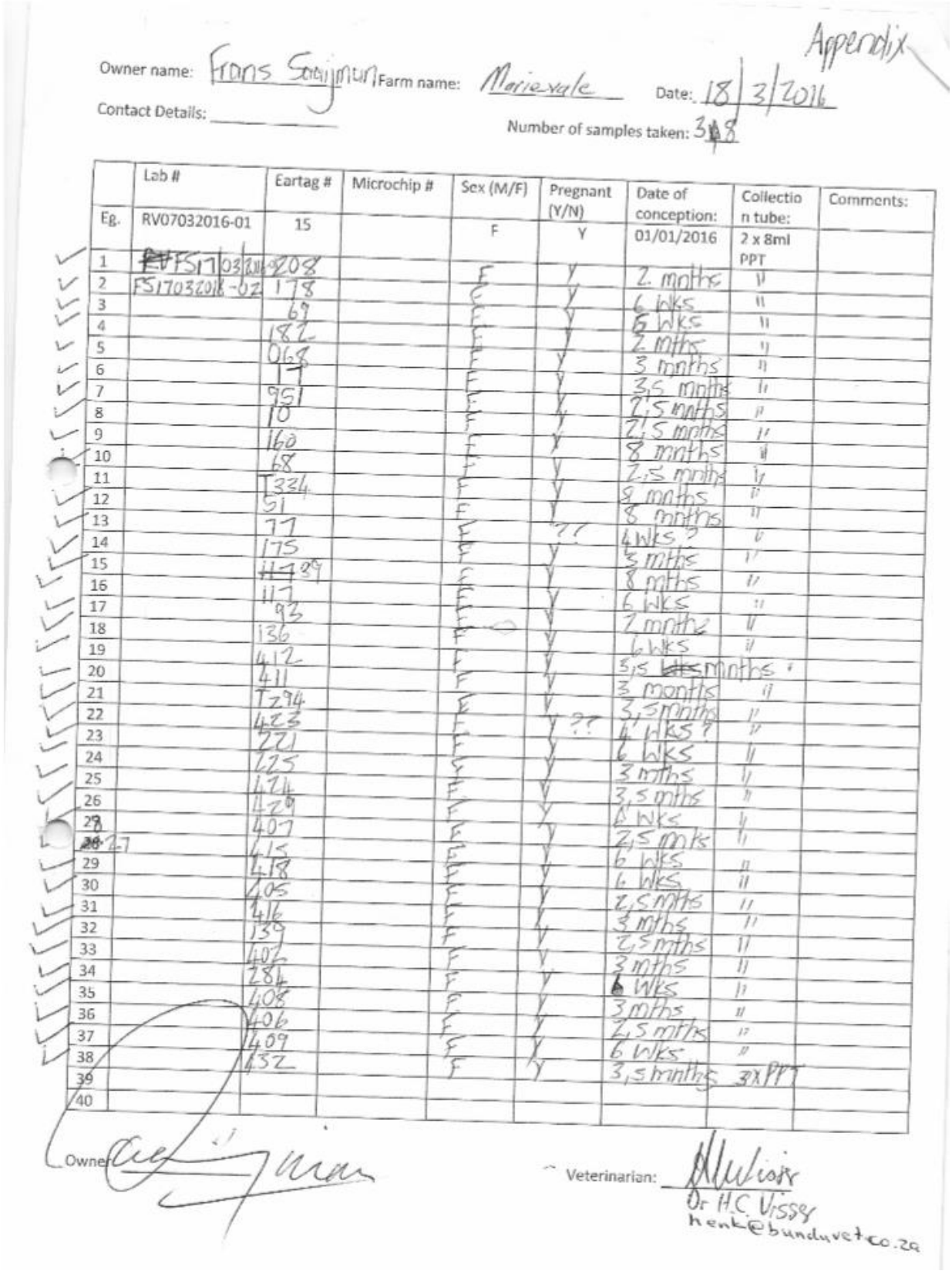
APPENDICES



Appendix

Owner name: Frans Snijman Farm name: Marievale Date: 18/3/2016

Contact Details: _____ Number of samples taken: 38

	Lab #	Eartag #	Microchip #	Sex (M/F)	Pregnant (Y/N)	Date of conception:	Collection tube:	Comments:
Eg.	RV07032016-01	15		F	Y	01/01/2016	2 x 8ml PPT	
1	~~RV~~FS17032018208			F	Y	2 maths	V	
2	FS17032018-02	178		F	Y	6 wks	V	
3		69		F	Y	6 wks	V	
4		182		F	Y	2 mths	V	
5		068		F	Y	3 months	V	
6		17		F	Y	3,5 mths	V	
7		951		F	Y	2,5 mths	V	
8		10		F	Y	2,5 mths	V	
9		160		F	Y	8 months	V	
10		68		F	Y	2,5 months	V	
11		T334		F	Y	8 months	V	
12		51		F	Y	8 months	V	
13		77		F	??	4 wks ?	V	
14		175		F	Y	3 mths	V	
15		~~117~~ 39		F	Y	8 mths	V	
16		117 93		F	Y	6 wks	V	
17		136		F	Y	2 months	V	
18		412		F	Y	6 wks	V	
19		411		F	Y	3,5 ~~wks~~ months	V	
20		T294		F	Y	3 months	V	
21		423		F	Y	3,5 mths	V	
22		221		F	??	4 wks ?	V	
23		225		F	Y	6 wks	V	
24		424		F	Y	3 mths	V	
25		429		F	Y	3,5 mths	V	
26		407		F	Y	4 wks	V	
27 ~~28~~		415		F	Y	2,5 mths	V	
29		418		F	Y	6 wks	V	
30		405		F	Y	6 wks	V	
31		416		F	Y	2,5 mths	V	
32		139		F	Y	3 mths	V	
33		402		F	Y	2,5 mths	V	
34		284		F	Y	3 mths	V	
35		408		F	Y	4 wks	V	
36		406		F	Y	3 mths	V	
37		409		F	Y	2,5 mths	V	
38		432		F	Y	6 wks	V	
39						3,5 months	3x PPT	
40								

