MICROSATELLITE INSTABILITY IN COLORECTAL AND OESOPHAGEAL CANCER

by

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This thesis is dedicated to:

NIRI, KIMERA AND NARINA
WITH LOVE

PREFACE

The experimental work described in this thesis was carried out in the Department of Pathology and the Molecular Biology Research Facility, University of Natal Medical School, Durban, from March 1996 to June 1998, under the supervision of Professor Runjan Chetty.

The studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of others it has been duly acknowledged in the text.

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ABSTRACT

The development and progression of carcinogenesis is a major area of interest to many scientists. Numerous factors, including both environmental and genetic have been implicated in the causation of cancer. It is clear that both these factors and others contribute to neoplastic development and progression.

Microsatellites are short tandem repeat sequences which are located in the intron segments of the genome. These noncoding sequences range from 2 to 6 base pairs. An increase or decrease in the number of repeat sequences is referred to as microsatellite instability, also referred to as genetic instability.

It is thought that microsatellite instability arises as a result of defects in DNA repair process. During DNA synthesis, the DNA repair genes ensure that the correct nucleotide is incorporated into the newly synthesised DNA strand, so when a mismatch base is incorporated, this is promptly removed and replaced with the correct base. However, if the repair system is defective this would give rise to numerous genetic aberrations along that region of the genome.

Recently, microsatellite instability and allelic imbalance/loss of heterozygosity have been shown to play an important role in the development of many cancers, especially colorectal cancer (CRC) associated with the hereditary nonpolyposis colorectal cancer (HNPCC) syndrome.

This study was undertaken to investigate microsatellite instability and allelic imbalance in colorectal and oesophageal carcinomas in the KwaZulu Natal region of South Africa. The molecular analysis was correlated with clinicopathological data to establish a baseline level on which further studies could be performed. In addition, this study represents the first fluorescent based microsatellite analysis of these two common cancers in South Africa.

Normal and tumour DNA was isolated from formalin fixed paraffin embedded tissue. Fluorescent-based DNA technology using an automated DNA sequencer (Alf Express Automated DNA Sequencer) was employed. CY5 labelled primers for microsatellite markers (DCC, D18S34, D18S58, D3S659, D2S123 and D3S1255) were used. The data was captured and analysed using the Fragment Manager Software.

The informativity of the microsatellite markers used in this study ranged from 50% to 71.8%. LOH/AI in the region of the *DCC* gene in the under 35 years of age CRC was 39.1%, while MSI in this region occurred in 31.25% of cases. The DNA repair gene status in these young patients was as follows: LOH/AI: 31.3% and MSI: 40.4%.

In the over 50 years of age CRC, LOH/AI in the 18q region was 28% and MSI was 38%. The DNA repair genes (hMSH2 and hMLH1) in this cohort showed LOH/AI in 24% and MSI also in 24%.

As regards oesophageal cancer, LOH/AI in the 18q region was 20.5% and MSI 7.7%. The repair genes showed LOH/AI in 17.9% and MSI in 10.25% of cases. When the molecular events were correlated with clinicopathological features, no statistically significant pattern emerged. However, it must be remembered that relatively small numbers of cases (39) were analysed.

In conclusion:

- No statistical correlation was found between clinicopathological characteristics and the molecular analysis in either CRC and oesophageal cancer.
- LOH/AI and MSI was higher in the under 35 age group.
- LOH/AI and MSI in 18q, 2p and 3p in sporadic CRC were similar to other fluorescent-based studies in patients over 50 years of age.
- LOH/AI and MSI in 18q, 2p and 3p in oesophageal cancer was similar to studies from other geographical areas.
- Finally, fluorescent-based microsatellite PCR and analysis was found to be an objective and efficient technique.

PUBLICATIONS

1. Allelic imbalance and microsatellite instability of the DCC gene in colorectal cancer in patients under the age of 35 using fluorescent DNA technology
   R Chetty, **R Naidoo** and J Schneider
   J Clin Pathol (1998); **51**: 35-38.

2. Allelic imbalance and microsatellite instability at microsatellite loci located on chromosome 2p, 3p and 18q in oesophageal squamous carcinomas from South Africa
   R. Naidoo, M. Tarin, A. Reddi and R. Chetty
   Human Pathology (submitted for publication)

3. The application of microsatellites in Molecular Pathology. **R. Naidoo,** R. Chetty. **Pathology Oncology Research** (In Press).

4. DNA repair gene status in oesophageal cancer. **R. Naidoo**, R. Chetty. **Diagnostic Molecular Pathology** (In Press).

5. Genetic instability in colorectal cancer. **R. Naidoo**, M. Tarin, R. Chetty. **Journal of Pathology** (submitted).

PRESENTATIONS AT SCIENTIFIC MEETINGS

1. **R Naidoo** and R Chetty
   Microsatellite instability " Proof-reading gone wrong".
   36th Congress of the Federation of South African Societies of Pathology.
   Mpumalanga -June 1996.

2. **R. Naidoo** and R. Chetty
   Assessment of allelic imbalance (AI) and loss of heterozygosity in the DCC gene using fluorescent based DNA technology.
   37th annual Congress of the Federation of South African Societies of Pathology. Cape Town - June 1997.

3. **R. Naidoo** and R Chetty
   Assessment of allelic imbalance (AI), loss of heterozygosity (LOH) and microsatellite instability of the *DCC* gene in colorectal cancer under the age of 35 using fluorescent based DNA technology.
   Faculty Research Day, Natal University Medical School - September 1997
   PRIZE FOR THE BEST SCIENTIFIC PRESENTATION

4. **R. Naidoo** and R. Chetty
   Assessment of allelic imbalance (AI), loss of heterozygosity and microsatellite instability of the DCC gene in colorectal cancer under the age of 35 using fluorescent based DNA technology.
   4 th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association. Maui, Hawaii - February 1998.

5. **R. Naidoo** and R. Chetty
   The use of microsatellite PCR and fluorescent DNA technology to detect allelic loss/microsatellite instability in oesophageal cancer .
   FUTUREPATH 98 -38th annual Congress of the Federation of South African Societies of Pathology. Pretoria - July 1998.

6. **R. Naidoo,** M. Tarin, A. Reddi and R. Chetty
   Genetic instability in oesophageal cancer in 6 microsatellite loci located on chromosome 2p, 3p and 18q.
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GLOSSARY/ABBREVIATIONS

A,C,G,T,U	adenine, cytosine, guanine, thymine, uracil
Abs	absorbance
AI	allelic imbalance
APC	adenomatous polyposos coli
APS	ammonium persulphate
BER	base excision repair
CAT	chloramphenicol acetyltransferase
CRC	colorectal cancer
DCC	deleted in colorectal cancer
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylene diamine tetra-acetic acid
g	acceleration due to gravity
g	grams
H	homozygous
HMRDS	hereditory mismatch repair deficiency syndrome
HNPCC	hereditory non-polyposis colon cancer
L	litre
LOH	loss of hetreozygosity
mA	milliamps
MCC	mutated in colorectal cancer
mg	milligrams
ml	millilitre
MMP-2	metalloproteinase 2
MSI	microsatellite instability
NAI	no allelic imbalance

NER	nucleotide excision repair
nm	nanometres
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
pmol	picomoles
RER	replication error
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulphate
SSCP	single stranded conformational polymorphism
Taq	*thermus aquaticus*
TBE	tris borate ethylene diamine tetra-acetic acid
TE	tris/EDTA
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet
V	volts
VNTR	variable number tandem repeats
W	watts
μ	micron
μg	micrograms
μl	microlitre
μm	micrometre

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CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

We are in the midst of a biomolecular revolution in which DNA recombinant technology has spawned the domain of molecular medicine. This new discipline is a combination of modern medicine and basic science. It has led to an exponential increase in our understanding of the processes governing cell growth and differentiation and the defects that occur in this, normally, tightly regulated process.

The faithful transmission of genetic information from one generation to the next is paramount to both the normal functioning and survival of the cell. There are numerous mechanisms that exist in a cell's cycle to ensure that this control occurs. However, sometimes these control processes fail, resulting in genetic alterations which ultimately have deleterious consequences to the host cells.

One of the fundamental features of malignant cells is that they continue to divide while normal cells do not proliferate. It is clear that in the malignant cells the genetic control processes have failed. This may be due to aberrant expression of genes that are inactive in the normal cell. There are two kinds of regulatory genes that exist in the cell: those that promote growth, called oncogenes, and those that suppress growth, called tumour suppressor genes. Changes in the expression of these genes may lead to the development of cancer.

It is now known that one of the causes of tumour development and progression, is a result of numerous genetic changes that occur in the cell. Recently, there has been a significant increase in the number of studies which have focused on the molecular and genetic events in these disease conditions. This is due to the improved and increased understanding of the genetic basis of disease and new molecular biology techniques currently available to carry out such projects.

These genetic changes may be due to either activation or inactivation of the regulatory genes. However, the exact mechanism and molecular origins of such changes are still not clear. Recent studies show that alterations due to mutations in the simple repeat sequences or microsatellites are a feature in some tumours (Brentnall, 1995; McCarthy, 1995). It is envisaged that assaying for microsatellite instability at hot spots at different chromosome loci may be important in identifying a substantial fraction of human cancers in addition to gaining greater insight to the underlying pathogenic mechanisms. Further, it may be a good prognostic indicator in cancer patients.

With this in mind, a study analysing microsatellite instability and loss of heterozygosity (LOH) was carried out in sporadic cases of colorectal cancers in patients under the age of 35, patients over 50 years of age and oesophageal carcinomas seen at King Edward VIII Hospital, Durban, South Africa. Microsatellite analysis and LOH was done with the use of The ALFexpress DNA automated sequencer. The ALF Manager and Fragment Manager software packages were used to analyse the data obtained. An attempt to correlate the histopathological and clinical features with the microsatellite analysis and to compare these findings with other studies was undertaken. This represents the first study in South Africa utilising this technique in this group of tumours.

Important genes in tumour biology include tumour suppressor genes and the DNA repair genes. Alterations in these genes have been implicated in many cancers, but specifically the *DCC* (deleted in colorectal cancer), the *APC* (adenomatous polyposis coli) and DNA repair genes are important in the pathogenesis of both colorectal and oesophageal cancers. The genetics of cancer in South Africa cancers is largely unknown, and since this is an important geographical focus for oesophageal cancer, knowledge of molecular aberrations is important. Furthermore, it is important to know if there, indeed, is any geographic variation in the genetics of these cancers. An additional facet of the study is to explore the relationship, if any, with the traditional established pathological prognostic parameters.

1.2 HISTORICAL PERSPECTIVE

Biological science has been revolutionised by a series of new investigations and techniques which have developed over the last 20 years. These techniques allow scientists to manipulate molecules that are critical to many processes such as cell growth, division, differentiation and development.

Nucleic acids are composed of *ribonucleic acids* (RNA) and *deoxyribonucleic acids* (DNA). DNA together with RNA and the nuclear proteins interact to regulate all the activities in the cell. DNA has been referred to as the molecule of life. It is composed of four nucleic acids (adenine, A; cytosine, C; guanine, G and thymine, T), a deoxyribose sugar moiety and a phosphodiester linkage. Each strand is a string of nucleotides that are linked between the 5' carbon of the deoxyribose sugar of the one nucleotide to the 3'carbon of the sugar moiety of the neighbouring nucleotide.

This genetic information is arranged in units called genes, which contain the hereditary traits of an organism. The exact duplications of these genes ensure the genetic continuity from one generation to the next. The genetic code for any gene is copied or transcribed into RNA templates called messenger RNA (mRNA). The mRNA which carries the exact code ensures the accurate, stepwise assembly of amino acids into proteins. This process is referred to as translation. This entire process is highly regulated and accurate, and is responsive to the specific needs of the body. In the normal cell this status quo is maintained by multiple tumour suppressor genes, multiple oncogenes, cell cycle regulators and DNA repair genes.

Genetic alteration in any of these genes could trigger off a cascade of events which would eventually result in the growth of cells in inappropriate locations or to propagate indefinitely, ultimately resulting in the development of cancers (Land *et al.*, 1983; Fearon and Vogelstein, 1990).

A landmark discovery that changed the complexion of research, especially in pathology, was that of the polymerase chain reaction. This technique, now made it possible to amplify DNA from archival tissue (Bianchi *et al.*, 1991; Going and Lamb, 1996). Genes influencing tumourigenesis can be isolated from formalin-fixed paraffin embedded tissue. Researchers now had at their disposal a tremendous amount of material, that sparked the beginning of new ideas and protocols for investigation.

Routinely, the diagnosis of tumours is made by histological examination. This has been, and still is, the gold standard. However, with the advent of molecular biology techniques such as immunocytochemistry, *in-situ* hybridisation, sequencing, single-stranded conformational polymorphism and microsatellite analysis, "hot spots" (sites at which the frequency of mutations is increased and commonly found) in different chromosomes could now be examined, providing a greater understanding of the biology of the disease. Further, the collective results obtained from the histological examination, immunohistochemistry and microsatellite analysis could have prognostic significance.

1.3 COLORECTAL CARCINOGENESIS

1.3.1 DIETARY AND GEOGRAPHICAL FACTORS

It is clear that colorectal cancer is a major cause of death in the Western world and increasing in so-called Third World Countries. There is marked inter-population variation in the incidence of the disease, with the highest incidence rates recorded in populations with a high socio-economic standard and low rates in economically deprived areas, even in urban centres in the west.

Diet seems to play an important role in the causation of the disease. High fibre diets generally prevail in populations where colon cancer is infrequent. The faster transit time associated with such diets has been suggested to prevent the action of any lumenal carcinogens (Burkitt, 1971). Others, however, have failed to show an association between the amount of fibre in the diet, transit time and colon cancer (Ward *et al.*, 1973). Thus the effect of indigestible fibre in diets is still not clear.

On the other hand, high fatty diets have been shown to correlate with high incidence of the disease (Wynder, 1975). It has been suggested that high fat intake leads to greater production of cholesterol and cholesterol metabolites which may be carcinogenic. In countries such as the United States and Argentina, where there is a high consumption of meat, the colon cancer risk is exceptionally high. Faeces from persons in high-risk populations have higher counts of *Bacteroides* and lower counts of aerobic bacteria, than in populations where colorectal carcinoma is uncommon. Among the important metabolic functions of intestinal bacteria, particularly *Bacteroides*, is the degradation of bile salts with the production of potential carcinogens. Further, some species of *Clostridia* have been shown to be excessively frequent in patients with colon cancer (Hill *et al.*, 1971).

1.3.2 GENETIC FACTORS

In addition to dietary factors, genetic factors need to be built into the equation when considering the aetiology of colorectal carcinoma. Application of modern molecular techniques has significantly extended the understanding of the genetic events that underlie colorectal carcinogenesis (Fearon and Vogelstein, 1990; Aaltonen *et al.*, 1993; Cawkwell *et al.*, 1995; Chetty *et al.*, in press). These advances have already provided some new prognostic indicators for colorectal cancer.

Our understanding of colorectal cancer has been greatly facilitated by two important factors. Firstly, it has been recognised that colorectal carcinomas usually develop from adenomas. This is the so-called adenoma-carcinoma sequence (Morson, 1974). Secondly, relatively common inherited forms of colorectal cancer have been well described. In these cases, molecular analysis of colorectal carcinogenesis of the affected families would be ideal. Familial adenomatous polyposis (FAP) is one of the best characterised syndromes of inherited colorectal cancer (Bodmer *et al.*, 1987; Erisman *et al.*, 1989). This form of the disease is recognised by the widespread development of thousands of adenomatous polyps in the colon and rectum. If these polyps are not removed these individuals inevitably develop colorectal cancer, which is usually by the age of forty years.

Two other hereditary forms of colorectal cancer have also been described: site specific colon cancer and cancer family syndrome, referred to as Lynch type I and Lynch type II, respectively (Lynch *et al.*, 1993). Both these are collectively referred to as hereditary non-polyposis colon cancer (HNPCC). The condition is said to be inherited in an autosomal dominant fashion, and approximately 70 % of affected individuals develop colorectal cancer.

There are several types of genes that have been shown to be involved in the development and progression of cancer. Broadly, these are tumour suppressor genes, oncogenes and mismatch repair genes. Mutations in these genes through DNA damage have been implicated in the pathogenesis of cancers. However, it is clear that a single mutation alone is not responsible and/or sufficient, but a series or a total accumulation of mutations results in the final malignant state.

Knudson (1971), proposed his "two hit" hypothesis for the development of retinoblastoma, which states that in the hereditary form of retinoblastoma, inheritance of an inactivating germline mutation in one allele of the retinoblastoma gene together with a somatic mutation in the other allele, occurs in the same cell, and this could give rise to retinoblastoma. The development of sporadic retinoblastoma on the other hand, requires two somatic mutations.

Further, the genetic model for tumourigenesis by Fearon and Vogelstein (1990), indicates that tumours appear to arise from a series of mutational events. These events include activation of oncogenes and inactivation of tumour suppressor genes. Since tumour suppressor genes achieve their oncogenic effects by inactivation of both normal alleles, it is possible to identify tumour suppressor gene loci through loss of heterozygosity (LOH) studies. These investigations look at loss of allelic markers in tumour tissue as compared to normal tissue from the same patient.

Since the progression of colorectal cancer is thought to arise as a result of a series of mutations, it is necessary to investigate a number of genes, both tumour suppressor genes and oncogenes, which are located on various loci on different chromosomes. The most commonly investigated genes in colorectal cancer are *p53, DCC, MCC, K-ras,* and *APC.* An outline of the molecular events in the development of colon cancer is shown below (Nofsinger and Fenoglio-Preiser, 1995).

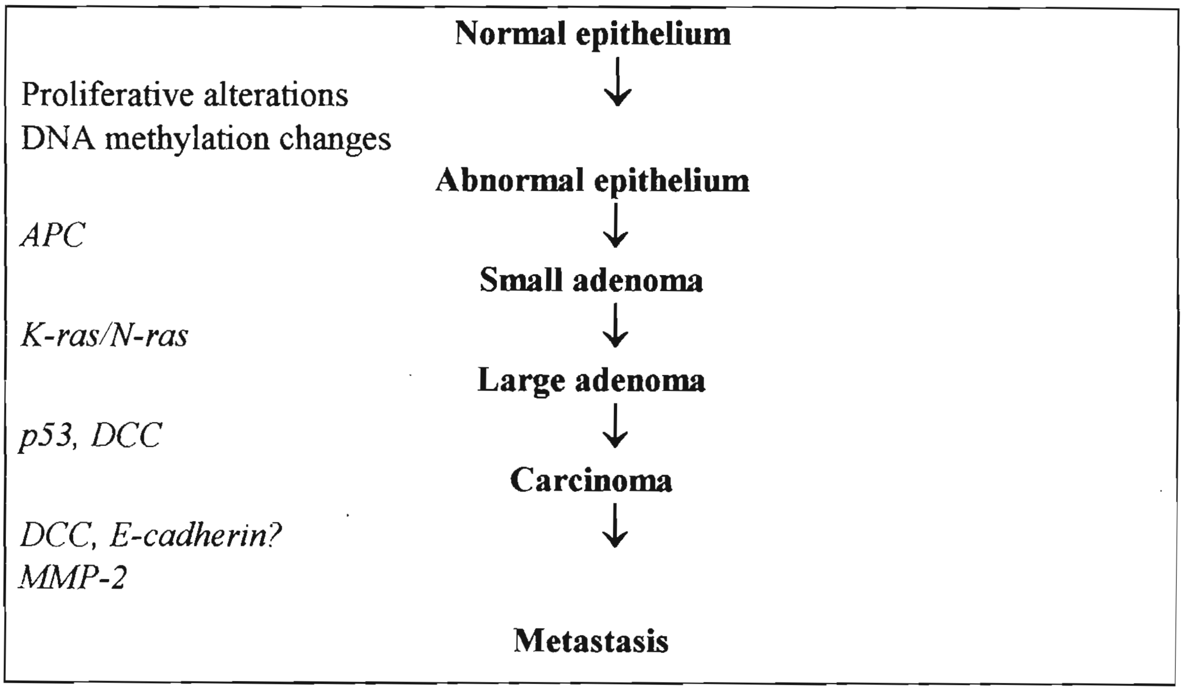


Figure 1: Diagrammatic representation of the molecular events which are known to be significant in the development of colorectal cancer. The earliest changes involve alterations in proliferation and DNA methylation. Mutations and/or allelic loss in *APC* gene probably play a role in the transition from hyperproliferative mucosa to adenoma. Mutations in the *ras* gene are thought to play a role in the growth and progression of the adenoma. *p53* and *DCC* mutations are late events in neoplastic progression. Less is known about other factors important in the progression to malignancy.

1.3.2.1 *APC* GENE

The clinical condition of FAP has been linked to mutations in the *APC* gene. This gene has also been directly implicated in colorectal carcinogenesis, where it is thought to be capable of initiating colorectal adenoma formation.

Early studies by Herrera *et al.* (1986), showed that the gene was located on the long arm of chromosome 5. Cytogenetic analysis revealed a large deletion on the long arm of chromosome 5 (5q21-22) and this region provided a strong candidate region for the *APC* gene. Later, RFLP analysis (Bodmer *et al.*, 1987; Leppert *et al.*, 1987) in FAP families using polymorphic DNA probes, mapped the region to chromosome 5. The probes were mapped to the 5q21 region of chromosome 5, using *in situ* hybridisation techniques.

The *APC* gene was also investigated in sporadic and inherited colorectal cancers using polymorphic probes which were mapped to chromosome 5. Allelic deletions were found in twenty two percent (10/44) of sporadic tumours. (Solomon *et al.*, 1987). Deletions in the region of the *APC* gene were shown in 29% of colorectal adenomas and 36% of colorectal carcinomas (Vogelstein *et al.*, 1988). In subsequent studies, using probes closer to the *APC* locus, it was shown that deletions occurred in over 50% of colorectal cancers (Miki *et al.*, 1991). Detectable changes in the *APC* gene were also observed by Miyoshi *et al.*, (1992) in approximately 60% of both adenomas and carcinomas. However, in a study of 26 cases of colorectal adenocarcinomas, allelic loss at the *APC* locus was found in 40% (Cawkwell *et al.*, 1993). In the latter study, fluorescent-based DNA technology was adopted to analyse the data.

These studies clearly indicate that the *APC* gene is located on chromosome 5 and that deletions in the gene are a common occurrence in colorectal adenomas and carcinomas.

1.3.2.2 *p53*

The tumour suppressor gene, *p53* which is located on the short arm of chromosome 17, is one of the genes most commonly involved in human malignancy (Harris, 1993).

p53 controls cell proliferation and alteration of growth regulatory functions. During DNA damage *p53* mediates arrest at the G1 phase of the cell cycle in order to allow repair to occur, however if the damage is too great, then *p53* induces apoptosis (programmed cell death). In this way mutations are prevented from being passed on to the daughter cells. Therefore, loss of normal *p53* function, will prevent this important check-point function causing genetic instability to the cell.

Mutant p53 protein can be demonstrated in both the nucleus (Fearon and Vogelstein, 1990) and cytoplasm (Bosari *et al.*, 1995). Mutant p53 contributes to the process of cell transformation by loss of gain of function. Further, it has a dominant negative effect on wild-type p53, and therefore promotes cell proliferation rather than overriding the normal suppressor function (Milner *et al.*, 1995). Loss of normal p53 function results in decreased DNA repair and consequently increases mutagenesis.

This gene consists of at least 8 exons, with exons 5-8 the so-called "hot spots" of this gene, where most point mutations occur. The *p53* gene encodes a 53 kD nuclear phosphoprotein which is involved in cell cycle regulation. The protein is 393 amino acids in length and exists as a complex composed of four molecules (tetramer). It is postulated that the binding of the tetramer to a p53 binding site, activates the expression of adjacent genes, which in turn inhibit growth or invasion.

Structural studies indicate that the p53 protein has several functional domains that are involved in regulation. The N-terminal domain is said to have a transcriptional activator function. Fields and Jang (1990) noted that there was an acidic domain close to the N-terminus which was similar to other well described transcription factors. DNA binding studies show that the acidic domain could activate transcription from a *GAL4* operon. Further, the activation domain has been mapped to the region lying between codon 20 and 42 (Miller *et al.*, 1993; Unger *et al.*, 1992). These findings suggest that p53 is involved in transcriptional control, either via a complex formation with other proteins that bind to specific genes, or directly on its own.

There are several ways in which normal *p53* expression is lost. Mutations in the *p53* gene are common, resulting in the production of a truncated protein, which is unable to form the tetramer. A deletion in one or both of the alleles will result in a decrease in the concentration of p53 protein, which would be below that required for normal function. Mutations in the *p53* gene, resulting in alteration of the alleles could give rise to the production of a protein tetramer which is inactive.

Genetic alterations in the *p53* gene have been demonstrated by the use of various molecular biology techniques. Immunohistochemical staining, which detects p53 protein expression is perhaps the most popular method used. Mutations in the gene are detected with the use of PCR, SSCP and sequencing analysis.

Mutations in the *p53* gene have been identified in colorectal cancer and other cancers and are said to occur in specific conserved regions of the gene. These mutations are present in well over 50% of colorectal cancers (Nigro *et al.*, 1989). Gross overexpression of the protein was demonstrated using immunohistochemical staining of primary colorectal carcinomas with antibodies specific to p53. Over 50% of the malignant tissue examined showed overexpression of the protein and this was thought to be synonymous with mutations in the gene (Rodrigues *et al.*, 1990). However, subsequently it had been shown that overexpression of the p53 protein does not necessarily reflect abnormalities of the gene. This was shown in a study in which p53 protein expression was seen in sporadic colorectal cancers with and without microsatellite instability (Ilyas *et al.*, 1996).

In colorectal carcinomas, it has been shown by the use of monoclonal antibodies that the p53 protein expression was 46.9% (39/83) (Bertorelle *et al.*, 1995). Further, the p53 positive tumours showed a higher incidence of lymph node and liver metastases. The use of multiplex PCR to analyse 20 cases of colorectal adenomas indicate that allelic loss in the *p53* locus is 66% (Cawkwell *et al.*, 1994). Further, other markers used in this study were: *DCC, APC, RB1, NF1, WT1* and *NM23*.

Whilst most studies of colorectal carcinomas found *p53* aberrations to be around 50%, Froggatt *et al.* (1995) found the incidence of mutations and deletions to be low (22% mutations and 36% deletions). This study was carried out on 35 paired normal and tumour colorectal carcinomas. Also, an interesting finding was that the point mutations were all GC to AT transitions, with 7/8 occurring at the CpG dinucleotides.

A study done on 50 colectomy specimens in patients over the age of 50 years showed an inverse relationship between p53 and retinoblastoma proteins (pRb) staining (Chetty *et al.*, in press). Twelve tumours, which showed low levels of pRb, showed high levels of p53 protein. Similarly, 7 tumours with high pRb levels displayed low p53 expression. In terms of immunohistochemical profile, p53 high/pRb low tumours tended to be poorly differentiated and had advanced stage.

1.3.2.3 *DCC* (DELETED IN COLORECTAL CANCER)

The *DCC* tumour suppressor locus on chromosome 18 is another important "hot spot" in colorectal cancer. The gene is extremely large and spans more that 1.35 million base pairs of DNA and encompasses at least 29 exons. It has been predicted that the protein product consists of 750 amino acids. The *DCC* gene codes for a protein product which is thought to be a cell-cell, cell-matrix adhesion molecule (Fearon *et al.*, 1990). Sequencing studies of the *DCC* cDNA indicate that the gene codes for a transmembrane protein with significant similarity to the neural cell adhesion molecule. One or two forms of the gene could occupy a given locus on the chromosome, which is also referred to as a single or double allele, or homozygous and heterozygous, respectively. The homozygous form is said to be non-informative while the heterozygous form is said to be informative. A deletion in parts or segments of the gene will result in loss of function or loss of tumour suppressor activity in that region. This is referred to as loss of heterozygosity (LOH) in genes that carry two alleles. Deletions in this gene are frequent in colorectal adenocarcinomas but infrequent in colorectal adenomas. Although allelic deletions involving chromosome 18 occur in more that 70 % of colorectal cancers (29/41 cases) (Fearon *et al.*, 1990), others show that allelic losses in adenomas range from 14% (1/7) (Maesawa *et al.*, 1995) to 33% of informative cases (Huang *et al.*, 1993). This would suggest that LOH in the *DCC* locus is a late event in colorectal carcinogenesis.

Since the *DCC* gene represents a cell adhesion molecule, any decrease in the expression of this gene will result in altered adhesion and in this way enhance tumour growth and metastatic spread of colonic tumours. LOH in the *DCC* gene was found in 75% of colorectal cancer and is said to occur at the transition point from adenoma to carcinoma (Delattre *et al.*, 1989).

In an attempt to assess the prognostic value of this locus, allelic loss of chromosome 18q was assessed in resection specimens of stage II and III colorectal carcinomas and was found to occur in 70% of the specimens (Jen *et al.*, 1994). Among patients with stage II disease, the five year survival rate was 93% in those that showed no allelic loss in chromosome 18q, however, this figure was 54% in those with allelic loss. However, in patients with stage III disease these figures were 52% and 38%, respectively. Therefore, the status of chromosome 18q has a strong prognostic value in the stage II colorectal carcinomas. The prognosis of stage II cancer with allelic loss was similar to that of stage III cancer without allelic loss. In addition, these patients are thought to benefit from adjuvant chemotherapy. In contrast, patients with stage II disease with no chromosomal aberration have a survival rate similar to that of patients with stage I disease. These patients may not require additional therapy.

In a most recent study of LOH on chromosome 18q (Martinez-Lopez *et al.*, 1998), in which one hundred and forty-four consecutive patients with CRC were analysed at three dinucleotide CA-repeat microsatellites, the incidence of allelic loss at one or more loci was found to be 45%. For the individual markers, LOH was found in 30% of the cases at locus D18S68, 27% at D18S474 and 27% at D18S58. Further, the conclusions from this study indicate that allelic deletions on chromosome 18q are linked to a shorter survival in stage II disease and therefore may provide a useful tool for selecting patients with stage II colorectal cancer for adjuvant chemotherapy. This study confirms the work by Jen and co-workers (1994).

Decreased levels of mRNA expression in the *DCC* gene which was cloned from deleted regions of chromosome 18 was assessed in resected specimens of colorectal cancer by RT-PCR (Itoh *et al.*, 1993). Semi-quantitative measurements of the mRNA indicated a lower expression for the *DCC* gene in 57% of the 30 samples used in this study, when compared to the adjacent noncancerous tissue. Further, all 4 specimens in which liver metastasis was also observed, showed decreased *DCC* mRNA expression.

The analysis of two microsatellite markers for the *DCC* gene in a panel of 35 pairs of normal and neoplastic human colorectal tissue showed low frequency of deletions (41% informative cases) (Froggatt *et al.*, 1995). This was associated with the progression of early (not invading beyond the muscularis mucosae) to late carcinoma (invasion beyond the muscularis propria), rather than with increasing adenoma size. The *DCC* deletions were detected at a higher frequency in distal tumours than in proximal tumours. Further, the two cases in which microsatellite instability was found in both markers, were in tumours of proximal origin.

Deletions in the *DCC* gene have also been noted in 45% of male germ cell tumours (Murty *et al.*, 1994). Two homozygous deletions affecting the *DCC* gene were observed in the 91 tumours studied, and reduced or absent *DCC* gene expression was seen in a subset of germ cell tumour samples. The data suggest that inactivation of the *DCC* gene may contribute to the development of a subset of germ cell tumours.

Allelic losses affecting the *DCC* gene were seen in only 6% of 64 leukaemias (Miyake *et al.*, 1993). Only one case showed a homozygous loss of the *DCC* gene whilst, a reduction or absence of expression was seen in eight of 26 patients with acute myelogenous leukaemia, three of nine patients with acute lymphoblastic leukaemia and seven of 29 patients with chronic myelogenous leukaemia. These observations suggest that *DCC* inactivation may contribute to the development of leukaemia.

1.3.2.4 *Ras* GENE

The *ras* gene is another gene that has been implicated in the multistage progression to cancer. Alterations in the *ras* gene have been studied extensively in colorectal cancer. Messenger RNA transcript levels, the p21 protein production and DNA mutations being the focus of attention at different times. Alterations in the p21 protein levels have been studied using either western blotting or immunohistochemistry. Overall these studies have shown that increased levels of p21 are associated with proliferation of colon cancer.

There are three forms of the *Ras* gene, *K- ras, N-ras and H-ras,* in both man and rats. The three forms of *Ras* are closely related and code for a 21 kDa protein. The Ras proteins are small monomeric proteins that bind GTP. Ras protein bound to GDP is inactive while Ras carrying GTP is active. Descrete domains of Ras are responsible for guanine nucleotide binding and membrane attachment.

All three forms of the gene have been shown to be mutated in colorectal cancer. Activation of *K-ras* in colorectal cancer is usually by point mutation and almost always occurs at the 12th codon (Bos, 1989). Mutations in the *ras* gene have been found in 37-60% of colorectal carcinomas (Bos *et al.*, 1987; Forrester *et al.*, 1987; Vogelstein *et al.*, 1988). In a study of 99 colorectal cancers it was shown that an increase in mutations in *K-ras* correlated with increasing aggressiveness of the tumours (Laurent-Puig *et al.*, 1991).

Whilst mutations in the *K-ras* or *H-ras* account for 32% of colorectal Dukes' B cancers (Oudejan *et al.*, 1991), they occur infrequently (9%) in small adenomas (less than 1 cm), however in adenomas greater than 1 cm there is an increase in the number of mutations (41%) (Vogelstein *et al.*, 1988). In a study by Sasaki *et al.* (1990), *K-ras* mutations were shown to occur in 36% of colorectal cancers and in 12% of FAP cases.

In order to determine the role of *K-ras* and other tumour suppressor genes, a study was undertaken using SSCP, RT-PCR and sequencing techniques, and it was found that 40% of colorectal cancers from surgical specimens had *K-ras* gene mutations (Suzuki *et al.*, 1994).

1.3.2.5 *MYC* GENE

The *myc* gene, which was identified as a cellular homologue of the transforming gene of the avian myeloblastosis virus, is located on chromosome 8q24 and produces two transcripts of 2.2 and 2.4 kb long. The protein products of these are 62 and 64 kD, respectively. Both the polypeptides are phosphorylated on serine and threonine residues and bind to DNA *in vitro*. The function of the protein product is still unknown, however, the structure suggests that they may be involved in transcriptional regulation or in some way function as an auxillary factor for DNA polymerase. *myc* mRNA and p62/64 are frequently increased in colorectal cancers (Finley *et al.*, 1989; Rowley *et al.*, 1990). Immunohistochemical studies using antibodies to p62 have confirmed the presence of high levels of *myc* mRNA. *myc* overexpression is much higher in left-sided colonic cancers and has been demonstrated in 22 out of 27 cases (Rothberg *et al.*, 1985). This study suggests that these may represent a subgroup of tumours with a different aetiology to right-sided lesions. Raised *myc* levels were also shown in 50 % of colorectal tumours which also showed deletions in the region of the *APC* gene (Erisman *et al.*, 1989). However, these *APC* deletions were not seen in tumours which did not show raised *myc* levels. Significant increases in *myc* mRNA expression was shown in about two-thirds of adenomatous polyps (Finley *et al.*, 1989), and this was also confirmed by *in situ* hybridisation. Increased transcription of *myc* is associated with an increased tendency to malignancy of polyps (Imaseki *et al.*, 1989; Pavelic *et al.*, 1992).

1.3.2.6 *MCC* GENE (MUTATED IN COLORECTAL CANCER)

The *MCC* gene was initially thought to be responsible for FAP and was identified by Kinzler *et al.* (1991). Using RFLP analysis, rearrangements in the 5q21 region was identified in colon tumours. This region was subsequently shown to lie within the *MCC* gene. Although this was obviously close to the *APC* gene, there was no evidence of mutations within FAP families, however, it was later demonstrated that mutations did occur in the *MCC* gene in this setting (Nishesho *et al.*, 1991). These mutations occur in at least 15% of colorectal carcinomas, and deletions in over 40% of cases. This was shown by LOH analysis (Miki *et al.*, 1991), however, this study was done prior to the realisation that *MCC* was in close proximity to the *APC* gene. The exact role or function of *MCC* in the development of colorectal carcinomas is not known. It may play a role in complex formation, together with *APC* through the same pathway.

1.3.2.7 MICROSATELLITES

Alterations due to mutations in the simple repeat sequences or microsatellites are a feature in many tumours (Todd, 1992; Horii *et al.*, 1994; Brentnall, 1995). It is envisaged that assaying for microsatellite instability may be important in identifying aberrations in a substantial fraction of human cancers (McCarthy, 1995).

The eukaryotic genome contains not only introns and exons, but also large numbers of copies of other seemingly nonessential DNA (over 90%). This is occasionally referred to as “junk DNA” in the literature. These DNA sequences do not code for any protein and in many regions are “repetitious”. The variation within these repetitious stretches of DNA are so great that each human, for example, can be distinguished by a “DNA fingerprint”. This “fingerprint” is based on the variation in the repetitive sequences.

The presence of these repeat sequences in higher eukaryotes was first revealed by hybridisation experiments. In this procedure the genome is mechanically broken into short fragments of double stranded DNA molecules which are about 1000 base pairs long. The fragments are then denatured to produce single-stranded DNA. Under conditions that favour reannealing, the speed with which these single-stranded DNA reanneal, is dependent on the number of complementary regions each fragment finds. This is usually a slow process, however, when the DNA from a human cell is analysed in this manner, about 70% of the DNA strands reanneal slowly, as is expected. The remaining 30% of the DNA strands anneal much more quickly. The reason for this is, that these strands contain sequences that are repeated many times in the genome and find their complementary strands relatively rapidly. About a third of all these repeat sequences are short tandem repeat sequences referred to as microsatellites which are scattered throughout the human genome. In one of the early studies (Hamada *et al.*, 1982), huge tracts of dT-dG alternating sequences were identified by Southern blotting and hybridisation analysis using ^{32} P- labelled poly (dT-dG).(dC-dA) as a probe. These sequences have been shown to adopt a left-handed DNA conformation, also referred to as Z-DNA. This conformation was thought to influence the expression of certain cellular genes. This hypothesis was tested in a CAT assay system (Hamada *et al.*, 1984) and their results indicate that the poly (dT-dG).(dC-dA) sequence is capable of enhancing gene expression *in vivo*, however, the exact mechanism by which this enhancer activity is brought about is not clear.

The repeating units may be as short as one, two or three nucleotides; the most common being the dinucleotide repeat, for example the CA dinucleotide in a $(CA)_n$ repeat (Thibodeau *et al.*, 1993). These microsatellites are non-transcribable and constitute 10-15 percent of the total mammalian genome. The length of these microsatellites are unique to each individual and vary between individuals (Beckman and Weber, 1992), however, there is no variation between different cells in the same individual. In addition, these sequences are highly conserved and are stably inherited (Weber and May, 1989).

Novel or new alleles at (CA)/(TG) microsatellites and at tetranucleotide repeats are known to be formed without exchange of flanking markers. This means that they are not generated by unequal crossover. Since the mutant allele has been observed to differ by a single repeat unit from the original parent allele, the most likely mechanism to explain this length polymorphism is due to DNA slipped strand mispairing also referred to as DNA slippage (Figure 2).

This occurs when the normal base pairing between the two complementary strands is altered by staggering of the repeats on the two strands, leading to incorrect pairing of repeats (Umar and Kunkel, 1996). The mechanism for DNA slippage is illustrated in figure 2.

