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Towards the optimisation of a detection system for markers linked to neural tube defects

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TOWARDS THE OPTIMISATION OF A DETECTION SYSTEM FOR MARKERS LINKED TO NEURAL TUBE DEFECTS

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Declaration

I, Louise Cloutier, hereby declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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I thank them all.

Dedicated to my father.

Table of Contents

List of Abbreviations

A – Adenine
ABI – Applied Biosystems
AChE - acetylcholinesterase
AD – Anno Domini
AFAFP – amniotic alpha fetoprotein
AFP - Alpha fetoprotein
APS – ammonium persulfate
BPD – biparietal diameter
bp – base pairs
C – Cytosine
CBS – cystathionine beta-synthase
CCR5 - chemokine (C-C motif) receptor 5
CDC – Centers for Disease Control and Prevention
CSF – Cerebrospinal fluid
CNS – central nervous system
ddH_20 – double distilled water
Dept – department
DNA – deoxyribonucleic acid
dNTP – deoxynucleotide triphosphate
ddNTP – dideoxynucleotide triphosphate
EDTA – ethylenediaminetetraacetic acid
EtBr – ethidium bromide
EXOI – exonuclease I
F - forward
G - Guanine
GuSCN – guanidinium thiocyanate
Hb^S – sickle hemoglobin
HGP – Human Genome Project
ICD – International classification of Diseases
Ins – insertion
LCN – low copy number
NTD – neural tube defect
MSAFP - Maternal serum alpha-fetoprotein
$MgCl_2$ – Magnesium chloride
MoM – multiples of the normal median
MS – methionine synthase
MTHFR – methylenetetrahydrofolate reductase
MTRR – methionine synthase reductase
NaCl – sodium chloride
NaI – sodium iodide
PAGE – polyacrylamide gel electrophoresis
PCR – polymerase chain reaction
pg – pictogram
PK – Proteinase K
Ptaq – Platinum *Taq* DNA polymerase
R - reverse
RNA – ribonucleic acid
rpm – revolution per minute
RRM – ready reaction mix
SAP – shrimp alkaline phosphatase
SB – spina bifida
SBE – Single Base Extension
SBC- spina bifida cystica
SBO – spina bifida occulta
SDS – Sodium Dodecyl sulphate
SNE – Single Nucleotide extension
SNP – single nucleotide polymorphism
T - Thymine
TBE – Tris, borate, EDTA solution
TM - trademark
U – units
UK – United Kingdom
US – United States
UV – ultra-violet
WWB – working wash buffer

List of Figures

List of Tables

Abstract

Neural tube defects are the second most common birth defect of the central nervous system. The aim of this research is to design and optimise a novel detection system to analyse five markers that have been shown to be linked to neural tube defects. The developed detection system consists of three steps: an initial multiplex PCR, a hemi-nested multiplex PCR, and a multiplex SNE-PCR using the ABI Prism® SNaPshot™ kit. The additional step of a hemi-nested PCR has been incorporated to increase the sensitivity and specificity of the test. By detecting all five markers simultaneously, the linkage between each of the mutations can be ascertained more rapidly than if detected independently. Four different sample types were analysed, modern DNA, experimentally degraded DNA, medical specimens and archeological samples. The genotypes were successfully generated for all the samples except the archeological samples, where only partial genotypes were achieved. The designed detection system can be applied to archive medical specimens (biopsies, smears, and blood donor cards), archeological material, and other degraded samples. Future applications of this technique can include medical screening, population mapping, association studies, familial pedigrees, and the study of the evolution of diseases. This developed three step methodology can be applied to the detection of other diseases and conditions.

1 Introduction

The term 'genome' was first introduced by Hans Winkler (1920), a botanist at the University of Hamburg in Germany, by combining the sounds and meanings of the two words *GENe* and *chromosOME.* The genome of an organism is its entire hereditary information encoded as DNA and includes both coding and non-coding sequences or, for some viruses, it's RNA. In eukaryotes the genome is organised into chromosomes, the first genetic map of a *Drosophila melanogaster* chromosome was accomplished by the American geneticist Alfred Sturtevant (1913) based on his observations of linkage analysis. The most influential breakthrough for genome research was the discovery of the double helical structure of the DNA molecule in 1953 by Francis Crick and James Watson (Watson and Crick 1953a; Watson and Crick 1953b). DNA is an anti-parallel double stranded helical macromolecule in which the backbone consists of alternating sugar and phosphate groups joined by nitrogenous bases. The genetic code or DNA is made up of four nitrogenous bases: Adenine (A), Cytosine (C), Guanine (G), and Thymine (T). Each base from one strand binds to a base on the other strand; this is called complementary base pairing. In order to maintain a balance between the two complementary strands, a purine must bond with a pyrimidine via hydrogen bonds. Two hydrogen bonds form between A and T, while three hydrogen bonds are present between the C and G. In 1975, Frederick Sanger developed the chain termination technique to sequence DNA, also known as the dideoxy-termination method or the Sanger method (Sanger *et al.* 1977b). Two years later, Sanger *et al.* (1977a) successfully completed the sequencing of the 5368bp genome of the phage Φ-X174, the first genome to be sequenced. In 1976, Walter Fiers completed the sequence of the

first RNA based genome of the bacteriophage MS2 (Fiers 1976). The polymerase chain reaction (PCR) was developed by Kary Mullis which allows the amplification of specific regions of DNA and has fuelled the development of modern molecular techniques (Mullis *et al.*, 1986). Automated sequencing followed along with the possibility of analysing the entire human genome. The human genome consists of 23 pairs of chromosomes, with each parent contributing one chromosome to each pair. The entire genome of eukaryotes typically includes the nuclear and the mitochondrial genomes. The human mitochondrial genome was sequenced in 1981 (Anderson 1981). The $3-billion dollar 'Human Genome Project' (HGP) began in 1990, to map and sequence the human genome. It was estimated to take 15 years to complete (U.S. Dept. of Health and Human Services 1990). However, widespread international effort and cooperation comprising geneticists from China, France, Germany, Japan, and the United Kingdom greatly aided the project's progress. As well as rapid technological advances in the field of genomics such as: (1) the use of restriction fragment-length polymorphisms; (2) the polymerase chain reaction; (3) developments in sequence analysis; (4) development of bacterial and yeast artificial chromosomes, which are now preferred in sequencing procedures because of their superior stability and larger size. These technological advances accelerated the completion of HGP producing a 'rough or working draft' of the human genome in November 2000 (November 2000; White House Press Release June 26, 2000) and published in *Nature* (International Human Genome Mapping Consortium 2001) and *Science* (Celera Genomics Sequencing Team 2001). These drafts represented 90% of the entire genome. The remaining 10% included the telomeres, and around the centromeres, where few if any genes are expected to be located. The

announcement of the completed genome in April 2003, coinciding with the 50th anniversary of the discovery of the DNA double helix, two years ahead of expectation and significantly under original spending projection (Henry 2003). All three billion base pairs in the human genome were sequenced creating a "blueprint" that contains all the information required for the construction, assembly, and operation of the human body.

The HGP has lead to the fields of genomics and proteomics where over 20,000 genes have been identified and their biological functions are being determined (International Human Genome Sequencing Consortium 2004). The project has become a landmark to an ever expanding field of genomics leading to the consideration of genomic sequencing of other organisms, such as commercially important species (i.e. crop plants and livestock) (Honeybee Genome Sequencing Consortium 2006; Ihara *et al.* 2004; International Rice Genome Sequencing Project 2005) species that have a relevance to human health (i.e. pathogenic bacteria or vectors of diseases) (Gardner *et al.* 2002; Holt *et al.* 2002; Parkhill *et al.* 2003; Shimizu *et al.* 2002; Waterston *et al.* 2002), and species that could help answer evolutionary questions (i.e. chimpanzee) (Cheng *et al.* 2005; Chimpanzee Sequencing and Analysis Consortium 2005; Hughes *et al.* 2005; Khaitovich *et al.* 2005). The HGP has lead to numerous related projects such as: the SNP Consortium; the NIH Cancer Genome Project; HapMap; and the Human Genome Diversity project.

The genomic era has created a revolution in biology by seeking to correlate genetic variations with phenotypic differences. In humans, these traits range from hair colour, eye colour, skin colour, and blood group, to disease susceptibility. Some variations in the DNA sequence do not produce a detectable change in biological

function while others do with either positive or negative consequences. The positive effects can include a mutational change that causes an increased resistance to an infection or disease, such as the Δ32 deletion of the chemokine (C-C motif) receptor 5 (CCR5) gene. Homozygotes for the allele exhibit a strong, although incomplete, resistance to HIV infection, whereas heterozygotes display delayed progression to acquired immunodeficiency syndrome (AIDS) (Blanpain *et al.* 2002). Negative consequences include diseases, syndromes or conditions and there are many human diseases that have a genetic cause. Some diseases have a large genetic component such as Huntington's disease, Tay-Sachs disease, or cystic fibrosis, while cancer, diabetes and heart disease possess a more modest genetic component because humans inherit only a predisposition and not a certainty of developing the disease. Genetic disorders can be grouped into three categories: single gene disorders, chromosome disorders, and multifactorial inheritance disorders. Single gene disorders are the product of a single gene mutation that can be present on one or both chromosomes. They may be autosomal or X-linked and are usually found as recessive, dominant or co-dominant. Examples of single gene disorders are Duchenne's muscular dystrophy and Huntington's disease (Emery 2003; Gusella and Macdonald 2006). Chromosome disorders are caused by an excess or deficiency of the genes located on chromosomes or structural changes within the chromosomes. An extra copy of chromosome 21, with no abnormal individual gene, causes Down's syndrome (Capone 2001). Multifactorial inheritance disorders or polygenic diseases are caused by the interaction of a number of genes in conjunction with the environment. Heart disease, most cancers, obesity and alcoholism are examples of these multifactorial disorders.

Some diseases, even when they cause morbidity and mortality can be kept in the population by selective pressures. An example is sickle hemoglobin (Hb^S) causing sickle cell anaemia in which the heterozygous state provides a selective advantage in environments affected by malaria (Aidoo *et al.* 2002; Williams *et al.* 2005). Likewise, cystic fibrosis can confer resistance to cholera (Bijman *et al.* 1988; Rodman and Zamudio 1991). In the right environment, the incidence of these diseases can remain quite high, while elsewhere in the world where there are no selective pressures the incidence drops.

The complete human genome has motivated research on understanding complex genetic diseases, disorders, and conditions. In order to associate specific genetic variations with conditions, genome-wide association studies are necessary. Researchers compare the genomes of affected and unaffected (control) individuals, and identify specific regions that differ between the two groups. The genetic differences can identify individuals with an increased risk for developing the condition which can then be exacerbated by environmental triggers. Genes associated with Duchenne's muscular dystrophy (Koenig *et al.* 1987), retinoblastoma (Dryja *et al.* 1986; Lee *et al.* 1987), and cystic fibrosis (Kerem *et al.* 1989; Riordan *et al.* 1989; Rommens *et al.* 1989) were the first to be mapped and cloned. Recently, researchers have announced the discovery of new genes associated with breast and ovarian cancer (Hughes-Davies *et al.* 2003); type-2 diabetes (Gunton *et al.* 2005); and Alzheimer's disease (Rogaeva *et al.* 2007).

There are many benefits of studying human genetic variations and their phenotypic manifestations. The ability to associate certain mutations and genes with

specific conditions can shed light on the causes and factors of these diseases. Such knowledge can lead to the development of new therapies and strategies to treat or even prevent these diseases. By recognising the genotypic differences and their implications towards treatments, researchers can determine how individuals may respond to treatments based on their genotype and which strategies are more effective for their recovery. Understanding of such correlations can result in a new generation of medicines that target diseases with increased precision and reduced risks, a developing field that is known as pharmacogenomics. Expanding our biochemical knowledge of disease-related genes will lead to the development of innovative drugs that target theses specific biological functions.

1.1 Genetic Mutations

A mutation can be defined as any heritable change in the sequence of nucleotides in DNA that causes a permanent alteration of genetic information (Horton 2002). Mutations contribute to phenotypic variation and influence an individual's anthropometric characteristics, risk of disease and response to the environment (International SNP Map Working Group *et al.* 2001). A mutation can involve insertions, deletions or substitutions of one or more bases in the DNA molecule.

Deletions and insertions range from changes of a single nucleotide to ones that involve a genetic region. A frameshift mutation, where one to three bases are added or removed from the DNA molecule, causes a shift in the codon reading frame (Tamarin 1991). These are potentially the most devastating in their effects on an organism because the sequence of a gene changes from the site of mutation onward. Deletions

and insertions can also cause large-scale changes in the amino acid composition of the polypeptide chain and can lead to non-functional genes (Cummings 1994).

A nucleotide substitution, also referred to as a single nucleotide polymorphism (SNP) (International SNP Map Working Group *et al.*), does not modify the number of nucleotides but entails a variation within the sequence in which a single nucleotide is exchanged for another. The most common form of sequence variation, nucleotide substitutions consist of three types of mutations: a missense mutation, a nonsense mutation and a sense mutation (Cummings 1994). A missense mutation is a single nucleotide change that results in the substitution of one amino acid for another in a protein. A nonsense mutation changes an amino acid-specifying codon to one of the three terminating codons; resulting in a shorter polypeptide chains due to the premature termination of translation. A sense mutation produces elongated proteins since a terminating codon has been changed into one that codes for an amino acid. Nucleotide substitutions may affect gene functionality, gene presence, alter the morphology or physiology of the organism resulting in phenotypic consequences, or have no noticeable phenotypic effects.

1.1.1 Single Nucleotide Polymorphisms

A single nucleotide polymorphism (SNP) is usually a bi-allelic nucleotide substitution that exists in normal individuals in some populations. A true SNP designation requires the allele to be present at or above 1% frequency in the tested population (Brookes 1999). Human genomes are 99.9% identical and of the 0.1% difference, 90% are SNPs (Collins *et al.* 1998; Li and Sadler 1991; Wang *et al.* 1998). Phenotypic differences can be attributed to SNPs; and this is the basis for genetic

diversity found among humans. Single nucleotide polymorphisms occur on average every 1,000 bases when any two chromosomes are compared making them more prevalent in the genome than microsatellites (Cargill *et al.* 1999; Kwok *et al.* 1996; Li and Sadler 1991; Wang *et al.* 1998). Currently, more than 1.4 million SNPs have been identified in the human genome (International SNP Map Working Group *et al.* 2001). There are two types of SNPs: transitions (C↔T or G↔A) and transversions (C↔A, C↔G, T↔A and G↔T). Approximately two thirds of SNPs involve transitions (Brookes 1999). There are three possible genotypes when a SNP occurs; in the case of a C-G mutation, there could be a CC, CG and GG genotype. The nomenclature for this bi-allelic system is CC when both alleles have a cytosine in that SNP position. Alternatively, a CG represents a heterozygote where one allele has a cytosine and the other allele has a guanine.

Their extreme abundance in the genome indicates that SNPs can occur in both coding and non-coding regions (Syvanen 2001). This allows linkage between SNP variations within gene sequences and observed changes in gene function. Consequences at the phenotypic level depend on the location of the SNP. The SNPs located in non-coding regions have no direct known impact on the phenotype of an individual and can serve as biomarkers. Whereas, SNPs found in coding regions that alter the function or structure of the encoded proteins are a necessary and sufficient cause of most of the known inherited monogenic diseases (Syvanen 2001). These SNPs are important for diagnostic purposes.

SNPs are also considered to be very stable heritable markers with relatively low mutation rates in comparison with other types of genetic markers (Carlson *et al.* 2001)

and seem to mutate randomly (Crow 1995; Li *et al.* 1996a). These characteristics render SNPs a valuable tool for a broad range of disciplines. The analysis and identification of multiple SNPs can generate a SNP profile. This SNP profile can be applied for identification purposes in forensic criminal profiling for differentiating between individuals, populations and ethnic groups (Chakraborty *et al.* 1999; Collins *et al.* 1997; Risch and Merikangas 1996). The stability of SNPs allows a SNP profile to be used in the study of human history and evolution (Hacia *et al.* 1999; International SNP Map Working Group *et al.* 2001; Jorde *et al.* 2000). A generated SNP profile can be used in conjunction with mitochondrial DNA haplotyping (Nelson *et al.* 2007) to follow inheritance patterns in humans over time or to identify inheritance patterns of chromosomal regions from generation to generation (Mountain *et al.* 1992). Single nucleotide polymorphisms are also of interest in the field of pharmacogenomics for predicting individual variations in drug response and identifying new targets for drug design (Poste 2001). The specificity and sensitivity of SNPs enables the study of archeological specimens that can provide insight into the evolution of diseases such as the analysis of cytokines by Turner *et al.* (2002).

The study of SNPs is important because they contribute to our understanding of the underlining genetic causes of diseases and conditions that manifest in the clinical phenotypes. Researchers have shown that many clinical phenotypes have a considerable genetic component like SNPs (Brookes 1999). Some SNPs located in genes can be expected to directly affect protein structure or expression levels, and these may represent candidate alterations for genetic mechanisms in disease (Landegren *et al.* 1998). Attempts to correlate individual and combination of SNPs with

phenotypes that act either as markers and/or are causative for common multifactorial disorders such as cancer, cardiovascular disease, mental illness, auto-immune states, and diabetes, who are expected to be heavily influenced by the individual's SNP patterns in certain key susceptibility genes (Brookes 1999). However, the effect of genotype on disease phenotype does vary between disorders and populations owing to genetic and environmental heterogeneity (Weiss and Terwilliger 2000). It is the combined effect of a collection of SNP alleles in sets of key genes, plus environmental factors, that together determine whether an individual suffers from a particular disease (Brookes 1999). Eventhough SNPs contribute to conditions and diseases have environmental factors and the study of SNPs requires extensive research.

SNPs can therefore be used to identify genetic components and factors associated with complex diseases and identify individuals who are predisposed and at risk (Chakraborty *et al.* 1999; Collins *et al.* 1997; Johnson and Todd 2000; Risch and Merikangas 1996). If a SNP contributes an increased risk for disease occurrence, that polymorphism should therefore be found at a higher frequency in individuals with that disease compared to non-disease controls, associated with the phenotype (Brookes 1999). Assays that could analyse all the SNPs in large sets of patients and matched non-disease controls for all the complex phenotypes should be developed (Brookes 1999).

After the completion of the HGP, a SNP revolution began in which SNPs were the focus of much attention as a race to identify and associate SNPs motivated rapid technological progress. The unique characteristics of SNPs allow them to be amenable to automated, large-scale, high-throughput genotype analysis. This has been the

driving force to develop new methods and techniques in the quest to better understand complex diseases which will have a tremendous impact on human biology.

1.2 Birth Defects

A birth defect, or congenital malformation, is defined as an abnormality of structure, function or metabolism present at birth that results in physical or mental disabilities or death (March of Dimes 2007). Birth defects are the result of an abnormality during the developmental process and can occur in any major organ or part of the body. Most birth defects occur during the first three months of pregnancy and their severity ranges from mild implications to serious complications. Some birth defects can be life threatening, lead to lifelong disability and require costly medical care and multiple hospitalisations. Some can be diagnosed through prenatal screening (e.g. Down syndrome), at birth (e.g. achondroplasia), within the first few years of childhood (e.g. phenylketonuria), or anytime later in life (e.g. Huntington's disease).

Approximately 3% of babies are born with birth defects (Centers for Disease Control and Prevention (CDC) 2006; Greenberg 1991; Moore 1988b). By the end of the first year, detection of malformations that were not obvious at birth increases to 6% (Moore 1988b). Birth defects are the leading cause of death in the first year of life in the developed world (Martin *et al.* 2005; Pinar 2004). Birth defects can occur as an isolated abnormality or in association with or combined with other abnormalities (Greenberg 1991). When multiple defects occur together and have a similar cause, they are called syndromes (Greenberg 1991). If two or more defects appear together but do not share the same cause, they are called associations (Greenberg 1991). Known causes of birth defects include single gene disorders, chromosome abnormalities and environmental

exposures such as infectious diseases or harmful chemical agents during pregnancy (Mitchell 2005). Birth defects can be grouped into four categories: structural and metabolic abnormalities; congenital infections; and other conditions (Copp 1994; March of Dimes 2007; Stevenson 1993).

Structural birth defects are the result of abnormal development in which an internal or external body part is missing or has formed incorrectly. Examples of a structural defect would be a cleft lip and a cleft palate (Mitchell 1997). Metabolic defects are caused by a biochemical disruption from missing or incorrectly formed enzymes which facilitate the conversion of various substrates into other products. An imbalance will create either a deficiency, or an accumulation of substrates. A deficiency may reduce the ability to synthesise essential compounds while substrate accumulation can be toxic or interfere with normal function (Cummings 1994). Gaucher's disease is an example of a metabolic birth defect (Brady 2006). This disease causes an abnormal build-up of a fatty substance called glucocerebroside throughout the body that becomes toxic to organs and tissues, resulting in progressive and permanent damage (Beutler 1995).

Birth defects resulting from congenital infections appear when a mother acquires an infection prior to or during pregnancy and it can cause deafness, blindness, mental retardation, or heart defects. Cytomegalovirus and rubella are common congenital infections (Moore 1988b). Other conditions that cause birth defects are fetal alcohol syndrome, illicit drug use, and Rhesus disease. To date, several thousand different birth defects have been identified, however, the causes of 70% of birth defects are still unknown (Centers for Disease Control and Prevention (CDC) 2006).

1.3 Neural Tube Defects

Neural tube defects (NTDs) are an important category of birth defects. These congenital malformations of the central nervous system (CNS) are the second most common birth defects, the first being congenital cardiovascular abnormalities (Manning and Archer 2001). Congenital defects of the CNS arise from complex embryological malformations related to the growth and development of the brain, skull and spinal cord. Defects may include all congenital abnormalities which involve the failure of the neural tube to properly close during the fourth week of human embryogenesis, with defects occurring at any point along the formation of the spinal cord, either rostrally from the developing brain and caudally to the sacrum (Finnell *et al.* 2003). Their severity depends on the size, level or location of the defect along the neuroaxis of the embryo and whether the defect crosses CNS segmental boundaries.

Many NTDs often results in exposure of neural tissue to the environment. All NTDs appear to be etiologically heterogeneous (Holmes *et al.*, 1976) and multifactorial in origin; their cause is attributed to a combination of genetic and environmental factors. The most important environmental factors include geography, month of conception, epidemic trends, maternal age, birth order, socio-economic class, maternal diet, maternal diabetes, maternal obesity, and maternal illness and drug exposure (Elwood 1992).

A genetic predisposition for NTDs has been supported by several lines of evidence. The incidence of NTDs varies greatly among populations and ethnic groups, suggesting that there are genetic factors that may explain the differences in prevalence (Copp and Bernfield 1994). There is a significantly higher prevalence of consanguinity

among parents of infants with NTDs, suggesting a clustering of affecting genes within families that may possibly contribute to NTD in some way (Elwood 1992). The risk of occurrence of NTDs in offspring of parents with prior affected pregnancies is substantially higher not only for those parents but also for their first-, second-, and third-degree relatives. The risk for NTDs is highest among first-degree relatives and decreases for more distant relatives. The risk of occurrence for siblings of an affected individual is 2-5%, representing a 25-50 fold increase compared to the general population (Elwood 1992). The increased risk also appears to extend to cousins of the affected individual, although possibly to only maternal cousins (Carter 1974). The risk of reoccurrence, for women who have had one affected infant, is increased by at least 1-3% above that of the general population (Papp *et al.* 1997; Pietrzyk 1983; Swerts *et al.* 1987; Thunem *et al.* 1988). In addition, females and monozygotic twins appear to be more prone to NTD (Hall 1998). Neumann *et al.* (1994) interpreted such data to argue that human NTDs exhibit approximately 60% heritability. These observations support a prominent role for genetically inherited components in the occurrence of NTDs.

The term neural tube defect encompasses a broad range of malformations, rendering this condition difficult to classify. The spectrum includes: anencephaly; spina bifida; encephalocele; iniencephaly and craniorachischisis (Figure 1) (Hall 1998). Classification of NTDs varies among the medical literature. NTDs can be grouped as "open" where the brain and/or spinal cord are exposed at birth through a defect in the skull or vertebrae, or "closed" where the spinal defect is covered by skin (Elwood 1992; Mitchell 2005). They can also be typed as NTDs that affect cranial or spinal structures (Frey and Hauser 2003). Another way of classifying NTDs is based on its position

along the length of the neural tube, whether it is high or low (Blatter *et al.* 1997). This classification is similar to that of the cranial/spinal grouping. Newer classification schemes attempt to divide these malformations by their locations rather than by gross anatomical characteristics.

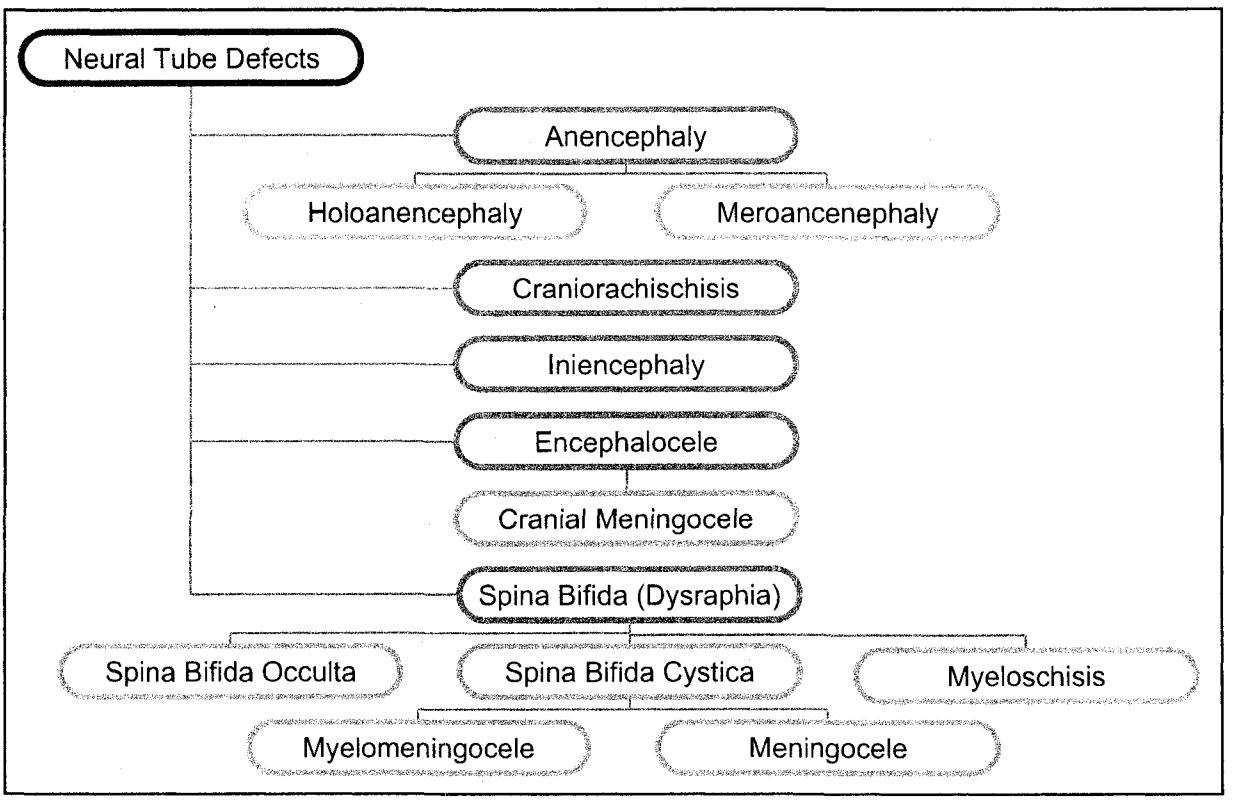

Figure 1 – Types of Neural Tube Defects

1.3.1 Anencephaly

The two most common types of NTDs observed in humans are anencephaly and spina bifida cystica (Detrait *et al.* 2005; Hall 1998). Anencephaly was first described by von Recklinghausen in 1886 in which he suggested that the birth defect is caused by improper closure of the neural tube (von Recklinghausen 1886). Anencephaly comprises 50-65% of all NTDs and is immediately noticeable at birth (Hunter 1993; Thomas *et al.* 1994). This condition is lethal; most infants are stillborn or die shortly

after birth, usually within 48 hours (Elwood 1992; Hunter 1993). This type of malformation results from incomplete closure of the cephalic portion of the neural tube leading to partial (meroanencephaly) or complete absence (holoanencephaly) of the cranial vault (skull), overlying tissues and cerebral hemispheres as well as an abnormal development of the skull base. The remaining degenerated brain tissue that consists of a small, spongy, vascular mass of disorganised neural tissue is referred to as the 'area cerebrovasculosa' (Elwood 1992). Holoanencephaly accounts for 65% of anencephaly cases and is characterised by a defect that extends through the foramen magnum (Myrianthopoulos and Melnick 1987). Meroanencephaly is a milder defect in which the area cerebrovasculosa protrudes through a partially formed skull (Myrianthopoulos and Melnick 1987). In both types, the eyes are bulging, giving a frog-like appearance and the neck is usually short (Figure 2) (El-Ghani 2006). The brain stem is present but is usually incomplete; the infant's face has the characteristic appearance of an elevated and pointed nose (Elwood 1992).

