Detection of *Toxoplasma gondii* in Fresh New Zealand Farmed Meat by the Polymerase Chain Reaction

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Abstract

Toxoplasma gondii is an obligate intracellular parasite that is estimated to infect one third of the world's human population. Upon infection this parasite causes the disease toxoplasmosis which in most healthy humans is chronic and often asymptomatic. If infection occurs in a human host without a competent immune system such as an unborn baby or infant or someone suffering from AIDS, toxoplasmosis can lead to permanent disability or death. A growing amount of evidence indicates a statistical relationship between chronic infection in healthy adults and neurological disorders leading to behavioural and psychological problems.

Toxoplasma gondii can be spread through contact with cat faeces. This was thought to be the main mode of human infection. Subsequent testing with murine and *Felidae* models has shown that the bradyzoite of *Toxoplasma gondii* within intracellular cysts in muscle and neural tissue are far more infectious than the oocyst morphology shed in cat faeces. This indicates that consumption of infected meat products may also play a major contributing role in the world's high rate of toxoplasmosis infection.

The chief aim of this study was to develop a sensitive PCR assay to detect the presence of *Toxoplasma gondii* in fresh raw meat products. Beef, chicken, lamb and pork mince were chosen as convenient forms of fresh raw meat as each pack available for purchase contains the meat from several animal's tissues (increasing the likelihood of contamination) and it has been partially homogenised allowing for easier sampling and DNA extraction.

Secondary aims of this study were to evaluate PCR primer sets targeting polymorphic regions to gain some understanding of what strains of *Toxoplasma gondii* are present in our meat and the prevalence in the local cat populations.

To accomplish these aims positive and negative PCR controls were also developed using a live toxoplasmosis vaccine and DNA extracted from Zebrafish. DNA extraction techniques were developed to purify DNA from both muscle

tissue and feline faecal samples and primer sets were selected from published literature as well as designed using NCBI primer BLAST.

Toxoplasma gondii specific DNA was detected in only one of the 25 meat samples purchased. This was achieved using a primer set targeting the P30 region of the *SAG1* gene and confirmed by DNA sequencing.

Using primer sets targeting polymorphic sites within the *Toxoplasma gondii* genome, sequencing and restriction enzyme digests it was shown that *Toxoplasma gondii* present in the feral *Felis catus* population in the raglan area is type II at the GRA6 and SAG3 loci. Amplification of polymorphic PCR targets from DNA extracted from meat samples could not be achieved.

Non-specific amplification occurred with a majority of primer sets trialled with meat DNA extracts indicating improvements are needed in primer design for the purpose of PCR detection of *Toxoplasma gondii* in meat. Nested PCR increased specificity however resulted in contamination in several instances.

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List of Abbreviations

16Sr	16S ribosomal RNA
µM	Micromole
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DNA	Dioxyribose Nucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
E	Expect value
ELISA	Enzyme linked immunosorbant assay
FeLV	Feline leukemia virus
g	Gram
GRA6	granule antigen protein gene
Hr	Hour
Ig	Immunoglobulin
IPA	Isopropyl alcohol
mg	Milligram
Mg	Magnesium
mL	Millilitre
mM	Millimole
mRNA	Messenger RNA
MQH_20	Milli-Q H_20

mV	Millivolts
NCBI	National Centre for Biotechnology Information
ng	Nanogram
nM	Nanomole
PCR	Polymerase Chain Reaction
SAG	Surface antigen gene
T. gondii	*Toxoplasma gondii*
UPRT	Uracil phosphoribosyltransferase gene
V	Volts
WW	World wide

Chapter One
Literature Review

1.1 Introduction

Toxoplasma gondii is an protozoan obligate intracellular parasite that infects a wide range of wild and domestic warm-blooded animals worldwide (Su et al., 2012). The primary hosts for *T. gondii* are the members of the *felidae* family including domestic and feral *Felis silvestris catus*. This parasite belongs belongs to the phylum *Apicomplexa*, subclass *Coccidiasina* and family *Sarcocystidae* and is responsible for the disease toxoplasmosis.

T. gondii was first described in 1908 after its discovery in the common gundi (*Ctenodactylus gundi*), a species of rodent (from which the term gondii is derived) (Nicolle & Manceaux, 1908). It was not until 1970 that oocysts were first found in *felidae* feaces and the full life cycle of *T. gondii* could be described (J. Dubey, 2009) (see Figure 1.1 below).

It is within the intestinal walls of a primary (*filidae*) host that *T. gondii* is able to complete sexual recombination (Figure 1.1) and it is the only region where it is able to form a diploid state, in its other morphologies (tachyzoites, bradyzoites and oocysts) *T. gondii* exists only in a haploid state (Figure 1.2).

In (primary) *felidae* hosts the parasite undergoes sexual reproduction (and recombination) in the epithelial cells lining the small intestine. This process is known as merogony (Figure 1.1), where merozoites replicate within enterocyte cells in the intestinal cell wall to produce gametes which are able to recombine giving rise to oocysts (Hunter & Sibley, 2012b). Oocysts are shed in *fealidae* faeces during a period of around two weeks between one and three weeks following infection. Upon shedding oocysts undergo sporylation creating a durable capsule that allows the oocysts to survive in soil and in water tables for prolonged periods of time.

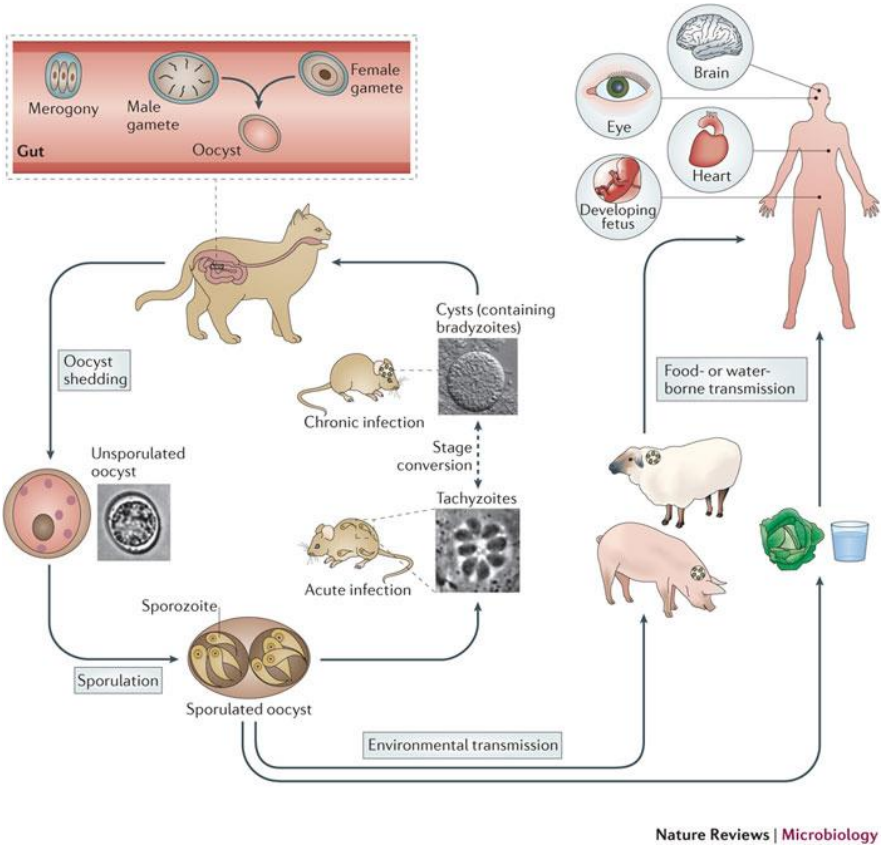

Figure 1.1 Life cycle of *Toxoplasma gondii* (Hunter & Sibley, 2012b)

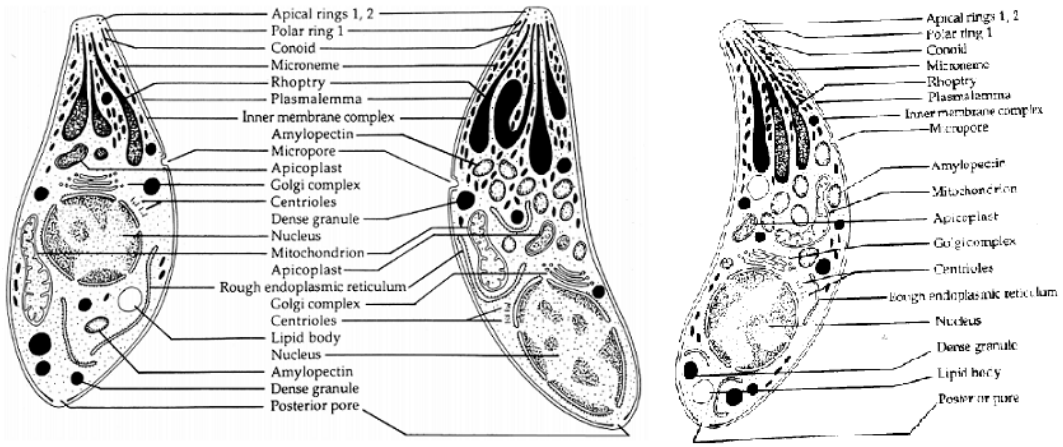

Figure 1.2 Schematic diagrams of *Toxoplasma gondii* morphologies. Tachyzoite (left), bradyzoite (centre), Sporozoite (right) (J. Dubey, Lindsay, & Speer, 1998). The localisation of the microneme, rhoptry, and dense granules are depicted**.**

1.2 *Toxoplasma gondii* infection

There are multiple potential routes of *T. gondii* infection. Consumption of contaminated meat containing bradyzoite cysts is thought to be the major cause of human infection. Alternatively, zoonotic transmission can occur after accidental ingestion of oocysts from cat faeces after cleaning a cat's litter box or gardening. Oocysts can survive in water tables and therefore can be contracted within drinking water. Contaminated water runoff is the likely source of oocysts found in mussel populations are thought to be a cause of otter and seal infections (Miller et al., 2008). Toxoplasmosis can also be contracted though blood transfusion and organ transplant and perhaps most dangerous to humans is congenital contraction where tachyzoites pass from the bloodstream of an infected mother to unborn child through the placental walls.

Infection with *T. gondii* is pervasive and potentially deadly in both animal and human hosts. Healthy humans infected by *T. gondii* may experience flu-like symptoms during the initial (acute) phase of the infection, however unborn children, cancer patients, AIDS and other patients without normal immune system function can quickly become seriously ill and die from rampant *T. gondii* infection.

New Zealand has a higher than average rate of chronic *T. gondii* infection (Lake et al., 2002; Zarkovic et al., 2007). A national serological study carried out in 2009 showed that aproxamately 20% of New Zealanders aged between 16 and 24 and 30% of New Zealanders aged between 25 and 44 have been exposed to *Toxoplasma gondii* (Weir, Jennings, Young, Brunton, & Murdoch, 2009). In 2002, the New Zealand Food Safety Authority carried out an assessment of the potential risk of contracting toxoplasmosis from contaminated meat. No information on the risk of infection from New Zealand meat products was available at that time (Lake et al., 2002).

According to the Centre for Disease Control (CDC), toxoplasmosis is the second most common foodborne killer in USA next to salmonella. It is estimated that 50% of these cases are due to consumption of contaminated food (Bayarri,

Gracia, Lázaro, Pérez-Arquillué, & Herrera). In New Zealand, between 2000 and 2006, congenital toxoplasmosis caused three deaths with seven cases discharged from hospital (ESR, 2010). During this same period, thousands of individuals would have contracted chronic toxoplasmosis but would only have noticed mild flu-like symptoms, if any.

Although it is often considered asymptomatic, chronic *T. gondii* infection causes increased levels of the neurotransmitter dopamine and the hormone testosterone. Chronic toxoplasmosis has been linked to increased aggressive behaviour and increased rates of mental illness such as schizophrenia, manic-depression, obsessive compulsive disorder, depressive states and other mental illnesses (Fekadu, Shibre, & Cleare, 2010; Jarolslav Flegr, 2013). Chronic *T. gondii* infection has also been linked with an increased rates of motor vehicle accident, with one study finding that *T. gondii* seropositive individuals are 2.3 times more likely to be involved in a car accident (Kocazeybek et al., 2009).

1.3 Host cell invasion

Although *T. gondii* has no flagellum, independent motility is achieved through writhing and actin-myosin powered mechanisms (Meissner, Schlüter, & Soldati, 2002). Upon contact with a target host cell an orchestrated cascade of secretion from three structurally and biochemically distinct organelles; the micronemes, rhoptries and dense granules (Figure 1.2). This facilitates parasitic invasion, the formation of a parasitiphorous vacuole (PV) and uptake of nutrients into the PV and protection from host cell immune responses (Figure 1.3). Host cell entry of the parasite is completed in 15–20 seconds (SAFFER, MERCEREAU-PUIJALON, DUBREMETZ, & SCHWARTZMAN, 1992).

Fusion with the host cell membrane occurs at the apical tip of the invading parasite. When this occurs, micronemes discharge their contents. Many of these proteins are predicted to be membrane associated, some of which target the host cell membrane and some of which remain lodged in the cell membrane of the

parasite. These factors are thought to play essential roles in creation of a moving junction ie., the interface between the host cell membrane and the developing PV that will completely enclose the parasite following invasion. This moving junction also provides a point of attachment for actin-myosin 'motors' that propel the parasite further into the host cell.

The club-shaped rhoptries are the next secretory organelle to discharge proteins from the apical tip of the invading parasite directly into the cytoplasm of the host cell. There are approximately 8–12 rhoptries per cell, occupying 10–30% of the total cell volume. They are the only known acidified organelles in *T. gondii* with a pH ranging from 3.5 to 7.0 (Weir et al., 2009). Known functions of rhoptry proteins are facilitation of biogenesis of the parasitophorous vacuole (PV) and host-PV interactions. These factors/enzymes/proteins have also been observed to play essential roles in supressing host cell immune responses.

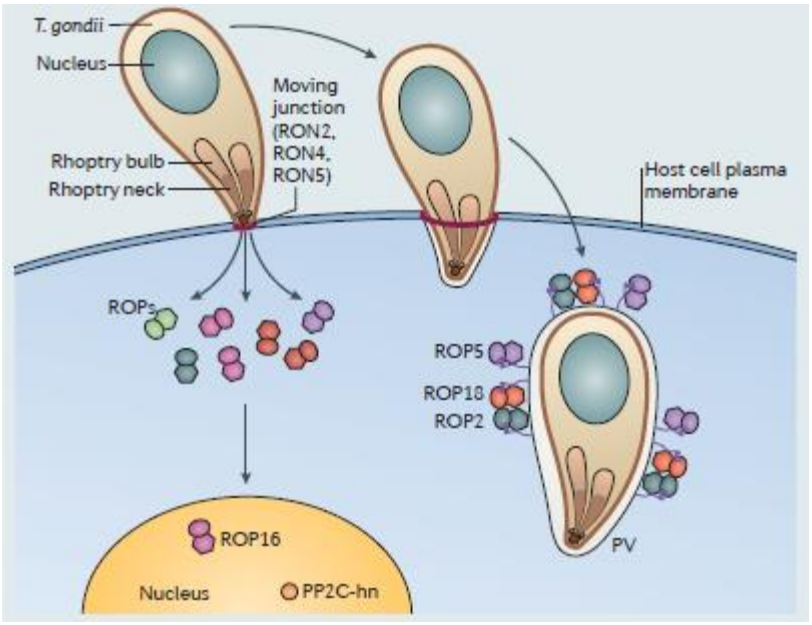

Figure 1.3 *Toxoplasma gondii* invasion of a host cell, formation of the parasitophorous vacuole and deployment of rhoptry proteins (Hunter & Sibley, 2012b). PV = parasitophorous vacuole; ROP = rhoptry protein; RON = rhoptry neck protein.

Dense granules are the last of the secretory organelles to release their contents. These enter the parasitiphorous vacuole once formed and are thought to facilitate the uptake of nutrients from within the cytoplasm of the host cell into the parasitiphorous vacuole. Dense granule proteins may also play a role in suppressing host immune responses and also inducing changes in host neurological function.

Once within the intestine of a secondary host (prey such as mice and birds or other warm blooded animals) the parasite reproduces as rapidly dividing tachyzoites which replicate in a clonal manor known as endodyogeny until causing host cell lysis and release of the tachyzoites contained within. Once tachyzoites reach the host circulatory system circulatory system they become are distributed throughout almost all the host's tissues. By invasion of leukocytes and manipulating gene expression, tachyzoites are able to penetrate through ordinarily impermeable membranes and barriers (such as the blood brain barrier) into almost all of bodies tissue, particularly the central nervous system and skeletal muscle. (Weidner & Barragan, 2014)

1.4 Development and perpetuation of chronic infection

As the host immune response becomes effective against the invading tachyzoites a small fraction of those tachyzoites differentiate into a much more slowly replicating bradyzoite morphology. These persist within intracellular cysts that can contain upto several thousand bradyzoites (Hunter & Sibley, 2012a) within a paracitophorous vacuole that is able to protect the encysted bradyzoites from intracellular immune responses (Hunter & Sibley, 2012a).

Immunoprivaleged tissues such as nervous and muscle tissues are most susceptible to cyst formation. These cysts are able to mask themselves from host immune responses through the formation of parasitiphorous vacuoles, organelle like membrane bound vesicles which are derived in part from host cell membrane (Figure 1.4) and through manipulation of host gene expression, inflammatory

responses, and intracellular immune response mechanisms via secretion of various proteins and enzymes (Melo, Jensen, & Saeij, 2011) (Zhou et al., 2005).

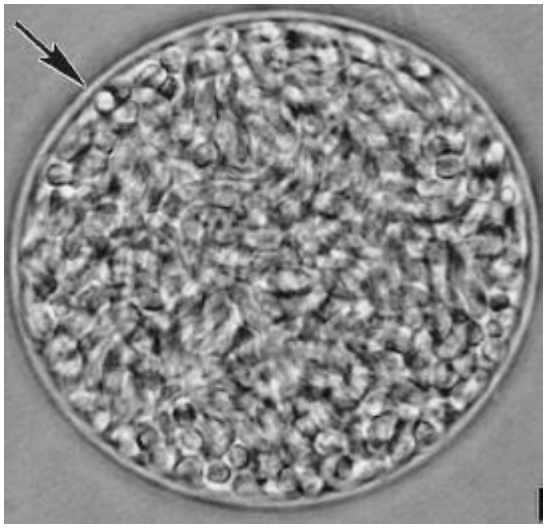

Figure 1.4 A bradyzoite cyst isolated from a mouse brain. The arrow shows the parasitiphous vacuole membrane (J. Dubey et al., 1998).

Through comparison of differential gene expression, polymorphisms between various *T. gondii* strains and genetic manipulation in concert with murine studies, researchers have demonstrated the function of numerous proteins responsible for TG virulence. For example, enzymes such as Rhoptry proteins.

Rhoptry protein RHOP18 is a kinase that inhibits interferon gamma IFNγ mediated killing of intracellular toxoplasma through phosphorylation of a nucleotide binding site of interferon regulated guanine triphosphatases (GTPases) (Melo et al., 2011). Other NTPases have also been observed to affect Toxoplasma virulence (Asai, Miura, Sibley, Okabayashi, & Takeuchi, 1995). Although the mechanism for this is unclear, it has been proposed that NTPases may reduce the amount of ATP available for induction of pyroapoptosis though activation of Nlrp3 inflammasome (Melo et al., 2011; Schroder & Tschopp, 2010). An inflammasome is a multiprotein oligomer.

RHOP5 is a duplicated polymorphic psuedokinase with the isoform/polymorphism is estimated to be responsible for 90% of the variance in

virulence between strains in murine models. The mechanism by which RHOP5 effects virulence is unknown but knockout studies show its presence or absence has no effect on invasion, PV formation, nutrient uptake, replication or egress from host cells (Reese, Zeiner, Saeij, Boothroyd, & Boyle, 2011).

ROP16 and dense granule protein GRA15 have both been observed to effect host cell gene transcription. Polymorphisms of ROP16 were observed to have a significant effect on *T. gondii* virulence. The mechanisms by which ROP16 affects virulence is unclear but ROP16 is known to stimulate STAT3 and STAT6 (signal transducers and activators of transcription) (Ong, Reese, & Boothroyd, 2010; Yamamoto et al., 2009). STAT3 is able to inhibit NF-κβ, transcription factors that play important roles in regulation of immune responses and may be one of the mechanisms responsible. ROP16 has also been detected within host cell nuclei suggesting that it may play other important roles in manipulating host gene expression.

Study of polymorphisms of the dense granule protein GRA15 has shown that this protein can modulate NF-κβ activation. GRA15 from the archetypal type II lineage shows significantly higher levels of NF-κβ activation leading to IL-12 (interleukin 12) and other pro-inflammatory cytokine synthesis than other GRA15 analogues (Robben et al., 2004; Rosowski et al., 2011).

1.5 The *Toxoplasma gondii* genome

The *Toxoplasma gondii* genome consists of 14 chromosomes containing approximately 65 million bases (Figure 1.8) ranging in size from 2 to 7 Mb, a 35 thousand base apicoplast circular chromosome as well as mitochondrial DNA. The number for genes is estimated to be 7793 (Khan et al., 2005).

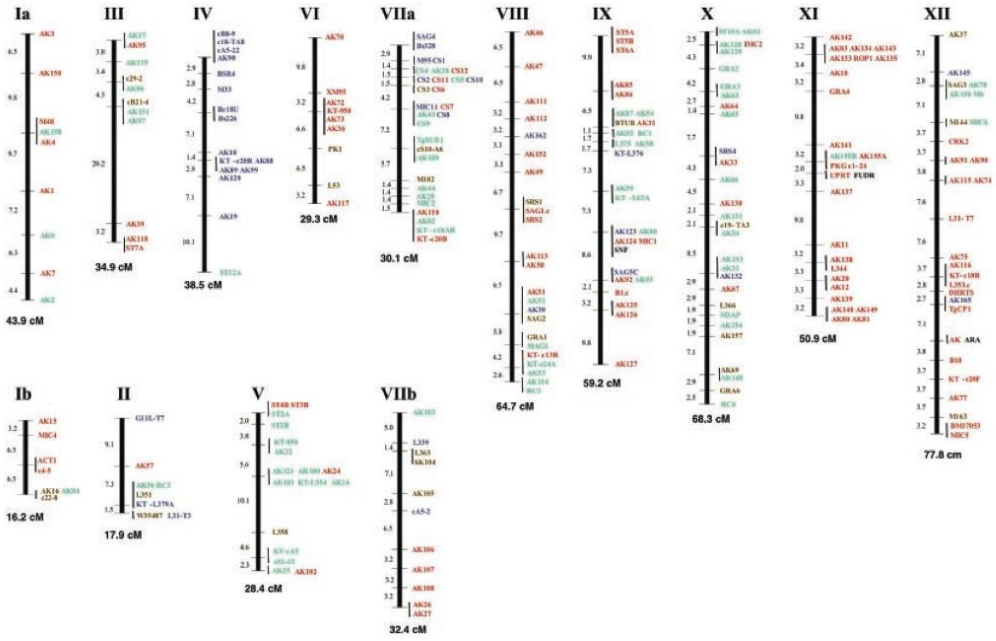

Figure 1.5 Linkage map of the 14 chromosomes of *Toxoplasma gondii* (Khan et al., 2005).

1.6 Population Genetics

Important with regard to the population genetics of *T. gondii* is that without ingestion by *felidae*, merogogy cannot occur and the parasite is only able to reproduce in a clonal mode, with evolution occurring only through random mutation. The rate at which random mutations occur within *T. gondii* subspecies and morphologies is an unknown and comlicating factor when considering its evolutionary history (Wendte, Gibson, & Grigg, 2011).

Significant genetic changes occur only when localised sub-species (largely clonal lineages) are able to recombine and evolve through independent assortment (admixture) when merogogy of two (or more) infections occur simultaneously within the intestine of the primary host. This could be a result of ingestion of

multiple prey animals or prey animals with infections of multiple *T. gondii* subspecies.

T. gondii subspecies all originate from three or four archetypal lineages that originated in South America and spread to North America and Europe around 10,000 years ago (Figure 1.5). Within South America greater genetic variation of T. Gondii has been observed than outside of South America where genotyping studies indicate all subspecies variation is a result of admixture of the three common *T. gondii* subspecies known (types i, ii, and iii) (Minot et al., 2012). There is a variance among these subspecies in virulence, due to differences in the way in which each subspecies interferes with host immune systems. This is due to differences in levels of secreted kinase and psuedokinase enzymes that the parasite employs to interfere with immune response systems and gene expression of host cells.

The importance of gaining an accurate measure of how we (New Zealanders and humans) contract toxoplasmosis cannot be underestimated. The parasite causes infant mortality, lifelong disease and is likely to contribute rates of mental illness and road toll. The effects on behaviour combined with the high rate of infection may have significant effects on community wellbeing.

The opening of global shipping lanes appears to have stimulated significant diversification of *T. gondii* subspecies through the transportation of infected mice, rats and cats from continent to continent. The result of this admixture can be observed in studies of global *T. gondii* diversity such as 'Globalisation and population structure of *T. gondii'* (Lehmann, Marcet, Graham, Dahl, & Dubey, 2006).

Phylogenetic analysis of *T. gondii* isolates have shown that the current global genetically (and morphologically) diverse distribution of *T. gondii* subspecies originated from a small number (likely four) ancestral lineages (Fig.1.5) which over recent centuries have recombined to give various localised populations. (Fig. 1.6) (Khan et al., 2005; Minot et al., 2012; Sibley, Khan, Ajioka, & Rosenthal, 2009; Su et al., 2012; Wendte et al., 2011).

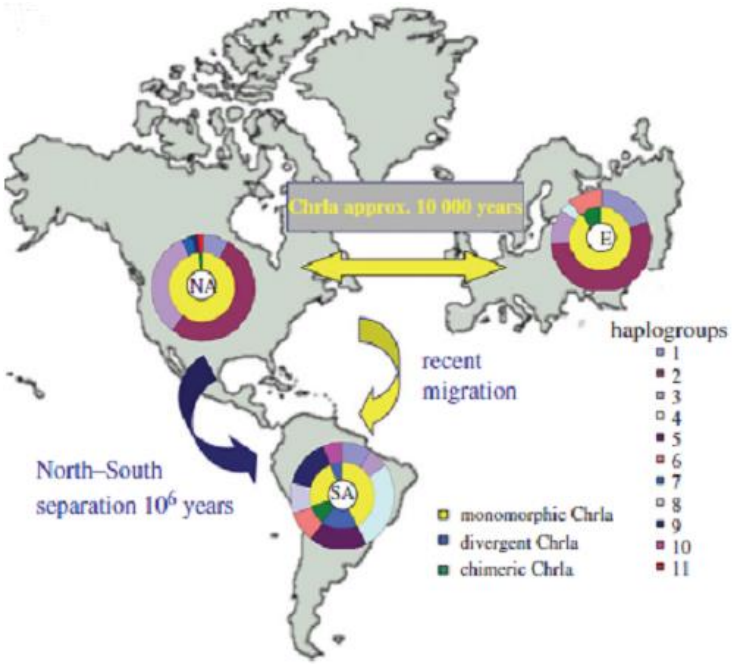

Figure 1.6. Estimated global distribution of *Toxoplasma gondii* lineages derived from phylogenetic analysis of eleven chromosome 1a haplotype blocks (Khan et al., 2006; Sibley et al., 2009).

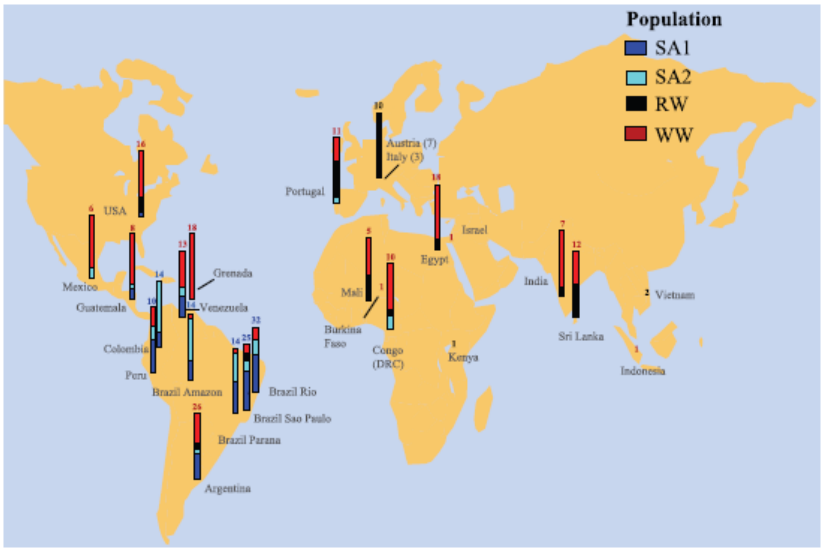

Figure 1.7 Global distribution of subspecies variation. This proposed model related to four ancient clonal lineages; two from South America (SA1 and SA2), one distributed throughout the rest of the world (RW), and one distributed worldwide (WW) (Lehmann et al., 2006).

1.7 Global distribution:

It is estimated that approximately one third of the world's population is thought to be infected with chronic toxoplasmosis but survey results of local infection rates around the world vary widely, from up to 100% in some areas in Brazil to 0% in parts of northern Europe (Pappas, Roussos, & Falagas, 2009).

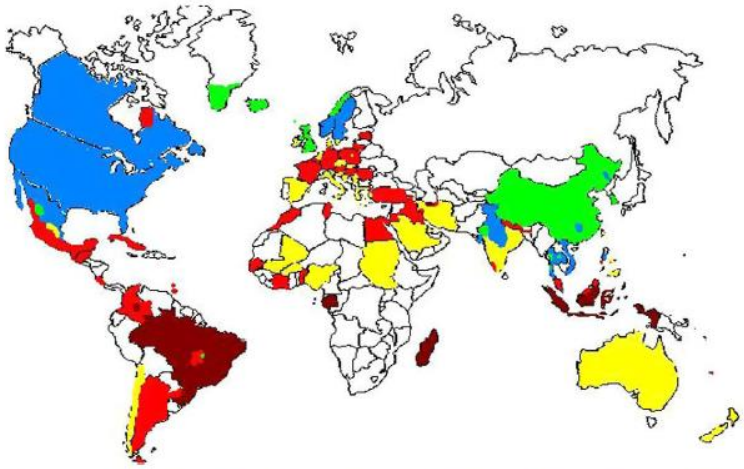

Fig. 1. Global status of *Toxoplasma gondii* seroprevalence. Dark red equals prevalence above 60%, light red equals 40–60%, yellow 20–40%, blue 10–20% and green equals prevalence <10%. White equals absence of data. Data as described in tables and text (Section 3).

Figure 1.8 Global status of *Toxoplasma gondii* seroprevalance. Colour shading represents prevalence levels: Dark red: greater than 60%; Light red: from 40% to 60%; Yellow: from 20% to 40%; Blue: 10% to 20%; Green: less than 10%; white: absence of data (Pappas et al., 2009).

1.8 Methodology used to detect *T. gondii*

Seroepidemiological methods can detect whether humans or animals have been exposed to *T. gondii* using the Sabin-Feldman Dye Test developed in 1949 (Grillo et al., 1999) and the enzyme linked immunosorbent assay (ELISA) or the . The Sabin-Feldman Dye Test is the gold-standard for detection of chronic *T. gondii* infection. *T. gondii* cells are incubated in (test) serum. If immunoglobulin G (IgG) antibodies are present in the test serum, they will cause lysis of the parasite cells. To test whether lysis has occurred, the nuclear stain

methylene blue is applied. If cell lysis has occurred, the stain will be able to access the parasite nucleus and therefore stain the cells blue. If lysis has not occurred, the parasite remains unstained. ELISA involves the detection of anti-*T. gondii* antibodies on plastic plates coated with *T. gondii* surface proteins (antigenic proteins) or surface polysacharides. Anti-*T. gondii* antibodies bind to these targets and can then visualised (and potentially quantified) using enzyme linked or fluorescently labelled secondary antibodies (that bind to the heavy chain end of the anti *T. gondii* antibodies. This technique is useful for detecting chronic *T. gondii* infection where no parasite DNA is available and using quantification methods can also give an indication of the length of time since infection as antibody concentration will increases over time. In comparison, these two techniques are unable to determine the subspecies of a chronic *T. gondii* infection. To achieve this, a sample containing tachyzoites or bradyzoite cysts is required.

Polymerase chain reaction (PCR) detection methods can be used to detect active tachyzoite infection as live parasite is present within the bloodstream, and other fluids including amneotic and cerebrospinal fluid. These situations occur when the host's immune response is either compromised pharmaceutically, for example with immunosuppressant drugs for organ transplant recipients, through infection (typically due to AIDS) or due to underdevelopment in the case of congenital or early childhood infection (Costa et al., 2013).

Although more difficult and more invasive, PCR methods can be used to detect chronic *T. gondii* infection in both muscle and neural tissue and could potentially also be used to analyse the subspecies present (Jauregui, Higgins, Zarlenga, Dubey, & Lunney, 2001). This would be advantageous for physicians treating these cases as genotyping can give valuable insights into the virulence and antibiotic resistance and/or susceptibility (Aspinall, Marlee, Hyde, & Sims, 2002) of the infection and hence assist in choosing the optimal course of treatment. A variety of treatment options are used for acute toxoplasmosis infection, strategies vary widely depending on the way in which toxoplasmosis presents, (ie: prenatal/neonatal/ocular/cerebral lesions) these strategies generally consist of

prolonged high doses of a variety of antibiotics but may include surgical options such as drainage where encephalitis has occurred.

As a diagnostic tool, PCR reactions employing primers to target genomic DNA repeat regions such as *B1* gene and 529-bp repeat element *AF146527* provide high sensitivity. *B1* is thought to be repeated between 2.5 and 10.2 times (relative to the single copy target, *P30*)(Costa & Bretagne, 2012) within the toxoplasmosis genome and AF146527 between 21.1 and 64.3 times(Costa & Bretagne, 2012). There can be significant variation in copy number depending upon the type and strain of *T. gondii* infection (Costa & Bretagne, 2012; Reischl, Bretagne, Krüger, Ernault, & Costa, 2003).

Nested PCR techniques are generally used for amplification of single copy targets due to their high specificity and amplification power. Nested PCR employs a second set of "internal" primers to amplify a specific region from within the product or products from the "outer" set.

A study of human blood donors from the Waikato region in 2007 gave an estimated infection rate of 42.9% ± 8% (Zarkovic et al., 2007), but infection rates among the general population may be even higher. In a study by RTM Cursons et al. at Waikato hospital, infection rates among pregnant women were measured at between 58.5% (15-20 yrs old) and 68.5% (31-35yrs old) (Lake, Hudson, & Cressey, 2002).

T. gondii has generally been associated with exposure to cat faeces but studies overseas have shown that consumption of undercooked infected meat may be a major contributor to the rate of human infection. For example a UK study (Aspinall et al., 2002) found 38% of their samples of 71 different meat products were contaminated with *T. gondii.*

Because *T. gondii* isolates itself from the circulatory system by forming intracellular cysts it is impossible to determine which subspecies has caused cases of chronic toxoplasmosis without a tissue biopsy.

1.9 Aims and Objectives

In this study, we propose to measure the rates at which New Zealand's most commonly consumed meats are contaminated with infectious *T. gondii* cysts through PCR analysis of meat samples taken at random from Hamilton meat outlets. If possible we will also attempt to genotype any *T. gondii* detected using PCR primer sets targeting polymorphic regions of the *T. gondii* genome.

The main aim of the research undertaken in this thesis was to develop a PCR assay to detect *Toxoplasma gondii* in fresh New Zealand farmed meat samples. To complete these aims, the following five objectives were fulfilled:

Development and optimisation of DNA extraction methods for
tachyzoite DNA from within a live *T. gondii* vaccine solution;
oocyst DNA from within cat faecal samples; and
bradyzoite DNA from within various types of animal muscle tissue.
Development of reliable PCR assays for the detection of *T. gondii* specific DNA sequences;
Assessment the effectiveness of developed PCR assays using positive controls and feline faecal extracts;
Application of optimised PCR assays to test for the presence of (highly infectious) *T. gondii* bradyzoite cysts within fresh meat available in supermarkets and butcheries in the Hamilton area; and
To confirm the results of PCR assays by DNA sequencing and restriction enzyme digest analysis.