Owner: _____ Veterinarian: _____

Dr H.C. Vissr

henk@bunduvet.co.za

Appendix 2
Artikel 20
DAFF
Animal Health

Owner name: Mamie Farm name: _____ Date: _____

Contact Details: _____ Number of samples taken: ____

	Lab #	Eartag #	Microchip #	Sex (M/F)	Pregnant (Y/N)		Collection tube:	Comments:
Eg.	RV07032016-01	15		F	Y	Early/Mid/Late	2 x 8ml PPT	
1	9[illegible]	[illegible] 26		F	Y	Mid	2 x 10ml	Steck
2	9[illegible]	[illegible] 7		F	Y	Mid	"	
3	7[illegible]	[illegible] 16		F	Y	8 weeks	"	
4	2[illegible]							
5	9[illegible]	[illegible] G19		F	Y	5 mnths	"	
6	9[illegible]	[illegible] G15		F	Y	4 mnths	"	
7								
8	9[illegible]	[illegible] W15		F	Y	7+ mnths	"	
9	7[illegible]	[illegible] G22		F	Y	7 mnths	"	
10	[illegible]	[illegible] G4		F	Y	3,5 mnths	"	
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Hereby the owner of the above mentioned animals acknowledges that blood samples were taken for the sole purpose of developing a test to determine fetal sex. The owner understands the risks involved in the collection of the samples and that the North-West University may not be held liable for any damages during the process. The owner also acknowledges that no harm was done to the animals in the process of collecting these samples and that the animals were in a healthy state after collection of the samples.