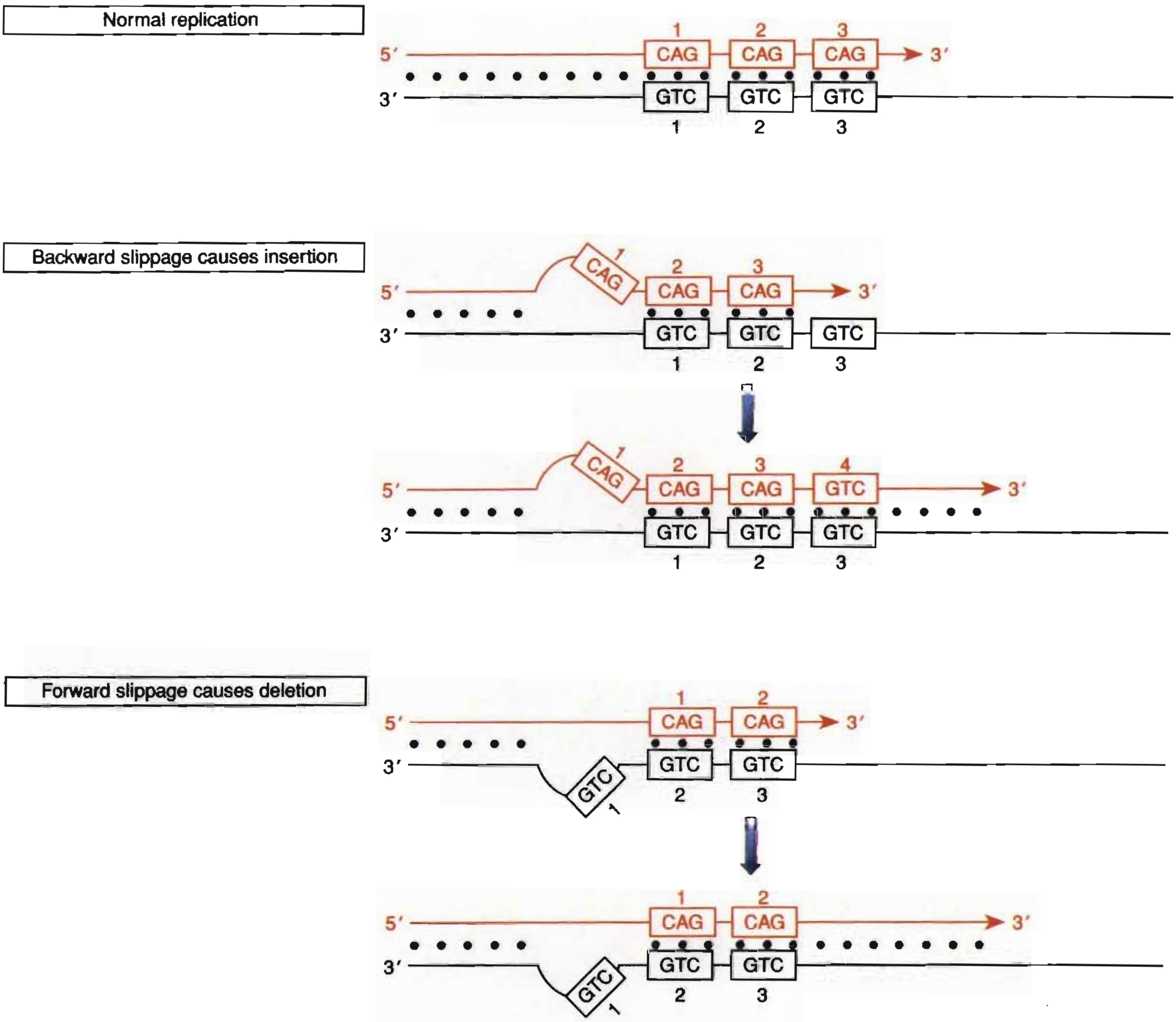


Figure 2: The examples show how DNA slippage occurs during replication. The upper strand is the newly synthesised complementary strand and the lower strand represents the parent strand. During backward slippage there is a region of nonpairing (shown as a bubble) containing one or more repeats of the newly synthesised strand. This would result in increased repeat units or insertions. During forward slippage there is a region of non pairing (shown as a bubble on the parent strand), which results in a decrease in the number of repeating units or deletions in the newly synthesised strand.

Microsatellites are located in the heterochromatin near chromosomal centromeres and telomeres. In the eukaryotic genome, microsatellites are identified as stretches of dT-dG sequences with varying length. Microsatellites are estimated to occur between 55 000 and 100 000 copies in the human genome, providing a marker density of one microsatellite every 100 000 base pairs, even by the most conservative estimates (Koreth *et al.*, 1996). However, although widely distributed, microsatellites are not regularly spaced in the chromosome.

Weissenbach *et al.* (1992) isolated and mapped a large number of microsatellites in order to construct a linkage map of the human genome with an average resolution of 5 centimorgans (cM). The sequence data obtained from this study is widely used for various microsatellite applications. Microsatellites may be used for personal identification, population genetic analysis (Di Rienzo *et al.*, 1994) and in the construction of the human evolutionary tree. Further, they are located and linked to several important gene loci, thus they are associated with human diseases not only as markers but also directly in disease aetiopathogenesis, providing insight into the replication, repair and mutation of eukaryotic DNA (Meltzer, 1995). Microsatellites were initially thought to play a functional role in the genome, either directly in gene regulation, or indirectly as hot spots for recombination, however, their exact function still remains elusive. Recent studies have shown that mutations occur in these microsatellites, due to mismatch repair errors termed RERs. These mutations are due to an increase or decrease in the number of repeats. As a result, different lengths of DNA are produced which arises directly from the defective repair process. The mutations that occur are referred to as microsatellite instability (MSI), which have been implicated in a host of human disorders, both hereditary and non-hereditary, including tumours, e.g. colorectal carcinomas, breast cancer and prostate cancer.

Over the past five years there has been a dramatic increase in the number of studies which have focused on changes in the molecular mechanisms surrounding tumour biology and tumourigenesis. However, more recent reports seem to indicate a greater involvement of microsatellites in the initiation and development of cancers (Loeb, 1994; Bedi *et al.*, 1995; Keller *et al.*, 1995).

1.3.2.7.1 ANALYSIS OF MICROSATELLITES

The most common way to analyse DNA polymorphisms is by examining RFLPs, in which base pair substitution between the two alleles alter a restriction enzyme consensus sequence, however with microsatellite markers the difference is not a base pair substitution, but rather an alteration in the length of the sequence. Such differences in sequence length can be detected by using PCR. Currently there are over 2000 polymorphic microsatellite markers identified in the human genome (Weissenbach, 1992), some of which have been used in genetic linkage studies.

The analysis of microsatellites is conventionally done using radioactively labelled primers in the PCR reaction (Gruis *et al.,* 1993; Halling *et al.,* 1995; Keller *et al.,*1995; Tomlinson *et al.,* 1995). The microsatellite PCR products are resolved on sequencing gels which are fixed and dried. The dried gels are then autoradiographed. However, this method has a number of disadvantages, most of all the hazard of working with radioactivity. The major technical problem in the analysis of microsatellites is the resolution and detection of the amplified DNA. This is due to the presence of additional bands which appear in addition to the microsatellite band, creating ambiguity in the analysis of the results. These bands are referred to as "stutter bands". This occurs when there are multiple repeat units in the template, which gives rise to the formation of secondary structures. The DNA polymerase fails to read through the repeating units within the secondary structure, thus producing a shorter product. Since polymorphisms between alleles occur as multiples of two base pairs, high resolution of acrylamide gels are required for the separation of these fragments. These fragments are visualised as bands by ethidium bromide staining or silver staining.

The problem with the former technique is that it is often not sensitive enough to detect the quantity of amplified DNA products, whereas the latter technique is more sensitive. There is however, conflicting reports on the use of silver staining. Koreth *et al.* (1995) found that the band intensity on silver staining does not correlate well with radioactive RFLP quantitation, while Schwengel *et al.* (1994) found this technique adequate.

Recently the use of fluorescence based technology was shown to be a sensitive and useful tool for the detection and analysis of microsatellite PCR products. The advantages of using the automated sequencer for the analysis of microsatellites were described by Skolnick and Wallace in 1988. Ziegle *et al.* (1992), reported the use of automated DNA sizing technology for genotyping microsatellites. Fluorescently labelled primers were used in the PCR assay. The labels contained FAM (blue), JOE (green), TAMRA (yellow) and ROX (red). The fluorescently labelled PCR products were separated on polyacrylamide gels and the fluorescent products were detected by laser and analysed by computer software.

Since then, this technology has been used more often and the results clearly show an improvement in the assessment and analysis of the data. A fluorescent based assay for the detection of MSI in sporadic colorectal carcinomas was demonstrated by Cawkwell *et al.* (1995). Fifty four cases were used in their study, and of these 22% showed MSI for at least one marker. Six of the tumours showed MSI at high frequency, which meant that at least 63% of the markers were affected.

Multiplex polymerase chain reaction is another important technique which has become increasingly popular in the analysis of microsatellites in cancers. In this technique, more than one set of primers are used in the same PCR tube, allowing for co-amplification of multiple products. This method has been used by Cawkwell *et al.* (1994), in which multiple markers were analysed using fluorescent based DNA technology to analyse 20 cases of colorectal carcinomas. The findings from this study indicate allelic loss to be 29% for *DCC*, 66% for *p53* and 50% for the *APC/MCC* region. A slightly different technique was used to analyse microsatellite instability in 21 gastric cancers and their corresponding normal tissue, using the automated DNA sequencer (Toh *et al.,* 1996). The procedure adopted in this study was different in that the two amplified products from both tumour and normal tissue were co-loaded into a single lane of the DNA sequencer and analysed. The results of this study are in keeping with other studies in that microsatellite instability was found to occur in 22,7% of the cases, however, the technique employed was unique, accurate and proved to be an efficient method for detection of microsatellite instability.

The advantages of using fluorescent based DNA technology for the analysis of microsatellites are very clear. It allows for easy interpretation of the data which can be captured and stored using the DNA software linked to the DNA sequencer. Accurate and impartial scoring of alleles, including interpretation of heterozygous and homozygous copies, has significantly increased the accuracy of the data produced. Further, a high throughput of samples is an added advantage, especially for large scale linkage studies. The technology also allows for assaying for multiple loci as in multiplex PCR. The use of internal and external standards eliminates lane to lane variation arising as a result of uneven mobility of the PCR products through the gel. The use of the fluorescent tag for detection is highly sensitive, so only 1-3 µl of PCR product needs to be run on the gel. The cost of the reagents required in the PCR reaction is reduced and therefore there is tremendous cost saving.

1.3.2.8 MISMATCH REPAIR GENES

Recently, a new class of genes, known as the mismatch repair genes has been identified. These genes were originally identified in bacteria and yeast (Modrich and Laheu, 1996), and their human homologue has been implicated in the pathogenesis of HNPCC, as well as a host of different sporadic cancers that exhibit MSI.

1.3.2.8.1 DNA DAMAGE

Mismatched base pairs are generated by:

- damage to nucleotide precursors
- errors that occur during replication
- genetic recombination
- double strand DNA breaks which lead to chromosome disruption (Chu, 1997).

Damage due to any of the aforementioned mechanisms results in marked cellular dysfunction. The errors arise as a result of environmental insults that damage specific nucleotides. An example of this is UV damage to DNA. Here, UV light can lead to cross-linking of pyrimidine (thymidine or cytosine) residues. The dimers that form as a result, block normal DNA replication. Another type of injury to the DNA is due to the covalent addition of chemical groups to the nucleotides (ie. alkylation). In addition, purines (adenine or guanine) spontaneously break off from the DNA backbone due to intrinsic thermal decompression. Finally, spontaneous deaminations occur at cytosine residues resulting in the formation of uracil residues. Since uracil is not one of the bases that ordinarily occurs in DNA, transcription is blocked when DNA polymerase encounters one of these altered residues. DNA damage as a result of these errors are corrected by the process of nucleotide excision repair (Wood, 1996).

Microsatellite instability is said to be indicative of a malfunction in the replication or repair of DNA and therefore, is referred to as replication error or replication error phenotype (RER). Mismatch repair has long been known to play two major roles in the cell: as a repair mechanism for errors arising during DNA synthesis and in genetic recombinations which result in new genetic markers.

Mismatch repair genes play the role of 'proofreaders' by ensuring that the copies of new DNA produced in the cell have the same genetic make-up or sequence as the parent strands. The repair of biosynthetic errors such as alterations in microsatellite length and single base mismatches is a highly conserved cellular function which is carried out by proteins that recognise these defective sequences, and excise and replace them with the correct ones. Enzyme systems that faithfully repair these aberrations have been identified in a wide variety of organisms (Fishel and Kolodner, 1995). In humans, defective DNA mismatch repair genes have been linked to HNPCC as well as to sporadic cancers that exhibit microsatellite instability (Smyrk, 1994; Liu *et al.*, 1995b). However, mutations in the genes coding for these proteins result in defective proteins which fail to correct replication errors. This has a cascading effect, producing secondary mutations further downstream from the initial mutation site. Sometimes these secondary mutations could occur in oncogenes and tumour suppressor genes. At present, there are six genes that code for mismatch repair proteins.

1.3.2.8.2 MECHANISMS OF DNA REPAIR

1.3.2.8.2.1 EXCISION REPAIR

Excision repair is classified into two distinct modes called base excision repair (BER) and nucleotide excision repair (NER). During BER damaged bases are excised as free bases, whereas in NER, the offending base is excised as a free nucleotide. NER, sometimes referred to as short-patch repair is said to operate efficiently in DNA which has been damaged due to chemical modifications such as cyclobutane pyrimidine dimers, which causes distortion of the helix. Mismatch repair has been intensively investigated in *Escherichia coli* and the yeast *Saccharomyces cerevisiae* systems. The relationship in defects in mismatch repair and human disease has been examined closely, with particular interest in the development of cancer (Kolodner, 1995).

The mechanism of action in NER is outlined in figure 3. There are two proteins UvrA and UvrB that bind to structurally damaged DNA in an ATP-dependent manner. The UvrC protein then binds and cleaves the damaged strand at two sites, 12 base pairs apart flanking the damaged nucleotides. Helicase II unwinds the DNA, and then polymerase I excises the nucleotides between the two nicks and resynthesises the strand. The final step in the process is closure of the gap, which is accomplished by DNA ligase.

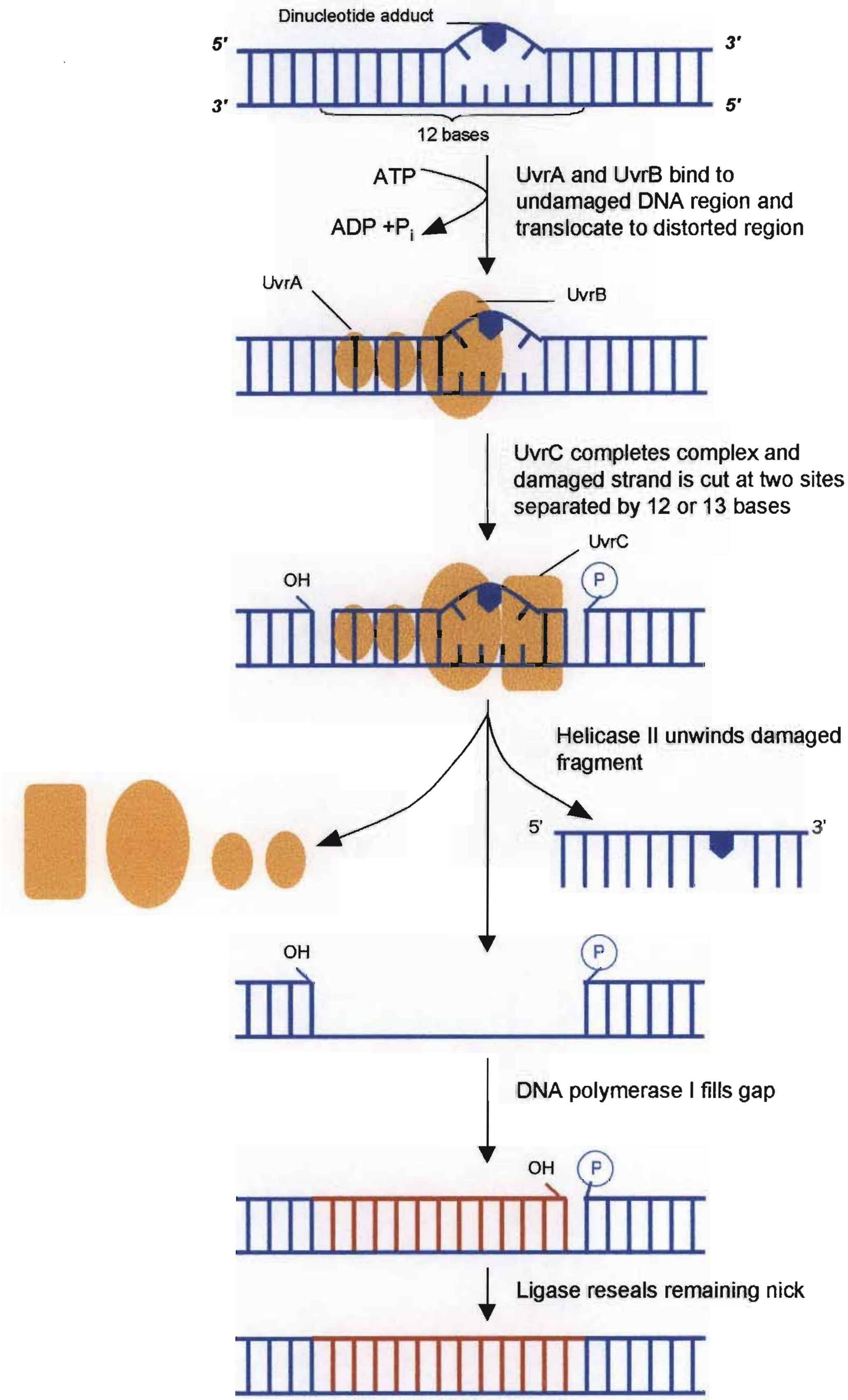


Figure 3: Mechanism of NER (Adapted from Sancar, 1996).

The second way by which DNA can be altered is via mismatching of nucleotides. Here DNA duplication occurs with every mitotic division. Errors could be introduced during this process of duplication, and therefore an extremely precise proofreading mechanism must exist. Although very precise, DNA polymerase is not 100 % accurate.

In the *E. coli* system, the mismatch repair pathway recognises all single base mispairs, except possibly C-C. In addition, all small insertion mispairs are repaired, however sections that have more than four mispaired bases are not efficiently recognised by this system. This system recognises the mispaired bases that arise as a result of misincorporation during DNA synthesis, and in cells that lack this repair system pathway, there is a high rate of accumulation of mutations.

The MutHLS repair pathway is unique in that it is suited to correct DNA replication errors. In the *E. coli* system, the GATC sites are normally methylated by the enzyme Dam methylase, and these sites exist as methylated sites. However, after replication the daughter strand is transiently unmethylated. The repair system recognises these unmethylated sites on the daughter strand and selectively corrects this.

The two most extensively studied repair genes are, *hMSH2,* which is located on chromosome 2p and *hMLH1,* located on chromosome 3p. Both *hMSH2* and *hMLH1* act essentially as tumour suppressor genes with loss of both copies of the gene resulting in abnormalities in the mismatch repair system. It is now known that both *hMLH1* and *hMSH2* represent homologues to bacterial mismatch repair genes. These genes are known to be mutated in hereditary non-polyposis colorectal cancer (HNPCC).

The normal function of these genes in the bacterial system is well established and is outlined in Figure 3. The correction of a replication error is initiated by the binding of the 97 kD MutS protein which recognises and binds to the mismatched DNA sequence. The DNA exists as a heteroduplex because of the lack of complementarity of the bases. The MutL (a 70 kD dimer) and the MutH (a 25 kD monomer) proteins, then function in concert with already bound MutS protein. MutH induces a nick in the strand containing the incorrect nucleotide. The enzyme helicase II then unwinds the DNA strand on both sides of the nick. This is followed by the bidirectional removal of bases in between the nick and the mismatched base. The gap is then filled with the correct nucleotide sequence by DNA polymerase and the newly synthesised strand is finally sealed by DNA ligase.

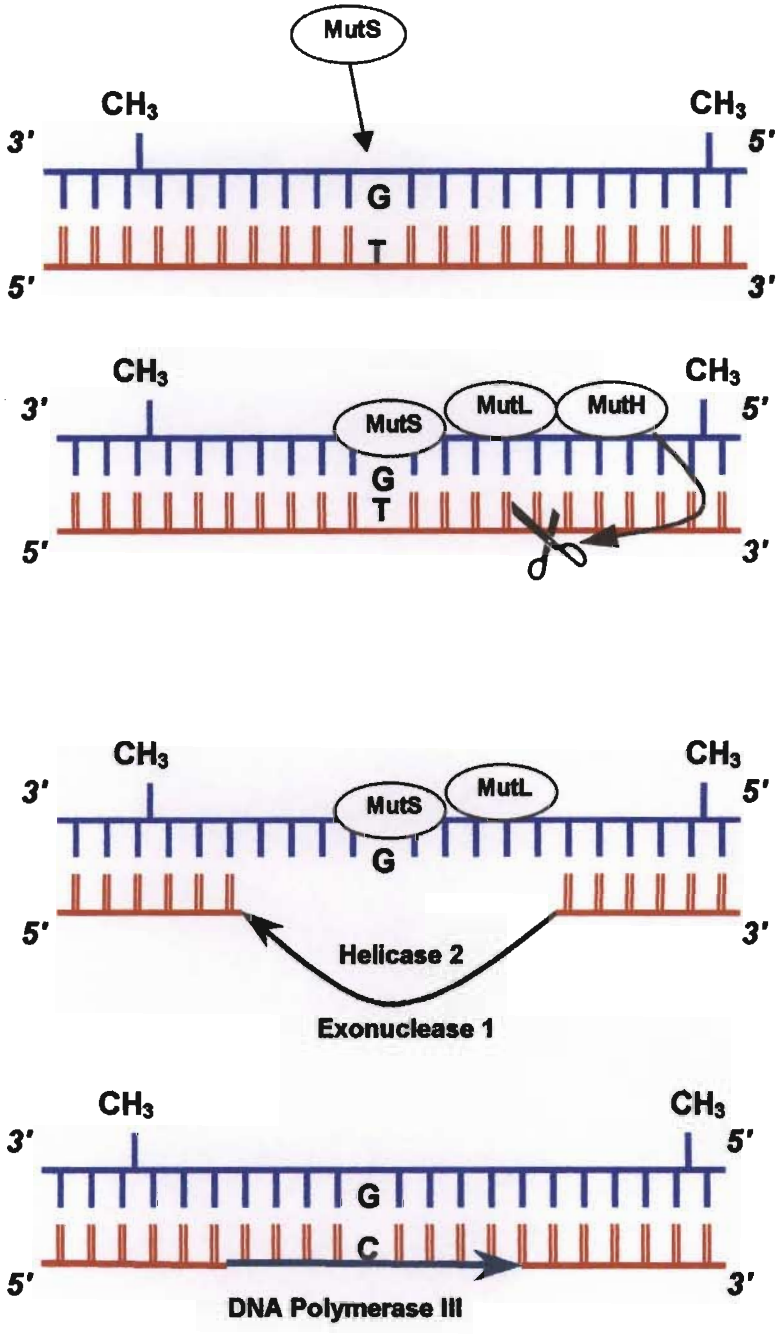


Figure 4. Diagrammatic representation of mismatch repair in bacteria. MutS and MutL which are homologues of the human hMLH1 and hMSH2, respectively, bind to the DNA at the site of a mismatched nucleotide. MutH protein induces a nick in the DNA molecule near the mismatch site. The abnormal area is then excised with the aid of a DNA helicase 2 and an exonuclease 1. The resultant gap in the DNA is then filled by the DNA polymerase III enzyme.

Less is known about the homologues *hPMS1, hPMS2* and *GTBP* (Fishel and Kolodner, 1995). These genes are thought to act as a complex and mutations in any of these genes also give rise to MSI. It is envisaged that these genetic alterations act as a trigger for the initiation and pathogenesis of many tumours.

1.3.2.8.3 MSI AND COLORECTAL CARCINOMA

Microsatellite instability was first described in patients with hereditary non-polyposis colorectal cancer (HNPCC) and sporadic colorectal cancers. Patients with the HNPCC syndrome have an increased risk of developing colorectal cancers and this has been linked to germline defects of the DNA repair genes. These cancers tend to present earlier than the sporadic colorectal carcinomas. Further, in patients with HNPCC the tumours are right-sided and mucinous, in contrast to sporadic colorectal carcinoma. These features would suggest that these two conditions are biologically different.

MSI in HNPCC has been extensively investigated (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Lothe *et al.*, 1993; Peltomaki *et al.*, 1993) and Aaltonen *et al.* (1993) reported the occurrence of MSI in this condition to be greater than 70%.

1.3.2.9 HNPCC AND HMRDS

1.3.2.9.1 HNPCC

Hereditary non-polyposis colorectal cancer is an autosomal dominant disorder which is characterised by the occurrence of colorectal cancer within a family. It was described in 1895 by Alfred Warthin, whose seamstress told him that she would die of colon cancer at an early age, because most of her family had died of these cancers. She did die of endometrial carcinoma and this prompted Warthin and co-workers to investigate her family, referred to as “family G”. In the initial report in 1913, “family G” included 10 subjects with uterine and seven with gastric cancer. However, these aggregations of cancer of the colon, stomach and endometrium in “family G” only impacted many years later when a cancer prone syndrome was noted in two extended kindreds under the cancer family syndrome (CFS). This syndrome was later called HNPCC and is also referred to as Lynch syndromes I and II.

HNPCC is subclassified on the basis of either an absence or presence of extracolonic malignancies. Lynch syndrome I is consistent with an autosomal dominant inheritance pattern and an early age onset, whilst Lynch syndrome II has all the features of Lynch syndrome I and also shows frequent carcinomas of the endometrium and other organs. A distinct feature in this type of cancer is the absence of gastrointestinal polyposis.

An International Collaborative Group was established and defined the Amsterdam criteria for the identification of HNPCC kindred. The criteria are as follows:

- at least three relatives should have histologically verified colorectal cancer with at least two of them being first degree relatives.
- at least two successive generations should be affected.
- at least one of the relatives should be diagnosed with colorectal cancer under 50 years of age.

However, despite the above criteria, there still remains a great deal of phenotypic variation between families. So, pedigree analysis does have drawbacks and studies that address gene analysis must be taken into account in these tumours.

Although colon cancer is a major cancer in HNPCC families, 35-40% of the people in these families have other types of tumours, of which endometrial and ovarian tumours are the commonest. Further, it is also common for HNPCC patients to develop more than one tumour.

In very recent studies significant progress has been made towards elucidating the underlying molecular mechanisms involved in HNPCC. Genetic linkage studies have identified two HNPCC loci which account for 90% of HNPCC. The locus thought to be involved was mapped to chromosome 2p15-16 (Peltomaki *et al.*, 1993), however this was later mapped with greater resolution to chromosome 2p21 (Fishel *et al.*, 1993). This region is thought to account for 60% of HNPCC. Another locus which accounts for up to 30% of HNPCC has been mapped to chromosome 3p21.

To date six human DNA mismatch repair genes (3 homologues of bacterial *MutS*: *hMSH2*, *hMSH6* (*GTBP*), and *hMSH3*; and 3 homologues of bacterial *MutL*: *hMLH1*, *hPMSI1*, and *hPMSI2*) have been identified (Chung and Rustgi, 1995; Eshleman and Markowitz, 1996).

Although the term HNPCC was developed to encompass Lynch I and Lynch II syndromes, it has been suggested that both these disorders may arise as a result of mutations of the same DNA mismatch repair genes. So, HNPCC describes a single hereditary disorder associated with cancers that occur in multiple sites, and share the features of DNA mismatch repair deficiency.

1.3.2.9.2 DIAGNOSTIC FEATURES OF HMRDS

Although the Amsterdam criteria were developed to standardise HNPCC families, a problem arises when grouping or classifying families with germline mutations in the DNA repair genes that do not meet the Amsterdam criteria or in individuals at a young age with no family history of colorectal cancer (Liu *et al.*, 1995a). For these reasons, it has been suggested that a more appropriate term would be hereditary mismatch repair deficiency syndrome (HMRDS) (Jass, 1998).

The diagnostic features of hereditary mismatch repair deficiency syndrome (HMRDS) are shown in Table 1 (Jass, 1998).

Table 1: Diagnostic features of HMRDS

- Family history of cancer in the following sites:
    - Colorectum (principal site)
    - Endometrium (major site)
    - Ovary, small intestine, pelvi-ureter, stomach, brain, skin (minor site)
- Onset of cancer at a young age
- Multiple cancers
- Pathology spectrum
    - Site: Predilection for proximal colon
    - Type: mucinous carcinoma and undifferentiated carcinoma
    - Grade: poor differentiation
    - Growth pattern: Expanding or circumscribed
    - Lymphocytes: Tumour infiltrating (intraepithelial) and peritumoural
    - Adenoma: Villous morphology
- Behaviour of colorectal cancer less likely to show distant spread
- DNA microsatellite instability implicating mono- and dinucleotide repeats at, at least two out of five loci tested in cancers from at least two affected members of a family
- Immunohistochemistry- loss of expression of hMLH1 or hMSH2
- Germline mutations

1.3.2.9.3 GENETIC DIAGNOSIS OF HMRDS

A germline mutation within the DNA repair gene which segregates within the affected family has to be demonstrated for the patient to be classified into the HMRDS category (Jass, 1998). At least seven different mechanisms may underlie a precancerous "field change" in HMRDS. These changes are listed below:

- In members of HMRDS families carrying germline mutations, there is no evidence of either an abnormal labelling index or shift in location of the proliferative compartment.
- So-called transitional mucosa may be found adjacent to cancers in HMRDS. This is now widely regarded as a secondary form of mucosal remodelling resembling the changes of mucosal prolapse.
- Functional changes within otherwise normal-appearing mucosa have been described in subjects with hereditary bowel cancer. The findings of increased binding by the lectin UAE-1 or reduced binding by the lectin DBA could be reproduced in affected members of HMRDS families.
- Clonal patches with an underlying mutation of a cancer gene may occur in epithelial surfaces, but it is arguable if this should be described as a "field". Even if the altered clone has a growth advantage, its extent would not be much more than microscopic. Epithelial surfaces exposed to environmental mutagens are littered with such clones.
- Altered DNA methylation could be conceived as a blanket change, but has not been shown to be of diagnostic value in HMRDS.
- A field may be neoplastic and arise through either lateral spread of a mutant clone or through confluence of discrete patches of neoplasia. Whilst this mechanism has been described in ulcerative colitis, no such mechanism has been described in HMRDS.

- The presence of two or more cancers within the colorectum invites the possibility of an underlying field change. The explanation would be more convincing if the discrete lesions were shown to harbour identical mutations (other than a germline mutation) but this has been shown in HMRDS. The explanation for multiple neoplasia in HMRDS presumably lies in the removal of a rate-limiting step in neoplastic progression.

One of the major clues in identifying HNPCC tumours is that they exhibit microsatellite instability. However, sporadic tumours also exhibit microsatellite instability. One of the first demonstrations that microsatellite instability was associated with sporadic tumours was by Ionov *et al.* (1993).

The examination of repeat sequences on chromosomes 2, 5, 11, 13, 18 and 20 on 23 cases of colorectal carcinomas demonstrated sequence alterations in 12 carcinomas at one or several loci (Remvikos *et al.*, 1995). In a very large study conducted by Hoff-Olsen *et al.* (1995), 244 colorectal carcinomas were examined for mutations in the D1S7 locus (a nine base pair repeat sequence). Somatic mutations in this region of the genome was found to be high (37 out of 244 cases). Further, to test whether these mutations mirror microsatellite instability, these 37 cases were examined at four microsatellite loci. There was a strong association between MSI and mutations at the D1S7 locus, suggesting this locus as a marker for genetic instability for colorectal cancer.

In a study of 108 colorectal adenocarcinomas for microsatellite instability, 6.5% of these cases were RER+ (Young *et al.*, 1993). Instability was noted at 4-15 microsatellite markers. However, in the same study, the examination of 46 colorectal adenomas showed no evidence of genomic instability.

Another study of 137 sporadic stage II and stage III colorectal carcinomas showed that 18 out of the 137 cases (13%) were RER+ (Kim *et al.*, 1994). In addition, the RER+ cancers were found in the younger patients in this study. A number of extra-colonic cancers of the gastrointestinal tract, urological and female reproductive tract have been associated with HNPCC, and hence, there have been many studies which have investigated instabilities in these tumours. Since it has been recognised that the proximal and distal gastric tumours exhibit distinct epidemiological features, Gleeson *et al.* (1996) undertook a study to investigate microsatellite instability in 38 gastric adenocarcinomas from the proximal stomach. Both dinucleotide and tetranucleotide microsatellite markers (138 in total) were used in this study. They showed that only one case demonstrated MSI in 62% of the markers, while MSI was found to range from 0.8% - 11.4% of the loci tested, demonstrating that low levels of microsatellite alterations are found in proximal gastric carcinomas.

Several studies have demonstrated genetic aberrations in colorectal cancer. An investigation of the short arm of chromosome 1 of 70 sporadic polyps showed allelic imbalance in 14 of the cases (20%) (Lothe *et al.*, 1995), demonstrating that alterations in microsatellites can de detected in large bowel polyps. It was also noted that alterations in chromosome 1p locus were equally distributed among small (<1 cm) and large polyps. Alterations in other loci (5q, 8p, 10p, 11p and 17q) were less frequent and confined to large polyps (>2cm).

One hundred cases of colorectal carcinomas were analysed to determine the frequency of allelic loss in the region of chromosome 11q (Bodmer and Tomlinson, 1996). Six highly informative microsatellite markers were used in this study in order to determine whether lesions in this region of the chromosome were common to all cancers or peculiar to colorectal cancer. Allelic loss was found to be high (20-29%) in colorectal cancer.

In order to assess pathological characteristics in RER+ CRC, a study was undertaken on 303 fresh colorectal cancer specimens (Jass *et al.*, 1998). Microsatellite instability was studied at nine loci that included *TGFβRII, IGRFIIR* and *BAX.* In this study RER+ cancers were termed mild RER, if up to two out of six loci, or extensive RER, if three or more positive loci were present. The results of this study showed that 61 cases were RER+ and 63 cases were RER-. Further, 37 cases showed extensive RER and 34 cases had mild RER. Weakly RER positive (RER+/-) cancers were indistinguishable from RER negative CRC.

The *Bax* gene which is linked to apoptosis, was found to be frequently mutated in HNPCC. In a study which included 13 adenomas and 24 adenocarcinomas from patients with HNPCC, frame-shift mutations were noted in 54.2% of these cases (Yagi *et al.*, 1998), suggesting that mutations in the *Bax* gene contribute to the adenoma-carcinoma sequence. Similar results were obtained for the *Bax* gene from a study in which the DNA mismatch repair genes *hMSH3* and *hMSH6* were also investigated (Yamamoto *et al.*, 1998).

However, in another study in which 15 cases of HNPCC were analysed, no mutations were found in the *Bax* gene, despite the presence of MSI in these cases (Sakakibara *et al.*, 1998)

1.4 OESOPHAGEAL CARCINOGENESIS

1.4.1 DIETARY AND RELATED FACTORS

There are a number of factors that influence the onset of oesophageal carcinoma. Certain countries seem to have a higher incidence of the disease and these include, China, Japan, Switzerland, France, Finland, certain parts of Africa and Iran. There are also local variations within a single country (Morson and Dawson, 1979).

The ratio of males to females for squamous carcinoma of the middle and lower thirds varies among the different series between 4:1 and 6:4 (Miller, 1962). Further, the overall average age range of the patients for squamous oesophageal carcinoma and carcinoma of the gastro-oesophageal junction is 57-67 years in men, and in women is between 54-62 years (Morson and Dawson, 1979).

There are a number of predisposing factors that seem to influence oesophageal carcinoma (Stemmermann *et al.*, 1994). However, in most reports there is no clear separation of the speculative from the proven aetiological factors. Poor oral hygiene, mechanical trauma, dietary intake, physiological narrowing of the oesophagus, syphilis and megaoesophagus have all been advanced, among others, as predisposing factors without convincing proof.

It is now believed that one third of all cancers are related to what we eat. Diets with a high salt content, smoked and salt-pickled foods are said to be high risk factors for oesophageal carcinoma. Nitrates and nitrosamines, which are contained in many preserved foods are said to be potent carcinogens. Increased intake of foods containing these compounds is said to promote the development of oesophageal tumours.

Another factor which has been allied to the development of many cancers, including oesophageal cancer is tobacco smoking. Tobacco contains more than 4000 chemical compounds, of which 43 are confirmed carcinogens (published by American Association for Cancer Research). The higher incidence of oesophageal carcinoma among those who smoke or chew tobacco, suggests that the high incidence of the disease is due to the ingestion of nicotine and other carcinogenic agents contained in tobacco (Winawer and Shike, 1995).

Oesophageal carcinoma is significantly more common in people who consume large quantities of alcohol, particularly spirits. There is also some evidence that a combined heavy intake of alcohol and tobacco is especially carcinogenic. A recent publication by the American Association for Cancer Research, outlined a number of mechanisms by which alcohol contributes to cancer development (Winawer and Shike, 1995).

Alcohol damages many cells, and this stimulates cell division, and anything that stimulates cell division is a factor which promotes cancer. Many chemical carcinogens, in order to become carcinogenic need to be activated. It also causes nutritional deficiencies, by depleting vitamin A, which has a general protective effective against a wide range of cancers. Also, alcohol depletes the body of folic acid, trace minerals and thiamine, factors which also have an overall protective role. In addition, alcohol impairs the capacity of the DNA repair enzymes to function optimally. Further, it is said to have a suppressive effect on the immune system, which affects the defence against carcinogens. Beer contains potent carcinogens, like nitrosamines, while bourbon, sherry and fruit brandies have carcinogenic agents like urethane. Alcohol also irritates the mucosa of the mouth and oesophagus, and may be a contributing factor.

Oesophageal cancer remains the most common malignancy encountered in South African males in the Transkei/KwaZulu-Natal region (Haffejee, 1998). The aetiopathogenesis of this cancer varies geographically, with alcohol and cigarette smoking being implicated in North America and Europe. However, in Central Asia, China, and South Africa, vitamin deficiencies, lack of protective antioxidants and diets high in carcinogens are thought to play a pivotal role in the development of the disease (Dayne *et al.*, 1982). In South Africa, the uneven geographical distribution of oesophageal cancer indicates that dietary and local cultural practices play an important role in the development of oesophageal cancer in these regions. A vastly increased incidence of the disease in the Transkei region of South Africa, may be related to a deficiency of molybdenum or a disturbance of the zinc/copper ratio in the soil. Further, epidemiological studies suggest that about 80-90% of cancers are attributed to lifestyle, of which 50% is diet related. Various genotypes of human papilloma virus have also been detected in oesophageal cancers, however their specific role in the development of this cancer is not clear.

1.4.2 GENETIC FACTORS

Allied to these environmental risk factors is the influence of molecular genetic alterations that may accrue from the aforementioned factors or precede these environmental considerations.

Several genes have been implicated in the causation of oesophageal carcinoma: *p53, APC, MCC* and *DCC* (Hollstein *et al.*, 1990; Huang *et al.*, 1993), including defects in the DNA repair genes.

In oesophageal carcinomas, *p53* gene abnormalities are said to occur in approximately 33% to 50% of cases. These aberrations occur as a result of both point mutations and loss of heterozygosity. Although most point mutations are as a result of the missense type, nonsense mutations have also been detected. Most missense mutations involve exons 5 to 8, with the preponderance of abnormalities affecting exons 5 and 6. Mutations, as a result of transversions and chain-termination are also very frequent. There have also been reports of loss of heterozygosity on chromosome 17p13 which harbours the *p53* gene. The figure ranges from 10% to 80% in oesophageal cancer. Hollstein and co-workers (1990) were one of the first to show the role of specific *p53* mutations in the tumourigenic process of oesophageal cancer. The patients examined in their study were from high risk geographical areas. The mutations occurring in these patients were distributed over the midrange of the gene, and the base substitutions in oesophageal cancer were different from that of gastric cancers. Alcohol and tobacco use was prevalent in this study population, which may suggest that the pattern of mutation may result from exposure to one or both of these carcinogenic risk factors.

Alterations on microsatellites occur at relatively high frequencies in sporadic and hereditary colorectal cancer, gastric and pancreatic cancer, and at lower frequencies in endometrial, ovarian and other cancers. Microsatellite instability in oesophageal cancer, however, has not been extensively investigated. A study recently undertaken by Meltzer *et al.* (1994) to determine MSI in oesophageal adenocarcinoma, showed that microsatellite instability occurred frequently in this group of patients. The 106 patients used in this study included 28 subjects with Barrett's metaplasia, 36 with Barrett's-associated adenocarcinoma, and 42 with primary oesophageal squamous cell carcinoma. Microsatellite instability was noted in 11 of the 106 patients (10%), in one or more chromosomal loci. In Barrett's metaplasia alone MSI was 7% (2 out of 28 cases), while this figure was 2% for squamous cell carcinomas and 22% in the adenocarcinomas. This study suggests that MSI in oesophageal adenocarcinomas develops as an early event in Barrett's metaplasia.