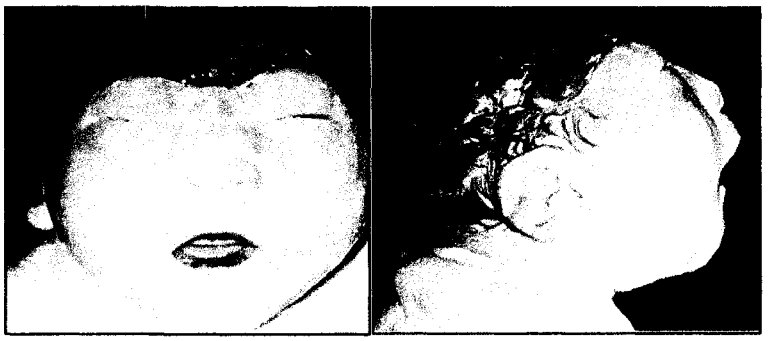

Figure 2 – Infant affected with anencephaly (Cohen 2002)

1.3.2 Craniorachischisis

Craniorachischisis, sometimes considered a subtype of anencephaly, is a rare and severe condition in which the neural folds never elevate to fuse along the entire

length of the embryo causing incomplete closure of the skull and spinal column (Finnell *et al.* 2003). This type of malformation is characterised as anencephaly with continuous involvement of the spine and no meninges covering the neural tissue. Like anencephaly, this condition is lethal (Hall 1998) and 80% of holoanencephaly cases are associated with craniorachischisis (Melnick and Myrianthopoulos 1987). The defect can be limited to the cervical region or the entire spine resulting in the CNS being exposed to the surrounding amniotic fluid, thus causing necrosis, degeneration or angioma-like formations. The condition is sometimes associated with severe anterior flexion of the spine, causing the infant to gaze upward (Figure 3) (Hunter 1993).

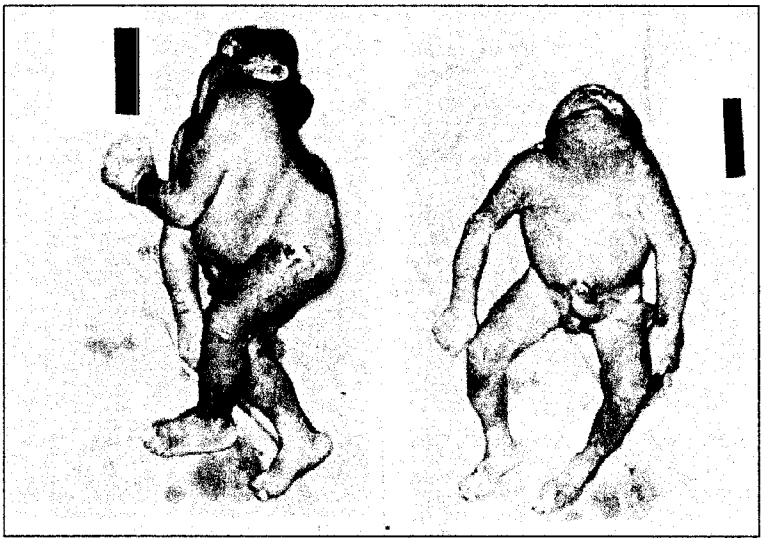

Figure 3 – Child affected with craniorachischisis (Moore *et al.* 1997)

1.3.3 Iniencephaly

Iniencephaly is a very rare NTD that combines extreme retroflexion of the head with severe distortion of the spine. Affected newborns do not generally live more than a few hours. The disorder is more common among females by a ratio of 10:1 (Hunter 1993). Iniencephaly is characterised by a defect in the occipital region accompanied by

cervical and thoracic vertebrae that are partially or completely absent, with the remaining vertebrae irregularly fused and with defective closure of the vertebral arches. This malformation results in fusion of the cranial area to the caudal area, causing continuity of the skin of the face to the chest and the skin of the scalp to the back. This condition is similar to craniorachischisis with its retroflexion of the spine; however, in the latter the cervical and thoracic vertebrae remain identifiable. Most infants tend to be short with a disproportionately large head and an absent neck (Figure 4). Individuals with iniencephaly usually have other associated anomalies such as anencephaly and/or a meningomyelocele present (Hunter 1993).

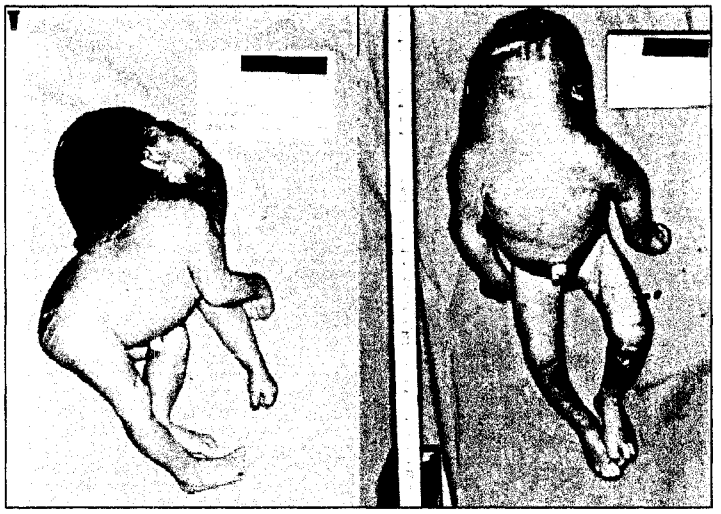

Figure 4 – Newborns with iniencephaly (Moore *et al.* 1997)

1.3.4 Encephalocele

Encephalocele is apparent upon examination of the newborn and is relatively less common than spina bifida and anencephaly (Hunter 1993). This birth defect is thought to be due to the failure of closure of the anterior neural tube at a slightly later stage of embryogenesis than in anencephaly. This condition is characterised by the incomplete development of the skull which results in a saccular herniation that extends

through the midline defect in the calvaria (Figure 5). Pressure within the skull subsequently pushes cranial tissue out through the opening (El-Ghani 2006). The contents of the sac may contain one or all of the following intracranial contents: the protective membranes normally covering the brain known as the meninges;

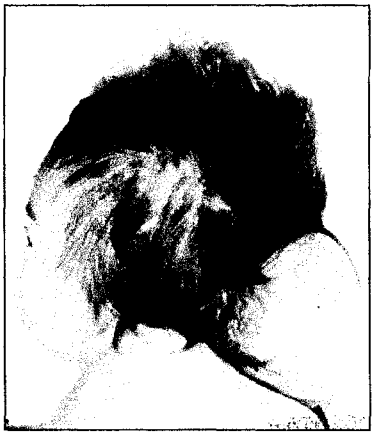

Figure 5 – Child with encephalocele (Cohen 2002)

cerebrospinal fluid (CSF); and/or some brain tissue. Herniation of the meninges through the cranial defect is termed 'cranial meningocele', whereas herniation of brain tissue, meninges and CSF through the bony defect is called an 'encephalocele' (El-Ghani 2006). The meninges can grow to cover the protrusion as skin does in some cases. The severity of the protrusion can vary in size; varying from very small to larger than the head of the infant. In either case, the sac must be excised so that the skin does not erode and cause meningitis. Encephalocele can be classified as occipital, anterior or parietal (Cohen 2002); the frequencies being about 75%, 10% and 15% respectively within the US population (Lemire 1988). The herniated sac can also be covered with either normal skin or a thin epithelium (Campbell *et al.* 1986). The neurological impairment depends on the extent of brain involvement; there can be little to no brain tissue or the protrusion may contain a significant fraction of the brain

resulting in severe brain damage. Thus, the greater the neural abnormalities, the greater the rate of mental retardation (Bozinov *et al.* 2005).

1.3.5 Spina Bifida

Spina bifida (SB), also known as spinal dysraphism, means 'split spine' in Latin and can be referred to as 'open spine' (Freeman 1980). This term is used to describe many varieties of lesions due to midline separation of the vertebrae (Elwood 1992). It is a posterior NTD characterised by caudal lesions affecting the spinal cord, vertebrae and skin (Botto *et al.* 1999). This birth defect presents at birth with a lesion on the back (Freeman 1980). SB varies in severity, ranging from asymptomatic to clinically significant types. This condition results from failure of the halves of the vertebral arch of one or more vertebrae to develop normally and fuse in the median plane (Moore 1988). Lesions can occur at any location along the spine and vary in size from a single vertebra to the entire neuroaxis (Hunter 1993). SB is subdivided into three main classes: spina bifida occulta (SBO), spina bifida cystic or aperta and myeloschisis.

SBO, often called 'hidden' spina bifida, is the mildest form of spina bifida in which disability is rarely seen (Northrup and Volcik 2000). This malformation usually affects only one or two vertebrae. The split in the vertebrae is so small that the spinal cord and the meninges are in their normal position within the vertebral canal and are usually not involved or herniated. Thus there is no opening in the back or sac protruding and the defect is completely covered by skin (Figure 6). Spina bifida occulta occurs mostly in the lumbosacral region of the spine in the L5, S1 and/or S2 vertebrae in about 10-25% of healthy individuals and is regarded as a normal variation in the population (Botto *et al.* 1999; Moore 1988b; SBHAC 2000). In many cases, it is neurologically

asymptomatic and is often diagnosed incidentally during a radiographic exam of the lower spine (El-Ghani 2006). However, if several vertebrae are affected bowel, bladder and motor problems, weakness and numbness of lower limbs, as well as foot deformities may arise (SBHAC 2000).

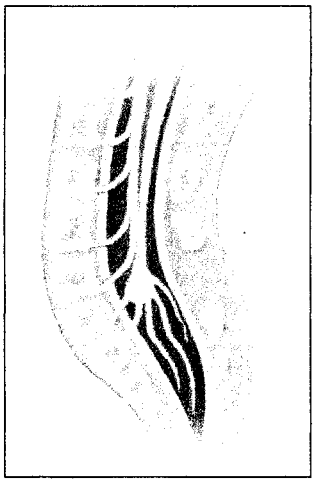

Figure 6 – Spina Bifida Occulta (Botto *et al.* 1999)

Approximately 80% of individuals with spinal cord malformations exhibit some type of skin abnormality overlying the defect which can also be indicative of the condition (Kinsman 2001; van der Put *et al.* 2001). These skin defects include: a hairy patch; fatty lump (lipoma); hemangioma; dark spots or birthmarks; skin tract or sinus (i.e. a deep dimple); and hypopigmented spots (Figure 7) (Kinsman 2001).

Spina bifida cystica or aperta is a severe open neural tube defect because it involves the protrusion of a herniated sac containing meninges or meninges and spinal cord tissue through a defect in the vertebral arches (Northrup and Volcik 2000). It is termed cystica because of the cyst-like sac that is associated with this type of malformation. Individuals affected can survive into adulthood with considerable disability depending on the severity, type and location of the lesion. Spina bifida cystica has two subclasses: myelomeningocele or meningomyelocele and meningocele. Although this

condition can occur at any level, it most commonly occurs in the lumbar or lumbosacral regions of the spine (Moore 1988b).

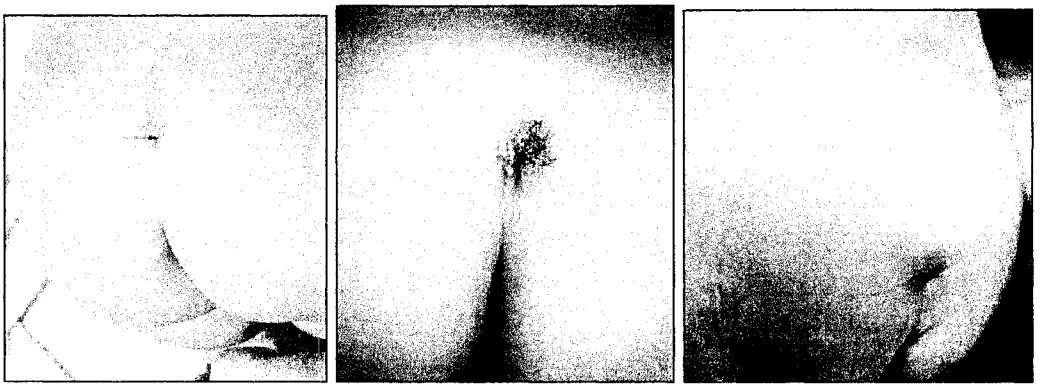

Figure 7 – Examples of skin abnormalities associated with Spina Bifida such as a dimple (Moore 1988b); a tuft of hair and lipoma (Pierre-Kahn *et al.* 1997)

In about 10% of SB cases the malformation consists of a dorsal protrusion of a fluid-distended sac through the incompletely formed vertebrae. This meningocele contains only meninges and CSF (Hunter 1993). The spinal cord and spinal roots are in their normal position and the sac is usually, but not always, covered by normal skin (Figure 8) (Northrup and Volcik 2000). The defect can be surgically repaired with little nerve damage.

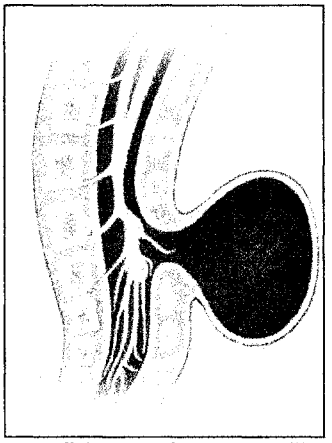

Figure 8 – Spina bifida meningocele (Botto *et al.* 1999)

About 90% of SB cases in neonates are myelomeningoceles (Hunter 1993). This condition is characterised by saccular herniation containing the spinal cord and/or nerve

roots, CSF and the meninges through a large vertebral defect of the spine (Figure 9) (Botto *et al.* 1999). The lesion may be covered by a thin layer of skin or a transparent membrane (Kaplan *et al.* 2005). The top of the protruding sac is often a soft, fragile, "area medullavasculosa" consisting of blood vessels, pia mater and neural remnants (Hunter 1993).

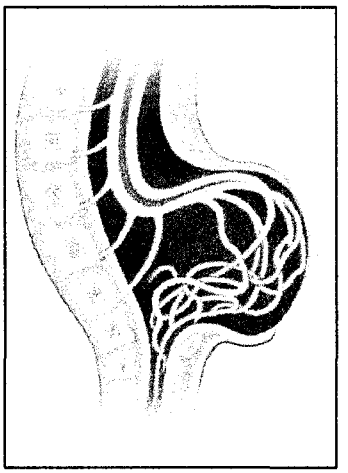

Figure 9 – Spina bifida myelomeningocele (Botto *et al.* 1999)

Meningomyelocele is more severe than meningocele because the condition results in a marked neurological deficit inferior to the level of the protruding sac (Northrup and Volcik 2000). SB myelomeningocele demonstrates variable degrees of neurological impairment based on three factors: location of lesion; extent of lesion; and interruption of the spinal cord or spinal cord and tissue involvement. In fact, 99% of children born with an open NTD have some type of handicap which can include: motor and sensory dysfunction or paralysis of lower limbs; anaesthesia of the skin; poor or nearly absent bladder and bowel control, and abnormalities of hips, knees, or feet (e.g. club foot and rocker bottom foot) (El-Ghani 2006; Northrup and Volcik 2000; Padmanabhan 2006). Other complications commonly seen are severe forms of scoliosis, kyphosis or lordosis as well as meningitis, tethered cord syndrome and

diastematomyelia (Kaplan *et al.* 2005). Patients with more severe forms of SB have Arnold-Chiari type II malformation of the cerebellum which is believed to be a secondary effect of hydrocephalus (Lemire 1988). Hydrocephalus is the accumulation of CSF in the ventricles of the brain and/or between the brain and dura mater (Moore 1988). As a result of the NTD, the CSF is unable to drain normally causing the head to become enlarged. All individuals with thoracic SB and most of the patients with lumbosacral SB are at increased risk for hydrocephalus and Arnold Chiari type II malformation (McLone and Dias 2003; Rintoul *et al.* 2002). Hydrocephalus is associated with 90% of lumbosacral SB; cervical, thoracic, and sacral lesions are less often complicated with hydrocephalus (Figure 10) (Hunter 1993; Lemire 1988).

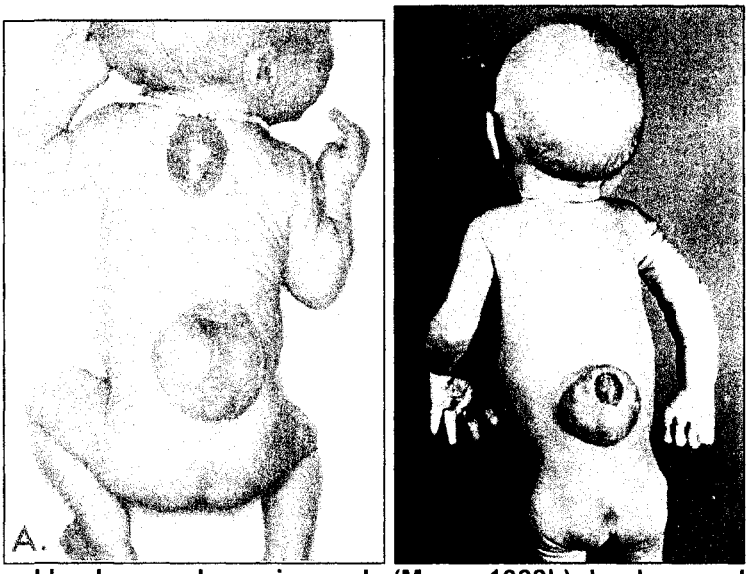

Figure 10 – Thoracic and lumbar myelomeningocele (Moore 1988b); lumbar myelomeningocele (Davies and Duran 2003).

The third and most severe type of SB is called myeloschisis. This defect occurs when the developed spinal cord is exposed to the external environment, because the dermal or meningeal covering is absent. There is no cystic sac observed in this condition and hair is usually found growing around the defect. CSF leaches from the

opening of the affected area and the spinal cord is represented as a raw, red, flattened mass of nervous tissue (Figure 11) (Moore 1988b). This causes paralysis of the lower limbs and pelvic organs (Moore 1988). Like meningomyelocele, there are the same associated complications and chance for severe infection and if not corrected can cause death (Kaplan *et al.* 2005).

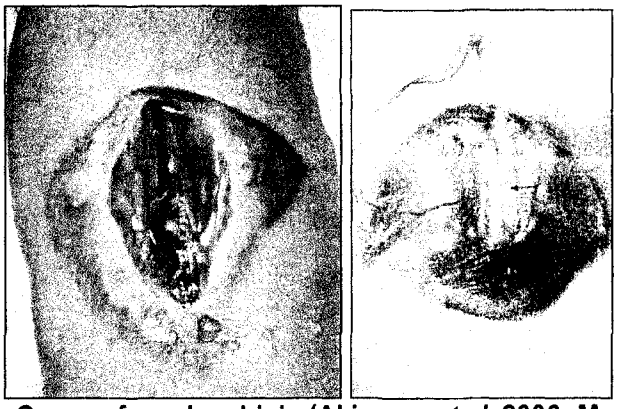

Figure 11 – Cases of myeloschisis (Akiyama *et al.* 2006; Moore 1988b)

1.4 Screening for Neural Tube Defects

Screening programs aid in identifying pregnant women with increased risks for various conditions. Screening can lead to four possible outcomes: true positives which correctly identifies an individual as affected with the condition; false positives which incorrectly identifies an unaffected person as affected; true negative which correctly identifies an individual as unaffected and a false negative which incorrectly identifies an affected person as unaffected with the condition (Ormond 1997). Women who show positive results are offered a specific diagnostic test such as a targeted ultrasound examination, amniocentesis, or other obstetrical interventions. Today's screening techniques identify fetuses with chromosomal abnormalities (i.e. Down syndrome) and women at high risk for obstetrical complications during the third trimester (Rose and Mennuti 1993). The detection of maternal serum α-fetoprotein (MSAFP) is the earliest

non-invasive procedure that can be used during pregnancy to acquire information about the fetus (Rose and Mennuti 1993). α-Fetoprotein (AFP) is the first embryonal serum protein that is produced during the stages of fetal life (Muller 2003). It is filtered by the fetal kidneys and excreted through fetal urine into the amniotic fluid and then enters into maternal circulation by diffusion through the membranes or the placenta (Fuhrmann and Weitzel 1985). Brock and Sutcliffe (1972) first discovered that there was a significantly elevated amount of AFP in women carrying fetuses affected with open NTDs. The association between NTDs and increased AFP values in maternal blood has been shown in many studies (Brock *et al.* 1973; Brock *et al.* 1974; Harris *et al.* 1974; Seller *et al.* 1974; Wald *et al.* 1974). In the presence of an open NTD, large amounts of AFP is released into the amniotic fluid from the exposed lesion, causing an increased diffusion into the maternal serum (Fuhrmann and Weitzel 1985). Studies have lead to routine laboratory testing for prenatal diagnosis of NTDs (Wald *et al.* 1977).

MSAFP concentration peaks in the second trimester to a maximum at about 30 weeks of gestation, and then declines until term (Drugan *et al.* 2001; Seppala and Ruoslahti 1973). For screening in the UK (Wald *et al.* 1977), a collaborative study determined that the optimal time for increased sensitivity to perform this test is between 16 to 18 weeks gestation. During this period, the AFP levels are about four times greater in open NTD pregnancies than in unaffected pregnancies (Wald 1992). AFP levels can, however, be measured between 14 to 22 weeks of completed pregnancy but with lowered sensitivity (Ormond 1997; Rose and Mennuti 1993). As the fetus matures, AFP gradually decreases since albumin replaces AFP as the principal fetal plasma protein (Drugan *et al.* 2001). Due to the variation of AFP concentrations, reference

ranges at each gestational age must be clearly determined prior to maternal serum or amniotic fluid testing (Muller 2003). Closed SB and encephaloceles are difficult to detect since MSAFP levels are often not elevated because the defect is skin covered causing AFP not to be released as much as an open NTD (Ormond 1997).

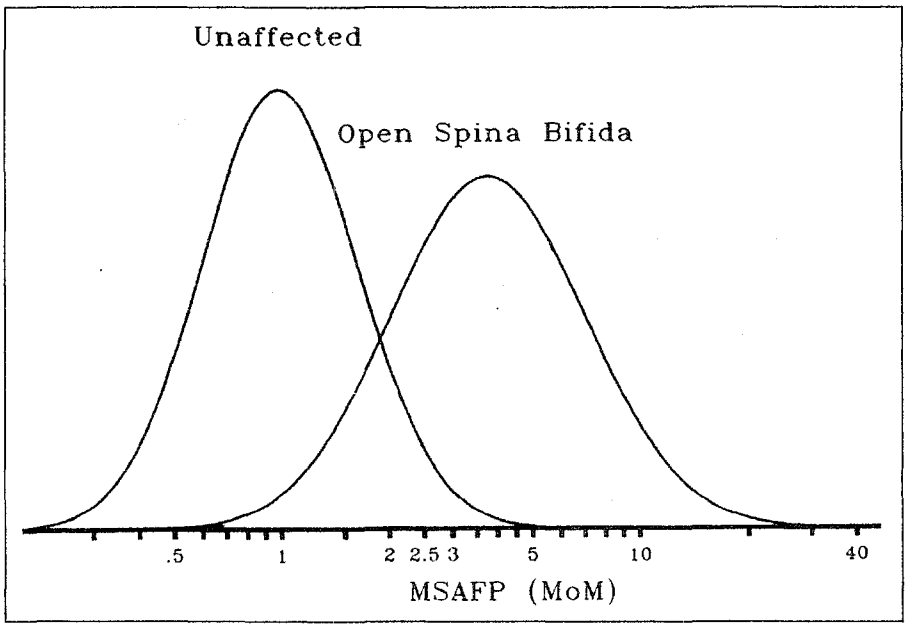

Figure 12 – Distribution curves of MSAFP in MoM at 16-18 weeks gestation among singleton unaffected and open Spina Bifida pregnancies (Wald 1992)

A normal MSAFP result does not ensure an infant without abnormality nor does abnormal values conclusively indicate an abnormality present (Figure 12). Rather, elevated values indicate an increased risk that warrants further testing. False positives results may also be due to various factors such as: fetal demise, blood contamination, or serious malformations other than a NTD (Wald 1992). Abnormal MSAFP levels are not exclusively observed in NTDs, they can also be seen in several other congenital abnormalities such as abdominal wall defects (omphalocele and gastroschisis), skin defects like epidermolysis bullosa and aplasia cutis, congenital nephrosis, renal agenesis, sacroccycgeal teratoma, hemangioma, and bladder and cloacal exstrophy

(Drugan *et al.* 2001; Harmon *et al.* 1995; Nadel *et al.* 1990). Abnormal MSAFP value can also be caused by other reasons such as: the presence of multiple gestations, incorrect assessment of fetal age, and pregnancies at high risk for poor outcomes.

MSAFP levels in twin pregnancies are approximately double those observed in singleton pregnancies, and they are even higher for three or more fetuses (Wald *et al.* 1975). Elevated levels can be explained by the greater resorption area for transamniotic AFP transfer (Fuhrmann and Weitzel 1985). This may cause interpretation to be difficult resulting in false positive or false negative results (Johnson *et al.* 1989). Underestimation of gestational age is the most common reason for an elevated maternal MSAFP (Rose and Mennuti 1993). Patients with inaccurate gestation times can result in either false positive or false negative results. Gestational age is usually verified during a subsequent ultrasound examination.

Increased AFP levels may also indicate a higher risk for spontaneous abortion, impeding intra-uterine death (Fuhrmann and Weitzel 1985). Akinbiyi (1996) demonstrated that women with unexplained elevated MSAFP are at increased risk of pre-term labor, ante-partum hemorrhage and intra-uterine growth restriction. Early identification of these high risk patients warrants close monitoring and supervision of the pregnancy. Furthermore, other factors that affect the calculation of MSAFP levels and should be considered during the interpretation of results include: maternal ethnicity, maternal weight, and insulin-dependant diabetes. Women of Afro-Caribbean origin have AFP levels about 10-15% higher than other women but have a lower prevalence of NTDs (Crandall *et al.* 1983; Cuckle 1994). This factor can cause a disproportionate number of women to have raised AFP levels. Women of Asian background have

MSAFP levels slightly elevated levels while Hispanic women have decreased MSAFP levels (Benn *et al.* 1997; Crandall *et al.* 1983).