Chapter Two

Materials and Methods

2.1 Preparation of materials and solutions:

All glassware and spatulas used in preparation of solutions and meat sampling were cleaned and disinfected with a Miele® Professional G7839 washer disinfector using Asepti® medical and dental (disodium trioxosilicate) dishwasher powder. Certified DNase, RNase, human DNA and PCR inhibitor free Multimax® centrifuge tubes and pipette tips used were all further sterilised with 72hours at 80°C (dry) heat. Certified DNase, RNase, human DNA and PCR inhibitor free Neptune 2mL conical base screw top tubes were used for all proteinase K meat and GITC faecal DNA extractions. Biospec® Glass and Zircon beads were used in all 'beadbeater' homogenisation after 72hours at 80°C dry heat treatment. Product details for all supplies and reagents along with recipes for all solutions and used can be found in appendices A1.

2.2 DNA extraction of THP-1 Toxovax™ live *T. gondii* vaccine

Aliquots of 1.5 mL of Toxovax™ were centrifuged at 16,000x*g* and the supernatant was removed. The resulting cellular pellet was suspended in 350µL SDS lysis solution and 10µL (60U/mL) proteinase K. This solution was then incubated at 55°C overnight.

Toxovax™ digests were combined with an equal volume of 5M LiCl (350µL), mixed and then added to 700µL chloroform and further mixed by vigorous shaking for 20s followed by 10 minutes on a mixing wheel. This mixture was then separated by centrifugation at 16,000G for 5 minutes. The supernatant was then removed using a fine tip dispensing pipette and combined with an equal volume

of IPA. The IPA/supernatant solution was then chilled at -16°C overnight to induce DNA precipitation. After centrifugation at 16,000x*g* the supernatant was removed and the remaining DNA pellet washed with 1mL 70% ethanol then dried and suspended in 1X TE buffer. The DNA was then quantified by analysis at 260/280nm and 230/260nm using a Thermo Scientific Nano2000 spectrophotometer.

2.3 Faecal DNA Extraction

2.3.1 Procurement of Faecal Samples

Faecal samples were obtained in from various sources: Most samples were provided from veterinary clinics from around the Waikato following a cold-calling campaign; the majority of these were from Raglan vets who deal with relatively large numbers of feral and stray cats. Other samples were brought in by friends and classmates from urban litterboxes and 4 samples were obtained from a freshly dug garden in a Hamilton suburb.

2.3.2 GTIC – Charcoal extraction of faecal DNA

An effective faecal DNA extraction method was developed owing to the presence of inhibitory factors in faeces. Initial trials produced DNA pellets containing unknown ionic or protein inhibitors of Taq polymerase function when tested with bacterial specific 16S primers. Chelex treatment (see below) was incompletely successful in solving this problem. Treatment of DNA suspended in TE with 1% activated charcoal was also inconsistent. This GITC method consisted of using small quantities ~ (0.1 to 0.2 grams dependant on water content/viscosity) extracted from sample vials with sterile swab into a 1.7 mL screw top eppendorf tube (containing 350µL SDS lysis solution) and heated at 95°C for 10 minutes. 600µL GTIC solution was then added and solids homogenised using the stick from a sterile swab followed by 10 minutes at 95°C and 900rpm agitation. Treatment with 1% activated charcoal was then carried out by addition of 60µL (10%) activated charcoal solution followed by mixing by vortex and 5 minutes rest. An equal volume of phenol chloroform was then added, mixed by vigorous shaking and 20min on a mixing wheel. The mixture was the centrifuged at 16,00G for ten minutes and the supernatant transferred to a 1.5mL

centrifuge tube using a fine tip dispensing pipette. The DNA was pelleted by adding an equal volume of IPA and centrifuged at 16,00x*g* for 15min. The pellet was then dissolved in 100µL SDS lysis solution followed by the addition of 100µL 5M NaCl and 80µL 10% CTAB were added and incubated at 65°C for 10min (at 900rpm). After cooling an equal volume of chloroform was added and shaken vigorously by hand for 40s or until any wax-like deposits had dissolved. The solutions were then put on a mixing wheel for 10min. After centrifugation at 16,000G for 10 minutes the aqueous phase was removed into a second 1.5mL centrifuge tube using a fine tip dispensing pipette to which an equal volume of IPA was added. The solution was then cooled to -16ºC overnight to induce the precipitation of DNA. The following morning a DNA pellet was produced by centrifugation and washed with 70% ethanol (as in section 3.1). The final DNA pellet was then dissolved in 1XTE in preparation for gel purification.

Ten microliters of loading buffer was added to each 50µL faecal DNA sample which was then divided into two thin 12 wells in a 1% agarose-superbuffer gel containing 3µL of ethidium bromide (10mg/mL) and electrophoresed for 10 minutes at 200V and 500mA. Genomic high molecular weight DNA bands were then visualised using blue light and cut from the gel using the thin end of cut 200µL sterilised micropipette tips and stored in weighed 1.5mL centrifuge tubes. DNA was then purified from the gel plugs using a DNA pure© gel extraction kit (see materials). Two microliters of cresol red was added to the gel extraction buffer to ensure pH<7.5.

2.4 Extraction of DNA from Meat Samples

2.4.1 Procuring meat samples

Fresh mince was purchased from butchers shops and supermarkets in the central Hamilton and east Hamilton areas. From each meat tray/bag samples were taken in a randomised fashion to make up two 20 gram aliquots of each mince type to be stored at -16°C in 50mL raliegh tubes.

2.4.2 Proteinase K digestion (hydroxylation)

Three 0.1 to 0.17 gram samples taken at random and added to 1.5mL screw top eppendorf tubes containing ~ 0.1mL of 0.5mm zircon beads and 6 or 7 2.5mm glass beads. Seven hundred microliters of SDS lysis solution was added to each tube and homogenisation using an eppendorf minibead beater for one minute. Each tube was then spun down for 30 seconds to reduce foam content and 25µL (60 units/mL) proteinase K added. Each digest was then incubated in an eppendorf thermomixer for one hour at 55°C (at 750 rpm) followed by a further 2.5 hours at 65°C (at 750rpm). For chicken mince and fish negative controls (Dr S Bird, UoW) a significantly greater DNA yield was achieved when incubated overnight at 65°C. To stop enzyme activity each tube was heated to 95°C for 15 minutes.

To each cooled digest-lysis solution an equal amount of 50/50 phenol chloroform solution was added and mixed vigorously for 20s by hand before a further 20min mixing on a mixing wheel. After mixing the aqueous and non-polar phases were separated by centrifugation for 10 minutes at 16,000G. Six hundred microliters of the supernatant aqueous phase was then removed to a 1.5mL centrifuge tube using a fine tip pasture/dispensing pipette. 100µL 5M NaCl and 80µL 10% CTAB were added and incubated 15min at 55°C. After cooling an equal volume of chloroform was added and shaken vigorously by hand for 40 seconds or until any wax like deposits were dissolved. The solutions were then put on the mixing wheel for at least 10 minutes. The aqueous supernatant phase was again separated into a separate 1.5mL centrifuge tube and an equal volume IPA added. Tubes were then cooled to -15°C overnight to induce DNA precipitation. The chilled DNA precipitate was centrifuged at 16,000G for 15 minutes to form a pallet and the supernatant discarded. The pellets were then washed with 1mL 70% ethanol. The supernatant was then removed using a fine tip dispensing pipette and the pellets allowed to dry (1Hr in fume hood). The pellets were then suspended in 50µL TE buffer for storage prior to purification by gel electrophoresis.

To separate large molecular weight DNA and remove potentially inhibitory contaminants total DNA extract (suspended in 1X TE buffer) was mixed

thoroughly with 6X GelRed™ DNA stain and loaded into ‘thick 10’ wells before being electrophoresed through a 1% agarose TAE gel for 40 minutes at 90V and 500mA. The resulting dispersion of nucleic acids through the agarose gel left large high weight DNA fragments along the edge of the wells (see Figure 2.1 below).

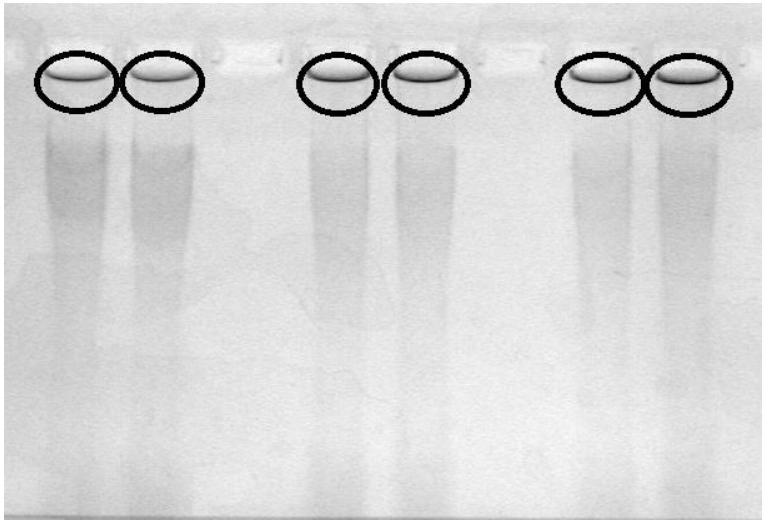

Figure 2.1 Beef5 Purification of high molecular weight DNA by gel electrophoresis. High molecular weight DNA can be observed as the dark stripes close to the edge of each well. Approximate cut area is indicated by the black rings.

The high molecular weight DNA was the cut from the gel using the wide end of sterilised (cut) 200µL micropipette tips and stored in pre-weighed 1.5mL centrifuge tubes and purified using the Geneaid quickclean II™ gel extraction kit. Best results were obtained when 25% (of volume) IPA was added to the binding buffer/gel mixture prior to being passed through the spin column and higher yields were obtained when the elution buffer was allowed to stand in the spin column for several minutes during the final (elution) step.

2.4.3 Poly ethylene glycol precipitation of high molecular weight DNA

Unpurified (total) DNA in 1X TE (140µL at ~ 300ng/µL) was combined with 1/4th volume 20% PEG solution (+2.5M NaCl) mixed thoroughly and allowed to

sit at room temperature for 15 minutes. Solution was then centrifuged at 13,000rpm. The supernatant was then removed and the pellet washed with 1mL 100% ethanol, centrifuged and washed again with 1mL 70% ethanol. After drying the resulting purified DNA was solubilised in (15µL) TE buffer (giving a nanodrop reading of 17.8ng/µL).

2.4.4 Pepsin / proteinase k digestion

A 1g pork mince (Pork1) was combined with 5ml (0.85%) NaCl and digested by addition of an equal pepsin-HCl solution (5mL0.1M HCl, 50mg NaCl, 0.7mg Pepsin) and incubated at 37°C for 1 hour. The solution was then centrifuged for 10 minutes at 1800g and the supernatant was removed using a dispensing pipette. The pallet suspended in 1mL SDS lysis solution and 50µL (60U/mL) proteinase K. and incubated overnight at 37°C. The digest was then transferred to a 2mL minicentrifuge tube and incubated in an eppendorf thermomixer for 1hour at 80°C with 750rpm agitation. The solution was then divided into two equal volumes and incubated at 95°C for a further 10 minutes with 850rpm agitation. DNA was extracted and purified using the phenol chloroform method (as in section 2.22 and 2.23).

2.4.5 Chelex treatment

DNA extracts suspended in TE buffer were diluted 1:5 (DNA + TE : Chelex solution) then mixed thoroughly and rested at room temperature. The resulting solutions were then trialled as templates for 16Sr DNA primer PCR reactions.

2.5 PCR Protocols

All meat and Toxovax™ DNA PCR reactions were carried out with approximately 2ng template DNA /µL reaction mixture. Faecal DNA extract concentrations meant significantly lower template DNA concentrations (as low as 0.4ng/µL in some cases). Forward and reverse primer concentrations of

0.2pmol/µL reaction mixture was used in all cases. See materials for PCR reaction mixture details.

Thermocycler Protocols		Initial Melt	Touchdown Cycles				Standard PCR cycles				Final Extension	Finish
		1	2	3	4	5	6	7	8	9	10	11
		95°C	95°C	Aneal time and temp -1°C/cycle	72°C	GOTO 2 cycles	95°C	Anneal (Time @ °C)	72°C	GOTO 6	Final Extension	room temperature
Protocol	1	15 min	20s	20s @ 70	30s	10X	20s	20s @ 66	30s	40X	10min @ 68	20°C
	2	15 min	20s	20s @ 70	30s	10X	20s	20s @ 60	30s	40X	10min @ 68	20°C
	3	15 min	20s	20s @ 70	30s	10X	20s	20s @ 58	30s	40X	10min @ 68	20°C
	4	15min	20s	20s @ 70	30s	10X	20s	20s @ 60	30s	36X	10min @ 68	20°C
	5	15min	15s	15s @ 70	10s	5X	15s	15s @ 60	10s	35X	5min @ 72	20°C
	6	15min	15s	15s @ 66	10s	5X	15s	15s @ 63	10s	35X	5min @ 72	20°C
	7	15min	15s	15s @ 70	10s	10X	15s	15s @ 62.7	10s	40X	10min @ 68	20°C
	8	15min	15s	15s @ 70	10s	10X	15s	15s @ 60	10s	40X	10min @ 72	20°C
	9	15 min	20s	20s @ 70	30s	10X	20s	20s @ 60	30s	36X	10min @ 68	20°C
	10	15min	20s	20s @ 68	30s	10X	20s	20s @ 58	30s	40X	10min @ 72	20°C
	11	15min	20s	20s @ 71	40s	10X	20s	20s @ 61	40s	40X	10min @ 72	20°C
	12	15min	20s	20s @ 70	40s	10X	20s	20s @ 60	40s	40X	10min @ 72	20°C
	13	15min	20s	20s @ 70	20s	11X	20s	20s @ 62	20s	40X	10min @ 72	20°C
	14	15min	20s	20s @ 70	20s	11X	20s	20s @ 61.5	20s	40X	10min @ 72	20°C
	15	15min	20s	20s @ 70	20s	11X	20s	20s @ 58.5	20s	40X	10min @ 72	20°C
	16	15min	20s	20s @ 70	20s	11X	20s	20s @ 58	20s	40X	10min @ 72	20°C
	17	15min	20s	20s @ 67	20s	10X	20s	20s @ 60.5	20s	40X	10min @ 72	20°C
	18	15min	20s	20s @ 67	20s	10X	20s	20s @ 56	20s	40X	10min @ 72	20°C
	19	15min	20s	20s @ 65	20s	10X	20s	20s @ 56.5	20s	40X	10min @ 72	20°C
	20	15min	20s	20s @ 65	20s	10X	20s	20s @ 54.3	20s	40X	10min @ 72	20°C
	21	15min	20s	20s @ 67	20s	10X	20s	20s @ 62	20s	40X	10min @ 72	20°C
	22	15 min	20s	20s @ 65	30s	9X	20s	20s @ 55	30s	35X	10min @ 72	20°C
	23	15min	15s	15s @ 67.5	10s	10X	15s	15s @ 57.5	10s	40X	10min @ 72	20°C
	24	15min	15s	15s @ 61	15s	10X	15s	15s @ 50	15s	40X	10min @ 72	20°C
	25	15min	15s	15s @ 63	20s	12X	15s	15s @ 58	20s	40X	10min @ 72	20°C
	26	15min	15s	15s @ 63	20s	12X	15s	15s @ 57.4	20s	40X	10min @ 72	20°C
	27	15min	15s	15s @ 63	20s	12X	15s	15s @ 56.4	20s	40X	10min @ 72	20°C
	28	15min	15s	15s @ 63	20s	12X	15s	15s @ 54.9	20s	40X	10min @ 72	20°C
	29	15min	15s	15s @ 63	20s	12X	15s	15s @ 53.1	20s	40X	10min @ 72	20°C
	30	15min	15s	15s @ 63	20s	12X	15s	15s @ 51.5	20s	40X	10min @ 72	20°C
	31	15min	15s	15s @ 63	20s	12X	15s	15s @ 50.5	20s	40X	10min @ 72	20°C
	32	15min	15s	15s @ 63	20s	12X	15s	15s @ 50	20s	40X	10min @ 72	20°C
	33	15min	15s	15s @ 70	20s	10X	15s	20s @ 60	20s	40X	10min @ 72	20°C
	34	15min	15s	15s @ 70	20s	10X	15s	20s @ 58	20s	40X	10min @ 72	20°C
	35	15min	15s	15s @ 69	25s	10X	15s	15s @ 59	25s	40X	10min @ 72	20°C
	36	15min	15s	15s @ 57	25s	10X	15s	15s @ 57	25s	40X	10min @ 72	20°C
	37	15min					15s	15s @ 59	25s	40X	10min @ 72	20°C
	38	15min					15s	15s @ 58	25s	40X	10min @ 72	20°C
	39	15min					15s	15s @ 57.1	25s	40X	10min @ 72	20°C
	40	15min					15s	15s @ 55.9	25s	40X	10min @ 72	20°C
	41	15min	15s	15s @ 66	25s	10X	15s	15s @ 59	25s	40X	10min @ 72	20°C
	42	15min	15s	15s @ 66	25s	10X	15s	15s @ 58	25s	40X	10min @ 72	20°C
	43	15min	15s	15s @ 66	25s	10X	15s	15s @ 57.1	25s	40X	10min @ 72	20°C
	44	15min	15s	15s @ 66	25s	10X	15s	15s @ 55	25s	40X	10min @ 72	20°C
	45	15min	15s	15s @ 70	25s	10X	15s	15s @ 60	25s	40X	10min @ 72	20°C
	46	15min	15s	15s @ 65	25s	10X	15s	15s @ 55	25s	40X	10min @ 72	20°C
	47	15min	15s	15s @ 65	20s	10X	15s	15s @ 59	20s	40X	10min @ 72	20°C
	48	15min	15s	15s @ 65	20s	10X	15s	15s @ 58	20s	40X	10min @ 72	20°C
	49	15min	15s	15s @ 65	20s	10X	15s	15s @ 57.1	20s	40X	10min @ 72	20°C
	50	15min	15s	15s @ 65	20s	10X	15s	15s @ 55	20s	40X	10min @ 72	20°C
	51	15min	15s	15s @ 60	20s	10X	15s	15s @ 50	20s	36X	5min @ 68	20°C
	52	15min	15s	15s @ 65	20s	10X	15s	15s @ 55	20s	30X	10min @ 68	20°C
	53	15min	15s	15s @ 62	20s	12X	15s	15s @ 55	20s	36X	5min @ 68	20°C
	54	15min	15s	15s @ 65	20s	10X	15s	15s @ 55	20s	30X	5min @ 68	20°C
	55	15min	15s	15s @ 68	20s	10X	15s	15s @ 58	20s	40X	10min @ 72	20°C
	56	15min	15s	15s @ 65	20s	9X	15s	15s @ 55	20s	35X	10min @ 72	20°C
	57	15min	15s	15s @ 62	25s	10X	15s	25s @ 52	20s	34X	10min @ 72	20°C
	58	15min	15s	15s @ 65	15s	10X	15s	15s @ 60	15s	40X	10min @ 72	20°C
	59	15min	15s	15s @ 65	15s	10X	15s	15s @ 55	15s	40X	10min @ 72	20°C
	60	15min	15s	15s @ 68	20s	10X	15s	15s @ 58	20s	36X	10min @ 68	20°C
	61	15min					15s	15s @ 50	25s	40X	10min @ 68	20°C
	62	15min					15s	15s @ 55	20s	40X	10min @ 68	20°C
	63	15min	15s	15s @ 66	20s	10X	15s	15s @ 56	20s	35X	10min @ 72	20°C
	64	15min	15s	15s @ 66	30s	10X	15s	15s @ 56	30s	35X	10min @ 72	20°C
	65	15min	15s	15s @ 64	30s	10X	15s	15s @ 54	30s	35X	10min @ 72	20°C
	66	15min	15s	15s @ 64	25s	10X	15s	15s @ 54	25s	35X	10min @ 72	20°C
	67	15min	15s	15s @ 66	25s	10X	15s	15s @ 56	25s	25X	10min @ 72	20°C
	68	15min	15s	15s @ 64	30s	10X	15s	15s @ 54	30s	25X	10min @ 72	20°C
	69	15min	15s	15s @ 65.8	25s	10X	15s	15s @ 59.4	25s	40X	10min @ 72	20°C
	70	15min	15s	15s @ 65.8	25s	10X	15s	15s @ 55.8	25s	40X	10min @ 72	20°C
	71	15min	15s	15s @ 66	25s	10X	15s	15s @ 56	25s	45X	10min @ 72	20°C

Table 2.1 Thermocycler protocols. Times and temperatures for initial melt, touchdown and standard PCR cycles and final extension.

2.6 Sequencing and sequence analysis

One microlitre of alkaline phosphate and 1µL of exonucleaseI was mixed with 30µL of PCR product and incubated at 37^{o}C for 15 minutes. The solution was then heated at 55^{o}C for 15 minutes to inactivate the enzymes and then sent for DNA sequencing.

DNA sequences were resolved using a 3130XL Genetic Analyzer System fitted with 50 cm capillary arrays (Life Technologies Corporation) loaded with POP-7 polyacrylamide matrix (Life Technologies Corporation). DNA templates were prepared using Big Dye v3.1 terminator chemistry (Life Technologies Corporation).

Sequence analysis was carried out using Biomatters Genious© R7 software package.

Chapter Three

Results

3.1 Positive and negative controls:

Positive and negative controls were developed to assess the effectiveness of the primer sets shown in Table 3.1. The positive control was effective in almost all cases but non-specific amplification was a factor in the negative control developed.

PCR TARGETS	Primer Name	Location			Expected product size	Primer details	
		Chromasome / Locus	Start	Finish		Primer sequence	Reference
TGR1E (tandem repeat)	TGR1E-1	(RH)AJ288978	7		192	atggtccggccggtgtatgatatgcg	Turcekova *et al.* 2013
	TGR1E-2			199		tccctacgtggtgccgcattgcct	
AF (tandem repeat)	TgRE1 (AF-F)	(RH)AF487550	87		163	agggacagaagtcgaagggg	Falahi *et al.* 2014
	TgRE2 (AF-R)			250		gcagccaagccggaaacatc	
B1 (tandem repeat)	B22	9	5049218		1776	tgttctgtcctatcgcaacg	Alfonso *et al.* 2013
	B23			5050994		tgggtctacgtcgatggcatg	
SAG1	SAG1 F	8	2664589		127	ctgatgtcgttcgtcggtgt	Yu, Huang, Zhou, Chen and Du 2013
	SAG1 R			2664718		gtgaagtggttctccgtcggtgt	
P30	P30-1	8	2664921		125	agttccaatcgagaagttccc	Costa & Bretagne 2011
	P30-2			2665037		ttattgacgaccgatgaggc	
SAG3	SAG3F	7	510875		291	caactctcaccattcttccaccc	Su, Zhang, Dubey 2011
	SAG3R			510565		gcgcgttgttagacaagaca	
NN (18S internaly transcribed sequence)	NN1	(ME49) AY259045	1		393	cctttgaatcccaagcaaaacatgag	Halova *et al.* 2011
	NN2			393		cgagccaagacatccattgctga	
ITS (18S internaly transcribed sequence)	ITSfw	(ME49) AY259045	79		301	gatttgcattcaagaagcgtgatagta	Halova *et al.* 2011
	ITSrev			380		agtttaggaagcaatctgaaagcacat	
L358	L358-F2	5	2241763		304	aggaggcgtagcgcaagt	Su, Zhang, Dubey 2011
	L358-R2			2241407		ccctctggctgcagtgct	
GRA6	GRA6-F1	10	7273239		345	tttccgagcaggtgacct	Su, Zhang, Dubey 2011
	GRA6-R1x			7272915		tcgccgaagagttgacatag	
UPRT	UPRT-F	11	2722303		346	actgcgacgacatactggagaac	Wu, Chen, Jiang, Cao 2009
	UPRT-R			2722649		aagaaaacaaagcggaacaacaa	
API (apicoplast)	API FWD	apicoplast	14907		305	tctattgcaatggaaaaaggtatg	Wu, Chen, Jiang, Cao 2009
	API REV			15211		tcaatggtagagcaaaggactg	
API-184	API 184 F	apicoplast	5951		184	tggttttaaccctagattgtggt	designed using NCBI primer blast
	API 184 R			6134		aagtccgctgtgtgtttaccaa	
B1-463	B1-463 F	9	4975767		463	cggagttcttcccagacgtg	designed using NCBI primer blast
	B1-463 R			4975305		tcatttgcaaaacagcggca	
SAG1-449	SAG1-449 F	8	2664527		449	acgagtatgtttccgaaggca	designed using NCBI primer blast
	SAG1-449 R			2664974		tctcccttgatgcaaccgac	
SAG2	SAG2 F	8	4757938		334	gctacctcgaacaggaacac	Aspinal, Marlee, Hyde and Sims 2002
	SAG2 R			4758272		gcatcaacagtcttcgttgc	
449F/P30R	SAG1-449 F	8	2664527		510	acgagtatgtttccgaaggca	NCBI Primer Blast
	P30-2			2665037		ttattgacgaccgatgaggc	Costa & Bretagne 2011
SAG1F/449R	SAG1 F	8	2664589		385	ctgatgtcgttcgtcggtgt	Yu *et al.* 2010
	SAG1-449 R			2664974		tctcccttgatgcaaccgac	NCBI Primer Blast

Table 3.1 Primer target regions, expected product sizes, sequences and references.

Table 3.1 shows the primers selected from literature and generated using NCBI blast which were selected for this study based upon the criteria of sensitivity, specificity and previous success's.

3.1.1. Positive controls:

DNA was extracted and purified from Toxovax™, the MSD Animal Health® live toxoplasmosis vaccine for sheep (see Figure 3.1) using the proteinase K digestion and phenol/chloroform extraction (see methods). A DNA extract was also purified from Toxovax™ which had been grown in THP-1 (human monocytic) cell culture. These extraction results are summarised in Table 3.1

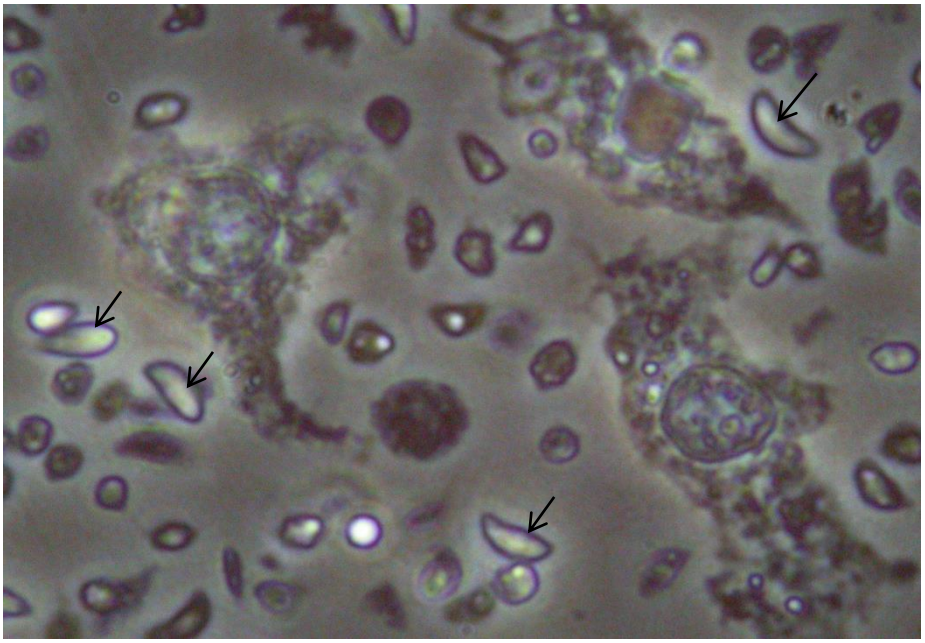

Figure 3.1 Toxovax™ under 1000X magnification. No stain. Viewed with a Zeiss Axiostar™ plus transmitted light microscope fitted with a Canon Coolpix™ 4500 digital camera. Live tachyzoites can be seen indicated by arrows.

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
1	Toxo Vacc 1	341.5	ng/µl	6.83	3.454	1.98	2.04	DNA	50
2	Toxo vacc 2	100.6	ng/µl	2.012	0.94	2.14	2.11	DNA	50
3	Toxo vacc 3	68.9	ng/µl	1.379	0.637	2.17	2.36	DNA	50
4	Toxo vacc 4	55.9	ng/µl	1.118	0.512	2.18	2.46	DNA	50
5	Toxo vacc 5	363.3	ng/µl	7.266	3.862	1.88	2.24	DNA	50
6	Toxo vacc 6	78.1	ng/µl	1.561	0.707	2.21	2.46	DNA	50
1	Toxo THP-1	1722.5	ng/µl	34.45	16.413	2.1	2.14	DNA	50

Table 3.2 Nanodrop concentration readings of purified Toxovax™ (MSD animal health®) DNA extracts.

Toxovax™ THP-1 DNA extract was diluted to 100ng/µL in 1X TE buffer as a positive control for PCR reactions. This control was first used to test the specificity (and functionality) of sixteen primer sets and combinations of primer sets (eg: SAG449F/ P30R) that targeted the same region.

PCR reactions were all carried out using 100ng template (Toxovax™ DNA extract) and 2µL 5pM forward and reverse primers. The thermocyler protocol 63 (see methods), a ten cycle touchdown to 56°C followed by 35 standard cycles was used to amplify the PCR products visible in Figures 3.2 and 3.3.

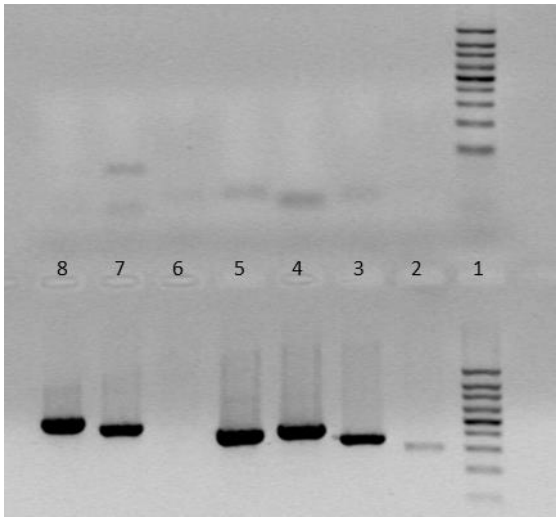

Figure 3.1.2 Toxovax™ THP1 positive control primer test 1. Lane1: Solis Biodyne 100bp ladder. Lane2: API. Lane 3:GRA6. Lane 4:L358. Lane5:NN1/2. Lane6:SAG2. Lane7:SAG449. Lane8:449F/P30R. Negatives run in top lanes. Gel: 1% agarose in SB + 3µL Ethidium bromide (10mg/mL)

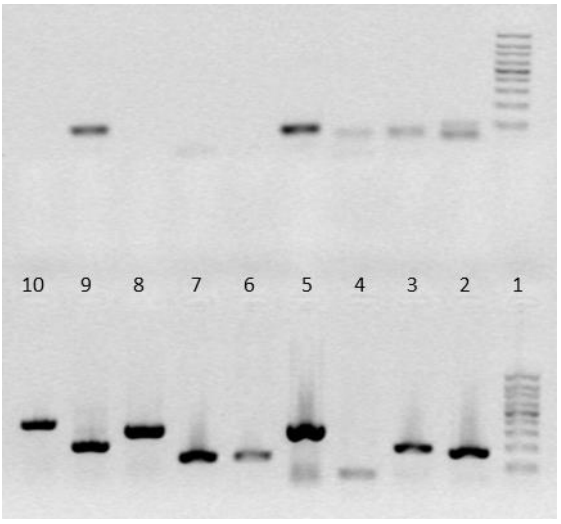

Figure 3.1.3 Toxovax™ THP1 positive control primer test 2. Lane1: Solis Biodyne™ 100bp DNA ladder. Lane2:AF. Lane3:API184. Lane4:B1. Lane5:ITS. Lane6:P30. Lane7:SAG3. Lane8:TGR1E. Lane9:UPRT. Negatives run in top lanes. Gel: 1% agarose in SB + 3µL Ethidium bromide (10mg/mL)

All reactions gave product bands of the expected size with the exception of SAG2 and B22. It was discovered late in the study that owing to a misprint in (Alfonso et al., 2013) in which resulted in a primer set targeting a much larger (1776bp) region of B1 than intended had been ordered. The tabulated primer sequences (Alfonso et al., 2013) indicate a 190bp PCR product should be produced and reference Bretagne et.al. 1993 (Bretagne, Costa, Vidaud, Van Nhieu, & Feith, 1993) however only one of these primers is a match for primer set indicated in the original 1993 study. The reason for lack of SAG2 amplification was unknown but may be a reflection of the attenuation of the Toxovax© genome or scientific error.

The primer sets targeting, SAG3 and GRA6 and SAG1nested set of outer primers: SAG449F/P30R and inner primers:SAG1F/SAG449R were used to test for the presence of *T. gondii* within faecal samples owing to the high product concentration achieved in the initial tests against the Toxovax™ DNA extract and the potential for polymorphism between *T. gondii* strains at these loci/targets.

Sequencing of SAG1(nest) PCR product from Toxovax™ template gave the following BLAST hits:

SAG1F:

Toxoplasma gondii strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds. Evalue = 1.30e-140. Grade =100%. Hit start:280, Hit end: 554.

P30R:

Toxoplasma gondii strain P-Br SAG1 protein (SAG1) gene, complete cds. Evalue=0, Grade = 99.9. Hit start:470, Hit end:57.

(See Appendix 2 for chromatograms)

3.1.2. Negative controls:

Negative controls were generated by the extraction of high molecular weight DNA from three fish species as cold blooded animals are not known to be hosts for *T. gondii* (Dubey, 2008). The scales, fins and digestive tract were removed from each specimen and the remaining tissues were homogenised for proteinase K / phenol-chloroform DNA extraction followed by gel purification of high molecular mass DNA fragments (See methods section 2.3)

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
1	Fish1	4.8	ng/µl	0.096	0.038	2.54	0.15	DNA	50
2	Shark1	60.5	ng/µl	1.21	0.65	1.86	0.4	DNA	50
3	Zebra Fish	549.6	ng/µl	10.992	5.912	1.86	1.74	DNA	50

Table 3.3. Negative control DNA extraction results. Shows the gel purified nucleic acid yield resulting from each fish meat digest trialled.

The first (misc.) fish sample was a freshwater fish of unknown species sourced from a small lake in the Waikato area by the Waikato universities Aquatic Research Centre This specimen was in poor condition and provided only a small amount of gel purified DNA, which was later found to test positive for *T. gondii* SAG1 DNA when tested with nested PCR indicating probable contamination within these DNA extracts or the primer sets used in these reactions.

The second fish sample (shark) came from the fin of a shark caught in the Kaipara Harbour (cc. Dr Stevin Bird) and stored frozen at low temperature. Although this sample gave a greater DNA yield it was also found to test positive for *T. gondii* SAG1 DNA when tested with nested PCR.