Loss of heterozygosity or allelic imbalance is indicative of somatic deletions on the chromosome. LOH in the vicinity of the *DCC* locus in oesophageal cancer has been seen in 23% of cases (Miyake *et al.*, 1994) and is said to be associated with increased metastatic potential. In this study 44 out of the 51 cases (86%) were informative for the *DCC* markers. Further, allelic deletions were observed only in moderately and poorly differentiated squamous cell carcinomas, but not in well differentiated ones. A study by Maesawa *et al.* (1996), however, showed that only 10 out of the 61 informative cases (16%) displayed LOH. The polymerase chain reaction together with VNTR and RFLP was used to analyse these cases. The results of this study suggest that LOH in the region of the *DCC* locus is not crucial for the acquisition of metastatic potential in oesophageal carcinoma.

Genetic instability analysed at five different loci, on the other hand was observed in 18% of the 32 cases studied (Nakashima *et al.*, 1995a). Although no significant correlation was shown between MSI analysis and clinicopathological findings, some of their cases correlated with DNA replication error. Ogasawara *et al.* (1995) on the other hand, detected LOH in 12 of the 35 cases studied (34%). This study looked at genetic alterations of the DNA repair genes located on chromosome 3p. The results suggest that RER at the 3p locus is an early event and that a tumour suppressor gene which is involved in the progression of oesophageal cancer may exist in the vicinity of the 3p25 locus.

A recent study from a Chinese group found the incidence of LOH on chromosome 3 and 9 to be high in their cohort of patients investigated (Wang *et al.*,1996). Microsatellite instability was observed in 24 of the 36 cases (66.7%) at one or more chromosomal loci. Loss of heterozygosity was observed in 9 of the 18 cases for marker D9S156 and in 8 of the 19 cases for marker D3S1480. A total of 12 microsatellite markers were investigated.

Although many studies demonstrate microsatellite instability in oesophageal cancers, a recent finding by Muzeau *et al.* (1997) showed that microsatellite instability was found to be infrequent in their cohort of 20 squamous cell carcinomas and 26 Barrett's adenocarcinomas studied. Their study analysed 39 poly-CA microsatellite loci in the French population.

Table 2: Genetic instability and LOH in recent studies on squamous cell oesophageal carcinoma

AUTHORS	**CASE NUMBERS**	**LOCUS (2P)**	**LOCUS (3P)**	**LOCUS (18q)**
Wagata *et al.*, (1991)	35	NOT DONE	LOH - 10%	NOT DONE
Huang *et al.*, (1992)	72	NOT DONE	NOT DONE	LOH - 24%
Meltzer *et al.*, (1994)	106	MSI - 8.5%	NOT DONE	NOT DONE
Aoki *et al.*, (1994)	93	NOT DONE	LOH - 35%	LOH - 38%
Miyake *et al.*, (1994)	51	NOT DONE	NOT DONE	LOH - 23%
Mori *et al.*, (1994)	21	NOT DONE	LOH - 64%	LOH - 22%
Shibagaki *et al.*, (1994)	35	LOH - 41.1%	LOH - 17.6%	LOH - 45.7%
Ogasawara *et al.*, (1995)	35	LOH - 3% MSI - 20%	LOH - 3-35% MSI - 13-40%	NOT DONE
Nakashima *et al.*, (1995a)	32	LOH - 3% MSI - 3%	LOH - 0% MSI - 9%	NOT DONE
Maesawa *et al.*, (1996)	111	NOT DONE	NOT DONE	LOH - 16%
Wang *et al.*, (1996)	34	NOT DONE	LOH - 12-30% MSI - 5.7-30%	NOT DONE

1.5 MICROSATELLITE INSTABILITY IN OTHER CANCERS

1.5.1 GASTRIC CANCER

In addition to colorectal cancers, the microsatellite status of other tumours located along the GIT have also been investigated since they share numerous molecular abnormalities with the colon. Gastric cancers have been extensively investigated in various geographical settings, and the role of microsatellite instability in gastric cancer has recently been reviewed (Hayden *et al.*, 1998). The high prevalence of gastric cancer in Japan prompted many researchers to investigate microsatellite instability and its association with gastric cancer in this country. Examination of 25 gastric cancers showed microsatellite instability in 4 cases (16%). MSI was observed in both the elderly and in lymph node metastasis-negative patients (Nakashima *et al.*, 1995a). A recent study by Toh *et al.* (1996), using automated fluorescent DNA technology to analyse 22 gastric cancers for microsatellite instability, showed that 5 of 22 cases (22,7%) displayed microsatellite instability. Another study analysed 43 sporadic gastric cancers for mutations in microsatellite sequences and found that 12 tumours (28%) showed MSI in at least 1 locus (Ohue *et al.*, 1996).

In order to investigate the genetic susceptibility of gastric cancers, Shinmura *et al.* (1995) carried out a study to determine the frequency of mutator phenotype in two groups of patients with gastric cancer. One group had multiple gastric carcinomas and the other had solitary gastric lesions. Their findings indicate that MSI was greater in patients with multiple gastric cancers than those with solitary gastric cancers, suggesting that defects in the DNA repair genes contribute to the occurrence of gastric cancer in Japanese.

A more recent study of genetic instability in multiple gastric cancers by Nakashima *et al.* (1995b) showed that MSI occurred in 11 out of 14 cases (78.5%), while MSI in solitary gastric cancers occurred in 5 out of 24 cases (20.8%). These findings suggest that MSI plays an important role in the development of multiple gastric cancers.

Another investigation of microsatellite instability in a subset of primary gastric adenocarcinomas arising in the proximal stomach showed that a high percentage of gastric tumours exhibit a low level of microsatellite instability at dinucleotide and tetranucleotide repeat loci (Gleeson *et al.*, 1996). A study of 78 gastric adenocarcinomas from a population of Texan Hispanics and Anglo-Saxons, two ethnic groups which have a high incidence of gastric cancer, showed that a tumour suppressor gene on chromosome 3p was involved in the development of a subset of gastric cancers (Schneider *et al.*, 1995).

Gastric carcinomas were also investigated by Dos Santos *et al.* (1996), in which microsatellite loci were analysed. The findings from this study indicate that microsatellite instability occurs in 34.4% (21 out of 61) cases. Five different microsatellite loci were investigated for instability in 52 cases of gastric cancers (Rhyu *et al.*, 1994). MSI was found at one or more loci in 31% of the cases.

1.5.2 ENDOMETRIAL CANCER

Endometrial carcinoma is a tumour which is commonly associated with HNPCC. Genetic instability in this tumour was investigated in 36 cases and the results from this study indicate that 17% of these cases show microsatellite instability (Risinger *et al.*, 1993). Another study investigating the incidence of microsatellite instability in this tumour showed that 23% of the 30 cases investigated were positive (Burks *et al.*, 1994).

1.5.3 PROSTATE CANCER

Microsatellite instability has been observed in human prostate cancers (9/21) (Watanabe *et al.*, 1995). Loss of heterozygosity was noted in 24% of these cases. Although MSI did not correlate with the clinical staging, these observations indicate that MSI might play a role in a subset of prostate cancers.

1.5.4 BREAST CANCER

An investigation into whether LOH was present at various loci (16q, 17q, 17p, and 13q) in invasive breast cancer and *in situ* ductal carcinoma, showed that the frequency of LOH ranged from 8% in the 17p locus to 50% in the 17q locus (Lakhani *et al.*, 1995). These results indicate that invasive and *in situ* ductal carcinoma are genetically different, in addition to their clinical and morphological differences. Further, analysis for LOH in five loci on chromosome 11q done in 41 cases of sporadic breast carcinoma (Tomlinson *et al.*, 1995), showed a high frequency of LOH (59%) in this region of the chromosome. This indicates the importance of this chromosomal location in the pathogenesis of breast cancer. Allelic deletions in the 7p, 16q, 17p and 17q region were observed in (25-30%) of human breast carcinomas (Aldaz *et al.*, 1995). Microsatellite instability was found to occur at a high frequency in invasive lobular breast carcinomas (39%), in which 9 out of 23 cases showed MSI.

1.5.5 MISCELLANEOUS CANCERS

Microsatellite instability has been investigated in a host of tumours, in addition to those occurring in the gastrointestinal tract (Tahara, 1995) and HNPCC (Peltomaki, 1995).

An investigation of 29 testicular germ cell tumours showed MSI in 21% of cases (Haddart *et al.*, 1995). Microsatellite instability in human skin cancers was found to occur at low frequency (Halling *et al.*,1995). Other tumours investigated include, preinvasive and invasive head and neck squamous carcinoma (El-Naggar *et al.*, 1996), non-small cell cancer of the lung (Fong *et al.*, 1995) and transitional cell carcinoma of urinary bladder (Li *et al.*, 1995). Homozygous deletions and LOH on chromosomes 6 and 9 have also been demonstrated in primary human small cell lung cancer (Merlo *et al.*, 1994).

However, a study in uterine serous carcinoma showed that microsatellite instability was uncommon (Tashiro *et al.*, 1997). In a study by Mark *et al.* (1998), it was suggested that MSI played an important role in the pathogenesis of Hodgkin's disease.

Although microsatellite instability has been noted in varying degrees in a number of tumours, several studies on different tumours have shown an absence of MSI. These figures are listed in table 3.

Table 3: Non-familial solid tumours showing no microsatellite instability

Tumour site	**Number of cases**	**Reference**
Brain	0/15	(Amariglio *et al.*, 1995)
Pancreas	0/26	(Seymour *et al.*, 1994)
Testis	0/85	(Peltomaki *et al.*, 1993)
Ovary	0/20	(Wooster *et al.*, 1994)
Lung (Small cell)	0/37	(Adachi *et al.*, 1995)
Lung (non-small cell)	0/85	(Peltomaki *et al.*, 1993)

CHAPTER 2

MATERIALS AND METHODS

2.1 COLORECTAL CANCER

2.1.1 CASE SELECTION

Seventy nine colectomy specimens of sporadic colorectal cancer over the age of 50 years and 37 colectomy specimens of colorectal cancer under the age of 35 were selected for this study. Stage D tumours were omitted from this study in the CRC over 50 group, because in many instances total colectomies with attached lymph nodes were not submitted for histological examination. In addition, only a few cases of stage D colorectal cancers with complete clinicopathological correlation were available, thus for simplicity, stage D cases were omitted. Furthermore it is well known that stage D tumours have an extremely poor prognosis. The thrust of this study being on the identification of molecular events in earlier stage lesions also influenced the cohort of cases selected. In the under 35 and over 50 groups no family history of CRC or any other cancer was documented in the clinical notes.

The tissue used in this study was obtained from the archives of the Department of Pathology at the University of Natal Medical School, Durban and the Department of Anatomical Pathology at Tygerberg Hospital, Cape Town. Surgical resection specimens of colorectal carcinomas were submitted to the Department of Pathology from King Edward VIII Hospital.

2.1.2 TISSUE PREPARATION AND SECTION CUTTING

All specimens were fixed in 10% buffered formalin. The colorectal resection specimens were routinely sampled and included sections of the carcinoma and normal colonic mucosa. Regional lymph nodes were also dissected and processed. All tissue sections were routinely processed and embedded in paraffin wax blocks. Tissue sections were cut at 3 μm thickness with disposable microtome blades, floated onto a waterbath (60°C) and collected on glass slides.

The sections were stained with haematoxylin and eosin (H and E) for histological examination. The tissue sections used in our study were selected after histological examination of a range of H and E slides. In this way sections containing maximal tumour could be selected. However, in some sections that did not contain 100% tumour, the slides containing a minimum of 80% tumour were selected. In these samples the tumour was demarcated from stroma, with a Sharpie pen. Tumour tissue was microdissected away from the normal tissue in these cases. Three sections of 6 μm thickness were cut from the selected blocks. These sections were used for the DNA extraction.

2.1.3 HISTOLOGICAL ASSESSMENT

2.1.3.1 GRADING

The tumours were classified as well differentiated (greater than 75% of the tumour containing glandular structures), moderately differentiated and poorly differentiated (less than 25% of the tumour containing glandular structures) on the basis of the degree of glandular formation. In this study, mucinous and signet ring carcinomas were regarded as synonmous.

2.1.3.2 STAGING

The Astler-Coller modification of the Dukes' classification for grading of the colorectal cancer was adopted. This is based on the extent of local spread of the tumour and the presence of distant visceral metastasis. A schematic representation of the Astler-Coller classification is illustrated in figure 5.

Stage A - Local spread of the tumour up to the muscularis propria
Stage B - Local spread of the tumour through the muscularis propria
Stage C - Local spread of the tumour including lymph nodes
Stage D - Metastases to distant viscera

2.1.4 CLINICAL FOLLOW-UP

Unfortunately, the study was hampered by lack of availability of adequate follow-up on the majority of the cases. The patients' post-operative records were purged and only very scanty and patchy information was available. As a result, no attempt was made to correlate the molecular findings with patient outcome.

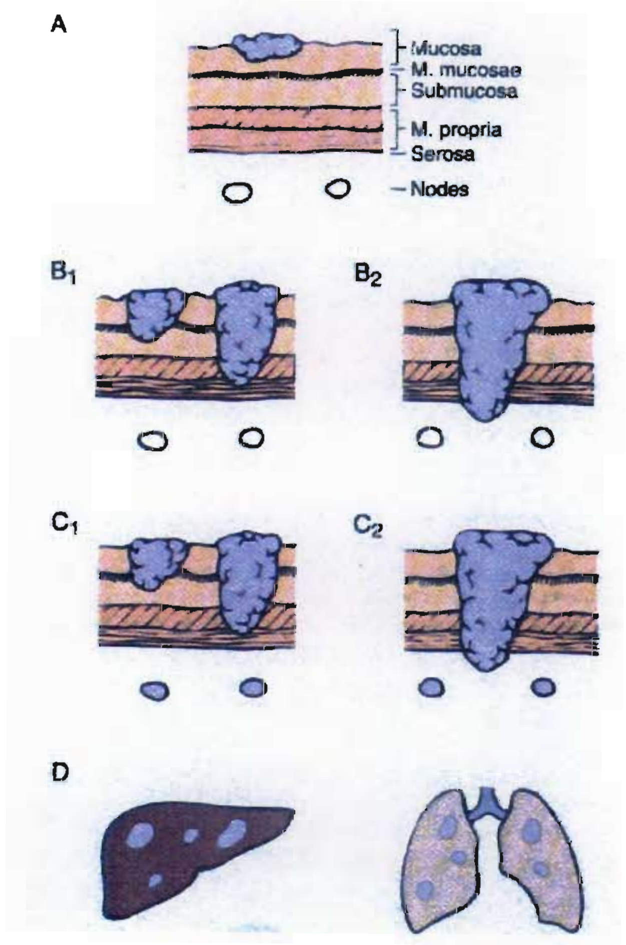


Figure 5: The Astler-Coller modification of Dukes' classification (Astler and Coller, 1954). Stages B1 and B2 were grouped as stage B and stages C1 and C2 were grouped as stage C.

2.2 OESOPHAGEAL CANCER

2.2.1 CASE SELECTION

Forty seven oesophagectomy specimens of oesophageal squamous cancer were accessed for this study. Surgical resection specimens were sent to the Department of Pathology at the University of Natal Medical School, Durban. The tissue was fixed and processed as discussed in section 2.1.2.

2.2.2 HISTOLOGICAL ASSESSMENT

2.2.2.1 GRADING

The squamous carcinomas were graded in a conventional fashion as: well, moderate and poorly differentiated.

2.2.2.2 STAGING

The tumours were staged according to the UICC/TNM system

	Primary tumour (T)
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma *in situ*
T1	Tumour invades lamina propria or submucosa
T2	Tumour invades muscularis propria
T3	Tumour invades adventitia
T4	Tumour invades adjacent structures

Lymph node (N)

NX	Regional lymph nodes cannot be assessed
N0	No regional node metastasis
N1	Regional lymph node metastasis

Distant metastasis (M)

MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Stage grouping

0	Tis	N0	M0
I	T1	N0	M0
IIA	T2	N0	M0
	T3	N0	M0
IIB	T1	N1	M0
	T2	N1	M0
III	T3	N1	M0
	T4	Any N	M0
IV	Any T	Any N	M1

2.2.3 CLINICAL FOLLOW-UP

Follow-up was available from the Department of Cardio-thoracic Surgery, courtesy of Mr Anunathan Reddi. The follow-up period ranged from 1 month to 1 year.

2.3 DNA EXTRACTION

It was particularly important to obtain good quality DNA since this ultimately determines the results of the microsatellite PCR analysis. Several methods were used in optimising the DNA extraction procedure. These include the methods of Maniatis *et al.* (1982), Sambrook *et al.* (1989), Chetty *et al.* (1995), Zhuang *et al.* (1995), Morgan *et al.*, 1996 and Moskaluk and Kern (1997). The DNA extraction and precipitation was carried out as described in Appendix 1.

2.3.1 REAGENTS

2.3.1.1 Proteinase K buffer

Proteinase K buffer was made fresh each time. The following solutions were added to a sterile disposable 10 ml tube:

200 µl of 100 mM NaCl
100 µl of 10mM Tris-HCl (pH 8.4)
500 µl of 25 mM EDTA
1 ml of 0.5% SDS

Proteinase K (Boehringer Mannheim) (0.1 mg), was added, vortexed briefly and the volume was brought up to 10 ml with distilled water.

2.3.1.2 Phenol (TE/water saturated)

Phenol (100 g) (Sigma), was dissolved in its bottle by heating at 65°C in a water bath. Sterile water was added up to the neck of the bottle before shaking thoroughly. Most of the water was removed and 15 ml of TE buffer was added. This solution was stored at 4°C.

2.3.1.3 Phenol/Chloroform/Isoamyl alcohol

Saturated phenol (75ml) was added to 75 ml of chloroform. This solution was mixed before adding 1 ml of isoamyl alcohol. The mixture was transferred to a clean dark bottle, overlayed with 15 ml of TE buffer and stored at 4°C.

2.3.1.4 3M Sodium Acetate

Sodium acetate (123g) was dissolved in 300 ml of distilled water in a clean 1 L Schott bottle. Glacial acetic acid (150 ml) was added and the volume brought up to 500 ml with distilled water. The solution was sterilised by autoclaving and stored at RT.

2.3.1.5 Ethanol (80%)

Absolute ethanol (Merck Chemicals) was diluted to 80% with sterile water and stored at -20°C.

2.4 ASSAY FOR CONCENTRATION AND PURITY OF DNA

The DNA concentration was estimated using ultraviolet spectrophotometry (Davis *et al.*, 1986) using a Gene Quant II DNA/RNA calculator (Pharmacia Biotechnology). The DNA sample (2μl) was mixed with 98μl of sterile water to give a dilution of 1:50. This was transferred to an acid washed quartz cuvette. The Gene Quant was blanked with sterile water before the absorbance was read. The data obtained for each sample was the absorbance at 260 nm, 260/280 ratio and the concentration (μg/ml). The concentration was determined by reading at 260 nm where an estimate of 50 μg/ml is equal to an absorbance of 1.

2.5 PCR REACTION USING THE INSULIN PRIMERS

PCR was carried out to ensure that the DNA isolated from paraffin embedded tissue was of acceptable quality to continue with, for the microsatellite analysis. This was done, using the ubiquitous insulin gene. Primers for exon 2 of the insulin gene were used (Olansky *et al.*, 1992).

2.5.1 INSULIN PRIMERS

The primers were synthesised for the exon 2 region, at the Department of Biochemistry, University of Cape Town, South Africa. In addition some primers were a gift, courtesy of Dr Fraser Pirie, from the Department of Medicine, University of Natal Medical School. The oligonucleotide sequences for the exon 2 region are as follows:

2R 5' AGG GGC AGC AAT GGG CGG TTG 3'
2F 5' ACC CAG ATC ACT GTC CTT CTG CC 3'

The PCR product size for these primers was 236 base pairs.

2.5.2 INSULIN PCR REACTION

PCR reaction was done as outlined below.

The PCR Core Kit (Boehringer Mannheim) was used for the PCR. The kit consisted of: 10x reaction buffer (containing 1.5 mM $MgCl_2$), dNTP mix and Taq DNA polymerase. The primers (20 pmoles) were used in the PCR in a total reaction volume 25 μl, containing 5 μl template DNA, 200 μM dNTPs, 50 mM PCR buffer containing 1.5 mM $MgCl_2$ and 0.2 units of Taq DNA polymerase.

The PCR was carried out in 200 μl thin-walled reaction tubes. An initial denaturing step was carried out at 94°C for 5 min. This was followed by 30 cycles, each cycle consisting of 1 min at 94°C, 1 min at 64°C and 2 min at 72°C. The last step was a final extension cycle at 72°C for 10 min. A Techne Progene Thermocycler was used for the PCR reaction. The PCR products (10 μl) were analysed using a 2% agarose gel.

2.6 AGAROSE GEL ELECTROPHORESIS OF DNA

The separation of PCR products was carried out as described previously by Maniatis *et al.* (1982).

2.6.1 REAGENTS

2.6.1.1 TBE Buffer (10x)

Tris base (26 g), boric acid (16 g) and EDTA (2 g) were dissolved in 400 ml of sterile water. The volume was then brought up to 500 ml, filtered and stored.

2.6.1.2 Ethidium Bromide Dye

Ethidium bromide (10 mg) was dissolved in 1 ml of sterile water. The container was covered in aluminium foil and kept at RT.

2.6.1.3 Bromophenol Loading Dye

0.02 % bromophenol blue, 0.02% xylene cyanol and 50 % glycerol

2.6.1.4 Agarose gel (2%)

Agarose (0.8 g) was dissolved in a 200 ml conical flask with 40 ml of TBE buffer (1x). This mixture was heated in a microwave oven for 2 minutes and then cooled to approximately 50 °C. Ethidium bromide (2 μl) was added and the gel was poured into the casting tray with the comb positioned approximately 10 mm from one end of the tray. The gel was allowed to polymerise for 30 to 60 min at RT.

2.6.2 SAMPLE PREPARATION

Bromophenol blue loading dye (1μl), was added to 10μl of PCR product. The DNA molecular weight marker (1μl), was added to 10μl of sterile water and 1μl loading dye.

2.6.2.1 PROCEDURE

Once the gel was completely polymerised the comb was removed and the gel was placed into the electrophoretic tank. TBE buffer (1x) was added to the buffer chamber until the gel tray was completely submerged. The DNA samples and the molecular weight markers were carefully loaded into the wells using a P20 pipetman. Electrophoresis was carried out at 100 V for 30 min at RT. After the electrophoretic run the gel was visualised using the UV transilluminator.

2.6.3 PHOTOGRAPHY OF THE GEL

The gel was placed on a Camag UV transilluminator and viewed at long wavelength (300 nm). The gel was scanned into a computer using a video camera. The appropriate light and contrast adjustment was made and the picture was then printed.

2.7 MICROSATELLITE PCR

2.7.1 CY5 LABELLED PRIMERS FOR *DCC* GENE

The CY5 labelled primers, which were 5' labelled, were purchased from Boehringer Mannheim. The microsatellite primers for the *DCC* gene used in this study were as follows.

Table 4 - Microsatellite PCR primer sequences

***DCC* gene locus**

Locus	**Sequence**	
DCC	5' GTGGTTATTGCCTTGAAAAG 3'	Primer 1
	5' GATGACATTTTCCCTCTAG 3'	Primer 2
D18S34	5' CAGAAAATTCTCTCTGGCTA 3'	Primer 1
	5' TCATGTTCCTGGCAAGAAT 3'	Primer 2
D18S58	5' GCTCCCGGCTGGTTTT 3'	Primer 1
	5' CAGGAAATCGCAGGAACTT 3'	Primer 2

Primer sequences were obtained from Boland *el al.* (1995) and Liu *et al.* (1995a). The DCC marker spans 18q21.2 to 18q21.3.

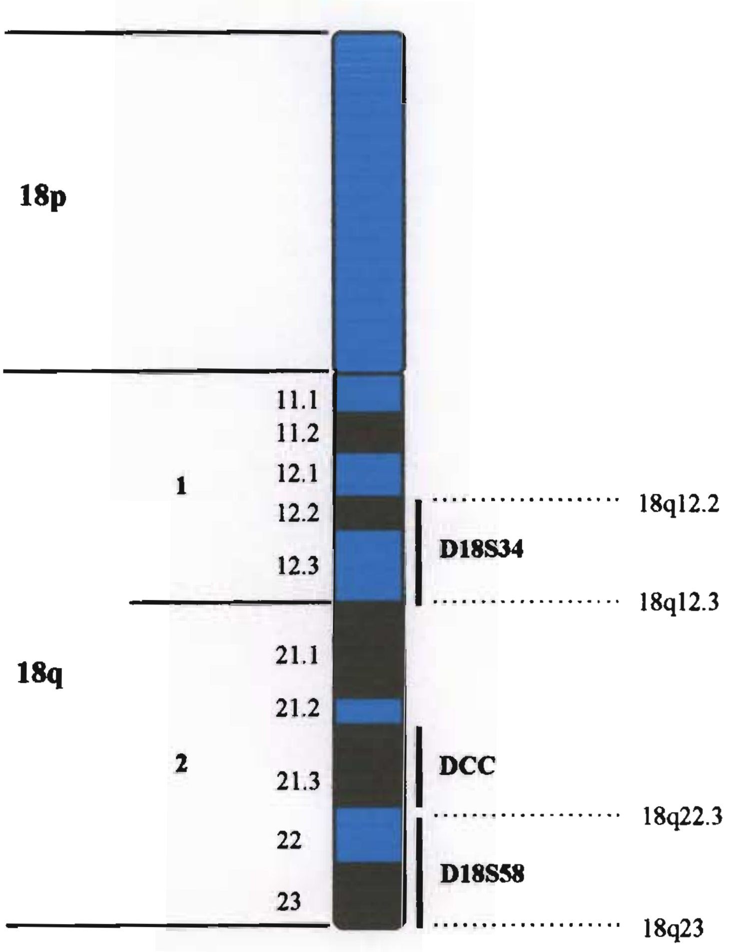


Figure 6: Ideogram of chromosome 18, showing the location of 3 loci: DCC (150-210 bp), D18S34(103-119 bp) and D18S58 (144-160 bp).

2.7.2 CY5 LABELLED PRIMERS FOR MISMATCH REPAIR LOCI

Locus	**Sequence**	
D2S123	5' AAACAGGATGCCTGCCTTTA 3'	Primer 1
	5' GAACTTTCCACCTATGGGAC 3'	Primer 2
D3S1255	5' CTCACTCATGAACACAGATGC 3'	Primer 1
	5' AACCCATCTTGTATTCTTGCAG 3'	Primer 2
D3S659	5' ATTCCAGGGACAAGTTCCCC 3'	Primer 1
	5' CTGCAAGGTCTGTTTAACAG 3'	Primer 2

Primer sequences were obtained from Weissenbach *el al.* (1992).

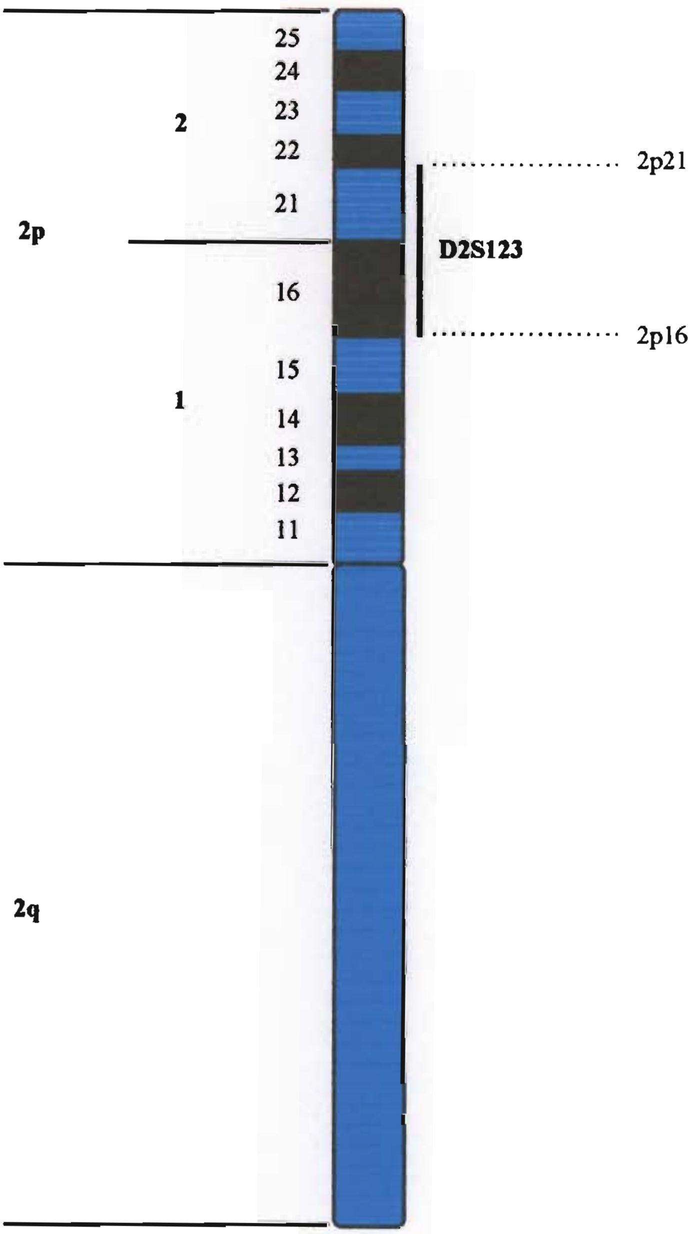


Figure 7: Ideogram of chromosome 2, showing the location of the D2S123 locus (198-228 bp).

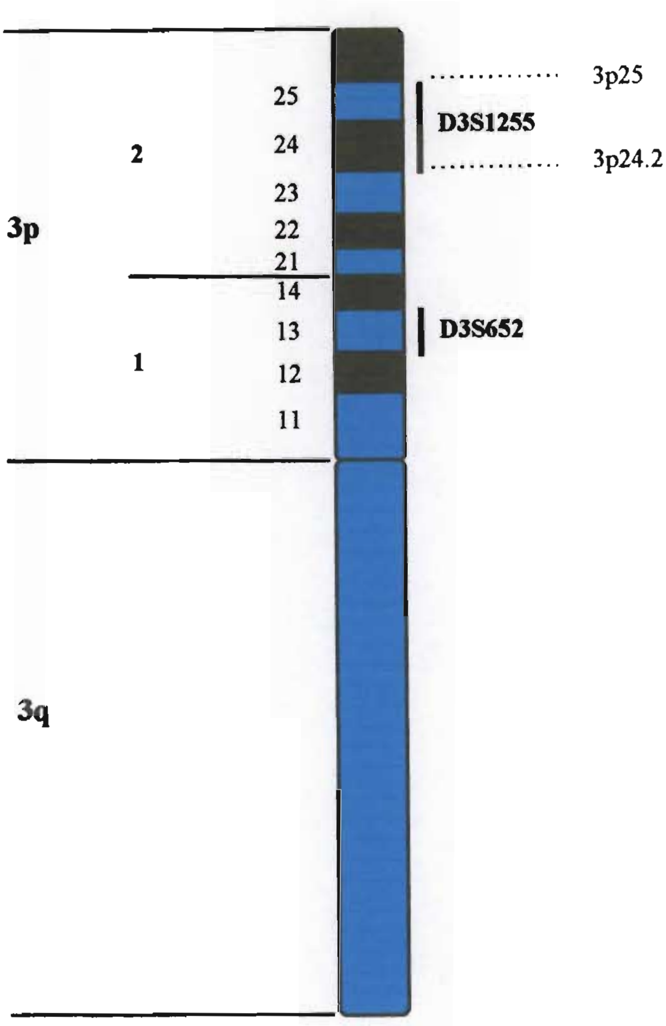


Figure 8: Ideogram of chromosome 3, showing the location of D3S1255 (140-160 bp) and D3S659 (110-150 bp).

2.7.3 MICROSATELLITE PCR REACTION

The polymerase chain reactions were carried out in 200 µl thin-walled PCR tubes. The PCR Core Kit (Boehringer Mannheim) was used for this procedure. The kit consisted of: 10x reaction buffer (containing 1.5 mM $MgCl_2$), dNTP mix and Taq DNA polymerase. The CY5 labelled primers (20 pmoles) were used in the PCR in a total reaction volume of 25µl, containing 5 µl template DNA, 200 µM dNTPs, 50 mM PCR buffer containing 1.5 mM $MgCl_2$ and 0.2 units of Taq DNA polymerase. The PCR amplification was performed using a Techne Progene Thermocycler. The PCR reaction mixture was initially denatured at 95 °C for 5 min. Thirty cycles were performed, each cycle consisting of 1 min at 94 °C, 20 seconds at 58 °C and 40 seconds at 72 °C. This was followed by a final extension step for 10 min at 72 °C. The PCR products were held in a foiled container at 4 °C before analysis on a sequencing gel.

2.8 AUTOMATED DNA FRAGMENT ANALYSIS

The microsatellite PCR products were analysed using a 6% Longranger sequencing gel.

2.8.1 PREPARATION OF 6% LONGRANGER GEL

Approximately 60 ml of gel was prepared as follows:
Ultra-pure urea (25.2 g) was weighed into a clean 250 ml beaker. MilliQ water (5ml) was added to the urea. The urea was dissolved using a magnetic stirrer and heat. TBE buffer (7 ml of 10x concentration), was added to the dissolved urea followed by 6 ml of Longranger gel. The volume was made up to 60 ml with distilled water and this solution was kept in a foiled plastic bottle at 4 °C. TEMED (40 µl), and 400 µl of APS (10%) was added before the gel was cast.

2.8.2 PREPARATION OF GEL CASSETTE (SHORT THERMOPLATE)

The short thermoplate (Pharmacia Biotech) was used to cast the gel. Disposable gloves were used at all times during the handling of the gel cassette. The plates, glass spacers (0.3 mm) and the comb (0.3 mm) were thoroughly cleaned with MilliQ water and 70% alcohol. All parts of the gel cassette were carefully examined under reflected light before assembly. This was done to ensure that the plates were free of any dust particles, which affects band migration and broadening during the electrophoretic run. This cleaning procedure was also adopted because the ALFexpress DNA Sequencer was highly sensitive and capable of detecting trace contaminants. The spacers were positioned carefully along the indented edges on the right and left sides of the thermoplates. Gentle pressure was applied to the spacers to secure them onto the silicone-rubber seals. The glass coverplate was positioned over the thermoplate and bound together with the gel clamps. The comb was positioned between the plates and the gel was cast from the lower edge. The gel solution was allowed to polymerise for 90 minutes.

2.8.3 SAMPLE PREPARATION

The microsatellite PCR products (3µl) were mixed with 3 µl of STOP solution (Blue dextran 2000, deionized formamide) (Pharmacia Biotech) and held on ice. The mixture was denatured at 96°C for 3 min in a Techne Progene thermocycler and held on ice before the samples were loaded on the gel.

2.8.4 PREPARATION OF SIZE MARKERS

External size markers were purchased from Pharmacia Biotech. A 50-500 CY5 labelled base pair standard was used as the external size marker.

2.8.5 PROGRAMMING AND RUNNING CONDITIONS ON THE ALFexpress DNA SEQUENCER

Once the instrument was switched on, the running conditions and the data were entered using the ALF Manager Software (Pharmacia). The following running parameters were used for the microsatellite analysis:

Sampling interval	1 second
Voltage	1600 V
Power	25 W
Current	60 mA
Temperature	55 °C
Running time	180 min

Figure 9: ALFexpress DNA automated sequencer

2.8.6 ATTACHMENT OF GEL CASSETTE ON TO THE ALFexpress DNA SEQUENCER

The outer surface of the glass plates were cleaned, especially around the area of the detectors. Any polymerised gel and urea present was also removed. The lower buffer chamber was placed in front of the instrument before attaching the gel cassette to the instrument. The push-fit connectors of the instrument's thermocirculator was attached to the slots on the thermoplate to allow water to be automatically pumped through the thermoplates. One litre of TBE buffer (1x concentration) was added to both the upper and lower buffer chambers.

The comb was then removed and the wells cleaned of any unpolymerised acrylamide and urea. This was done by squirting buffer into the wells with a syringe. Once the temperature had reached 55°C the samples which were held on ice were loaded. Special elongated gel loading tips were used to load 6 µl of sample into each well.

2.8.7 DETECTION OF MICROSATELLITE PCR PRODUCTS

The amplification of each new microsatellite locus was verified by running the PCR products on an ethidium bromide-stained 2% agarose gel along with a molecular weight marker. In this way the production of an efficient fragment together with the predicted length of the fragment was established.

2.8.8 CALCULATION OF RATIOS FOR AI

Cases demonstrating homozygosity and/or MSI were excluded from the assessment of LOH/AI. The allele ratios were calculated by the method suggested by Canzian and colleagues, (1996). Briefly, the peak areas were expressed as ratios as follows: (T2 X N1)/ (T1 X N2), where T/N 1 and 2 are the first and second peaks of tumour and normal DNA respectively. If the ratio was < 0.6, then there was loss of the larger allele, and if the ratio was > 1.67, then the smaller allele was lost. With the exclusion of homozygous cases and those showing MSI, there was a reduction in the number of informative cases. The allele imbalance frequency was then calculated using the following formula (Canzian *et al.*, 1996) [AI/(AI+N)] X 100%, where AI is the number of cases with allelic imbalance and N is the number of cases not showing homozygosity or MSI. For microsatellite instability, a difference in the electrophoretogram patterns (that is, the appearance of novel alleles) between normal and tumour DNA was regarded as evidence of microsatellite instability for that marker (Cawkwell *et al.*, 1995).

2.9 STATISTICAL ANALYSIS

This was performed using the Chi squared test (two by two tables) with the Yates' correction. (See Appendix F)

CHAPTER 3
RESULTS

3.1 CLINICAL DATA

3.1.1 COLORECTAL CANCER

3.1.1.1 LIGHT MICROSCOPIC EVALUATION OF CRC

The same histological parameters were used to grade the CRC from both age groups. More signet ring cancers were seen in the under 35 age group. The tumour cells were present singly and in groups suspended within pools of extracellular mucin (Figure 10). The signet ring cells were typified by intracytoplasmic mucin which displaced the nucleus to an eccentric location (Figure 11). Well differentiated cancers were composed of well developed glandular structures which constituted more than 75% of the tumour (Figure 12). Poorly differentiated carcinomas, on the other hand, showed little or no gland formation and were disposed in sheets (Figure 13).

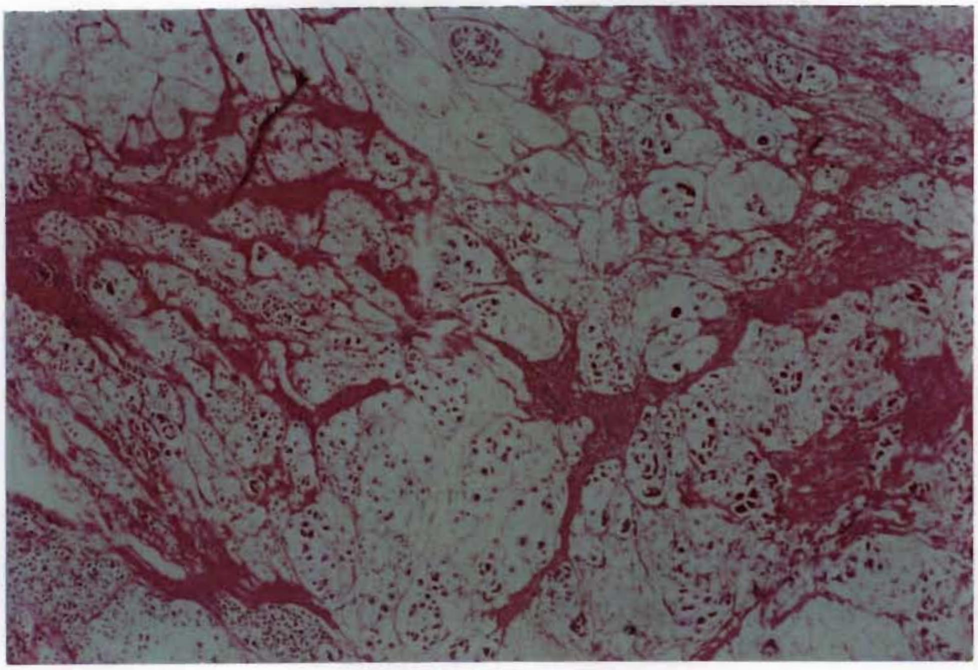


Figure 10: Low power view of a typical SR cancer with islands of tumour and single cells floating in lakes of extracellular mucin (H & E stain).