Maternal weight and MSAFP concentrations have been found to be inversely related (Crandall *et al.* 1983). In larger women, increased plasma volumes can dilute the amount of AFP present and can lead to an underestimation of the AFP value. The reverse can be seen in smaller women (Ormond 1997; Wald 1992). This can lead to misidentifying a pregnancy as a high or low risk for NTDs (Adams *et al.* 1984). Women with insulin-dependant diabetes are at an increased risk of having children with a NTD or other structural abnormalities (Milunsky 1982). MSAFP levels are reduced in diabetics by about 25% (Cuckle 1994; Wald *et al.* 1979b). This could lead to erroneous 'normal' values causing a decrease in the sensitivity of the test.

When abnormal MSAFP levels are encountered it is the preliminary indication of the presence of an abnormality and the expecting parents are usually provided with genetic counselling in which the next steps are discussed (Figure 13). An ultrasound examination is usually performed to identify multiple gestations, intra-uterine death, and to verify gestational age. The observation of multiple gestations and fetal demise require further special prenatal care. Approximately 40% of elevated MSAFP cases are caused by incorrect gestational dating, with most being underestimated. Gestational age is determined by measuring from one outer edge of the fetal skull to the other in a transverse section. This is called the biparietal diameter (BPD) (Nicolaides *et al.* 1986). Wald *et al.* (1980) demonstrated that fetuses with SB had a mean BPD 16% lower than unaffected pregnancies at the same gestational ages.

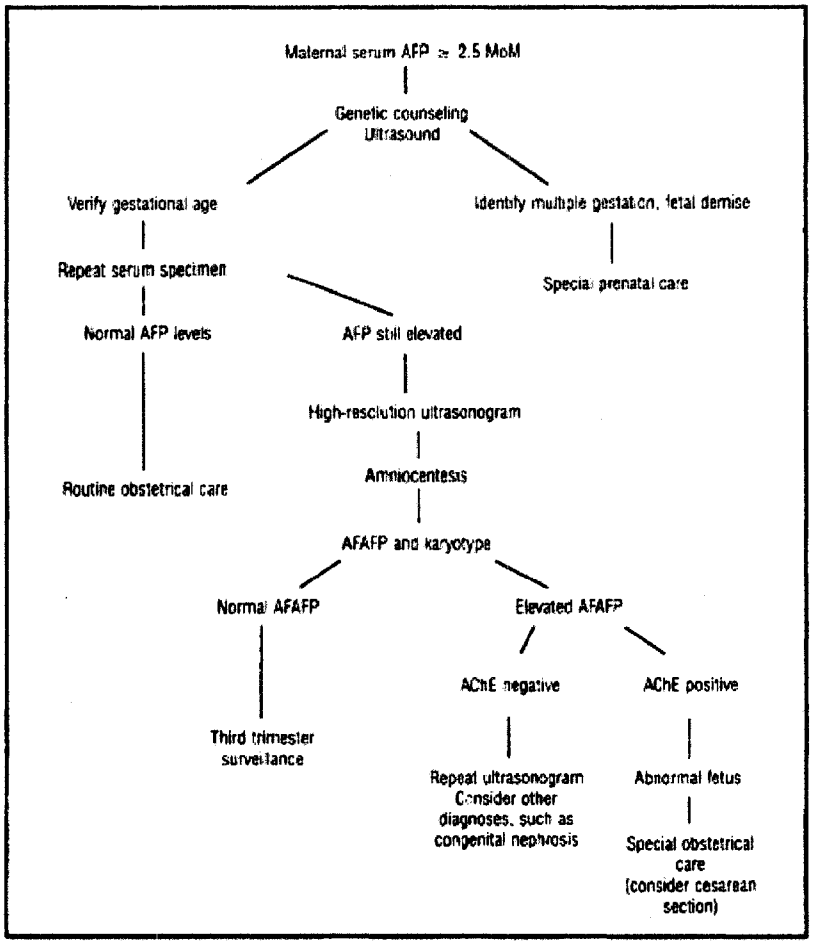

Figure 13 – Suggested Neural Tube Defect Determination Protocol. AFP- alpha-fetoprotein, AChE – acetylcholinesterase, AFAFP – amniotic fluid alpha-fetoprotein, MoM – multiple of the median (Rose and Mennuti 1993).

This factor would produce an apparently earlier gestational age thus making the MSAFP value appear even higher. The detection rate for open SB is then significantly increased to 90% or more (Wald *et al.* 1980). If there is a discrepancy of more than one or two weeks in gestational age, the MSAFP is recalculated (Canick 1992). For women with normal MSAFP levels based on ultrasound dating, no further testing is recommended. As gestational age increases, MSAFP values tend to regress toward the mean of their group (Knight *et al.* 1988). In NTD affected pregnancies, MSAFP levels would increase, however, unaffected pregnancies would result in decreased MSAFP levels (Ormond 1997). Those women who have their MSAFP test repeated,

30-40% of them would have a normal level on the second test, contributing to a lower risk for NTDs (Burton 1988). If levels are still elevated after a second test, the women are then offered a high resolution ultrasound examination to scan for anomalies.

Detailed ultrasound examination is an alternative approach for the evaluation of the patients with high MSAFP levels. When combined with MSAFP screening, NTD detection rates are greatly improved. Anencephaly was the first type of NTD to be observed by ultrasound (Stephenson 2003). Today, anencephaly is virtually impossible to miss during a sonographic examination and can be detected as early as 12-13 weeks (Aubry *et al.* 2003). At 22 weeks of gestation, it is possible to diagnose NTDs such as encephalocele, SB meningocele, SB myelomeningocele, and sometimes SBO (Aubry *et al.* 2003). Since SB can be missed by AFP screening and amniocentesis, sonography becomes a valuable tool in its identification (Wingate 2004). Sonography can visualise the cranial or spinal defect when the fetus is in a position with the amniotic fluid surrounding the protruding mass (i.e. Meningocele: Figure 14). Obvious disruption in the contour of fetal skin or bulging within the posterior contour of the fetal back are indicative of irregularities of the spine (Wingate 2004).

Scanning the spine is also necessary to verify proper fusion of the vertebral arches which appear U-shaped if open. Spina bifida occulta can be extremely difficult to identify in the fetus since the defect is skin-covered and without a mass protruding. SBC, conversely, is more recognisable due to these protrusions. Brain and cranial abnormalities signify the possibility of a neural tube malformation, making it possible to detect NTDs so tiny that the lesion itself is barely noticeable or when the spine is difficult to visualise adequately.

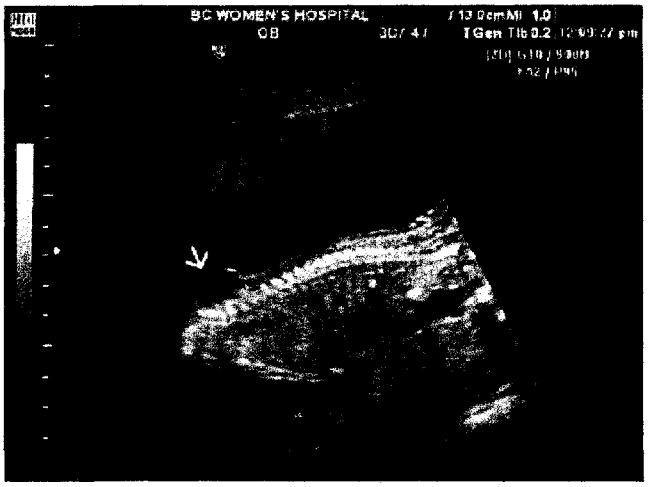

Figure 14 – Spina Bifida with meningocele image with a sac containing CSF and meninges protruding through a sacral defect (Stephenson 2003).

Nicolaides *et al.* (1986) first described cranial anatomy alterations that were consistent features present in virtually all fetuses with SB, termed the lemon and banana signs (Figure 15). The lemon sign is due to temporal bone malformation and is represented by the 'scalloping', or pinching, of the frontal bones of the fetal skull producing a lemon shaped skull (Wald 1992). The lemon sign is indicative of an closed NTD more frequently than the banana sign (Nyberg *et al.* 1988). The banana sign is caused by to the downward pulling of the cerebellum through the cisterna magna causing a distortion of the cerebellar hemispheres (Harmon *et al.* 1995). The cerebellum exhibits an abnormal anterior curvature causing it to appear banana shaped which would normally look like a dumbbell (Wingate 2004). This feature is associated with the Arnorld-Chiari type II malformation (Aubry *et al.* 2003). Recognition of either signs

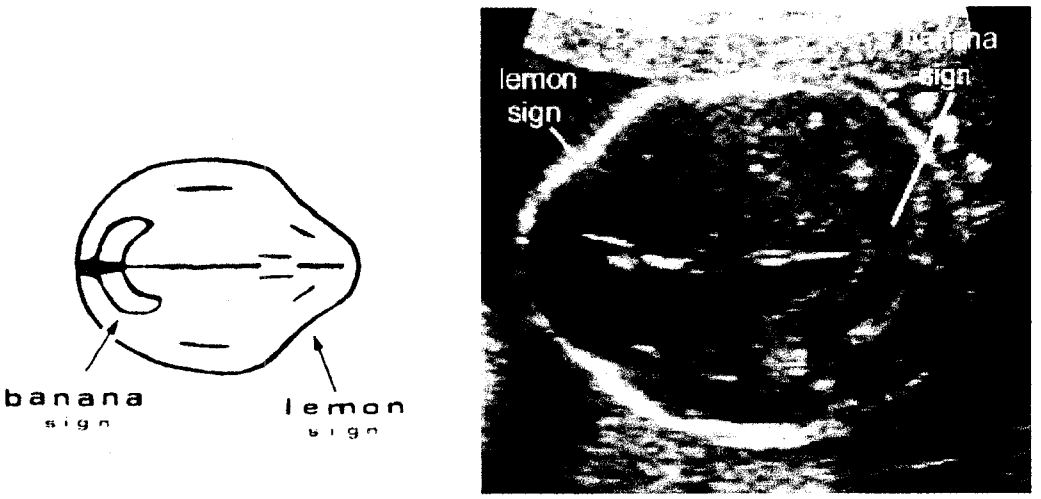

Figure 15 – Schematic representation (Left:(Nicolaides *et al.* 1986)) a sonographic illustration (Right: (Ghi *et al.* 2006)) of the banana and lemon signs in a fetus with SB.

is definitive evidence for the presence of a NTD in sonograms obtained before 24 weeks gestation (Nicolaides *et al.* 1986). Researchers have found that the lemon sign has a detection rate of 98% with a false positive rate of 0.8% for open SB; the rates for the banana sign are similar (Campbell *et al.* 1987; Nicolaides *et al.* 1986; Nyberg *et al.* 1988; Pilu *et al.* 1988; Van den Hof *et al.* 1990) . These markers will likely improve the diagnostic accuracy of ultrasonography in the evaluation of pregnancies at high risk for this fetal abnormality. Increasing resolution of sonography equipment and improved training of ultrasonographers will advance the sensitivity of ultrasound diagnosis. Van den Hof *et al.* (1990) reported accuracies as high as 100% for the sonographic diagnosis of open spina bifida in 130 fetuses. Targeted sonographic evaluation of the fetus for NTD, is remarkably accurate with a sensitivity of 97%, specificity of 100%, and the positive and negative predictive values are close to 100% (Lennon and Gray 1999). It must be noted, however, that sensitivity is not the same for each anomaly (Nadel *et al.* 1990).

Targeted ultrasound is sometimes performed in addition to or in place of amniocentesis (d'Ercole *et al.* 2003; Kooper *et al.* 2007; Nadel *et al.* 1990). There is a debate as to whether it is necessary to perform amniocentesis as the procedure carries significant risk of adverse pregnancy outcomes with an estimated 0.5% fetal mortality rate (Kolata 1980; Tabor *et al.* 1986). Nadel *et al.* (1990) suggest that a sonographic examination is a sufficient alternative to amniocentesis for the diagnosis of a NTD in patients with moderate elevations of the normal median (MoM) levels. AFP levels are expressed in multiples of the MoM established for each gestational week. Lennon and Gray (1999) believe that amniocentesis should be performed for the following situations: where the patient is at risk for a chromosomal disorder, an ultrasound finding suspicious for a chromosomal abnormality, greater than 3.0 MoM MSAFP results despite a normal scan, inability to properly visualise the fetal anatomy, or where the patient requests the procedure after adequate counselling. Samples of amniotic fluid can be assayed for amniotic fluid AFP (AFAFP), which will confirm the elevated MSAFP if a defect is present. Wald *et al.* (1989) developed an effective and economical procedure to perform both types of biochemical testing. If MSAFP levels are elevated, but AFAFP values are normal with a normal ultrasound, an open NTD can be excluded with a high degree of confidence. These women are at high risk for obstetrical complications and should be monitored during their third trimester (Rose and Mennuti 1993). A secondary important diagnostic test, to evaluate acetylcholinesterase (AChE) (Collins *et al.* 2003) levels, is performed concurrently with the AFAFP and enables the distinction between NTDs and other AFAFP related fetal defects (Kooper *et al.* 2007). Elevated acetylcholinesterase levels, which leak into the amniotic fluid also indicates an open

neural tube defect (Kolata 1980). Acetylcholinesterase is an enzyme that is contained in blood cells, muscle and neural tissue (Rose and Mennuti 1993). When amniotic fluid AFP and AChE determinations are combined and both show elevated levels, it is indicative of open NTD, thus eliminating false positive results of the AFP tests (Brock *et al.* 1985; Wald 1981). The combination of both measurements renders highly accurate diagnosis of open NTD via amniocentesis, with a 99% detection rate (Ormond 1997).

Nevertheless, cytogenetic evaluation could shed light on the genetic factors of NTDs and is recommended by some researchers (Babcook *et al.* 1995; d'Ercole *et al.* 2003; Harmon *et al.* 1995). Chromosomal abnormalities were diagnosed between 6.5-17% karyotypes of fetuses with NTDs (Babcook *et al.* 1995; Harmon *et al.* 1995; Kennedy *et al.* 1998; Sepulveda *et al.* 2004). The need to further investigate this area justifies the risk of amniocentesis as it would affect the prognosis, intervention, outcome, and recurrence risks for future pregnancies.

Early diagnosis decrease the morbidity and mortality rates by allowing preparation for the appropriate delivery strategies and post-delivery measures (Rose and Mennuti 1993). Cesarean section prior to labor has been reported to be more beneficial, for the infant who has greater neurological function, than those undergoing labor before cesarean section or vaginal delivery (Luthy *et al.* 1991). Expectant parents are also provided with the necessary information early enough to make an informed decision as whether to terminate a pregnancy or not. The combination of all or most of these detection methods gives a clear indication of abnormality with a high degree of confidence.

1.5 Mutations of Interest

1.5.1 Folic Acid and Homocysteine Metabolic pathway

Folate deficiency has been associated with the occurrence and reoccurrence of selected birth defects, and studies have demonstrated that the risk of NTD to be dramatically reduced (by up to at least 75%) by preconceptional dietary folic acid supplementation (Czeizel and Dudas 1992; MRC Vitamin Study Research Group 1991). Folic acid is known to contribute to three metabolic pathways: remethylation, the folate cycle, and transsulfuration (Cabrera *et al.* 2004). Although, the mechanism in which folic acid plays a protective role in preventing NTD is poorly understood, this suggests that folate-dependant enzymes may be functionally important in producing the disease phenotype.

It has been shown that concentrations of homocysteine are mildly elevated in some pregnant women who subsequently give birth to NTD affected babies (Kruger *et al.* 2000; Mills *et al.* 1995; Steegers-Theunissen *et al.* 1994; van der Put and Blom 2000). Genetic alterations in cystathionine ß-synthase (CBS) (Mudd 1989; Sebastio *et al.* 1995), in 5,10-methylenetetrahydrofolate reductase (MTHFR) (Goyette *et al.* 1995) and in methionine synthase (MS) (Leclerc *et al.* 1996) can cause elevated plasma homocysteine levels leading to homocystinuria. This suggests that defective genes in the folate-dependent homocysteine pathway (Figure 16) may be involved in the etiology of NTDs and are candidates for NTDs causes, from both a genetic and an environmental perspective.

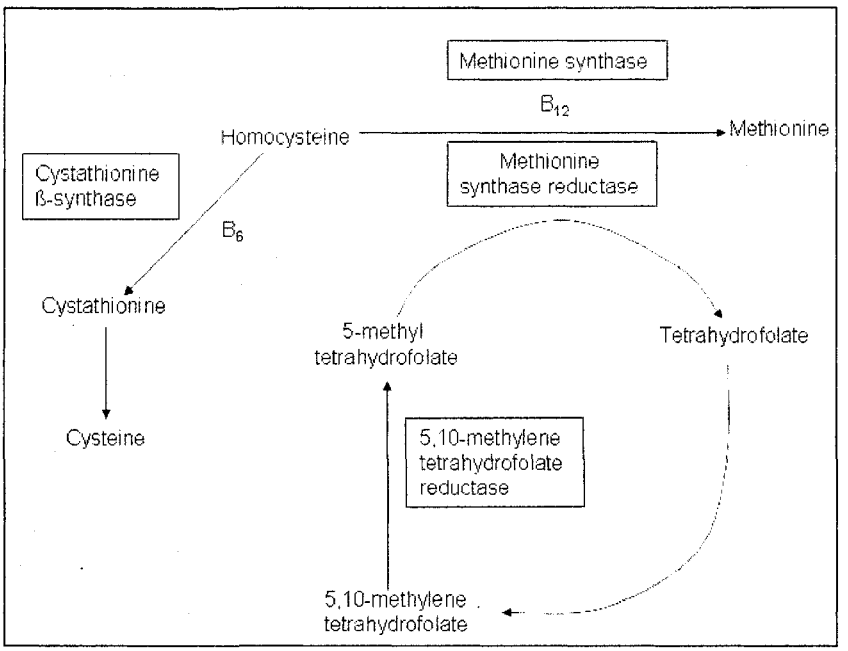

Figure 16 – Pathways of homocysteine metabolism

1.5.2 5,10-Methylenetetrahydrafolate reductase

The folate-dependant enzyme, 5, 10-methylenetetrahydrofolate reductase (MTHFR) (EC 1.5.1.20), is one of the principal means by which cells regulate intracellular concentrations of methionine and homocysteine. This enzyme catalyses the conversion of 5, 10-methylenetetrahydrofolate into 5-methylenetetrahydrofolate, the predominant circulatory form of folate, for use as a carbon donor for the remethylation of homocysteine to methionine (Frosst *et al.* 1995). The MTHFR gene was first isolated and mapped to chromosome 1p36.3 by Goyette *et al.* (1994) in which Frosst and colleagues (1995) first identified the cytosine to thymine (C→T) transition in nucleotide position 677. This polymorphism leads to the substitution of an alanine to valine residue (Frosst *et al.* 1995) and is considered an autosomal recessive mutation (Bailey and Gregory 1999b).

The presence of the mutation in the homozygous state correlates with increased MTHFR thermolability and a 50% reduction in enzymatic activity (Kang *et al.* 1991;

Rozen 1996; van der Put *et al.* 1996; Volcik *et al.* 2000). Decreased MTHFR activity is associated with elevated homocysteine and red blood cell folate concentrations and decreased plasma folate levels (Koch *et al.* 1998; Melnick and Marazita 1998; Molloy *et al.* 1997; Shaw *et al.* 1998a; van der Put and Blom 2000; van der Put *et al.* 1997b). Homozygous mutant (TT) individuals have been reported to have significantly elevated plasma total homocysteine concentration compared to those who are homozygous (CC) and heterozygous (CT) (Bailey and Gregory 1999b; Friedman *et al.* 1999; Frosst *et al.* 1995). High homocysteine levels have also been noted in children affected with SB and who are either heterozygous or homozygous for the polymorphism (Bjorke-Monsen *et al.* 1997). The allelic frequency of the mutation in North America is approximately 35%, predisposing individuals to mild hyperhomocysteinemia when folate status is low (Christensen *et al.* 1999; Frosst *et al.* 1995; Jacques *et al.* 1996). The mutation is the most frequent genetic cause for mild to moderate hyperhomocysteinemia especially during times of folate insufficiency (Clark *et al.* 1998; Engbersen *et al.* 1995; Frosst *et al.* 1995; Kluijtmans *et al.* 1996; van der Put *et al.* 1998; Weisberg *et al.* 2001).

The homozygote (TT) genotype has been associated as a risk factor for NTDs in several studies. A Dutch study found a higher incidence of the MTHFR C677T mutation in patients and parents of SB cases than in the control samples (van der Put *et al.* 1995). Studies by Whitehead *et al.* (1995), Shields *et al.* (1999), Ou *et al.* (1996), de Franchis *et al.* (2002; de Franchis *et al.* 1998a), Shaw *et al.* (1998b), Kirke *et al.* (1996) support their findings. Those individuals found to be homozygous for this mutation were found to have a three to sevenfold increased risk for a NTD, while the risk for heterozygous individuals for the mutation were only slightly elevated over those wild

type homozygotes (CC) (Kirke *et al.* 1993; Ou *et al.* 1996; van der Put *et al.* 1995; Whitehead *et al.* 1995). Botto and Yang (2000) concluded that homozygosity in infants is associated with approximately a 70% increased risk for SB.

Other studies that supported Botto and Yang's findings (2000) and have also demonstrated that there was a further increased risk for NTD associated with the MTHFR C677T polymorphism when the mother and child are homozygous for the variant allele (TT) (Christensen *et al.* 1999; Pietrzyk *et al.* 2003; Shields *et al.* 1999; van der Put *et al.* 1996; Volcik *et al.* 2000). Johanning *et al.* (2002) and Kirke *et al.* (2004) also concluded an increased risk for NTDs in individuals who were heterozygotes and mutant homozygotes. Posey *et al.* (1996) calculated that 13% NTD cases can be attributed to the MTHFR C677T mutation homozygote (TT); and a total of 24% if the allele contributes also in heterozygotes.

Other studies however, failed to find evidence of association with MTHFR C677T in different populations such as Turkish, German and Mexican populations (Boduroglu *et al.* 1999; Gonzalez-Herrera *et al.* 2002; Johnson *et al.* 1999; Koch *et al.* 1998; Stegmann *et al.* 1999). These discrepancies may be caused by the variation of the MTHFR C677T frequency amongst different ethnic groups (Barber *et al.* 2000; Botto and Yang 2000; de Franchis *et al.* 1998a; de Franchis *et al.* 1995; Franco *et al.* Jan 1998; Frosst *et al.* 1995; Monsalve *et al.* 2003; Mornet *et al.* 1997; Papapetrou *et al.* 1996; Pepe *et al.* 1998; Shaw *et al.* 1998b; Ubbink *et al.* 1999; van der Put *et al.* 1997a; Wilcken and Wang 1996). This observed high variability in the prevalence of the T allele suggests that the TT genotype may only be a risk factor for NTDs in some ethnic groups and not in others (Papapetrou *et al.* 1996).

The heterozygotes (CT) and mutant homozygotes (TT) for the MTHFR C677T polymorphism produces a 50-60% reduction in enzyme activity than the wild-type, however with an adequate level of folate in the diet, the mutant homozygotes (TT) can exhibit normal folate levels in the blood (Botto and Yang 2000). Therefore the mutation may only be a risk factor in populations with poor nutrition and low folate levels in the diet. This could explain the conflicting studies (Shields *et al.* 1999). Nevertheless, a study conducted by van der Put and colleagues (1997a) observed frequencies in different populations and found that 9.2% of controls are homozygous (TT), while 14.5% of all mothers with a NT-affected child are homozygous (TT), 15.5% of all fathers of a NT-affected child are homozygous (TT), and 16.4% of NT-affected individuals are homozygous (TT). Thus, the study demonstrated a statistically significant association of a 2-fold increased risk for NTD (van der Put *et al.* 1997a) and therefore should be regarded as a genetic risk factor for SB in some populations (Johnson *et al.* 1999).

A second polymorphism in the MTHFR gene has been characterised by van der Put and colleagues (1998) and Weisberg *et al.* (1998). The polymorphic variant is described as an A to C transition at nucleotide position 1298 (A1298C), resulting in a substitution of glutamate to alanine in the enzyme (van der Put *et al.* 1998). The mutation is also associated with decreased enzyme activity (van der Put *et al.* 1998; Weisberg *et al.* 1998; Weisberg *et al.* 2001) that is more pronounced in the homozygous genotype (CC) than in the heterozygous genotype (AC) or wild type (AA), and does not result in a thermolabile protein (Friedman *et al.* 1999). However, the effect of the MTHFR A1298C mutation on the enzyme activity and homocysteine concentration is less than that of the MTHFR C677T mutation (Dekou *et al.* 2001; Friedman *et al.* 1999;

van der Brandhof 2001; van der Put and Blom 2000; van der Put *et al.* 1998; Volcik *et al.* 2000; Weisberg *et al.* 1998). The allele frequency of the MTHFR A1298C polymorphism is similar to that of the MTHFR C677T polymorphism (Bailey and Gregory 1999a); however unlike the MTHFR C677T, the MTHFR A1298C mutation in either the heterozygous or homozygous state does not seem to be associated with elevated homocysteine levels (Isotalo *et al.* 2000; Medina and Amores-Sanchez 2000; van der Put *et al.* 1998; Weisberg *et al.* 2001).

Van der Put *et al.* (1998) observed an increase in the frequency of the MTHFR A1298C mutation in NTD-affected children. DeMarco *et al.* (2002) determined that the prevalence of heterozygotes (AC) and homozygotes (CC) is increased among NTD patients and mothers. Trembath *et al.* (1999) also demonstrated a significant association between the MTHFR A1298C allele and the risk of SB. Furthermore, individuals who are compound heterozygotes for both the MTHFR A1298C (AC) and MTHFR C677T (CT) alleles tend to have a biochemical profile similar to that seen among MTHFR C677T mutant homozygotes (TT); with increased homocysteine levels and decreased serum folate levels (Weisberg *et al.* 1998). The MTHFR 677T allele is also nearly always found in *trans* or *cis* configuration with the MTHFR 1298A allele and the MTHFR 1298C allele with the MTHFR 677C allele (Parle-McDermott *et al.* 2003b). The MTHFR C677T and MTHFR A1298C polymorphisms have never been observed in a compound homozygous state (MTHFR 677TT/1298CC) (Barber *et al.* 2000; van der Put *et al.* 1998). Compound heterozygosity was also observed to cause mildly elevated homocysteinemia (van der Put *et al.* 1998; Weisberg *et al.* 2001). Studies have reported an increased prevalence of the combined heterozygous genotype (MTHFR

677CT/1298AC) in NTD cases compared to controls (Akar *et al.* 2000; Barber *et al.* 2000; Dekou *et al.* 2001; Isotalo *et al.* 2000; Parle-McDermott *et al.* 2003b; Richter *et al.* 2001; Stegmann *et al.* 1999; Trembath *et al.* 1999; van der Put *et al.* 1998). Almost a three-fold increased risk for combined heterozygous-mutated genotype among NTD case compared with the MTHFR 677CC/1298AA genotype. Van der Put *et al.* (van der Put *et al.* 1998) calculated that combined heterozygosity for both mutations was correlated with 28% of NTDs. However, other studies fail to support their findings possibly due to comparatively small sample sizes (Parle-McDermott *et al.* 2003a; Yamada *et al.* 2001) .

1.5.3 Methionine synthase

Since both hyperhomocysteinemia and elevated vitamin B12 levels are independent indicators of NTD risk, focus has pointed towards a potential role for methionine synthase (Gaughan *et al.* 2001; Kirke *et al.* 1993; Scott 1994). The methionine synthase (MS) enzyme (EC 2.1.1.13), with the use of vitamin B_{12} as a cofactor and 5-MTHF as the methyl-group donor, catalyses the remethylation of homocysteine to tetrahydrofolate and methionine (Zhu *et al.* 2003). Thus, a deficiency in MS or vitamin B_{12} should theoretically lead to increased plasma folate and plasma homocysteine suggesting its possible involvement in the etiology of NTDs. As well, maternal preconceptional dietary intake of methionine was associated to reduce the risk of NTD, thus supporting the role of methionine in NTDs (Shaw *et al.* 2002).