The third fish DNA extract was obtained from a fresh Zebrafish harvested from the University of Waikato C.2.03 (molecular genetics lab) Zebrafish tank. This specimen was humanely euthanized using an anaesthetic solution. The gel purified high molecular weight DNA was found to test negative for *T. gondii* specific sequences using SAG1F/SAG449R (see figure 3.4), SAG 3 (see figure 3.5) and the NN1/2 outer and ITS inner nested primer sets. GRA6 primers gave a >500bp non-specific PCR product (see figure 3.6) which sequencing and BLAST analysis showed was clearly a Zebrafish specific sequence with a significant size difference (500bp vs an expected 343bp)

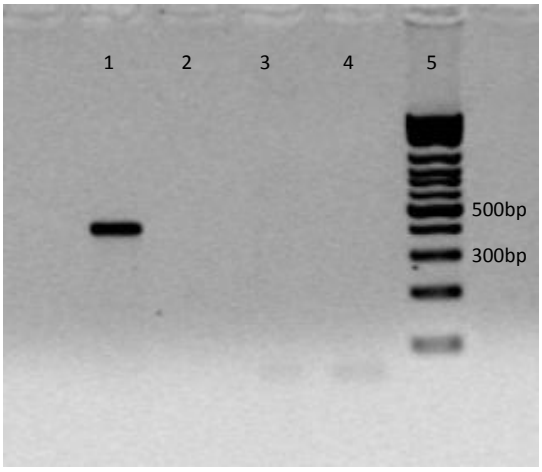

Figure 3.1.4 Toxovax™ THP-1 positive control and Zebrafish negative control amplified using SAG1F/SAG449 primers. Lane 1 Toxovax™ THP-1 positive control, Lane 2: Empty, Lane 3: Zebrafish, Lane 4: Blank (2µL 1XTE), Lane 5: Solis Biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide (10mg/mL)

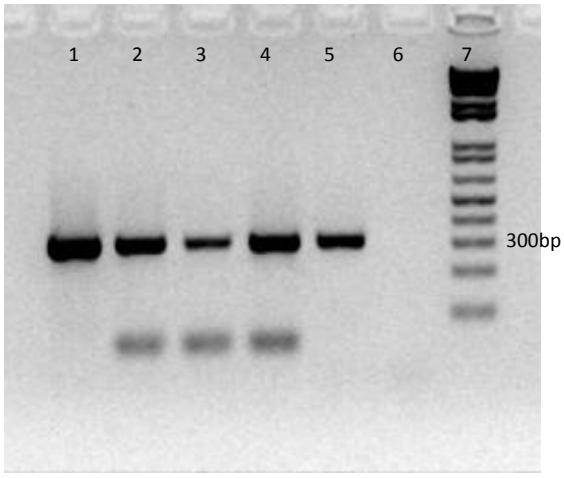

Figure 3.1.5 Toxovax™ THP-1 positive control and Zebrafish negative control amplified using SAG3 primers. Lane 1: Toxovax™ positive control, Lane 2: CatV, Lane 3: CatT, Lane 4:CatR, Lane 5: Cat P, Lane 6: Zebrafish. Lane 7: Solis Biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide (10mg/mL)

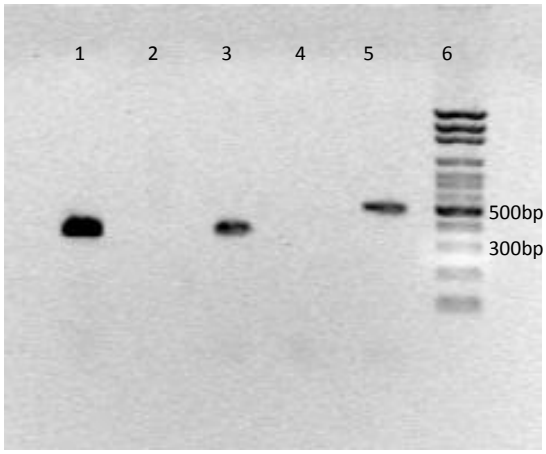

Figure 3.1.6 Non specific product resulting from Zebrafish DNA extract amplified using GRA6 primers. Lane 1: Toxovax™ positive control, Lane 2: Empty, Lane 3: Cat T, Lane 4: Cat J, Lane 5: Non specific Zebrafish product, Lane 6 Solis Biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide (10mg/mL)

3.1.3. Summary of results for positive and negative controls

Positive and negative controls		Expected product size (bp)	Observed			
			Toxovax	Misc. Fish	Shark	Zebra fish
Primer Set	TGR1E(RF)	192	Y			
	TGRE1(AF)	173	Y			
	B22/23(B1)	1778	N			
	SAG1	127	Y			
	P30	125	Y			
	SAG3	291	Y			N
	NN1/2	393	Y			
	ITS	301	Y			
	NN1/2(outer) ITS(inner) Nested.	301				N
	L358	304	Y			
	GRA6	343	Y			NS
	UPRT	346	Y			
	API	305	Y			
	API-184	184	Y			
	B1-463	463	Y			
	SAG-449	449	Y			
	SAG2	334	N			
	449F/P30R	510	Y			
	SAG1F/449R	385	Y			N
	449FP30R(outer) SAG1F449R(inner) Nested	385	Y	Y	Y	

Table 3.4 Summary of PCR results for positive and negative controls tested against various primer sets. Y= Product band of expected size observed. N= No product observed. NS= Product or products of incorrect size observed. (seq) indicates sequencing was carried out, see appendix for data. (cont.) indicates contamination of reaction.

3.2 Faecal DNA extraction and PCR results

A reliable extraction and purification technique was developed using GITC homogenisation followed by phenol chloroform extraction and gel purification. This overcame early extraction methods that proved unreliable at removing Taq polymerase inhibitory contaminants. Table 3.21 shows nucleic acid concentrations retrieved from all faecal samples. 260/280(nm) measurements close to 2.00 indicates a high percentage of DNA.

3.2.1 PCR analysis of feline faecal DNA extracts

To test for the presence of Taq polymerase inhibitory contaminants within the faecal DNA extracts, PCR reactions were attempted using recombinant 16Sr DNA bacterial specific primers to control for false negatives. If inhibitory contaminants are present no PCR product will be produced. A band of 220bp would indicate a positive amplification and hence a lack of inhibitory contaminants. A negative control, containing no extracted DNA but containing bacterial DNA from the recombinant manufacturing process should always result in a 220bp PCR product.

Initial results showed amplification from some DNA samples extracted via GITC homogenisation followed by phenol chloroform DNA extraction. However, results were inconsistent (see figures 3.2.1, 3.2.2 and 3.2.3).

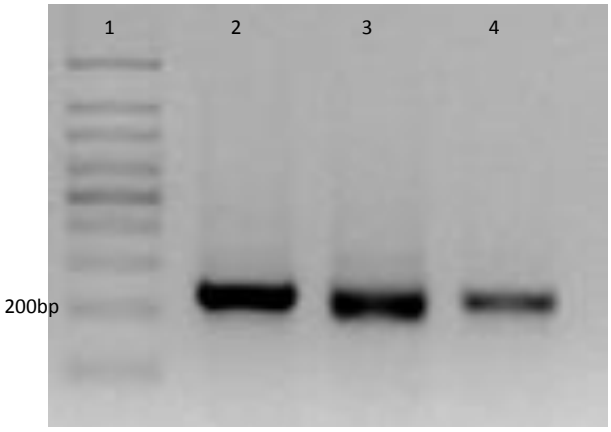

Figure 3.2.1 16Sr DNA primer amplification of unpurified faecal extracts. Lane 1: Invitrogen™ kb+ DNA ladder. Lane 2: 16Sr positive control amplification. Lane 3: Cat B, Lane 4: Cat A. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

	Extraction	Conc.		A260	A280	260/280	260/230	Sample	Factor
1	Cat A	79.4	ng/µl	1.589	0.82	1.94	2.66	DNA	50
2	Cat B	57.7	ng/µl	1.154	0.599	1.92	3.77	DNA	50
3	CatC	131.6	ng/µl	2.632	1.527	1.72	0.55	DNA	50
4	CatD	172.3	ng/µl	3.446	2.125	1.62	0.31	DNA	50
5	CatE	214.2	ng/µl	4.283	2.401	1.78	0.45	DNA	50
6	CatF	276.3	ng/µl	5.526	2.777	1.99	1.07	DNA	50
7	CatG	170.4	ng/µl	3.408	1.966	1.73	0.52	DNA	50
8	CatE	10.1	ng/µl	0.203	0.127	1.59	2.98	DNA	50
9	CatF	17.1	ng/µl	0.341	0.208	1.64	1.26	DNA	50
10	CatE	5.5	ng/µl	0.11	0.068	1.61	-0.91	DNA	50
11	CatH	107.1	ng/µl	2.142	1.259	1.7	0.76	DNA	50
12	CatI	135.1	ng/µl	2.703	1.589	1.7	0.52	DNA	50
13	CatJ	215.9	ng/µl	4.318	2.362	1.83	0.71	DNA	50
14	CatK	141.8	ng/µl	2.835	1.777	1.6	0.3	DNA	50
15	CatM	146.8	ng/µl	2.936	1.813	1.62	0.35	DNA	50
16	CatN	260.6	ng/µl	5.212	2.984	1.75	0.48	DNA	50
17	CatO	110.9	ng/µl	2.219	1.512	1.47	0.31	DNA	50
18	CatP	121.8	ng/µl	2.436	1.468	1.66	0.47	DNA	50
19	CatQ	194.9	ng/µl	3.899	2.281	1.71	0.46	DNA	50
20	CatR	152.7	ng/µl	3.054	1.933	1.58	0.33	DNA	50
21	CatS	367.9	ng/µl	7.359	4.157	1.77	0.51	DNA	50
22	CatT	153.5	ng/µl	3.069	1.915	1.6	0.35	DNA	50
23	CatU	188.5	ng/µl	3.771	2.177	1.73	0.4	DNA	50
24	CatV	57.1	ng/µl	1.142	0.64	1.79	0.71	DNA	50
25	CatV CC	64.5	ng/µl	1.289	0.669	1.93	0.93	DNA	50
26	CatP CC	41.7	ng/µl	0.835	0.587	1.42	0.33	DNA	50
27	Cat P	40.2	ng/µl	0.803	0.571	1.41	0.32	DNA	50
28	CatV CC Column	26.3	ng/µl	0.525	0.333	1.58	0.52	DNA	50
29	CatP CC Gel	4.4	ng/µl	0.088	0.05	1.78	0.07	DNA	50
30	CatV CC Gel	6.4	ng/µl	0.129	0.074	1.75	0.08	DNA	50
31	CatR (PEG)	0.6	ng/µl	0.011	0.013	0.9	0.03	DNA	50
32	CatT (PEG)	1.5	ng/µl	0.029	0.021	1.38	0.01	DNA	50
33	CatR CC Gel	9.7	ng/µl	0.193	0.124	1.56	0.03	DNA	50
34	CatT CC Gel	9.3	ng/µl	0.187	0.138	1.36	-0.62	DNA	50
35	CatM CC Gel	6.9	ng/µl	0.139	0.065	2.14	0.13	DNA	50
36	CatN CC Gel	8.6	ng/µl	0.172	0.081	2.12	0.41	DNA	50
37	CatQ CC Gel	11.9	ng/µl	0.238	0.119	1.99	0.33	DNA	50
38	CatU CC Gel	5.2	ng/µl	0.104	0.035	2.96	0.2	DNA	50
39	CatP CC Gel	8.3	ng/µl	0.165	0.086	1.91	0.45	DNA	50
40	CatR CC Gel	8.5	ng/µl	0.171	0.093	1.83	0.47	DNA	50
41	CatT CC Gel	9	ng/µl	0.181	0.086	2.1	0.34	DNA	50
42	CatV CC Gel	9.1	ng/µl	0.183	0.087	2.1	0.33	DNA	50
43	CatB CC Gel	9.8	ng/µl	0.195	0.1	1.96	0.72	DNA	50
44	CatC CC Gel	13.7	ng/µl	0.275	0.15	1.83	0.61	DNA	50
45	CatD CC Gel	4.8	ng/µl	0.097	0.035	2.78	0.49	DNA	50
46	CatE CC Gel	8.8	ng/µl	0.175	0.084	2.09	0.56	DNA	50
47	CatF CC Gel	15.9	ng/µl	0.317	0.175	1.82	0.76	DNA	50
48	CatG CC Gel	20	ng/µl	0.4	0.228	1.75	0.65	DNA	50
49	Cati CC Gel	12.3	ng/µl	0.246	0.126	1.95	0.58	DNA	50
50	CatJ CC Gel	13.5	ng/µl	0.269	0.114	2.35	0.03	DNA	50
51	CatK CC Gel	4.1	ng/µl	0.082	0.03	2.73	0.45	DNA	50
52	CatL CC Gel	6.4	ng/µl	0.128	0.071	1.81	0.37	DNA	50
53	CatS CC Gel	14.3	ng/µl	0.286	0.147	1.94	0.5	DNA	50
54	CatW CC Gel	5.2	ng/µl	0.104	0.032	3.3	0.09	DNA	50
55	CatX CC Gel	10.8	ng/µl	0.216	0.13	1.66	0.54	DNA	50
56	CatY CC Gel	7.8	ng/µl	0.157	0.089	1.77	0.45	DNA	50
57	CatZ CC Gel	3.3	ng/µl	0.067	0.02	3.27	0.46	DNA	50
58	CatA2 CC Gel	8.7	ng/µl	0.174	0.085	2.05	0.33	DNA	50
59	CatB2 CC Gel	1.9	ng/µl	0.037	-0.006	-6.75	0.5	DNA	50
60	CatC2 CC Gel	3.4	ng/µl	0.068	0.017	4.1	0.53	DNA	50
61	CatD2 CC Gel	11.9	ng/µl	0.238	0.113	2.11	0.03	DNA	50

Table 3.5 Faecal DNA extraction results. All faecal DNA extracts shown. CC= activated charcoal treatment, PEG= polyethylene glycol precipitation of high molecular weight DNA, Column= purified using DNA pure™ DNA purification kits, Gel= purified by gel electrophoresis and extraction from gel using DNA pure™ gel extraction kits.

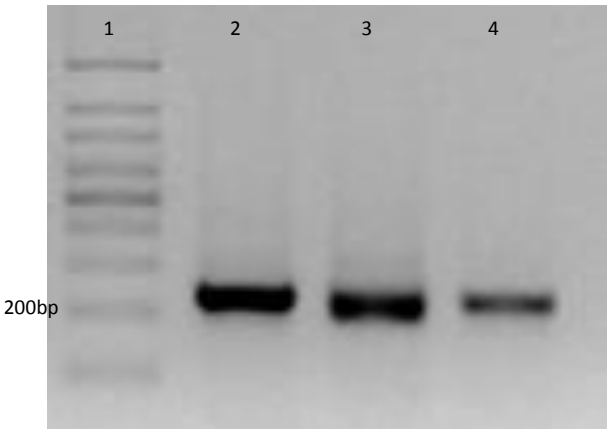

Figure 3.2.1 16Sr DNA primer amplification of unpurified faecal extracts. Lane 1: Invitrogen™ kb+ DNA ladder. Lane 2: 16Sr positive control amplification. Lane 3: Cat B, Lane 4: Cat A. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

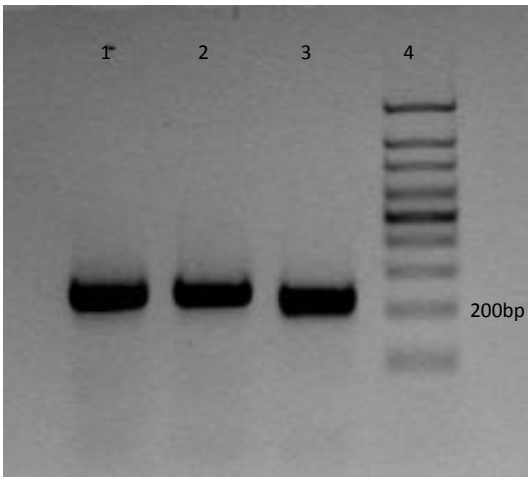

Figure 3.2.2 16Sr DNA primer amplification of faecal extracts. Lane 1: 16Sr positive control amplification. Lane 2: Cat D(unpurified), Lane 3: Negative control. Lane 4: Invitrogen™ kb+ DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

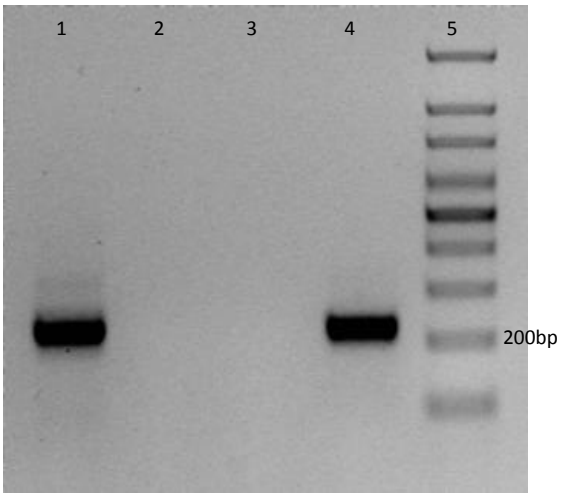

Figure 3.2.3 16Sr DNA primer amplification of faecal extracts. Lane 1: CatG(unpurified). Lane 2: Cat F(unpurified). Lane 3: Cat E(unpurified), Lane 4: 16Sr control amplification. Lane 5: Invitrogen™ kb+ DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

In an attempt to remove inhibitory ionic components, DNA extracts were treated with 5% (200 to 400) mesh chelex solution. This resulted in amplification in some of the previously inhibitory DNA samples (Cat E) but did not reliably remove all inhibitory contaminants (see figure 3.2.4 and 3.2.5 below).

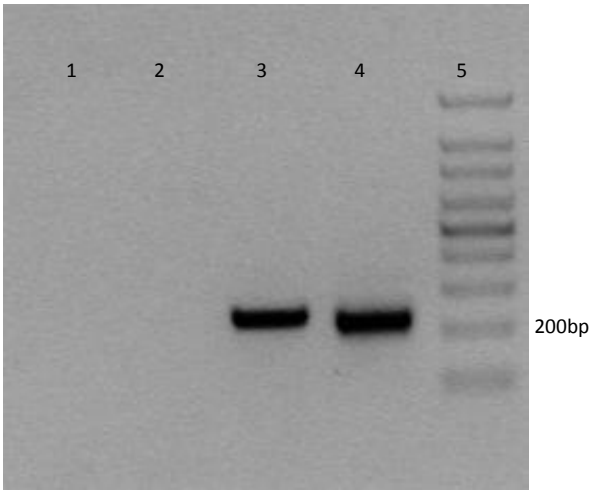

Figure 3.2.4 16Sr DNA primer amplification of faecal DNA extracts. Lane 1: Cat H(chelex). Lane 2: Cat F(chelex 1). Lane 3: Cat E(chelex 1), Lane 4: 16Sr control amplification. Lane 5: Invitrogen™ kb+ DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

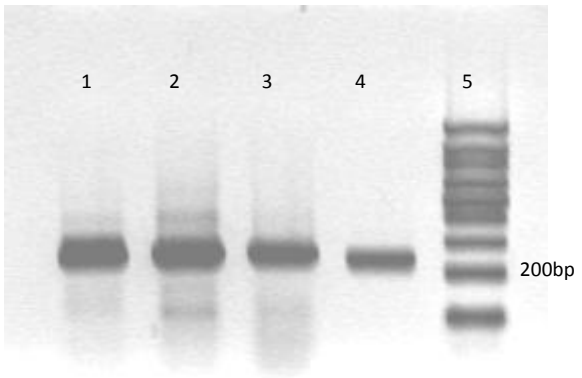

Figure 3.2.5 16Sr DNA primer amplification of faecal DNA extracts. Lane 1: Cat J(unpurified). Lane 2: Cat I(unpurified). Lane 3: Cat F(chelex 2), Lane 4: 16Sr control amplification. Lane 5: Invitrogen™ kb+ DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL).

The second method trialled in the attempt to eliminate inhibitory components was activated charcoal treatment (to remove organic contaminants). This method did not appear to be successful (see figures 3.2.7 and 3.2.8 below). These samples were sourced from Anexa animal health in Raglan and had been refrigerated, in some cases, for several months.

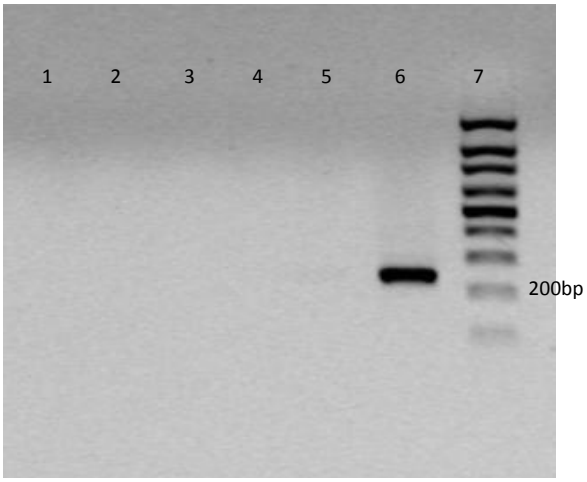

Figure 3.2.6 Attempted amplification of activated charcoal treated faecal DNA extracts. Lane 1: Cat N, Lane 2: Cat P, Lane 3: Cat Q, Lane 4: Cat R, Lane 5: Cat T, Lane 6: Cat U, Lane 6: 16Sr control amplification. Lane 7: Invitrogen™ kb+ DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL)

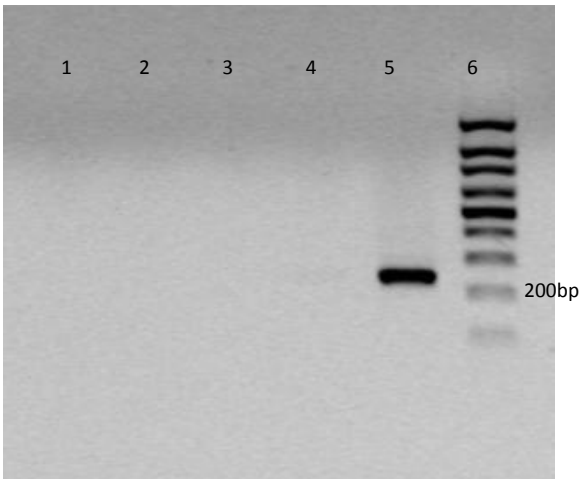

Figure 3.2.7 Lack of visible PCR products from both untreated and charcoal treated DNA extracts. Lane 1: CatV (charcoal treated), Lane 2: Cat V (Untreated), Lane 3: Cat P (charcoal treated), Lane 4: Cat P (Untreated). Lane 5: 16Sr control amplification, Lane 6: Invitrogen™ kb+ DNA ladder

When both chelex and activated charcoal treatment gave inconsistent results, gel purification was trialled. DNA extracts were elecrophosesed through a 1% agarose SB gel containing 2µL ethidium bromide for 10 minutes at 200 volts and 500mA. The resulting bands were punched out and purified using DNA pure™ gel extraction 'spin columns'. The resulting DNA was consistently amplifiable (see figures 3.2.9 and 3.2.10).

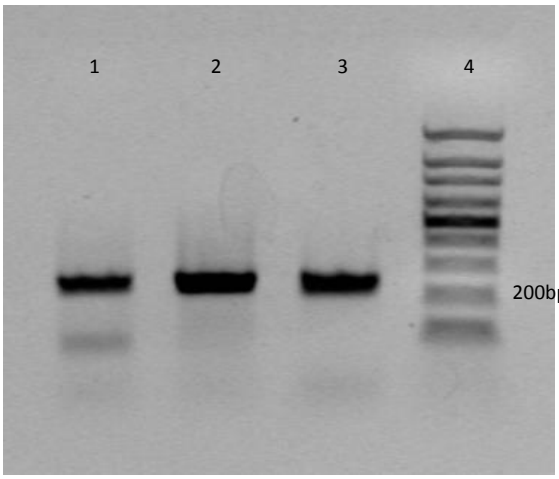

Figure 3.2.8 16Sr DNA primer amplification from gel purified faecal DNA extracts. Lane 1: Cat V (charcoal treated and gel purified). Lane 2: Cat P (charcoal treated and gel purified) Lane 3: 16Sr control amplification, Lane 4: Invitrogen™ kb+ DNA ladder. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide.

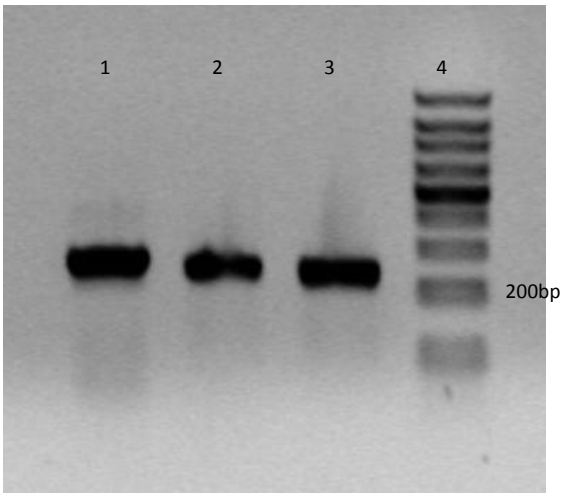

Figure 3.2.9 16Sr DNA primer amplification from gel purified faecal DNA extracts. Lane 1: Cat K(charcoal and gel purified). Lane 3: Cat L(charcoal and gel purified), Lane 4: 16Sr control amplification. Lane 5: Invitrogen™ kb+ DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL)

Low DNA yield presented some problems in that, at low concentrations (<10ng/µL, Table 3.21) the PCR reaction mixtures had to be diluted by up to 10% in some cases with DNA template solution. Amplification of *T. gondii* specific PCR products was achieved using three primer sets targeting regions thought to be polymorphic and useful as genotyping markers however inconsistencies were observed.

Specimens from 'at risk' felines, typically young feral cats or old unhealthy cats gave a particularly high rate of *T. gondii* positive results using SAG3 and GRA6 primer sets. All results for samples known to be from young domestic cats were *T. gondii* negative. There are likely several factors behind these observations ie: diet, hunting and feline leukemia virus (see discussion).

SAG2 was targeted in a preliminary trial giving a single product band close to the expected size of 334bp in one case and multiple products in one case. Surprisingly, amplification was achieved using these primers which had previously failed to produce a product when trialled with Toxovax™ DNA extract.

Single copy *SAG1*, *SAG3* and *GRA6* targets were reliable *T. gondii* specific targets. A broader sample set was covered with the latter two targets. Results were contradictory in three of the nine cases where both *SAG3* and *GRA6 t*argets were amplified from the same DNA extracts; the reason for this is unknown but is likely due to scientific error.

3.2.2 *SAG3* amplification:

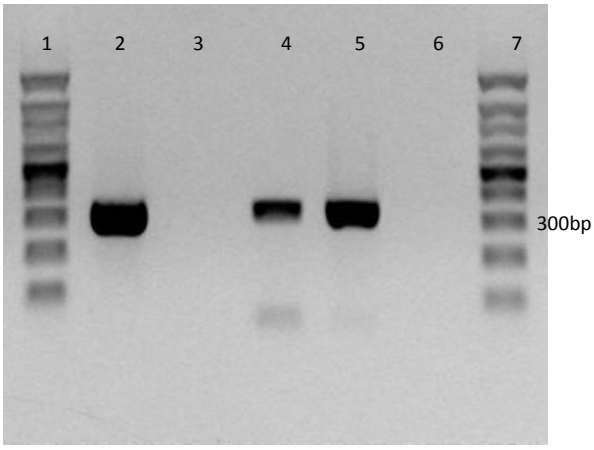

Figure 3.2.10 *SAG3* amplification of charcoal treated and gel purified faecal DNA extracts and positive control. Lane 1: Solis Biodyne™ 100bp DNA ladder, Lane 2: Toxovax™ positive control, Lane 3: empty, Lane 4: Cat V (charcoal and gel purified), Lane 5: Cat P(charcoal and gel purified), Lane 6: Negative control, Lane 7: Solis Biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL)

Figures 3.2.12 and 3.2.13 (below) demonstrate the potential inconsistencies between different primer sets (PCR targets). Cat R DNA extract gave a result consistent with the presence of *T. gondii* DNA when amplified using SAG3 primers however no product was observed when the same sample was amplified using SAG449F/P30R (*SAG1* locus) primers. Cat T DNA was not amplified due to the failure of a PCR tube but was successful in a later attempt.

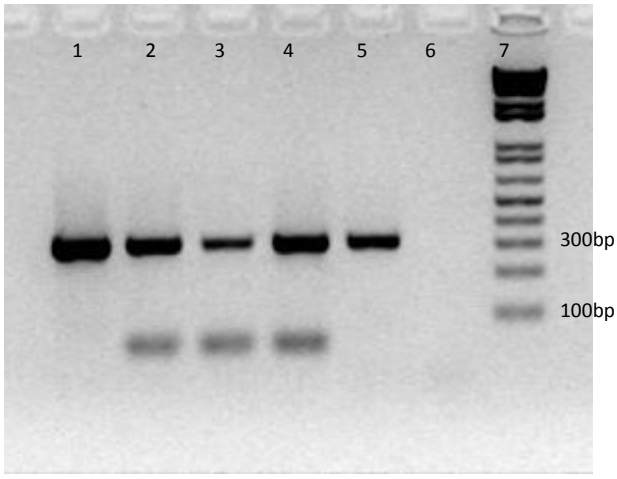

Figure 3.2.11 *SAG3* amplification of charcoal + gel purified faecal extracts with positive and negative controls. Lane 1: Toxovax™ positive control, Lane 2: Cat V, Lane 3: Cat T, Lane 4: Cat R, Lane 5: Cat P, Lane 6: Zebrafish negative control. Lane 7: Invitrogen™ Kb+ DNA ladder. Bands below 100 bp are primer dimers. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL)

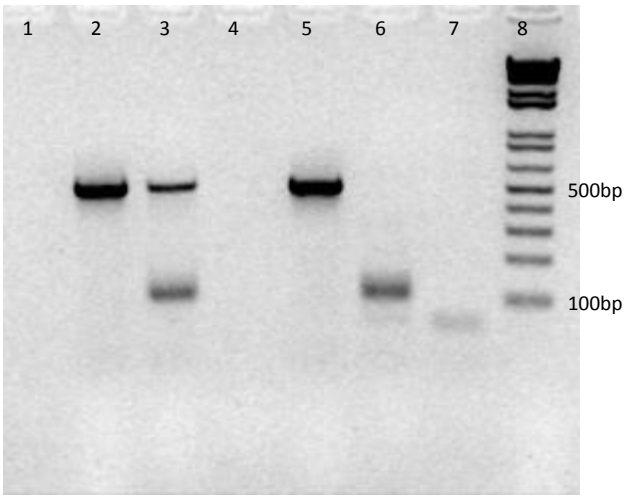

Figure 3.2.12 SAG449F/P30R amplification of four faecal extracts . Lane 1: empty, Lane 2: Toxovax™ positive control, Lane 3: Cat V, Lane 4: Cat T (evaporated reaction mixture), Lane 5:Cat R, Lane 6: Cat P, Lane 7: Zebrafish negative control, Lane 8: Invitrogen™ Kb+ DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL).

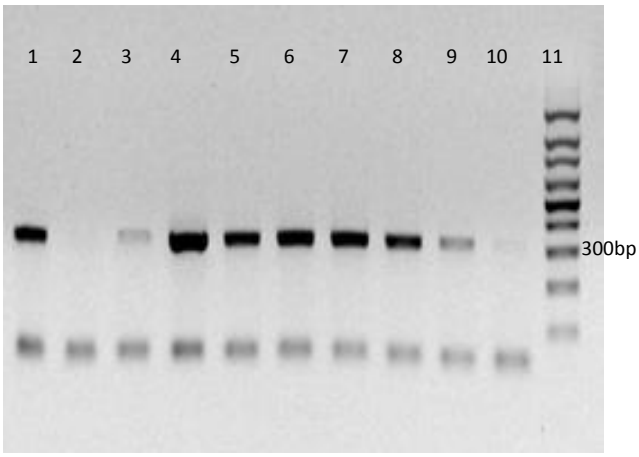

Figure 3.2.13 SAG3 amplification of faecal DNA extracts. Lane 1: Cat S. Lane 2: Cat L, Lane 3: Cat E Lane 4: Cat F, Lane 5: Cat G, Lane 6 Cat I, Lane 7: Cat J, Lane 8: Cat K, Lane 9: Cat L, Lane 10: Cat S. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide (10mg/mL).

Figure 3.2.14 demonstrates the high rate of results indicating the presence of *T. gondii* in faecal samples from feral cats in the Raglan area. Nine out of the ten samples obtained (a single consignment of samples) tested positive for the presence of *T. gondii* SAG3 DNA.

SAG1 amplification was achievable using primer sets SAG449F/P30R and SAG1F/SAG449R (see Figure 3.2.13). Positive results matched those for SAG3 primers in 3 of 5 cases and gave multiple products in a fourth. SAG3 and GRA6 targets were preferable overall because of higher product concentrations and greater probability of polymorphisms and were used in larger numbers trials. PCR results are summarised in Table 3.2 below.

3.2.3 GRA6 Amplification

GRA6 proved to be a reliable PCR target for faecal DNA extracts. Where amplification occurred, PCR products were specific giving product bands of the expected (343bp) size.

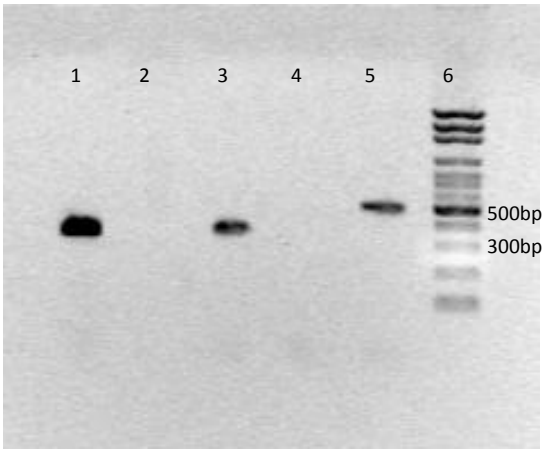

Figure 3.2.14 GRA6 amplification of faecal DNA extracts. Lane 1: 100bp Solis biodyne™ DNA ladder, Lane 2: Zebrafish mispriming product, Lane 3: CatJ, Lane 4 CatT, Lane 5: empty, Lane 6: Positive (Toxovax™) control. Amplified using protocol 67. Visualised using a 1% agarose SB gel containing 3µL ethidium bromide (10mg/mL).

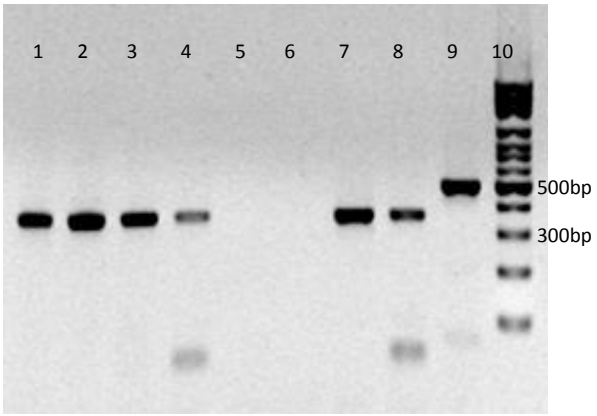

Figure 3.2.15 GRA6 amplification of faecal DNA extracts. Lane 1: Cat I, Lane 2: Cat H, Lane 3: Cat G, Lane 4: Cat F, Lane 5: Cat E, Lane 6:Cat D, Lane 7: Cat C, Lane 8: Cat B, Lane 9: Zebrafish mispriming product, Lane 10: Solis Biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 2µL Ethidium bromide (10mg/mL).

Faecal PCR results		Primer Set													
		16S		SAG449FP30R			SAG1FSAG449R				SAG1	SAG2	SAG3	GRA6	
Thermocycler protocol		46	56	61	64	65	45	60	62	66	45	55	64	64	71
Faecal DNA extract	1 CatA(1)	S					N	N			N				
	2 CatB(1)	S					S(seq)	N			N				
	43 CatB(2)														S
	3 CatC(1)		S					N				S			
	44 CatC(2)												S		S
	4 CatD(1)		S					N				MP			
	45 CatD(2)												S		N
	5 CatE(1)		N												
	5 CatE(+chelex)		S					N							
	46 CatE(2)												S		N
	9 CatF(1)		N												
	9 CatF(+chelex)		S					N							
	47 CatF(2)												S		S
	7 CatG(1)		S					N							
	48 CatG(2)												S		
	11 CatH							N							
	12 CatI(1)		S					N							
	49 CatI(2)												S		S
	13 CatJ(1)		S					N							
	50 CatJ(2)												S		S
	14 CatK(1)		S	N											
	51 CatK(2)												S		S
	52 CatL(1)		S	N									N		S
	35 CatM														S
	16 CatN(1)		N												N
	18 CatP(1)		N												
	26 CatP(2)		N												
	27 CatP(3)		N										S		
	39 CatP(5)		S			N							S(seq)		S
	19 CatQ(1)		N												N
	20 CatR(1)		N												
	33 CatR(2)				S	S							S,S(seq)		
	54 CatS(2)												S		
	23 CatT(1)		N												
	43 CatT(2)				S	S							S	S	
	23 CatU(1)		N												
	24 CatV(1)		N												
	25 CatV(2)														
	28 CatV(3)		N												
	31 CatV(4)		S			MP							S,S(seq)		
	55 CatW													S	

Table 3.6 Amplification of PCR products from faecal DNA extracts. S= single product amplified. N= No product amplified. MP= multiple products. (seq) = sequencing was attempted (see appendix for detail).