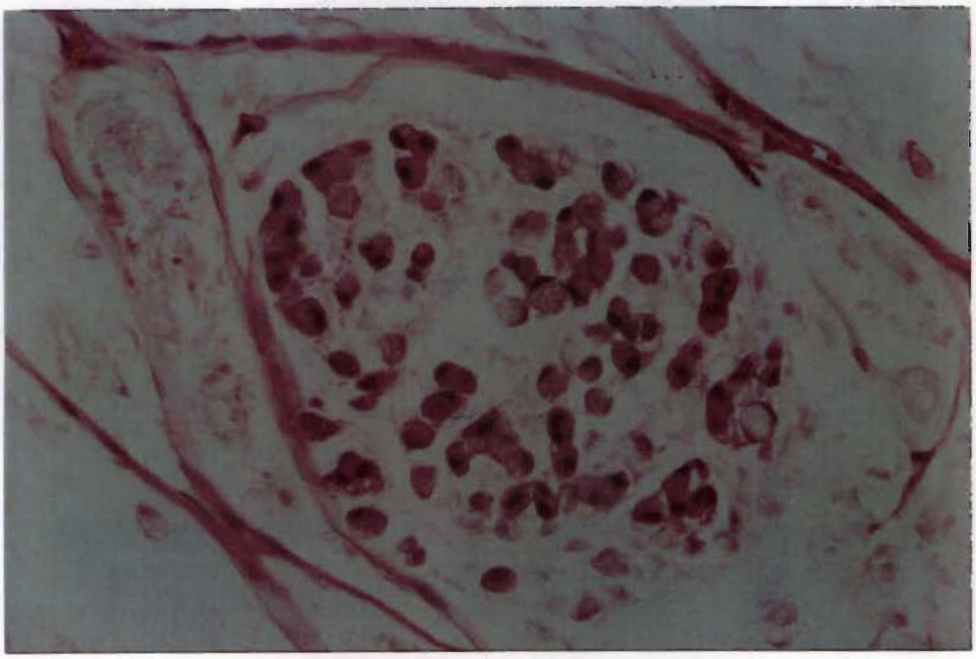


Figure 11: High power appearance of signet ring cells: intracytoplasmic mucin displacing the nucleus to an eccentric location (H & E stain).

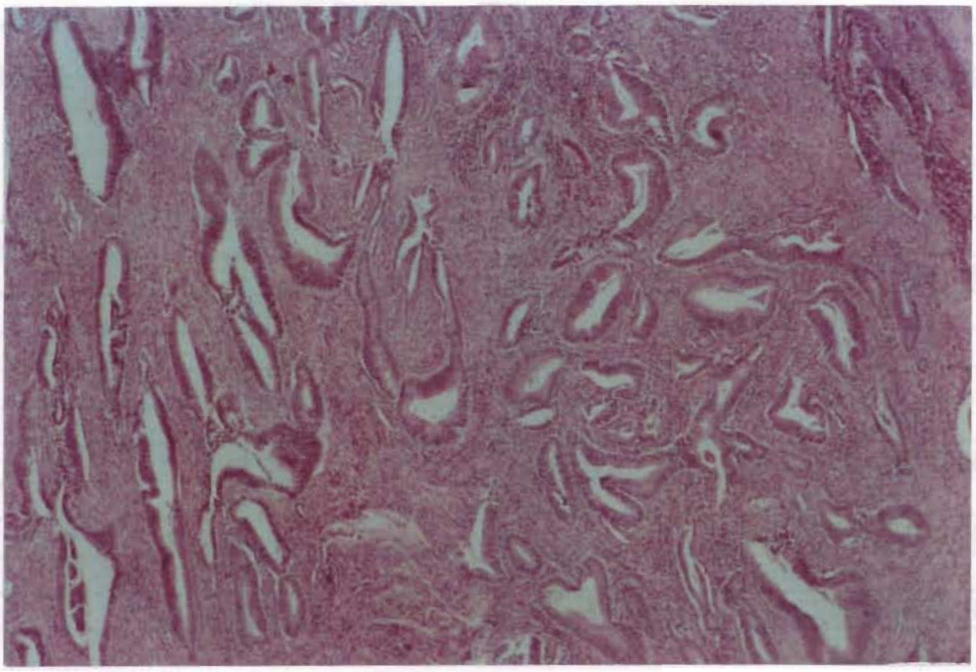


Figure 12: Well differentiated colorectal cancers contained well formed glandular structures infiltrating a desmoplastic stroma (H & E stain).

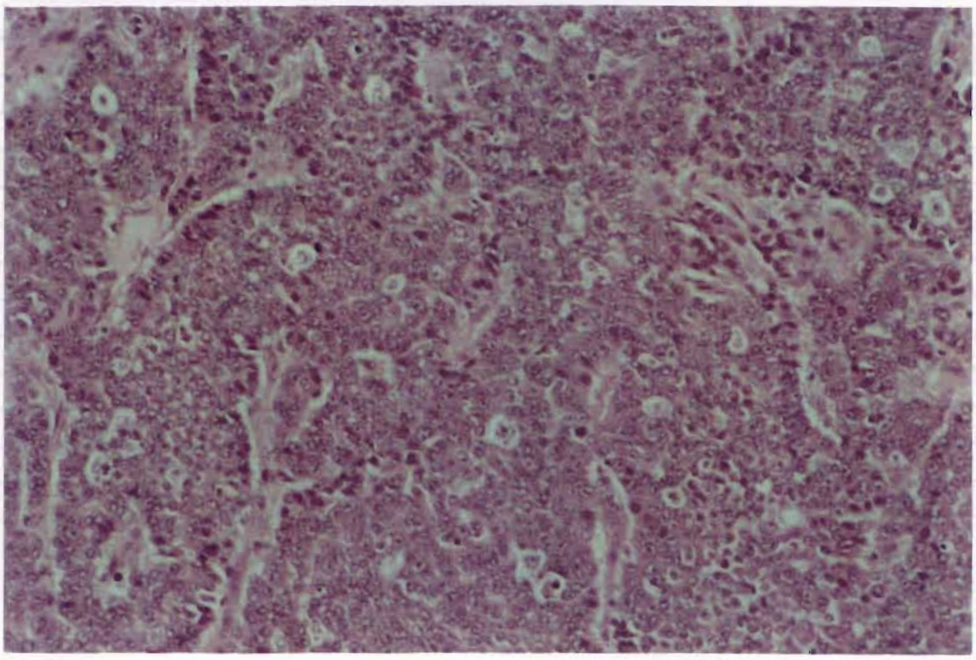


Figure 13: Poorly differentiated cancers contained sheets of tumour cells, with little attempt at glandular formation (H & E stain).

3.1.2 COLORECTAL CANCER - UNDER 35 YEARS

3.1.2.1 CLINICAL DATA

Table 5: Clinical data for colorectal cancer under the age of 35 years, showing age, sex and site of the tumours.

Case	Age	Sex	Site
1	34	M	ASCENDING COLON
2	27	F	RECTUM
3	33	M	COLON NOS
4	20	M	RECTUM
5	35	M	COLON NOS
6	34	F	ASCENDING COLON
7	21	M	RECTUM
8	28	M	ASCENDING COLON
9	21	F	SIGMOID
10	29	F	ASCENDING COLON
11	18	M	RECTUM
12	20	F	RECTUM
13	33	F	CAECUM
14	22	F	RECTUM
15	25	F	RECTUM
16	22	F	SIGMOID
17	26	M	RECTUM
18	23	F	SIGMOID
19	23	M	RECTUM
20	23	M	ASCENDING COLON
21	29	F	RECTUM
22	17	F	SIGMOID
23	23	F	RECTUM
24	29	F	CAECUM
25	28	F	RECTUM
26	24	M	RECTUM
27	23	M	CAECUM
28	29	F	COLON NOS
29	21	M	RECTUM
30	28	F	SIGMOID
31	25	F	CAECUM
32	24	F	COLON NOS

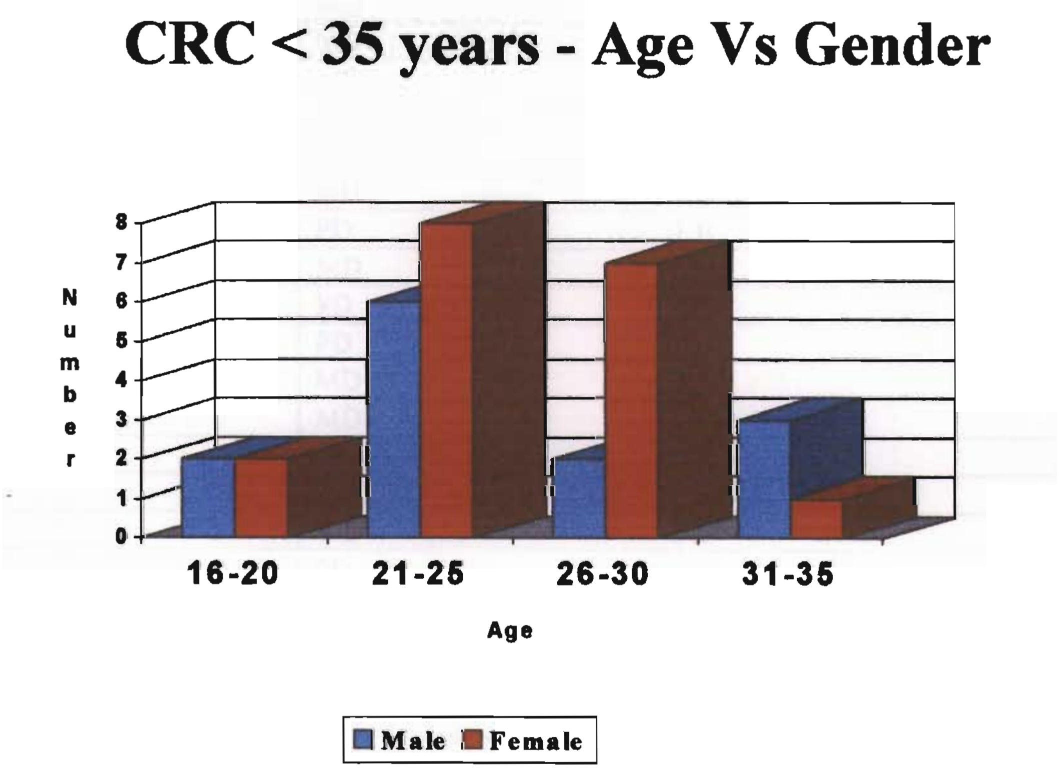


Figure 14: Graph of age and gender distribution for CRC under the age of 35

3.1.2.2 CRC < 35 - GRADE AND STAGE

Table 6: Grade and stage of colorectal tumours under the age of 35 years

Case	Grade	Stage
1	WD	C
2	MD	B
3	MD	C
4	MD	C
5	MUCINOUS	B
6	MUCINOUS	C
7	MD	B
8	PD	B
9	MD	B
10	PD	B
11	PD	D
12	MD	C
13	MD	B
14	WD	C
15	MD	A
16	WD	B
17	PD	C
18	MD	C
19	PD	C
20	PD	C
21	WD	D
22	MD	C
23	WD	B
24	MUCINOUS	C
25	WD	B
26	PD	C
27	MUCINOUS	C
28	MD	D
29	MUCINOUS	C
30	MD	B
31	WD	B
32	MD	B

3.1.3 COLORECTAL CANCER - OVER 50 YEARS

3.1.3.1 CLINICAL DATA

Table 7: Clinical data for colorectal cancer over the age of 50 years, showing age, sex and site of these tumours.

Case	Age	Sex	Site
1	63	F	RECTO-SIGMOID
2	63	F	RECTUM
3	56	M	RECTUM
4	51	M	RECTUM
5	68	F	RECTUM
6	65	M	ASCENDING COLON
7	57	M	ASCENDING COLON
8	55	F	ASCENDING COLON
9	72	F	DESCENDING COLON
10	60	F	CAECUM
11	74	M	RECTUM
12	60	F	RECTUM
13	65	M	RECTUM
14	60	F	RECTUM
15	70	F	RECTUM
16	59	M	SIGMOID
17	65	F	RECTUM
18	52	F	SIGMOID
19	52	M	RECTUM
20	61	F	RECTUM
21	72	F	RECTUM
22	53	M	SIGMOID
23	70	F	CAECUM
24	60	F	CAE /ASCENDING COLON
25	54	M	IL -CAECAL
26	66	F	SIGMOID
27	51	F	RECTUM
28	54	M	RECTUM
29	68	F	ASCENDING COLON
30	58	F	COLON NOS
31	55	F	RECTO/COLON
32	60	M	RECTUM
33	60	F	CAECUM
34	55	M	ASCENDING COLON
35	59	F	RECTUM
36	57	M	SIGMOID
37	50	M	RECTUM
38	74	F	DESCENDING COLON
39	73	F	ASCENDING COLON
40	62	F	SIGMOID
41	58	F	RECTUM
42	88	F	SIGMOID
43	67	F	ASCENDING COLON
44	81	F	RECTUM
45	73	F	RECTUM
46	61	M	ASCENDING COLON
47	84	M	SIGMOID
48	57	M	RECTUM
49	50	F	SIGMOID
50	53	F	CAE+ ASCENDING COLON

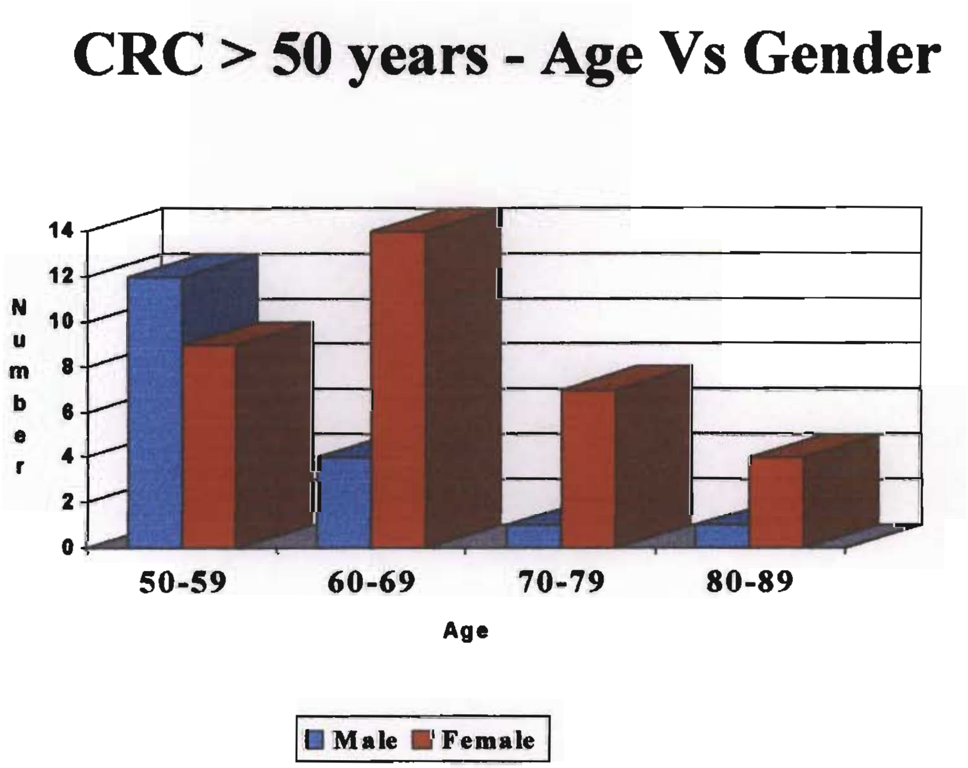

CRC > 50 years - Age Vs Gender

Figure 15: Graph of age and gender distribution in CRC over the age of 50

3.1.3.2 CRC > 50 - GRADE AND STAGE

Table 8: Grade and stage of colorectal cancer over the age of 50 years

Case	Grade	Stage
1	WD	B
2	MD	B
3	MD	C
4	MUCINOUS	B
5	MD	C
6	MD	B
7	MD	B
8	WD	B
9	WD	C
10	PD	C
11	MD	B
12	WD	C
13	WD	B
14	MD	C
15	MUCINOUS	C
16	PD	C
17	MD	B
18	WD	B
19	WD	B
20	MD	B
21	MD	B
22	MD	B
23	MUCINOUS	B
24	MUCINOUS	C
25	MD	C
26	WD	B
27	MUCINOUS	C
28	MUCINOUS	B
29	WD	C
30	MD	C
31	WD	B
32	PD	B
33	MUCINUOUS	B
34	WD	B
35	MD	B
36	MD	C
37	WD	C
38	MD	C
39	WD	C
40	WD	C
41	MD	C
42	WD	C
43	WD	B
44	WD	B
45	MUCINOUS	B
46	MD	B
47	MD	B
48	MUCINOUS	C
49	MD	C
50	MD	B

3.2.3 OESOPHAGEAL CANCER

3.2.3.1 LIGHT MICROSCOPIC EXAMINATION

All cases included in the study were squamous carcinomas that were one of 3 grades: well, moderate or poorly differentiated. The well differentiated cases were typified by abundant keratinisation (Figure 16) and an infiltrative pattern. Often surface mucosal dysplasia was also present (Figure 17).

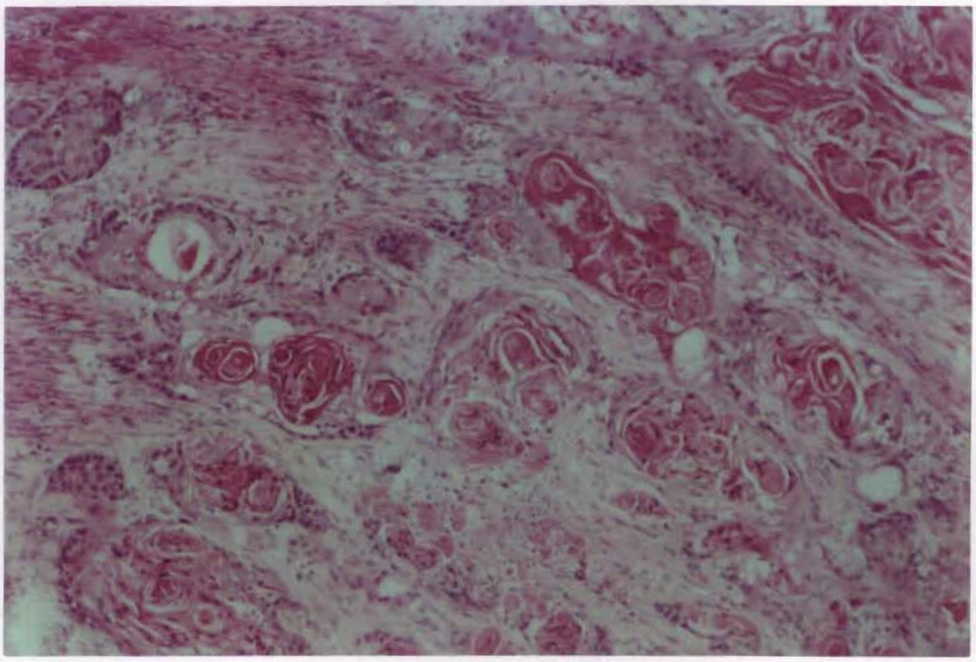


Figure 16: An example of a well differentiated infiltrating squamous cell carcinoma showing abundant keratinisation (H & E stain).

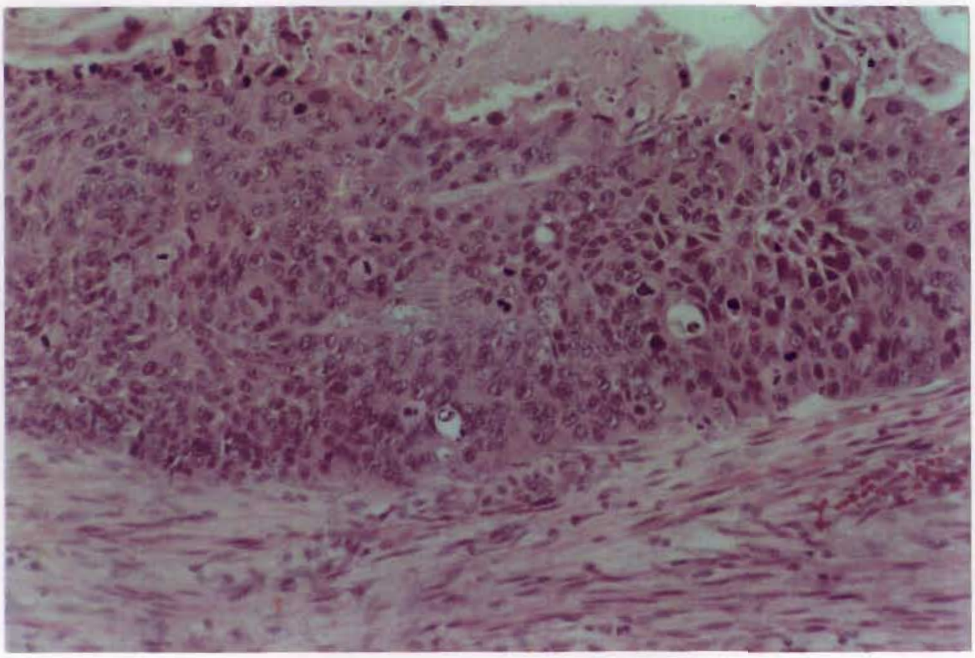


Figure 17: In all cases an *in situ* carcinoma component was present (H & E stain).

3.2.3.2 OESOPHAGEAL CANCER - CLINICAL DATA

Table 9: Clinical data for oesophageal cancer (Age, sex and site of tumour) are shown

Case	Age	Sex	Site
1	42	F	LOWER THIRD
2	54	F	LOWER THIRD
3	44	F	UPPER THIRD
4	49	F	LOWER THIRD
5	24	F	LOWER THIRD
6	62	F	MID THIRD
7	59	M	MID THIRD
8	71	M	LOWER THIRD
9	43	F	LOWER THIRD
10	55	M	MID THIRD
11	68	-	UPPER THIRD
12	68	F	LOWER THIRD
13	58	M	LOWER THIRD
14	77	M	UPPER THIRD
15	48	F	LOWER THIRD
16	58	F	LOWER THIRD
17	28	M	LOWER THIRD
18	54	M	LOWER THIRD
19	54	M	MID THIRD
20	63	M	LOWER THIRD
21	65	M	LOWER THIRD
22	48	M	MID THIRD
23	52	M	LOWER THIRD
24	53	F	MID THIRD
25	54	M	MID THIRD
26	44	F	LOWER THIRD
27	41	M	MID THIRD
28	54	F	LOWER THIRD
29	61	F	MID THIRD
30	68	F	LOWER THIRD
31	38	M	MID THIRD
32	63	F	MID THIRD
33	61	M	MID THIRD
34	33	M	LOWER THIRD
35	54	M	MID THIRD
36	50	F	MID THIRD
37	53	-	LOWER THIRD
38	50	M	LOWER THIRD
39	49	M	MID THIRD

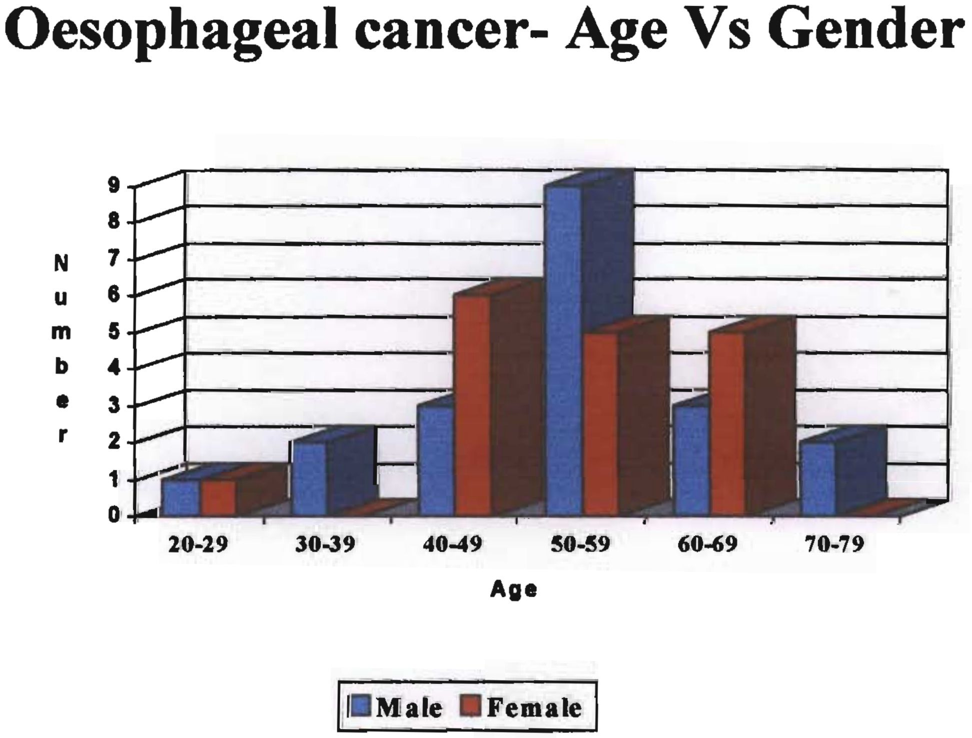


Figure 18: Graph of age and gender distribution in oesophageal cancer

3.2.3.3 OESOPHAGEAL CANCER - GRADE AND STAGE

Table 10: Grade and stage of oesophageal cancers

Case	Grade	Stage
1	MD	IIA
2	MD	IIB
3	WD	IIA
4	WD	IIA
5	PD	IIA
6	MD	IIB
7	PD	III
8	WD	IIA
9	WD	III
10	WD	IIA
11	MD	IIA
12	WD	III
13	PD	IIA
14	MD	IIA
15	MD	III
16	WD	IIA
17	WD	IIA
18	WD	IIA
19	PD	IIA
20	MD	IIB
21	WD	IIA
22	WD	IIA
23	MD	IIB
24	MD	IIB
25	MD	IIA
26	MD	IIB
27	WD	IIA
28	MD	III
29	WD	IIA
30	MD	IIB
31	MD	IIA
32	MD	IIB
33	WD	IIA
34	WD	IIA
35	MD	IIA
36	MD	IIA
37	MD	IIA
38	WD	IIB
39	WD	IIA

3.3 DNA EXTRACTION

Of the 79 colectomies in patients over the age of 50, only 50 cases yielded DNA of suitable quality for microsatellite PCR. 32 of the 37 colectomy cases from the under 35 age group were suitable for DNA analysis. In the oesophageal cancers, 39 of the 47 cases yielded amplifiable DNA.

3.3.1 AGAROSE GEL ELECTROPHORESIS

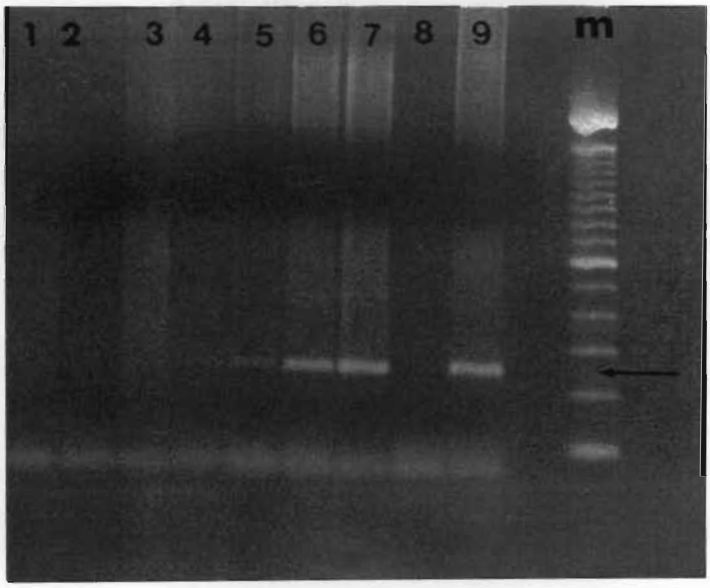


Figure 19: Representative results of a 2% agarose gel electrophoresis of DNA amplified across the insulin exon 2 promoter region from formalin-fixed paraffin-embedded sections of normal and microdissected tumour tissue. Lanes 1 to 7 contain PCR products. Lane 8 shows the negative control and lane 9 shows the positive control. Lane M shows the molecular weight marker (100 base pair ladder). Lanes 4, 5, 6, 7 and 9 show the amplified insulin fragment (arrowed), size 236 base pairs.

3.4 MICROSATELLITE ANALYSIS

3.4.1 COLORECTAL CANCER UNDER THE AGE OF 35 YEARS

Table 11: Results of the microsatellite analysis.

Case	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	AI	NAI	NAI	NAI	NAI	H
2	NAI	MSI	MSI	AI	MSI	NAI
3	NAI	NAI	NAI	AI	AI	H
4	NAI	H	NAI	NAI	MSI	H
5	MSI	H	NAI	NAI	AI	H
6	NAI	NAI	H	MSI	H	H
7	AI	NAI	NAI	H	H	H
8	NAI	NAI	NAI	H	NAI	NAI
9	MSI	MSI	H	NAI	AI	NAI
10	NAI	NAI	NAI	MSI	MSI	-
11	NAI	NAI	NAI	H	H	NAI
12	NAI	NAI	MSI	MSI	H	-
13	AI	NAI	NAI	H	H	NAI
14	H	MSI	H	NAI	NAI	NAI
15	NAI	NAI	MSI	MSI	H	AI
16	AI	NAI	NAI	NAI	H	NAI
17	MSI	MSI	MSI	H	MSI	H
18	H	NAI	NAI	NAI	NAI	H
19	NAI	AI	H	NAI	NAI	H
20	NAI	NAI	MSI	NAI	AI	MSI
21	NAI	H	NAI	MSI	MSI	NAI
22	AI	H	NAI	NAI	H	NAI
23	H	H	NAI	NAI	H	AI
24	AI	NAI	NAI	H	H	H
25	AI	H	NAI	NAI	H	NAI
26	H	H	H	NAI	MSI	NAI
27	H	H	NAI	MSI	AI	AI
28	NAI	AI	NAI	NAI	AI	NAI
29	NAI	H	NAI	H	H	NAI
30	MSI	MSI	MSI	MSI	MSI	NAI
31	MSI	MSI	MSI	NAI	MSI	NAI
32	NAI	NAI	H	NAI	AI	H

Table 11: Microsatellite analysis of colorectal cancer under the age of 35 years (NAI - No Allelic imbalance; H - Homozygous; AI - Allelic imbalance; MSI - Microsatellite instability; (-) Could not get a result for D3S1255.

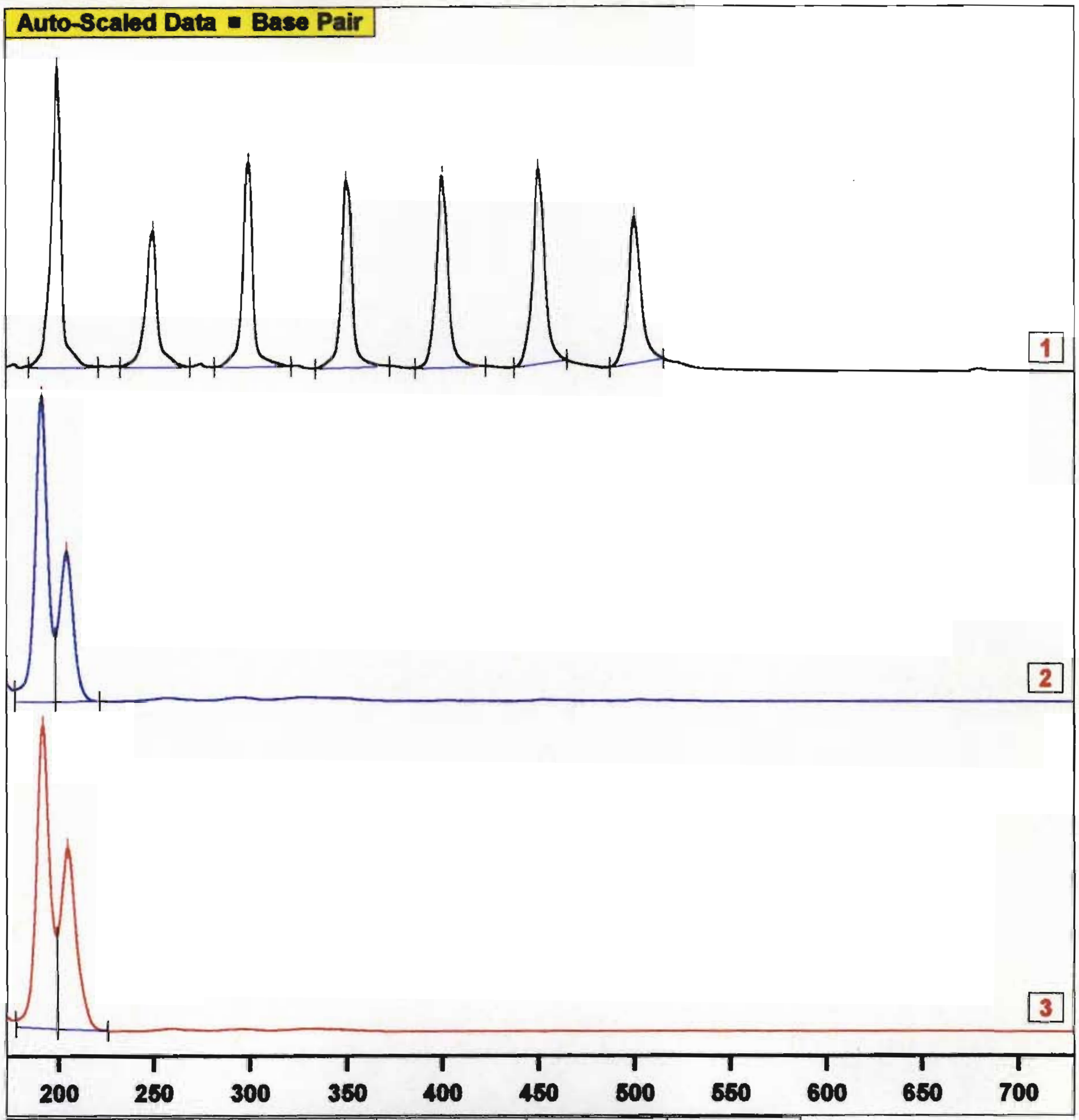
3.4.1.1 ANALYSIS OF ELECTROPHEROGRAMS

The Pharmacia Fragment Manager software version 1.0, was used to perform the microsatellite analysis. Each experiment was given a file name before the run on the automated sequencer. Normal and tumour DNA for each case was run in adjacent lanes on the gel. Once the electrophoretic run was completed, the data was automatically processed and stored under the relevant file name. The software detects every peak produced in each lane together with the run time, peak areas and the quantitative ratios when more than one peak is detected per lane. In addition, the size of the fragments (base pairs) are also produced when an external marker is used. All electropherograms illustrated include one lane with a 50-500 base pair external standard. The x-axis shows the product size in base pairs and the y-axis shows the peak height.

Electropherograms of representative examples from different microsatellite loci are shown in the following figures:

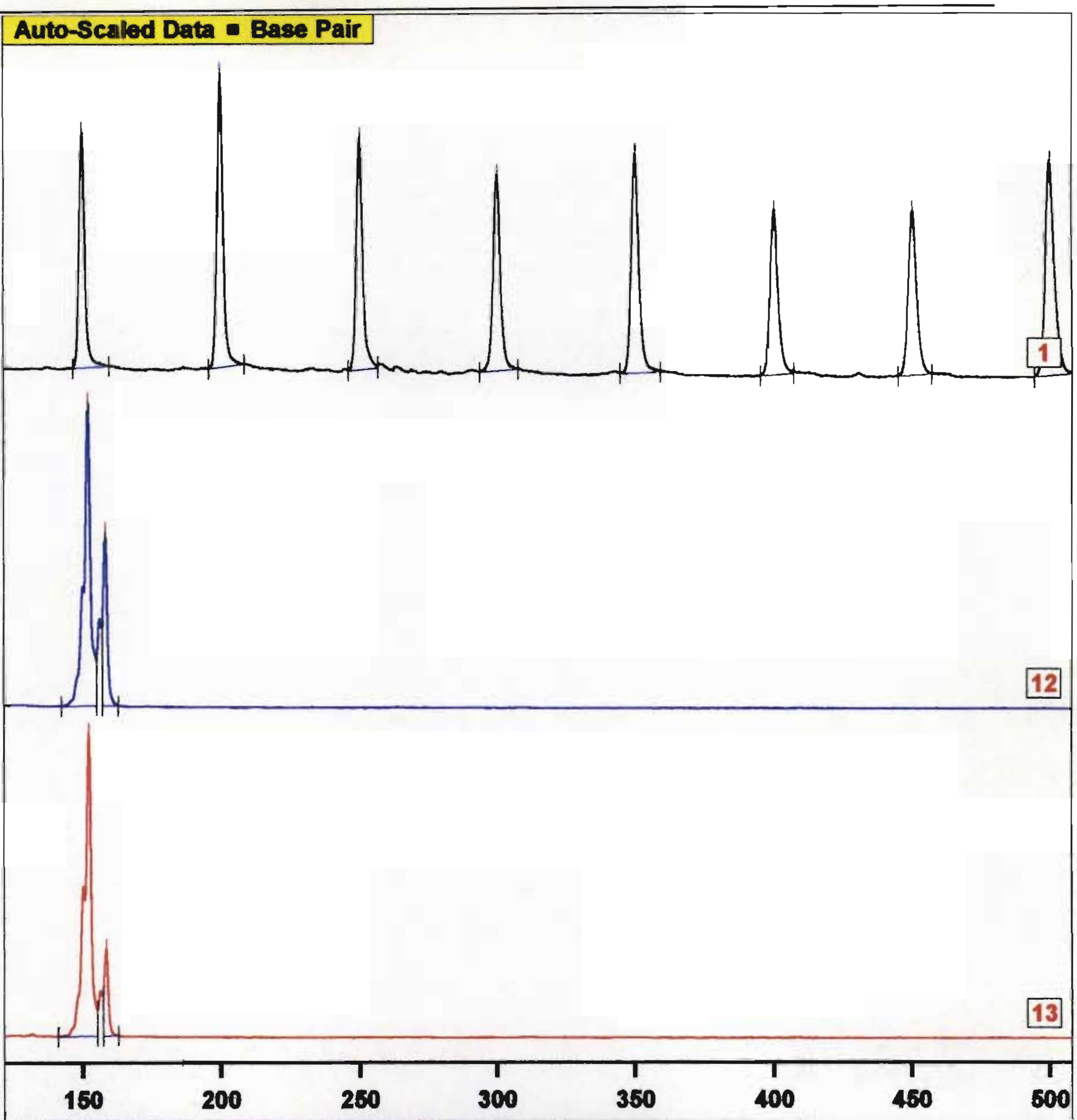
Figure 20 -	(Colorectal cancer < 35 years of age). NAI for the DCC locus
Figure 21 -	(Colorectal cancer < 35 years of age). AI for the D3S1255 locus
Figure 22 -	(Colorectal cancer < 35 years of age). Homozygous non informative case for the D18S34 locus
Figure 23 -	(Colorectal cancer < 35 years of age). MSI for D3S659 locus.
Figure 24-	(Colorectal cancer < 35 years of age). Showing LOH for the D2S123 locus

Additional electropherograms are shown in Appendix B



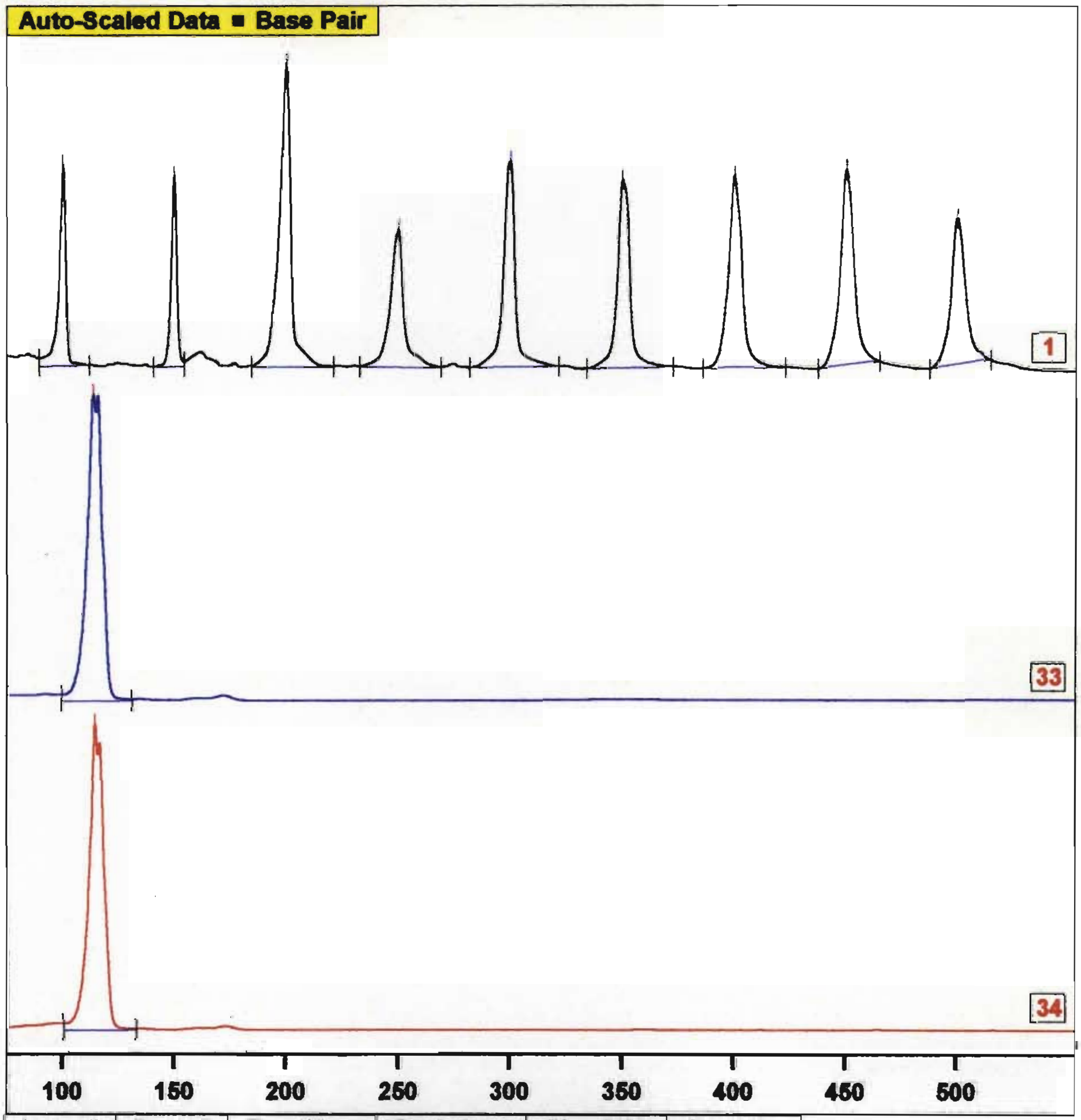
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	21:32	176.11	#50.0	0.30151
	2	24:53	152	#100.0	0.26024
	3	29:17	172.57	#150.0	0.29544
	4	35:03	584.09	#200.0	1
	5	40:39	309.4	#250.0	0.52971
	6	46:12	396.18	#300.0	0.67828
	7	51:57	404.75	#350.0	0.69296
	8	57:55	462.38	#400.0	0.79162
	9	63:55	473.05	#450.0	0.80988
	10	69:51	372.16	#500.0	0.63716
2	1	34:05	1140.8	191.6	1
	2	35:34	622.53	204.6	0.5457
3	1	34:09	1533.4	192.2	1
	2	35:38	1066.3	205.2	0.6954

Figure 20: Case 2; Showing NAI for the DCC locus. Lane 2 (normal DNA) and lane 3 (tumour DNA) show 2 peaks indicative of a heterozygous, informative case, with no difference in the peak area ratios. Lane 1 shows the 50 -500 base pair CY5 labelled external standard. The size range of the product is 150-210 base pairs.