The methionine synthase gene is located on chromosome 1q43sw and was first cloned, sequenced and the mutation identified by Chen, Leclerc, Li and colleagues (Chen *et al.* 1997; Leclerc *et al.* 1996; Li *et al.* 1996b). A commonly observed genetic

polymorphism was an A to G transition at base pair 2756 that converts an aspartic acid into a glycine residue. Individuals who are deficient in this enzyme have high levels of homocysteine, low levels of plasma methionine, and clinical implications similar to those brought about by a deficiency of MTHFR activity (Frosst *et al.* 1995; Harmon *et al.* 1999; Mills *et al.* 1995; Mills *et al.* 1996) which may indirectly contribute to NTD risk (van der Put *et al.* 1997c).

Van der Put *et al.* (2000) speculate that the combination of low vitamin B12, together with the MS A2756G mutation could result in a decreased methylation of homocysteine to methionine. The MS 2756AA genotype is found to be associated with a modest, but significant increase in homocysteine levels (Chen *et al.* 2001; Harmon *et al.* 1999). Christensen *et al.* (1999) indicate that the homozygous mutant genotype (GG) conferred an increased risk for NTDs and the risk was further increased if both mother and child had this genotype. The MS 2756G allele frequency was observed to be slightly higher in SB patients, with a difference at the limit of statistical significance (Gueant-Rodriguez *et al.* 2003). Zhu *et al.* (2003) demonstrated that there was a higher frequency of the mutant genotype (GG) among NTD cases.

Johanning *et al.* (2000) has reported that associations between the 677T MTHFR mutation and the A2756G MS polymorphism slightly increased NTD risk. Morrison *et al.* (1998) also made a possible association with MTHFR in which mothers who had both variants (MS A2756G/ MTHFR C677T) were at increased risk for having a child with SB. Some studies fail to associate a risk with NTD (Brody *et al.* 1999; De Marco *et al.* 2002; Morrison *et al.* 1997; Shaw *et al.* 1999; van der Put *et al.* 1997c) however; these studies were relatively small with only sufficient statistical power to detect large effects.

1.5.4 Methionine synthase reductase

Since methionine synthase reductase (MTRR) (EC 2.1.1.135) is responsible in maintaining methionine synthase in an active state, it has also been studied for its involvement in NTDs. A MTRR deficiency will adversely affect the function of methionine synthase (Brody *et al.* 1999). The enzyme has been cloned by Leclerc and colleagues (1998) and localized to chromosome 5p15.2-15.3. A common polymorphism of the gene (MTRR A66G) has been identified, which substitutes an A to a G in nucleotide position 66 causing the amino acid change from isoleucine to methionine.

Individuals homozygous for the MTRR A66G mutation (GG) have been found to have hyperhomocysteinemia (Lucock *et al.* 2001). This finding was supported by Gaughan *et al.* (2001) who observed that a MTRR A66G polymorphism significantly influences the circulating homocysteine concentrations. While the MTRR A66G mutation combined with a low level of vitamin B_{12} was found to increase the risk of NTDs (Gueant-Rodriguez *et al.* 2003; Wilson *et al.* 1999). Zhu *et al.* (2003) reported an association between the MTRR mutant genotype (AG or GG) and a threefold higher risk of NTD when compared to the AA genotype. Pietrzyk and colleagues (2003) demonstrated that the presence of the MTRR 66GG genotype increases the risk of SB. Doolin *et al.* (Doolin *et al.* 2002) found evidence of an increased risk of SB in the offspring of women with the MTRR AG and GG genotype, relative to the AA homozygotes. Wilson *et al.* (1999) observed that a combined genotype of MTHFR 677TT with MTRR 66GG in children and mothers have a fourfold and threefold increased risk for SB respectively (Wilson *et al.* 1999) which was supported by Pietrzyk *et al.* (2003). An Italian study found an increased risk of NTDs only when the MTHFR

677CC genotype was combined with the MTRR 66GG genotype (Gueant-Rodriguez *et al.* 2003) while Relton *et al.* (2003) also detected an interaction between MTHFR C677T and MTRR A66G in NTD cases, which supported the observations of Wilson *et al.* (Wilson *et al.* 1999). They also observed a maternal (MTRR A66G)-fetal (MTHFR C677T) interaction which elevated the NTD risk (Relton *et al.* 2003).

The presence of at least one high-risk allele (MS A2756G or MTRR A66G) is associated with an increased of SB in offspring and that this risk may be further increased when the mother is homozygous for the high risk allele (Doolin *et al.* 2002). Zhu *et al.* (2003) observed an exceptionally high frequency of double mutants of both MTRR and MS (AG and AG) among NTD cases. Some studies fail to make an association between MTRR variants in NTDs (Lucock *et al.* 2001), however their studies were limited and a larger population study is needed to confirm their results.

1.5.5 Cystathionine ß-synthase

Another key enzyme in homocysteine metabolism is cystathionine ß-synthase (CBS) (EC 4.2.1.22). CBS catalyses an irreversible condensation of homocysteine and serine to cystathionine using vitamin B_6 as a cofactor (Mudd 1989; Quere *et al.* 1999). Kraus and colleagues (1994) first sequenced the CBS gene located on chromosome 21q22.3. CBS controls homocysteine levels by degrading homocysteine into cystathionine (Borcsok and Abeles 1982; Morrison *et al.* 1998). Deficiency in enzyme activity leads to elevated levels of homocysteine as well as methionine in plasma and urine (Mudd 1989). Abnormalities of this gene may result in enzyme deficiency and hyperhomocysteinemia, which is now recognised as a risk factor for neural tube defects (De Franchis *et al.* 1998b; Mills *et al.* 1995; Sperandeo *et al.* 1996; Tsai *et al.* 1996).

The identification of a 68 base pair insertion in the coding region of exon 8 of the CBS gene (Figure 17) was first documented in a homocystinuria patient (Sebastio *et al.* 1995) which has led to the study for its involvement in the etiology of NTDs. Increased rate of fetal loss has been reported in women with homocystinuria due to CBS deficiency (Mudd *et al.* 1985) and CBS abnormalities have been reported in a minority of NTD cases (Steegers-Theunissen *et al.* 1991).

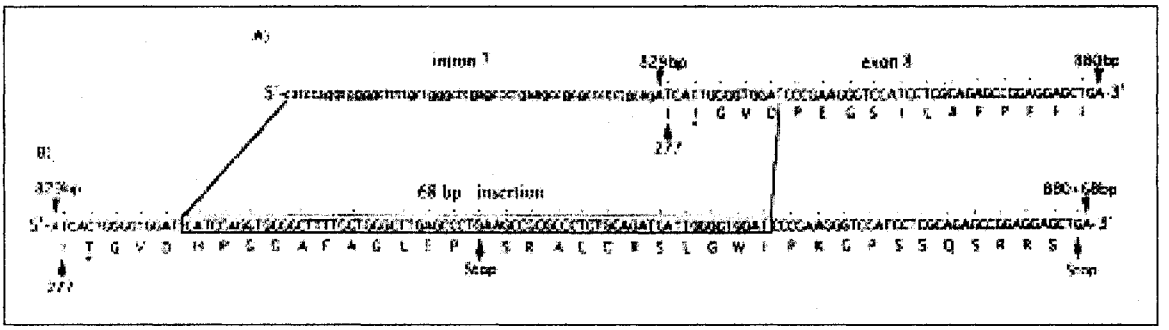

Figure 17 – Cystathionine ß-synthase Insertion

Only a handful of studies have been done in regards to this mutation and its association with NTDs. Ramsbottom *et al.* (1997) and Morrison *et al.* (1998) fail to provide evidence for the insertion being associated with NTD in Irish, Dutch and British populations. In an Italian study, NTD patients homozygous for the MTHFR 677TT mutation was significantly associated with the CBS 844ins68 allele than the MTHFR 677TT controls (de Franchis 1997). The majority of studies have focused on the CBS insertion alone and have not assessed the presence of the other mutations (gene-gene interaction) linked to NTDs. Sperandeo *et al.* (1995) speculate that this insertion, in combination with the MTHFR 677TT homozygote, might further influence homocysteine metabolism. Ramsbottom *et al.* (1997) reported a non-significant increase in the frequency of cases that were heterozygous for the CBS 844ins68 allele among those

who were MTHFR 677TT homozygotes. Botto and Mastroiacovo (1998) observed that homozygosity for the CBS mutation was not a NTD risk factor but they reported a five-fold increased risk for NTD in individuals homozygous for the MTHFR C677T mutation combined with the CBS mutation. Relton *et al.* (2004) found that the MTHFR C677T variant in combination with the CBS 844ins68 variant inflates the effect of the MTHFR C677T variant. This interaction is supported by Speer *et al.* (1999) and de Franchis *et al.* (2002).

The CBS insertion event occurred fairly recently but it is found in all human populations (De Stefano *et al.* 1998). It has been demonstrated that this mutation in its heterozygous state is highly prevalent (Tsai *et al.* 1996); however its frequency varies extensively between ethnic and geographical backgrounds (De Franchis *et al.* 1998b; Franco *et al.* 1998). The ethnic variation is evident in the heterozygosity of the CBS gene mutant, which was found to be 13.5% in Caucasians while it was absent among Asians (Franco *et al.* 1998). However the significant heterogeneity between populations provides the potential for this mutation to be used as an anthropogenetic marker that would be able to distinguish between some of the major human population groups (Pepe *et al.* 1999).

Gene-nutrient, gene-environment, and gene-gene interactions should be considered in the etiology of NTDs. Numerous studies have indicated that the possession of more than one polymorphism at folate-dependant loci can elevate the risk of NTD (Morrison *et al.* 1998; van der Put *et al.* 1998; Wilson *et al.* 1999). Gene-gene interactions of seemingly neutral mutations might be related to the etiology of NTD (van der Put *et al.* 2001).

1.6 Mutation Detection

The methodological approach I developed uses existing multiplex polymerase chain reaction technology and fluorescent single nucleotide extension (SNE) methods. The SNE system used here is the single nucleotide polymorphism detection analysis system, called SnaPshot™(Applied Biosystems). This approach includes the use of capillary electrophoresis coupled with Genescan® analysis software.

My project involves the optimisation of three multiplex PCRs including an initial multiplex PCR, a multiplex hemi-nested PCR and a multiplex single base extension or SnaPshot™ PCR. Each of the PCRs was performed consecutively and optimised to detect very low quantities of DNA and fragmented or damaged DNA. The fragment analysis was performed using capillary electrophoresis detection coupled with Genescan® analysis software. All three multiplex PCRs, used together, can serve as a novel tool to detect five of the mutations linked to neural tube defects rapidly with high specificity and sensitivity.

1.6.1 Nested and hemi-nested Polymerase Chain Reaction

Nested or hemi-nested PCR can be used following purification of the initial PCR product. The initial PCR product is used as template DNA for the successive round of PCR. The process can use one of the initial primers and a new primer (hemi-nested PCR) or two new primers (nested PCR), whose sequences are found inside the PCR product from the first reaction. This second PCR is intended to amplify a smaller secondary fragment primed within the first PCR product (Figure 18) (Mullis *et al.* 1986).

The nested PCR method, first demonstrated by Beck and Ho (1988), can be employed to improve sensitivity and specificity (Haqqi *et al.* 1988). Specificity can be increased as it would be unlikely that non-specific PCR products would contain the binding sites for the new primers (McPherson 2000). The second round of amplification increases sensitivity by producing a greater yield of the desired amplicon.

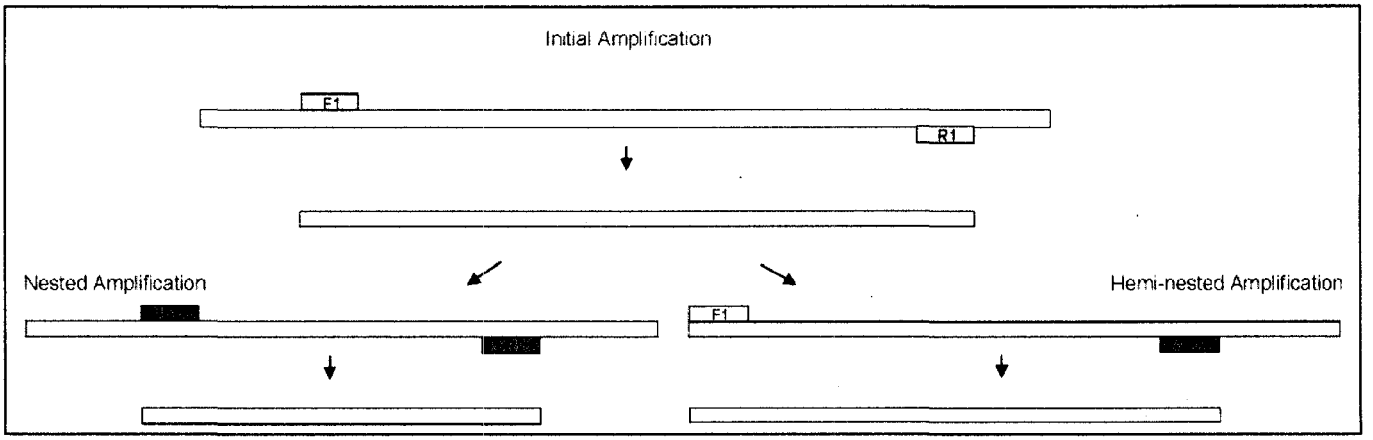

Figure 18 – Schematic of Nested and Hemi-nested PCR

An approximate 10^4 enhancement of the target region can be obtained (McPherson 2000). This step can overcome amplification failure due to degradation which is ideal when working with degraded or ancient DNA that do not contain large amounts of intact regions of DNA greater than 200 base pairs (Hummel 2003).

1.6.2 Sequencing Polymerase Chain Reaction

Sequencing analysis permits the identification of the succession of bases within an amplified DNA fragment detecting all types of polymorphisms (Hummel 2003). Many techniques have been developed, however the automated Sanger sequencing method, first described by Sanger (Sanger *et al.* 1977b), is the most commonly used today. A sequencing PCR requires a second amplification following the initial PCR that amplifies

the target region(s). A purification step is usually performed prior to and following the sequencing PCR to remove any unincorporated dNTPs, ddNTPs and primers. This PCR, sometimes referred to as an asymmetrical PCR, differs in that it requires only one primer resulting in a linear growth of single stranded DNA (Sanger *et al.* 1977b). The reaction mixture contains all the same reagents as the initial PCR plus fluorescently labelled dye-'terminating' 2', 3'-dideoxynucleotide triphosphates (ddNTPs). Thus, ddTTP, ddCTP, ddATP, ddGTP are labelled with a red, blue, green, and yellow fluorescing dye respectively. During polymerisation, the DNA polymerase adds dNTPs complementary to the template. When a ddNTP is incorporated rather than the

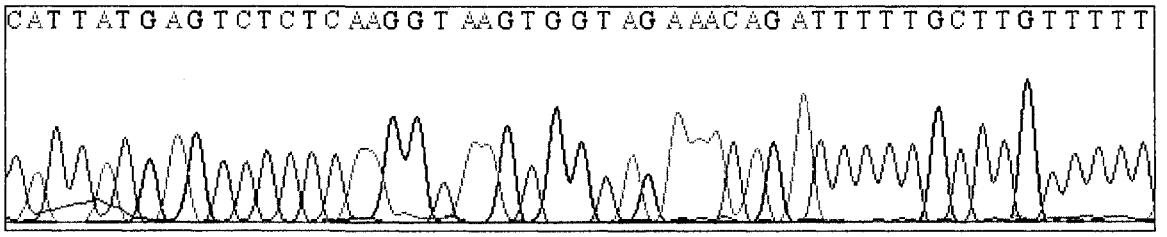

Figure 19 – An example of a sequencing electropherogram generated from the ABI 3100

corresponding dNTP, elongation terminates due to the absence of a hydroxyl group at the 3'-end of the ddNTP. This prevents the formation of the next phosphodiester bond necessary for DNA extension (McPherson 2000). This incorporation occurs randomly at all sites of the amplified PCR product resulting in single-stranded products of variable lengths representing all possible product length (primer +1 to primer +n). The single stranded amplification products are then electrophoresed via capillary electrophoresis and sorted by length, resulting in a succession of peaks of different colors representing the sequence of interest (Figure 19) (Li *et al.* 1999; Rosenblum *et al.* 1997).

1.6.3 Single Nucleotide Extension Polymerase Chain Reaction

A single nucleotide extension PCR (SNE-PCR), also known as mini-sequencing, involves the extension of an oligonucleotide by a single base with the aid of a DNA polymerase (Fiorentino *et al.* 2003; Syvanen *et al.* 1990; Tully *et al.* 1996). This project uses a commercially available kit from Applied Biosystems called SNaPshot™ (Applied Biosystems, Foster City, CA, USA) in which the methodology was first described in detail by Turner *et al.* (2002). This technique is employed to identify a known polymorphism without having to sequence the entire PCR product, while still maintaining the same qualitative characteristics of sequence analysis (Fiorentino *et al.* 2003). It requires an initial amplification of a DNA fragment containing the polymorphic site of interest, which serves as template for the SNE-PCR. Following the initial PCR, the amplification products are purified with shrimp alkaline phosphatase (SAP) and exonuclease 1 (EXO1) enzymes to remove any remaining single-stranded primers and unincorporated dNTPs that could interfere with the subsequent reaction (Butler 2005). The SNE-PCR requires the same cyclical steps as a typical PCR (Figure 20). The SNaPshot™ kit includes the fluorescent labelled ddNTPs, buffer and DNA polymerase compatible with any primer set. The SNE-PCR primers are specifically designed to be one base contiguous to the polymorphic site in either the forward or reverse orientation. Therefore, the primers anneal immediately 5' of the polymorphism and initiate the single-base extension reaction. The DNA polymerase extends the SNE primer by one base with a fluorescently dye-labelled terminating ddNTP, thus ending extension. Similar to sequencing PCR, each of the four nucleotides (ddNTPs) have their own dye color. The SNE-PCR undergoes 25 or more cycles to permit linear amplification of

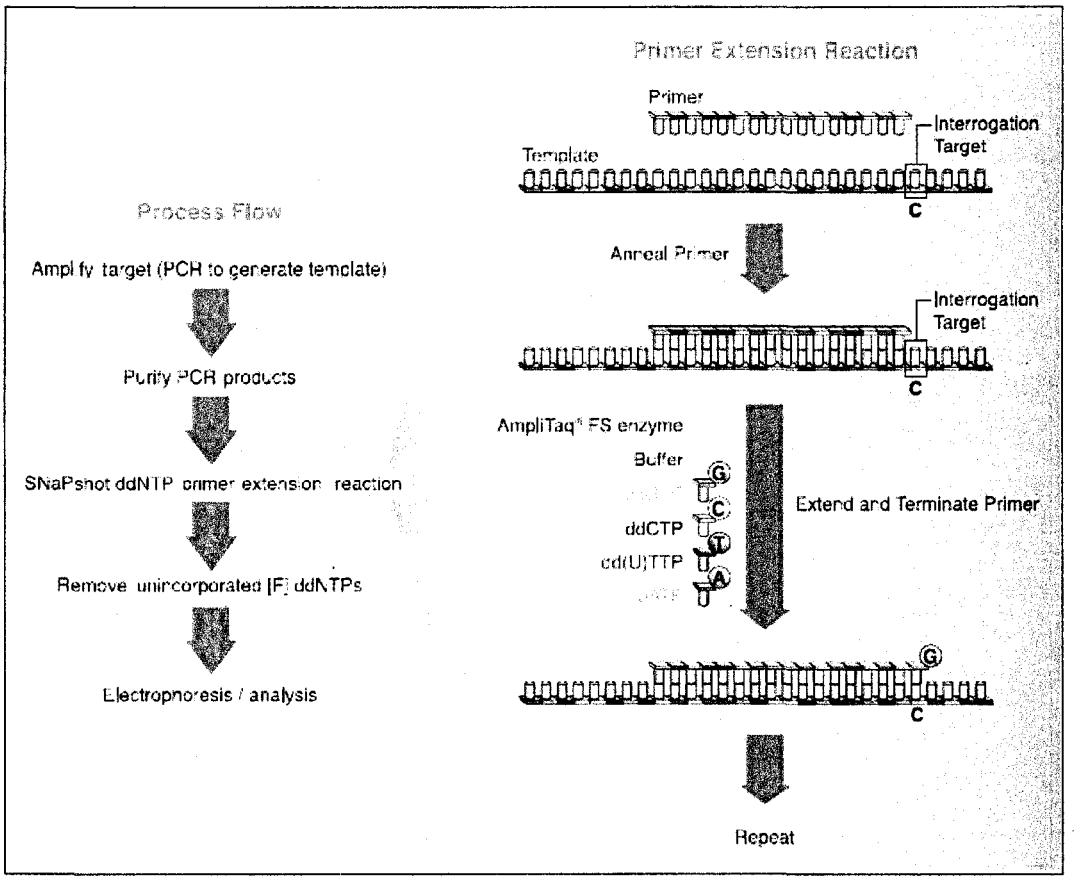

Figure 20 – SNaPshot™ PCR Kit process (Applied Biosystems 2001)

fluorescent ddNTP addition to the SNE primer by the DNA polymerase. After the SNE-PCR is complete, the PCR products are purified with SAP to remove unincorporated fluorescent ddNTPs that could cause dye blobs and artifacts during capillary electrophoresis rendering the electropherogram difficult to interpret. The final products are separated by size via capillary electrophoresis in which the fluorescence is detected. The products are then analysed and the genotype is determined by identifying which dideoxynucleotide was incorporated at the polymorphic site. The color of the peak indicates the nucleotide present at the site of interest while the position of the peak correlates to the SNE primer's size plus one base pair and is used to differentiate the various SNP primers. One of the advantages of the SNE-PCR is that

multiple polymorphisms can be analysed in parallel, known as a multiplex SNE-PCR, by separating the primers according to size and observing the incorporation of fluorescently-labelled ddNTPs (Tully *et al.* 1996).

1.6.4 Multiplex Polymerase Chain Reaction

A multiplex PCR uses two or more primer sets to amplify multiple target regions simultaneously within a single reaction (Edwards and Gibbs 1994). The primers amplify unique regions of DNA, both in individual pairs and in combinations of many primers, under a single set of reaction conditions (Markoulatos *et al.* 1999). This method was first described by Chamberlain *et al.* (1988) and has since proven a valuable tool in both clinical and research laboratories. The use of multiple primer sets saves sample material, time and effort, as well as cost. This approach also reduces the risk of contamination and increases throughput. In order for an accurate multiplex to be successful, a prerequisite is optimisation that involves strategic planning and multiple attempts (Markoulatos *et al.* 2002).

1.7 Development and Optimisation of Multiplex Methods

The development of an efficient PCR system requires strategic planning and extensive optimisation. This may take multiple attempts and various experiments to optimise the reaction conditions. Optimisation demands a delicate balance between the consistent and equal amplification of specific products and avoiding the production of non-specific products (Harris and Jones 1997). Optimisation can be easily obtained with one set of primers; however, with multiple primer sets a substantial amount of

optimisation is required. The addition of each new primer in a multiplex PCR reaction exponentially increases the complexity of possible primer interactions as well as the

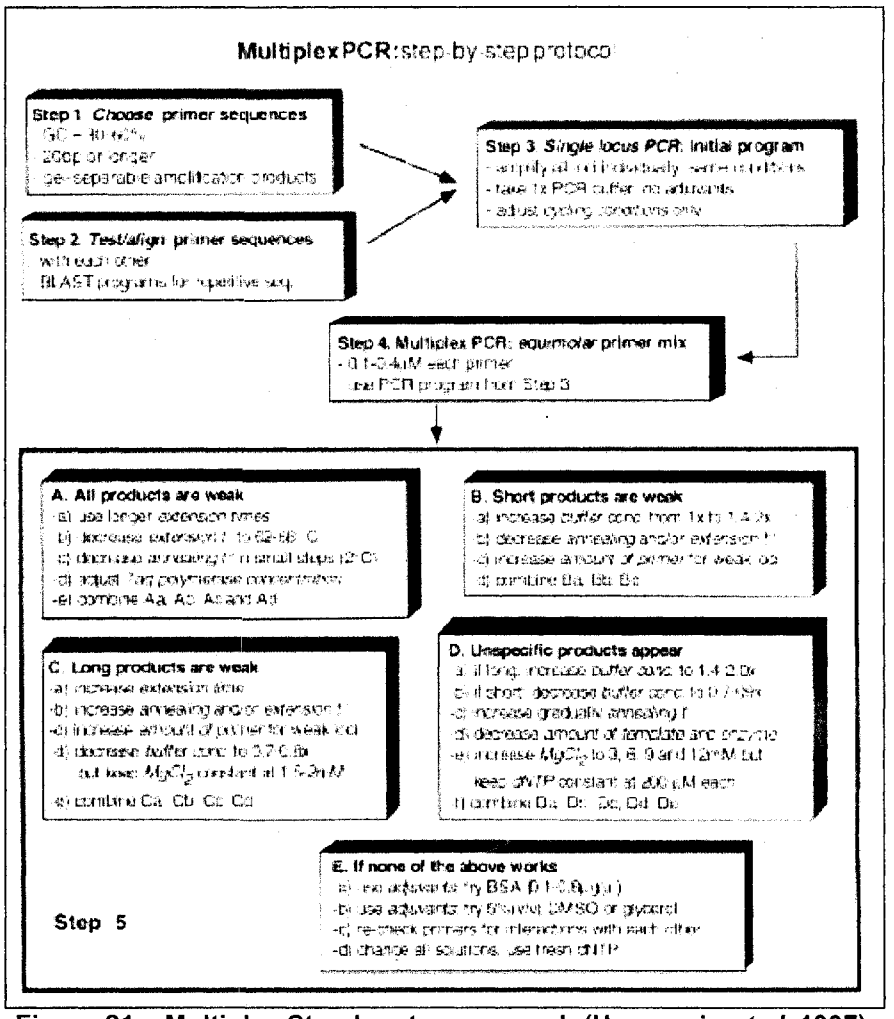

Figure 21 – Multiplex Step-by-step approach (Henegariu *et al.* 1997).

chance of generating non-specific artifacts (Brownie *et al.* 1997; Butler *et al.* 2001). These non-specific products may be amplified more efficiently than the desired target, consuming reaction components necessary for the desired targets. This not only decreases the PCR efficiency and sensitivity, but also makes analysis of the results more difficult. Thus, the optimisation of multiplex PCR should aim to minimise or reduce such non-specific interactions.

The development of an optimised multiplex PCR typically faces several challenges and difficulties including poor sensitivity and specificity, and/or preferential amplification of specific targets (Markoulatos *et al.* 2002). A successful multiplex PCR reaction requires the relative concentration of the primers, concentration of the PCR buffer, balance between the magnesium chloride and deoxynucleotide concentrations, cycling temperatures, and the amount of template DNA and DNA polymerase to be determined (Markoulatos *et al.* 2002). The two most critical factors in a highly specific and efficient multiplex PCR are primer design and optimal annealing temperature (Markoulatos *et al.* 2002).