3.2.4 Restriction digest of faecal GRA6 PCR products.

A restriction enzyme digest was used to confirm the identity of five GRA6 PCR products. The MseI cut pattern closely matches what one would expect for GRA6 originating from the type II archetypal strain of *T. gondii*.

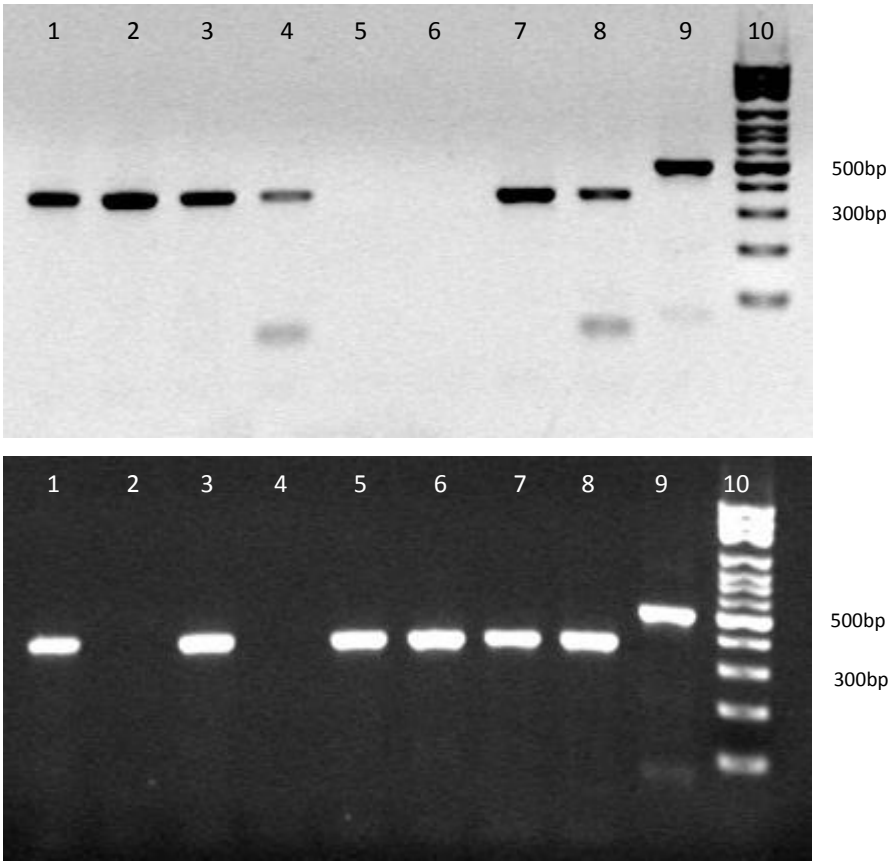

Figure 3.2.16 GRA6 PCR products from feline faecal samples. At top:Lane 1: Toxovax™ positive control, Lane 2: Cat I, Lane 3, Cat G, Lane 4 Cat F, Lane 5: Cat E, Lane 6: Cat D, Lane 7: Cat C, Lane 8: Cat B, Lane 9: Zebrafish mispriming product band, Lane 10: Solis Biodyne™ 100bp DNA ladder. At bottom: Lane 1: Toxovax™ positive control, Lane 2: Cat Q, Lane 3: Cat P, Lane 4: Cat N, Lane 5: Cat M, Lane 6: Cat L, Lane 7: Cat K, Lane 8: Cat J, Lane 9: Zebrafish mispriming product band, Lane 10: Solis Biodyne™ 100bp DNA ladder. Both images visualised using a 1% agarose SB gel containing 3µL ethidium bromide.

Amplified GRA6 products were selected for digestion on the basis of product concentration and greatest possible sample source distribution. Following twenty four hours incubation, all samples including Toxovax™ had undergone the same cut pattern giving distinct banding at approximately 280bp (see Figure 3.4) as would be expected from GRA6 sequences homologous with type II toxoplasmosis.

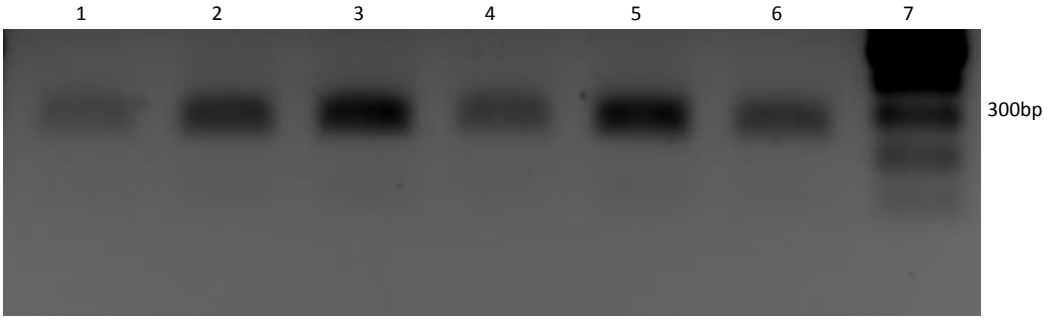

Figure 3.2.17 *MseI* restriction enzyme digest of GRA6 PCR products. Right to left: Lane 1: Solis Biodyne 100bp DNA ladder. Lane 2: CatC. Lane3: CatI. Lane 4: CatG. Lane 5: CatL. Lane6: CatP. Lane 7: Toxovax™ positive control. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide.

The high frequency at which *T. gondii* DNA was detected in feral feline faecal DNA extracts was unexpected given that *felidae* are thought to usually contract *T. gondii* at a young age when they will shed oocysts for a period of around two weeks following infection (J. Dubey, 2009) followed by a dormant period of up to 6 years during which very little or no shedding is expected. Immunosuppressive events have been shown to result in re-shedding (Malmasi, Mosallanejad, Mohebali, Sharifian Fard, & Taheri, 2009). In addition, Malmasi et al., 2009 demonstrated that rechallange by oral administration of *T. gondii* infected murine brain homogenate also resulted in re-shedding after 19 to 20 days.

Re-shedding may explain the high proportion of *T. gondii* positive results among faecal samples. All samples were obtained from veterinary clinics caring for either young, old or unhealthy cats, many of which were feral cats that were to be euthanized as part of a wildlife protection programme in the Raglan area.

These feral cats would be active hunters. By eating birds, mice and rat, these cats would be at significantly greater risk of toxoplasmosis infection and reinfection (rechallange) through bradyzoite consumption. Another contributing factor is that the dietary and physical stresses of a hunting and scavenging feral lifestyle could be great enough to compromise the immune system in host cats leading to 'flourishing' of existing infections and consequently oocyst shedding. Domestic cats on the other hand are generally healthier and their diets composed of processed foods that would not contain active oocysts (ie: cat biscuits and canned food).

The feline leukemia virus (FeLV) has become widespread throughout global *Felidae* populations. This virus has a significant impact upon its host's ability to respond to pathogenic challenge (such as toxoplasmosis) effectively. Due to its similarity in this respect FeLV is often referred to as cat AIDS. Domestic cats are now mostly immunised against this virus (especially younger cats) however the virus still infects a significant portion of the feral cat population. This compromised immune response of these infected cats is likely directly linked to reshedding of *T. gondii* oocysts as they consume prey animals with chronic (bradyzoite) toxoplasmosis infection.

3.2.5 SAG3 Sequencing results

Figures 3.2.19 and 3.2.20 show that the DNA extracted from Toxovax™ vaccine which was subsequently amplified using the SAG3 primers and sequenced was indeed *T. gondii*.

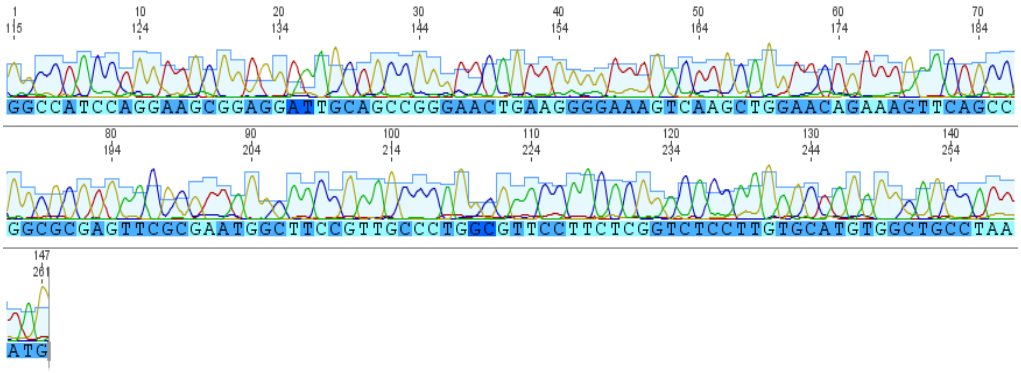

Figure 3.2.18 Chromatogram of Toxovax™ positive control amplified DNA using the SAG3F primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number L21720 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1303-1449) with an E value of 8.81e-70 and a Grade score of 100%.

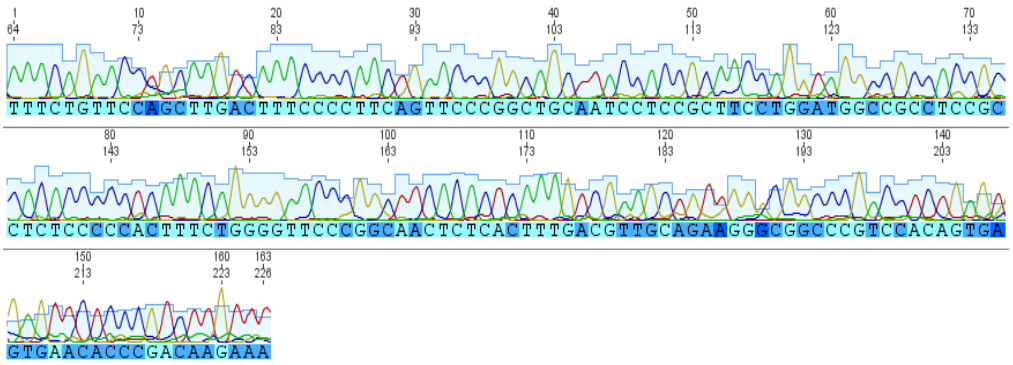

Figure 3.2.19 Chromatogram of Toxovax™ positive control amplified DNA using the SAG3R primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number AF340227 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1078-916) with an E value of 81.27e-78 and a Grade score of 100%.

The Expect (E) value represents the number of hits one can "expect" to see by chance when searching a database of a particular size. The grade score is a weighted score for the hit comprise of the E-value, the pairwise identity and the coverage.

Three PCR products (from catR, CatT and catV DNA extracts) amplified with SAG3 primers were confirmed as *T. gondii* SAG3 using sequencing and nr megablast analysis. The overall sequencing quality was high as demonstrated by clear distinct nucleotide peaks.

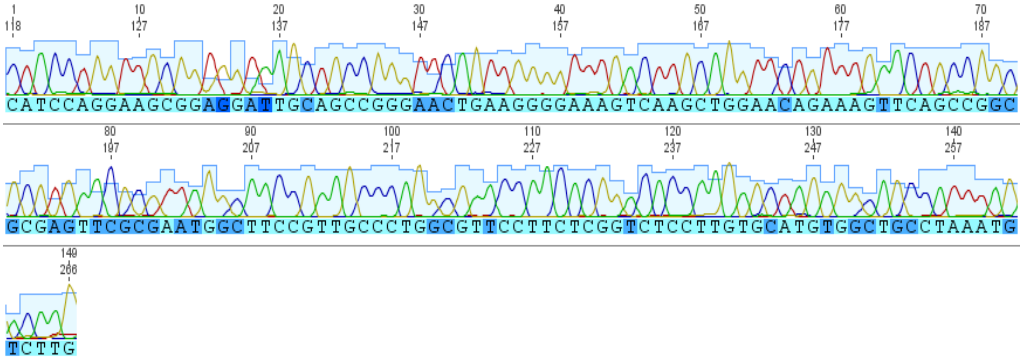

Figure 3.2.20 Chromatogram of CatR amplified DNA using the SAG3F primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number L21720 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1306-1454) with an E value of 6.93e-71and a Grade score of 100%.

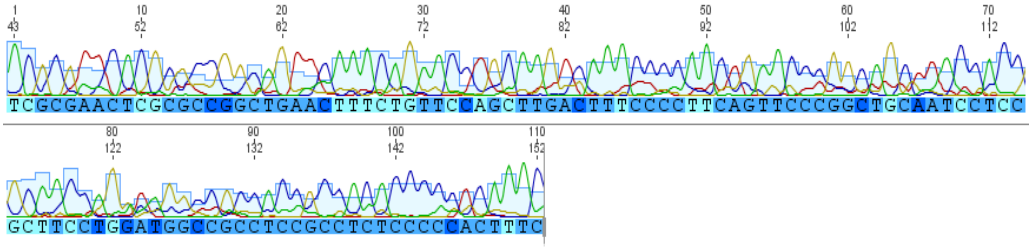

Figure 3.2.21 Chromatogram of CatR amplified DNA using the SAG3R primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number AF340227 (*T. gondii* surface antigen 43 (SAG3) mRNA).

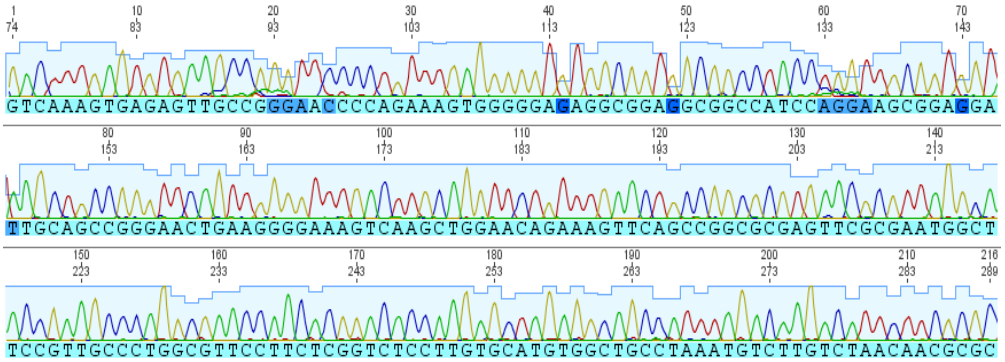

Figure 3.2.22 Chromatogram of CatT amplified DNA using the SAG3F primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number L21720 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1252-1467) with an E value of 6.20e-108 and a Grade score of 100%.

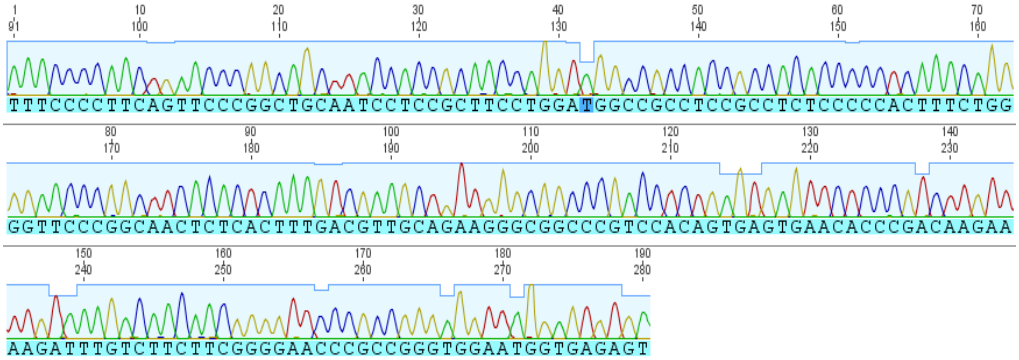

Figure 3.2.23 Chromatogram of CatT amplified DNA using the SAG3R primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number AF340227 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1060-871) with an E value of 1.51e-93 and a Grade score of 100%.

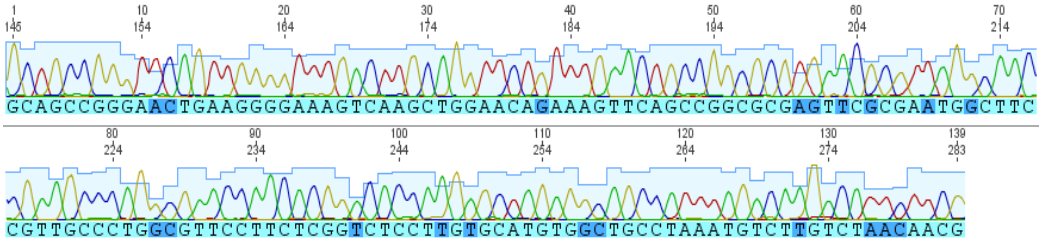

Figure 3.2.24 Chromatogram of CatV amplified DNA using the SAG3F primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number L21720 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1365-1464) with an E value of 2.29e-65 and a Grade score of 100%.

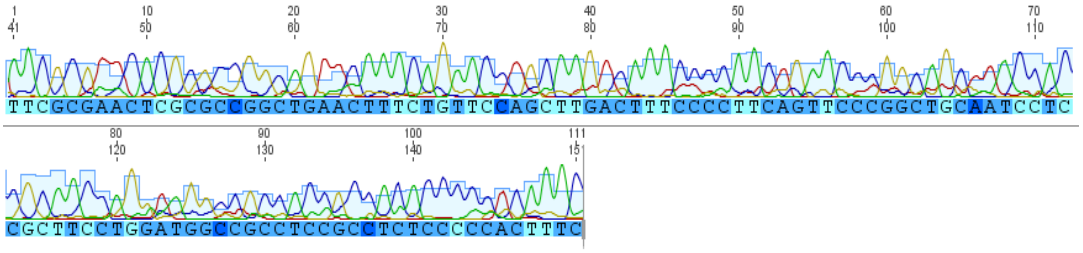

Figure 3.2.25 Chromatogram of CatV amplified DNA using the SAG3R primer. This sequence was analysed using NCBI nr Megablast and aligned with the top hit Genbank Accession number AF340227 (*T. gondii* surface antigen 43 (SAG3) mRNA from position 1102-992) with an E value of 6.31e-50 and a Grade score of 100%.

3.3 Meat DNA extraction

DNA was extracted from 25 samples of fresh mince from beef, lamb, pork and chicken. Table 3.3 shows that DNA was extracted from meat that was purchased from a single supermarket (Meat 1). For example, beef1(complete) refers to a sample of beef that was homogenised, and digested with proteinase K and purified using phenol:chloroform and the DNA concentration was 1173 ng/uL. The nanodrop reading suggests that the DNA is of good quality due to a absorbance reading of 1.98 at 260/280. Due to issues with PCR (Section 3.3), this DNA sample was later gel purified (Beef1 Gel) and as a result, the concentration was reduced to 173.4 ng/uL. In summary, nucleic acid concentration from the four extraction methods ranged from 3.3 ng/uL to 4971.6 ng/uL (Table 1 and Appendix 3)

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat1	beef1 (complete)	1173	ng/µl	23.459	11.85	1.98	1.72	DNA	50
	chicken1 (complete)	3937.7	ng/µl	78.755	38.479	2.05	2.02	DNA	50
	lamb1 (complete)	1876.4	ng/µl	37.527	18.363	2.04	1.95	DNA	50
	pork1 (complete)	857.8	ng/µl	17.156	8.547	2.01	1.99	DNA	50
	Beef1 Gel	173.4	ng/µl	9.454	4.893	1.93	1.49	DNA	50
	Lamb1 Gel	472.7	ng/µl	3.469	1.819	1.91	1.64	DNA	50
	Pork1 Gel	538.6	ng/µl	10.731	5.596	1.92	1.76	DNA	50
	pork1(pepsin)	4971.6	ng/µl	99.432	48.754	2.04	2.15	DNA	50
	pork1(pepsin)	1634.3	ng/µl	32.686	16.083	2.03	2.18	DNA	50
	pork1(pepsin) Gel	16.4	ng/µl	0.327	0.164	2	0.16	DNA	50
	pork1(pepsin) PEG	17.8	ng/µl	0.357	0.117	3.04	-3.36	DNA	50
	Beef1 Gel	54.5	ng/µl			1.95	1.44	DNA	50
	Chicken1 Gel	3.3	ng/µl			2.87	0.14	DNA	50
	Lamb1 Gel	69.1	ng/µl			1.93	0.94	DNA	50
	Pork1 Gel	39.2	ng/µl			1.95	0.54	DNA	50

Table 3.7 Nanodrop of extracted DNA of meat sourced from supermarket 1.

3.4. PCR analysis of purified meat DNA extracts.

3.4.1 Repeat region PCR targets:

Initial attempts were made to amplify *T. gondii* specific PCR targets using high sensitivity PCR primers targeting repeat regions within the *T. gondii* genome. Although these attempts using these primers with DNA extracted from four meat varieties gave product amplification, results were poor in most cases, ie: weak amplification levels with often multiple product bands indicated mispriming due to non-specific primers or potentially multiple infecting strains polymorphic at these target sites. (See figure 3.4.1 below.)

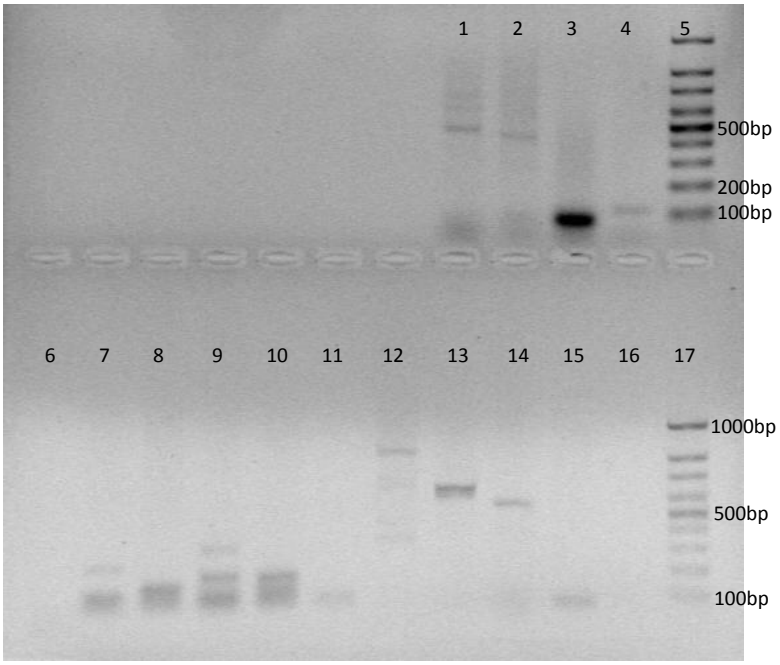

Figure 3.4.1. AFRE, B22/23 and TGR1R repeat region amplification from purified Meat1 DNA. Lanes 1-4 B22/23 amplification of: Lane 1: Pork1, Lane 2: Lamb1, Lane 3: Chicken 1, Lane 4, Beef1. Lane 5: Genescript 100bp DNA ladder. Lane 6: B22/23 negative. Lane 7 to 11 AFRE amplification of: Lane 7: Pork1, Lane 8: Lamb1, Lane 9: Beef1. Lane 10: Chicken 1. Lane 11: Negative. Lanes 12 to 15: TGR1E amplification, Lane12:Pork1, Lane 13:Lamb1 Lane 14: Beef1, Lane 16: TGR1E negative. Visualised on a 1% agarose TAE gel containing 2µl Ethidium bromide (10mg/mL).

Gel purification of complete DNA extracts gave improved results (see Figure 3.4.2), however chicken DNA had seemed to have been degraded to a state where only a negligible amount of high molecular weight DNA could be retrieved which was at a concentration that was not practical for PCR reactions.

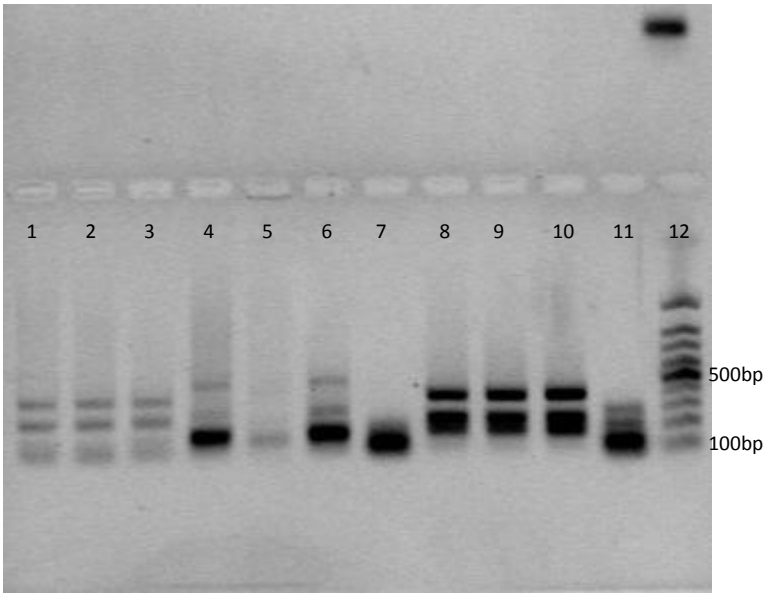

Figure 3.4.2. AFRE, B22/23 and TGR1R repeat region amplification from purified Meat1 DNA. Top right: B22/23 primer dimer from negative. Lanes 1-3 B22/23 amplification of: Lane 1: Pork1, Lane 2: Lamb1, Lane 3: Beef1. Lanes 4-7: AFRE amplification of: Lane 4: Pork1, Lane 5: Lamb1, Lane 6: Beef1, Lane 7: Negative. Lanes 8-11: TGR1E amplification of: Lane 8: Pork1, Lane 9: Lamb1, Lane 10: Beef 1. Lane 11 TGR1E negative. Lane 12 Genescript™ 100bp DNA ladder. Visualised on a 1% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

3.4.2 Single copy PCR targets:

Gel purification of Meat1 DNA extracts along with modified thermocycler protocols (to suit smaller PCR products) gave improved results for single copy P30 and SAG1 PCR targets. The difference can be observed in Figure 3.4.3 (unpurified) *cf.* Figures 3.4.4 and 3.4.5 (both gel purified).

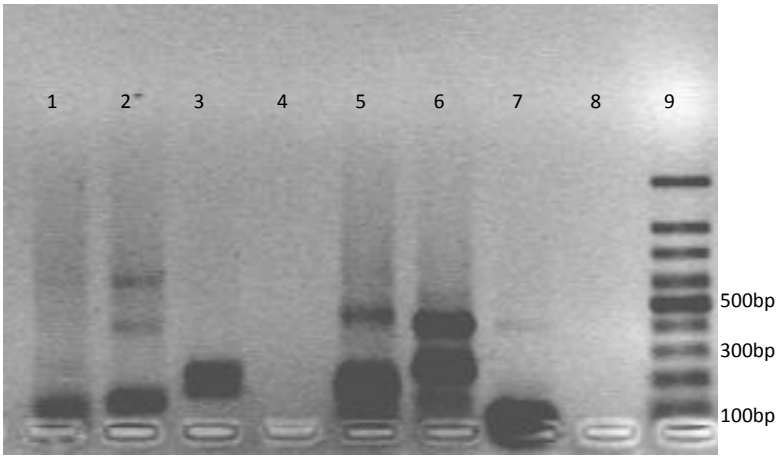

Figure 3.4.3 P30 and SAG1 amplification of Meat1 DNA extracts. Lanes 1-4 SAG1 amplification of: Lane 1: Pork1, Lane2: Lamb1, Lane 3: Beef3, Lane 4: SAG1 negative, Lane 5-8: P30 amplification, Lane 5 Pork1, Lane 6: Lamb1, Lane 7: Beef1. Lane 8: P30 negative control. Lane 9: Genescript™ 100bp DNA ladder. Visualised on a 1% agarose gel containing 2µL Ethidium bromide (10mg/mL).

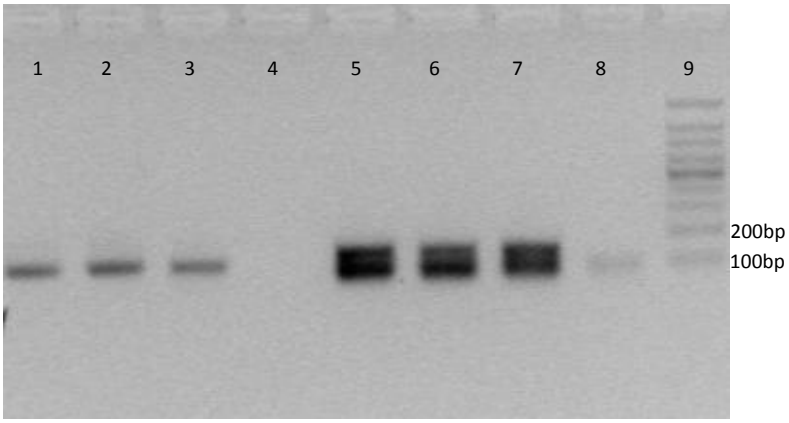

Figure 3.4.3 P30 and SAG1 amplification of Meat1 DNA extracts. Lanes 1-4 SAG1 amplification of: Lane 1: Pork1, Lane2: Lamb1, Lane 3: Beef3, Lane 4: SAG1 negative, Lane 5-8: P30 amplification, Lane 5 Pork1, Lane 6: Lamb1, Lane 7: Beef1. Lane 8: P30 negative control. Lane 9: Genescript™ 100bp DNA ladder. Visualised on a 1% agarose gel containing 2µL Ethidium bromide (10mg/mL).

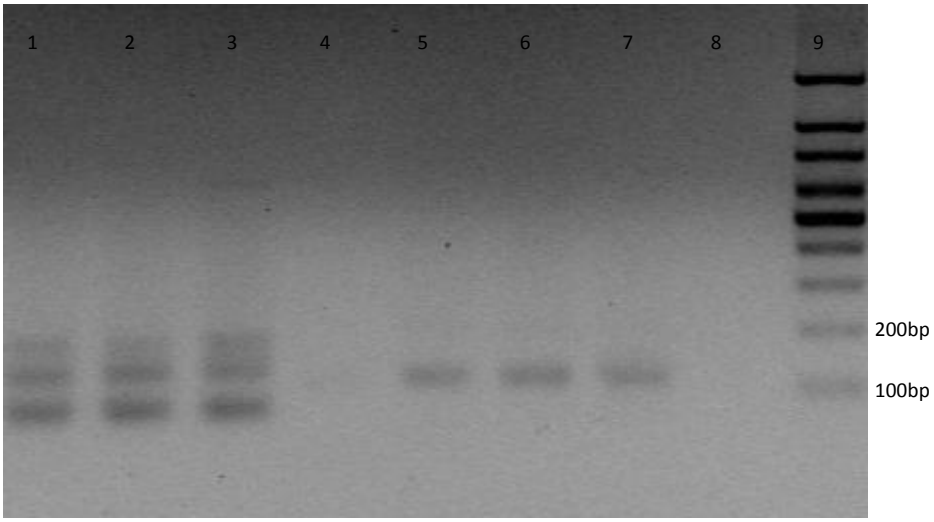

Figure 3.4.4 P30 and SAG1 amplification of Meat1 gel purified DNA. Lane 1-4 SAG1 amplification: Lane 1: Pork1, Lane 2: Lamb, Lane 3: Beef1, Lane 4 SAG1 negative control. Lanes 5-8 P30 amplification: Lane 5: Pork1, Lane 6: Lamb1, Lane 7, Beef1. Lane 8: Genescript™ 100bp DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

In all but one occurrence (New Zealand farmed pork mince) *T. gondii* DNA could not be detected in meat DNA extracts using standard PCR protocols. This positive result was confirmed through sequencing (see appendix). An attempt was made to replicate this result using the same pork DNA extract (Pork1) along with Lamb1 and Beef1 DNA. The original positive result could not be replicated (see sequence results in appendix). No further *T. gondii* specific DNA sequence was amplified. This shows that the DNA extraction method used is variable, this could be due to scientific error and or low bradyzoite cyst density within the muscle tissues tested.

In a surprising turn of events DNA sequencing of amplified products indicated that mispriming regularly resulted in incorrect products of a very similar size to those expected from amplification of *T. gondii* template. For example, both P30 and SAG1 primer sets amplifies nonspecific products of almost identical size (125

and 127 base pairs, respectively) from beef, lamb and pork DNA extracts (see Figure 3.4.3 and 3.4.4 and sequence results in appendix).

3.4.3 Genotyping PCR targets:

Initial attempts at amplification of *T. gondii* targets using genotyping targets such as SAG3, GRA6, NN1/2 and L358 failed to produce PCR products. By lowering the anneal temperature and increasing anneal time to 15s it was possible to produce PCR products but sequencing results (where possible) were poor and showed no evidence of the presence of *T. gondii* DNA.

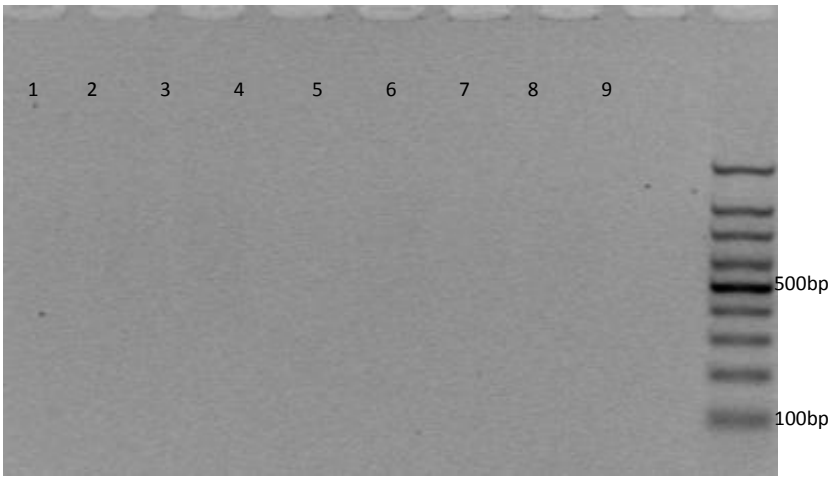

Figure 3.4.5 Lack of visible PCR products using Meat2 extracts with GRA6 and SAG3 primers at an annealing temperature of 58°C. Lane 1-4: GRA6: Lane 1: Pork2, Lane 2: Lamb2, Lane 3: Beef3, Lane 4: GRA6 negative control. Lane 5-8 SAG3: Lane 5 Pork2, Lane 6: Lamb2, Lane 7: Beef2, Lane 8 SAG3 negative control, Lane 9: Genescript™ 100bp DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

Based on the melt points for each primer (IDT scitools), 'Genotyping' primers were tested against Meat2 template DNA. Annealing temperatures used were as close to 5C below the expected melt point as possible. This resulted in expression of L358 products only (see figure 3.4.7). Sequencing results for L358 products were poor and did not indicate the presence of *T. gondii*, but that mispriming had occurred.