Owner: Veterinarian:

Appendix 3

Owner name: Wian vd Linde Farm name: Wintershoek Date: 30/10/17

Contact Details: _____ Number of samples taken: ____

	Lab #	Eartag #	Microchip #	Sex (M/F)	Pregnant (Y/N)		Collection tube:	Comments:
Eg.	RV07032016-01	15		F	Y	Early/Mid/Late	2 x 8ml PPT	
1	WV30102017-01	Red 6		F	Y	Mid	2x5 freck	10 ml
2	02	Red 30		F	Y	Mid	"	
3		Red 34		F	Y	2-3 Months	"	
4		Blue 33	710305959	F	Y	Late	"	
5		Blue 34		F	Y	Mid-late	"	
6		Blue 31		F	Y	Mid-late	"	
7		Red 27		F	Y	Mid-late	"	
8		Red 20		F	Y	Mid	"	
9		Red 17		F	Y	Late	"	
10		Red 37		F	N?	Very early	"	
11		[illegible] Wetty		F				
12		No tag / Red 109		F	N?	Very early	"	
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Veterinarian:

Steps data

Tip1		KingFisher Duo 6 tip comb	
	Pick-Up	Plate 1	(D) - Tip Comb
	Mix1-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:10, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix1-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:10, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix2-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:10, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix2-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:10, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix3-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No

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	Mix3-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix4-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix4-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix5-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix5-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No

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	Mix6-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix6-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix7-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix7-2	Plate 1	(B) - Bind 2
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
	Mix8-1	Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No

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Mix8-2		Plate 1	(B) - Bind 2
Beginning of step		Precollect	No
		Release beads	No
Mixing / heating:		Mixing time, speed	00:00:30, Fast
		Heating during mixing	No
End of step		Postmix	No
		Collect beads	No
		Post-temperature	No
Mix9-1		Plate 1	(A) - Bind 1
Beginning of step		Precollect	No
		Release beads	No
Mixing / heating:		Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
End of step		Postmix	No
		Collect beads	No
		Post-temperature	No
Mix9-2		Plate 1	(B) - Bind 2
Beginning of step		Precollect	No
		Release beads	No
Mixing / heating:		Shake 1 time, speed	00:00:30, Fast
		Shake 2 time, speed	00:00:15, Bottom mix
		Heating during mixing	No
End of step		Postmix	No
		Collect beads	No
		Post-temperature	No
Mix-Collect 1		Plate 1	(A) - Bind 1
Beginning of step		Precollect	Yes
		Release beads	No
Mixing / heating:		Mixing time, speed	00:04:30, Slow
		Heating during mixing	No
End of step		Postmix	No
		Collect beads	No
		Post-temperature	No
ReleaseBeads1		Plate 1	(C) - 1mL Wash 1
		Release time, speed	00:00:05, Fast
Mix-Collect 2		Plate 1	(B) - Bind 2
Beginning of step		Precollect	Yes
		Release beads	No
Mixing / heating:		Mixing time, speed	00:04:30, Slow
		Heating during mixing	No
End of step		Postmix	No
		Collect beads	No
		Post-temperature	No

	Wash 1	Plate 1	(C) - 1mL Wash 1
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:01:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	3
		Collect time [s]	1
		Post-temperature	No
	1st 80p EtOH	Plate 2	(A) - 1mL 80% EtOH
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:00:20, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No
	2nd 80p EtOH	Plate 2	(B) - 500uL 80% EtOH
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:00:20, Medium
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No
	Dry	Plate 2	(B) - 500uL 80% EtOH
		Dry time	00:02:00
		Tip position	Outside well / tube
	Elute	Plate 2	(D) - 350µl Elution
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:05:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No
	Leave	Plate 1	(D) - Tip Comb

Appendix 5.1
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Steps data

Tip1		KingFisher Duo 6 tip comb	
Pick-Up		Plate 1	(D) - Tip Comb
Mix1-1		Plate 1	(A) - Bind 1
	Beginning of step	Precollect	No
		Release beads	No
	Mixing / heating:	Shake 1 time, speed	00:00:50, Fast
		Shake 2 time, speed	00:00:10, Bottom mix
		Loop count	5
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
Mix-Collect 1		Plate 1	(A) - Bind 1
	Beginning of step	Precollect	Yes
		Release beads	No
	Mixing / heating:	Mixing time, speed	00:04:30, Slow
		Heating during mixing	No
	End of step	Postmix	No
		Collect beads	No
		Post-temperature	No
Wash 1		Plate 1	(C) - 1mL Wash 1
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:01:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	3
		Collect time [s]	1
		Post-temperature	No
1st 80p EtOH		Plate 2	(A) - 1mL 80% EtOH
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:00:20, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No