1.7.1 Primer Design

A successful multiplex PCR enables similar amplification efficiencies for all the primer pairs and their respective targets. Each additional primer set in the reaction provides challenges to optimising the technique, challenges such as primer interference and the increase of non-specific priming. However, these difficulties can be overcome with the design of compatible and specific primer sets that achieve a balance between specificity and efficiency (Dieffenbach *et al.* 1993). Thus, primer sensitivity and specificity are the most important aspects of multiplex development. Many factors must be considered while designing primers (Dieffenbach *et al.* 1993; Nicodeme and Steyaert 1997; Robertson and Walsh-Weller 1998; Shuber *et al.* 1995; Wu *et al.* 1991). Here are a few suggested primer design guidelines:

a) Primers should be sequence specific and should fully match the intended hybridisation sites. Detailed sequence information for primer binding sites is important in order to avoid non-specific amplification at other sites with similar

sequences and primer-template mismatch sites (Dieffenbach *et al.* 1993). To examine the similarity of the primer to other known sequences that may result in multiple binding sites for the primer and to verify the uniqueness of the primer sequence, a BLAST search can be conducted in GeneBank via the internet at http://www.ncbi.nlm.nih.gov/BLAST (Altschul *et al.* 1997) (Butler 2005).

b) Given that all primer sets must function under a single set of experimental conditions it is essential that they are designed to have nearly identical melting temperature (Dieffenbach *et al.* 1993).

c) Primers should be between 18-30 bp in length (Harris and Jones 1997) and similar in size and nucleotide ratios. The shorter the primer, the more quickly it will anneal to the target DNA and form a stable double-stranded molecule to which the DNA polymerase can bind and begin synthesis (Dieffenbach *et al.* 1993). However, long primers allow a higher annealing temperature and produce less non-specific products.

d) The G/C content should range from 35-60% (Gibbs 1989; Henegariu *et al.* 1997). Lower G/C contents lead to poor amplification, while higher contents lead to non-specific amplification products.

e) Primers within the multiplex should not display significant homology either internally (hairpin structure) or to one another (primer-primer interactions). Complementarity at the 3'ends of any of the primers should be avoided as this promotes the formation of undesirable primer-dimer artifacts (Dieffenbach *et al.* 1993; Henegariu *et al.* 1997). These primer artifacts, which deplete the reaction of dNTPs and primers and compete for the DNA polymerase, the multiplex's

desired amplicons for polymerase, result in a lower yield of the desired product (Bourque *et al.* 1993; Vandenvelde *et al.* 1990).

f) If possible, primers should contain a 3'-terminal "G/C clamp" (one or two Gs and/or Cs at the 3' end) (Sharrocks 1994). A 3' G/C clamp is to ensure the 3'end has a stronger bonding than the rest of the primer due to the 3 hydrogen bonds that form between G's and C's. Without a 3' terminal G/C clamp there is a risk that the DNA polymerase will not bind and extend the product efficiently.

g) However, runs of three or more Cs or Gs within the primer should be avoided especially at the 3'end. Homopolymeric stretches of G or C could promote rich sequences, primer dimer or the formation of secondary structures within the primer. Along with homopolymeric stretches of the four bases, repeated motifs or palindromic sequences should also be avoided (Sharrocks 1994).

1.7.2 Cycling Parameters

Each step of the PCR influences the efficiency and specificity of the amplification and therefore has a direct relation on the yield of the desired amplicon.

Denaturation

Denaturation is influenced by the sequence of the DNA template. Templates that are G/C rich require a higher denaturation temperature due to the increase in hydrogen bonds between the nucleotides. Incomplete denaturation of the target template and/or the PCR product is the most likely cause for PCR failure. If the temperature is too low, the DNA template fails to denature completely and returns to its more stable double-stranded configuration (Harris and Jones 1997). This reassociation prevents efficient primer annealing and DNA extension. However, if the temperature is too high or

denaturation length is too long, DNA polymerase activity will be lost due to temperature inactivation. Both extremes reduce the yield of the desired amplicon. An initial hot start step can be used prior to the regular PCR cycling to ensure that all large molecules are denatured and to eliminate non-specific reactions (production of primer-dimers) (Chou *et al.* 1992).

Anneal

The annealing step is considered to be one of the most important parameters during PCR optimisation (Markoulatos *et al.* 2002). The success of a PCR relies heavily on the specificity with which a primer anneals to its target sequence therefore it is important to optimise this molecular interaction. Stringent annealing temperatures, especially during the first several cycles help to increase specificity, thus increasing the overall yield of amplicon. The annealing temperature (T_a) and length of time required is dependent on the melting temperature (T_m), base composition, length and concentration of the primers (Harris and Jones 1997). Determining an optimal T_a begins with calculating the melting temperatures (T_m), the simplest method being T_m= [(number of G+C) x 4˚C + (number of A+T) x 2˚C] (McPherson 2000). This calculation is an approximation because T_a is affected by buffer components and primer and template concentrations (Roux 1995). When employing multiple primer sets, a similar T_a is important to avoid preferential amplification. If one primer anneals preferentially at the temperature chosen, the desired exponential amplification would not occur. The T_a is usually about 5˚C below the true T_m of the primers (Henegariu *et al.* 1997) followed by performing temperature gradients to determine experimentally the optimal T_a. The optimal T_a is a compromise between hybridisation specificity of the

actual primers and full reaction efficiency (Hummel 2003). A low T_a tolerates more mismatches between the template and primers, resulting in a increased amplification of different products (artifacts) due to mis-priming to non-complementary sequences and non-specific amplification (McPherson 2000). These products are observed as PCR products or bands seen above or below the targeted amplicon on gel electrophoresis (Harris and Jones 1997). A higher T_a adds more specificity to the process by reducing the amount of incorrectly annealed primers. However, it also reduces the overall efficiency because primers with a lower T_a fail to anneal and thus, do not produce amplification product (Dieffenbach *et al.* 1993; Harris and Jones 1997). A rule of thumb in choosing the optimal temperature is to select the highest temperature possible without reducing the sensitivity (Markoulatos *et al.* 1999).

Extension

This step is traditionally performed at 72°C because it is the optimal temperature for *Taq* DNA polymerase activity. However the specific temperature can change depending on the DNA polymerase as DNA polymerases from other species have different optimal extension temperatures. The extension time depends upon the length and concentration of the target sequence. In multiplex PCR, as more loci are simultaneously amplified, the pool of DNA polymerase and nucleotides becomes the limiting factor and more time is necessary for the DNA polymerase to complete synthesis of all the products (Henegariu *et al.* 1997). Therefore, longer extension times can be added at the end of cycling to ensure complete extension of all amplified products (Harris and Jones 1997). Longer extension times are also helpful in early cycles if the substrate concentration is very low and in later cycles when product

concentration exceeds enzyme concentration. Longer extension and annealing times can result in non-specific amplification (Chamberlain 1991).

Number of Cycles

The number of cycles influences both specificity and efficiency and the optimal number of cycles varies depending on the amount of the initial template, the primers used and the success of each amplification step. Increasing the number of PCR cycles increases the efficiency of amplification but reduces the overall specificity (Harris and Jones 1997). It can lead to the generation of both specific and non-specific products such as smears composed of high molecular weight products rich in single-stranded DNA (Bell and DeMarini 1991). Too many cycles can increase the amount and complexity of non-specific background products while too few cycles give low product yield. Between 25 and 35 cycles are usually used for modern samples while 40-60 cycles can be used for ancient samples. After 60 cycles, the DNA polymerase activity decreases as the enzyme looses activity. Greater sensitivity can be achieved by using nested PCR rather than performing more than 40 cycles (Brisco *et al.* 1990).

1.7.3 PCR components

After cycling parameters are optimised, the concentration of each PCR reagent such as DNA template, primers, DNA polymerase, buffer, dNTPs and $MgCl_2$ is investigated experimentally.

Template Amount

Ideally, the initial template should be void of any PCR inhibitor, which could negatively affect amplification efficiency, and should not contain high concentrations of chelating agents such as EDTA, or negatively charged ionic groups such as

phosphates, which can reduce the available Mg^{2+} ions (Harris and Jones 1997). As well, the template should be intact over the length of the region to be amplified, a challenge often encountered when working with degraded DNA. During optimisation, the primer-to-template ratio may need to be adjusted. Primers must usually be in a 10^7 molar excess with respect to template (Markoulatos *et al.* 2002). If the ratio is too high, primer-dimers form, this occurs with low template concentrations or excess primer concentrations. High concentrations of DNA can also lead to non-specific amplification products (Harris and Jones 1997) as well as the generation of smears (Roux 1995). Too much DNA also disrupts the relative intensity of the bands and increases the hazard of false-positive amplification, while too little DNA produces weak or undetectable bands (Chamberlain 1990). It must be noted that degraded templates will give weaker signals for longer amplicons (Chamberlain 1992).

Primers

Primer pairs that function well in a singleplex reaction can be combined in a multiplex reaction and the relative performance of each primer set monitored. Primer concentration is a critical parameter for a successful PCR and must be refined experimentally in a multiplex when adding new primer sets (Markoulatos *et al.* 2002). Preferential amplification of one target sequence over another is a typical occurrence when optimising a multiplex PCR (Mutter and Boynton 1995). The most common approach to balancing the amount of each product is to simply begin with equimolar primer concentrations. If the targeted amplicons do not produce uniformly intense bands for all fragments, the concentration of the relevant primer sets in relation to the others should be adjusted by increasing the amount of primers for the "weak" loci and

decreasing the amount for the "strong" loci (Henegariu *et al.* 1997; Markoulatos *et al.* 2002). The final concentrations of primers may vary considerably among loci (Markoulatos *et al.* 1999). Higher primer concentrations may promote mis-priming and accumulation of non-specific product and may increase the probability of generating primer-dimers (Markoulatos *et al.* 2002). As well, increasing the concentration of the primers does not necessarily improve the yield of specific amplicons, but amplification efficiency is compromised if concentration is too low (Harris and Jones 1997).

Empirical testing have to be used when testing several primer pairs, because there are no means to predict the performance characteristics of a selected primer pair even among those that satisfy the general parameters of primer design (Henegariu *et al.* 1997).

Magnesium ions and dNTPs

Magnesium ions and dNTPs influence both efficiency and specificity of the amplification process. Their requirements generally increase with the number of target amplicons in the multiplex, therefore, the concentrations must be optimised since each primer pair may have different requirements (Chamberlain 1991). Optimal Mg^{2+} will depend on the dNTP concentration, specific template DNA, and sample buffer composition; if they contain chelators such as EDTA, the optimal Mg^{2+} chloride concentration will change (Markoulatos *et al.* 2002). The concentrations of $MgCl_2$ and dNTP concentrations are dependent on each other as well as on the concentrations of primers. Magnesium chloride concentration needs only to be proportional to the amount of dNTPs (Henegariu *et al.* 1997; Markoulatos *et al.* 2002) The $MgCl_2$ concentration must be a few millimolars higher than the nucleotide concentration (Harris and Jones

1997) since dNTPs and primers bind to free Mg^{2+} ions (Markoulatos *et al.* 2002). In addition, *Taq* DNA polymerase is a magnesium-dependant enzyme that requires available Mg^{2+} ions for proper activity. Therefore, any change in dNTP concentration affects the concentration of available Mg^{2+} ions (Roux 1995). Lower dNTP concentrations can lead to a considerable increase in the formation of primer artifacts as a consequence of excess free Mg^{2+} and to inconsistent amplification results which will frequently produce only a portion of the targeted amplicons (Markoulatos *et al.* 1999). The best results were found to be between 200 and 400 µM per each dNTP (Markoulatos *et al.* 2002). Increasing $MgCl_2$ concentration improves the efficiency but reduces the specificity of amplification (Harris and Jones 1997). Excessive Mg^{2+} concentration stabilises the DNA double strand and prevents complete denaturation of DNA, which reduces yield. It can also reduce the fidelity of *Taq* DNA polymerase and lead to non-specific amplification products (McPherson 2000). On the other hand, inadequate Mg^{2+} reduces the amount of product (Markoulatos *et al.* 2002).

Buffer

Taq DNA polymerase suppliers provide an appropriate buffer which has already been optimised. Increasing the concentration can sometimes improve efficiency of the multiplex reaction. Primer pairs with longer amplification products work better at lower salt concentrations; whereas primer pairs with short amplification products work better at higher salt concentrations (Henegariu *et al.* 1997).

***Taq* DNA Polymerase**

Enzyme requirements may vary with respect to individual target templates or primers and in particular the quality of the extracted DNA (Harris and Jones 1997).

High concentrations of DNA polymerase do not necessarily result in higher yields of the desired amplicons (Harris and Jones 1997). It can result in unbalanced amplification and a slight increase in non-specific background products (Henegariu *et al.* 1997). Insufficient amounts of the desired product will be produced if the enzyme is in low concentrations. No product bands or weak bands may indicate too little enzyme (McPherson 2000).

Additives

Poor or non-existent amplification may indicate the presence of inhibitors in the DNA sample (Roux 1995). PCR additives can be introduced in the reaction mixture to enhance primer annealing specificity, reduce mismatch primer annealing and improve product yield and length (McPherson 2000). Additives can be chemicals or commercially available reagents that influence the secondary structure of DNA, the hybridisation of the primers, the removal of inhibitors and the activity of DNA polymerase (McPherson 2000). The formation of secondary structures within regions of template DNA during the extension process can be prevented by the addition of additives which reduce DNA polymerisation (Hengen 1997). Further action of these additives, as it interacts with DNA, is to modify hybridisation which could benefit by reducing the melting temperature of GC-rich sequences and increasing the resistance of the DNA polymerase to denaturation (Hengen 1997; Rees *et al.* 1993). Available PCR additives that have been reported to improve the performance of multiplex PCR include: bovine serum albumin (BSA), glycerol, dimethyl sulfoxide (DMSO), formamide, and betaine (Bachmann *et al.* 1990; Hung *et al.* 1990; Markoulatos *et al.* 2002; Pomp and Medrano 1991; Sarkar *et al.* 1990; Seto 1990). These additives can benefit

multiplex PCRs but should be tested under different conditions to assess their usefulness in each case (Henegariu *et al.* 1997).

1.7.4 Troubleshooting

The prerequisite to a successful multiplex PCR is optimisation. There are many articles, books and protocols available as aids in optimizing a multiplex reaction (Figure 21) (Henegariu *et al.* 1997; Markoulatos *et al.* 2002; McPherson 2000; Römpler *et al.* 2006).

1.8 Project Aim

There are numerous genetic mutations and polymorphisms that have been linked to NTDs. The aim of my research is to design a novel detection system to analyse five single nucleotide polymorphisms that have been shown to be linked to NTDs. The objective is to design, develop and optimise a method for detecting molecular variations that may cause NTDs by using the latest technology of mutation detection analysis. The developed genetic technique must be rapid, specific and sensitive. The single nucleotide extension (SNE) method is extremely rapid as it only requires the extension of one single base to the amplification product. It is highly specific depending on the primer system being used. Here additional steps of hemi-nested PCR have been incorporated to increase test specificity. The addition of the hemi-nested PCR step will also increase the sensitivity allowing application to degraded DNA samples. As part of the development and optimisation of this technique, four different types of samples were analysed, modern DNA, experimentally degraded DNA, archived medical specimens and archeological samples.

The simultaneous detection of five markers linked to NTDs has not been previously developed and this study would be the first of its kind. The advantage for detecting all five markers simultaneously is that the linkage between each of the mutations and the etiology of the associated NTD can be ascertained much faster than if independent detection systems were performed. The comparison of various types of sample sources should aid in establishing a relationship between the mutations and the condition thus, explaining why certain individuals are more prone to inherit this complex multifactorial genetic condition than others. Also, with samples of different chronological state, the methodology must be sensitive in order to analyse allelic sequence variations on degraded or damaged DNA. This type of analysis could also aid in understanding the evolutionary associations of the disease and identify the historical and contemporary distributions and abundances of the disease in human populations. This genetic research could one day lead to gene therapy and new practices that can alleviate the suffering associated with this condition. It could also be the basis for a novel genetic test with the purpose of facilitating clinical diagnosis and prenatal testing.

1.8.1 DNA Damage and Degraded DNA

In living organisms, DNA is continuously being damaged. This damage is repaired by enzymatic repair processes (Lindahl 1993). However, after death, these repair and maintenance processes cease and many destructive processes continue resulting in rapid DNA degradation. DNA degradation rates are dependant on the manner in which the organism dies and the post mortem taphonomy (Hofreiter *et al.* 2001a; Hoss *et al.* 1996; Lindahl 1993; Paabo 1989). In the case of medical specimens,

biopsies and test samples, DNA degradation depends on the method of preservation employed and the storage conditions (Greer *et al.* 1991); Kim 2003; Taubenberger *et al.* 1997).

Post-mortem and extracorporeal DNA is subject to degradation by cellular nucleases, lytic enzymes, lytic chemicals and hydrolytic damage based on cellular water content, free radical and oxidative damage (Hofreiter *et al.* 2001a; Lindahl 1993; Paabo *et al.* 2004). Many features can affect the rate of DNA degradation like exposure, water content and temperature (Lindahl 1993). While rapid dessication can occur at low temperature or high salt concentration which can slow the destructive processes and DNA degradation (Hofreiter *et al.* 2001b). DNA damage includes either base modification (oxidation and deamination), base loss, strand breaks or crosslinks (Hoss *et al.* 1996; Lindahl 1993; Paabo 1989). The resulting DNA is degraded and/or fragmented which can interfere with PCR amplification. DNA damage studies have shown that the molecules are reduced to a size of some hundred base pairs with most ancient DNA extracts not revealing significant amounts of intact targets exceeding 200 bp (Hofreiter *et al.* 2001a; Hummel 2003; Paabo 1989). Previously, when ancient DNA was identified to be fragmented to less than 80-100 bp it was deemed unsuitable for analysis. However, with SNE-PCR what previously was deemed unsuitable is now viable for informative analysis. However after long enough time, no useful molecules will remain due to the cumulative effects of DNA damage (Hofreiter *et al.* 2001b).

The invention of PCR (Mullis *et al.* 1986) has made it possible to study and recover information from degraded and fragmented samples as well as low copy number DNA samples. Low copy number (LCN) DNA is often defined as samples

containing less than 100 pg of genomic DNA that is below the stochastic threshold level where PCR amplification is not as reliable for normal interpretation (Butler 2006). Characteristics of degraded and LCN DNA make it almost impossible to obtain long amplification products (Handt *et al.* 1994). Therefore, the use of smaller amplicons enhances DNA recovery and the small amplicons are more likely to be amplified in samples containing degraded DNA (Butler 2005; Chung *et al.* 2004; Wiegand and Kleiber 2001).

One of the challenges of working with degraded DNA is the increased chance for contamination (Gill 2001). A sterile laboratory environment is necessary to reduce contamination from reagents, exogenous DNA from casual contact or secondary transfer of samples, and from laboratory aerosols or amplification products. Contamination can be either systematic, from contaminated water or PCR buffer, or sporadic, from contaminated individual PCR tubes or individual samples. There are many published guidelines to working with degraded and LCN DNA that should be followed to reduce or prevent contamination (Butler 2005; Hofreiter *et al.* 2001b; Paabo *et al.* 2004; Poinar 2003).

Data interpretation is also more complicated due to the stochastic variation during PCR amplification. Difficulties include allelic drop-out, allelic drop-in (from contamination), and increased stutter products (Gill 2001; Gill *et al.* 2000). Allelic drop-out, also known as heterozygote imbalance, may occur if one allele of a heterozygote locus is preferentially amplified due to stochastic effects resulting in the complete loss of the other allele and is therefore interpreted as a homozygote genotype (Gill 2001). Allele drop-in, can sometimes be called stutter false alleles and can be produced by

contamination or non-specific amplification products due to an unoptimised amplification method (Butler 2005). Stutter products can be preferentially analysed and can be greater than the true allele (Gill 2001; Gill *et al.* 2000). Typically an allele drop-in is not reproducible and thus by repeating the process multiple times without obtaining identical results, the problem can be identified as allele drop-in. This phenomenon is usually due to sporadic contamination (Budowle 2001).

2 Materials and Methods

2.1 Samples

DNA extracts from four types of samples were used: modern samples, experimentally degraded samples, medically archived samples and archeological samples. The modern sample was used primarily to develop and optimise the methods. The modern extracts are all from hair samples with a visible root sheath, from one individual with a known genotype for all of the markers being analysed. Various degraded samples were used to assess and ensure the sensitivity and specificity of the optimised protocol. The experimentally degraded samples were used in the optimisation stage predominantly, while the medical and archeological samples were used to assess the sensitivity and specificity of the developed method (Table 1). The experimentally degraded samples were blood samples from various donors, not known to suffer from NTDs that were treated to mimic degraded samples. Hydrolytic damage was generated by exposure to water at acidic pH (5.0). Oxidative damage was generated by exposure to bleach and ultra-violet radiation. The experimentally degraded samples were stored at -20°C. The preparation of these samples was not performed as part of this thesis and the resulting degraded DNA was provided. Ten samples in total were used. The medical archive samples were blood samples collected on FTA gene cards stored at -20°C. There were five medically archived specimens in this study. My project included samples demonstrating both physiological aspects of NTD and samples without any manifestations in order to establish a link. The samples exhibiting physical signs of being affected with NTD include ancient skeletal and tooth samples.

Table 1 – Sample Information

Sample #	Sample Type	Tissue	Source
1	Modern Sample	Hair	LC
2	Experimentally Degraded Sample	Blood	TJ
3	Experimentally Degraded Sample	Blood	RS
4	Experimentally Degraded Sample	Blood	AL
5	Experimentally Degraded Sample	Blood	RCP
6	Experimentally Degraded Sample	Blood	AT
7	Experimentally Degraded Sample	Blood	AH
8	Experimentally Degraded Sample	Blood	LI (1/10)
9	Experimentally Degraded Sample	Blood	CB (1/10)
10	Experimentally Degraded Sample	Blood	R (1/10)
11	Experimentally Degraded Sample	Blood	CH
12	Medical Archive Sample	Blood on FTA Card	AD
13	Medical Archive Sample	Blood on FTA Card	DD1
14	Medical Archive Sample	Blood on FTA Card	DD2
15	Medical Archive Sample	Blood on FTA Card	JZ
16	Medical Archive Sample	Blood on FTA Card	YB
17	Ancient Sample	Bone, Tooth	Burial #20
18	Ancient Sample	Bone, Tooth	Burial #37
19	Ancient Sample	Bone, Tooth	Burial #40
20	Ancient Sample	Bone, Tooth	Burial #41
21	Ancient Sample	Bone, Tooth	Burial #6

The ancient skeletal and teeth samples were archeologically recovered by Richard Stallings, from two archeological sites in the Tunica County, Mississippi: the Austin site and the Bonds site (Table 2).

Table 2 – Detailed information of Ancient samples

Sample	Site	Burial #	Sex	Estimated Age	Additional Information
17	A22-TU-549	20	F	11-12	Pathological hands and feet; has spina bifida occulta (SBO)
18	A22-TU-549	37	M	30 +/-	Has spina bifida occulta
19	A22-TU-549	40	M	18-19	Has an open neural arch on his fifth lumbar; extensive SBO
20	A22-TU-549	41	Unknown child	9	Has spina bifida occulta
21	B22-TU-530	6	M	17-18	Has spina bifid occulta

The Austin Site (-TU-549) is a ca. AD 1100-1250 village site that sits adjacent to an old Mississippi River channel cut-off. The Bonds site (-TU-530) is a sister site to the

Austin site and is located on the same Mississippi River cut-off a few miles north The Bonds site dates to the same time frame as the Austin site and it is suspected that the inhabitants may have had contact. Both sites yielded a number of individuals who had sacra which had unfused vertebral bodies as adults, although the neural arches were closed.

2.2 Optimised Protocol

2.2.1 Extraction and purification

The extraction and purification procedures were performed at the Paleo-DNA Laboratory at Lakehead University in Thunder Bay, Ontario. The proteinase K method was used to extract DNA from the modern sample, the medical archived specimens and some of the experimentally degraded samples while the guanidinium thiocyanate method was used to extract some of the experimentally degraded samples and the archeological samples.

2.2.1.1 Proteinase K and Modified Ethanol Precipitation Method

Samples were extracted by using an overnight incubation on an Eppendorf Thermomixer™ at 56˚C in 500 µL Extraction buffer (10% SDS, 0.5 M NaCl, 0.5 M EDTA, Tris-HCl 1 M pH=8.0) (Hansen 1974). The following day, a 10% volume of 3 M sodium acetate was added to the sample and mixed. Following the addition of a 2.5X volume of cold 95-100% ethanol, the tube was placed on ice for 30 minutes. The sample was then centrifuged for 5 minutes 13,000 r.p.m. and the supernatant discarded. Afterwards, 500 µL cold 95-100% EtOH was added to the tube and vortexed for one minute. The sample was then centrifuged for 10 minutes at 13,000 r.p.m. and

the supernatant discarded. The resulting pellet was allowed to dry for 30 minutes and was then resuspended in water or TE buffer and incubated at 37˚C for 15 minutes (Hayter 2007).

2.2.1.2 GuSCN and Silica-bead Purification Method

Samples were extracted by using an overnight incubation on an Eppendorf Thermomixer™ at 56˚C in 500 µL of GuSCN solution (Boom *et al.* 1990). The following day, the lysed samples were incubated at 94˚C for 10 minutes and then centrifuged for one minute at 13,000 r.p.m. The supernatant was removed and placed into a sterile 1.5 mL tube in which 900 µL of GuSCN solution and 12-15 µL of silica beads were added and vortexed briefly. The samples were placed on ice for 60 minutes and inverted every 15 minutes to resuspend the silica beads. The samples were then zip-spun (approximately 10 seconds) until clear and the supernatant removed and discarded. The silica beads were washed with 500 µL of working wash buffer (WWB), resuspended, zip-spun and the supernatant removed and discarded. The WWB wash step is repeated if required. The silica beads were washed with 150 µL of 100% ethanol. The silica beads were resuspended, zip-spun until clear and the supernatant removed and discarded. The DNA/silica bead suspension was then air-dried and resuspended using 150 µL ddH_20 via vortexing. The samples were subsequently incubated at 56˚C for one hour and stored at 4˚C for short term usage and -20˚C for long term storage. Prior to each amplification, the extracts were incubated for approximately 30 minutes at 56˚C and centrifuged at 13,000 r.p.m. for one minute (Hoss 1993).

2.2.2 Genetic Amplification

Amplification was performed using the polymerase chain reaction (PCR) in order to amplify specific regions of interest as defined by forward and reverse primers. An initial multiplex PCR, a hemi-nested PCR, and a single base extension PCR were used to amplify the five polymorphisms of interest. Primers were designed (Table 3) and purchased from Operon (Alameda, CA, USA). Oligonucleotides were delivered and re-suspended by adding the calculated volumes of deionized water. A working solution at 100 µM was prepared and aliquoted.

Table 3 – Primer Information for Multiplex PCR

Gene	Forward Primer (5'→3')	Primer Type	Reference	GeneBank Accession#	Position
CBS-1	TATTGGCCACTCCCATAATAGAA	Forward	(Barbaux *et al.* 2000)	AF042836	17974-17996
CBS-2	CGGCTCTGCGAGGATGGACCCTT	Reverse	(Barbaux *et al.* 2000)	AF042836	18101-18079
MS-A	TGTTCCCAGCTGTTAGATGAAAATC	Forward	(Barbaux *et al.* 2000)	AL359259	43622-43646
MS-B	AGTCACATTAAAAACAAGCAAAA	Reverse	(Barbaux *et al.* 2000)	AL359259	43762-43740
MTHFR-0	GAATGTGTCAGCCTCAAAGAAAAG	Reverse	(Barbaux *et al.* 2000)	AY338232.1	8790-8767
MTHFR-1	AGGGAGCTTTGA GGCTGACCTGAA	Forward	(Barbaux *et al.* 2000)	AY338232.1	8694-9717
MTHFR-E	GGAGCTGCTGAAGATGTGGGGGG	Forward	(Barbaux *et al.* 2000)	AY338232.1	10608-10630
MTHFR-D	GTAAAGAACAAAGACTTCAAAGAC	Reverse	(Barbaux *et al.* 2000)	AY338232.1	10677-10654
MTRR-F1	GCTACACAGCAGGGACAGGC	Forward	This study	AF121202	4175-4195
MTRR-R1	GTAACGGCTCTAACCTTATCGG	Reverse	This study	AF121202	4293-4272

2.2.2.1 Multiplex Polymerase Chain Reaction

The PCR setup was performed under sterile conditions to prevent contamination and performed in low light conditions on ice to prevent reagent and DNA extract

degradation. Reactions with a 25 µL volume were aliquoted into 0.2 mL tubes from a prepared mastermix (Table 4).