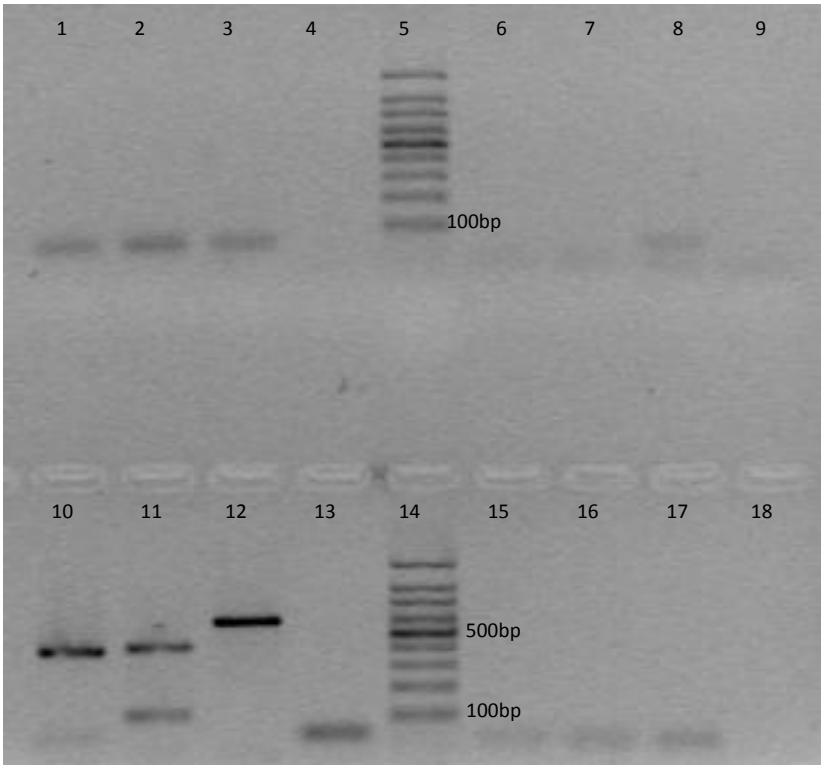

Figure 3.4.6 Touchdown and gradient PCR of SAG3 NN1/2, L358 and GRA6 primers with Meat2 extracts. Lanes 1-4 SAG3: Lane 1: Pork2, Lane 2: Lamb2, Lane 3: Beef2, Lane 4: negative control. Lane 5: Genescript ™ 100bp DNA ladder. Lanes 6-9: NN1/2: Lane 6: Pork2, Lane 7: lamb2, Lane 8: Beef2, Lane 9: NN1/2 negative control. Lanes 10-13 L-358: Lane 10 Pork2, Lane 11: Lamb2, Lane 12: Beef2. Lane 14: Genescript™ 100bp DNA ladder, Lanes15-18: GRA6: Lane 15: Pork2, Lane 16: Lamb2, Lane 17: Beef2, Lane 18 GRA6 negative control. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

By further decreasing annealing temperatures it was possible to obtain PCR products using GRA6 and SAG3 primer sets (see Figures 3.4.7 and 3.4.8 below), however multiple product bands indicate that these reactions are due to mispriming at suboptimal anneal temperatures.

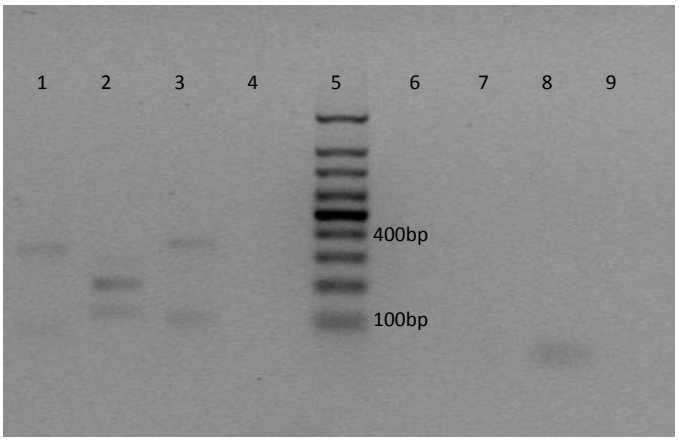

Figure 3.4.7 Attempted amplification of Meat2DNA extracts using SAG3 and GRA6 primers. Lanes 1-4 GRA6: Lane 1: Pork2, Lane 2: Lamb2, Lane 3: Beef2, Lane 4: GRA6 negative control. Lane 5: Genescript™ 100bp DNA ladder, Lanes 6-9: SAG3: Lane 6: Pork2, Lane 7: Lamb2, Lane 8 Beef2, Lane 9:SAG3 negative control. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

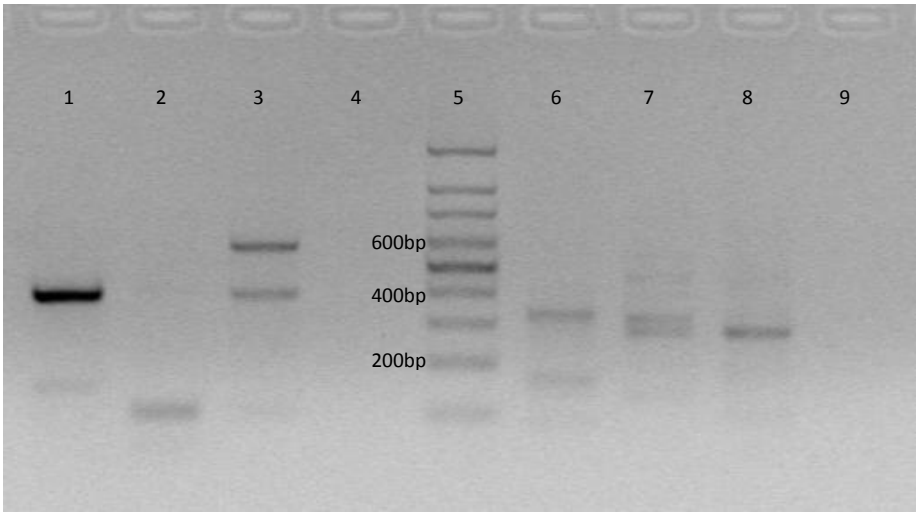

Figure 3.4.8 L358 and SAG3 amplification from Meat2 DNA extracts. Lanes 1-4: L358 amplifications. Lane 1 Pork2, Lane 2: Lamb2, Lane 3: Beef2, Lane 4: L358 negative control. Lane 5: Genescript™ 100bp DNA ladder. Lanes 6-9: SAG3 amplification. Lane 6: Pork 2, Lane 7: Lamb 2, Lane 8: Beef 2, Lane 9: SAG3 negative control. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL)

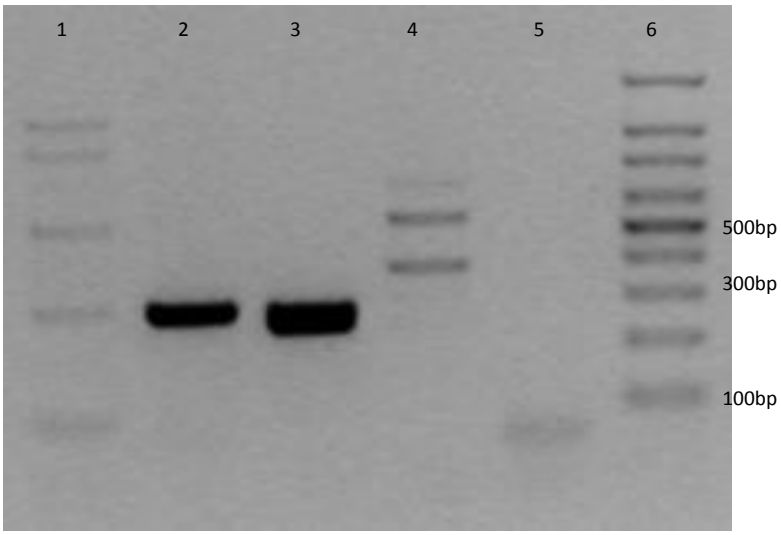

Figure 3.4.9 Amplification of Meat3 DNA extracts using SAG2 primers. Lane 1: Pork3, Lane 2: Beef3, Lane 3: Beef3, Lane 4: Chicken 3. Lane 5 SAG2 negative control. Lane 6 Solis biodyne™ 100bp DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

Beef and Lamb SAG2 products were sequenced but results were again disappointing, indicating amplification of host DNA not *T. gondii* (see sequencing results in appendix 2). Where multiple distinct product bands were obtained attempts were made to gel purify these product bands. All such attempts at column purification of PCR product bands gave low quality sequence data, none of which corresponded to *T. gondii* target sequences (see the SAG2 example below and sequencing data in Appendix).

When it was found that genotyping primers were acting in nonspecific manner, giving multiple PCR product bands that were not useful for sequencing and where sequencing was possible, giving PCR products matching the host tissues primers specific to the apicoplast were trialled. The first primer set (API) targeting the apicoplast genome initialy gave encouraging results. Figure 3.4.11 (below) shows product bands aproximate in size to the 304bp expected product. However sequencing results were both indicative of mispriming with host DNA. (see Appendix 2).

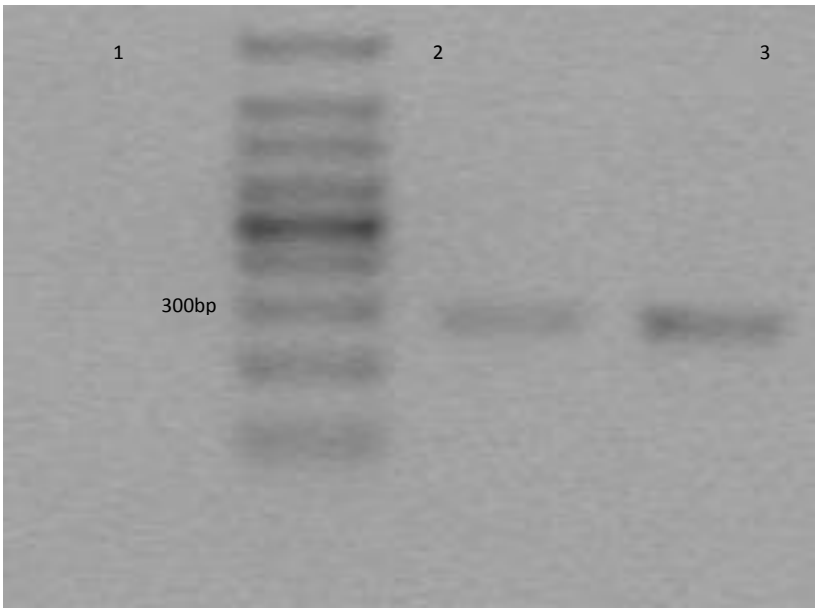

Figure 3.4.10 API amplification of Meat2 DNA extracts. Lane 1: API Negative control, Lane 2: Genescript™ 100bp DNA ladder. Lane 3: Lamb2, Lane 4: Beef2. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

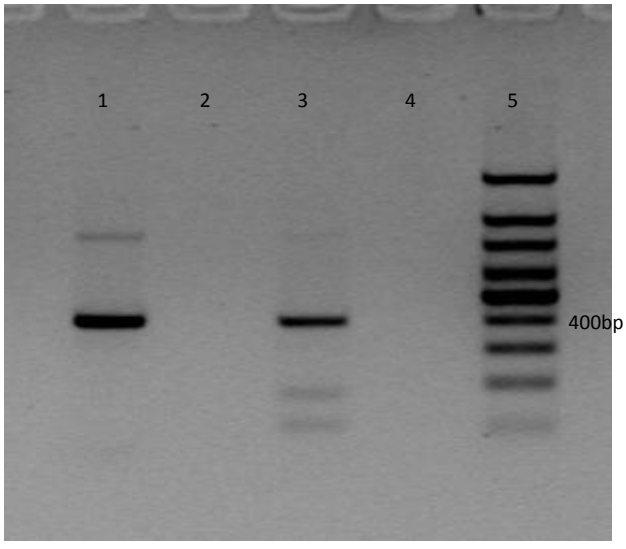

Figure 43.4.11 API-184 amplification of Meat2 DNA extracts. Lane 1: Pork2, Lane 2: Lamb2, Lane 3: Beef2, Lane 4: API-184 negative control. Lane 5: Genescript™ 100bp DNA ladder. . Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

A second and (according to primer blast) more specific apicoplast primer set (API-184) was used giving the results visible in Figure 3.4.11 (above). Although a faint product band of the expected 184bp) size is visible, multiple product bands indicates that significant mispriming has occurred.

In an attempt to increase the concentration of a PCR product band (Lane 2, Figure 3.4.12 below) attempts were made to re-amplify this product using a temperature titration to maximize specificity. The resulting PCR products can be seen in figure 3.4.13 below. Although a product band at 300bp was expected all reactions gave multiple products at a wide range of sizes. This indicates that gel purification of product bands does not preclude the presence of contamination with PCR products of unwanted sizes. This could in part explain the poor sequence data obtained using this method previously. No further attempts were made to sequence PCR product bands extracted from gel slices.

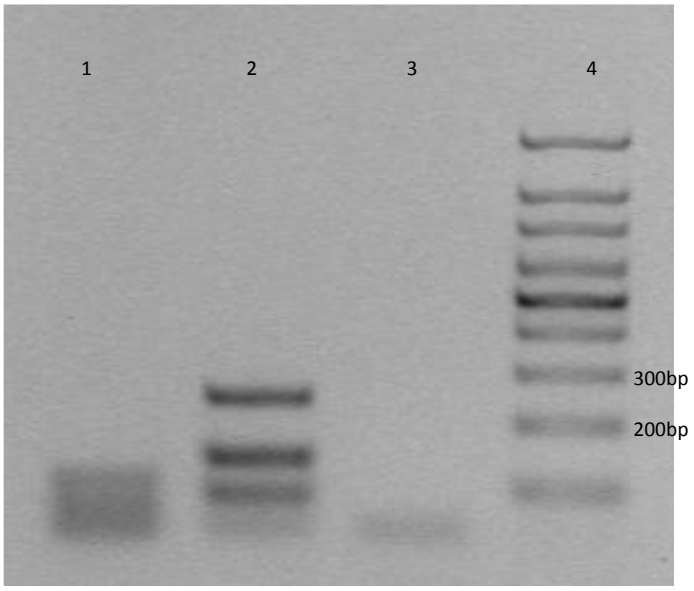

Figure 3.4.12 SAG2 amplification of Beef2 and Lamb2 DNA extracts: Lane 1: Lamb2, Lane 2: Beef2, Lane 3 SAG2 negative control. Lane 4: Genescript™ 100bp DNA ladder. Visualised on a 2% agarose TAE gel containing 2µL Ethidium bromide (10mg/mL).

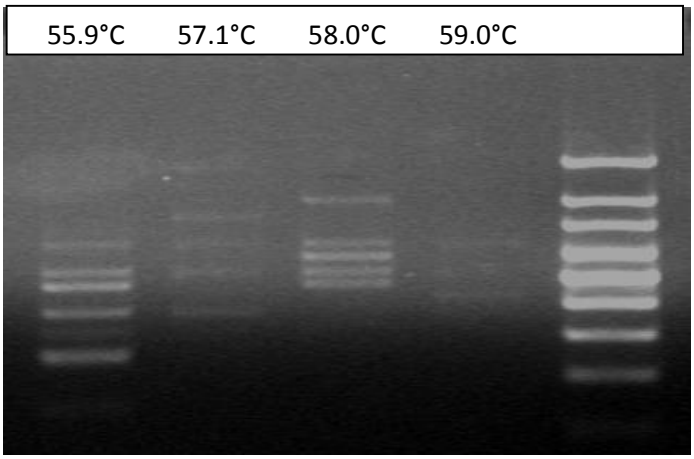

Figure 3.4.13 Reamplification of gel purified high molecular mass SAG2 PCR product including a temperature titration. Visualised using a 2% agarose TAE gel containing 2µL Ethidium bromide. Annealing temperature at top. Ladder: Genescript™ 100bp DNA ladder.

3.4.5 Nested PCR reactions:

Increased sensitivity and specificity were achieved using nested primer sets targeting regions of the SAG1 (SAG449FP30R/SAG1FSAG449R) and *18S* (NN1-2/ITS) loci (see methods). However nested PCR carries with it a much greater chance of contamination. Because of this any positive results were checked with a second nested set which had previously been shown to work well with the Toxovax™ positive control.

Amplification of SAG1 from DNA extracted from Beef6, Lamb6, Beef7 and Lamb7 was achieved using touchdown PCR protocols 71 and 70 (see figure 3.5). This result could not be confirmed using the second (NN1-2/ITS) nested PCR reaction (see figure 3.6). This discrepancy is likely due to contamination of the first nested reaction as both primer sets gave strong amplification with Toxovax© positive control.

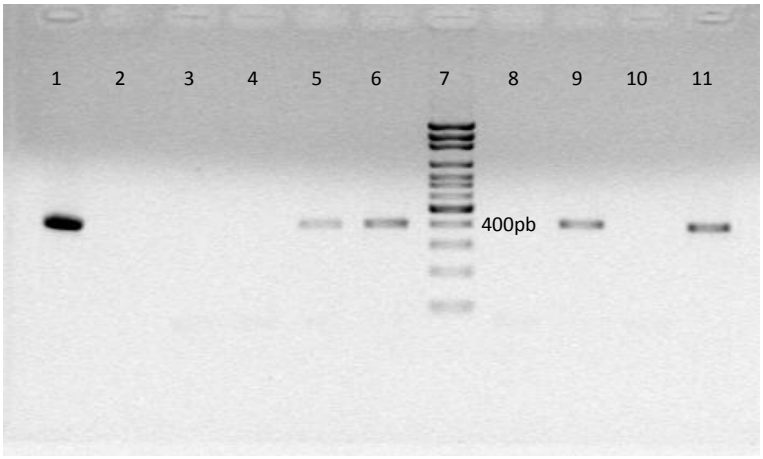

Figure 3.4.14 SAG1 nested PCR products amplified from Meat6 and Meat7 DNA extracts. Lane 1: Toxovax™ positive control, Lane 2: empty, Lane 3: Zebrafish negative control, Lane 4: Pork7, Lane 5: Lamb7, Lane 6: Beef7, Lane 7: Solis biodyne™ 100bp DNA ladder, Lane 8: Zebrafish negative control, Lane 9: Pork6, Lane 10 Lamb6, Lane 11: Beef6.Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide.

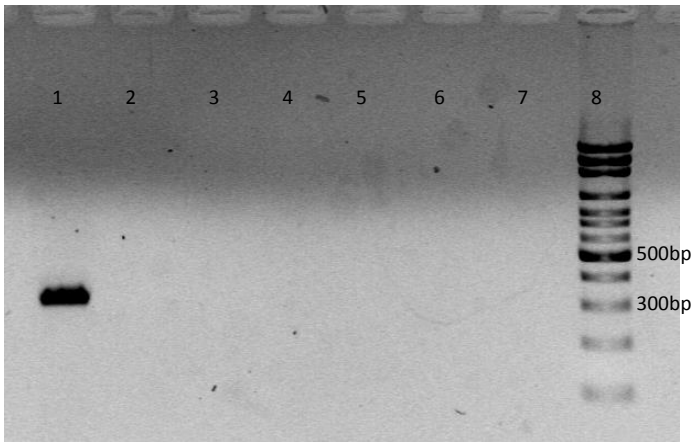

Figure 3.4.15 NN1/2-ITS nest of BEF6, Lamb6, Beef7 and Pork7.. Lane 1: Toxovax™ positive control, Lane 2: empty, Lane 3: Lamb7, Lane 4: Beef7, Lane 5: Pork 6, Lane 6: Beef6, Lane 7: Zebrafish negative control. Lane 8: Solis biodyne™ 100bp DNA ladder. Visualised on a 1% agarose SB gel containing 3µL Ethidium bromide.

3.4.6 Overall Meat DNA Extract PCR results:

Amplification of *T. gondii* specific nucleotide sequences were attempted on gel purified (see methods) DNA extracts from 25 portions of beef, chicken, lamb and pork mince purchased from butchers and supermarkets from within the Hamilton area. The results are summarised in Tables 3.3 to 3.26 below.

Genotyping of *T. gondii* from commercially available meat samples using amplified polymorphic PCR targets such as SAG3, L368 and GRA6 was not possible, either owing to non-specific primer binding, the complete lack of *T. gondii* template DNA or extremely low *T. gondii* template concentration.

Overall these results show that if *T. gondii* is present in NZ farmed meat for sale in the Hamilton region, cyst density is lower than would have been predicted by overseas studies conducted in the United Kingdom (Aspinall et al., 2002) and in the USA (J. Dubey et al., 2005).

Beef1		Primer Set						
		AFRE	B22	TGR1E	P30	SAG1	SAG449FP30R	SAG1FSAG449R
Thermocycler Protocol	1	MP						
	2		MP					
	3			MP				
	4				MP,N,S	MP,N,N		
	5				S(seq-),S	S(seq-),N		
	6				N	N		
	65						MP(1)	
	66							N(1)

Table 3.8 A summary of Beef1 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions. (seq+) and (seq-) indicate positive and negative sequencing results (see Appendix 2 for detail).

Lamb1		Primer Set						
		AFRE	B22	TGR1E	P30	SAG1	SAG449FP30R	SAG1FSAG449R
Thermocycler protocol	1	S						
	2		MP					
	3			MP				
	4				MP,N,N,MP	MP,N,N,MP		
	5				MP,S	N,N		
	6	S(seq-)	MP	MP	N	N		
	8	N			N			
	65						MP(1)	
	66							N(1)

Table 3.9 A summary of Lamb1 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions. (seq+) and (seq-) indicate positive and negative sequencing results (see Appendix 2 for detail).

Pork1		Primer Set									
		AFRE	B22	TGR1E	P30	SAG1	SAG449FP30R	SAG449	SAG1FSAG449R	SAG1(nested)	SAG2
Thermocycler Protocol	1	MP									
	2		MP								
	3			MP							
	4				MP,N,S	MP,N,N					
	5				S(seq+),S	S(seq-),N,N					
	6	S(seq-)	N	MP	N	N					
	69						N(1)		N(1)		
	70							N(2)		N(2)	
	47										N
	48										N
	49										N
	50										N
	51							N(3)			
	52									N(3)	
	51							S(4)			
	52									S(4)(seq-)	
	53						MP(5)				
	54								N(5)		
	55										MP
	65						MP(6)				
	66								N(6)		

Table 3.10 A summary of Pork1 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions. (seq+) and (seq-) indicate positive and negative sequencing results (see appendix for detail).

Although the original Pork1 extract tested positive for *T. gondii* DNA, a further 3 extracts were carried out (approximately 0.5 grams per extraction), no *T. gondii* DNA was detected using both nested and conventional PCR reactions.

Beef2		Primer Set															
		AFRE	P30	SAG1	SAG1FP30R	P30(seminest)	SAG1(seminest)	GRA6	SAG449	NN1/2	ITS(nested)	L358	SAG3	API	UPRT	API184	SAG2
Thermocycler Protocol	8	MP	S(seq-),MP(seq-)	MP		S(1)	MP(1)										
	9				N(1)												
	10							N	N								
	11									N(1)							
	12										N(1)						
	13							N									
	14											S					
	15												N				
	16									N(2)							
	17										N(2)						
	18							N				N					
	19												N				
	20							MP									
	21																
	22													S(seq-)	N		
	23															N	
	24															MP	
	25															N	
	26															N	
	27															S	
	28															MP	
	29															N	
	30															N	
	31															N	
	32															N	
	33															MP(seq-)	
	35																S,N
	36																MP

Table 3.11 A summary of Beef2 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. (seq+) or (seq-) show sequencing results. Bracketed numbers show nested PCR reactions.

Lamb2		Primer set															
		AFRE	P30	SAG1	SAG1FP30R	P30(seminest)	SAG1(seminest)	GRA6	SAG449	NN1/2	ITS(nested)	L358	SAG3	API	UPRT	API184	SAG2
Thermocycler protocol	8	MP	S(seq-)	MP		S(1)	S(1)										
	9				N(1)												
	10							N	N			MP					
	11									N(1)							
	12										N(1)						
	13												N				
	15																
	16									N(2)		MP					
	17										N(2)		MP				
	18												N				
	20							MP									
	19											N					
	21												MP				
	22													S(seq-)	N		
	23															N	
	24															N	
	35																S,N
	36																MP
	41																N
	42																N
	43																N
	44																N

Table 3.12 A summary of Lamb2 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions. (seq+) and (seq-) indicate positive and negative sequencing results.

Pork2		Primer Set																
		AFRE	P30	SAG1	TGR1E	SAG1FP30R	P30(seminest)	SAG1(seminest)	GRA6	SAG449	NN1/2	ITS(nested)	L358	SAG3	API	B1-463	SAG449	SAG2
Thermocycler Protocol	8	MP	S(seq-)	MP			S(1)(seq-)	MP(1)										
	9					N(1)												
	10								N	N								
	11										N(2)							
	12											N(2)						
	13								N									
	14												S					
	15													N				
	16										N(3)							
	17											N(3)						
	18								N									
	19													N				
	20								MP									
	21													MP				
	22																	
	23														N			
	24														MP			
	33															N	N	
	34															N	N	
	35																	N

Table 3.13 A summary of Pork2 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions. (seq+) and (seq-) indicate positive and negative sequencing results.

Beef3		Primer Set					
		16S	SAG449FP30R	SAG1FSAG449R	SAG1	P30	SAG2
Thermocycler protocol	45	S					
	47						MP
	54			N(2)			
	57		N(1),MP(2)				
	58				N(1)		
	59					MP(1)	

Table 3.14 A summary of Beef3 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions.

Chicken3		Primer Set					
		16S	SAG449FP30R	SAG1FSAG449R	SAG1	P30	SAG2
Thermocycler Protocol	45	S					
	47						s(seq-)
	54			N(2)			
	57		S(1),MP(2)				
	58				N(1)		
	59					MP(1)	
	65			N(3)			
	66				N(3)		

Table 3.15 A summary of Chicken3 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions. (seq+) and (seq-) show positive and negative sequencing results (see Appendix 2 for detail).

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Lamb3		Primer Set					
		16S	SAG449FP30R	SAG1FSAG449R	SAG1	P30	SAG2
Thermocycler protocol	45	S					
	47						S(seq-)
	54			N(2)			
	57		S(1),MP(2),				
	58				N(1)		
	59					MP(1)	

Table 3.16 A summary of Lamb3 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. (seq+) or (seq-) show sequencing results (see Appendix 2 for detail). Bracketed numbers indicate nested PCR reactions.

Pork3		Primer Set					
		16S	SAG449FP30R	SAG1FSAG449R	SAG1	P30	SAG2
Thermocycler Protocol	45	S					
	47						MP
	54			N(2)			
	57		S(1),MP(2)				
	58				N(1)		
	59					MP(1)	

Table 3.17 A summary of Pork3 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions.

Beef4		Primer Set		
		16S	SAG449FP30R	SAGF449R
Thermocycler protocol	54		N(1)	
	56			N(1)
	59	S		
	61		N(2)	
	62			N(2)
	65		N(3)	
	66			N(3)

Table 3.18 A summary of Beef4 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions.

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Lamb4		Primer Set		
		16S	SAG449FP30R	SAGF449R
Thermocycler protocol	54		N(1)	
	56			N(1)
	59	S		
	61		N(2)	
	62			N(2)
	65		N(3)	
	66			N(3)

Table 3.19 A summary of Lamb4 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions.

Pork4		Primer Set		
		16S	SAG449FP30R	SAGF449R
Thermocycler Protocol	54		N(1)	
	56			N(1)
	59	S		
	65		N(2)	
	66			N(2)

Table 3.20 A summary of Pork4 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers indicate nested PCR reactions.

Beef5			Primer set		
			SAG449FP30R	SAG1	SAG1FSAG449R
Extract1 (contaminated)	Theromocycler protocol	65	MP(1)		
		66		S(1)(seq+)	S(1)(seq+)
Extract2					

Table 3.21 A summary of Beef5 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions. (seq+) and (seq-) show positive and negative sequencing results (see Appendix 2 for detailed sequence data).

Chicken5			Primer set		
			SAG449FP30R	SAG1	SAG1FSAG449R
Extract1 (contaminated)	Theromocycler protocol	65	MP(1)		
		66		S(1)(seq+)	S(1)(seq+)
Extract2					

Table 3.22 A summary of Chicken5 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions. (seq+) and (seq-) show positive and negative sequencing results (see Appendix 2 for detailed sequence data).

Lamb5			Primer set		
			SAG449FP30R	SAG1	SAG1FSAG449R
Extract1 (contaminated)	Theromocycler protocol	65	MP(1)		
		66		S(1)(seq+)	S(1)(seq+)
Extract2					

Table 3.23 A summary of Lamb5 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions. (seq+) and (seq-) show positive and negative sequencing results (see Appendix 2 for detailed sequence data).

Pork5			Primer set		
			SAG449FP30R	SAG1	SAG1FSAG449R
Extract1 (contaminated)	Theromocycler protocol	65	MP(1)		
		66		S(1)(seq+)	S(1)(seq+)
Extract2					

Table 3.24 A summary of Pork5 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Beef6			Primer Set				
			SAG449FP30R	SAG1FSAG449R	GRA6	NN1/2	ITS
Extract1 (contaminated)	Thermocycler Protocol	67	MP(1)				
		68		S(1)			
Extract2		64			N		
		67		S(2)			N(3)
		68	N(2)			N(3)	

Table 3.25 A summary of Beef6 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Lamb6			Primer Set				
			SAG449FP30R	SAG1FSAG449R	GRA6	NN1/2	ITS
Extract1 (contaminated)	Thermocycler Protocol	67	MP(1)				
		68		S(1)			
Extract2		64			N		
		67		S(2)			N(3)
		68	N(2)			N(3)	

Table 3.26 A summary of Lamb6 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Pork6			Primer Set		
			SAG449FP30R	SAG1FSAG449R	GRA6
Extract1 (contaminated)	Thermocycler Protocol	67	MP(1)		
		68		S(1)	
Extract2		64			N
		67		N(2)	
		68	N(2)		

Table 3.27 A summary of Lamb6 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Beef7			Primer Set			
			SAG449FP30R	SAG1FSAG449R	NN1/2	ITS
Extract1 (contaminated)	Thermocycler Protocol	67		S(1)		
		68	S(1)			
Extract2		67		S(2)		N(3)
		68	N(2)		N(3)	

Table 3.28 A summary of Beef7 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Chicken7			Primer Set	
			SAG449FP30R	SAG1FSAG449R
Extract1 (contaminated)	Thermocycler Protocol	67		S(1)
		68	S(1)	
Extract2		67		N(2)
		68	N(2)	

Table 3.29 A summary of Chicken7 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Lamb7			Primer Set			
			SAG449FP30R	SAG1FSAG449R	NN1/2	ITS
Extract1 (contaminated)	Thermocycler Protocol	67		S(1)		
		68	S(1)			
Extract2		67		S(1)		N(2)
		68	N(1)		N(2)	

Table 3.30 A summary of Lamb7 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions

Pork7			Primer Set	
			SAG449FP30R	SAG1FSAG449R
Extract1 (contaminated)	Thermocycler Protocol	67		S(1)
		68	S(1)	
Extract2		67		N(2)
		68	N(2)	

Table 3.31 A summary of Pork7 PCR results with each primer set and thermocycler protocol trialled. N= no visible product. MP= multiple product bands visible. S= single product band visible. Bracketed numbers show nested PCR reactions.

Chapter Four

Discussion

4.1 Summary of DNA extraction and PCR assays

4.1.1 Development of positive and negative PCR controls

A positive PCR control from a sample of Toxovax™ live toxoplasmosis vaccine for sheep was kindly donated by MSD animal health. Using proteinase K digestion followed by phenol-chloroform extraction DNA from this sample was gel purified, resulting in a DNA solution containing a large proportion of *T. gondii* DNA. This positive control was found to reliably amplify all but two of the primer sets selected for this study. A total of 18 primer sets were investigated. Failed amplification in one case (*B1*) was caused by an incorrect primer order (resulting from a misprint in reference literature), in the second case (*SAG2*), for unknown reasons.

The development of a negative control was more problematic. Proteinase K digestion followed by phenol-chloroform extraction and purification by gel electrophoresis was used to develop three separate negative PCR controls using DNA extracted from fish tissue. The first sample was from a freshwater fish of unknown species, the second from a section shark fin from a spotty dogfish, and the third from a Zebrafish. Contamination problems rendered to the first two specimens (miscellaneous fish and shark fin) were not overcome with PCR optimisation and good laboratory practice. The third negative control developed from purified Zebrafish DNA extract was more successful. This negative control showed no evidence of *T. gondii* sequence amplification with all primers tested however non-specific priming with GRA6 primers resulted in a PCR product (of a significantly different size to the *T. gondii* product expected). This non-specific product was confirmed as a Zebrafish specific sequence using DNA sequencing.

4.1.2 Feline Faecal DNA extraction

Development of a reliable faecal DNA extraction method was achieved only after methods were found to remove (Taq polymerase) inhibitory components. Section 3.2 provides a detailed description of the development of a feline faecal DNA extraction method. Initial trials demonstrated significant DNA yields but were not reliably amplifiable owing to unknown inhibitory contaminants. Treatment with chelex resin chelating solution was successful in some cases in removing these contaminants but this method was not consistently successful. Treatment with 1% activated charcoal was also found to be unsuccessful. A reliable DNA purification method was found to be treatment with 1% activated charcoal followed by purification by gel electrophoresis. No sample purified in this manor failed to produce PCR products when trialled with 16Sr DNA bacterial specific primers to test for inhibition.

4.1.3 Amplification of Faecal DNA extracts

Amplification of *T. gondii* specific sequences from faecal DNA extracts using SAG3 and GRA6 primer sets demonstrated a higher than expected rate of oocyst shedding in faecal samples (Section 3.2.1). This high rate of shedding could be explained by presence of feline leukaemia virus among feral cat populations. This virus compromises a cat's ability to produce an effective immune response. This can lead to flourishing of existing chronic infection into secondary acute infections. An impaired immune response can also make felines much more susceptible to secondary infection upon consumption of infected prey leading to further oocyst shedding.

4.1.4 Fresh Meat DNA extraction

DNA was extracted from twenty five mince samples (beef, chicken, lamb and pork) purchased from supermarkets and butchers shops from central Hamilton and Hamilton East. DNA was extracted from these samples using proteinase K digestion followed by phenol-chloroform extraction (Section 3.3.1). Initial DNA extracts were amplified using primer sets targeting tandem repeat regions such as *B1*, *AF* and *TGR1E* and single copy PCR targets P30 and SAG1 of the *SAG1* gene. When electrophoresed, these PCR products showed broad product bands and

multiple bands of unexpected sizes. Results were significantly improved when (total) DNA extracts were purified by gel electrophoresis to give high molecular weight DNA fragments only. These purified DNA samples gave much clearer PCR product bands and far fewer bands of unexpected sizes. DNA yield was highest and most consistent from beef and lamb mince samples and more variable from pork mince. It was found that DNA yields from chicken mince was greatly improved by increasing proteinase K digest incubation time from three hours to 20 hours.

4.1.5 Amplification of DNA from fresh meat

Once a DNA extraction method had been settled upon, PCR products giving single bands of the expected size were purified and then sent for DNA sequencing analysis. Sequencing of one PCR product from the first pork DNA extract (pork1) amplified using primers targeting the P30 region of the *SAG1* gene was found to be specific for *T. gondii* (99.9% grade). Although PCR products from other meat samples were obtained that appeared to be the same size as this product, sequencing results (although low quality) indicated that these were very probably *Bos taurus* sequences likely resulting from nonspecific primer binding. This initial positive result could not be repeated despite multiple further attempts.

Although amplification using primer sets targeting tandem repeat regions produced PCR products multiple product bands were always produced which made confirmation of these products impossible. Also, the banding patterns resulting from these PCR reactions did not match those produced when the same primer sets were trialled with the positive PCR control.

Multiple attempts were made to amplify *T. gondii* specific sequences using primer sets (sourced from literature) which targeted polymorphic regions used in genotyping studies. These primer sets (GRA6, SAG3, SAG2, L358) produced poor results in that where PCR reactions were achieved they regularly resulted in multiple products or where single products were obtained, sequencing results indicated that these were a result of non-specific primer binding. Attempts were made to cut product bands from gels and purify them for sequencing. These attempts resulted in very poor quality sequence data, if any.

An apicoplast specific DNA sequence was targeted for PCR using the primer set API. Sequencing results from the PCR products produced using these primers indicated amplification had occurred as a result of non-specific primer binding.

As primer sets sourced from literature had produced multiple products or products shown to be a result of non-specific primer binding, NCBI primer blast was used to design new specific primer sets (API-184, B1-463 and SAG1-449) with the intention of preventing amplification from any of the host tissues (*Bos taurus*, *Gallus gallus*, *Aries ovis* and *Sus scrofa* as well as *Homo sapiens)*. Although API-184 produced PCR products, sequencing results showed these were a result of non-specific primer binding. B1-463 and SAG1-449 primers did not produce any PCR products in the small number of reactions attempted; however, SAG449 forward and reverse primers were useful in developing a nested PCR method to target the *SAG1* gene.

4.1.6 Nested PCR

Both *SAG1* and *18S* regions were targeted using nested PCR reactions (Section 3.3.5). The nested set, (outer)SAG-449F/P30R and (inner)SAG1F/449R was chosen above other options for amplifying SAG1 as this gave a larger product size than other options available. Thus, making confirmation of PCR product identity by sequencing more reliable. This set was shown to work well with Toxovax™ positive PCR. The nested set, (outer)NN1/2 and (inner)ITS was used to target an internally transcribed region of the *18S* gene and was also found to work well with Toxovax™ positive PCR control.