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	2nd 80p EtOH	Plate 2	(B) - 500µl 80% EtOH
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:00:20, Medium
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No
	Dry	Plate 2	(B) - 500µl 80% EtOH
		Dry time	00:02:00
		Tip position	Outside well / tube
	Elute	Plate 2	(D) - 350µl Elution
	Beginning of step	Precollect	No
		Release time, speed	00:00:05, Fast
	Mixing / heating:	Mixing time, speed	00:05:00, Fast
		Heating during mixing	No
	End of step	Postmix	No
		Collect count	2
		Collect time [s]	1
		Post-temperature	No
	Leave	Plate 1	(D) - Tip Comb

No lot numbers have been defined.

Appendix 6.1
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Table 6 is a summary of the samples collected with their concentrations after cfDNA isolation

Sample #	Species	Gestation status (weeks)	DNA [ ] (ng/ul)
423	Cattle	4	8.52
77	Cattle	4	16.7
69	Cattle	6	
117	Cattle	6	
136	Cattle	6	
407	Cattle	6	29.4
418	Cattle	6	36.4
284	Cattle	6	5.1
409	Cattle	6	3.24
178	Cattle	6	5.36
221	Cattle	6	6.66
429	Cattle	6	1.71
93	Cattle	8	
182	Cattle	8	3
208	Cattle	8	9.4
10	Cattle	10	
68	Cattle	10	
951	Cattle	10	7.02
405	Cattle	10	31.6
415	Cattle	10	5.42
406	Cattle	10	3.44
139	Cattle	10	0.582
411	Cattle	12	2.3
416	Cattle	12	2.02
408	Cattle	12	14.1
068	Cattle	12	2.36
175	Cattle	12	9.42
225	Cattle	12	2.68
402	Cattle	12	2.74
412	Cattle	14	
T294	Cattle	14	0.4
424	Cattle	14	14.8
432	Cattle	14	
17	Cattle	14	8.52
51	Cattle	32	
39	Cattle	32	
160	Cattle	32	3.14
T334	Cattle	32	21.8
FS-13	Cattle	4	16.7
FS-02	Cattle	6	5.36
FS-23	Cattle	6	6.66
FS-26	Cattle	6	1.71
FS-27	Cattle	6	5.42
FS-29	Cattle	6	36.4
FS-34	Cattle	6	5.1
FS-37	Cattle	6	3.24
FS-01	Cattle	8	9.4

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FS-04	Cattle	8	3
FS-07	Cattle	10	7.02
FS-30	Cattle	10	31.6
FS-32	Cattle	10	0.582
FS-36	Cattle	10	3.44
FS-05	Cattle	12	2.36
FS-14	Cattle	12	9.42
FS-24	Cattle	12	2.68
FS-33	Cattle	12	2.74
FS-35	Cattle	12	14.1
FS-06	Cattle	14	8.52
FS-09	Cattle	32	3.14
FS-11	Cattle	32	21.8
Rooi 26	African buffalo	~22	1.72
Pers 7	African buffalo	~22	5.46
Geel 16	African buffalo	8	1.65
Groen 19	African buffalo	20	3.2
Groen 18	African buffalo	16	1.03
Wit 25	African buffalo	>28	3.02
Groen 22	African buffalo	>28	0.816
Groen 4	African buffalo	14	2.42
Rooi 6	African buffalo	~22	1.29
Rooi 30	African buffalo	~22	0.218
Rooi 34	African buffalo	10	3.54
Blue 33	African buffalo	>28	0.918
Blue 34	African buffalo	~25	2.16
Blue 37	African buffalo	~25	3.06
Red 27	African buffalo	~25	3.80
R00	African buffalo	~22	1.10
Red 17	African buffalo	>28	0.344
Red 37	African buffalo	4	0.876
White	African buffalo	4	0.742
Red 49	African buffalo	4	2.54