The cycling parameters were as follows: two minutes of initial hot start denaturation at 94°C followed by 30 cycles of denaturation for one minute at 94°C, one minute of annealing at 60°C, and two minutes extension time at 72°C. For the ancient samples, the number of cycles was increased to 45.

Table 4 – Initial Multiplex PCR amplification concentrations and volumes

Reagent	Initial Concentration	Final Concentration	µL per reaction
PCR buffer	10 X	1 X	2.5
dNTP	10 mM	0.2 mM	0.5
$MgCl_2$	50 mM	2.0 mM	1.0
CBS-1	10 µM	0.06 µM	0.15
CBS-2	10 µM	0.06 µM	0.15
MS-A	10 µM	0.1 µM	0.25
MS-B	10 µM	0.1 µM	0.25
MTHFR-1	10 µM	0.032 µM	0.08
MTHFR-0	10 µM	0.032 µM	0.08
MTHFR-E	10 µM	0.072 µM	0.18
MTHFR-D	10 µM	0.072 µM	0.18
MTRR-F1	10 µM	0.16 µM	0.4
MTRR-R1	10 µM	0.16 µM	0.4
Platinum®Taq	5 U/µL	1.25 U	0.25
DNA extract	-	-	0.2-18.63 µL (ancient)
ddH_20	-	-	Brought up to 25 µL

After amplification, 6-7 µL of the PCR product and 3 µL of 6 X loading buffer was mixed and electrophoresed on a 6% polyacrylamide gel (PAGE) (Appendix – B). The electrophoresis tank was filled with 1X TBE running buffer and the gel was run for 30 minutes at 135 volts. The PCR products were visualised using ethidium bromide staining and photographed on a UV-B trans-illuminator. The size of the band was used to interpret the results (Table 5).

Table 5 – Initial PCR Amplification Target size

Polymorphism	Primers (F/R)	Amplicon Size (bp)
MS A2756G	MS-A/B	141
CBS 844ins68	CBS-1/2	128 (without insertion) 196 (with insertion)
MTRR A66G	MTRR-F1/R1	119
MTHFR C677T	MTHFR-1/0	97
MTHFR A1298C	MTHFR-E/D	73

2.2.2.2 Hemi-nested Polymerase Chain Reaction

A hemi-nested PCR was performed on degraded or LCN DNA samples to increase sensitivity and specificity. Prior to the hemi-nested PCR, the remaining PCR products were purified using DyeEx^{TM} spin columns (Qiagen, Maryland, USA) following the manufacturer's protocol (Appendix – C). The hemi-nested PCR was optimised on the modern DNA sample (Table 6 before being applied to the degraded and LCN DNA samples.

Table 6 – Primer Information for Hemi-nested Multiplex PCR

Gene	Forward Primer (5'→3')	Primer Type	Reference	GeneBank Accession#	Position
CBS-2	CGGCTCTGCGAGGATGGACCCTT	Reverse	(Barbaux *et al.* 2000)	AF042836	18101-18079
CBS-3	GCTTTTGCTGGCCTTGAGCC	Forward	This study	AF042836	18019-18036
MS-A	TGTTCCCAGCTGTTAGATGAAAATC	Forward	(Barbaux *et al.* 2000)	AL359259	43622-43646
MS-C	CAAGCAAAATCTGTTTCTACCACTTAC	Reverse	This study	AL359259	43721-43748
MTHFR-0	GAATGTGTCAGCCTCAAAGAAAAG	Reverse	(Barbaux *et al.* 2000)	AY338232.1	8790-8767
MTHFR-2	GCTGACCTGAAGCACTTGAAGG	Forward	This study	AY338232.1	8707-8738
MTHFR-D	GTAAAGAACAAAGACTTCAAAGAC	Reverse	(Barbaux *et al.* 2000)	AY338232.1	10677-10654
MTHFR-F	TGGGGGGAGGAGCTGACC	Forward	This study	AY338232.1	10624-10641
MTRR-F1	GCTACACAGCAGGGACAGGC	Forward	This study	AF121202	4175-4195
MTRR-R2	GCAGAAAATCCATGTACCACAGC	Reverse	This study	AF121202	4258-4236

Hemi-nested PCR amplifications were prepared from a mastermix and aliquoted into 25 µL reaction volumes like the initial multiplex PCR amplification (Table 7).

Table 7 – Hemi-nested PCR amplification concentrations and volumes

Reagent	Initial Concentration	Final Concentration	µL per reaction
PCR buffer	10 X	1.6 X	4.0
dNTP	10 mM	0.2 mM	0.5
$MgCl_2$	50 mM	1.8 mM	0.9
CBS-2	10 µM	0.1 µM	0.25
CBS-3	10 µM	0.1 µM	0.25
MS-A	10 µM	0.12 µM	0.3
MS-C	10 µM	0.12 µM	0.3
MTHFR-0	10 µM	0.08 µM	0.2
MTHFR-2	10 µM	0.08 µM	0.2
MTHFR-F	10 µM	0.08 µM	0.2
MTHFR-D	10 µM	0.08 µM	0.2
MTRR-F1	10 µM	0.1 µM	0.25
MTRR-R2	10 µM	0.1 µM	0.25
Platinum®Taq	5 U/µL	1.25 U	0.25
Purified PCR product	-	-	0.2-7.0
ddH_20	-	-	Brought to 25 µL

The samples were then placed in the thermal cycler under the following parameters: 2 minutes of initial denaturation at 94˚C (Hot Start) followed by 30-45 cycles of denaturation for 1 minute at 94˚C, 1 minute of annealing at 58˚C, and 2 minutes extension time at 65˚C.

After amplification, 6-7 µL of the PCR product and 3 µL of 6 X loading buffer was mixed and then electrophoresed on a prepared 6% polyacrylamide gel (PAGE) (Appendix – B). The electrophoresis tank was filled with 1 X TBE running buffer and the gel was run for 30 minutes at 135 volts. The PCR products were then visualised using ethidium bromide staining and photographed on a UV-B trans-illuminator (Table 8).

Table 8 – Hemi-nested PCR amplification Target sizes

Polymorphism	Primers (F/R)	Amplicon Size (bp)
MS A2756G	MS-A/C	126
MTHFR C677T	MTHFR-2/0	84
CBS 844ins68	CBS-3/2	83
MTHFR A1298C	MTHFR-F/D	83
MTRR A66G	MTRR-F1/R2	54

2.2.2.3 Snapshot Polymerase Chain Reaction

Prior to the SNaPshot™ PCR, the remaining PCR products were purified using DyeEx™ spin columns (Qiagen, Maryland, USA) following the manufacturer's protocol. A second purification step was performed by transferring 15 µL of the purified PCR product to a sterile 0.2 mL tube in which 5 U SAP (Waterston *et al.*) (Promega, Madison, Wisconsin, USA) and 6 U Exonuclease I (EXO1) (BioLabs, Ipswich, MA, New England) was added. The samples were incubated at 37°C for 60 minutes followed by 20 minutes at 80°C to inactivate the enzymes. Following purification the SNE-PCR was performed on the digested and purified PCR products (Table 9).

Table 9 – Single nucleotide extension primer sequences

Gene	Forward Primer (5'→3')	Reference	GeneBank Accession#	Position
α-CBS	TGCAGATCATTGGGGTGGATC	This study	AF042836	18055-18075
α-MS-F1	GGAAATCATGGAAGAATATGAAGATATTAGACAGG	This study	AL359259	43666-43700
α-MTHFR-F1	GAAGGTGTCTGCGGGAG	This study	AY338232.1	8730-8746
β-MTHFR-R1	GAGGTAAAGAACAAAGACTTCAAAGACACTT	This study	AY338232.1	10680-10650
α-MTRR (F2)	CAGGCAAAGGCCATCGCAGAAGACAT	This study	AF121202	4190-4212

To prepare the SNE-PCR, 0.3-0.5 µL of the digested PCR product was mixed with 4.5 µL SnaPshot™ PCR Ready Reaction mix (Applied Biosystems, Foster City, CA, USA), 1 µL of each 10 mM extension primer (Table 9) adding ddH_20 to bring the final volume to 10 µL. This mixture was placed in the thermal cycler under the following parameters: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30

seconds. When completed, 1 U of SAP was added and the reaction mixture was incubated for 60 minutes at 37°C and 20 minutes at 80°C to deactivate the enzymes.

2.2.3 Data Analysis

The samples were electrophoresed on the ABI Prism 3100 Genetic Analyser (Applied Biosytems) at the Paleo-DNA Laboratory. Prior to loading, 9 µL HI-DI formamide and 0.1 µL of Genescan™ 120 LIZ™ size standard were added to 1 µL of reaction mixture. The samples underwent denaturation by heating to 95°C for 5 minutes. Products generated were then analysed with GeneScan® Analysis Software in which genotypes of either homozygotes or heterozygotes were determined based on the location and fluorescence color of peaks (Table 10 and Table 11).

Table 10 – Extension primers for the multiplex detection of the five markers

Locus	Primer sequence	Length	Reference
MS A2756G	GGAAATCATGGAAGAATATGAAGATATTAGACAGG	35bp	This study
MTHFR C677T	GAAGGTGTCTGCGGGAG	17bp	This study
MTHFR A1298C	GAGGTAAAGAACAAAGACTTCAAAGACACTT*	31bp	This study
CBS 844ins68*	TGCAGATCATTGGGGTGGATC	21bp	This study
MTRR A66G	CAGGCAAAGGCCATCGCAGAAGACAT	26bp	This study

*This is the only reverse primer.

Table 11 – Determination of genotype

Locus	Wild Type Genotype	Heterozygous Genotype	Mutant Type Genotype
MS A2756G	AA	AG	GG
MTHFR C677T	CC	CT	TT
MTHFR A1298C*	AA (TT)	AC (TG)	CC (GG)
CBS 844ins68**	No insertion -/- (CC)	+/- (AC)	Insertion +/+ (AA)
MTRR A66G	AA	AG	GG

* Brackets indicate the nucleotide detected by SNE due to a reverse primer being used.
** The CBS marker is an insertion, + indicates the presence of the insertion while – indicates its absence.

3 Results

3.1 Gel Electrophoresis

3.1.1 Initial Multiplex

Optimised annealing temperature, magnesium chloride ($MgCl_2$) and *Taq* DNA polymerase concentrations were determined by performing gradient PCRs. The optimal condition was determined visually. Densitometry is able to identify the quantity and thus most efficient amplification but visually I was able to identify the presence of non-specific product and other PCR artefacts.

The temperature gradient PCR for the optimisation of each of the singleplex reactions were performed first so that the optimal conditions could be determined. The optimal annealing temperature for each genetic target was determined (Figures 22-26). The temperature gradient PCR for the MTHFR C677T (Figure 22) target and the CBS (Figure 25) target produced a large optimal temperature range from 58-62˚C while the others all produced a smaller optimal temperature range between 60-61˚C (Figure 23, 24, and 26).

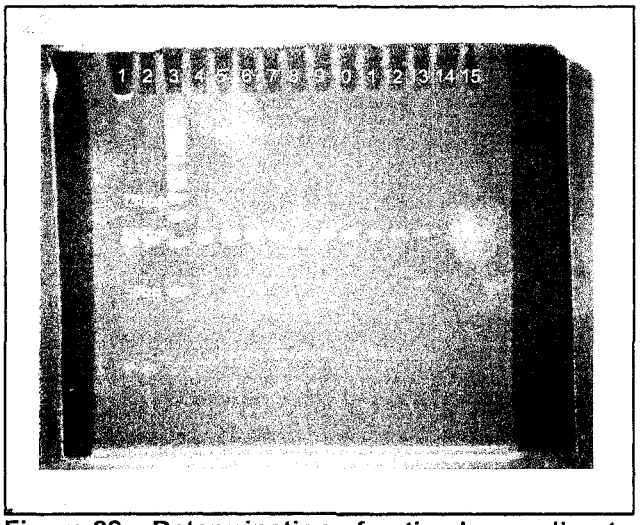

Figure 22 – Determination of optimal annealing temperature for the MTHFR C677T mutation (Initial PCR)

The product is at 97bp. Lane 1) 58.1˚C; 2) 58.2˚C; 3) 50bp Molecular marker; 4) 58.6˚C; 5) 59.3˚C; 6) 60.2˚C; 7) 61.3˚C; 8) 62.4˚C; 9) 63.5˚C; 10) 64.5˚C; 11) 65.4˚C; 12) 66.1˚C; 13) 66.4˚C; 14) Empty and 15) Empty.

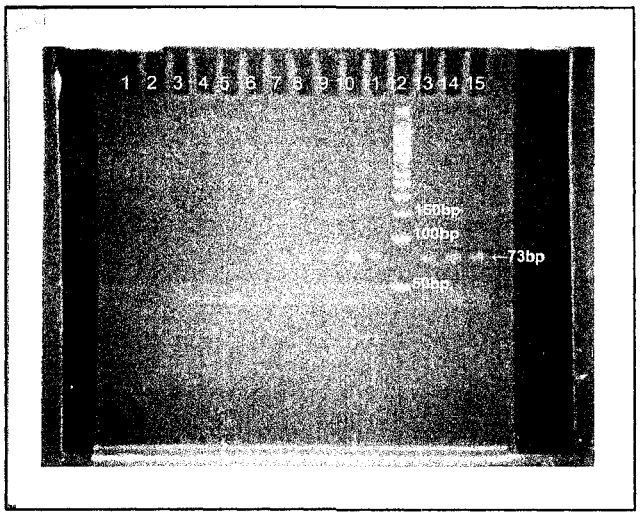

Figure 23 – Determination of optimal annealing temperature for the MTHFR A1298C mutation (Initial PCR)

The product is at 73bp. Lane 1) Empty; 2) Empty; 3) 66.4˚C; 4) 66.1˚C; 5) 65.4˚C; 6) 64.5˚C; 7) 63.5˚C; 8) 62.4˚C; 9) 61.3˚C; 10) 60.2˚C; 11) 59.3˚C; 12) 50bp Molecular marker; 13) 58.6˚C; 14) 58.2˚C; 15) 58.1˚C.

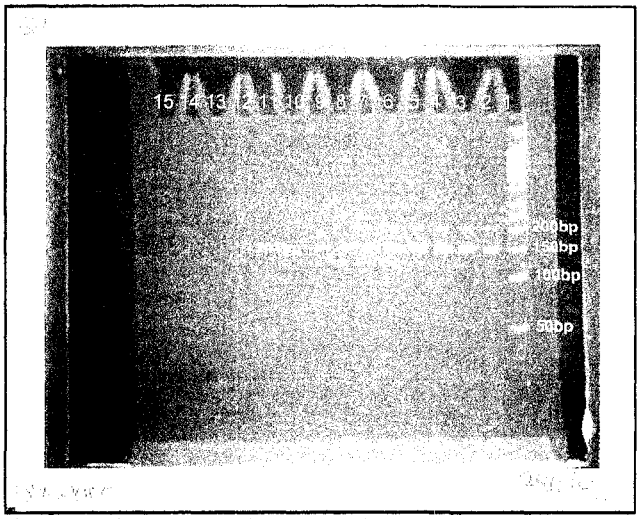

Figure 24 – Determination of optimal annealing temperature for the MS A2756G mutation (Initial PCR)
The product is at 141bp. Lane 1) 50bp Molecular marker; 2) 58.1˚C; 3) 58.2˚C; 4) 58.6˚C; 5) 59.3˚C; 6) 60.2˚C; 7) 61.3˚C; 8) 62.4˚C; 9) 63.5˚C; 10) 64.5˚C; 11) 65.4˚C; 12) 66.1˚C; 13) 66.4˚C; 14) Empty and 15) Empty.

A gradient temperature PCR will identify which targets may be prone to the generation of non-specific product. Non-specific amplification products were observed predominantly in the MS (Figure 24) and the MTRR (Figure 26) amplifications. These products were produced below 60˚C. All of the other targets demonstrated some degree of primer dimer formations but not at the level that would interfere with the efficiency of the PCR.

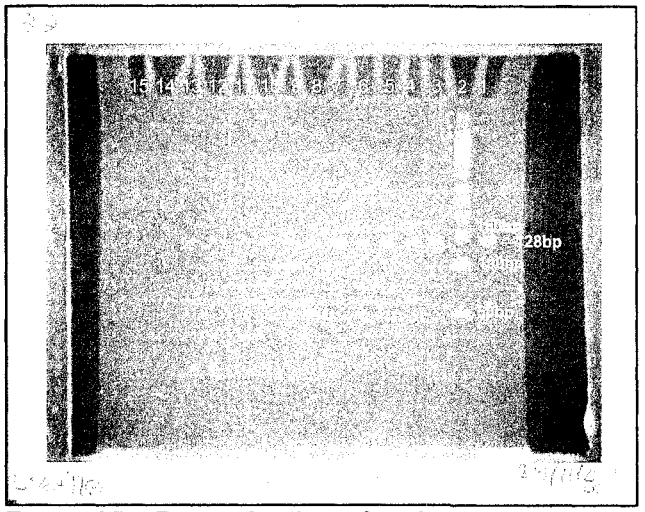

Figure 25 – Determination of optimal annealing temperature for the CBS 844ins68 mutation (Initial PCR)

The product size is 128bp. Lane 1) 58.1˚C; 2) 50bp Molecular marker; 3) 58.2˚C; 4) 58.6˚C; 5) 59.3˚C; 6) 60.2˚C; 7) 61.3˚C; 8) 62.4˚C; 9) 63.5˚C; 10) 64.5˚C; 11) 65.4˚C; 12) 66.1˚C; 13) 66.4˚C; 14) Empty and 15) Empty.

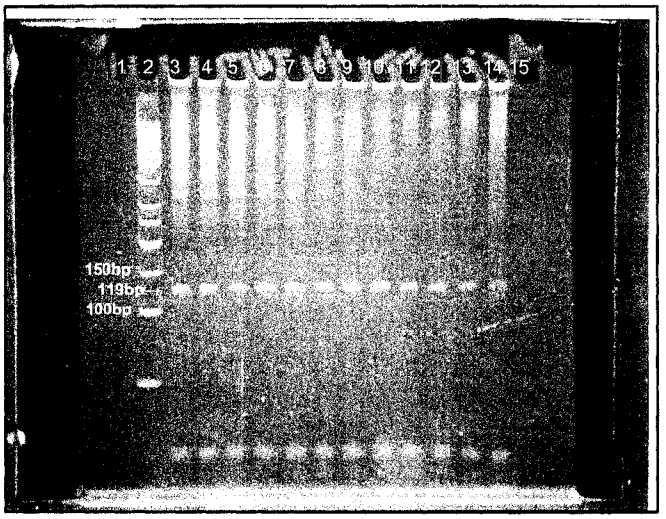

Figure 26 – Determination of optimal annealing temperature for the MTRR A66G mutation (Initial PCR)

The product is 119bp. Lane 1) Empty; 2) 50bp Molecular marker; 3) 55.0˚C; 4) 55.2˚C; 5) 55.7˚C; 6) 56.6˚C; 7) 57.7˚C; 8) 59.0˚C; 9) 60.4˚C; 10) 61.8˚C; 11) 63.1˚C; 12) 64.2˚C; 13) 65.0˚C; 14) 65.4˚C; 15) Empty.

A gel was performed to compare the amplicon sizes to one another (Figure 27). This gel confirms that all of the amplicons can be resolved from one another, there is no size overlap of any of the amplicons and all of the amplicons are the expected sizes. This gel shows each singleplex amplicon side by side and will help to identify the expected size of each band in the multiplex reaction in relation to each other.

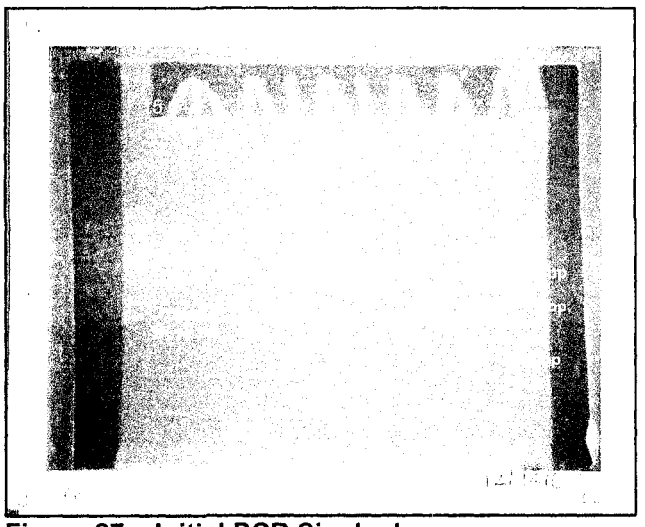

Figure 27 – Initial PCR Singleplexes,
The product sizes are the following: CBS 844ins68 128bp; MS A2756G 141bp; MTHFR C677T 97bp; MTHFR A1298C 73bp. Lane 1) 50bp Molecular marker; 2) Extraction Negative; 3) PCR Negative; 4) CBS 844ins68; 5) CBS 844ins68; 6) CBS 844ins68; 7) MS-A2756G; 8) MS-A2756G; 9) MS-A2756G; 10) MTHFR C677T; 11) MTHFR C677T; 12) MTHFR C677T; 13) MTHFR A1298C; 14) MTHFR A1298C and 15) MTHFR A1298C.

The next step of the optimisation is to add all of the primer sets together in a single multiplex reaction. Since all of the singleplex reactions were optimal at 60°C then this temperature was chosen for the first stages of the initial multiplex optimisation. The primers were added in equimolar concentration and then each primer concentration was changed depending on the relative intensity of the product band. The optimisation of primer concentration took many PCR reactions and gels to determine (Figure 28, 35, 37 and many more not shown). An example of this optimisation process is shown in Figure 28 where one of the gels for primer concentration changes is shown. Two experiments are shown in triplicate on the left and on the right hand side of the gel. Two of the bands on left hand side of the gel are faint (lane 4: 141 and 128 bp) while on the right the concentration of the primers have been increased to produce a relatively equal intensity of the product band. The optimal concentration of all of these primers was determined (Table 4).

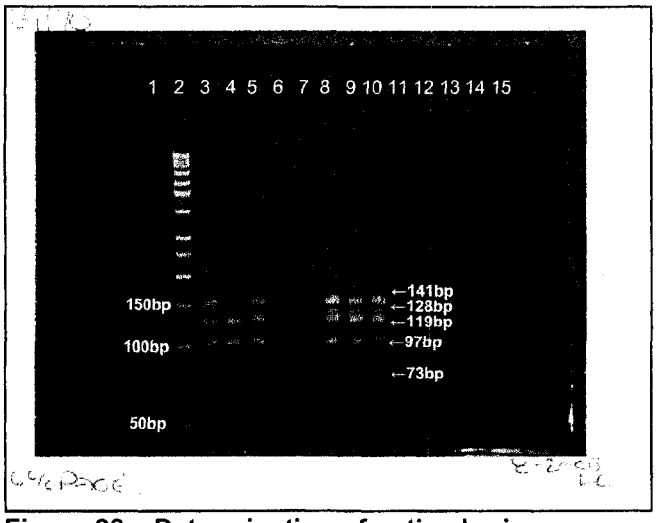

Figure 28 – Determination of optimal primer concentrations for the initial Multiplex
The product sizes for each target are: MS 2756G 141bp; CBS 844ins68 128bp; MTRR A66G 119bp; MTHFR C677T 97bp; MTHFR A1298C 73bp. Lane 1) Empty; 2) 50bp Molecular marker; 3) to 5) Initial multiplex; 6) PCR Negative; 7) Empty; 8) to 10) Initial multiplex; 11) PCR Negative; 12) to 15) Empty.

3.1.2 Hemi-nested Multiplex

The optimisation of the hemi-nested PCR followed by using the initial PCR products of the control DNA. Again all of the temperature gradient singleplex reactions were performed (Figure 29-33). All of the singleplex targets except the MTHFR C677T target (Figure 29) produced non-specific product and smearing (Figure 30-33). However, this was not of such great concern as this multiplex in the final procedure will be performed as a hemi-nested PCR using the PCR product from the initial multiplex. All of the non-specific product and smears would be eliminated due to the successful competition between specific template and non-specific product as none of the non-specific product will be present from the initial multiplex reaction. The optimal annealing temperature for the hemi-nested PCR was determined to be 58˚C.

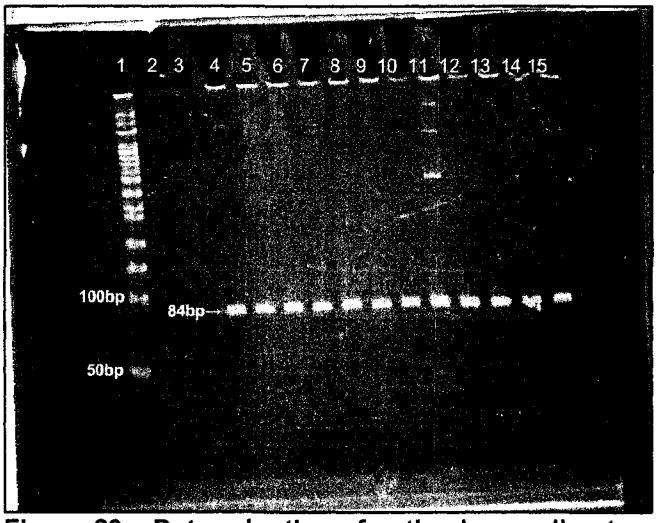

Figure 29 – Determination of optimal annealing temperature for the MTHFR C677T mutation (Hemi nested)

The amplicon size is 84bp. Lane 1) 50bp Molecular marker; 2) Empty; 3) Empty; 4) 55.0˚C; 5) 55.1˚C; 6) 55.4˚C; 7) 55.8˚C; 8) 56.4˚C; 9) 57.0˚C; 10) 57.7˚C; 11) 58.4˚C; 12) 59.1˚C; 13) 59.6˚C; 14) 60.0˚C; 15) 60.3˚C.

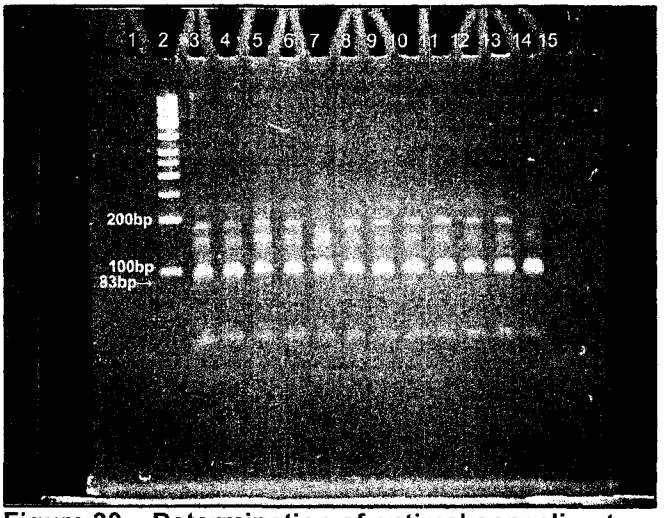

Figure 30 – Determination of optimal annealing temperature for the CBS 844ins68 mutation (Hemi-nested)

The amplicon size is 83bp. Lane 1) Empty; 2) 100bp Molecular marker; 3) 57.0˚C; 4) 57.2˚C; 5) 57.7˚C; 6) 58.6˚C; 7) 59.8˚C; 8) 61.1˚C; 9) 62.5˚C; 10) 63.8˚C; 11) 65.1˚C; 12) 66.2˚C; 13) 67.0˚C; 14) 67.5˚C; 15) Empty.