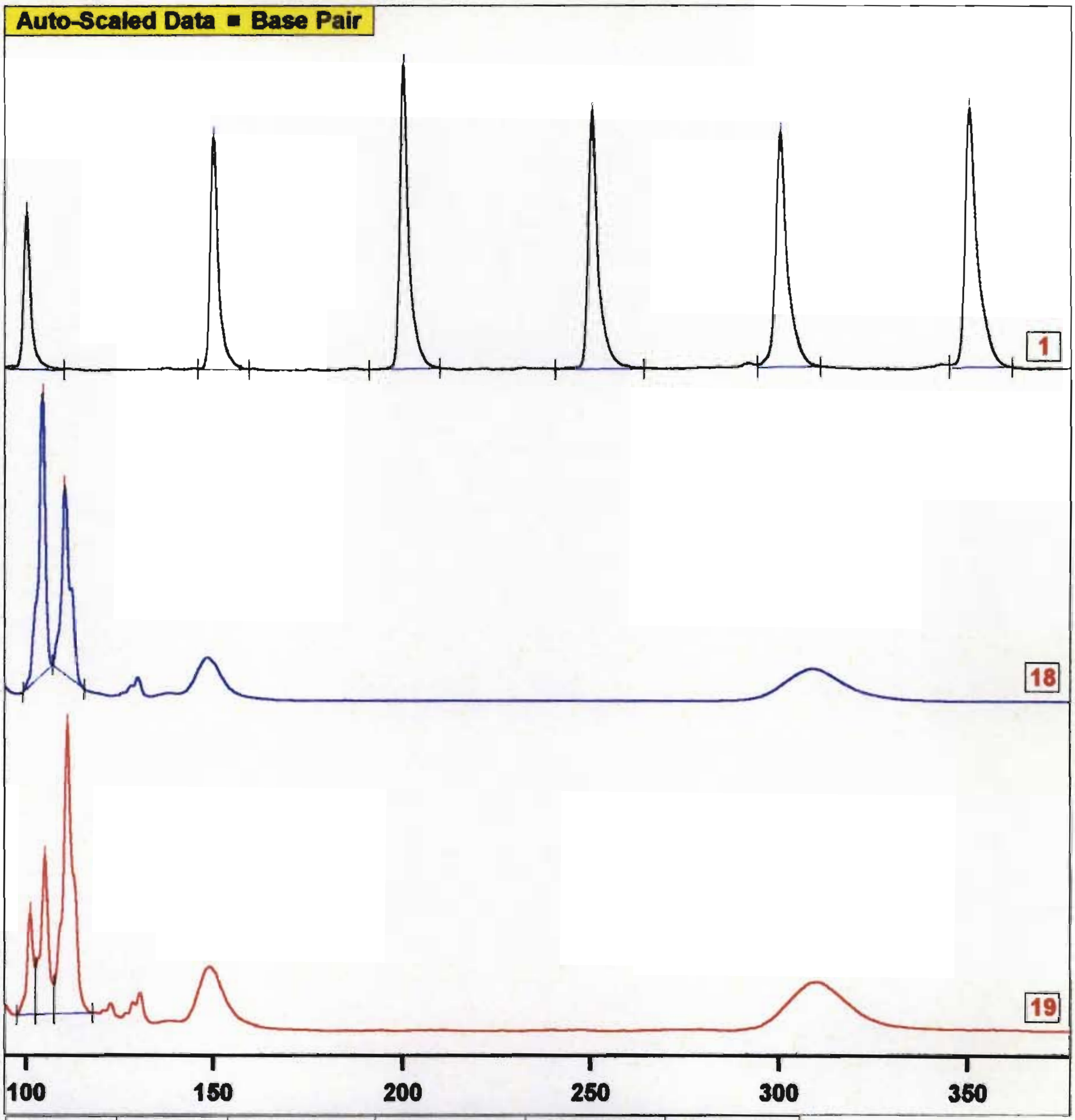
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	30:13	24.04	#50.0	0.24973
	2	37:19	33.308	#100.0	0.34602
	3	45:14	50.451	#150.0	0.5241
	4	53:52	72.38	#200.0	0.7519
	5	63:16	65.717	#250.0	0.68269
	6	73:11	64.907	#300.0	0.67428
	7	83:31	78.519	#350.0	0.81568
	8	94:10	64.089	#400.0	0.66578
	9	105:01	68.066	#450.0	0.70709
	10	115:54	96.262	#500.0	1
12	1	45:35	249.73	152.1	1
	2	46:37	94.274	158.3	0.3775
13	1	45:37	325.65	152.3	1
	2	46:39	58.16	158.5	0.1786

Figure 21: Case 15; Showing AI for the D3S1255 locus. The upper trace shows normal DNA with 2 peaks, representing 2 alleles. Lower trace shows tumour DNA with a difference in peak area ratios indicating AI. The ratio was 0.47 indicating loss of the larger allele. The ratio was calculated as described in section 2.8.8. The size range of the product is 140-160 base pairs



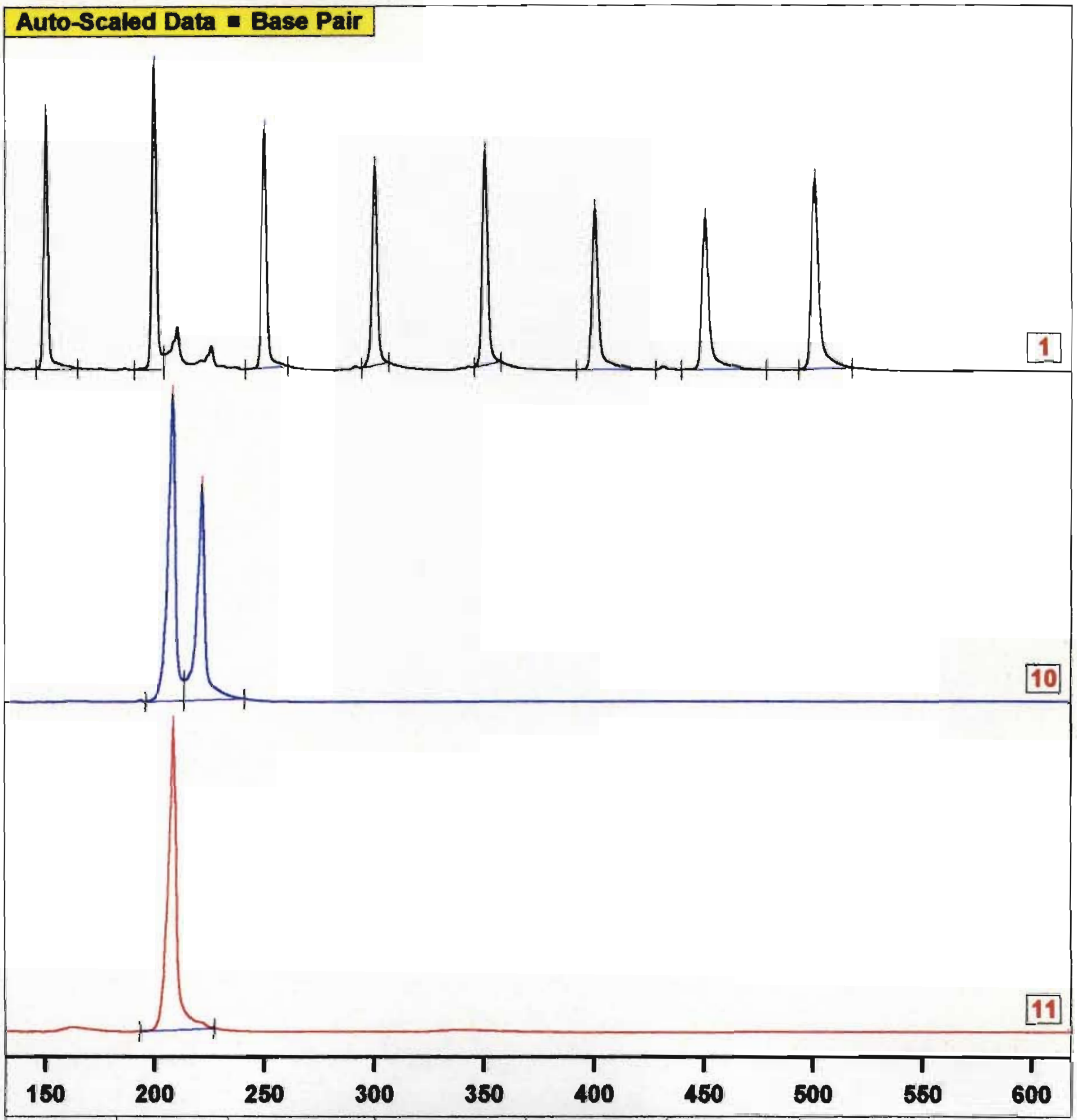
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	21:32	176.11	#50.0	0.30151
	2	24:53	152	#100.0	0.26024
	3	29:17	172.57	#150.0	0.29544
	4	35:03	584.09	#200.0	1
	5	40:39	309.4	#250.0	0.52971
	6	46:12	396.18	#300.0	0.67828
	7	51:57	404.75	#350.0	0.69296
	8	57:55	462.38	#400.0	0.79162
	9	63:55	473.05	#450.0	0.80988
	10	69:51	372.16	#500.0	0.63716
33	1	25:56	3953.4	113.4	1
34	1	26:00	3553	114.2	1

Figure 22: Case 29; Showing a homozygous case for locus D18S34, with 1 peak present in both the normal and tumour DNA samples. The size range of the products is 103-119 base pairs.



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	16:23	19.976	#50.0	0.14616
	2	20:56	40.404	#100.0	0.29564
	3	25:48	66.455	#150.0	0.48625
	4	30:58	102.65	#200.0	0.75112
	5	36:21	99.768	#250.0	0.73001
	6	41:50	103.28	#300.0	0.7557
	7	47:17	120.15	#350.0	0.87912
	8	52:40	98.957	#400.0	0.72407
	9	57:55	99.278	#450.0	0.72642
	10	63:01	136.67	#500.0	1
18	1	21:21	161.37	104.4	1
	2	21:54	139.12	110.2	0.86214
19	1	21:02	31.543	101.1	0.21988
	2	21:24	59.812	104.9	0.41693
	3	21:58	143.46	110.9	1

Figure 23: Case 30; Showing MSI for the D3S659 locus. The upper trace with normal DNA (lane 18) shows 2 peak, however the lower trace (lane 19) shows 3 peaks of, 101.1; 104.9 and 110.9 base pairs. The novel peak in the tumour DNA sample is at 101.1 base pairs. The size range of the product is 110-150 base pairs.



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
10	1	35:51	2795.2	208.3	1
	2	37:24	2161.2	221.8	0.77316
11	1	35:52	729.72	208.5	1

Figure 24: Case 28; Showing LOH/AI for the D2S123 locus. The upper trace (lane 10) shows normal DNA with 2 peaks, representing 2 alleles. Lower trace shows tumour DNA with loss of the second peak (lane 11). Lane 1 shows the 50 -500 base pair CY5 labelled external standard. The size range of the product is 198-228 base pairs.

3.4.2 COLORECTAL CANCER OVER THE AGE OF 50 YEARS

Table 12: Microsatellite analysis of colorectal cancer over the age of 50 years

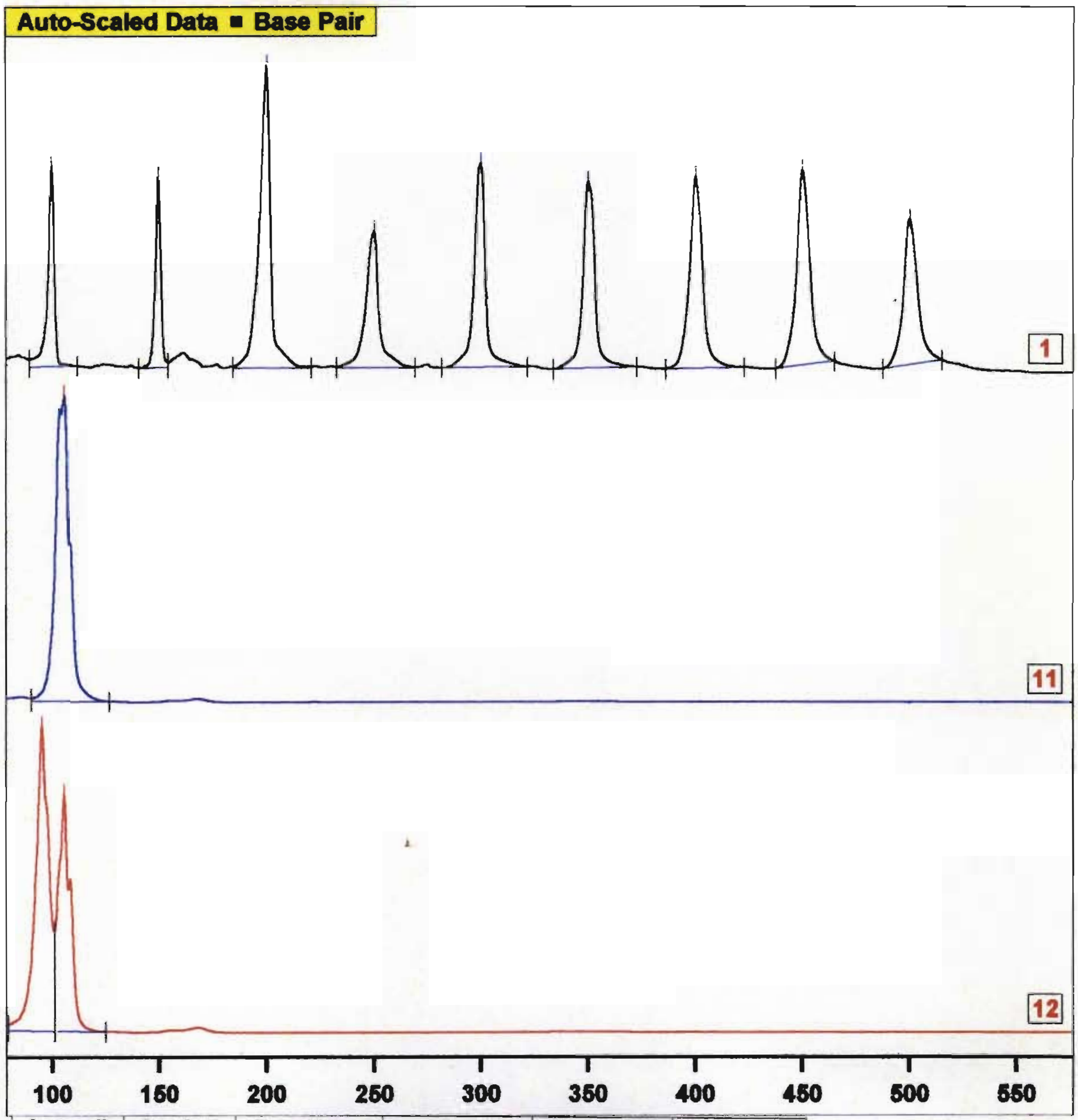
Case	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	MSI	H	NAI	NAI	H	NAI
2	NAI	NAI	NAI	NAI	H	H
3	MSI	NAI	H	NAI	H	AI
4	H	MSI	NAI	NAI	MSI	NAI
5	NAI	NAI	NAI	NAI	H	AI
6	NAI	NAI	NAI	H	NAI	AI
7	H	NAI	NAI	MSI	H	AI
8	H	AI	NAI	NAI	H	NAI
9	MSI	NAI	NAI	NAI	H	H
10	H	NAI	MSI	NAI	NAI	NAI
11	NAI	NAI	NAI	NAI	H	H
12	H	H	NAI	H	NAI	MSI
13	AI	NAI	NAI	NAI	H	H
14	NAI	H	MSI	H	H	H
15	H	H	MSI	NAI	NAI	NAI
16	NAI	H	H	H	H	MSI
17	NAI	H	MSI	H	NAI	H
18	H	MSI	MSI	NAI	H	NAI
19	NAI	NAI	NAI	NAI	NAI	NAI
20	NAI	H	H	MSI	AI	AI
21	MSI	H	H	H	H	NAI
22	NAI	H	H	MSI	NAI	H
23	NAI	H	MSI	NAI	NAI	NAI
24	H	AI	H	H	H	H
25	NAI	H	NAI	MSI	NAI	H
26	H	NAI	AI	NAI	NAI	NAI
27	MSI	NAI	AI	NAI	NAI	MSI
28	NAI	AI	NAI	MSI	MSI	H
29	AI	NAI	NAI	H	H	H
30	MSI	NAI	NAI	H	MSI	H
31	H	NAI	H	NAI	H	NAI
32	MSI	NAI	NAI	NAI	H	NAI
33	H	H	H	NAI	NAI	H
34	NAI	MSI	MSI	H	H	AI
35	NAI	NAI	NAI	NAI	NAI	NAI
36	H	H	H	H	NAI	NAI
37	NAI	AI	H	NAI	H	NAI
38	H	MSI	H	NAI	NAI	NAI
39	H	AI	MSI	H	NAI	H
40	H	H	MSI	NAI	H	AI
41	H	NAI	AI	NAI	NAI	NAI
42	H	H	H	H	NAI	AI
43	H	H	NAI	H	H	MSI
44	NAI	H	AI	NAI	NAI	H
45	NAI	H	H	H	NAI	AI
46	H	H	H	H	NAI	NAI
47	MSI	H	H	H	AI	NAI
48	AI	H	H	H	AI	AI
49	AI	NAI	H	AI	MSI	NAI
50	AI	H	NAI	H	NAI	NAI

3.4.2.1 ANALYSIS OF ELECTROPHEROGRAMS

Electropherograms of representative examples for CRC over the age of 50 are shown in the following figures:

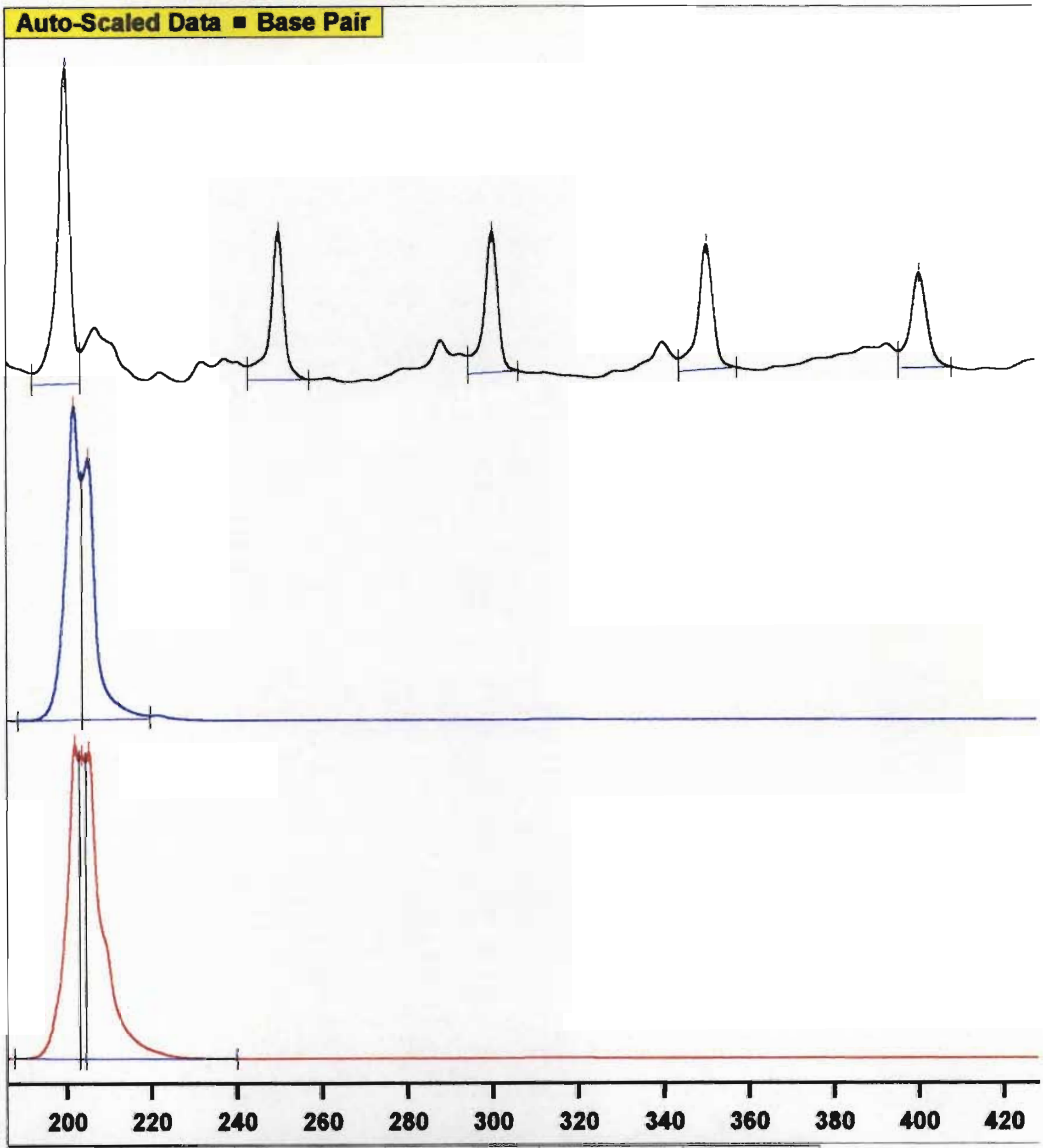
Figure 25	- (Colorectal cancer > 50) - MSI for D18S34 locus
Figure 26	- (Colorectal cancer > 50) - D2S123 locus with stutter band (NAI)
Figure 27	- (Colorectal cancer > 50) - LOH for D2S123 locus

Additional electropherograms are shown in Appendix C



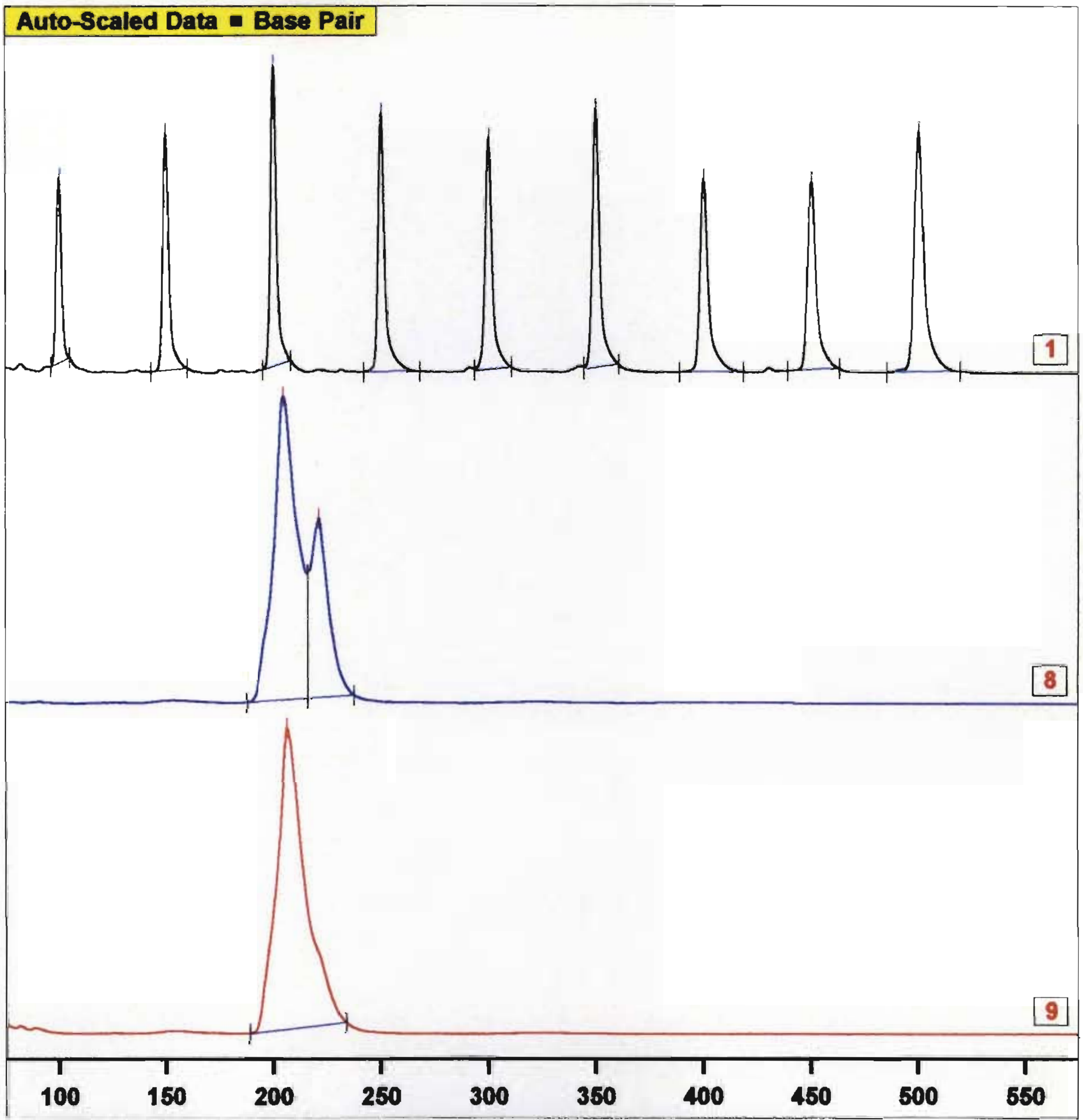
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	21:32	176.11	#50.0	0.30151
	2	24:53	152	#100.0	0.26024
	3	29:17	172.57	#150.0	0.29544
	4	35:03	584.09	#200.0	1
	5	40:39	309.4	#250.0	0.52971
	6	46:12	396.18	#300.0	0.67828
	7	51:57	404.75	#350.0	0.69296
	8	57:55	462.38	#400.0	0.79162
	9	63:55	473.05	#450.0	0.80988
	10	69:51	372.16	#500.0	0.63716
11	1	25:20	3658.4	105.9	1
12	1	24:33	2631.5	95.5	1
	2	25:20	1976.1	105.9	0.75096

Figure 25: Case 18; Showing MSI for locus D18S34. Lane 11 (normal DNA) with 1 peak and the tumour DNA in lane 12 with 2 peaks, the novel peak is seen at 95.5 base pairs in the tumour sample. Lane 1 shows the 50-500 CY5 external standard. The size range of the product is 103-119 base pairs



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:52	178.23	#50.0	0.38253
	2	36:08	162.79	#100.0	0.34939
	3	43:58	199.18	#150.0	0.42749
	4	51:12	465.94	#200.0	1
	5	59:26	249.64	#250.0	0.53579
	6	68:04	251.3	#300.0	0.53934
	7	76:56	251.12	#350.0	0.53897
	8	85:57	208.39	#400.0	0.44726
	9	95:04	292.77	#450.0	0.62835
	10	104:05	315.51	#500.0	0.67715
5	1	51:32	3210.8	202.0	1
	2	52:06	2643.1	205.5	0.8232
6	1	51:33	3074.7	202.1	0.73915
	2	51:50	1333.8	203.8	0.32065
	3	52:06	4159.8	205.5	1

Figure 26: Case 15; Showing NAI with a stutter band for the D2S123 locus. Lane 5 (normal DNA) with 2 peaks at 202 and 205.5 base pairs. Lane 6 (tumour DNA) show 3 peaks at 202.1, 203.8 and 205.5 base pairs. The 203.8 base pair peak in the tumour DNA is a stutter band and not a novel peak since it is less than 2 base pairs in size compared with the other 2 peaks in



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:18	100.98	#50.0	0.41157
	2	24:20	83.328	#100.0	0.33963
	3	29:35	133.16	#150.0	0.54271
	4	35:13	178.84	#200.0	0.72892
	5	41:08	181.39	#250.0	0.7393
	6	47:14	171.56	#300.0	0.69923
	7	53:23	199.1	#350.0	0.81148
	8	59:32	172.98	#400.0	0.70505
	9	65:38	166.78	#450.0	0.67976
	10	71:32	245.35	#500.0	1
8	1	35:45	902.12	204.6	1
	2	37:41	408.53	221.1	0.45286
9	1	35:56	1432.7	206.2	1

Figure 27: Case 20; Showing AI for locus D2S123. The normal DNA shows 2 peaks in lane 8, representing 2 alleles, while there is a loss of the second allele in the tumour DNA in lane 9. Lane 1 shows the 50-500 CY5 external standard.

3.4.3 OESOPHAGEAL CANCER

Table 13: Microsatellite analysis of oesophageal cancer

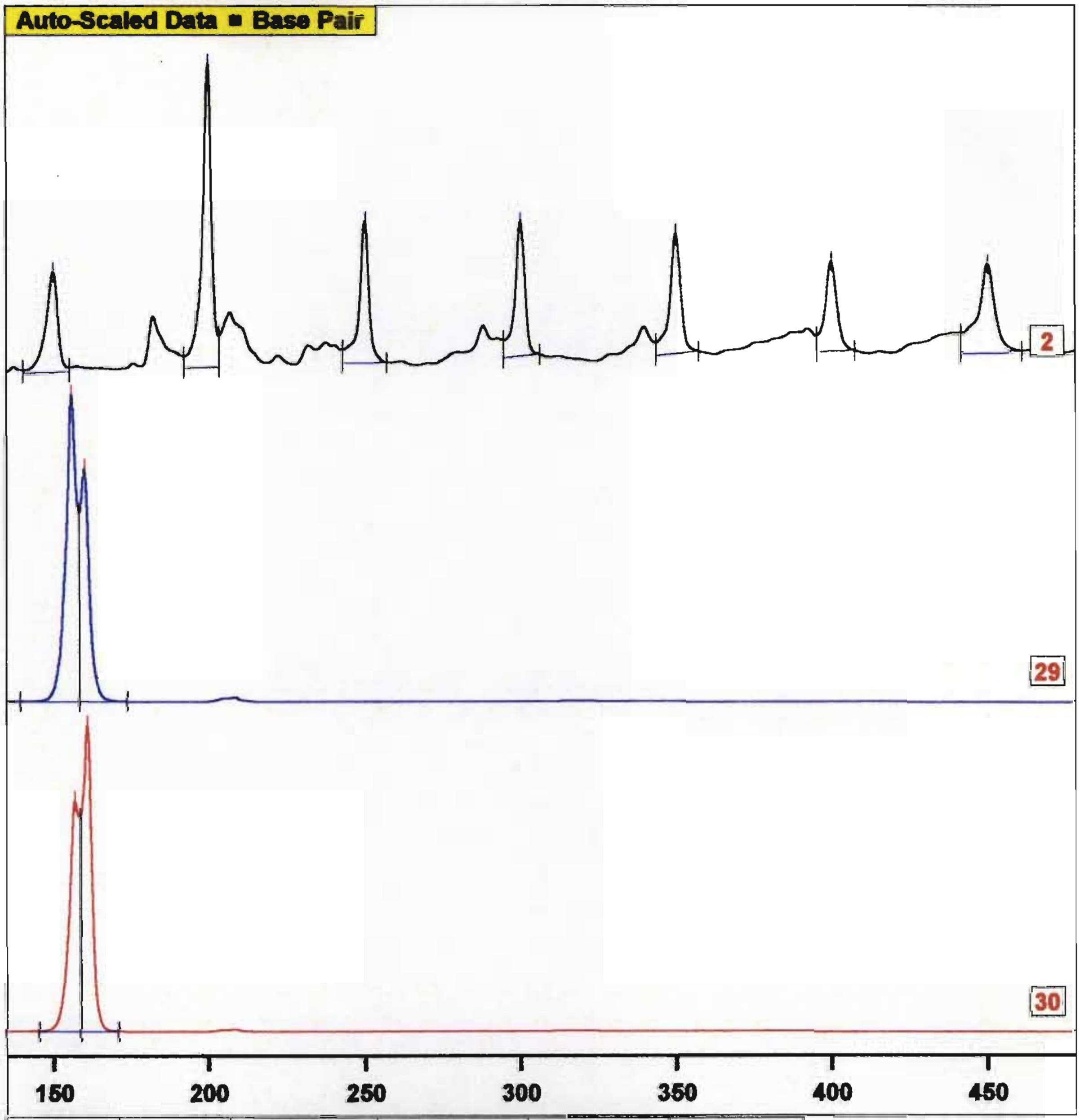
Case	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	NAI	H	NAI	NAI	H	NAI
2	H	H	NAI	AI	NAI	MSI
3	H	NAI	MSI	H	AI	MSI
4	AI	H	H	H	MSI	H
5	H	H	H	H	NAI	MSI
6	H	MSI	H	NAI	NAI	H
7	AI	H	NAI	NAI	NAI	H
8	H	H	NAI	AI	AI	H
9	NAI	H	NAI	H	MSI	AI
10	AI	AI	AI	NAI	NAI	NAI
11	H	AI	MSI	AI	NAI	H
12	H	H	H	NAI	AI	H
13	AI	H	AI	AI	NAI	NAI
14	AI	MSI	NAI	NAI	NAI	H
15	AI	H	NAI	H	NAI	H
16	NAI	NAI	NAI	H	NAI	H
17	H	NAI	AI	AI	NAI	NAI
18	NAI	H	H	AI	NAI	NAI
19	H	NAI	NAI	NAI	H	NAI
20	H	AI	AI	NAI	MSI	NAI
21	NAI	AI	AI	H	MSI	H
22	H	AI	H	NAI	H	H
23	H	H	NAI	H	H	H
24	MSI	H	NAI	H	H	NAI
25	H	NAI	AI	H	NAI	H
26	NAI	H	NAI	NAI	H	AI
27	MSI	NAI	NAI	AI	H	AI
28	H	NAI	H	H	NAI	NAI
29	AI	H	H	NAI	AI	AI
30	AI	NAI	H	NAI	H	NAI
31	H	H	NAI	H	H	H
32	H	H	H	H	AI	NAI
33	NAI	AI	H	NAI	H	NAI
34	H	H	MSI	H	H	H
35	H	H	H	H	H	-
36	NAI	H	NAI	H	H	-
37	AI	H	H	NAI	AI	-
38	H	H	H	H	H	H
39	H	H	NAI	H	H	NAI

3.4.3.1 ANALYSIS OF ELECTROPHEROGRAMS

Electropherograms of representative examples for oesophageal cancer are shown in the following figures:

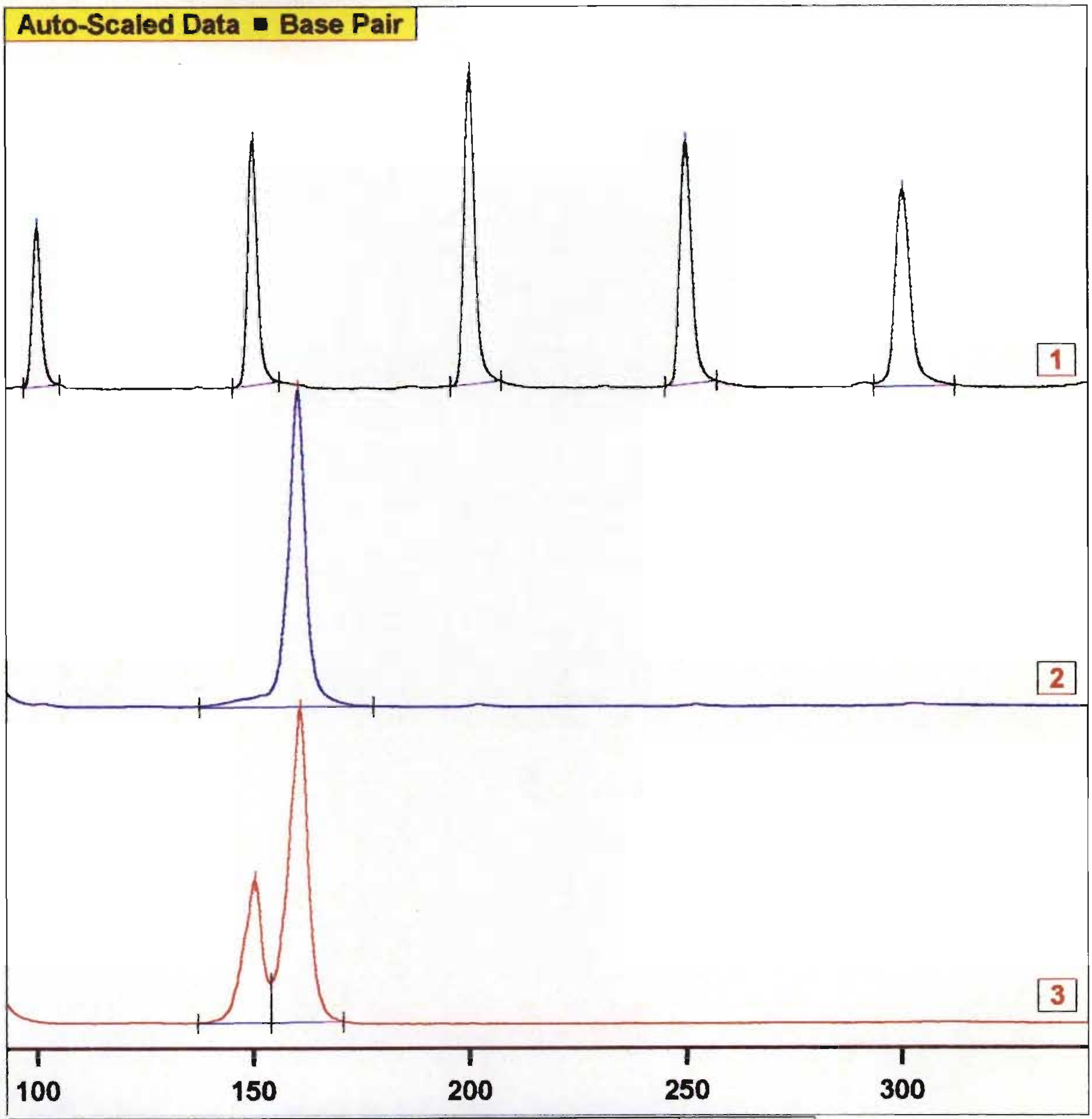
Figure 28	- (Oesophageal cancer) - AI for D18S58 locus
Figure 29	- (Oesophageal cancer) - MSI for D3S1255 locus
Figure 30	- (Oesophageal cancer) - AI for D3S659 locus