When used with meat DNA extracts these (nested) primer sets gave negative results in most cases. Contamination of eleven meat DNA extracts resulted in strong positive results which were later discounted following a repeat of DNA extractions using fresh reagents. In four cases (Beef6, Pork6, Beef7 and Lamb7) positive results were obtained using the nested set targeting SAG1 and were confirmed by sequencing. These results were discounted as the result of contamination when negative results were obtained using the nested set targeting the *18S* region gave negative results. This discrepancy in results highlights the additional risk of nested PCR techniques relative to single stage PCR.

4.2 Conclusions

Given the findings of this study it can be concluded that:

Toxovax™ cultured in THP-1 human monocytic cell culture provides an adequate *T. gondii* DNA yield to act as an effective positive PCR control for 16 of the 18 primer sets trialled.

GITC homogenisation followed phenol chloroform DNA extraction, charcoal treatment and gel purification allowed the extraction of sufficient DNA from the faeces of both domestic and feral *Felis catus* to allow testing for the presence of *T. gondii* oocyst shedding using PCR techniques.

PCR results showing amplification of both SAG3 and GRA6 target sequences which were confirmed using sequencing and restriction enzyme digests demonstrated that DNA extracts from *Felis catus* faecal samples contained a much greater than anticipated rate of oocyst shedding among the feral cat population from the Raglan area than would be expected in healthy populations.

Proteinase K digestion of raw meat samples followed by phenol chloroform DNA extraction and gel purification provides a reliable method for the extraction and purification of high molecular weight DNA from raw *Areis ovis*, *Bos taurus*, *Gallus gallus* and *Sus scrofa* muscle tissue. When applied to muscle tissue samples ranging in mass from 0.3g to 0.5g this method of DNA extraction was not able to consistently produce a DNA template that could provide evidence of the presence of intracellular bradyzoite *T. gondii* cysts using the 18 primer sets trialled.

T. gondii DNA was detected in one of the raw New Zealand farmed meat samples tested however which shows that *T. gondii* contamination within New Zealands domestic meat supply is possibly rare. This observation may reflect an inefficient DNA extraction procedure and less than optimal primer choice for DNA amplification for meat extracts.

4.3 Future recommendations

For this thesis, four DNA extraction protocols were tested and optimised for PCR detection of detection of *T. gondii* in fresh New Zealand farmed meat. In addition, suitable negative and positive controls were developed. Out of the twenty five meat samples tested using our developed PCR assay, only one sample was positive for *T. gondii.* Therefore, it is recommended that a larger sample set be analysed along with alternative DNA extraction methodologies that will efficiently extract DNA from larger sample sizes to increase the likelihood of including the DNA from what may be relatively widely distributed muscle cell bradyzoite cysts. In addition, use of deliberately infected livestock or livestock that have been determined to be carrying a chronic *T. gondii* infection through serological testing as positive controls to test for PCR assay effectiveness may be extremely valuable.

Although a large amount of research has been carried out studying the parasite *Toxoplasma gondii* since its discovery over a century ago (J. P. Dubey, 2008) there is much still to be learned about this small but complex organism. Areas still not well understood are virulence factors such as the means by which this parasite is able to permeate throughout the tissues of the host and mask itself from host immune responses(Lambert, Hitziger, Dellacasa, Svensson, & Barragan, 2006), the mechanisms by which the parasite is able to effect host neurology to alter host behaviour (Jaroslav Flegr & Markoš, 2014) and how it has become so widespread throughout animal populations (Jensen, Aars, Lydersen, Kovacs, & Åsbakk, 2010).

The mechanisms that determine virulence are only partially understood. Key to *T. gondii* virulence are the mechanisms by which the parasite is able to manipulate host immune responses. These have been examined through comparison of strains and crosses have demonstrated the effect of several genetic polymorphisms which influence the relative virulence of various strains (Hunter & Sibley, 2012a). However, these techniques are somewhat limited when compared with the potential of forward genetic approaches such as gene knockout or targeted mutation of existing genes. Relative virulence is also influenced by the ability of *T. gondii* tachyzoites to influence the gene expression of host leukocytes in order

to permeate into host tissues such as the central nervous system and the retina which would ordinarily remain insulated from blood born infections. Although this effect has been observed (Weidner & Barragan, 2014), the induced changes in gene expression responsible are yet to be elucidated. Careful comparison of transcriptomes from both infected (with known *T. gondii* genotypes) and uninfected leukocyte and microglia populations may in future demonstrate if not quantify the changes in gene expression responsible for these changes in cell activity and the mechanisms responsible for these changes.

The variation in virulence between the myriad strains/admixtures of *T. gondii* makes the examination of population distributions an important consideration as data from such studies could play important roles in treatment strategies in cases of congenital toxoplasmosis infection, infections resulting from organ transplants, and toxoplasmosis infections in patients with HIV/AIDS.

Variations in virulence also play an important role when considering the effects of *T. gondii* upon wildlife populations. The development of the Toxovax™ live vaccine for sheep relied upon such species specific differences in virulence. This strain of *T. gondii* was sourced from the coteledon of an aborted lamb and over many generations of culturing in murine cells the strain evolved in such a way that while remaining pathogenic to mice was no longer dangerous to gestating lambs and forms no observable chronic (bradyzoite cyst) infection.

Recent research has shown that *T. gondii* infects many species of marine mammals (Jardine & Dubey, 2002; Lambourn, Jeffries, & Dubey, 2001) and may be playing a significant role in the decline in New Zealand native Hectors and Maui dolphins (Roe, Howe, Baker, Burrows, & Hunter, 2013). An ongoing study into deaths of native Hector and Maui dolphins has shown that a majority of these native cetaceans are infected with *T. gondii* and in many cases is thought to be the cause of death. At present, researchers in this area speculate that these species become infected due to feeding in shallow waters around river mouths carrying oocysts in water runoff (Roe et al., 2013).

Other whale and dolphin populations have also been observed to carry toxoplasmosis infections (including the arctic beluga whale). The mode of parasite transmission to such a seemingly isolated (from *felidae* primary hosts) population bears the question of how *T. gondii* can be transmitted throughout food chains. Observations so far have shown that bicuspid filter feeders such as mussels can accumulate sporylated oocysts and this is thought to be the mode of infection of pacific sea otters (Miller et al., 2008). For toothed whales such as the beluga to become infected implies that they are either preying upon migratory secondary hosts such as other marine mammals or that *T. gondii* oocysts are able to accumulate in fish populations which has never been observed. (currently *T. gondii* is not known to infect any cold blooded species). Although soil and water testing for sporylated oocysts has been demonstrated a much greater understanding of modes of environmental transmission provides many potential areas for further research.

As filter feeders have been shown to accumulate oocysts, such species may provide an convenient avenue of investigation for the presence of oocysts within hector and maui dolphin feeding grounds. By strategically placing colonies of such organisms in such areas for short periods, large volumes of water would be filtered and oocysts present could be tested for subsequently either though bioassay or through PCR.

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Appendix 1 – Recipes and consumables

Geneaid DNA pure gel extraction kit

http://www.geneaid.com/products/dna-ultra-pure/dna-pure-kit-dp100-dp300

SDS Lysis Solution

5 mls 1M Tris (APS) solution, pH 9

5 mls 0.5 M EDTA (Ethylenediamine tetra acetic acid , Scharlau) solution, pH 8

5 mls 10 % SDS (Roche) solution

1 ml 5 M NaCl solution

Made up to 50 ml with MQ-H2O

5M GITC Solution

295.4 g Guanidine thiocyanate (Sigma)

2.5 g Sakanosyl (aka: N-Lauroylsarcosine sodium salt, Sigma)

3.9 g tri-Sodium citrate (BDH)

3.6 ml 2-mercaptoethanol (Scharlau)

Made up to 500 ml with MQ-H2O

pH to 7.0 with ~ 0.5 ml 1M NaOH

Pepsin HCl

5mL 0.1M HCl

50mg NaCl

0.7mg Pepsin

Proteinase K 60U/ml working solution

Proteinase K (recombinant) PCR grade Roche® 30U/mg

For 5mL @ 60 U/mL:

2.5ml glycorol

0.05ml Tris

0.0145g CaCl

2.5ml H2O

10mg Proteinase K

CTAB (10%)

5g CTAB

MQ H_20 to 50mL

5M LiCl

212 g LiCl / litre MQ H_20

TE buffer

Tris stock solution 1M: 6.07g/50mL H2O

EDTA solution 0.5M 9.3125g/50mL H20

TE buffer : 10mM Tris + 1mM EDTA + 10mM tris (500µL 1M Tris + 100µL 0.5M EDTA + 50mL H2O)

Agarose

Metaphor® agarose, FMC bioproducts

TAE electrophoresis buffer

TAE Running Buffer
50 x stock solution
242 g Tris (MW 121.14 g/mol, APS)
100 ml 0.5 M EDTA (Ethylenediamine tetra acetic acid , Scharlau) solution, pH 8
57.1 ml acetic acid (MW 60.05 g/mol, Ajax)
Made up to 1 L in ddH2O
Diluted to 1 x in ddH2O for working solution
Working solution final concentrations:
40 mM Tris
1 mM EDTA
20 mM acetic acid

SB electrophoresis buffer

SB Running Buffer
10 x stock solution
56 g boric acid (MW 61.83 g/mol, Ajax)
10 g NaOH (sodium hydroxide, MW 40.0 g/mol, Ajax)
Made up to 2 L in ddH2O
pH should be at 8.5
Dilute to 1 x in ddH2O for working solution
Working solution final concentrations:

45 mM boric acid
12.5 mM NaOH

Chelex solution (5%)

40ml TE

40ml MQ H_20

4g Chelex resin (200-400 mesh)

Taq polymerase and mastermix !!!

Primer suppliers and preparation:

IDT® integrated DNA technologies

Solubilised in MQ H_2O to give 200pmol/L

F=R primers diluted 1:50 with TE buffer to give 5pmol/L working solution

Quickclean II gel extraction kit

DNA pure gel extraction kit

Genescript® 6x loading buffer with Gelred

(http://www.genscript.com/molecule/M00120-6X_Loading_Buffer_with_GelRed.html)

PEG solution

20%/vol polyethylene glycol

2.5M NaCl

(other stock solutions?)

Alkaline phosphate and exonuclease

ROCHE rAPid alkaline phosphatase

Thermo Fisher Scientific Fermentas exonuclease 1

Appendix 2 – PCR target sequences

Expected PCR product sequences sourced from reference genome ME49 where possible:

TGR1E

ATGGTCCGGCCGGTGTATGATATGCGATTCGTCGAGTGCATGCACGGA
TGGGTGAGAGGTTACTGGTTGTGTGTCGTTATGCAGTCTGTCTGGGAG
ATGGTCGGGCGTATTGCCCAGGCGCCGGAGATGTTAGAAGAAAAGGT
TGCGGGACGTGTGGTGTGTCTCGAGGCAACTGCGGCACCACGTAGGGA

TGRE1

AGGGACAGAAGTCGAAGGGGACTACAGACGCGATGCCGCTCCTCCAG
CCGTCTTGGAGGAGAGATATCAGGACTGTAGATGAAGGCGACGGTGA
GGATGAGGGGGTGGCGTGGTTGGGAAGCGACGAGAGTCGGAGAGGGA
GAAGATGTTTCCGGCTTGGCTGC

B1: AF179871

TGTTCTGTCCTATCGCAACGGAGTTCTTCCCAGACGTGGATTTCCGTTG
GTTCCGCCTCCTTCGTCCGTCGTAATATCAGGCCTTCTGTTCTGTTCGCT
GTCTGTCTAGGGCACCCTTACTGCAAGAGAAGTATTTGAGGTCATATC
GTCCCATGAAGTCGACCACCTGTTTCCTCTCTTCACTGTCACGTACGAC
ATCGCATTCAAGGGAAGAGATCCAGCAGATCTCGTTCGTGTATTCGAG
ACAAGAGAGGTCCGCCCCCACAAGACGGCTGAAGAATGCAACATTCT
TGTGCTGCCTCCTCTCATGGCAAATGCCAGAAGAAGGGTACGTGTTGC
ATCATAACAAGAGCTGTATTTCCCGCTGGCAAATACAGGTGAAATGTA
CCTCCAGAAAAGCCACCTAGTATCGTGCGGCAATGTGCCACCTCGCCT

CTTGGGAGAAAAAGAGGAAGAGACGCTGCCGCTGTTTTGCAAATGAA
AAGGATTCATTTTCGCAGTACACCAGGAGTTGGATTTTGTAGAGCGTC
TCTCTTCAAGCAGCGTATTGTCGAGTAGATCAGAAAGGAACTGCATCC
GTTCATGAGTATAAGAAAAAAATGTGGGAATGAAAGAGACGCTAATG
TGTTTGCATAGGTTGCAGTCACTGACGAGCTCCCCTCTGCTGGCGAAA
AGTGAAATTCATGAGTATCTGTGCAACTTTGGTGTATTCGCAGATTGGT
CGCCTGCAATCGATAGTTGACCACGAACGCTTTAAAGAACAGGAGAA
GAAGATCGTGAAAGAATACGAGAAGAGGTACACAGAGATAGAAGTCG
CTGCGGAGACAGCGAAGACTGCGGATGACTTCACTCCCGTCGCACCAG
CAGCAGAGGAGTGCCGGGCAAGAAAATGAGATGCCTAGAGGAGACAC
AGCGTGTTATGAACAAATCTATTGAGGTTTCGCGAAGAGGAGGGAAC
ATATTATATACAGAAGAAGAACAAGAGACGTGCCGCATGTCGCTAAG
CCATCGGAAGGGATGCTCAGAAAATGGCACAGTATCACATTACAGTTC
CGTTGATTCGTCTGATGGTGACGAAAGGGGAAGAATAGTTGTCGCACC
AAAACTGGCTAGTTGTTATTTTGAAGAAGACGAGAGATGGAGTGAACC
ACCAAAAATCGGAGAAAATCGATGGTGTCACGTTTTTTGTCAGACTTC
ACTTTGTGCAGAAGCATTGCCCGTCCAAACTGCAACAACTGCTCTAGC
GTGTTCGTCTCCATTCCGTACAGTCTTCAAAAATACAAAAGAGAACAT
TCCAGCAACTTCTGCCTTTGTTCTTTTAGCCTCAATAGCAGGATGACGC
CTCCCTCCTATCTTTCAGCCAACCCAGCAAACACCGACGAACTCTCTGT
AGAGTAACAAAGAGAAGGCAAAACGCGCCATCACGAACACTCGCAGA
GATGATACAGAGACGTGTCATCAGGACAAGGTTGGTCGCTTAATTTTC
TGTATATAGCATTTTTAGAATGCACCTTTCGGACCTCAACAACCGTGCA
AAAGGATCGCCACCTGGTGTCTCTTCAAGCGTCAAAACGAACTATCTG
TATATCTCTCAAGGAGGACTGGCAACCTGGTGTCGACAACAGAACAGC
TGCAGTCCGGAAATAGAAAGCCATGAGGCACTCCAACGGGCGAGTAG
CACCTGAGGAGATACAAACTGCTAAACGGTCCGGGTGAAACAATAGA
GAGTACTGGAACGTCGCCGCTACTGCCCAGTTGTCATGCCATCGACGT
AGACCCA

SAG1:

CTGATGTCGTTCTTGCGATGTGGCGCTATGGCATCGGATCCCCCTCTTG
TTGCCAATCAAGTTGTCACCTGCCCAGATAAAAAATCGACAGCCGCGG
TCATTCTCACACCGACGGAGAACCACTTCAC

P30

AGTTCCAATCGAGAAGTTCCCCGTGACAACGCAGACGTTTGTGGTCGG
TTGCATCAAGGGAGACGACGCACAGAGTTGTATGGTCACAGTGACAGT
ACAAGCCAGAGCCTCATCGGTCGTCAATAA

SAG3:

CAACTCTCACCATTCCACCCGGCGGGTTCCCCGAAGAAGACAAATCTT
TTCTTGTCGGGTGTTCACTCACTGTGGACGGGCCGCCCTTCTGCAACGT
CAAAGTGAGAGTTGCTGGGAACCCCAGAAAGTGGGAGAGGGGCGGAG
GCGGCCATCCAGGAAGCGGAGGATTGCAGCCAGGAACTGACGGGGAA
ACTCAAGCTGGAACAGGAAGTTCAGCCGGCGCGAGTTCGCGAATGGC
TTCCGTTGCCCTGGCGTTCCTTCTCGGTCTCCTTGTGCATGTGGCTGCCT
AAATGTCTTGTCTAACAACGCGC

NN1/2:

CCTTTGAATCCCAAGCAAAACATGAGTTTGCATCTCTCTCCATTGGAG
AGATTTGCATTCAAGAAGCGTGATAGTATCGAAAGGTATTATTGCCTT
CTTCATGTTGGATATCCTGCGCTGCTTCCAATATTGGAAGCCAGTGCAG
GTATCCGGGGGTGCACAGCGAAGGGGCTCAATTTCTGGAAATTCGTGT
CTCTGTTGGGATACTGATTTCCAGGAGTTTCTTCAGTGTGCATTCTTTTT

TCCCACACCGTTATTTCAAACAACAAATCTGAGGAACATTTGAGAGAG
AGTGAAAGATTGTATCTTTCTGCATCTCTCTCGATGTGCTTTCAGATTG
CTTCCTAAACTATAATGTTATTTTAAATTTTCAGCAATGGATGTCTTGG
CTCGC

L358:
CCCTCTGGCTGCAGTGCTGCGTCTTGGCCCTGCAAGGGTGCGAGTCTCT
GCTCTTCCGGACGACACACTCGCATGCATGTCCTCTTTCTGCCTTCGGC
TTGTCGGATGCGTCCTGTCGGGGGAACGGAGCCTCTTCGTCTTCCCCGC
TTCCTTCGCTCGGCTGCGCGTCGACGCTGCAATGTCGCGCGGCCACCC
CGCTGATAGACAAGAAGGCGAGGAGAGAGGCAGAGATCAGTTTCTCC
TGCATTCGCCTGCCGCCTCTCTCGCGACTCGTTTCGGACGGAGAAGCG
GGGAGAGAAGCGAGCGACGAGACAGCGACGGAAACCGCGCAGCAGG
AAGGTTTCGCTTTCTCCGCCCGTGTCGAGGAAGAGAACATTCCATTCTT
CGCGGCATGCTGCGACTTGCGCTACGCCTCCT

UPRT:
ACTGCGACGACATACTGGAGAACTCGAGCATCCTTTATGGGTTGGTGT
TGATGAACGTAGGACTACCAGATGTCATTTCTTCATTGGAACACTTTAT
TGTACACGCATGCCGTTTATGCGCTGCTTATCCGAGGGTGGCATCACA
GGGACCCTACAAGAAGCTGACGGGGCACCTCCACTATGAAGTGACAT
GCCAATTCATGCCCAGGTTGCATGTTCCAAGTGTCTGGTATCTTTGGCT
ACGCTGTCTTTTACTAATCGCAAGAAACAGTGCTCGTCATGGGTGGTA
ACTCCTTGGTGCCACCAGAACGCATGTGGTGCGTTTGTTGTTCCGCTTT
GTTTTCTTG

API:

TCTATTGCAATGGAAAAAGGTATGAGATTTGCTATTAGAGAAGGAGGT
CGTACTATAGGAGCAGGTATAATTACTGATATTATAAAATAATAATTT
TATTATGAATAAAAAAAAAGTTATTATAAATAAATTAAAAAAACAAA
AAAATTTTTATTTAAATTTAAGATATTTATATTTAAAACAACTATCTAA
TTGAAATAATAAAATTTTTAATTTTATTAAATTAAAATAATTATAATTA
AAATGTTGAAAATCAGAATTGAACTGATAACTTAAGGATCTTCAGTCC
TTTGCTCTACCATTGA

API-184:

TGGTTTTAACCCTAGATTGTGGTTCTAGTTATAGCAAGTTCAATTCTTG
TCATTTATCAAAATATAAATGATATAATTTAATTAGGTAAAATAAAGA
ATTGCAAATTCTTGAATTCTCAGTTCAAATCTGAGTATCATTTTTTATA
AAAAGGATGTGGTGAAATTTGGTAAACACAGCGGACTT

B1-463:

CGGAGTTCTTCCCAGACGTGGATTTCCGTTGGTTCCGCCTCCTTCGTCC
GTCGTAATATCAGGCCTTCTGTTCTGTTCGCTGTCTGTCTAGGGCACCC
TTACTGCAAGAGAAGTATTTGAGGTCATATCGTCCCATGAAGTCGACC
ACCTGTTTCCTCTCTTCACTGTCACGTACGACATCGCATTCAAGGGAAG
AGATCCAGCAGATCTCGTTCGTGTATTCGAGACAAGAGAGGTCCGCCC
CCACAAGACGGCTGAAGAATGCAACATTCTTGTGCTGCCTCCTCTCAT
GGCAAATGCCAGAAGAAGGGTACGTGTTGCATCATAACAAGAGCTGT
ATTTCCCGCTGGCAAATACAGGTGAAATGTACCTCCAGAAAAGCCACC
TAGTATCGTGCGGCAATGTGCCACCTCGCCTCTTGGGAGAAAAAGAGG
AAGAGACGCTGCCGCTGTTTTGCAAATGA

SAG1-449(TgCatBr1)

ACGAGTATGTTTCCGAAGGCAGTGAGACGCGCCGTCACGGCAGGGGT
GTTTGCCGCGCCCACACTGATGTCGTTCTTGCGATGTGGCGTTATGGCA
TCGGATCCCCCTCTTGTTGCCAATCAAGTTGTCACCTGCCCAGATAAAA
AATCGACAGCCGCGGTCATTCTCACACCGACGGAGAACCACTTCACTC
TCAAGTGCCCTAAAACAGCGCTCACAGAGCCTCCCACTCTTGCGTACT
CACCCAACAGGCAAATCTGCCCAGCGGGTACTACAAGTAGCTGTACAT
CAAAGGCTGTAACATTGAGCTCCTTGATTCCTGAAGCAGAAGATAGCT
GGTGGACGGGGGATTCTGCTAGTCTCGACACGGCAGGCATCAAACTCA
CAGTTCCAATCGAGAAGTTCCCCGTGACAACGCAGACGTTTGTGGTCG
GTTGCATCAAGGGAGA

SAG2:

GCCACCTCGAACAGGAACACAAAGGGAACAGAAATGTTTCAGGTTGC
TGCAGTGACCCATCTGCGAAGAAAACGAGCGCTGCTTGCGATTCTGTG
TGTTGTTTCACGGGTTGCAGTTCTAGGAACTGAGTTGTGATTGTGCACA
ATTGCGGTGTGACACCTTCTGTCTCGTTCCAATCTTTGTCTTGTCGGAA
CTATGAGTTTCTCAAAGACCACGAGCCTAGCGTCGCTAGCGCTCACGG
GCTTGTTTGTTGTGTTCAAGTTCGCTCTTGCGTCCACCACCGAGACGCC
AGCGCCCATTGAGTGCACTGCCGGCGCAACGAAGACTGTTGAGGC

GRA6 type I and type II polymorphisms demonstrating restriction cut sites

Predicted PCR product (ME49)(typeII)

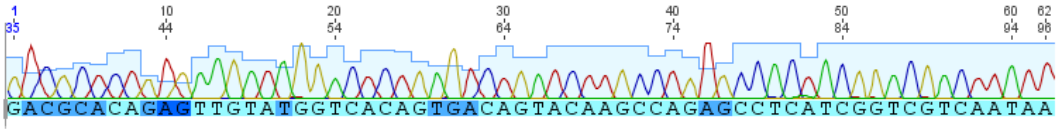
TTTCCGAGCAGGTGACCTGGGTCGCTTTTTTGAAACAGCAGGAAAACA
GCTTCGTGGTGCCACGTAGCGTGCTTGTTGGCGACTACCTTTTTTTCTT
GGGAGTGTCGGCGAAATGGCACACGGTGGCATCTATCTGAGGCAGAA
GCGTAACTTCTGTCCT***T^TAA***CTGTCTCCACAGTTGCTGTGGTCTTTGTA
GTCTTCATGGGTGTACTCGTCAATTCGTTGGGTGGAGTCGCTGTCGCAG
CAGACAGCGGTGGTGTTAGGCAGACCCCTTCGGAAACCGGTTCGAGCG
GTGGACAGCAAGAAGCAGTGGGGACCACTGAAGACTATGTCAACTCT
TCGGCGA

L33814.1 GI:609619 (Type1)
TTTCCGAGCAGGTGACCTGGGTCGCTTTTTTGAAACAGCAGGAAAACA
GCTTCGTGGTGCCACGTAGCGTGCTTGTTGGCGACTACCTTTTTTTCTT
GGGAGTGTCGGCGAAATGGCACACGGTGGCATCCATCTGAGGCAGAA
GCGTAACTTCTGTCCTGTAACTGTCTCCACAGTTGCTGTGGTCTTTGTA
GTCTTCATGGGTGTACTCGTCAATTCGTTGGGTGGAGTCCGTGTCGCAG
CAGACAGCGGTGGTG***T^TAA***GCAGACCCCTTCGGAAACCGGTTCGAGC
GGTGGACAGCAAGAAGCAGTGGGGACCACTGAAGACTATGTCAACTC
TTCGGCGA

X96720.1 GI:1255983 (Type1)
TTTCCGAGCAGGTGACCTGGGTCGCTTTTTTGAAACAGCAGGAAAACA
GCTTCGTGGTGCCACGTAGCGTGCTTGTTGGCGACTACCTTTTTTTCTT
GGGAATGTCGGCGAAATGGCACACGGTGGCATCCATCTGAGGCAGAA
GCGTAACTTCTGTCCTGTAACTGTCTCCACAGTTGCTGTGGTCTTTGTA
GTCTTCATGGGTGTACTCGTCAATTCGTTGGGTGGAGTCGCTGTCGCAG
CAGACAGCGGTGGTG***T^TAA***GCAGACCCCTTCGGAAACCGGTTCGAGC
GGTGGACAGCAAGAAGCAGTGGGGACCACTGAAGACTATGTCAACTC
TTCGGCGA

Appendix 3 – Sequencing results

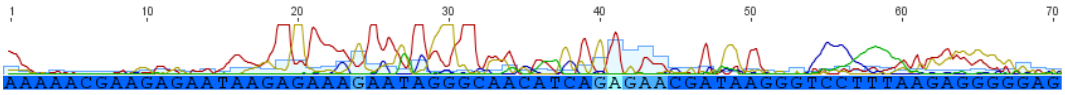
P30F Pork1 extraction: Positive *T. gondii* result.



Nr megablast result: AF110182 *Toxoplasma gondii* major surface antigen p30 gene, partial cds

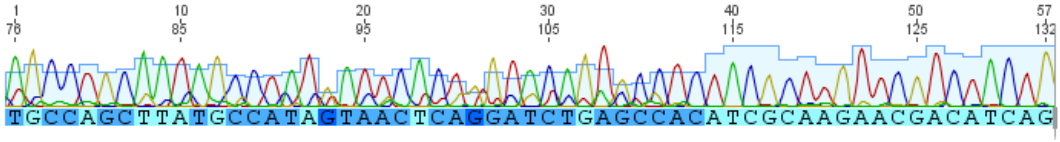
E Value: 2.92e-23 Grade:99.9% Hit:265-326

SAG1F Pork1

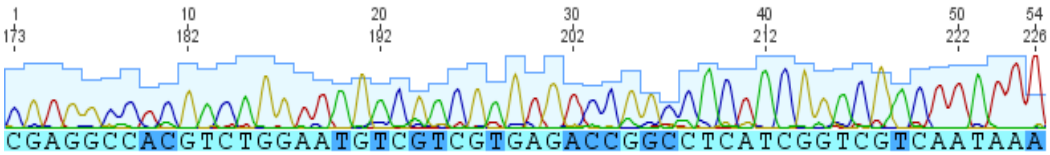


Nr megablast result: no matches found

SAG1R Pork1 extraction:



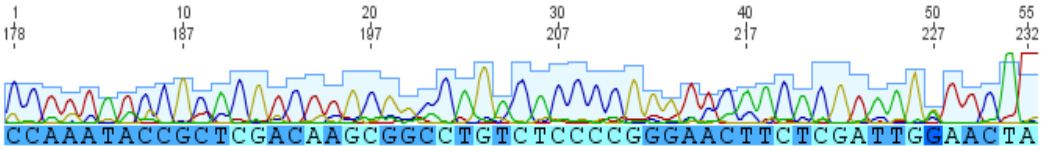
P30F beef1 extraction:



Nr megablast result: K00133 bovine 1.711 g/ml satellite dna, f fragment

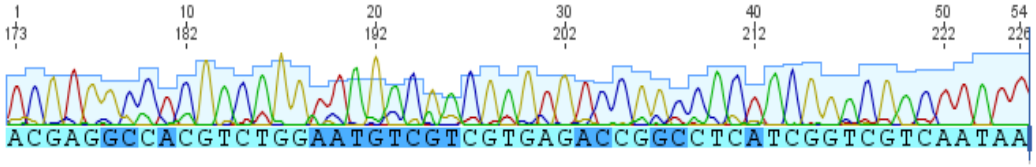
E value: 1.56e-11 Grade:63.0% Hit:636-676

P30R beef1 extraction:



Nr megablast result: No match found

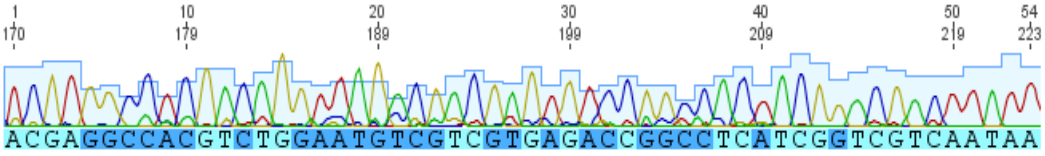
P30F Lamb1 extraction



Nr megablast result: K00133 bovine 1.711 g/ml satellite dna, f fragment

E value: 4.33e-12 Grade: 63.9% Hit: 635-676

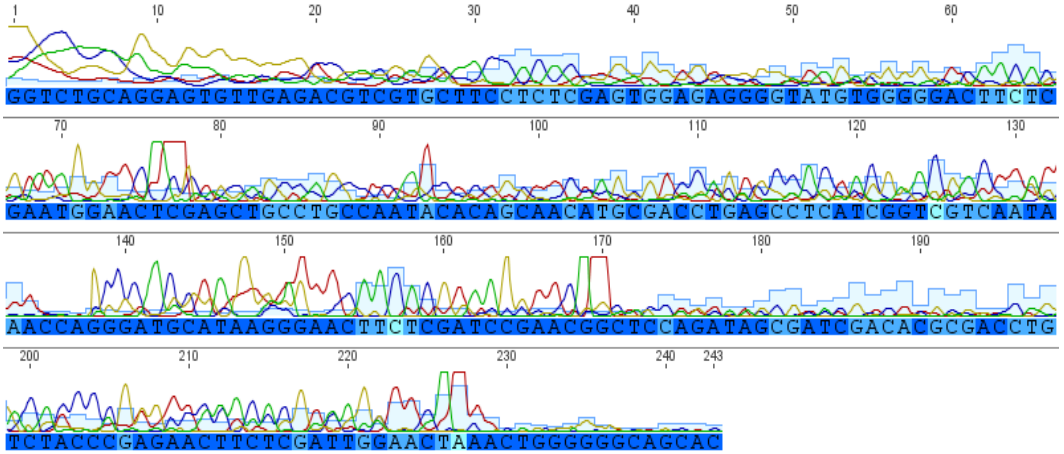
Pork1 P30F:



Nr megablast result: K00133 bovine 1.711 g/ml satellite dna, f fragment

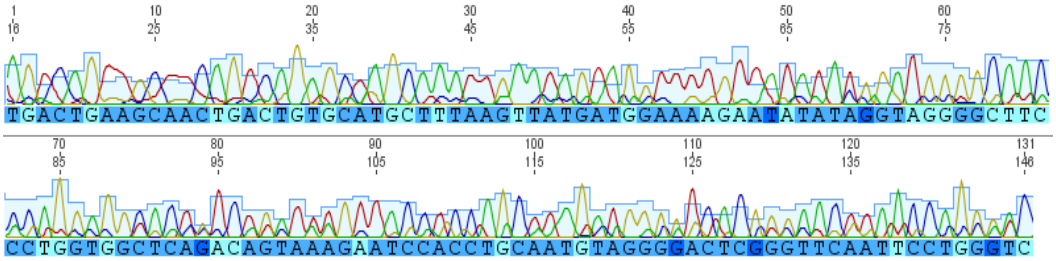
E value: 4.33e-12 Grade:63.9% Hit:635-676

Pork1 P30R:



No blast query carried out due to poor quality.