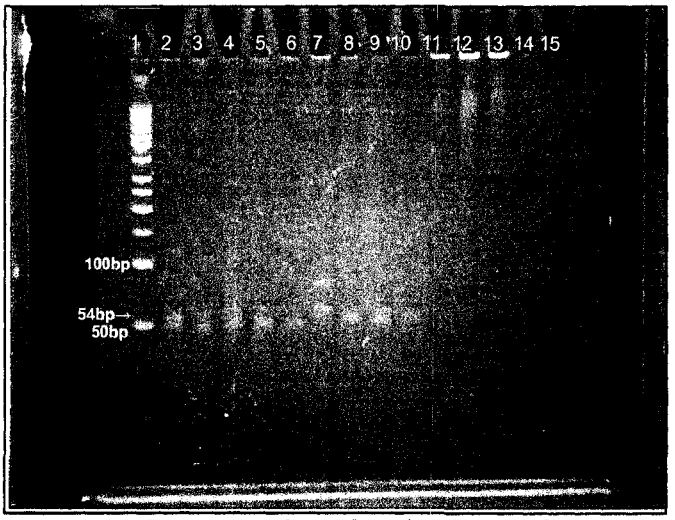

Figure 31 –Determination of optimal annealing temperature for the MTHFR A1298C mutation (Hemi-nested)

The amplicon size is 54bp. Lane 1) 100bp Molecular marker; 2) 54.9˚C; 3) 55.2˚C; 4) 56.0˚C; 5) 57.4˚C; 6) 59.1˚C; 7) 61.1˚C; 8) 62.5˚C; 9) 63.8˚C; 10) 65.1˚C; 11) 66.2˚C; 12) 67.0˚C; 13) 67.5˚C; 14) and 15) Empty.

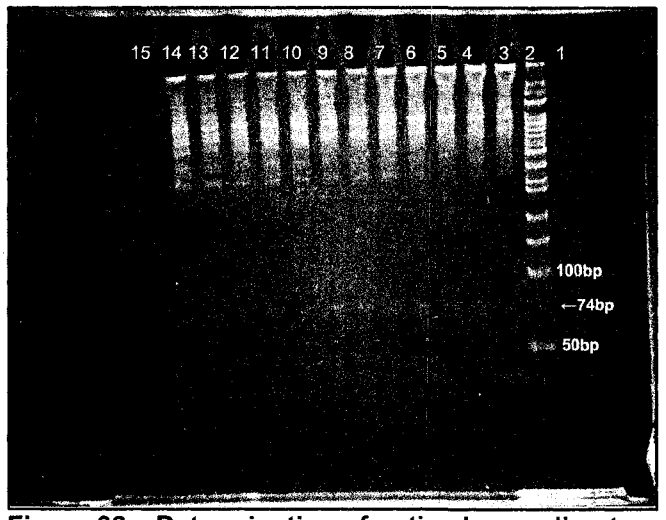

Figure 32 – Determination of optimal annealing temperature for the MTRR A66G mutation (Hemi-nested)

The amplicon size is 74bp. Lane 1) Empty; 2) 50bp Molecular marker; 3) 55.0˚C; 4) 55.1˚C; 5) 55.4˚C; 6) 55.8˚C; 7) 56.4˚C; 8) 57.0˚C; 9) 57.7˚C; 10) 58.4˚C; 11) 59.1˚C; 12) 59.6˚C; 13) 60.0˚C; 14) 60.3˚C; 15) Empty.

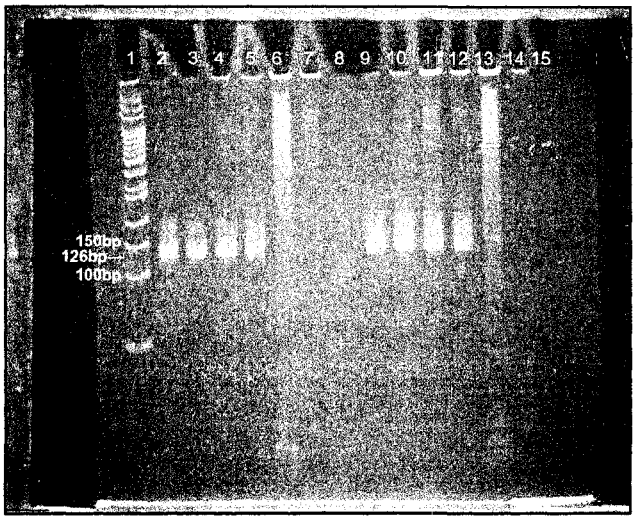

Figure 33 – Hemi-nested singleplex of MS A2756G
The amplicon size is 126bp. Lane 1) 50bp Molecular marker; 2) to 6) MS A2756G; 7) PCR Negative; 8) Empty; 9) to 13) MS A2756G; 14) PCR Negative; 15) Empty.

Once the singleplex reactions were optimised, a gel was performed to compare each of the resulting PCR products (Figure 34; not all products shown on this gel). This determined that each band could be resolved and helped to identify each band in the multiplex reaction in relation to each other. Throughout this research, contamination was observed (Figure 34). On the gel in the Figure 34 in lane 5 and 13, contamination was observed; however, this was in the optimisation stages. Lane 5 shows contamination producing a band at exactly the expected size for that amplicon. This band was produced by contamination of that PCR from modern DNA while lane 13 shows a smear and faint bands above and below the specific amplicon size. This contamination could be from modern DNA but has produced a great deal of non-specific product and could be from another animal source. It is more likely a mixture of background laboratory contamination of human and animal DNA as the specific band of interest is present though faint.

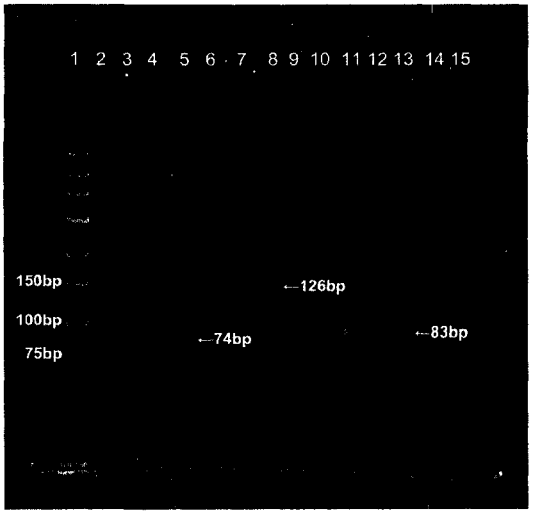

Figure 34 – Hemi-Singleplex
The amplicon sizes are: MTRR A66G F1/R2 74bp; MSA2756G-A/C 126bp; CBS 844ins68 2/3 83bp. Lane 1) 50bp Molecular marker; 2) Empty; 3) and 4) MTRR A66G; 5) PCR Negative; 6) Empty; 7) and 8) MS A2756G; 9) PCR negative; 10) Empty; 11) and 12) CBS 844ins68; 13) PCR negative;14) and 15) Empty.

Like the first multiplex reaction, the primer concentrations for the hemi-nested multiplex PCR had to be optimised. Figure 35 and 37 presents examples of this optimisation process. The optimal primer concentrations were determined (Table 7). Comparison of the initial multiplex results and the hemi-nested multiplex results can be compared in Figure 35 and 37.

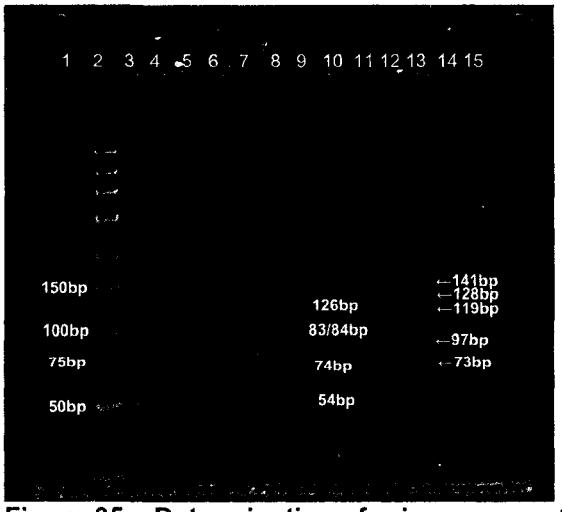

Figure 35 – Determination of primer concentrations in hemi-nested multiplex and initial multiplex PCR
The hemi-nested multiplex PCR sizes are: MS 2756G 126bp; MTHFR C677T 84bp; CBS 844ins68 83bp; MTRR A66G 74bp; MTHFR A1298C 54bp and the initial multiplex

PCR sizes (MS 2756G 141bp; CBS 844ins68 128bp; MTRR A66G 119bp; MTHFR C677T 97bp; MTHFR A1298C 73bp. Lane 1) Empty; 2) 50bp Molecular marker; 3) to 8) Hemi-nested multiplex; 9) PCR Negative; 10) Empty; 11) to 13) Initial multiplex; 14) and 15) Empty.

In both multiplex reactions, some of the reagents were tested under a concentration gradient PCR. The optimal $MgCl_2$, *Taq* DNA polymerase and buffer concentrations were determined to be 2.0 mM, 1.25 U and 1 X respectively for the initial PCR and 1.8 mM, 1.25 U and 1.6 X respectively for the hemi-nested PCR. An example of one of these gradient PCRs is presented as Figure 36 where the DNA polymerase concentration gradient is presented. The optimal concentration for the DNA polymerase was found to be 1.25 U for both reactions.

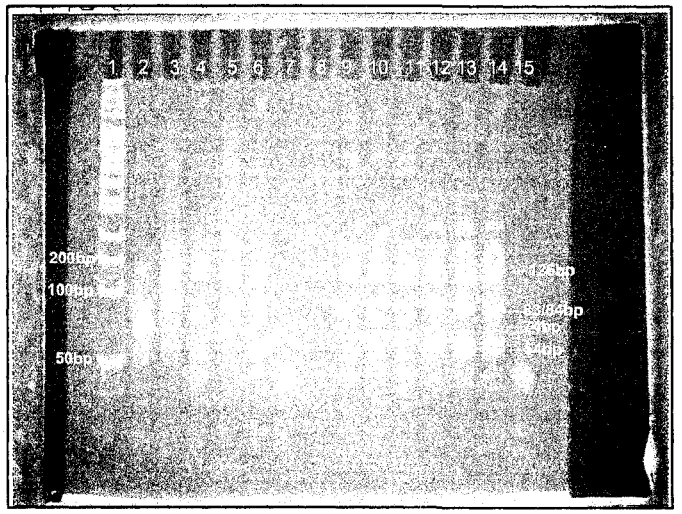

Figure 36 – Determination of optimal Taq and template concentrations - Hemi-nested Multiplex
The product sizes for each target are the following: MS 2756G 126bp; MTHFR C677T 84bp; CBS 844ins68 83bp; MTRR A66G 74bp; MTHFR A1298C 54bp. Lane 1) 50bp Molecular marker; 2) to 6) Decreased Taq concentration; 7) PCR negative; 8) Empty; 9) to 14) Template gradient; 15) Empty.

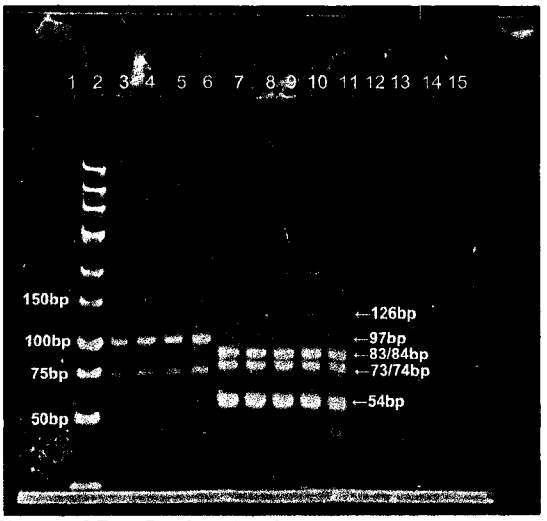

Figure 37 – Optimal primer concentrations determination of initial and hemi-nested multiplex The amplicon sizes of the initial multiplex are: MS 2756G 141bp; CBS 844ins68 128bp; MTRR A66G 119bp; MTHFR C677T 97bp; MTHFR A1298C 73bp while the amplicon sizes for the hemi-nested multiplex PCR are MS 2756G 126bp; MTHFR C677T 84bp; CBS 844ins68 83bp; MTRR A66G 74bp; MTHFR A1298C 54bp. Lane 1) Empty; 2) 25bp Molecular marker; 3) to 5) Initial multiplex; 6) Initial multiplex PCR negative; 7) to 10) Hemi-nested multiplex; 11) Hemi-nested multiplex PCR negative; 12) to 15) Empty.

3.2 Sequencing Data of Modern Sample

The following sequencing results consist of the data obtained from the modern known sample which was subsequently used to optimise the methodology. The genotype of the modern sample was determined through sequencing and SNE (Table 12). From the sequence data, the genotype at each loci can be determined (Figure 38, 40, 42, 44, and 46). Each of the sequences generated were aligned to the corresponding sequence in GeneBank (Figure 39, 41, 43, 45, and 47).

3.2.1 Cystathionine beta-synthase CBS 844ins68

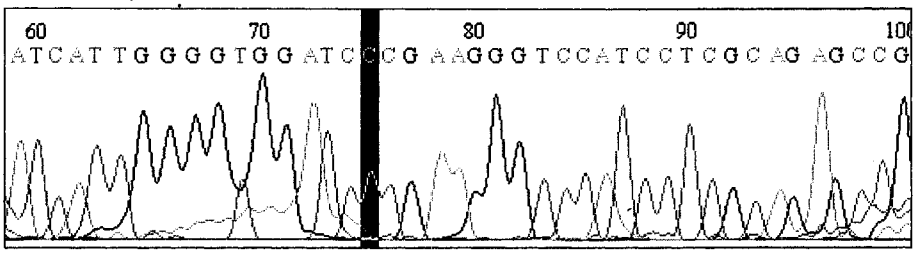

Figure 38 – CBS 844ins68 generated sequence for modern sample and sequence alignment with GeneBank Assession#AF042836.1 (with highlighted SNP)

The genotype for the CBS target in the modern sample is homozygote without the insertion (Figure 38 and 39). The insertion, if present, would have an extra 68 bp of sequence inserted at the point where the arrow indicates (Figure 39).

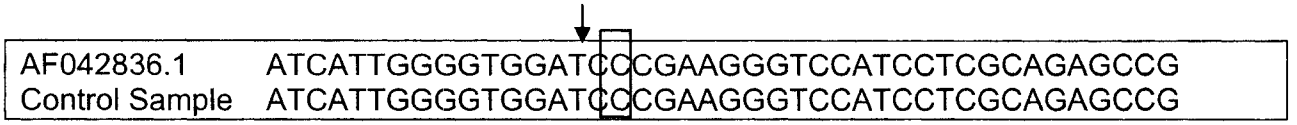

Figure 39 – The sequence alignment of the modern control sample to the reference sequence of Cystathionine beta-synthase.
The reference sequence is from GeneBank with accession number #AF042836.1. The arrow indicates where the insertion if present would be found. The box indicates the target for the SNE detection.

3.2.2 Methionine Synthase MS A2756G

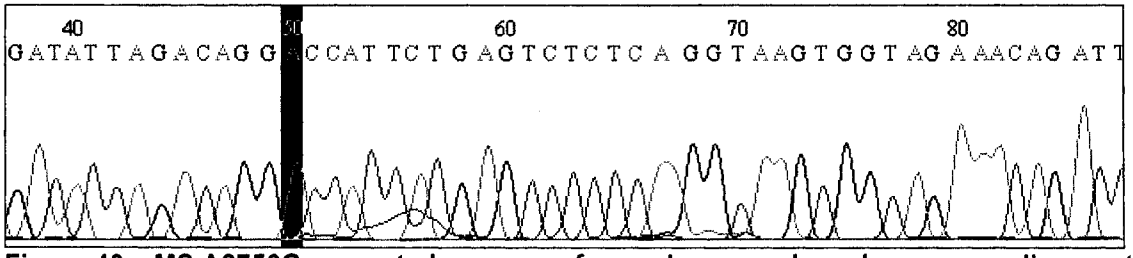

Figure 40 – MS A2756G generated sequence for modern sample and sequence alignment with GeneBank Assession#AL359259 (with highlighted SNP)

The sequence generated for the modern sample from the MS amplicon identified the modern sample as a homozygote (AA) (Figure 40 and 41). However, the SNE results indicated that this sample was a heterozygote (AG) (Table 12). The sequencing was replicated numerous times and still produced a homozygote indicating some of the

errors that can occur when using one method alone and the competition that occurs between both alleles of the target region.

AL359259	GATATTAGACAGGACCATTATGAGTCTCTCAAGGTAAGTGGTAG
Control Sample	GATATTAGACAGGACCATTATGAGTCTCTCAAGGTAAGTGGTAG

Figure 41 – The sequence alignment of the modern control sample to the reference sequence for Methionine Synthase
The reference sequence is from GeneBank with accession number #AL359259. The box indicates the target for the SNE detection.

3.2.3 Methionine synthase reductase MTRR A66G

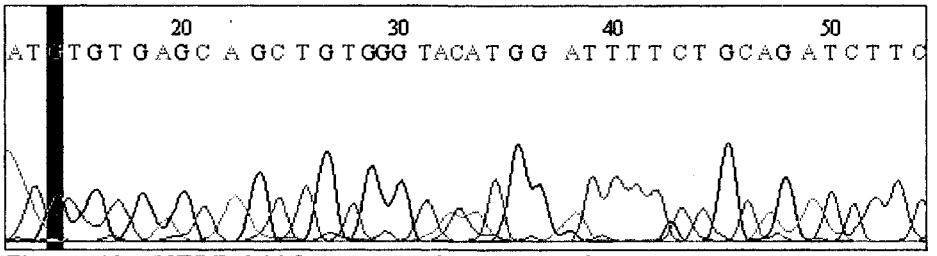

Figure 42 – MTRR A66G generated sequence for modern sample and sequence alignment with GeneBank Assession#AF121202 (with highlighted SNP)

A heterozygous genotype (AG) was identified for the modern sample at the MTRR loci. The sequence here indicates two bases overlapping the A and the G present on the electropherogram (Figure 42). This result was confirmed by the SNE detection (Table 12). The R in the alignment (Figure 43) denotes the presence of the A and the G in that nucleotide position.

AF121202	ATGTGTGAGCAAGCTGTGGTACATGGATTTTCTGCAGATCTTC
Control Sample	ATRTGTGAGCAAGCTGTGGTACATGGATTTTCTGCAGATCTTC

Figure 43 – The sequence alignment of the modern control sample to the reference sequence for Methionine Synthase Reductase
The reference sequence is from GeneBank with accession number #AF121202. The box indicates the target for the SNE detection. The R denotes the presence of an A or a G in that position, representing a heterozygote.

3.2.4 5, 10-Methylenetetrahydrofolate reductase MTHFR C677T

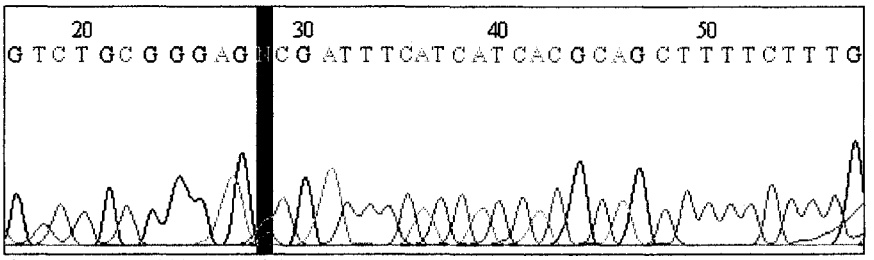

Figure 44 – MTHFR C677T generated sequence for modern sample and sequence alignment with GeneBank Assession# AY338232.1 (with highlighted SNP)

For the MTHFR C677T marker, the modern sample was determined to be a heterozygote (CT). The electropherogram shows two nucleotides present (black highlighted section) a C and a T (Figure 44). In the alignment this is represented as a Y (Figure 45).

AY338232.1	GTCTGCGGGAGCCGATTTCATCATCACGCAGCTTTTCTTTG
Control Sample	GTCTGCGGGAGYCGATTTCATCATCACGCAGCTTTTCTTTG

Figure 45 – The sequence alignment of the modern control sample to the reference sequence for 5, 10-Methylenetetrahydrofolate reductase C677T polymorphism
The reference sequence is from GeneBank with accession number #AY338232.1. The box indicates the target for the SNE detection. The Y denotes the presence of a C or a T in that position, representing a heterozygote.

3.2.5 5, 10-Methylenetetrahydrofolate reductase MTHFR A1298C

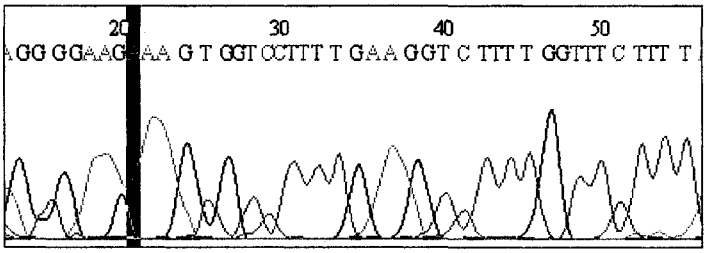

Figure 46 – MTHFR A1298C generated sequence for modern sample and sequence alignment with GeneBank Assession# AY338232.1 (with highlighted SNP)

The modern sample was identified as a heterozygote for the MTHFR A1298C polymorphism. In the sequence electropherogram, the presence of an A and a G can be seen (black highlighted section) (Figure 46). These two nucleotides are represented in the alignment of this sequence as an R using the usual FASTA format (Figure 47).

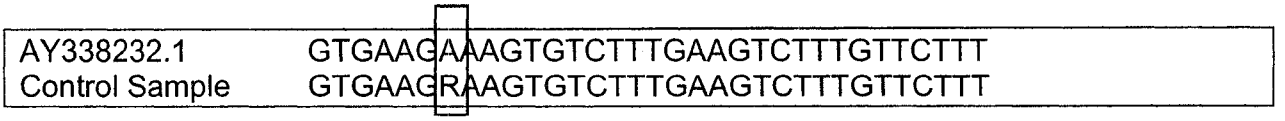

Figure 47 – The sequence alignment of the modern control sample to the reference sequence for 5, 10-Methylenetetrahydrofolate reductase A1298C polymorphism
The reference sequence is from GeneBank with accession number #AY338232.1. The box indicates the target for the SNE detection. The R denotes the presence of an A or a G in that position, representing a heterozygote.

3.3 Snapshot Data

Table 12 – Modern Sample Genotype

Sample Name	Genotype				
	MTRR A66G	MTHFR C677T	CBS 844ins68	MTHFR A1298C	MS A2756G
1-LC	AG	CT	-/-	AC	AG

*verified sequencing

The known modern control exhibited a heterozygous genotype for mutations MTRR A66G, MTHFR C677T, MTHFR A1298C and MSA2756G using the SNE typing method (Table 12). The CBS 844ins68 was the only mutation to be observed in a homozygous state (-/-) with no insert in either allele present (Table 12).

Table 13 – Experimentally Degraded Samples Genotypes

Sample Name	Genotype				
	MTRR A66G	MTHFR C677T	CBS 844ins68	MTHFR A1298C	MS A2756G
2-TJ	AG	CT	-/-	AC	AG
3-RS	AG	CT	+/-	AC	AG
4-AL	AG	CT	-/-	AC	AG
5-RCP	AG	CT	-/-	AC	AG
6-AT	AG	CT	+/-	AC	GG
7-AH	AG	CT	-/-	AC	AG
8-LI (1/10)	AG	CT	-/-	AC	AG
9-CB (1/10)	AG	CT	-/-	AC	AG
10-R (1/10)	AG	CT	-/-	AC	AG
11-CH	AG	CT	-/-	AC	AA

Full genotypes were determined for all of the experimentally degraded samples (Table 13). The experimentally degraded samples were heterozygous for the MTRR A66G (AG); MTHFR C677T (CT) and the MTHFR A1298C (AC) mutation. In the case

of the MS A2756G mutation, all samples were heterozygous (AG) except two samples which were homozygous: sample 6 (GG) and sample 11 (AA). The CBS 844ins68 insertion was observed in a heterozygous state (+/-) in samples 3 and 6.

Table 14 – Medical Archive Samples Genotypes

Sample Name	Genotype				
	MTRR A66G	MTHFR C677T	CBS 844ins68	MTHFR A1298C	MS A2756G
12-AD	AG	CT	-/-	AC	G
13-DD1	AG	CT	+/-	AC	AG
14-DD2	AG	CT	+/-	AC	AG
15-JZ	GG	CT	-	AC	GG
16-YB	GG	CT	+/-	AC	G

Most of the medical archive specimens produced full genotypes with the exception of samples 12, 15 and 16 which may have experienced allelic drop-out (MS A2756G and CBS 844ins68) (Table 14). Heterozygosity was observed for both MTHFR C677T and MTHFR A1298C mutations in all medical archive samples. The insertion allele (CBS 844ins68) was noted in three samples (sample 13, 14 and 16). In respect to the MTRR A66G mutation, two samples (samples 15 and 16) exhibited the mutant genotype (GG) while the other three samples were heterozygous (AG). The G allele was observed in all the samples either in heterozygous (AG) in samples 13 and 14; homozygous in sample 15.

Table 15 – Archeological Samples Genotypes

Sample Name	Genotype				
	MTRR A66G	MTHFR C677T	CBS 844ins68	MTHFR A1298C	MS A2756G
17-Burial #20				A	
18-Burial #37	G			AC	
19-Burial #40				A	
20-Burial #41		CT		A	
21-Burial #6	G	CT	+/-	AC	G

Of the five archeological samples, only one sample (sample 21) produced a full genotype (Table 15). Possible allelic drop-out was observed in all the samples. One

allele (G) was noted in the MTRR A66G and MSA2746G mutations in samples 18 and 21; while the A allele was noted in samples 17, 19 and 20 for the MTHFR A1298C mutation. Heterozygosity was observed in samples 20 and 21 for the MTHFR C677T mutation, samples 18 and 21 for the MTHFR A1298C mutation, as well as sample 21 for the CBS 844ins68 mutation. It must be noted that the samples were replicated at least twice depending on the amount of sample available. The concentration of certain samples was not high enough to allow for many replicates. The multiple results were compared and allowed an accurate genotype profile to be determined.

4 Discussion

4.1 Optimisation

A novel methodology was successfully designed and optimised in which five mutations were detected simultaneously in samples of various levels of degradation. Single nucleotide extension can be performed in two ways. It can be used directly on the DNA extracts or it can be used after an initial PCR of the desired target. By performing an initial PCR you are increasing the template concentration in relation to the DNA in the extract, thus increasing the sensitivity and the specificity of the SNE-PCR. Direct SNE-PCR cannot be used on degraded or ancient DNA as sensitivity and specificity of the analytical procedure is critical to these studies.

This method is not suitable for the detection of LCN DNA. Even though the technique is sensitive the LCN DNA may be in much lower concentration than can be reliably detected. These very low levels of DNA concentration can generate stochastic variation which will preferentially amplify different alleles and allelic or loci drop-out can occur (Gill 2001). This would result in inaccurate genotyping. These problems were encountered in this research.

In order to overcome this challenge, in addition to an initial PCR to increase sensitivity and specificity a hemi-nested PCR was developed. A hemi-nested PCR is often quite successful in reducing or eliminating unwanted products while at the same time increasing specificity (Gibbs 1990; Mullis 1991; Mullis and Faloona 1987; Zhang and Ehrlich 1994; Zimmermann *et al.* 1994). This hemi-nested approach followed by SNE-PCR to increase sensitivity and specificity for degraded DNA was introduced by

Larcombe *et al.* (2005). In this research one set of primers were designed to amplify the target region and a third primer was designed and optimised to perform a hemi-nested PCR which was then followed by the SNE-PCR. This was able to increase the sensitivity. However time did not permit the assessment of this three step methodological approach to overcome allelic drop-out.