Additional electropherograms for oesophageal cancer are shown in Appendix D



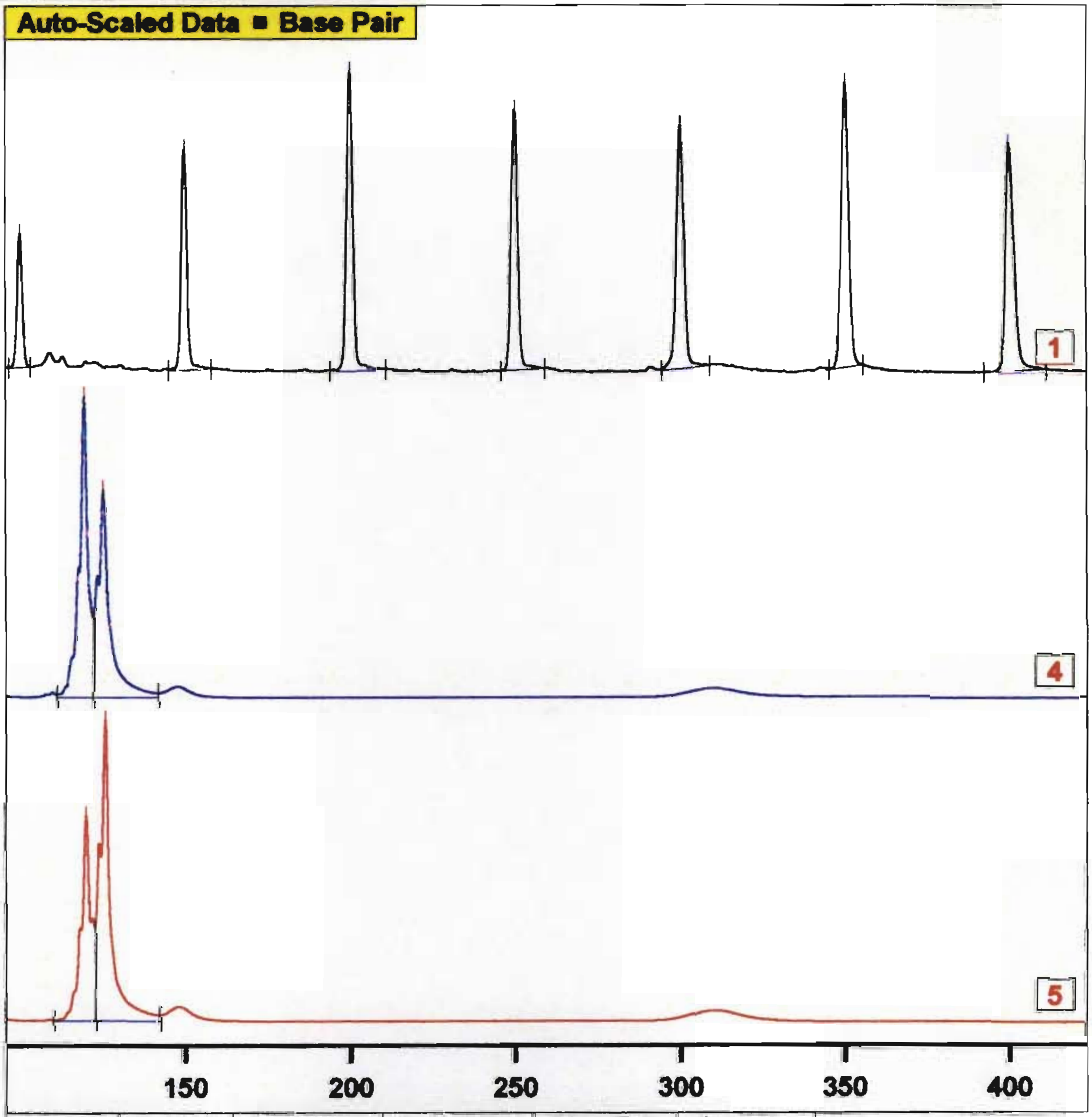
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:52	178.23	#50.0	0.38253
	2	36:08	162.79	#100.0	0.34939
	3	43:58	199.18	#150.0	0.42749
	4	51:12	465.94	#200.0	1
	5	59:26	249.64	#250.0	0.53579
	6	68:04	251.3	#300.0	0.53934
	7	76:56	251.12	#350.0	0.53897
	8	85:57	208.39	#400.0	0.44726
	9	95:04	292.77	#450.0	0.62835
	10	104:05	315.51	#500.0	0.67715
29	1	44:50	3339.9	156.0	1
	2	45:26	2145.7	160.1	0.64244
30	1	45:00	1676.9	157.1	0.75664
	2	45:33	2216.3	160.9	1

Figure 28: Case 25; Showing AI for the D18S58 locus. The upper trace shows normal DNA with 2 peaks, representing 2 alleles. Lower trace shows tumour DNA with a difference in peak area ratios indicating AI. The ratio was 2.05 indicating loss of the smaller allele. The ratio was calculated as described in section 2.8.8. The size range of the product is 144-160 base pairs



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	22:45	26.012	#50.0	0.30935
	2	28:43	31.341	#100.0	0.37273
	3	34:59	55.94	#150.0	0.66526
	4	41:41	83.839	#200.0	0.99706
	5	48:48	76.505	#250.0	0.90984
	6	56:10	83.758	#300.0	0.9961
	7	63:38	84.086	#350.0	1
2	1	36:19	312.98	160.2	1
3	1	35:02	117.62	150.4	0.42233
	2	36:24	278.49	160.9	1

Figure 29: Case 2; Showing MSI for the D3S1255 locus. The upper trace with normal DNA (lane 2) shows 1 peak, however the tumour DNA (lane 3) shows 2 peaks. The novel allele is 150.4 base pairs. Lane 1 shows the 50-500 base pair external standard. The product size range is 140-160 base pairs



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	17:07	6.743	#50.0	0.086525
	2	21:46	16.338	#100.0	0.20964
	3	26:45	32.788	#150.0	0.42073
	4	32:00	50.289	#200.0	0.6453
	5	37:30	47.242	#250.0	0.6062
	6	43:04	49.966	#300.0	0.64116
	7	48:39	58.086	#350.0	0.74535
	8	54:10	51.119	#400.0	0.65596
	9	59:35	53.631	#450.0	0.68819
	10	64:51	77.931	#500.0	1
4	1	23:42	1545.7	119.8	1
	2	24:16	1450.2	125.5	0.93821
5	1	23:45	860.19	120.3	0.60439
	2	24:20	1423.2	126.1	1

Figure 30: Case 27; Showing AI for the D3S659 locus. Two peaks in lane 4 (normal DNA) and 2 peaks in lane 5 (tumour DNA). Lower trace shows tumour DNA with a difference in peak area ratios indicating AI. The ratio was 1.76 indicating loss of the smaller allele. The ratio was calculated as described in section 2.8.8. The size range of the product is 110-150 base pairs

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Table 14: Colorectal cancer under the age of 35 years. Summary table showing details of the molecular analysis with clinicopathological data.

Case	Age	Sex	Grade	Stage	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	34	M	WD	C	AI	NAI	NAI	NAI	NAI	H
2	27	F	MD	B	NAI	MSI	MSI	AI	MSI	NAI
3	33	M	MD	C	NAI	NAI	NAI	AI	AI	H
4	20	M	MD	C	NAI	H	NAI	NAI	MSI	H
5	35	M	PD	B	MSI	H	NAI	NAI	AI	H
6	34	F	PD	C	NAI	NAI	H	MSI	H	H
7	21	M	MD	B	AI	NAI	NAI	H	H	H
8	28	M	PD	B	NAI	NAI	NAI	H	NAI	NAI
9	21	F	MD	B	MSI	MSI	H	NAI	AI	NAI
10	29	F	PD	B	NAI	NAI	NAI	MSI	MSI	-
11	18	M	PD	D	NAI	NAI	NAI	H	H	NAI
12	20	F	MD	C	NAI	NAI	MSI	MSI	H	-
13	33	F	MD	B	AI	NAI	NAI	H	H	NAI
14	22	F	WD	C	H	MSI	H	NAI	NAI	NAI
15	25	F	MD	A	NAI	NAI	MSI	MSI	H	AI
16	22	F	WD	B	AI	NAI	NAI	NAI	H	NAI
17	26	M	PD	C	MSI	MSI	MSI	H	MSI	H
18	23	F	MD	C	H	NAI	NAI	NAI	NAI	H
19	23	M	PD	C	NAI	AI	H	NAI	NAI	H
20	23	M	PD	C	NAI	NAI	MSI	NAI	AI	MSI
21	29	F	WD	D	NAI	H	NAI	MSI	MSI	NAI
22	17	F	MD	C	AI	H	NAI	NAI	H	NAI
23	23	F	WD	B	H	H	NAI	NAI	H	AI
24	29	F	PD	C	AI	NAI	NAI	H	H	H
25	28	F	WD	B	AI	H	NAI	NAI	H	NAI
26	24	M	PD	C	H	H	H	NAI	MSI	NAI
27	23	M	PD	C	H	H	NAI	MSI	AI	AI
28	29	F	MD	D	NAI	AI	NAI	NAI	AI	NAI
29	21	M	PD	C	NAI	H	NAI	H	H	NAI
30	28	F	MD	B	MSI	MSI	MSI	MSI	MSI	NAI
31	25	F	WD	B	MSI	MSI	MSI	NAI	MSI	NAI
32	24	F	MD	B	NAI	NAI	H	NAI	AI	H

WD: well differentiated; MD: moderately differentiated; PD: poorly differentiated; AI: loss of heterozygosity; NAI: no loss of heterozygosity; H: homozygous, uninformative case; MSI: microsatellite instability

Percentage cases positive for AI/MSI - (28/32) - 87.5%. There were 18F and 10M. (18F - 2 > 30 years and 16 <30 years); (10M - 3 > 30 years and 7 < 30 years)

Table 15: Colorectal cancer over the age of 50. Summary table showing details of the molecular analysis with clinicopathological data.

Case	Age	Sex	Stage	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	63	F	B	MSI	H	NAI	NAI	H	NAI
2	63	F	B	NAI	NAI	NAI	NAI	H	H
3	56	M	C	MSI	NAI	H	NAI	H	AI
4	51	M	B	H	MSI	NAI	NAI	MSI	NAI
5	68	F	C	NAI	NAI	NAI	NAI	H	AI
6	65	M	B	NAI	NAI	NAI	H	NAI	AI
7	57	M	B	H	NAI	NAI	MSI	H	AI
8	55	F	B	H	AI	NAI	NAI	H	NAI
9	72	F	C	MSI	NAI	NAI	NAI	H	H
10	60	F	C	H	NAI	MSI	NAI	NAI	NAI
11	74	M	B	NAI	NAI	NAI	NAI	H	H
12	60	F	C	H	H	NAI	H	NAI	MSI
13	65	M	B	AI	NAI	NAI	NAI	H	H
14	60	F	C	NAI	H	MSI	H	H	H
15	70	F	C	H	H	MSI	NAI	NAI	NAI
16	59	M	C	NAI	H	H	H	H	MSI
17	65	F	B	NAI	H	MSI	H	NAI	H
18	52	F	B	H	MSI	MSI	NAI	H	NAI
19	52	M	B	NAI	NAI	NAI	NAI	NAI	NAI
20	61	F	B	NAI	H	H	MSI	AI	AI
21	72	F	B	MSI	H	H	H	H	NAI
22	52	M	B	NAI	H	H	MSI	NAI	H
23	70	F	B	NAI	H	MSI	NAI	NAI	NAI
24	55	F	C	H	AI	H	H	H	H
25	54	M	C	NAI	H	NAI	MSI	NAI	H
26	66	F	B	H	NAI	AI	NAI	NAI	NAI
27	51	F	C	MSI	NAI	AI	NAI	NAI	MSI
28	64	M	B	NAI	AI	NAI	MSI	MSI	H
29	68	F	C	AI	NAI	NAI	H	H	H
30	58	F	C	MSI	NAI	NAI	H	MSI	H
31	55	F	B	H	NAI	H	NAI	H	NAI
32	60	M	B	MSI	NAI	NAI	NAI	H	NAI
33	60	F	B	H	H	H	NAI	NAI	H
34	55	M	B	NAI	MSI	MSI	H	H	AI
35	59	F	B	NAI	NAI	NAI	NAI	NAI	NAI
36	57	M	C	H	H	H	H	NAI	NAI
37	50	M	C	NAI	AI	H	NAI	H	NAI
38	74	F	C	H	MSI	H	NAI	NAI	NAI
39	73	F	C	H	AI	MSI	H	NAI	H
40	62	F	C	H	H	MSI	NAI	H	AI
41	58	F	C	H	NAI	AI	NAI	NAI	NAI
42	88	F	C	H	H	H	H	NAI	AI
43	67	F	B	H	H	NAI	H	H	MSI
44	81	F	B	NAI	H	AI	NAI	NAI	H
45	73	F	B	NAI	H	H	H	NAI	AI
46	61	M	B	H	H	H	H	NAI	NAI
47	84	M	B	MSI	H	H	H	AI	NAI
48	57	M	C	AI	H	H	H	AI	AI
49	50	F	C	AI	NAI	H	AI	MSI	NAI
50	53	F	B	AI	H	NAI	H	NAI	NAI

Percentage cases positive for AI/MSI - (42/50) 84%. There were 28 F and 14 M. 28 F - (20 > 60 years and 8 < 60 years); 14 M- (5 > 60 years and 9 < 60 years)

Table 18: Oesophageal cancer. Summary table showing details of the molecular analysis with clinicopathological data.

Case	Age	Sex	Stage	DCC	D18S34	D18S58	D3S659	D2S123	D3S1255
1	42	F	IIA	NAI	H	NAI	NAI	H	NAI
2	54	F	IIB	H	H	NAI	AI	NAI	MSI
3	44	F	IIA	H	NAI	MSI	H	AI	MSI
4	49	F	IIA	AI	H	H	H	MSI	H
5	24	F	IIA	H	H	H	H	NAI	MSI
6	62	F	IIB	H	MSI	H	NAI	NAI	H
7	59	M	III	AI	H	NAI	NAI	NAI	H
8	71	M	IIA	H	H	NAI	AI	AI	H
9	43	F	III	NAI	H	NAI	H	MSI	AI
10	55	M	IIA	AI	AI	AI	NAI	NAI	NAI
11	68	-	IIA	H	AI	MSI	AI	NAI	H
12	68	F	III	H	H	H	NAI	AI	H
13	58	M	IIA	AI	H	AI	AI	NAI	NAI
14	77	M	IIA	AI	MSI	NAI	NAI	NAI	H
15	48	F	III	AI	H	NAI	H	NAI	H
16	58	F	IIA	NAI	NAI	NAI	H	NAI	H
17	28	M	IIA	H	NAI	AI	AI	NAI	NAI
18	54	M	IIA	NAI	H	H	AI	NAI	NAI
19	54	M	IIA	H	NAI	NAI	NAI	H	NAI
20	63	M	IIB	H	AI	AI	NAI	MSI	NAI
21	65	M	IIA	NAI	AI	AI	H	MSI	H
22	48	M	IIA	H	AI	H	NAI	H	H
23	52	M	IIB	H	H	NAI	H	H	H
24	53	F	IIB	MSI	H	NAI	H	H	NAI
25	54	M	IIA	H	NAI	AI	H	NAI	H
26	44	F	IIB	NAI	H	NAI	NAI	H	AI
27	41	M	IIA	MSI	NAI	NAI	AI	H	AI
28	54	F	III	H	NAI	H	H	NAI	NAI
29	61	F	IIA	AI	H	H	NAI	AI	AI
30	68	F	IIB	AI	NAI	H	NAI	H	NAI
31	38	M	IIA	H	H	NAI	H	H	H
32	63	F	IIB	H	H	H	H	AI	NAI
33	61	M	IIA	NAI	AI	H	NAI	H	NAI
34	33	M	IIA	H	H	MSI	H	H	H
35	54	M	IIA	H	H	H	H	H	-
36	50	F	IIA	NAI	H	NAI	H	H	-
37	53	-	IIA	AI	H	H	NAI	AI	-
38	50	M	IIB	H	H	H	H	H	H
39	49	M	IIA	H	H	NAI	H	H	NAI

Percentage cases positive for AI/MSI - (30/39) 76.9%. There were 14F and 14M. (14 F - 9 < 60 years and 5 > 60 years);(14 M - 9 < 60 years and 5 > 60 years).

Survival analysis:

Two patients (cases # 9 and 14) died of causes unrelated to oesophageal cancer. However, a longer follow-up period is required to adequately assess the effect of genetic abnormalities on survival in oesophageal cancer.

CHAPTER 4

DISCUSSION

4.1 COLORECTAL CANCER UNDER THE AGE OF 35 YEARS

The use of semi- and fully- automated assessment of LOH and MSI or replication errors (RER) with fluorescent technology has been advocated by Cawkwell and colleagues (Cawkwell *et al.*, 1993). The advantage of this technique over conventional radioactive methods includes the use of paraffin embedded tissue, is less time consuming and introduces computer-assisted objectivity (Cawkwell *et al.*, 1993). This technology can be adapted to analyse both AI and MSI, two important mechanisms in the pathogenesis of colorectal cancer (Chetty *et al.*, 1998).

The phenomenon of MSI results from strand slippage due to defects in the DNA repair genes, which are meant to maintain the integrity of DNA during replication. Failure to do so results in the production of new alleles, which are detected by different peak patterns on electrophoretograms. MSI is found in a wide range of malignancies, but is characteristic of CRC associated with the HNPCC syndrome. Not many studies analysing AI and MSI of the *DCC* gene in patients under 35 years of age with CRC, exist. However, *DCC* has been reasonably intensively investigated in sporadic CRC in patients over the age of 50 years (Delattre *et al.*, 1989; Fearon *et al.*, 1990; Cawkwell *et al.*, 1995; Martinez-Lopez *et al.*, 1998).

Allelic losses of chromosome 18q have been reported to occur in up to 70% of sporadic colorectal cancers in the elderly (Cho and Fearon, 1995). However, studies looking specifically at 18q21, the putative site of the *DCC* gene, show AI or LOH to occur in 29 to 52% of sporadic cases (Cawkwell *et al.*, 1995; Law *et al.*, 1988).

The *DCC* gene is a tumour suppressor gene that encodes a cell adhesion molecule, and is thought to be absent or reduced in high stage colorectal cancers

(Kolodziej, 1997). Indeed, allelic loss of chromosome 18q is thought to have prognostic ramifications in CRC in the elderly (Jen *et al.*, 1994). Stage II (or stage B) CRC with 18q losses behave like stage III (stage C) cancers. In the analysis of our cases, AI and MSI did not correlate with any clinicopathological parameter in the under 35 age group, but it should be noted that the numbers within the different stages were small. Thirty nine percent of the cases in this study demonstrated AI for the 2 markers closest to the *DCC* gene. This figure is in keeping with the rates seen in other studies of sporadic CRC over 50 years.

As mentioned earlier, MSI in multiple loci, including the 18q region, is seen in approximately 80% of CRC associated with the HNPCC syndrome (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993; Thibodeau *et al.*, 1993; Peltomaki, 1995). However, only 15% of sporadic CRC demonstrate this phenomenon (Peltomaki, 1995). In this study we have shown that 31.25% of the cases demonstrated MSI at the 3 loci centromeric (2 markers) and telomeric (1 marker) to the *DCC* gene. The DNA repair gene status of patients under 35 years have shown a statistically significant difference in the incidence of MSI compared to those over 35 (Liu *et al.*, 1995a). The implications of this are manifold. Many of these cases could be undiagnosed cases of HNPCC (unlikely), or they may result from de novo germline mutations in the DNA repair genes. This latter suggestion is not applicable to sporadic CRC in old patients, because germline mutations in the DNA mismatch repair genes were detected in only 1 of 10 sporadic CRC showing MSI (Liu *et al.*, 1995b). Perhaps the crux of the situation lies with the ages of the patients in this study. Liu and colleagues found that 58% of patients under the age of 35 without the HNPCC syndrome showed MSI, many of which were due to germline mutations in the DNA repair genes (Liu *et al.*, 1995a)

In this study, investigation of the status of the DNA repair genes in the 2p and 3p regions showed MSI in 13 out of 32 cases (40.6%), whilst AI was present in 10 cases (31.3%).

When all six loci were analysed for MSI in at least 1 locus, 16 cases (50%) showed MSI. Further, 10 (31.3%) of these cases showed MSI in two or more loci, and these were classified as RER+ tumours. Allelic imbalance for all six markers was found in 18 cases (56.3%).

Molecular analysis of the 18q region and the DNA repair genes in the under 35 cases was compared to age, gender, site, grade, stage and lymph node status. No statistically significant correlation emerged from this study. However, it must be remembered that a larger cohort of cases needs to be analysed to verify this. Of interest, 59.3% of cases under 35 showed abnormalities in the 18q as well as the 2p and 3p regions. Nine out of the 12 (75%) signet ring (mucinous) and poorly differentiated colorectal cancers showed at least one marker to be abnormal (that is, showing MSI and/or AI). Whilst this did not correlate with any clinicopathological parameter, it does reflect that a high percentage of the high grade cancers in the under 35 age group harbour genetic abnormalities in either the 18q region and/or the DNA repair genes.

This study has shown that LOH/AI in the region of the *DCC* gene occurs in 39.1% of the patients under the age of 35 with CRC, while MSI in this region is 31.25%. The incidence of LOH/AI in the 2p and 3p regions is 31.3%, and of MSI 40.6%. These figures are in keeping with that of Liu *et al.* (1995a) who found CRC in patients under 35 to have similar results. In addition, 58% of their patients were categorised as RER+ (ie. showing MSI for two or more markers). However, in the current study, 31.3% of the cases were RER+. This discrepancy may be a true reflection of the different genetic background of the two studies, that is, United States/Scotland as opposed to South Africa. Alternatively, the different techniques employed: radioactivity (Liu *et al.*, 1995a) versus fluorescent based (current study), may be responsible for the differing results.

4.2 COLORECTAL CANCER OVER THE AGE OF 50

Lesions in colorectal carcinoma are readily recognised by histological characteristics, however the molecular changes associated with these changes are still unclear. Since the initial discovery of microsatellites and the link to the pathogenesis of colorectal carcinoma (Peltomaki *et al.*, 1993; Thibodeau *et al.*, 1993), there has been a plethora of information on the microsatellite status in different chromosomal loci.

Several studies have shown that allelic deletions occur in large areas of the chromosome and that these changes are associated with the pathogenesis of colorectal cancer (Jen *et al.*, 1994; Cho and Fearon, 1995; Froggatt *et al.*, 1995). One particular region in which allelic loss has frequently been noted in colorectal carcinomas is the 18q region (Delattre *et al.*, 1989; Fearon *et al.*, 1990; Martinez-Lopez *et al.*, 1998). This locus putatively harbours the *DCC* gene. The incidence of allelic loss in this locus has ranges from 29% to 75 %.

In this study, allelic imbalance/LOH, at one or more loci in the vicinity of the *DCC* gene was found in 14 cases (28%). At the DCC and D18S34 loci, allelic imbalance was observed in 5 out of the 50 cases (10%) for each of these markers. At the D18S58 locus, allelic imbalance was seen in only 4 cases (8%). Previous reports of AI in the 18q region have ranged from 33% (Huang *et al.*, 1993) and 45% (Martinez-Lopez *et al.*, 1998) to 70% (Jen *et al.*, 1994) and 75% (Delattre *et al.*, 1989). However, the findings of the present study (28%) are very similar to that of Cawkwell *et al.* (1994), who found AI in only 29% of their cases. This correlation is perhaps due to the use of fluorescent based DNA technology, a highly sensitive technique employed in both these studies. Further, the Fragment Manager software allows for easy interpretation of data over conventional radioactivity. The lower frequency of AI in our study could also be due to true differences between our series and other published series.

Microsatellite instability, on the other hand, in the 18q region was seen in 19 cases (38%) in at least one of the 3 loci investigated. As far as MSI at the individual loci is concerned, the results are as follows: at DCC, 16% (8 out of 50), at D18S58, 18% (9 out of 50) and at D18S34, 8% (4 out of 50). The frequency of MSI in this study is similar to that of other reports in sporadic colorectal carcinomas, which is approximately 15% (Cho *et al.*, 1994).

MSI in the mismatch repair genes was seen in at least one locus in 12 (24%) cases. MSI was slightly higher (10%) at the D3S659 locus, whilst for the D2S123 and D3S1255 loci, this figure was 8%. Allelic imbalance was also found in 12 (24%) cases in at least one locus. Two cases cases showed AI/LOH in more than one locus. AI/LOH for the individual markers is as follows: D3S659 (2%), D2S123 (6%) and D3S1255 (20%)

Six cases were categorised as RER+ (12%) when analysing all 6 markers. Further, 3 of these 6 cancers were mucinous tumours. Although only 6 mucinous carcinomas were encountered, it is interesting to note that 3 of these showed MSI in the mismatch repair loci. This association of mucinous carcinomas with MSI has also been observed by others (Kim *et al.*, 1994; Messerini *et al.*, 1997; Jass *et al.*, 1998).

Fifteen out of 28 stage B cases, and 16 out of 22 stage C cases showed abnormalities in the 18q region. When comparing stage B tumours with and without 18q abnormalities, with stage C with and without 18q abnormalities, no statistically significant difference was apparent. However, it can be seen, once again, a high percentage (73%) of the cases with lymph node spread (ie. stage C) harbour molecular alterations in the 18q locus. This increased propensity for wider dissemination in the group C tumours with 18q aberrations can be explained by the loss of *DCC* gene function. In other words, there is loss of cell to cell adhesion allowing for spread to occur.

Once again, no statistical correlation between molecular abnormalities and individual clinicopathological features was seen.

4.3 COMPARISON OF CRC IN PATIENTS UNDER 35 WITH THOSE OVER 50

The molecular analysis of colorectal cancer in patients under 35 years showed a much higher percentage of both AI/LOH and MSI, as compared to the sporadic (over 50) cases. This is not a surprising finding given that the pathogenesis of colorectal cancer in the two age groups is somewhat disparate and distinct. A definite family history of colorectal cancer could not be obtained despite careful perusal of patient clinical notes. It is likely that the under 35 age group have a strong familial and/or genetic input because colorectal cancer in Black patients, generally, is uncommon and it is decidedly rare in young Black patients. This argument is strengthened by finding AI/LOH in the DNA repair genes in 31% of cases and MSI in 41% of tumours. This genetic profile is similar to the only other study performed on this cohort (Liu *et al.*, 1995a). Whilst the overall figure is similar, Liu *et al.* (1995a) did find a higher percentage of RER+ tumours. In contrast, microsatellite alterations in 2p, 3p and 18q are not frequently encountered in the sporadic cancers in the patients over 50. This study reinforces this fact and shows that environmental factors play more of a role in the pathogenesis of this cohort of colorectal cancer. With the wide range of figures quoted in the world literature, the figures found in the study being presented are within the quoted range.

4.4 OESOPHAGEAL CANCER

There are a number of factors that influence the onset of oesophageal cancer. These factors include diet, cigarette smoking, alcohol intake and exposure to environmental toxins. However, genetic factors and their influence in the development of these tumours have not been investigated in our environment. In this study microsatellite instability and LOH were evaluated in conjunction with clinicopathological characteristics.

Several genes have been implicated in the causation of oesophageal carcinoma: *p53*, *APC*, *MCC* and *DCC*. A recent and important discovery is the role of defects in DNA mismatch repair genes. The normal DNA mismatch repair genes maintain the integrity of the DNA by repairing errors that occur during DNA replication or incurred after exposure to genotoxic agents/events, eg., chemicals or toxins. Defects in these repair genes lead to genetic instability and this is, therefore, thought to play a pivotal role in oncogenesis.

Mismatch repair genes were originally identified in bacteria and yeast, and their human homologues have been implicated in the pathogenesis of HNPCC, as well as a host of different sporadic cancers that exhibit MSI including oesophageal cancer.

Recently there have been several studies from different geographical settings which looked at genetic instability in oesophageal carcinomas. Studies have emanated from Japan, one of the first studies examining the *DCC* gene and its metastatic potential in oesophageal carcinoma (Miyake *et al.*, 1994). Allelic loss was found in 23% of the 51 cases investigated. Other studies have reported deletions in the 18q region in 20-24% of oesophageal carcinomas (Huang *et al.*, 1992; Miyake *et al.*, 1994; Shibagika *et al.*, 1994; Nakashima *et al.* 1995a)

The findings from this study show that microsatellite instability ranged from 5.1% to 7.7% for the three loci in the 18q region. Microsatellite instability was seen in two cases (5.4%) for the DCC and D18S34 markers, and in 3 cases (7.7%) at D18S58. Loss of heterozygosity/AI in this region, on the other hand, ranged from 15.4% to 20.5%. Loss of heterozygosity/AI for the individual markers is as follows: DCC 15.4%; D18S34 15.4% and D18S58 20.5%. In addition, 4 cases showed LOH/AI in 2 or more markers. These figures are similar to other LOH/AI studies carried out on markers in the 18q region (see Table 2 - section 1.4.2).

Microsatellite instability was not found in the D3S659 locus, whilst for D3S1255 and D2S123, it was 7.7% and 10.25%, respectively. For the D2S123 marker, LOH/AI was found in 15.3%, and MSI was found in 10.8% of cases. However, for the 3p region LOH ranged from 10.25% (D3S1255) to 17.9% (D3S659).

Eighteen cases showed lymph node metastases, 13 (72.2%) of which showed some abnormality (MSI and/or AI) in the 18q and/or DNA repair gene loci. However, no statistically significant difference was found when looking at similar abnormalities in the lymph node negative cases.

In conclusion, in the 18q region, the MSI and AI/LOH findings are similar to other studies (Huang *et al*, 1992; Miyake *et al.*, 1994). Our figures for AI and MSI in the 2p and 3p regions are lower than most other studies (Table 2). However, there seems to be no consensus reached from other studies which quote a wide range from 0-35%. This is perhaps due to the difference in the geographical settings from which these studies have emanated. These findings may therefore be indicative of differing genetic instability in different population groups. Further, it must also be noted that fluorescent-based DNA technology was employed as the detection system in this study. This together with the Fragment Manager software, which was used to analyse the data, clearly distinguishes novel alleles from stutter bands, which may have erroneously been interpreted as novel alleles which could inflate MSI/AI figures.

Despite the high incidence of oesophageal cancer in South Africa, we have demonstrated that genetic instability at the 18q, 2p and 3p regions occur at a low frequency and is in keeping with the genetic profile from other geographic regions. In addition, no correlation was found between the molecular analysis and clinicopathological parameters.

CHAPTER 5

CONCLUSION

5.1 CONCLUSION AND FUTURE STUDIES

This study was carried out to establish a baseline on which future studies could be done. The data provides further evidence that genetic alterations in the 18q, 2p and 3p regions of the genome play a role in the progression of sporadic colorectal cancers seen in the South African setting.

In other words, higher stage tumours (especially stage C) harbour more abnormalities in these loci. Furthermore, more aberrations were noted in the under 35 age group. Although a family history of colorectal cancer was not obtained, it is likely that there is a major genetic input in these cases. Overall, the figures obtained in this study are similar in both under 35 and over 50 age groups for colorectal cancer as seen in other parts of the world.

Although when analysing the age, gender, grade and stage of individual tumours against all the markers no statistically significant correlation emerged, it was apparent that advanced stage tumours showed more molecular abnormalities.

Further, the results clearly show that assaying for genetic aberrations using fluorescent-based microsatellite PCR and the automated DNA sequencer is a powerful and convenient new tool available to scientists. The use of these techniques clearly demonstrates the ease and accuracy with which the data may be analysed and interpreted. The results also support previous LOH/AI and MSI studies using these markers.

The current literature, however, shows some discrepancy with regard to the incidence of these molecular alterations in different geographical settings. This is perhaps a true reflection of the genetic alterations seen in different population groups, or due to the variable number of cases and markers investigated in different studies. In addition, different techniques are employed when analysing microsatellites (radioactivity versus fluorescence).

It is intended that in the future, more cases of oesophageal cancer and more markers will be examined. Although the present study did not find prognostic indicators, it is still hoped that molecular biological investigations will in future studies achieve these goals. A prospective study analysing oesophageal cancer is underway. Detailed histories of patients' diets, alcohol consumption, family history and other demographic data are being compiled. The laboratory investigation is a multi-discipline one where kinins, HPV status, mycotoxins and DNA repair genes are being examined

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APPENDIX A

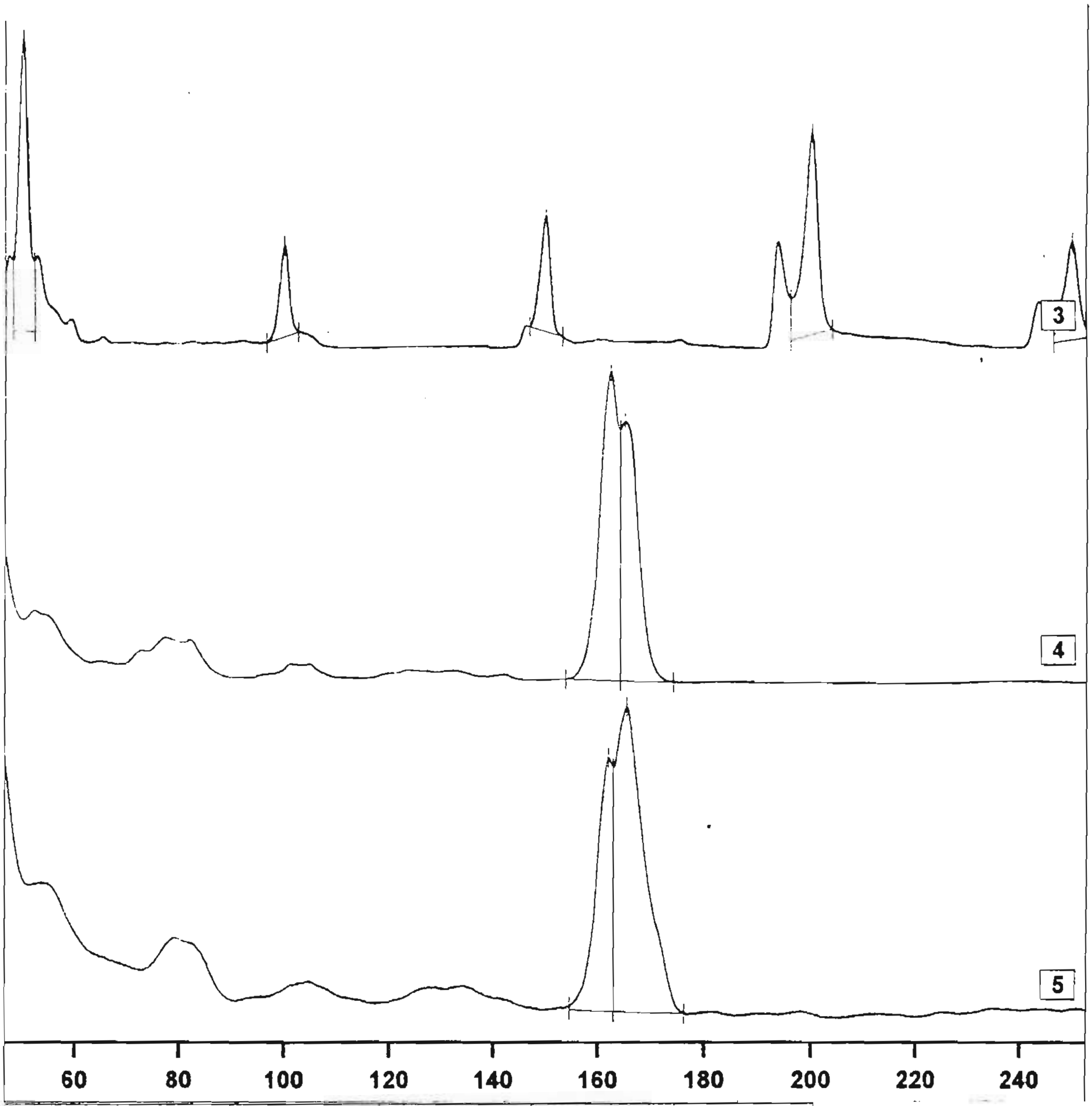
DNA Extraction

Tumour and normal tissue was scraped away from the glass slides using a sterile scalpel blade. The tissue was transferred into 1.5 ml Eppendorf tubes. The dewaxing and subsequent DNA extraction procedure was carried out as follows:

- Add 500 ml of warm xylene to 1.5 ml Eppendorf tubes containing the tissue.
- Incubate at 65°C for 10 min.
- Spin for 10 min at 10 000 rpm in a microfuge and discard supernatant.
- Repeat steps 1, 2 and 3 twice.
- Add 500 ml of ethanol and dry in a heating block.
- Add 200 µl proteinase K buffer to the dry pellet and incubate at 55°C for 48 hrs. Shake intermittently.
- Spin in a microfuge at 12 000 rpm and transfer supernatant to a clean Eppendorf tube. Discard pellet.
- Add 200 µl sterile water to the supernatant and then add 400 µl of saturated phenol. Vortex and spin in a nanofuge to separate the two phases.
- Carefully remove the upper aqueous phase with a sterile Pasteur pipette and transfer to a new Eppendorf tube.
- Add 400 µl of phenol/chloroform/isoamyl alcohol and vortex briefly.
- Spin in a nanofuge to separate the two phases. Transfer the upper aqueous phase to a new Eppendorf tube.
- Add 400 µl of chloroform, vortex briefly and spin in a nanofuge to separate the two phases.
- Transfer the upper aqueous phase to a clean Eppendorf tube.
- Add 2 volumes of cold (-20°C) absolute ethanol and 40 µl of 3 M sodium acetate. Vortex and place in a deep freeze (-20 °C) for 30 min.