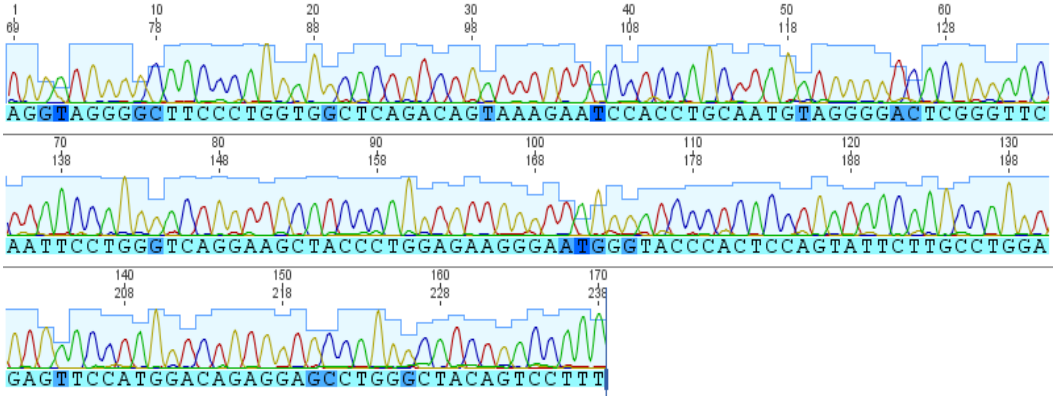
Beef API-F extraction



Nr megablast result: AC150847 Bos taurus BAC CH240-372I17 (Children's Hospital Oakland Research Institute Bovine BAC Library complete sequence

E value= 2.97e-19 Grade=48.6% Hit: 103175-103245

Lamb API-F extraction

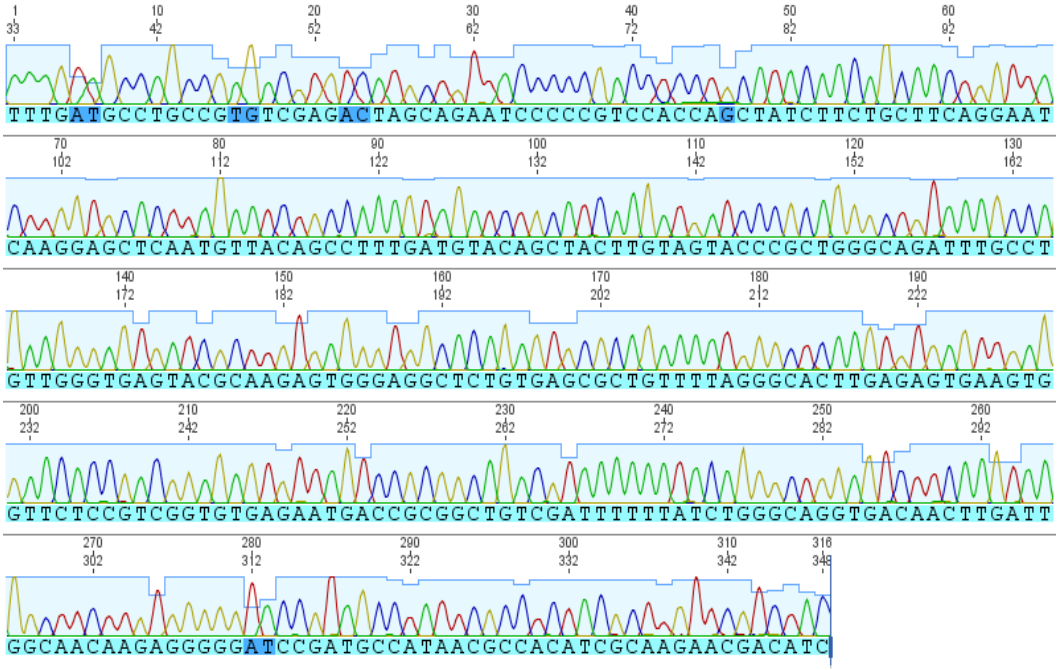


Nr megablast result: XM_005688682 PREDICTED: Capra hircus retinoblastoma-like 1 (p107) (RBL1), mRNA

E value:4.17e-47 Grade=87.4% Hit: 4944-4794

Contaminated SAG1 nest:

Beef5 449 R extraction

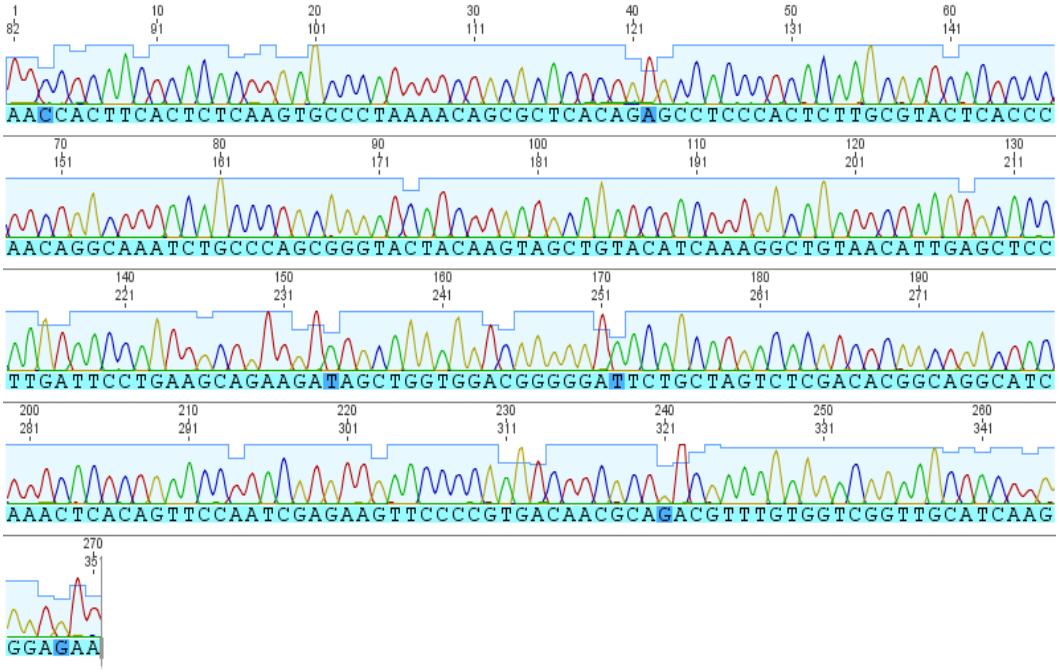


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value:2.26e-163 Grade = 100% Hit: 586-271

Contaminated SAG1 nest

Beef5 SAG1 F extraction

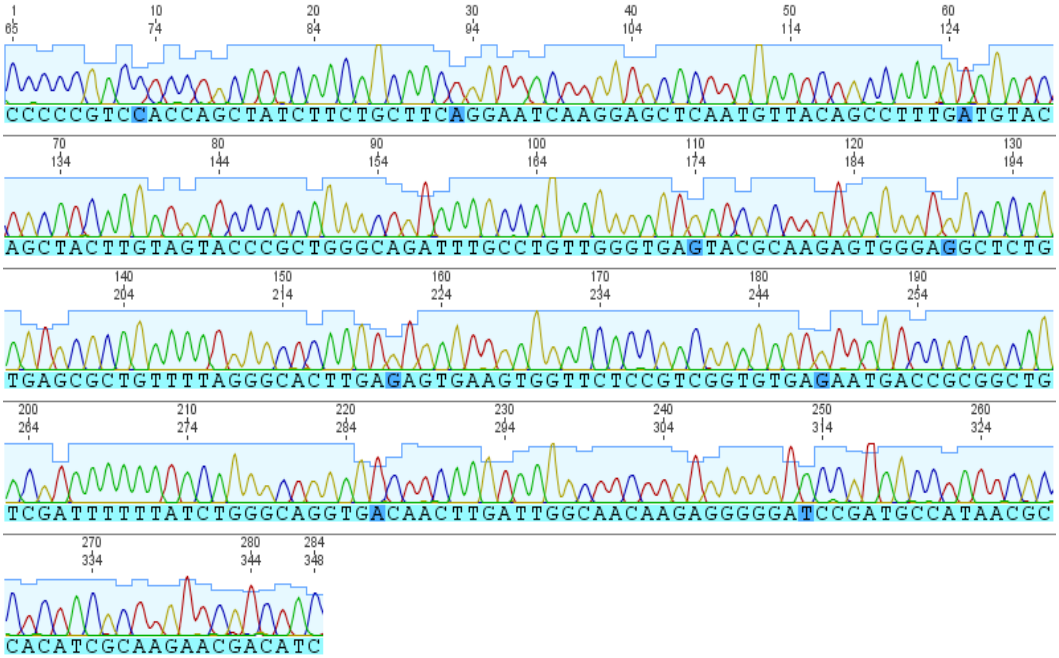


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value:2.27e-137 Grade=99.8% Hit:361-629

contaminated SAG1 nest

Lamb5 449F extraction

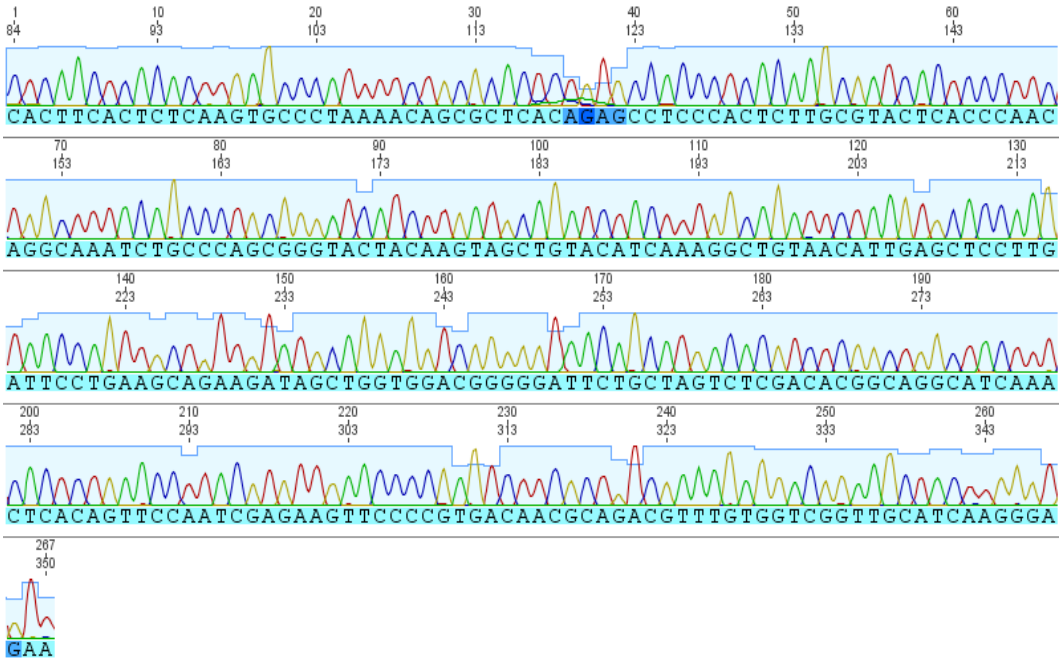


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value:1.45e-145 Grade=100% Hit:554-271

Contaminated SAG1 nest

Lamb5 SAGF extraction:

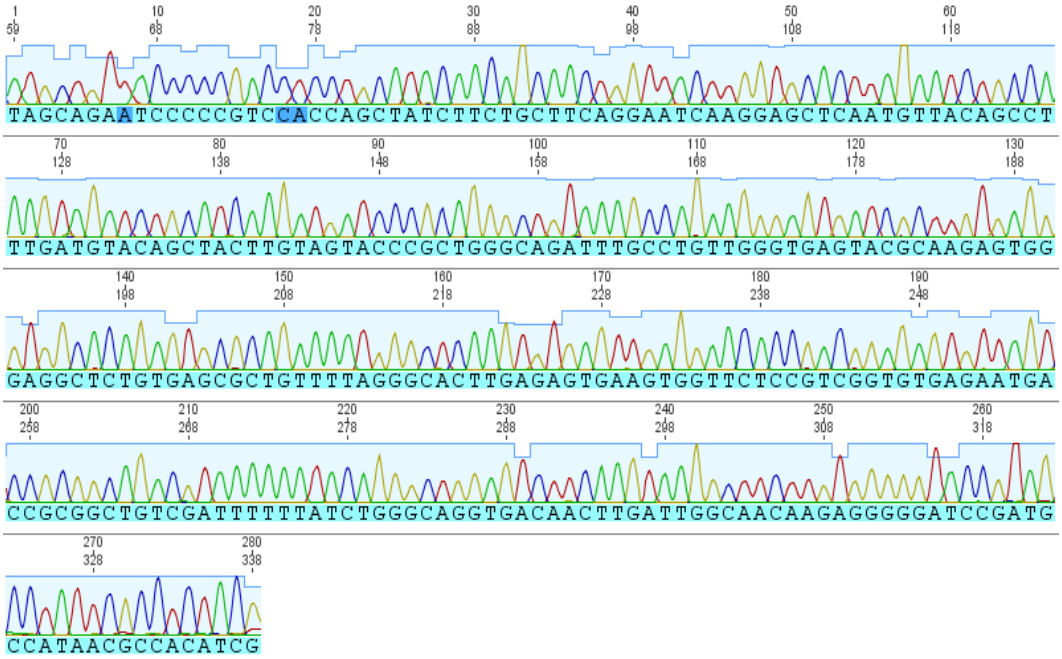


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value:1.28e-135 Grade=99.8% Hit:364-629

Contaminated SAG1 nest

Pork5 449R extraction:

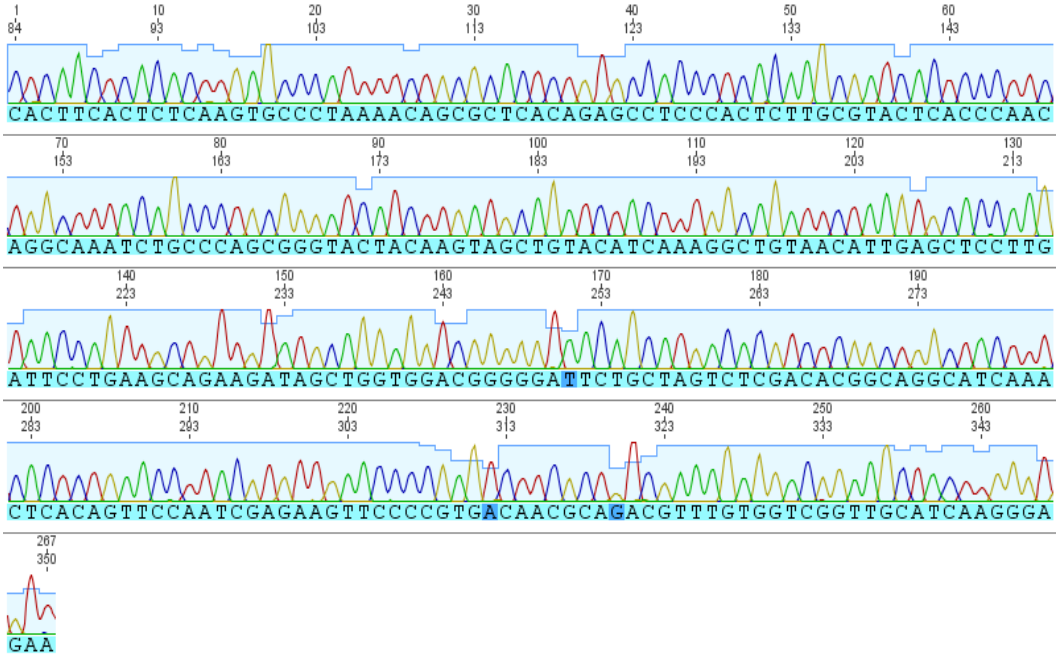


Nr megablast result: GQ253073 Toxoplasma gondii strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value:2.22e-143 Grade:100% Hit: 563-284

Contaminated SAG1 nest

Pork5 SAGF extraction:

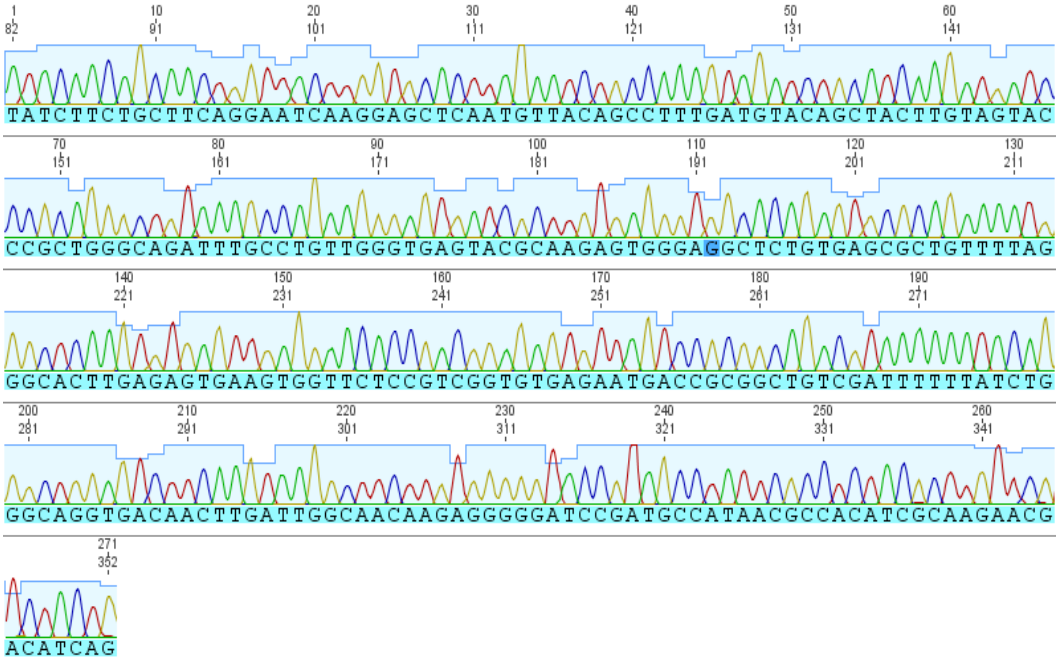


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value: 1.27e-135 Grade:99.8% Hit:364-629

SAG nest, dubious result:

Beef6 449R:

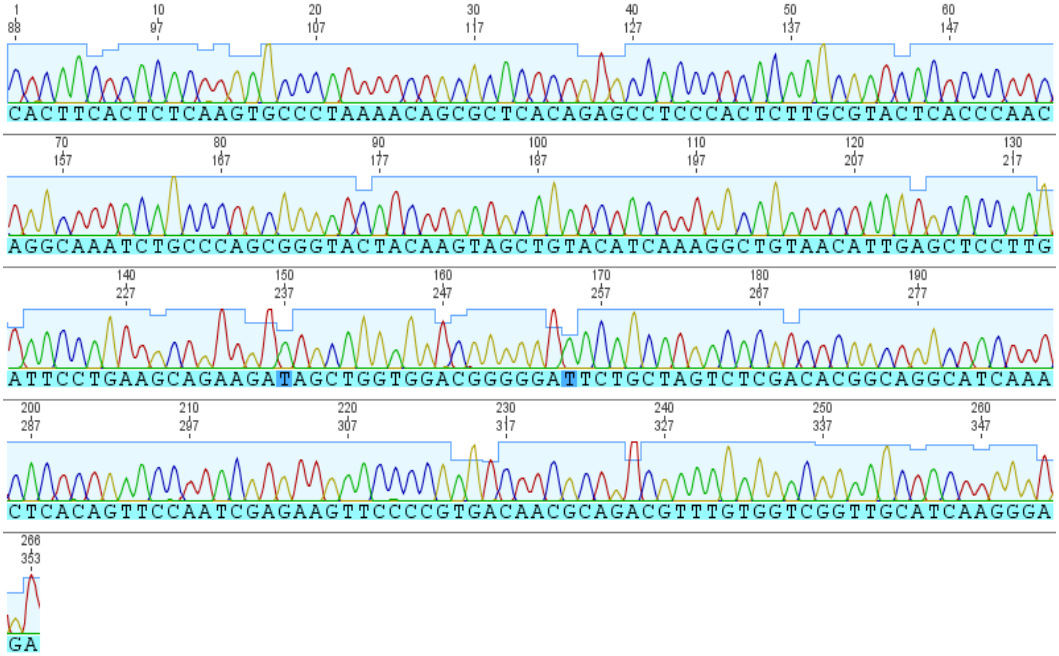


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value: 2.07e-138 Grade:100% Hit:539-269

SAG nest, dubious result

Beef6 SAG1F (extraction:

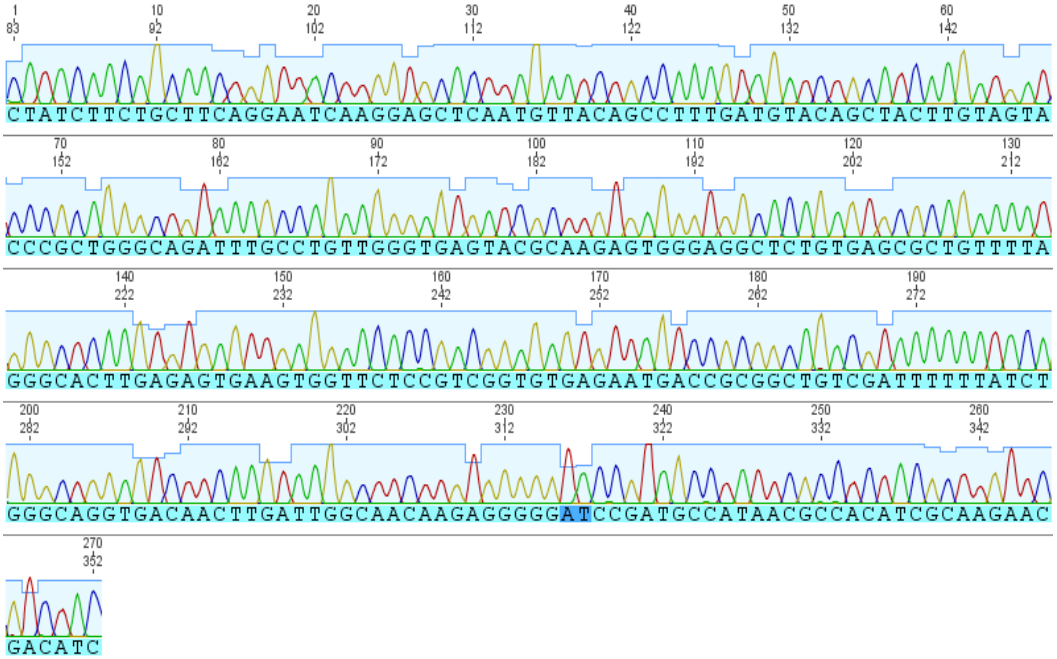


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E:1.22e-135 Grade:100% Hit:364-629

SAG nest, dubious result

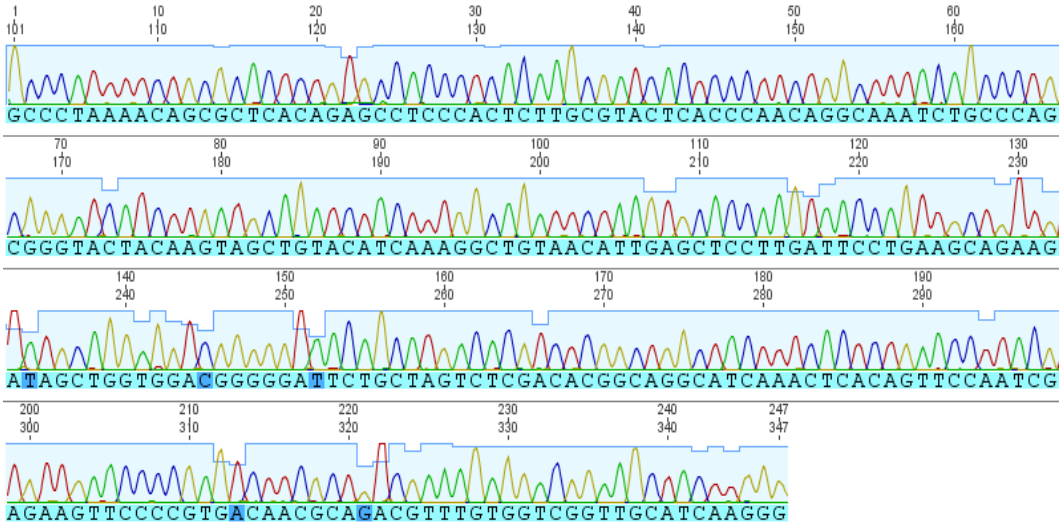
Lamb6 449R extraction:



Nr megablast hit: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value:7.41e-138 Grade=100% Hit:540-271

Lamb6 SAGF extraction

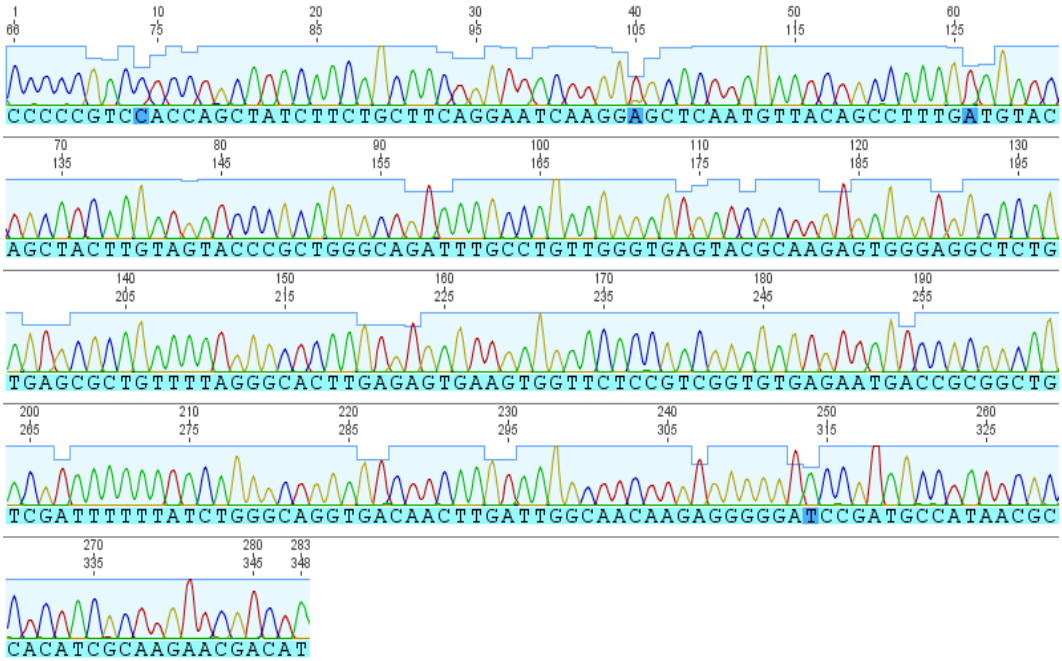


Nr megablast result: GQ253080 *Toxoplasma gondii* strain RMS-1999-BES major surface antigen (SAG1) gene, complete cds

E value: 4.08e-125 Grade:100% Hit:405-651

SAG nest, dubious result

Beef7 449F extraction:

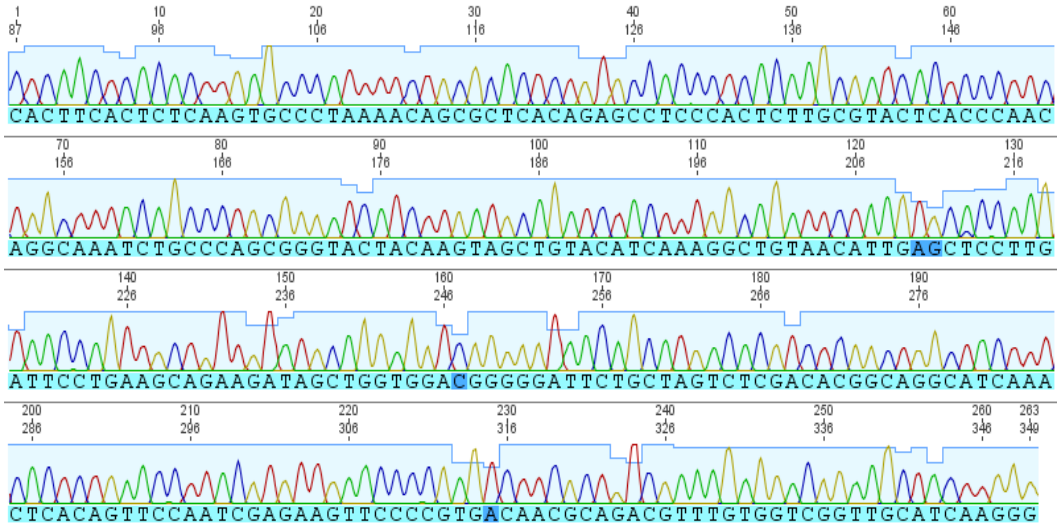


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value: 4.62e-145 Grade:100% Hit:554-272

SAG nest, dubious result

Beef7 SAGF extraction:

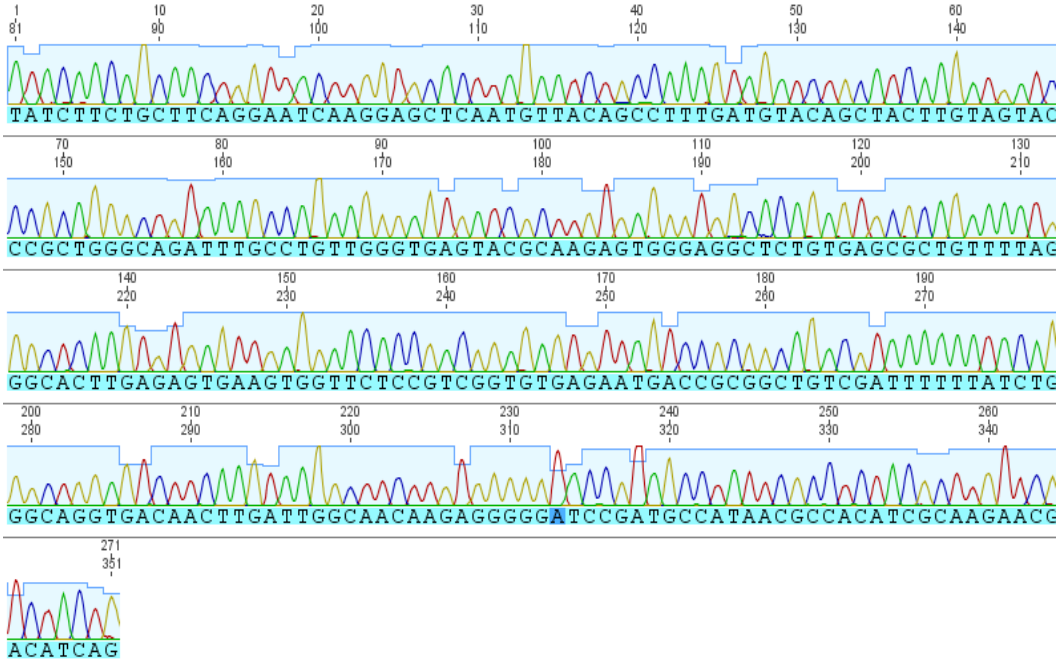


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value: 5.60e-134 Grade:100% Hit:364-626

SAG nest, dubious result

Lamb7 449R extraction

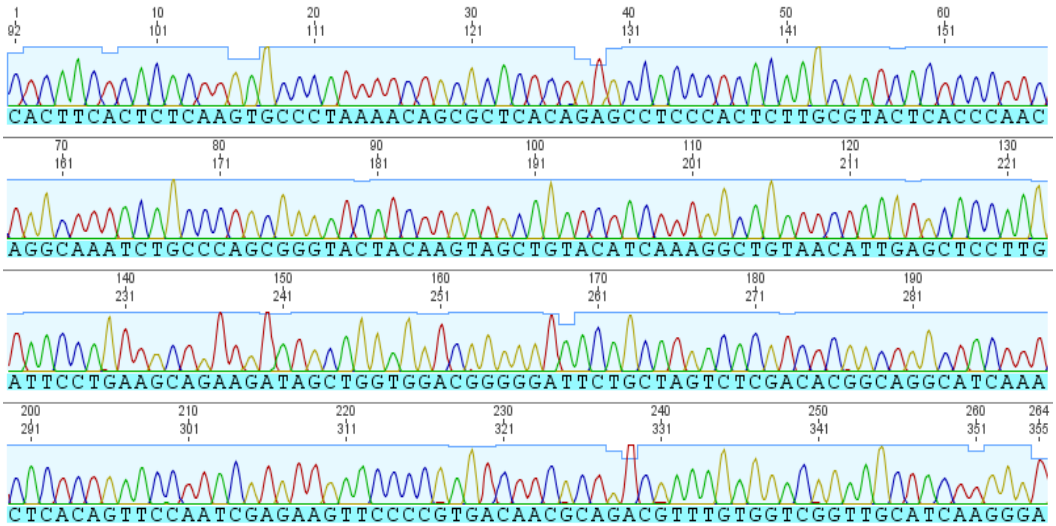


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value 2.07e-138 Grade:100% Hit:539-269

SAG nest, dubious result

Lamb7 SAGF extraction:

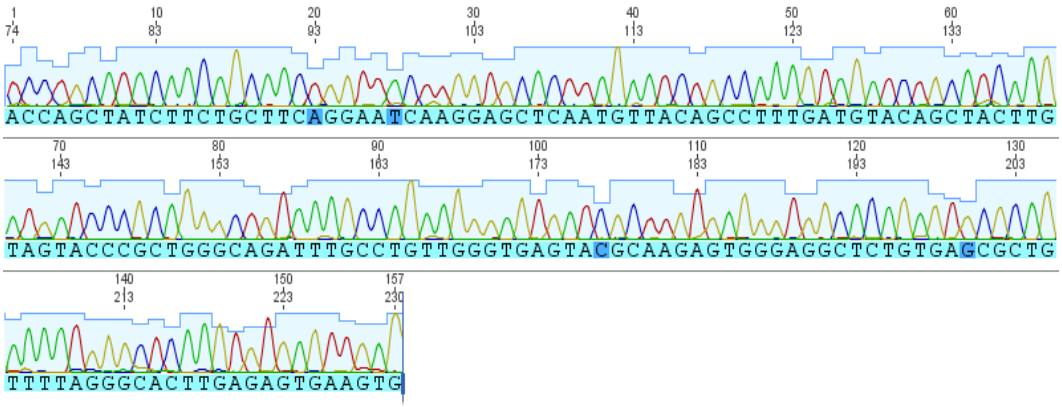


Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value: 1.56e-134 Grade:100% Hit:364-627

16-9-14 Meat5 contaminated:

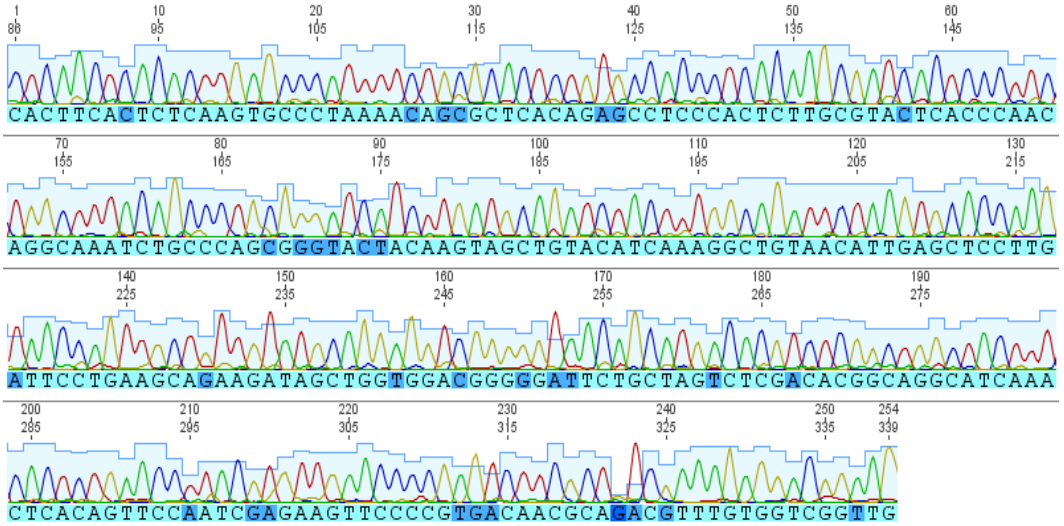
Beef5449R:



Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value:3.15e-120 Grade:100% Hit:520-283

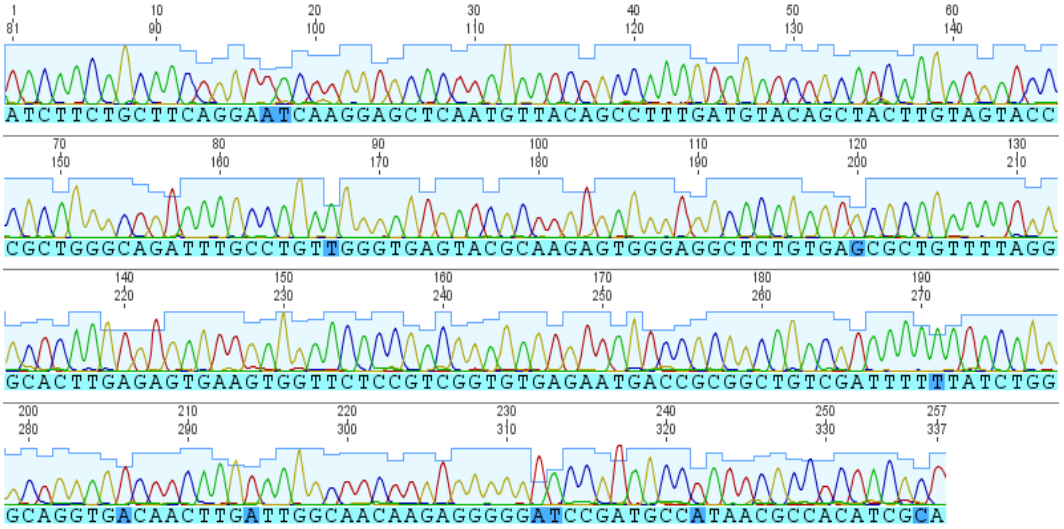
Beef5 SAGF extraction:



Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value:4.32e-129 Grade:100% Hit:364-617

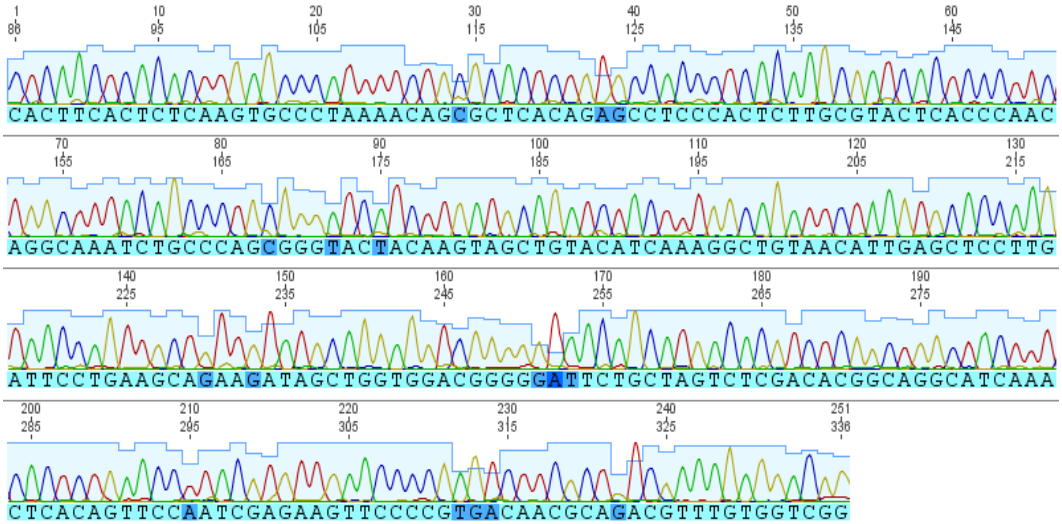
Chicken5 449R extraction:



Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value:9.42e-131 Grade:100% Hit:538-282

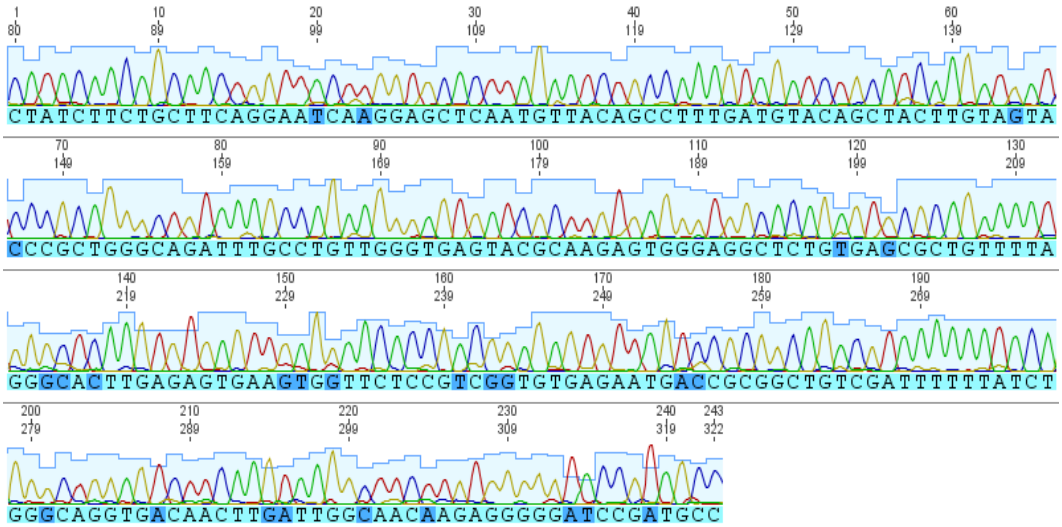
Chicken5 SAGF extraction:



Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

E value:2.59e-127 Grade:100% Hit: 364-614

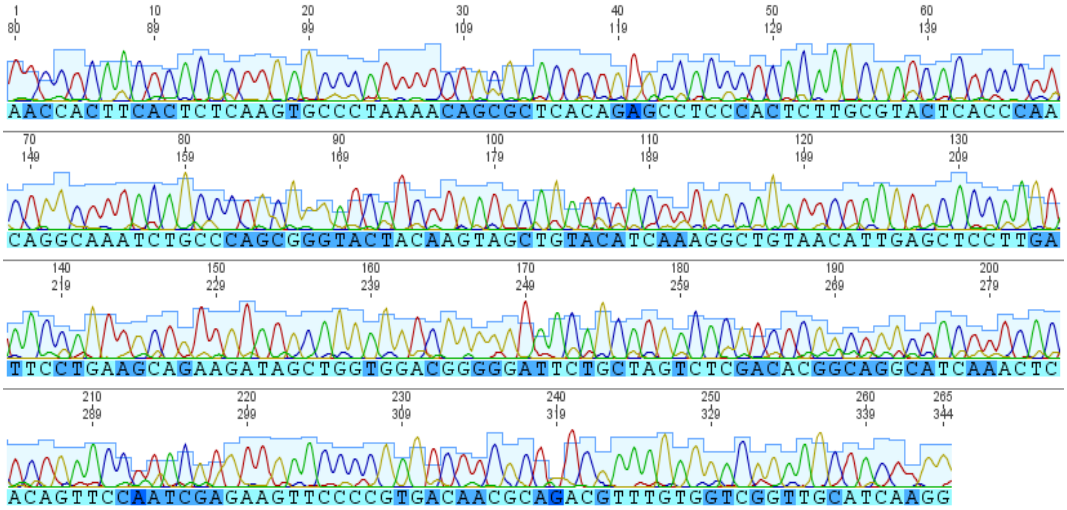
Lamb5 449R extraction:



Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

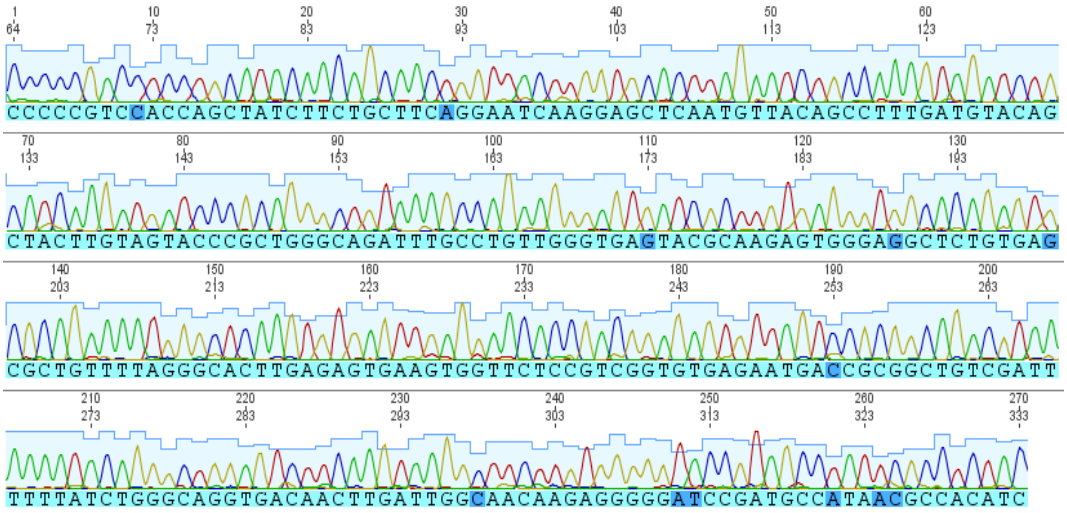
E value: 5.35e-123 Grade:100% Hit: 515-273

Lamb5 SAG F extraction:



Nr megablast result: AK317969 *Toxoplasma gondii* cDNA, clone: XTG02220.2, full cDNA, XTG Sugano cDNA library

Pork5 449R extraction:

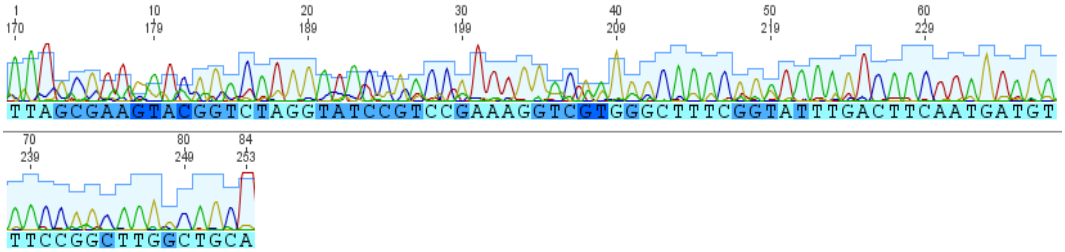


Nr megablast result: GQ253073 *Toxoplasma gondii* strain RMS-1994-LEF major surface antigen (SAG1) gene, complete cds

E value 5.91e-138 Grade:100% Hit:554-285

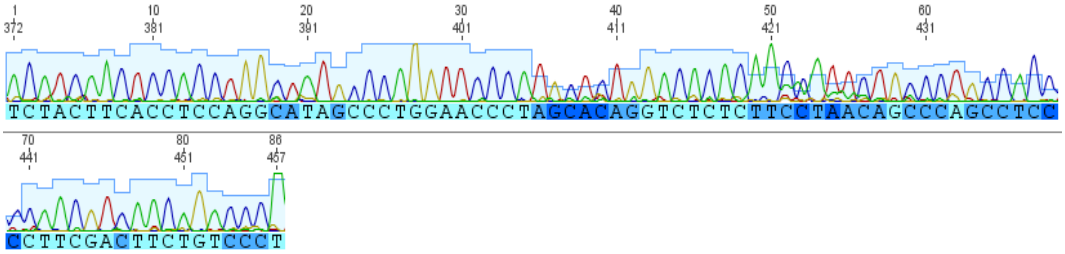
21-1-14 AF amplification:

AF-f (extraction)



Nr megablast result: No matches found

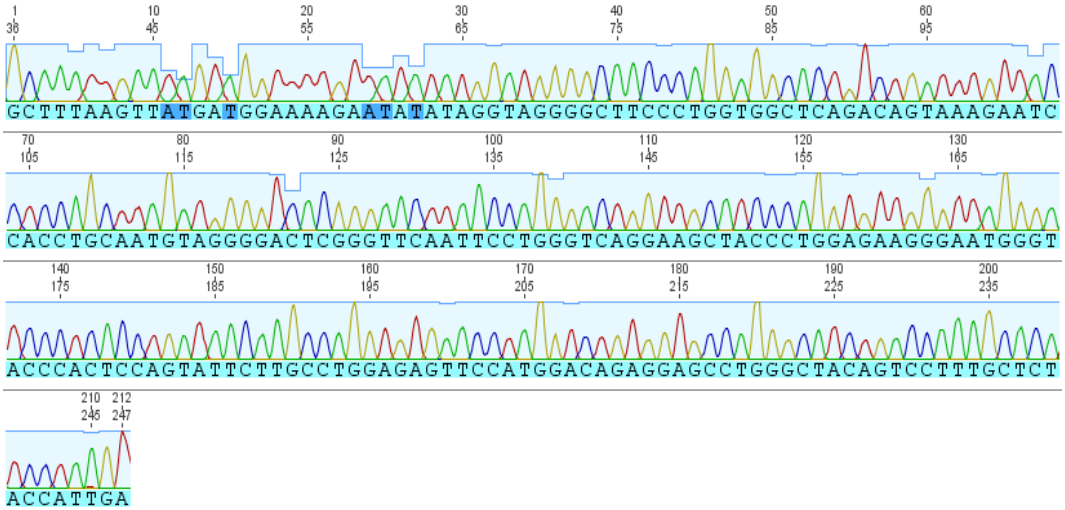
AF-r(extraction)



Nr megablast result: No matches found

31-3-15: API Beef

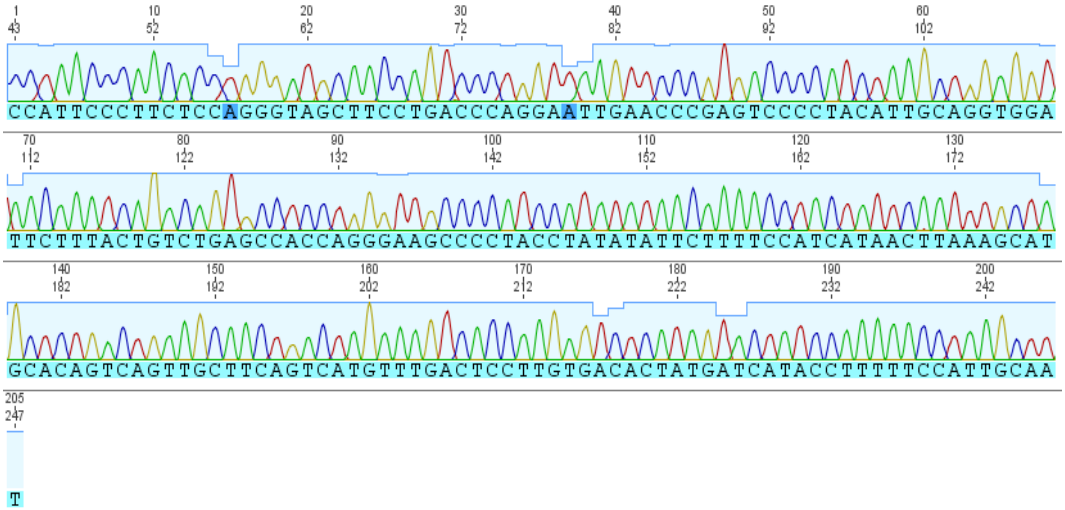
Beef API-F:



Nr megablast result: XM_005688682 PREDICTED: Capra hircus retinoblastoma-like 1 (p107) (RBL1), mRNA

E value: 8.58e-47 Grade:80.6% Hit:4944-4794

Beef API-R

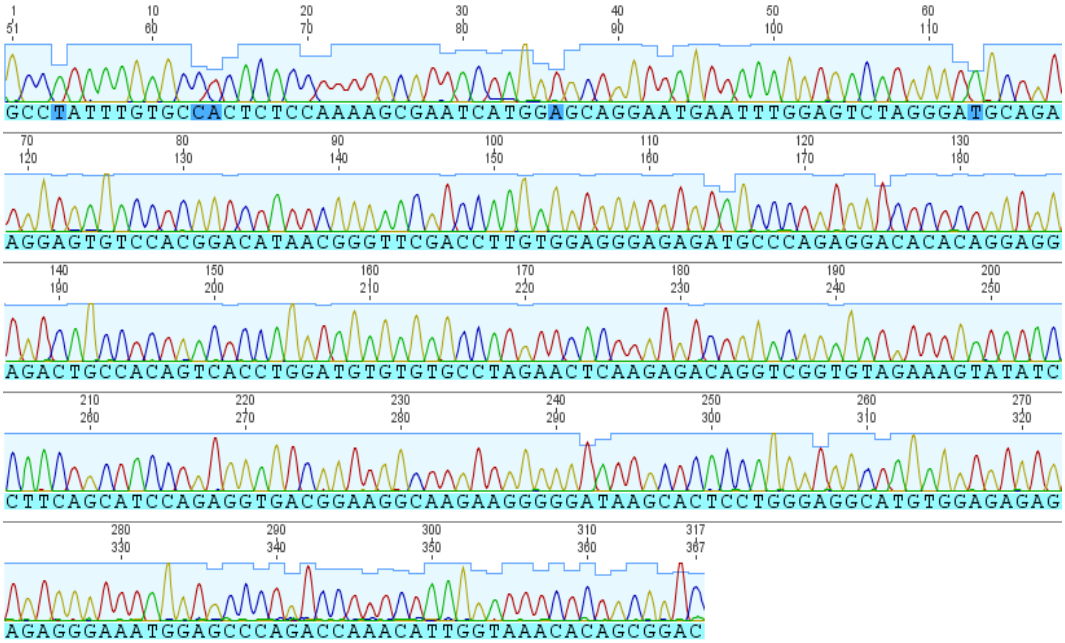


Nr megablast result: AC150847 Bos taurus BAC CH240-372I17 (Children's Hospital Oakland Research Institute Bovine BAC Library complete sequence

E value: 1.42e-24 Grade:68.6% Hit:103273-103175

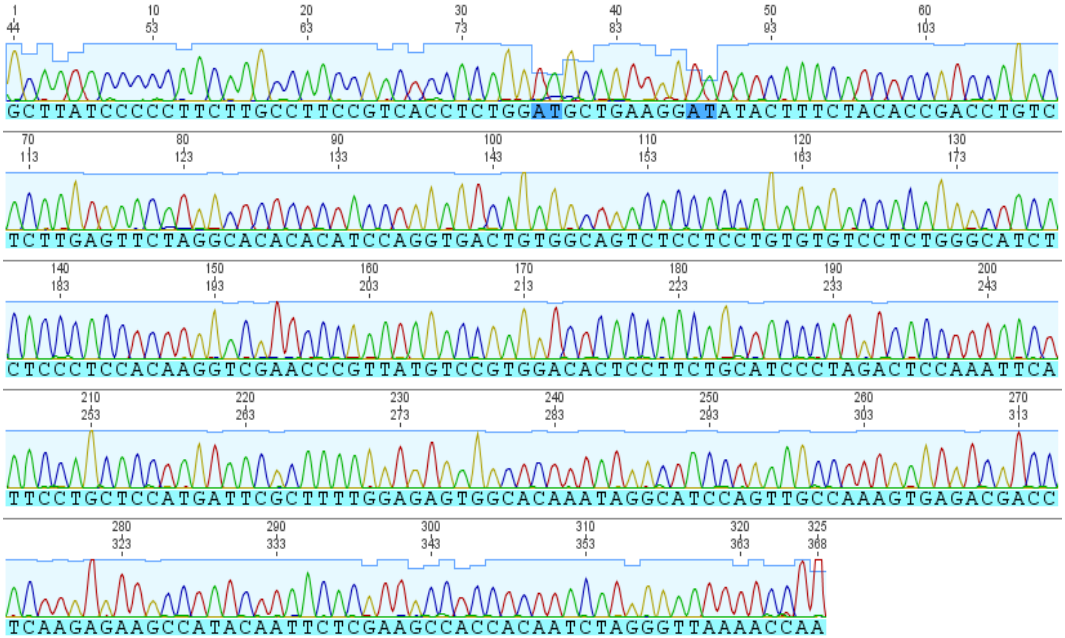
23-4-14 API-184

Beef API184 F extraction



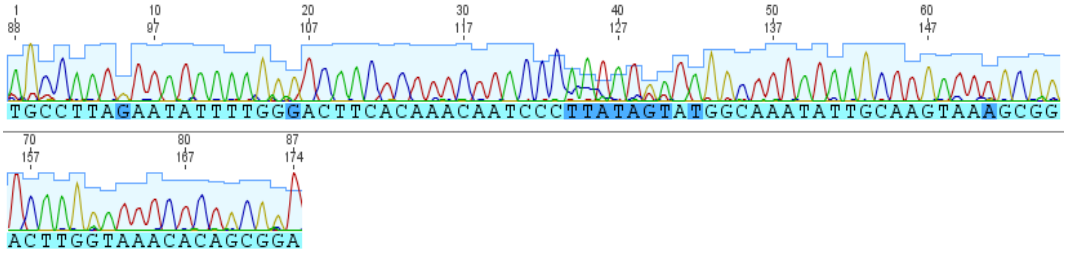
Nr megablast result: No match found

Beef API 184 R extraction



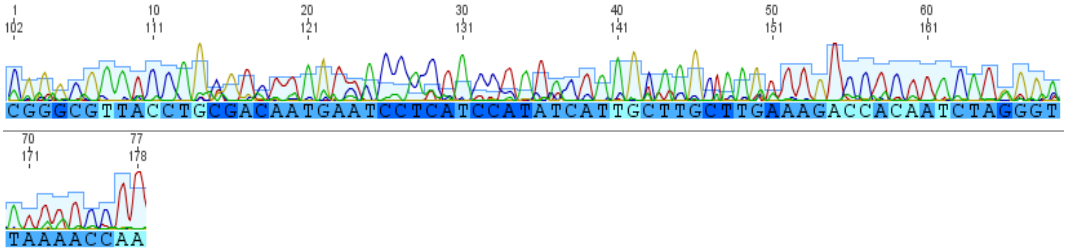
Nr megablast result: No matches found

Lamb API 184 F:



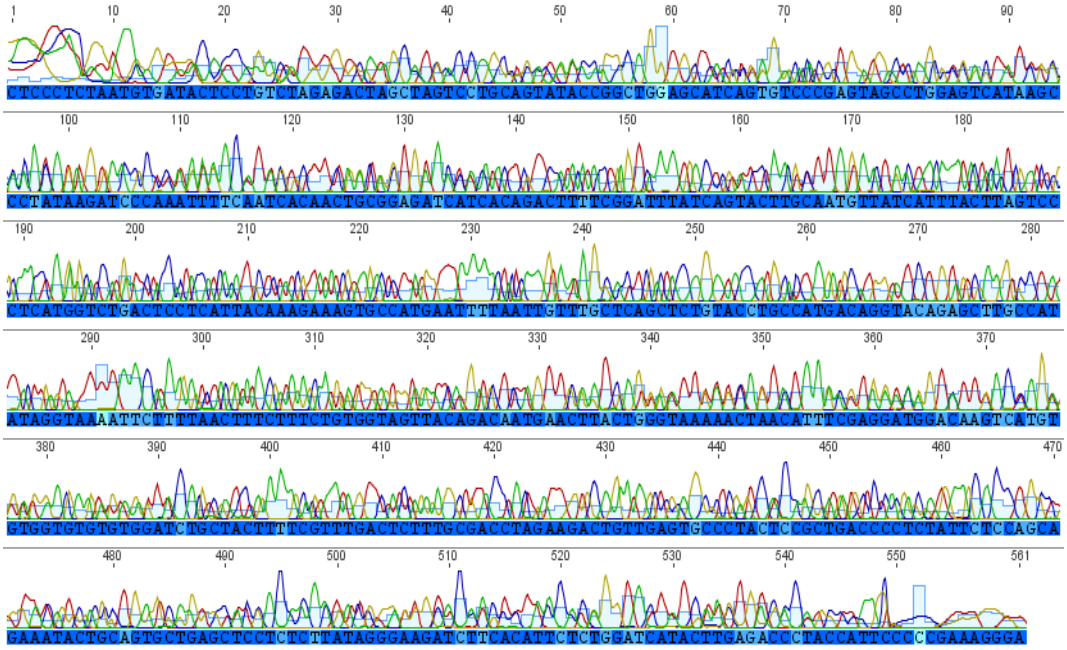
Nr megablast result- no matches found

Lamb API 184 R extraction:



Nr megablast result: no matches found

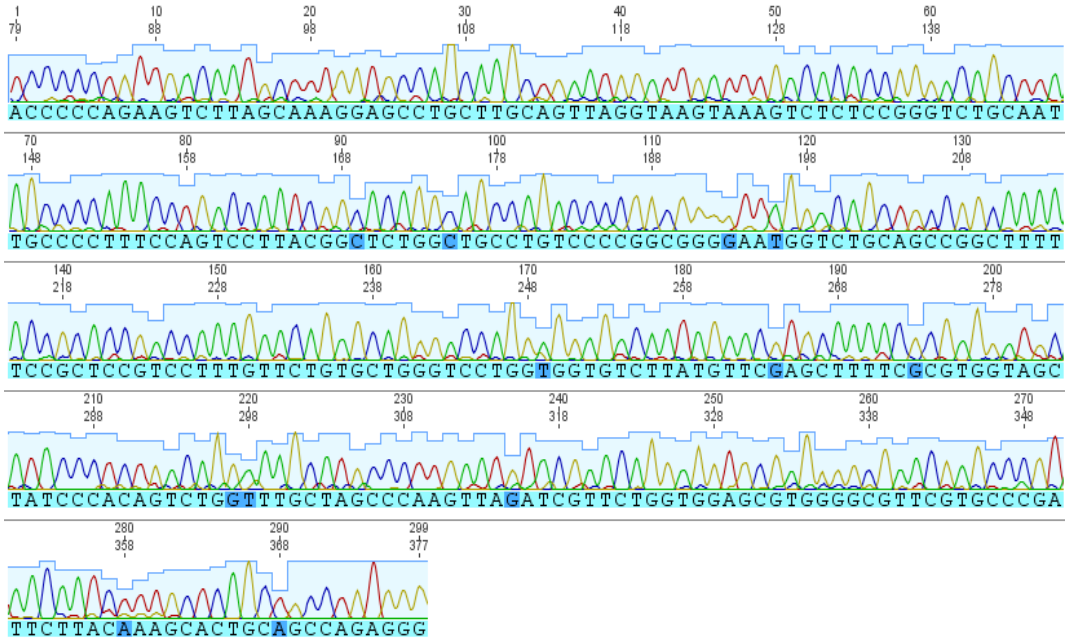
Beef L358 F (no extraction)



Nr megablast result: No matches found

Result appears to show multiple overlaid sequences- ie: multiple products

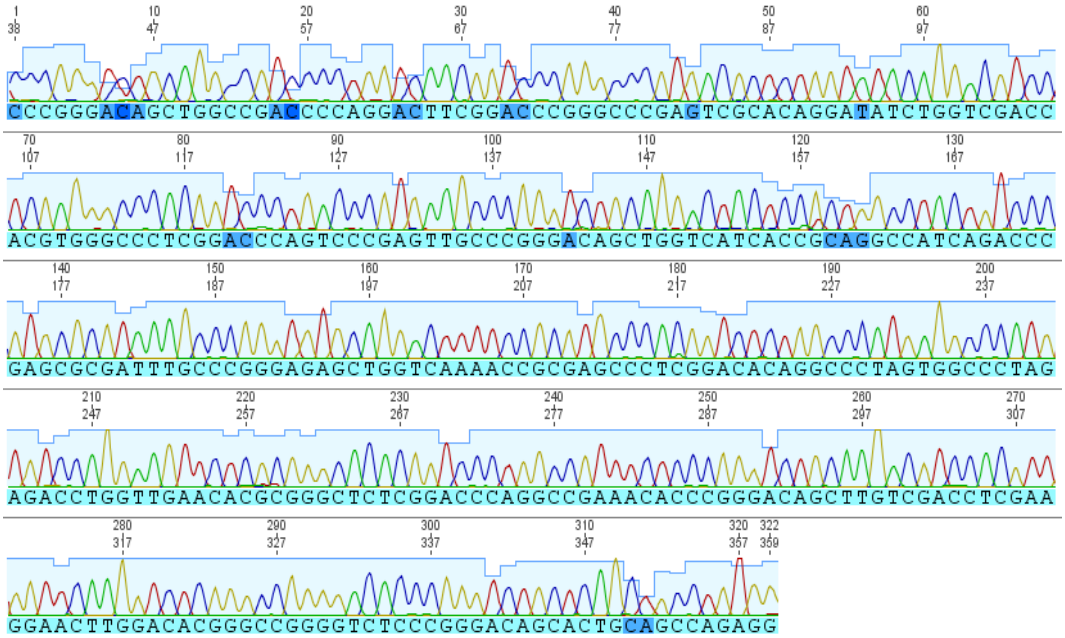
Lamb L358 F extraction:



Nr megablast result: AC169534 Bos taurus Y Chr BAC CH240-237I9 (Children's Hospital Oakland Research Institute Bovine BAC Library (male)) complete sequence

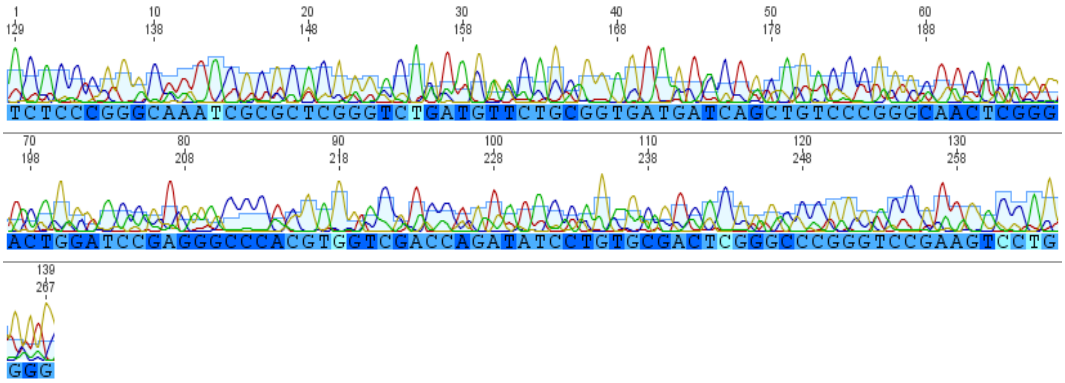
E value: 3.53e-47 Grade:86.9% Hit:41561-41836

Pork L358 F extraction:



Nr megablast result: No matches found

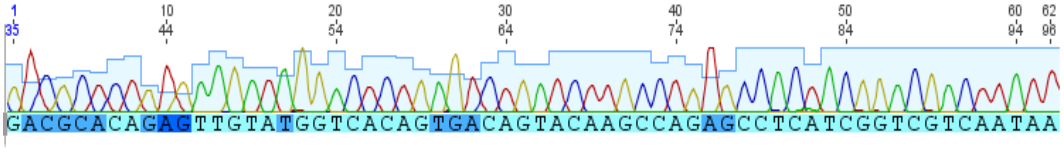
Pork L358 R extraction:



Nr megablast result: No matches found.

Result appears to show multiple overlaid sequences- ie: multiple products

Pork1 P30 F extraction:

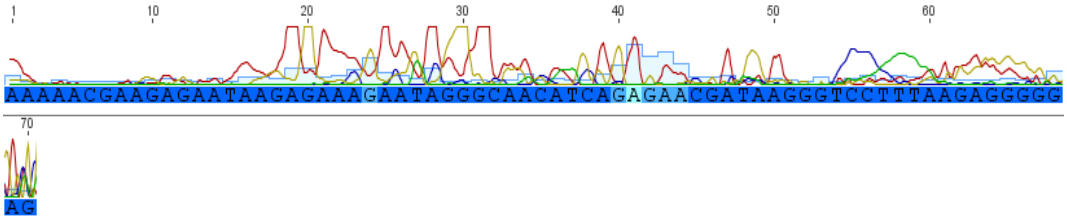


Nr megablast result: AF110182 *Toxoplasma gondii* major surface antigen p30 gene, partial cds

E value:2.92e-23 Grade:99.9% Hit:265-326

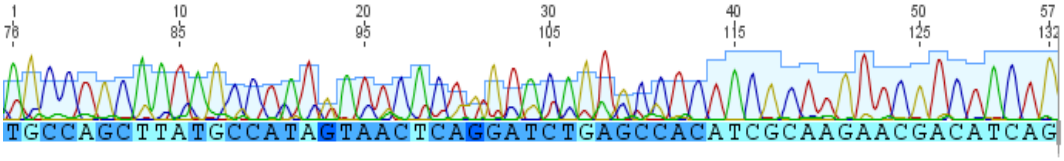
Pork P30R- Null result

Pork SAG F:



No blast search carried out.

Pork SAG R extraction:



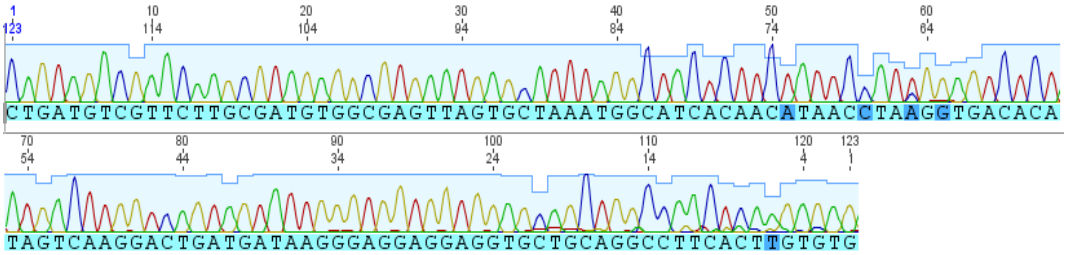
Nr megablast result: No matches found

Beef SAG449 SAG1 nest:

nest B SAGF:

Null result

Beef SAG nest B SAGR extraction:



Nr megablast result: No matches found

Appendix 3 – Nanodrop Results From Meat DNA Extractions

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat2	Beef2 complete	716	ng/µl	14.32	7.414	1.93	2.2	DNA	50
	Lamb2 complete	779.3	ng/µl	15.585	8.095	1.93	1.87	DNA	50
	Pork2 complete	924	ng/µl	18.479	9.373	1.97	2.15	DNA	50
	Beef2	73.8	ng/µl	1.475	0.804	1.84	0.34	DNA	50
	Lamb2	70.3	ng/µl	1.407	0.76	1.85	1.07	DNA	50
	Pork2	67.3	ng/µl	1.346	0.735	1.83	0.84	DNA	50
	Beef2(1)complete	168.8	ng/µl	3.375	1.711	1.97	2.3	DNA	50
	Beef2(2)complete	256.5	ng/µl	5.131	2.58	1.99	2.26	DNA	50
	Lamb2(1)complete	333.4	ng/µl	6.667	3.314	2.01	2.27	DNA	50
	Lamb2(2)complete	370.7	ng/µl	7.414	3.671	2.02	2.16	DNA	50
	Pork2(1)complete	377.6	ng/µl	7.553	3.754	2.01	2.31	DNA	50
	Pork2(2)complete	299	ng/µl	5.979	2.97	2.01	2.3	DNA	50
	Beef2	4.5	ng/µl	0.089	0.037	2.41	0.01	DNA	50
	Lamb2	8.4	ng/µl	0.168	0.074	2.28	0.01	DNA	50
	Pork2	2.7	ng/µl	0.054	0.008	6.96	0.01	DNA	50
	Beef2	85.8	ng/µl	1.716	0.022	76.25	-0.59	DNA	50
	Lamb2	74.4	ng/µl	1.489	0.016	91.68	-0.52	DNA	50
	Pork2	110.5	ng/µl	2.209	0.032	69.46	-0.73	DNA	50

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat3	Beef3	66.4	ng/µl	1.327	0.696	1.91	0.31	DNA	50
	Chicken3	10.2	ng/µl	0.203	0.105	1.94	0.09	DNA	50
	Lamb3	78.7	ng/µl	1.575	0.842	1.87	0.69	DNA	50
	Pork3	46.9	ng/µl	0.938	0.505	1.86	0.51	DNA	50

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat4	Beef4 complete	632.6	ng/µl	12.652	6.327	2	2.12	DNA	50
	Lamb4 complete	786.3	ng/µl	15.726	7.958	1.98	2.19	DNA	50
	Pork4 complete	778.3	ng/µl	15.566	7.745	2.01	2.24	DNA	50
	Beef4	118.3	ng/µl	2.367	1.251	1.89	0.65	DNA	50
	Lamb4	119.5	ng/µl	2.39	1.266	1.89	0.86	DNA	50
	Pork4	6.6	ng/µl	0.132	0.061	2.15	0.1	DNA	50
	Pork4	4.2	ng/µl	0.083	0.024	3.54	0.07	DNA	50
	Pork4	195.7	ng/µl	3.914	2.065	1.89	0.99	DNA	50

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat5	Beef 5	98.9	ng/µl	1.977	1.042	1.9	0.44	DNA	50
	Chicken 5	3.3	ng/µl	0.067	0.017	3.84	0.01	DNA	50
	Lamb 5	61.9	ng/µl	1.239	0.651	1.9	0.5	DNA	50
	Pork 5	6.2	ng/µl	0.123	0.048	2.54	0.09	DNA	50
	Beef5 (2)	320.9	ng/µl	6.418	3.403	1.89	0.88	DNA	50
	Chicken5 (2)	174.8	ng/µl	3.497	1.862	1.88	1.26	DNA	50
	Lamb5 (2)	153.6	ng/µl	3.073	1.635	1.88	0.59	DNA	50
	Pork5 (2)	152.9	ng/µl	3.058	1.625	1.88	1.3	DNA	50

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat6	Beef6	19.1	ng/µl	0.382	0.19	2.01	0.04	DNA	50
	Beef6	16	ng/µl	0.32	0.156	2.05	0.04	DNA	50
	Lamb6	37.4	ng/µl	0.747	0.394	1.89	0.24	DNA	50
	Pork6	19.1	ng/µl	0.382	0.206	1.86	0.16	DNA	50
	Lamb6 14-10	74.9	ng/µl	1.498	0.795	1.88	1.19	DNA	50
	Beef6 14-10	107.1	ng/µl	2.142	1.141	1.88	0.45	DNA	50
	Pork6 14-10	32.4	ng/µl	0.649	0.321	2.02	0.05	DNA	50
	Beef6	41.2	ng/µl	0.823	0.444	1.85	0.74	DNA	50
	Lamb6	99.1	ng/µl	1.982	1.062	1.87	1.01	DNA	50
	Pork6	33.7	ng/µl	0.673	0.365	1.84	0.96	DNA	50

#	Sample ID	Nucleic Acid Conc.		A260	A280	260/280	260/230	Sample Type	Factor
Meat7	Beef7	254.3	ng/µl	5.085	2.688	1.89	1.84	DNA	50
	Cicken7	23.4	ng/µl	0.469	0.232	2.02	0.17	DNA	50
	Lamb7	186.9	ng/µl	3.737	1.975	1.89	1.27	DNA	50
	Lamb7	80.9	ng/µl	1.618	0.864	1.87	1.07	DNA	50
	Pork7	81	ng/µl	1.619	0.86	1.88	0.94	DNA	50
	Beef7	50.3	ng/µl	1.005	0.544	1.85	0.5	DNA	50
	Lamb7	65	ng/µl	1.299	0.699	1.86	0.35	DNA	50
	Chicken7	147.8	ng/µl	2.957	1.556	1.9	1.04	DNA	50
	Pork7	106.1	ng/µl	2.121	1.131	1.88	0.34	DNA	50