The initial PCR, hemi-nested PCR and SNE-PCR were designed as a multiplex to gain more information from each analysis and to help in the authentication of the degraded DNA results. The design and optimisation process began with the design of the primers 3'or 5'of the SNP of interest. In the case of the CBS gene it was a 68 bp insertion and so primers were designed to identify the difference between the presence and absence of the insertion by SNE.

The design and optimisation of the initial PCR began with the design of the primers. For the initial PCR most of these primers were chosen from the literature (Table 3). All except for one set of primers for the MTRR target were chosen from the literature. All of the primers were tested using a computational simulator (Amplify™) (Appendix A) to determine their stability by avoiding the presence of secondary structures, primer-primer complementarity and calculate the melting temperature. The experimental annealing temperature was determined for the initial PCR. An initial multiplex PCR was performed of equimolar primer concentration including all five primer sets. Primer concentration adjustments were done to observe PCR products of equal intensity (Figure 27 and Figure 28). The primer concentration was increased for those genetic targets that produced a low intensity band and vice versa for the high intensity PCR products. $MgCl_2$, *Taq* DNA polymerase, and DNA template concentration gradients

were also performed to determine their optimal concentrations. Additives (i.e. DMSO, Q solution, BSA) were assessed to determine if the results could be improved. With the optimal concentrations and cycling parameters, the initial multiplex was optimised.

Prior to a hemi-nested PCR, the PCR products from the initial PCR must be purified. Several purification methods were tested such as Qiaquick spin columns, DTR spin columns, HiYield, silica bead purification; EtOH purification; NucleoSpin Extract spin column. The DyeEx purification column was determined to be the most effective method of purification. The hemi-nested multiplex was optimised in the same fashion as the initial multiplex. Primers were designed and evaluated on the computer before purchase (Table 6). Annealing temperature gradient singleplex PCRs were performed for each step, followed by a multiplex PCR containing equimolar primer concentrations. Primer, $MgCl_2$, buffer, *Taq* DNA polymerase, and template concentrations were also optimised with adjustments and gradients (Figure 36, Figure 35 and Figure 37). The use of additives such as 2%DMSO, betaine, and Q solution, were also applied with no success. Different types of DNA polymerase (Q *Taq*, Platinum *Taq*) were tried with no differences observed. Extension temperature gradients were performed and a 65˚C temperature demonstrated the best results, for most multiplex reactions the optimal extension temperature is usually about 5°C lower than the regular PCR.

The SNE primers were designed to ensure their product lengths would not overlap during data-analysis; the primers are separated by approximately 5bp in length (Table 10). As well possible genotypes had to be considered to avoid confusion during their interpretation. Each primer was tested individually and then combined at equimolar concentrations in a SNE multiplex PCR. The SNE multiplex PCR was

optimised with template volume, primer and SnaPshot™ reaction mixture at different concentrations. The optimisation of the SNE-PCR required the adjustment of primer concentrations and increase or decrease of template volume according to sample type (i.e. more DNA template for the archeological samples).

The optimisation of each method proved to be quite challenging. The presence of non-specific bands, amplification failure, primer interaction, preferential amplification, no detectable product, as well as a low yield of the desired product were some problems. This not only made the PCR less efficient, but also made analysis of the results more difficult. Adjustments of PCR components (i.e. primer concentration to overcome preferential amplification of specific targets) and parameters enabled their optimisation. It was essential during optimisation to maintain a balance between specificity and sensitivity. All three steps were optimised individually and then combined together to detect the mutations of interest (Section 2.2). As each step progressed, the specificity of the method subsequently increased. Thus, the product of the previous step was used as template for the next step with new primers of different sequences that exhibited complete homology.

Negative controls ensured that the results of the PCR amplifications were indeed from authentic DNA samples and not from PCR products of other samples that were amplified in the lab. A negative control was included for each step of the methodology and carried throughout the whole process. A total of four negatives were employed: an extraction negative, an initial PCR negative; a hemi-nested PCR negative; and a SNE-PCR negative. These controls would detect exogenous DNA from the extraction step until the final amplification and aid in identifying at which step contamination occurred.

Contamination did occur during optimization of the methodology however it was identified and addressed.

With LCN and degraded samples, the allelic balance deteriorates and the chance of allelic drop-out is increased compromising interpretation (Dixon *et al.* 2006). The difficulty of determining the homozygotes between allelic drop-out when analysing the results became the most challenging task. For this reason we cannot be certain that an individual is a true homozygote in the interpretation of the archeological samples (Table 15). Allelic drop-out is common due to the degradation level of the sample. Gill *et al.* (2000) suggest that duplication of each allele should be performed in order to adequately identify the genotype and because of stochastic variation, any apparent homozygote is considered a potential heterozygote.

In some cases the primers had to be redesigned. This was usually because the primers interacted with other primers in the multiplex reaction mix but was also due to data interpretation. As each amplicon was fluorescently labelled it could be detected using capillary electrophoresis but each of the amplicons exhibited some degree of electrophoretic run mobility shift depending on the fluorescent label. This change in the detected size of each of the amplicons caused difficulty in assigning the correct peaks. Shorter primers with poly-A tails added to modify the size of the primer and thus the mobility and separation on electrophoresis were evaluated. The primers with the poly-A tails produced less mobility shift and were slightly more reliable than the full length complimentary primers.

4.2 *Application of Methodology*

In this project, a novel multi-step methodology was developed, to assess simultaneously the genotype of five targeted areas within a sample of variable levels of degradation. Once the steps involved were optimised. The final methodology included three amplification steps: an initial multiplex PCR, a hemi-nested multiplex PCR, and a multiplex SNE-PCR using the ABI Prism® SNaPshot™ kit followed by detection with capillary electrophoresis (Figure 48). The most critical step was the purifications between each PCR reactions which can introduce contamination.

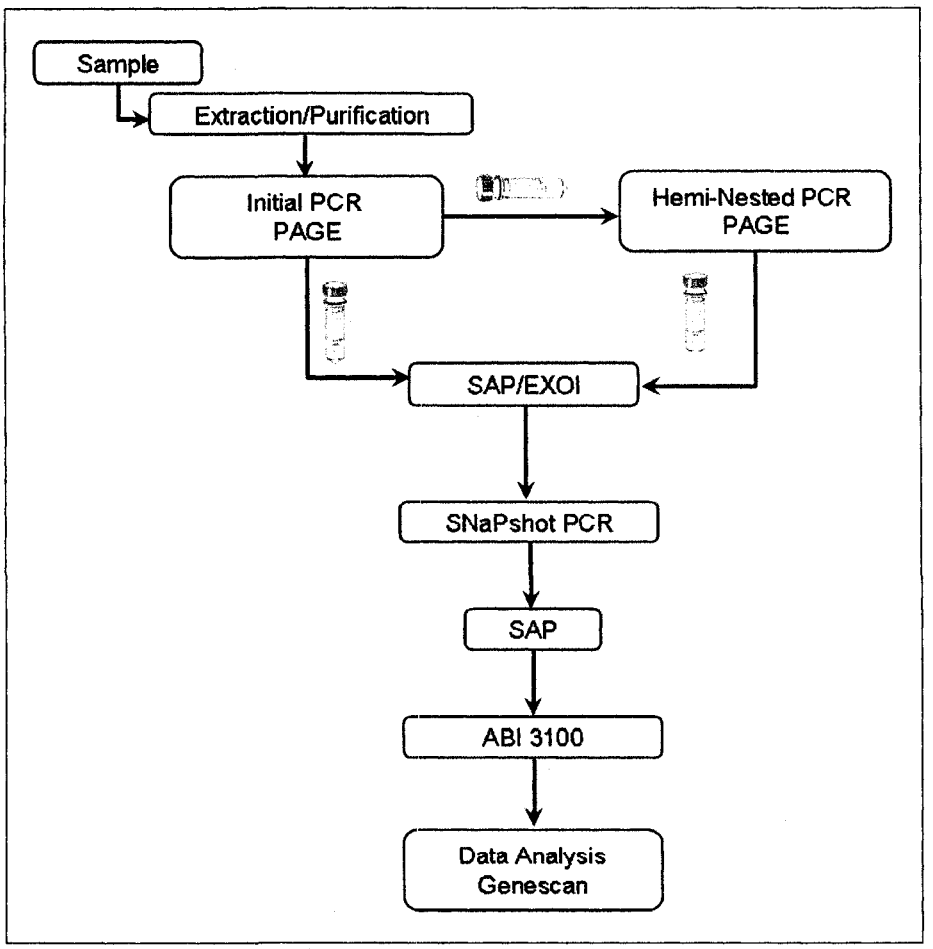

Figure 48 – Summary of Procedure

The genotype of a known modern sample was determined and used during optimisation of the methodology. The genotype of the modern sample (Table 12) at the

MS A2756G marker exhibited differences between the sequencing and SNE-PCR results. The sequence results indicated a homozygote while the SNE-PCR indicated a heterozygote. This is due to preferential amplification in the sequencing reaction amplifying only one allele. The results of the SNE-PCR were determined to be more reliable. Full genotypes were determined for all of the experimentally degraded samples (Table 13). While most of the medical archive specimens produced full genotypes with the exception of samples 12, 15 and 16 which may have experienced allelic drop-out (MS A2756G and CBS 844ins68) (Table 14). The majority of archeological samples, with the exception of sample 21, produced partial profiles (Table 15). This is to be expected due to the degradation of the samples. The different sample types demonstrated the test's sensitivity. As the sample's degradation increased, the more likely a partial profile would be detected. Applying a single base extension amplification directly on experimentally degraded DNA was determined to have failed 97% of the time (Beaulne 2004). This study demonstrates how a hemi-nested PCR is more senstitive and specific compared to applying the test directly to DNA. The table below (Table 16) contains the determined success rates.

Table 16 - Determined Success Rates

Sample Type	Methodology	Success Rate
Modern DNA	Direct SNaPshot™	18% (Larcombe 2005)
	Multiplex followed by SNaPshot™	86%
	Multiplex hemi-nested and SNaPshot™	98%
Experimentally degraded DNA	Direct SNaPshot™	3%
	Multiplex followed by SNaPshot™	61%
	Multiplex hemi-nested and SNaPshot™	93%
Degraded DNA	Direct SNaPshot™	Not determined
	Multiplex followed by SNaPshot™	6%
	Singleplex hemi-nested followed by SNaPshot™	60%
	Multiplex hemi-nested and SNaPshot™	20% (full profile) 60% (partial profile)

The challenge in determining an accurate degraded sample's genotype is differentiating between a true homozygote and allelic drop-out. It is for this reason that we cannot assume a homozygote is a true homozygote when it could possibly be heterozygote. Another difficulty experienced during genotype determination was the different mobility shift of the fluorescent tags. The red dye (T), was greatly displaced by up to 10bp; the blue dye (G) by -1 to 4bp; the green dye (A) by -2bp; while the black dye (C) showed no mobility shift.

All the samples exhibited heterozygosity (CT) for the MTHFR C677T mutation (Table 13, Table 14, and Table 15). Van der Put *et al.* (1995), Johanning *et al.* (2002) and Kirke *et al.* (2004) indicate that the NTD risk for heterozygous individuals is only slightly elevated over the wild type (CC). This mutation which is commonly found in approximately 35% in the North American population may predispose individuals to mild to moderate hyperhomocysteinemia when folate status is low (Christensen *et al.* 1999; Frosst *et al.* 1995; Jacques *et al.* 1996). However, this result in all the samples may simply be due to the low number of samples in the study. A more comprehensive genotyping study should be conducted with a larger number of samples including both control and individuals with a NTD and individuals with NTDs in the family. The increased risk of NTDs due to the presence of the MTHFR C677T mutation may be mitigated by an adequate level of folate on ones diet. The archeological samples (samples 20 and 21) were heterozygous for the MTHFR C677T mutation (Table 15) which may indicate that this mutation combined with dietary stress and lack of folate may explain the morphological manifestations consistent with SB.

All the experimentally degraded, medical archive samples, and two of the archeological samples (sample 18 and 21) contain the MTHFR A1298C heterozygous genotype (AC); while the rest of the archeological samples (17,19,20) exhibit the A allele at the MTHFR A1298C MTHFR mutation (Table 13, 14 and 15). De Marco *et al.* (2002) determined that the prevalence of heterozygotes is increased among NTD patients and mothers. All the samples contained both heterozygote mutant allele (MTHFR 677CT/MTHFR 1298AC) which may indicate an increased risk for NTDs as demonstrated by Van der put *et al.*(1998) and Weisberg *et al.* (1998).

In the case of the MS A2756G mutation, all the experimentally degraded samples contained the MS A2756G AG genotype except for samples 6 and 11, which had the MS A2756 GG and MS A2756G AA genotype respectively (Table 13). The medical archive samples consisted of two MS A2756G AG genotypes (samples 13 and 14), two MS A2756G GG genotypes (samples 12 and 15) and one sample with a MS A2756G G allele (Sample 16) for which the full genotype could not be determined (Table 14). The presence of the mutant allele demonstrates a deficiency in MS enzyme activity.

All the experimentally degraded and medical archive samples (except two samples) were found to be heterozygote MTRR A66G AG for the MTRR polymorphism (Table 13). Samples 15 and 16 contained the homozygous GG mutant allele and samples 18 and 21 of the archeological samples contained the G allele, the full genotype could not be reliably ascertained in this sample (Table 13). Wilson *et al.* (1999) detected a significant increase in NTD risk in heterozygotes of the MTRR 66G allele.

The CBS insertion was observed in 6 samples of the experimentally degraded, medical archive and archeological samples (3, 6, 13, 14, 16 and 21) (Table 13, Table 14, and Table 15). A homozygote for the CBS insertion was not observed in any of the samples analysed. The CBS insertion was not observed in combination with the MTHFR 677TT which would indicate an increased risk for NTDs. However, Relton *et al.* (2004) did demonstrate that the CBS insertion may inflate the individual effect of the MTHFR variant. Most of the experimentally degraded and medical archive samples were homozygous without the insertion (Table 13 and Table 14).

There is controversy regarding the linkage of these alleles to the risk of NTDs. There are no interpretations or conclusions presented here that link the specific genotype to NTDs. In my research the focus was on developing a methodology to analyse multiple mutant SNP that have been reported in the literature. Linking the genotype to NTDs would require larger sample numbers and more information on the part of the test subjects.

The archeological samples (Table 15) containing the mutation alleles (either in heterozygous or 'homozygous' state) may indicate that these mutations combined with dietary stress and lack of folate support the morphological manifestations indicating spina bifida. This is evident in sample 21 which contains all the mutant alleles.
A nutritional profile or the frequency of the alleles within the populations are not available for the archaeolgical samples and this study contains a relatively small sample size; therefore one must be cautious when drawing conclusions from these results regarding a link to NTDs.

5 Conclusion and Future Considerations

In this research I developed a novel methodology to identify simultaneously the genotype of five genetic target areas that have been linked to NTDs. In the past, researchers have studied either one to three mutations linked to NTDs at a time. This methodology is the first of its kind to detect all five mutations simultaneously. This approach allows the association between the mutations and NTDs as well as the association between each mutation to be determined. This methodology can therefore assess the compound effect of each marker studied here that has been linked to NTDs.

This method has also been assessed on samples exhibiting various levels of DNA degradation. Four different sets of samples exhibiting different amounts of degradation were analysed which tested the sensitivity and specificity of the technique. The designed method employs three steps of analysis: an initial multiplex PCR, a hemi-nested multiplex PCR and a multiplex SNE-PCR. High quality DNA samples may not require the nested-PCR step, as this step is present to increase the sensitivity and specificity which is not required in high quality DNA samples. The experimentally degraded samples, medical archive samples and archeological samples were also analysed. Full genotypes were generated for the experimentally degraded samples and the medical specimens however a full profile was generated for only one of the archeological samples.

More work could be done to further optimise the methodology especially on the archeological samples. To complete method validation, this study must be repeated using more samples and to specifically determine the matrix effects, limitations, marker frequency, sensitivity and specificity. Sequencing of one individual for testing and

optimizing PCR conditions, and for estimating effectiveness of the methodology could not eliminate the possibility of allelic drop-out. Therefore, in order to validate the method, numerous clinical samples with and without NTDs should ideally be sequenced and SNE genotyped to ensure that all alleles are represented in the test. This would also allow the rate of allelic drop-out to be calculated and the methods success to be evaluated. Furthermore, the method should be applied to a larger sample size of modern individuals affected by, or related to an individual with spina bifida, or various forms of NTD from various ethnic backgrounds. Once validated, medically archived and degraded samples from areas with high prevalence of NTD can be analysed. Finally, my research demonstrates how this methodology can increase the sensitivity and specificity of the detection of disease related genetic mutations. My research could be further expanded to include other conditions associated with point mutations allowing the technique to be applied to archived medical specimens, medical screening, association studies, population mapping and evolutionary disease studies. In particular, my technique optimised for degraded DNA can analyse medical archived specimens: old blood donor cards, archived medical biopsies and archived clinical slides. The ultimate generation of medical pedigrees can aid clinical medicine in developing patient's family history and possible treatment.

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Appendices

Appendix – A

Below are schematic representations of the initial and hemi-nested PCR primers and their genetic locations using the computational simulator. Figures 49 to 53 represent the position of the primers and the lengths of the amplicons for both the initial and hemi-nested singleplexes along the target regions. The blue and red arrows indicate the forward and reverse primers respectively. The black rectangle represents the amplicon amplified and the primers used. For example, in Figure 49, primers CBS-1 and CBS-2 are used for the initial PCR while primer CBS-2 and CBS-3 are used for the hemi-nested PCR producing an amplicon of 128 bp and 83 bp respectively.

Figure 49 – CBS Primer position

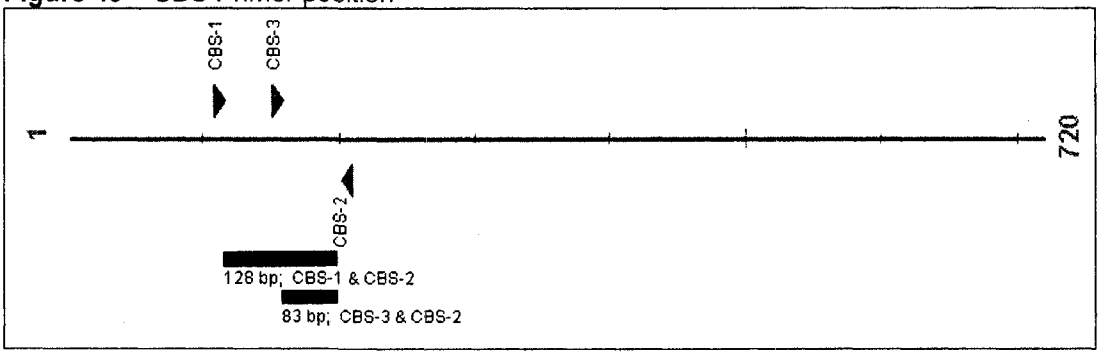


Figure 50 – MS Primer Position

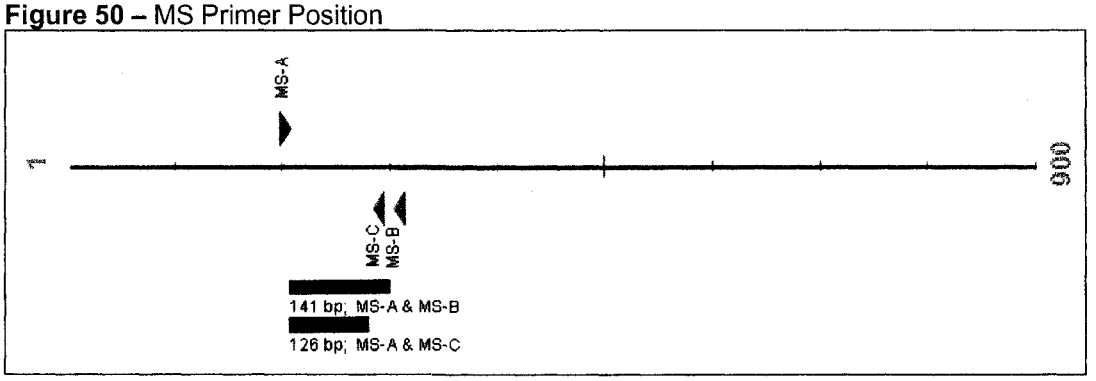


Figure 51 – MTHFR A1298C Primer Position

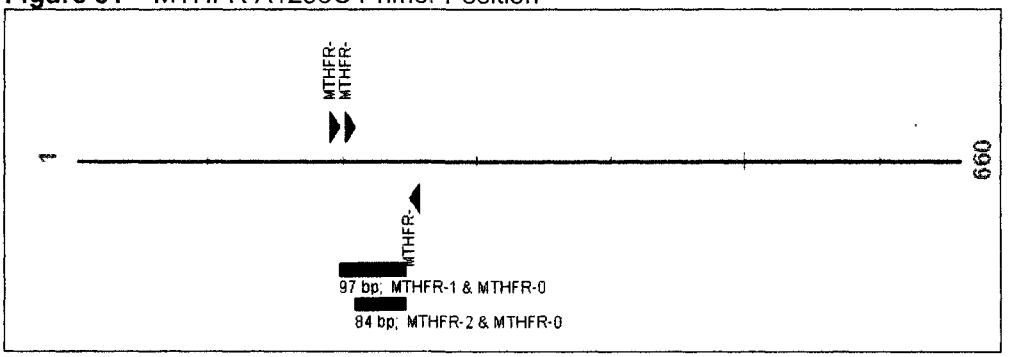


Figure 52 – MTHFR C677T Primer Position

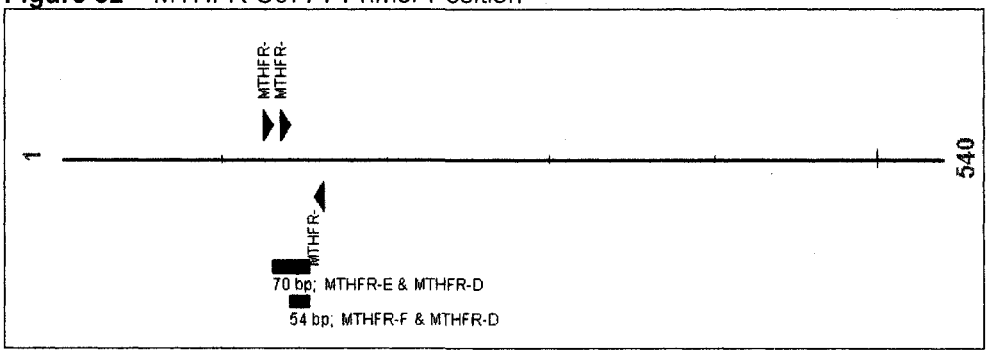
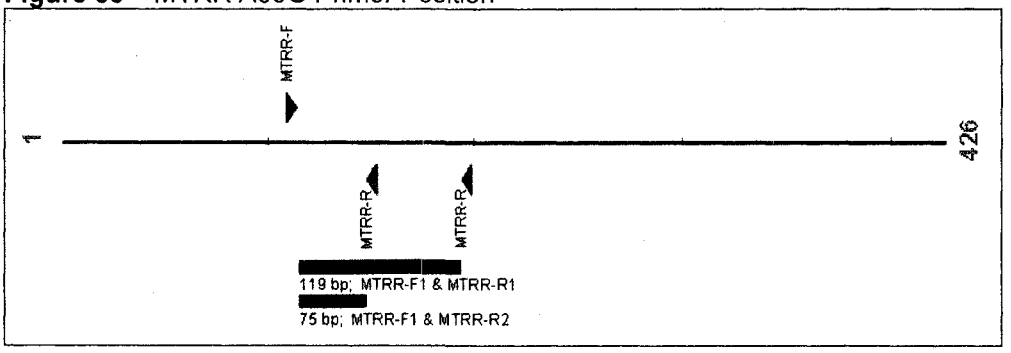


Figure 53 – MTRR A66G Primer Position



Appendix – B

Preparing a 6% Polyacrylamide gel (PAGE):

1. Keep on ice at all times!

Reagents	6 gels	12 gels
5 X TBE	10 mL	20 mL
Acrylamide	6.25 mL	15 mL
ddH_20	33.25 mL	64 mL
TEMED	45 µL	90 µL
10% APS	450 µL	900 µL

2. Prepare 10% APS solution by mixing 0.1 g. ammonium persulfate (APS) to 1 mL of ddH_20.
3. Place a 125 mL Pyrex flask in an ice box and allow cooling for approximately 20 minutes.
4. Set up gel cassettes and combs ahead of time.
5. Add reagents in the same order as they are listed above.
6. Swirl the flask to mix the reagents.
7. Fill gel cassettes from one corner using a transfer pipette.
8. Ensure the bottom cassette groove is completely filled and does not have any air bubbles.
9. Allow to solidify for 1 hour at room temperature, and then refrigerate.

The flask was swirled once all the reagents were added to mix them. A transfer pipette was used to fill the gel cassettes until the liquid reached the bottom cassette groove. Caution was taken to ensure that no bubbles were in the cassette. A gel comb was placed in the cassette to form the wells. The gels were allowed to solidify for 1 hour at room temperature then refrigerated. The electrophoresis tank was then prepared to run a gel. A prepared gel was taken and the strip of tape at the base of the cassette and the gel comb were removed. The gel cartridge was placed in the electrophoresis tank. Spacer blocks or empty cartridges were used if it was only necessary to run one gel. The tank was filled with 1 X TBE running buffer until the wells were covered.

10 X TBE, diluted to a 1 X concentration, was prepared using:

Reagent	Volume
ddH_20	1 L
TRIS	108.0 g
Borate	55.0 g
EDTA	5.8 g

The wells were cleaned out with a transfer pipette to ensure that no particles were in the wells that could impede the running of the samples. 6-7 µL of the amplified PCR products was mixed with 3 µL of 6 X loading buffer. The loading buffer contains ficoll, a substance that allows the DNA sample to sink to the bottom of the well, and a dye that allows visualisation of the sample being loaded. The 6 X loading buffer is prepared with the following reagents:

Reagent	Volume
2.5% xylene cyanol	12 µL
2.5% bromophenol blue	12 µL
Ficoll (35%)*	432 µL
1 X TBE	544 µL
*Mix 3.5g of solid Ficoll to 10mL ddH_20.	

A standard molecular marker is loaded on each gel for band size discrimination.

Loading and Running PAGE

1. Obtain gel cartridges and remove strip of tape of gel comb.
2. Place gel cartridge into gel apparatus.
3. Fill apparatus with 1 X TBE running buffer. To make 1 X TBE buffer dilute 10 X TBE (1 L ddH_20; 108 g Tris; 55 g borate; 5.8 g EDTA).
4. Make sure the running buffer covers the wells inside the apparatus and is 1/3 full in the outside cartridge compartments.
5. Clean out the wells with a transfer pipette.
6. Fill out gel loading sheet.
7. Fill in wells with sample and 6 X loading dye, and one lane of molecular marker.
8. Set voltage at 135 V and time at approximately 30 minutes.
9. After gel has run, stain with ethidium bromide (~15-20 minutes).
10. Wash gel with ddH_20 then viewed on the transilluminator (UV-B) and photograph.

Appendix – C

DyeEx™ purification spin column Protocol

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn to ensure the seal is broken. This is necessary to avoid a vacuum inside the spin column.
3. Snap off the bottom of the column and place the column into a collection tube (provided by manufacturer).
4. Centrifuge at 2800 r.p.m. for 3 minutes.
5. Discard the collection tube with the packing buffer.
6. Place the column into a sterile 1.5 mL tube.
7. Carefully add 10-20 μL of PCR solution (sequencing reaction) to the gel bed.
8. **Pipette the sequencing reaction directly onto the center of the slanted gel-bed surface. Do not allow the reaction mixture or the pipette tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flows down the sides of the gel bed. Avoid touching the gel-based surface with the pipette tip.
9. Centrifuge for 3 minutes at 2800 r.p.m..
10. Discard the column and kept the tubes.
11. Dry the samples in a vacuum centrifuge for sequencing. No need for dessication for Snapshot™.