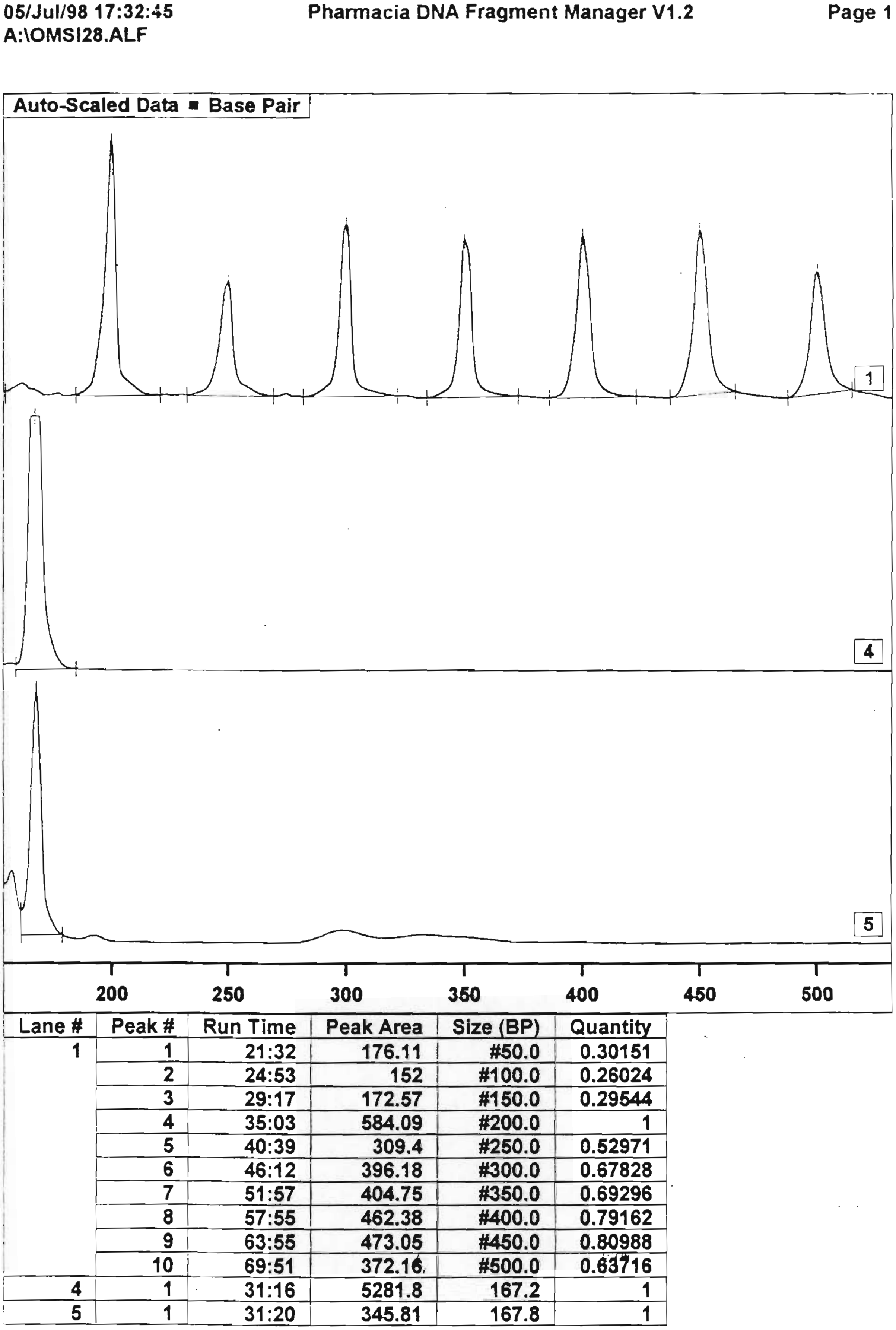
- Centrifuge for 20 min at 14 000 rpm in a microfuge. Remove the supernatant carefully without disturbing the pellet.
- Wash the pellet with 75 µl of 80% ethanol (-20°C), by centrifuging in the microfuge for 3 min.
- Remove the supernatant and air dry the pellet.
- Resuspend the dry pellet in 60 µl of sterile water.

APPENDIX B



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
3	1	100:13	291.42	#50.0	0.86193
	2	130:16	87.662	#100.0	0.25928
	3	162:47	128.29	#150.0	0.37943
	4	197:49	338.1	#200.0	1
	5	236:57	186.06	#250.0	0.55032
4	1	171:17	1380	162.5	1
	2	173:09	1117.3	165.2	0.80964
5	1	170:58	325.81	162.0	0.46025
	2	173:21	707.89	165.5	1

Appendix B - CRC <35: AI - DCC

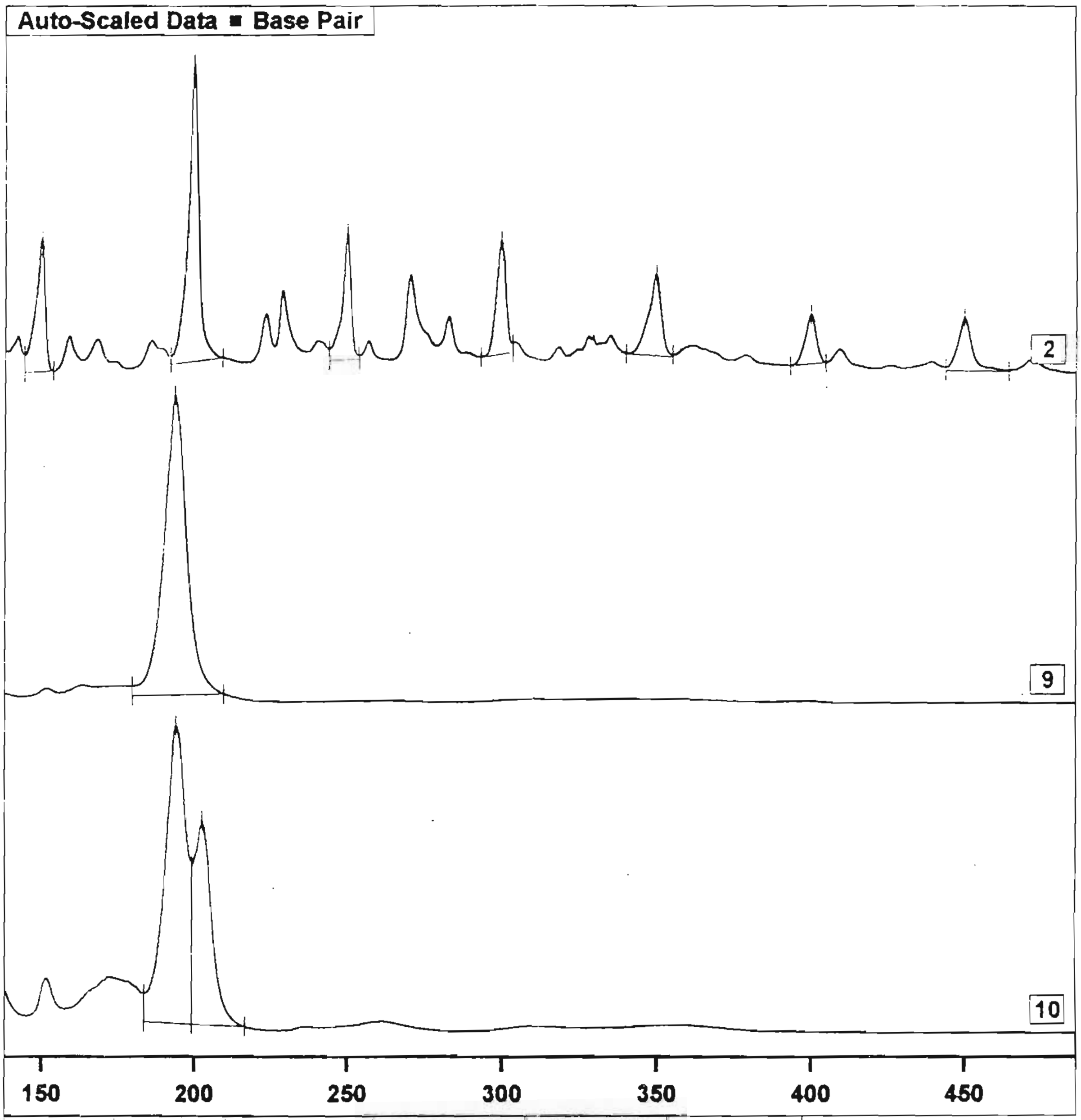


05/Jul/98 17:32:45 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMS128.ALF

Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	21:32	176.11	#50.0	0.30151
	2	24:53	152	#100.0	0.26024
	3	29:17	172.57	#150.0	0.29544
	4	35:03	584.09	#200.0	1
	5	40:39	309.4	#250.0	0.52971
	6	46:12	396.18	#300.0	0.67828
	7	51:57	404.75	#350.0	0.69296
	8	57:55	462.38	#400.0	0.79162
	9	63:55	473.05	#450.0	0.80988
	10	69:51	372.16	#500.0	0.63716
4	1	31:16	5281.8	167.2	1
5	1	31:20	345.81	167.8	1

Appendix B - CRC <35: H - DCC

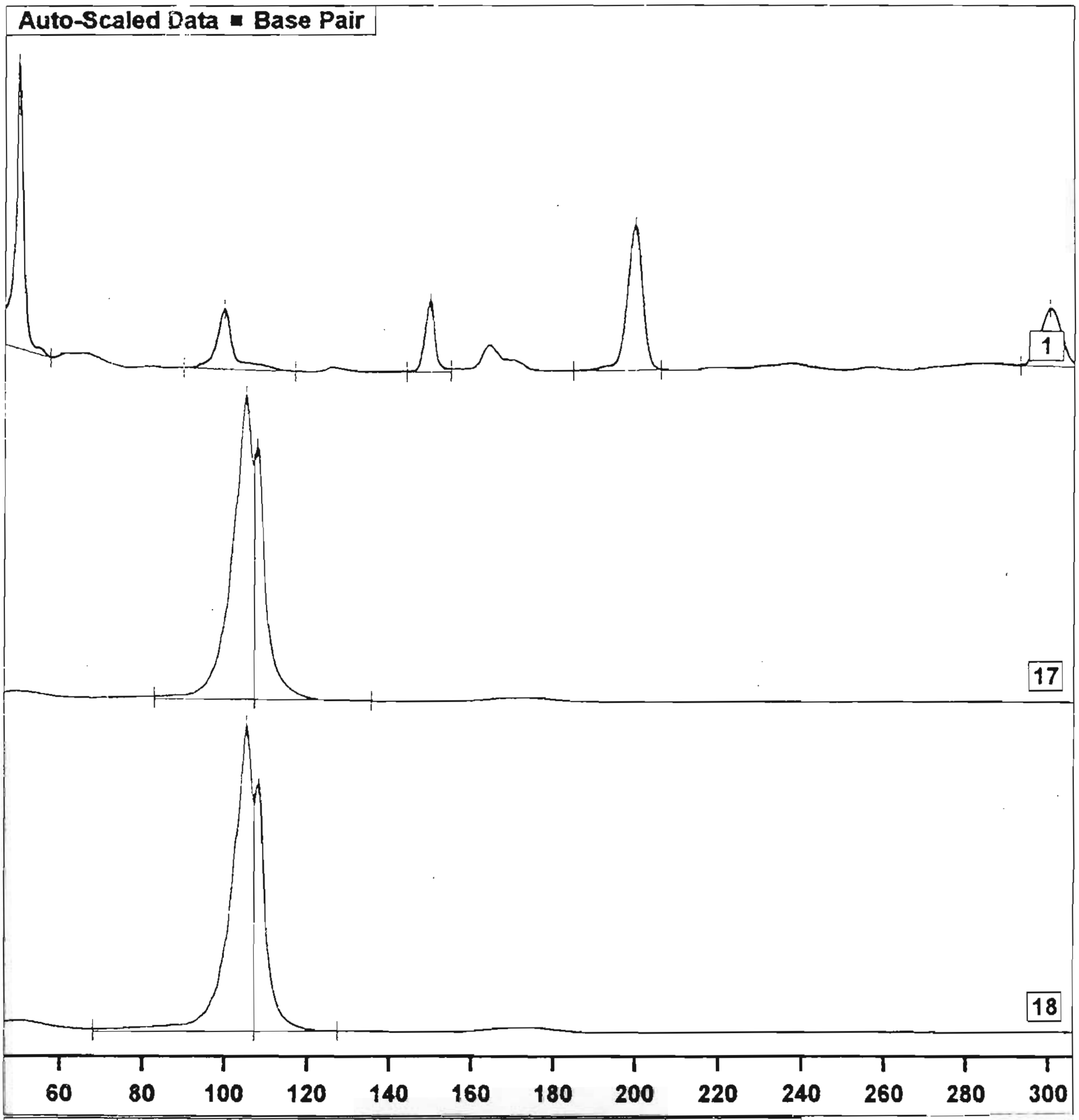
06/Jul/98 11:05:22 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\MSI697.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
9	1	45:41	1198.9	193.5	1
10	1	45:44	792.79	193.9	1
	2	46:54	515.1	202.5	0.64972

Appendix B - CRC <35: MSI - DCC

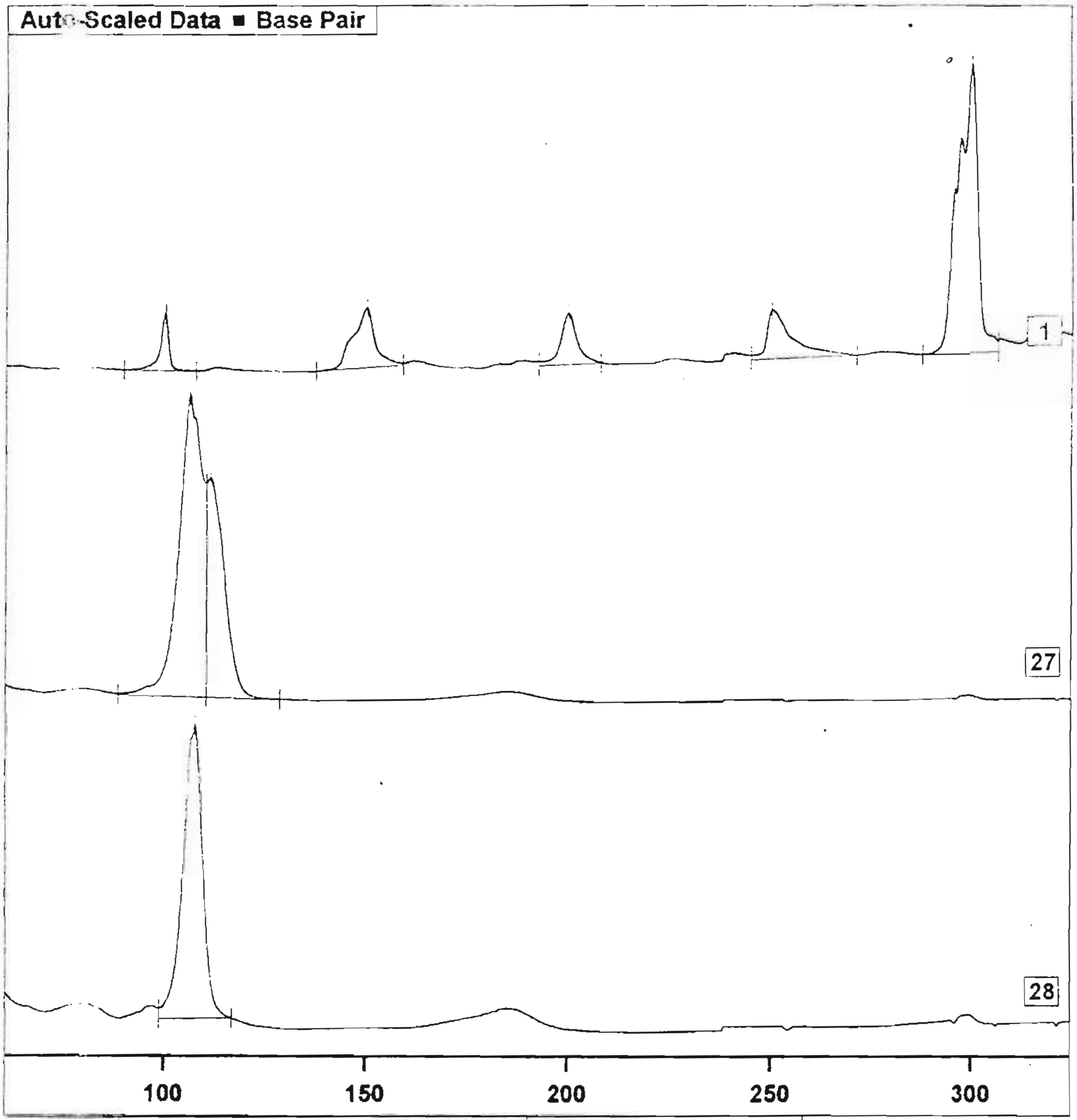
05/Jul/98 10:56:07 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\CRC711.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	32:25	228.27	#50.0	0.52794
	2	36:48	87.529	#100.0	0.20243
	3	43:10	91.455	#150.0	0.21151
	4	52:16	432.39	#200.0	1
	5	78:47	232.7	#300.0	0.53817
17	1	37:22	2059	105.3	1
	2	37:40	1011.4	108.1	0.49123
18	1	37:23	1745.8	105.5	1
	2	37:42	818.06	108.4	0.46859

Appendix B - CRC <35: NAI -D18S34

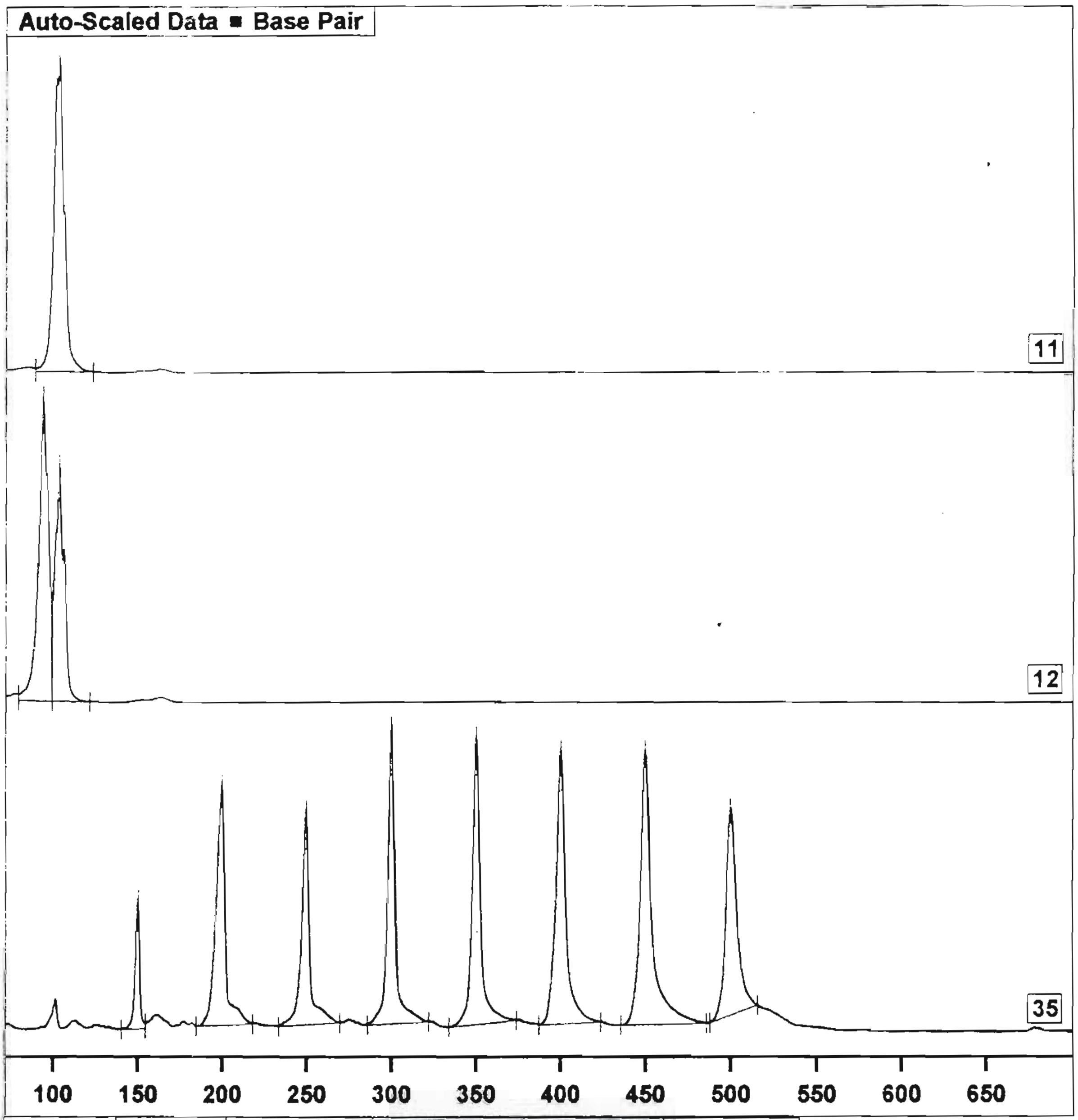
04/Jul/98 09:24:40 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI30.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	42:27	478.23	#50.0	0.37563
	2	47:54	155.54	#100.0	0.12217
	3	55:25	459.54	#150.0	0.36096
	4	63:47	337.67	#200.0	0.26523
	5	73:11	313.35	#250.0	0.24613
	6	77:54	1273.1	#300.0	1
	7	83:12	233.26	#350.0	0.18322
	8	88:04	425.36	#400.0	0.33411
	9	93:24	195.84	#450.0	0.15383
27	1	48:50	899.99	103.9	1
	2	49:36	493.63	110.0	0.54848
28	1	49:03	342.44	105.5	1

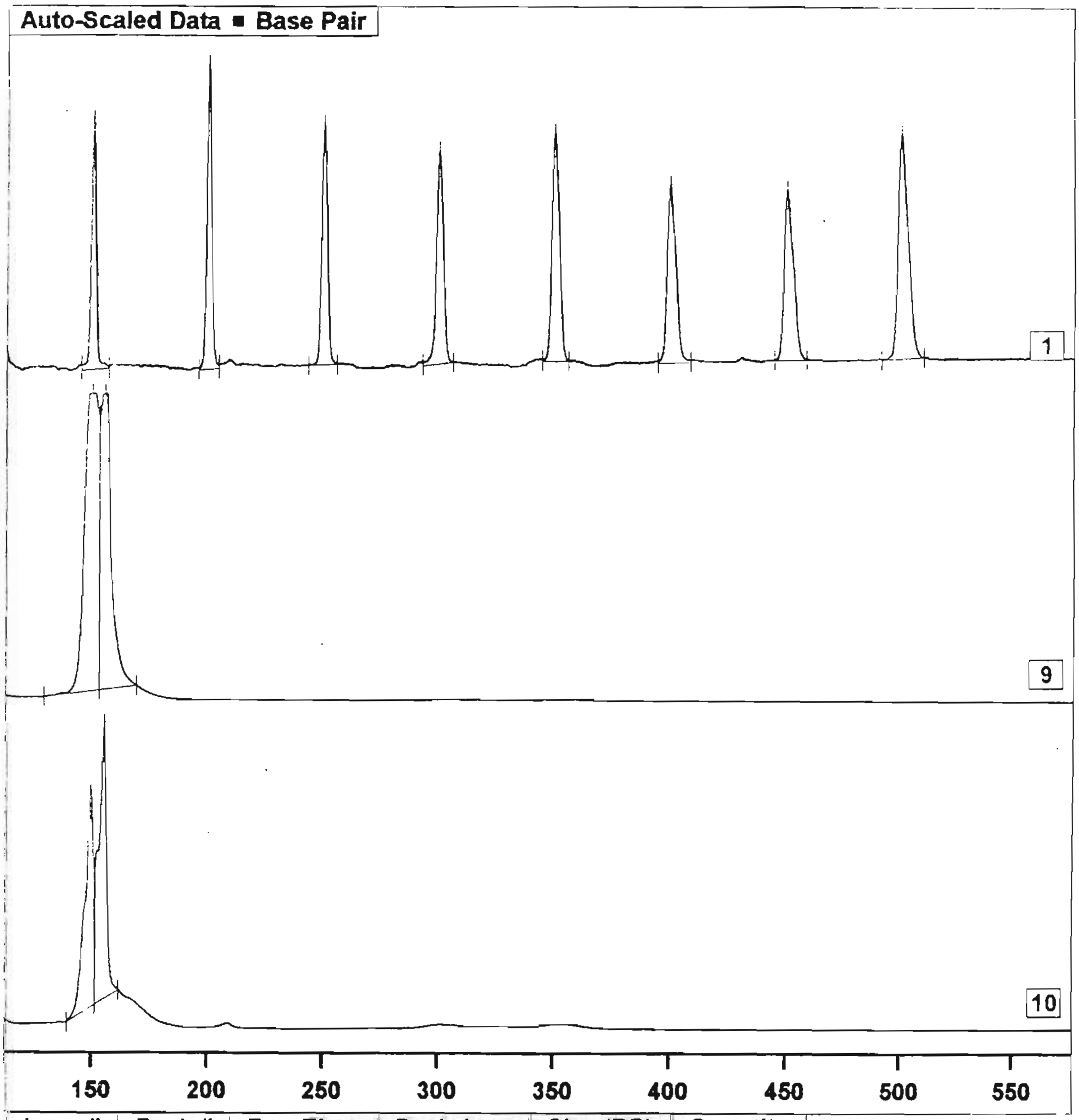
Appendix B - CRC <35: AI - D18S34

05/Jul/98 18:18:20 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI28.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
11	1	25:20	3658.4	103.8	1
12	1	24:33	2631.5	94.2	1
	2	25:20	1976.1	103.8	0.75096
35	1	21:27	37.019	#50.0	0.13884
	2	29:50	45.811	#150.0	0.17181
	3	35:43	174.29	#200.0	0.65368
	4	41:19	130.42	#250.0	0.48915
	5	47:06	176.12	#300.0	0.66053
	6	53:07	192.55	#350.0	0.72214
	7	59:16	227.51	#400.0	0.85326
	8	65:29	266.63	#450.0	1
	9	71:36	183.55	#500.0	0.68842

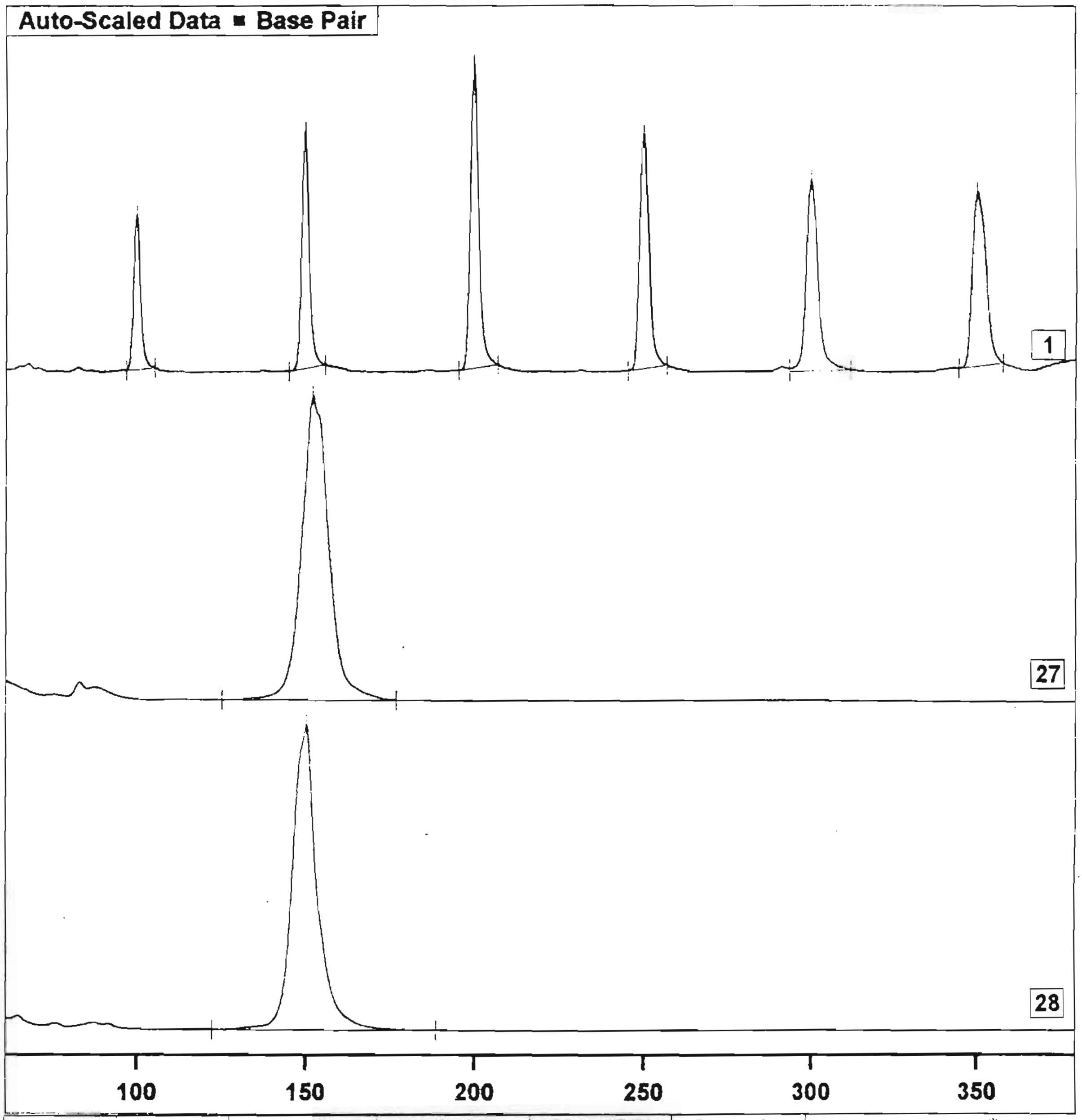
Appendix B - CRC <35: MSI -D18S34



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	20:12	12.879	#50.0	0.2036
	2	25:05	17.109	#100.0	0.27046
	3	30:12	28.422	#150.0	0.4493
	4	35:39	39.118	#200.0	0.61839
	5	41:26	36.654	#250.0	0.57943
	6	47:25	40.564	#300.0	0.64124
	7	53:32	47.216	#350.0	0.74641
	8	59:46	42.678	#400.0	0.67467
	9	66:01	43.775	#450.0	0.69201
	10	72:15	63.258	#500.0	1
9	1	30:12	4202.1	150.0	1
	2	30:48	3456	155.7	0.82246
10	1	30:11	406.51	149.8	0.79855
	2	30:48	509.05	155.7	1

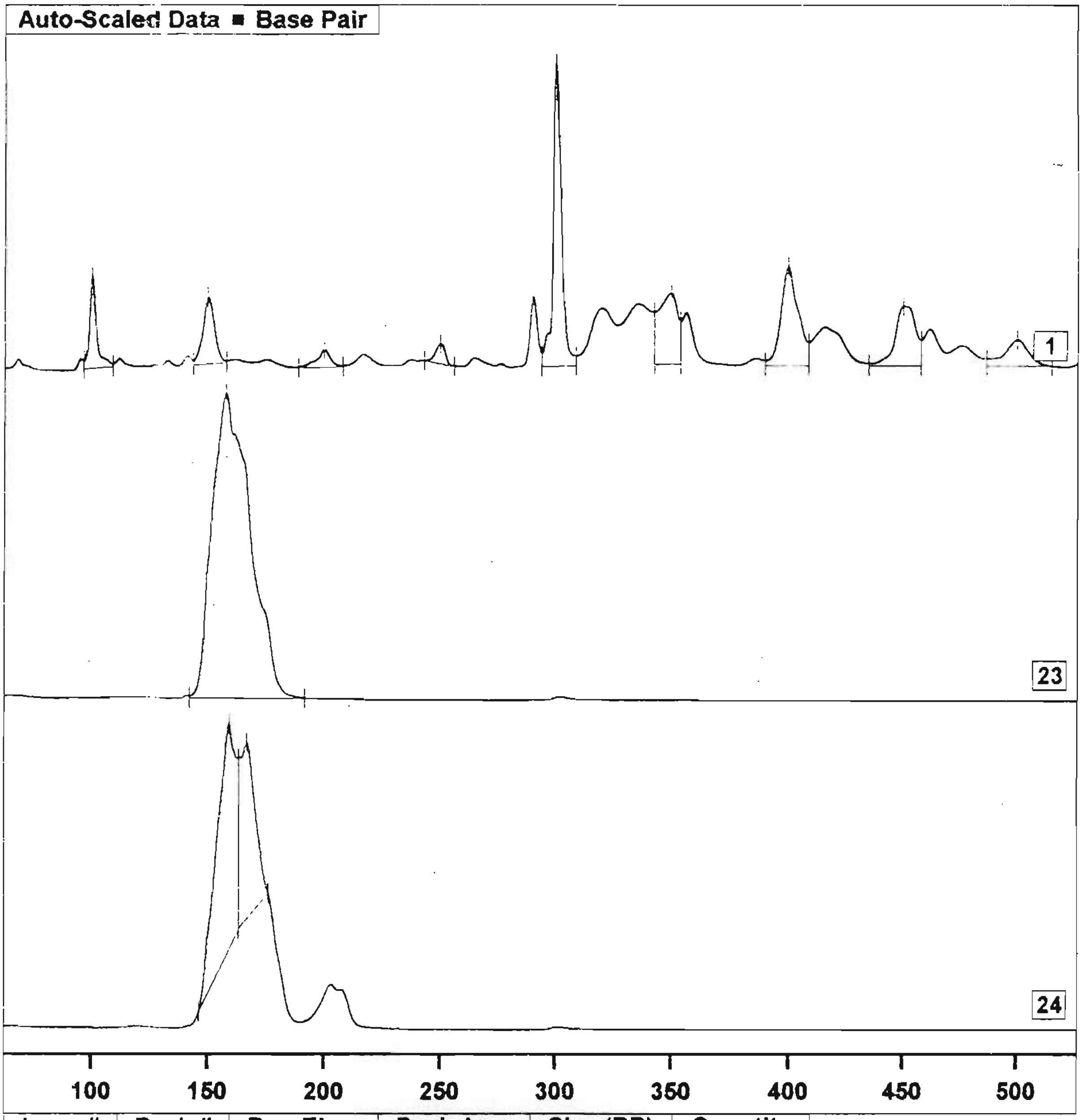
Appendix B - CRC <35: NAI - D18S58

04/Jul/98 14:25:26 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\63MSI98.ALF



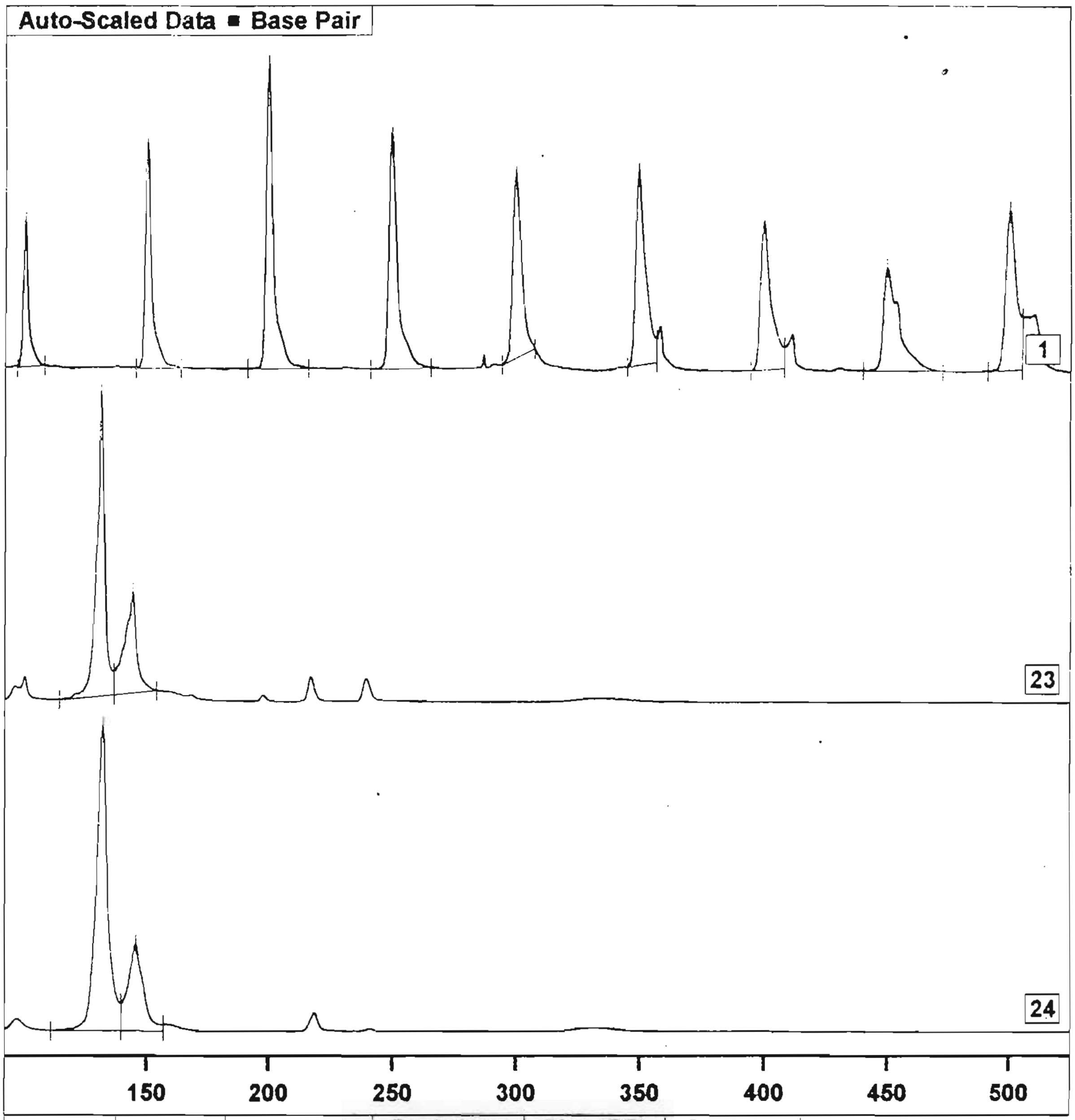
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	22:45	26.012	#50.0	0.30935
	2	28:43	31.341	#100.0	0.37273
	3	34:59	55.94	#150.0	0.66526
	4	41:41	83.839	#200.0	0.99706
	5	48:48	76.505	#250.0	0.90984
	6	56:10	83.758	#300.0	0.9961
	7	63:38	84.086	#350.0	1
27	1	35:17	1770.4	152.3	1
28	1	35:02	2439.2	150.4	1

Appendix B - CRC <35: H - D18S58



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	49:48	85.042	#50.0	0.31798
	2	57:07	149.62	#100.0	0.55944
	3	65:40	118.79	#150.0	0.44416
	4	70:13	25.874	#200.0	0.096747
	5	75:41	35.576	#250.0	0.13302
	6	82:35	267.44	#300.0	1
	7	85:31	124.24	#350.0	0.46455
	8	90:46	246.88	#400.0	0.92309
	9	96:34	169.68	#450.0	0.63446
	10	102:16	88.989	#500.0	0.33274
23	1	66:25	1604	158.2	1
24	1	66:32	688.56	159.5	1
	2	67:14	428.73	167.2	0.62265

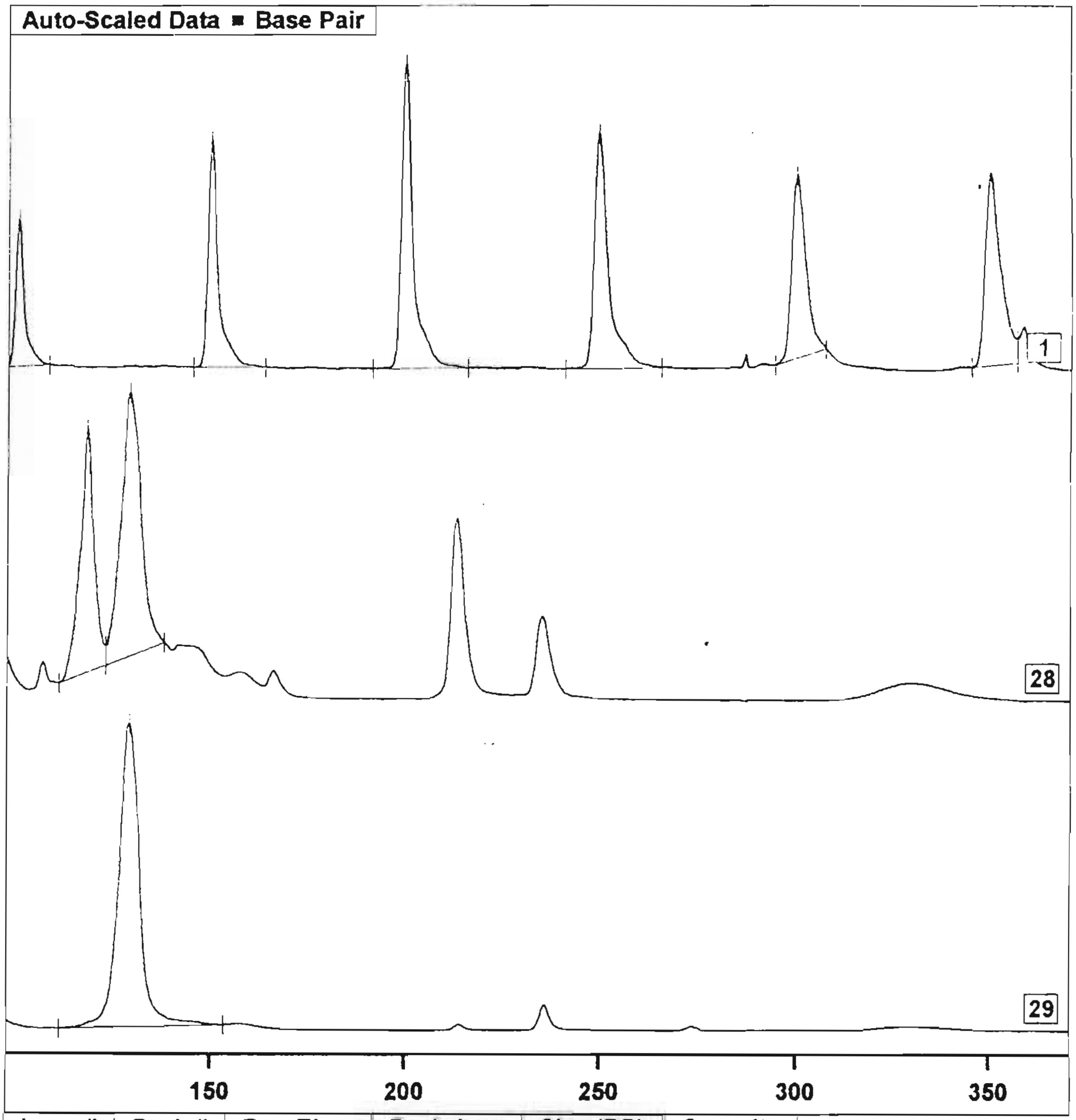
Appendix B - CRC <35: MSI - D18S58



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:49	20.434	#50.0	0.23147
	2	20:16	28.371	#100.0	0.32138
	3	24:51	55.392	#150.0	0.62747
	4	29:42	88.278	#200.0	1
	5	34:44	81.725	#250.0	0.92576
	6	39:46	63.272	#300.0	0.71673
	7	44:46	73.335	#350.0	0.83072
	8	49:39	59.771	#400.0	0.67708
	9	54:25	59.002	#450.0	0.66837
	10	58:58	60.638	#500.0	0.6869
23	1	23:07	1138.5	131.4	1
	2	24:19	591.91	144.3	0.51989
24	1	23:10	1480	131.9	1
	2	24:26	569.49	145.6	0.38479

Appendix B - CRC <35: NAI - D3S659

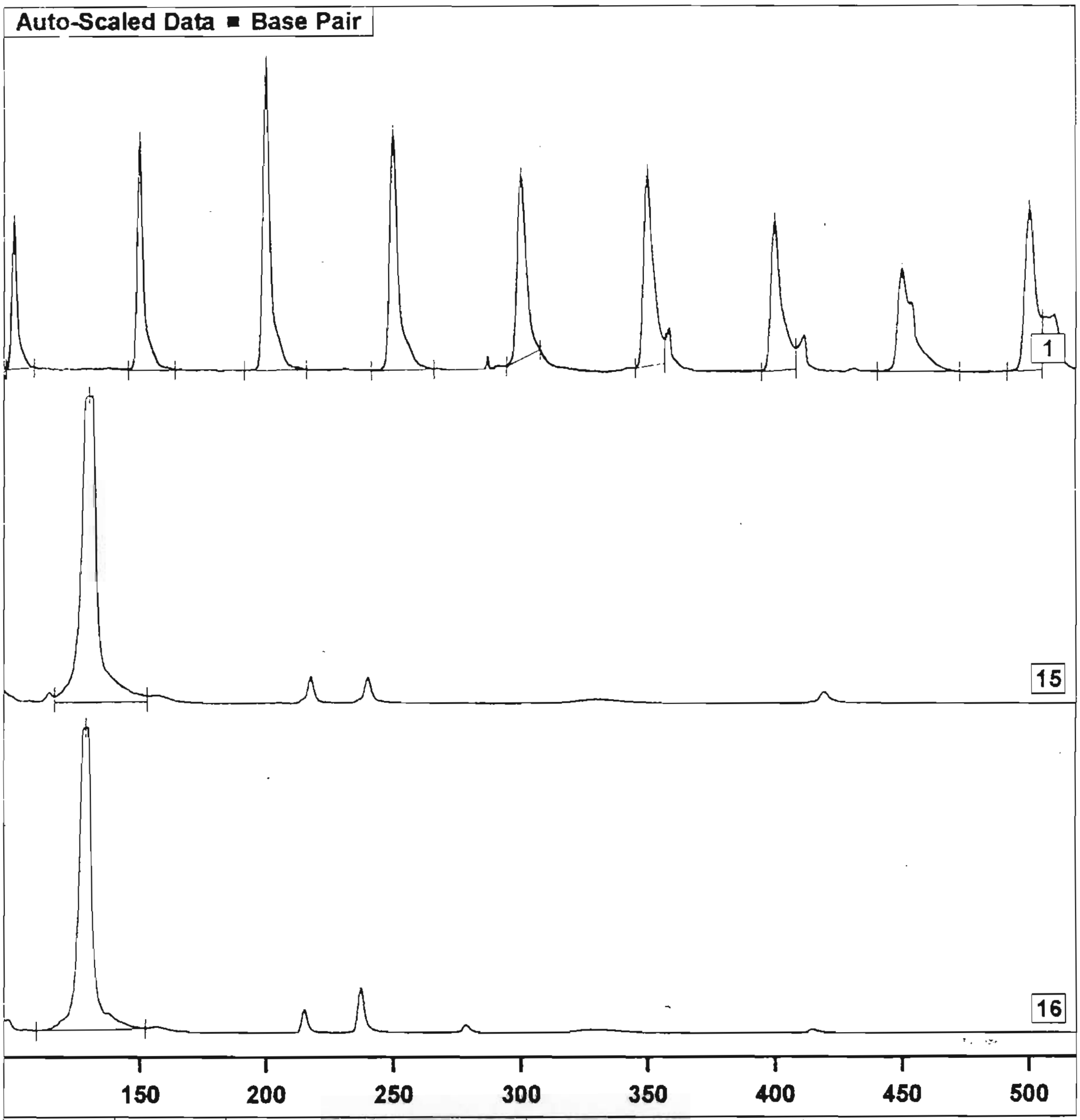
03/Jul/98 11:32:48 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\93MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:49	20.434	#50.0	0.23147
	2	20:16	28.371	#100.0	0.32138
	3	24:51	55.392	#150.0	0.62747
	4	29:42	88.278	#200.0	1
	5	34:44	81.725	#250.0	0.92576
	6	39:46	63.272	#300.0	0.71673
	7	44:46	73.335	#350.0	0.83072
	8	49:39	59.771	#400.0	0.67708
	9	54:25	59.002	#450.0	0.66837
	10	58:58	60.638	#500.0	0.6869
28	1	21:53	161.11	117.9	0.64099
	2	22:54	251.35	129.0	1
29	1	22:54	1132	129.0	1

Appendix B - CRC <35: AI - D3S659

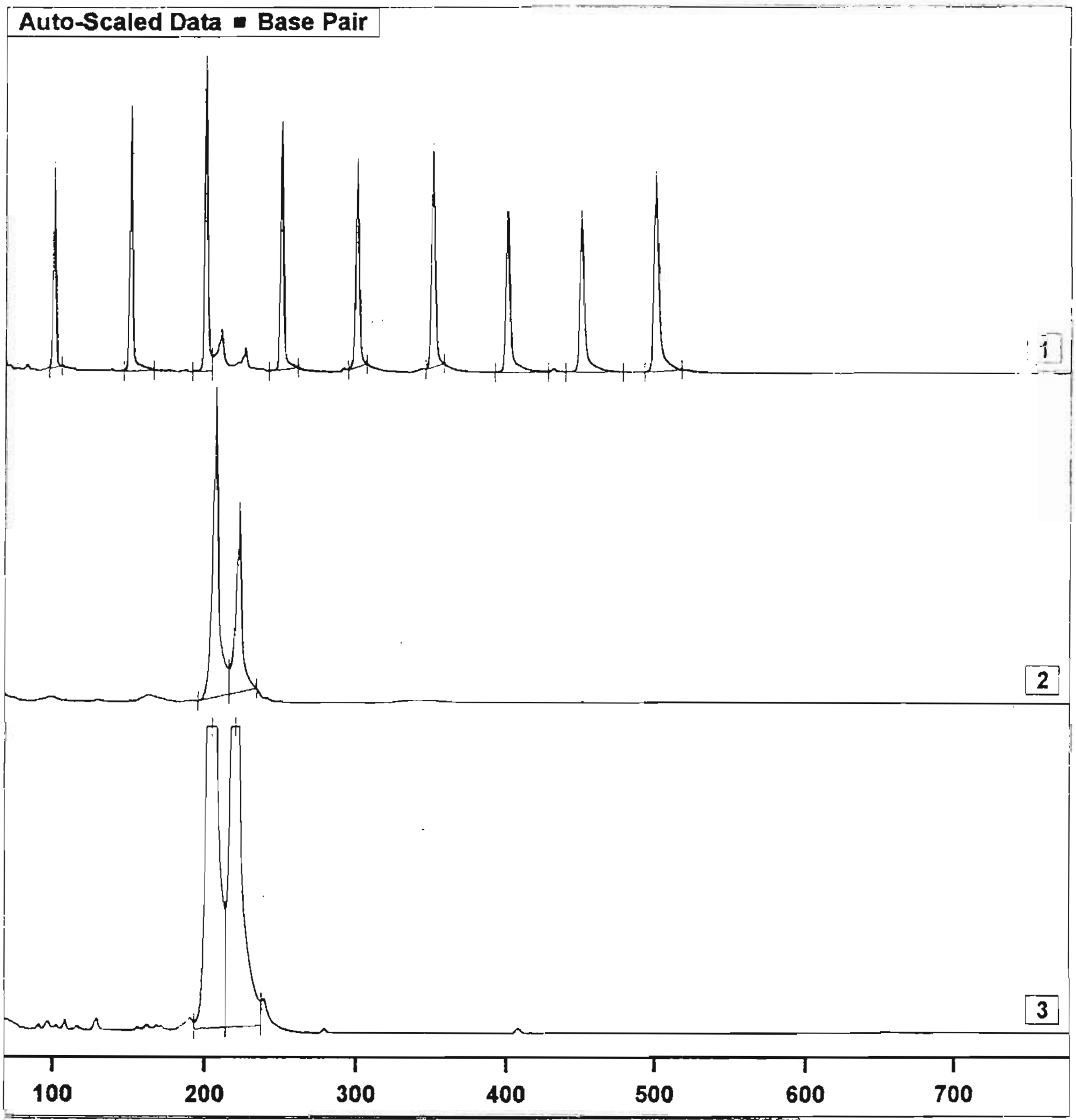
03/Jul/98 11:27:57 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\93MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:49	20.434	#50.0	0.23147
	2	20:16	28.371	#100.0	0.32138
	3	24:51	55.392	#150.0	0.62747
	4	29:42	88.278	#200.0	1
	5	34:44	81.725	#250.0	0.92576
	6	39:46	63.272	#300.0	0.71673
	7	44:46	73.335	#350.0	0.83072
	8	49:39	59.771	#400.0	0.67708
	9	54:25	59.002	#450.0	0.66837
	10	58:58	60.638	#500.0	0.6869
15	1	23:00	4280.3	130.1	1
16	1	22:52	3485.3	128.7	1

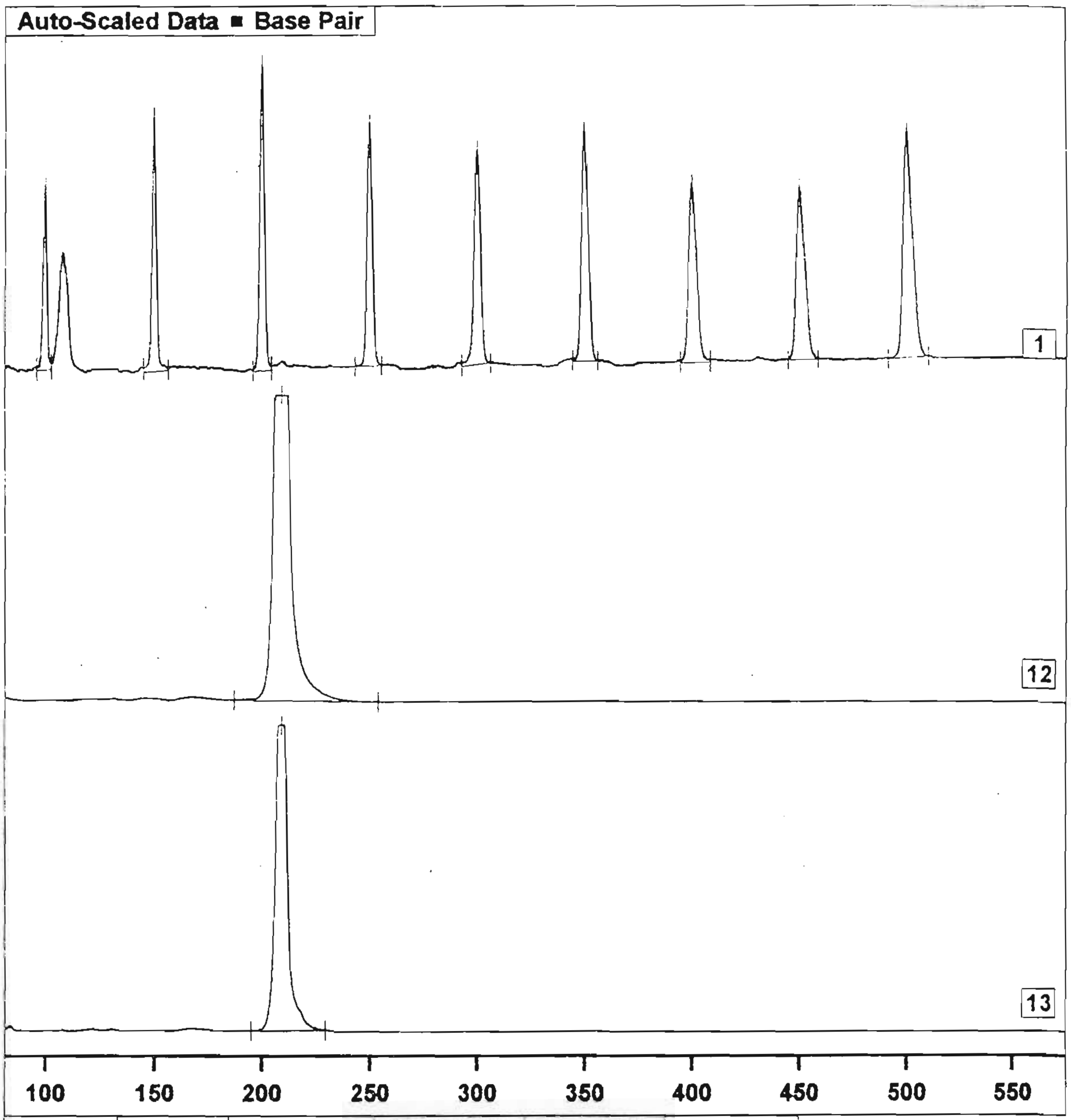
Appendix B - CRC <35: H - D3S659

02/Jul/98 18:08:30 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\19MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
2	1	35:44	722.5	207.3	1
	2	37:31	447.78	222.8	0.61976
3	1	35:26	7788.8	204.7	1
	2	37:13	7733.5	220.2	0.99291

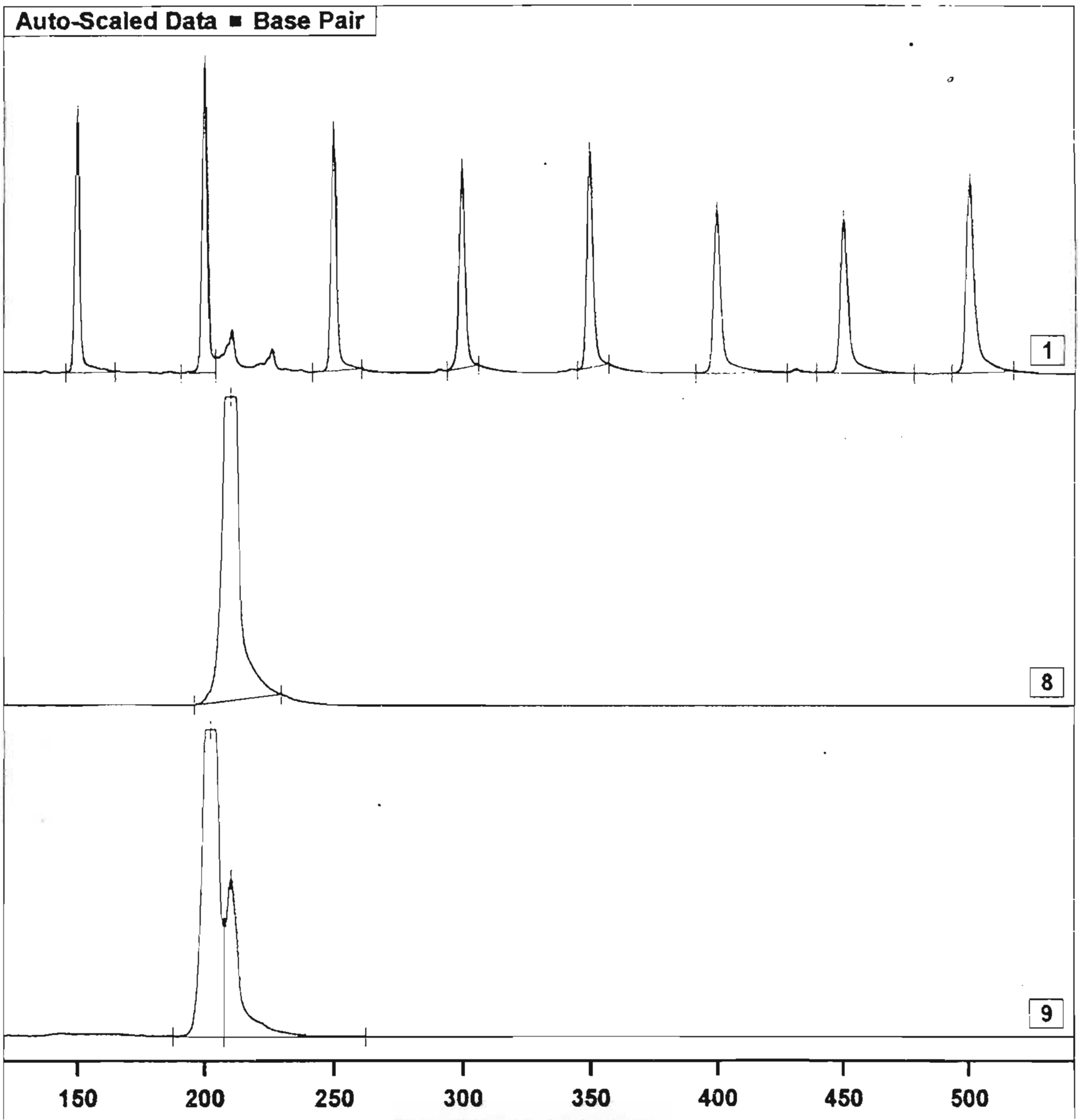
Appendix B - CRC <35: NAI - D2S123



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	20:12	12.879	#50.0	0.2036
	2	25:05	17.109	#100.0	0.27046
	3	30:12	28.422	#150.0	0.4493
	4	35:39	39.118	#200.0	0.61839
	5	41:26	36.654	#250.0	0.57943
	6	47:25	40.564	#300.0	0.64124
	7	53:32	47.216	#350.0	0.74641
	8	59:46	42.678	#400.0	0.67467
	9	66:01	43.775	#450.0	0.69201
	10	72:15	63.258	#500.0	1
12	1	36:41	7239.3	209.1	1
13	1	36:41	4720.3	209.1	1

Appendix B - CRC <35: H - D2S123

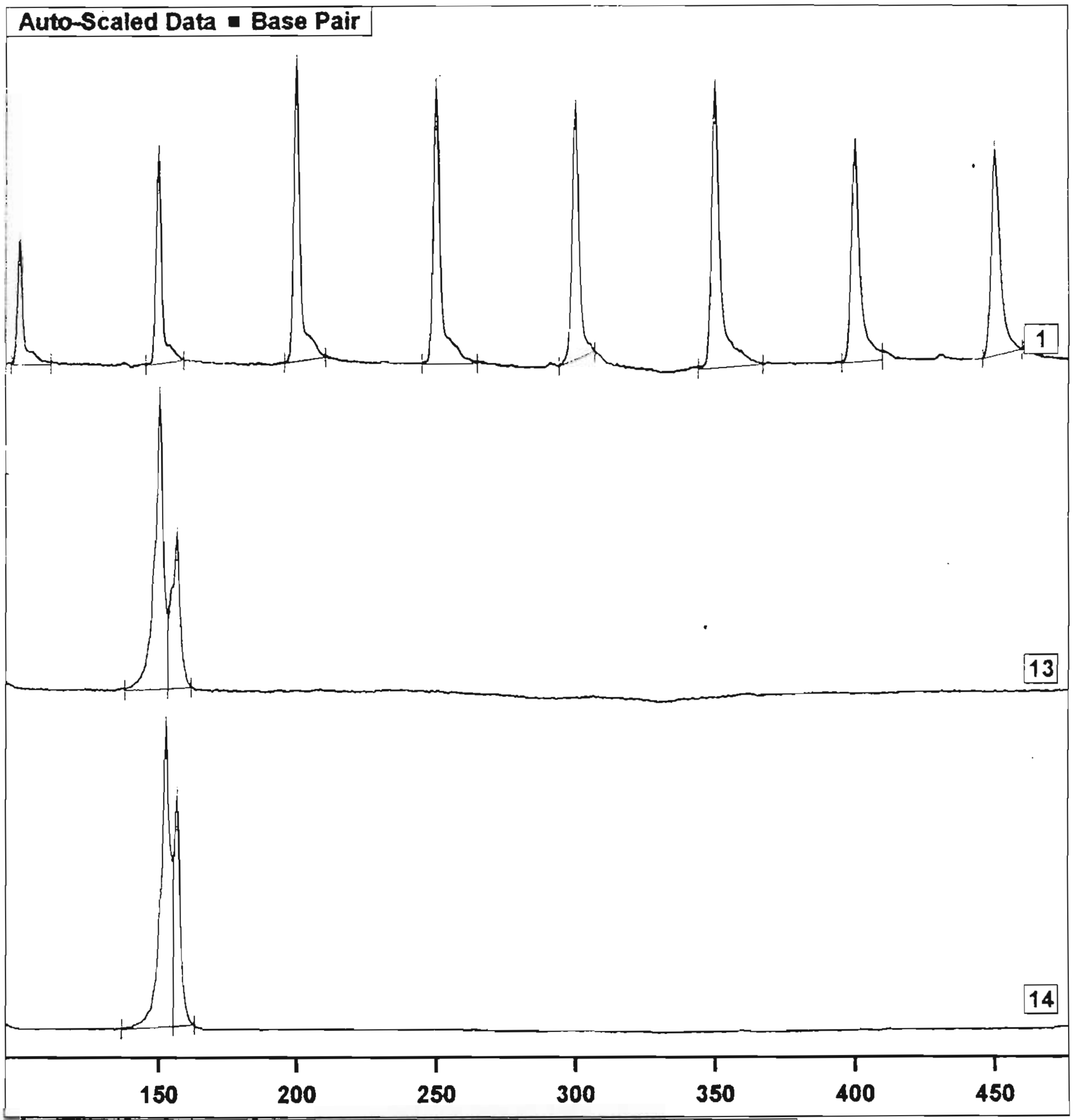
26/Jun/98 14:47:35 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\19MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
8	1	36:03	5746.5	210.1	1
9	1	35:09	5350.8	202.2	1
	2	36:03	2281	210.1	0.4263

Appendix B - CRC <35: MSI - D2S123

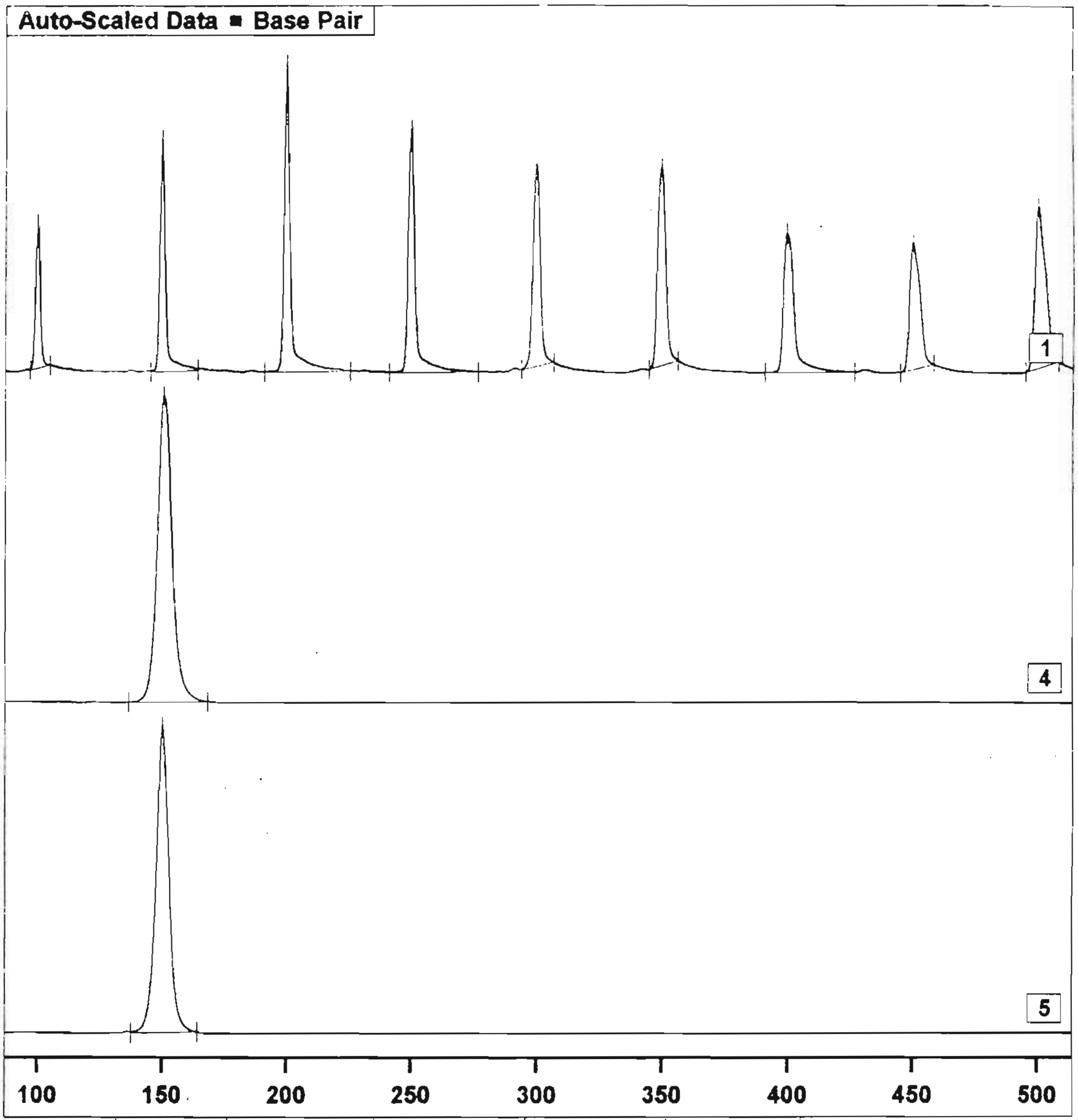
04/Jul/98 14:43:31 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\61MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	28:45	15.075	#100.0	0.23904
	2	35:05	25.953	#150.0	0.41151
	3	41:53	42.464	#200.0	0.67331
	4	49:07	46.961	#250.0	0.74462
	5	56:38	41.166	#300.0	0.65273
	6	64:19	59.523	#350.0	0.94381
	7	72:07	46.185	#400.0	0.73232
	8	79:57	44.125	#450.0	0.69964
	9	87:44	63.067	#500.0	1
13	1	35:09	45.001	150.5	1
	2	35:59	22.941	156.8	0.50979
14	1	35:26	88.184	152.7	1
	2	35:59	45.969	156.8	0.52129

Appendix B - CRC <35: NAI - D3S1255

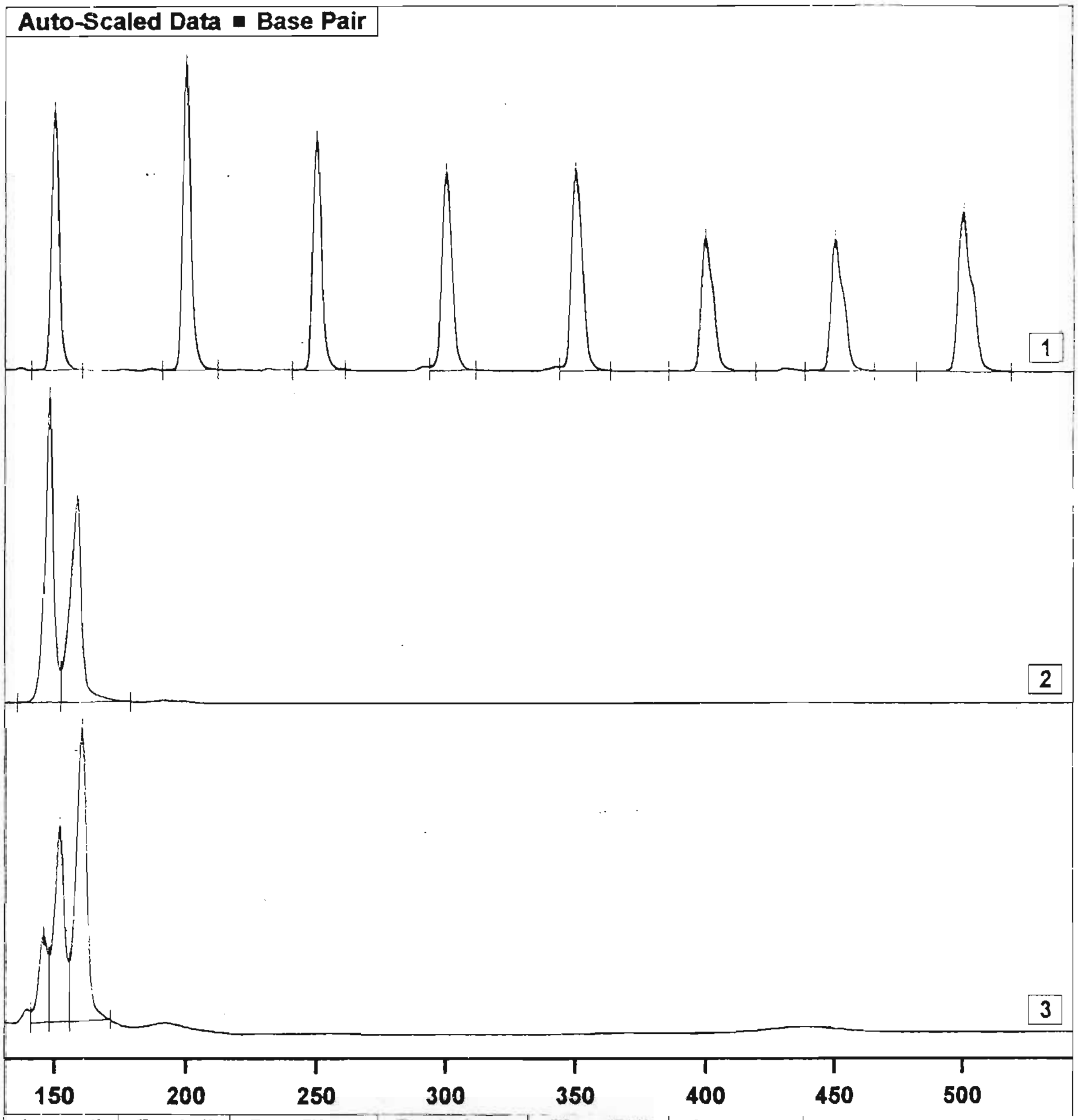
04/Jul/98 19:17:43 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\48MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	24:52	192.43	#50.0	0.23095
	2	30:52	251.34	#100.0	0.30165
	3	37:09	523.77	#150.0	0.62861
	4	43:54	833.23	#200.0	1
	5	51:05	756.16	#250.0	0.9075
	6	58:33	670.28	#300.0	0.80444
	7	66:12	750.91	#350.0	0.9012
	8	73:56	708.02	#400.0	0.84972
	9	81:47	605.59	#450.0	0.7268
	10	89:35	791.05	#500.0	0.94938
4	1	37:15	1182.4	150.8	1
5	1	37:11	1171.8	150.3	1

Appendix B - CRC <35: H - D3S1255

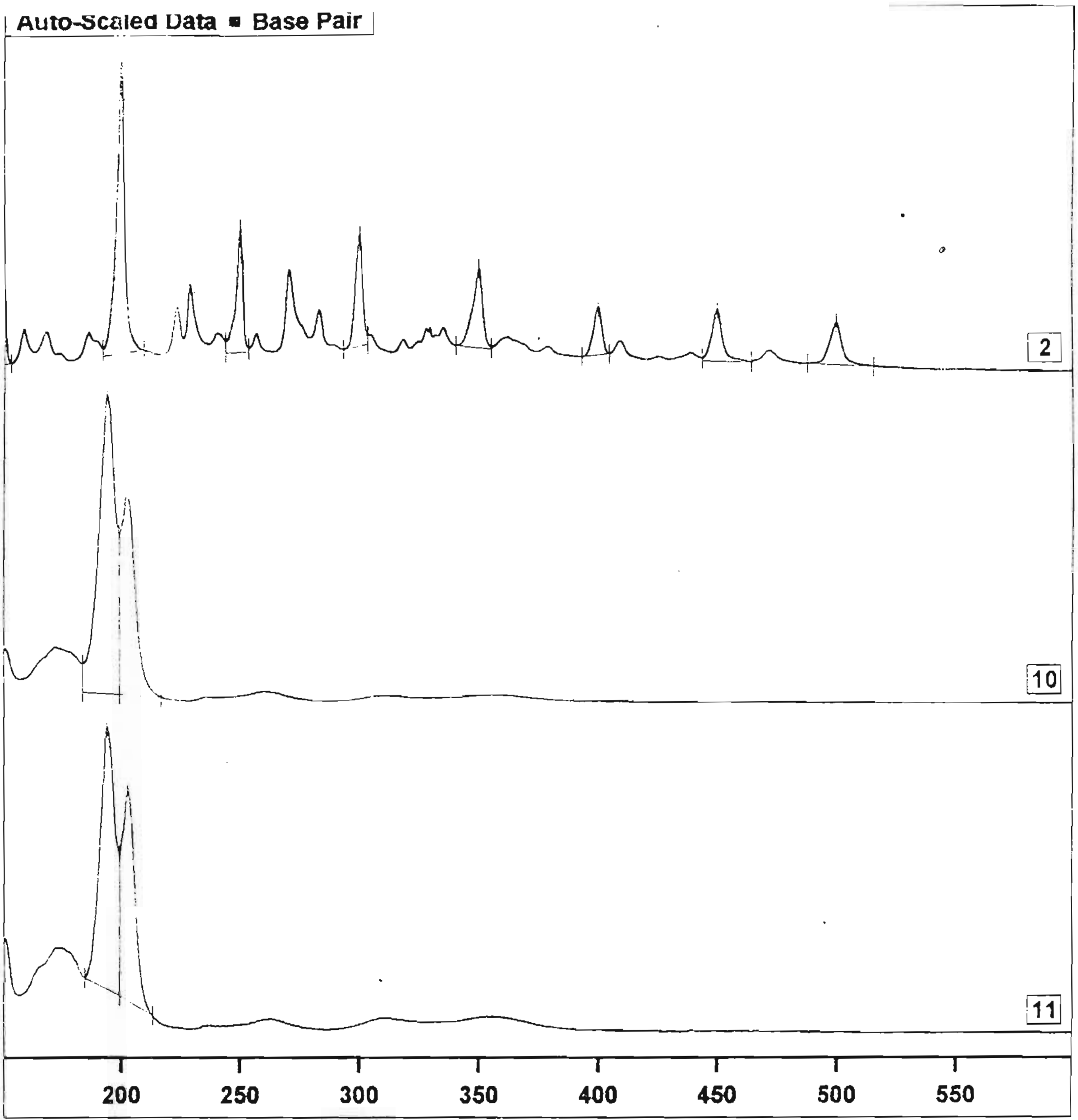
05/Jul/98 16:13:03 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\138MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	16:11	334.14	#50.0	0.55667
	2	19:38	307.05	#100.0	0.51154
	3	23:07	438.91	#150.0	0.73122
	4	26:46	600.24	#200.0	1
	5	30:36	512.22	#250.0	0.85336
	6	34:32	521.96	#300.0	0.86959
	7	38:33	583.86	#350.0	0.97271
	8	42:38	423.44	#400.0	0.70545
	9	46:45	444.1	#450.0	0.73988
	10	50:51	580.57	#500.0	0.96723
2	1	22:59	299.52	148.1	1
	2	23:43	279.56	158.4	0.93338
3	1	22:50	18.026	146.0	0.24427
	2	23:16	45.985	152.1	0.62314
	3	23:52	73.796	160.5	1

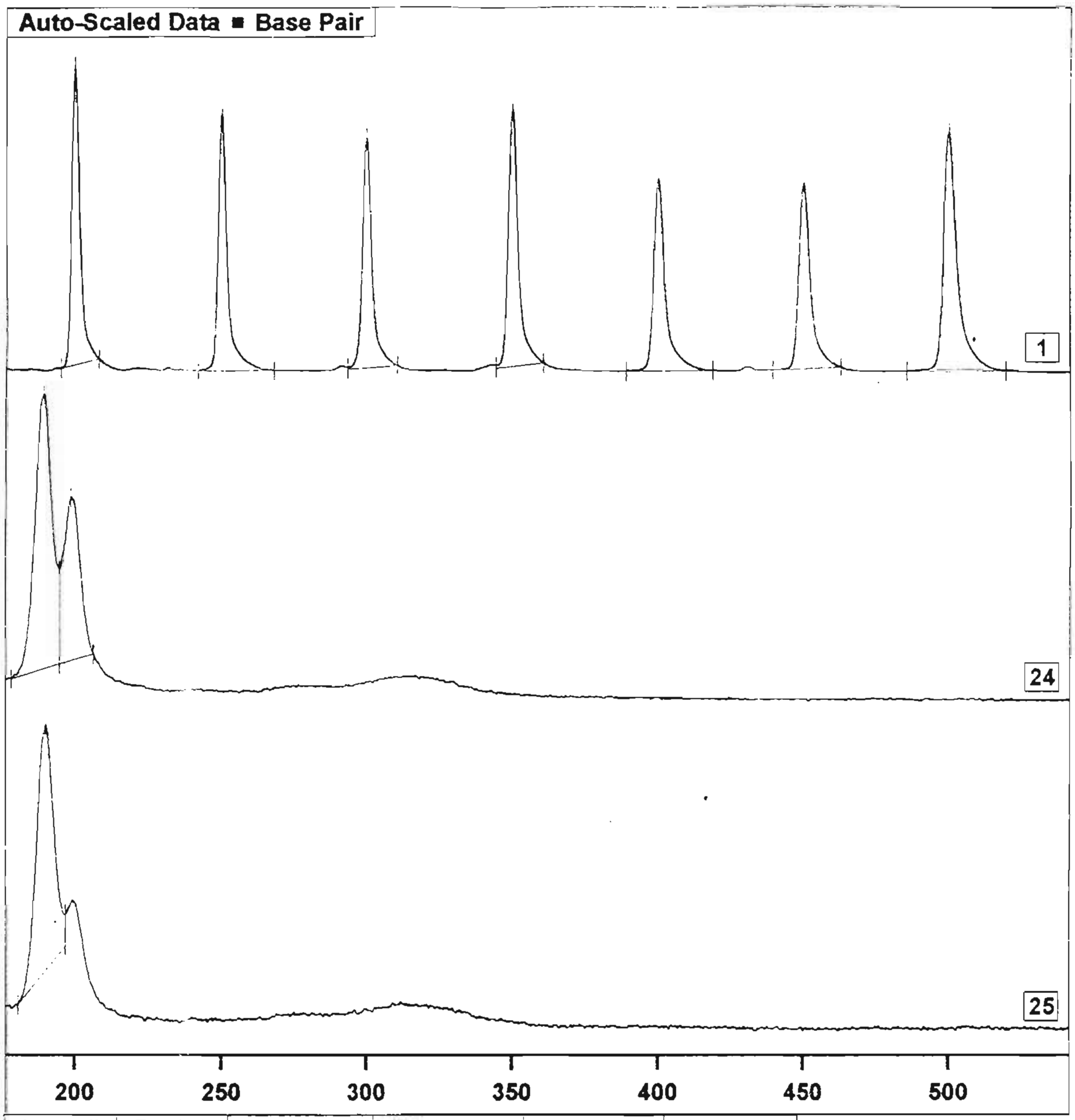
Appendix B - CRC <35: MSI - D3S1255

APPENDIX C



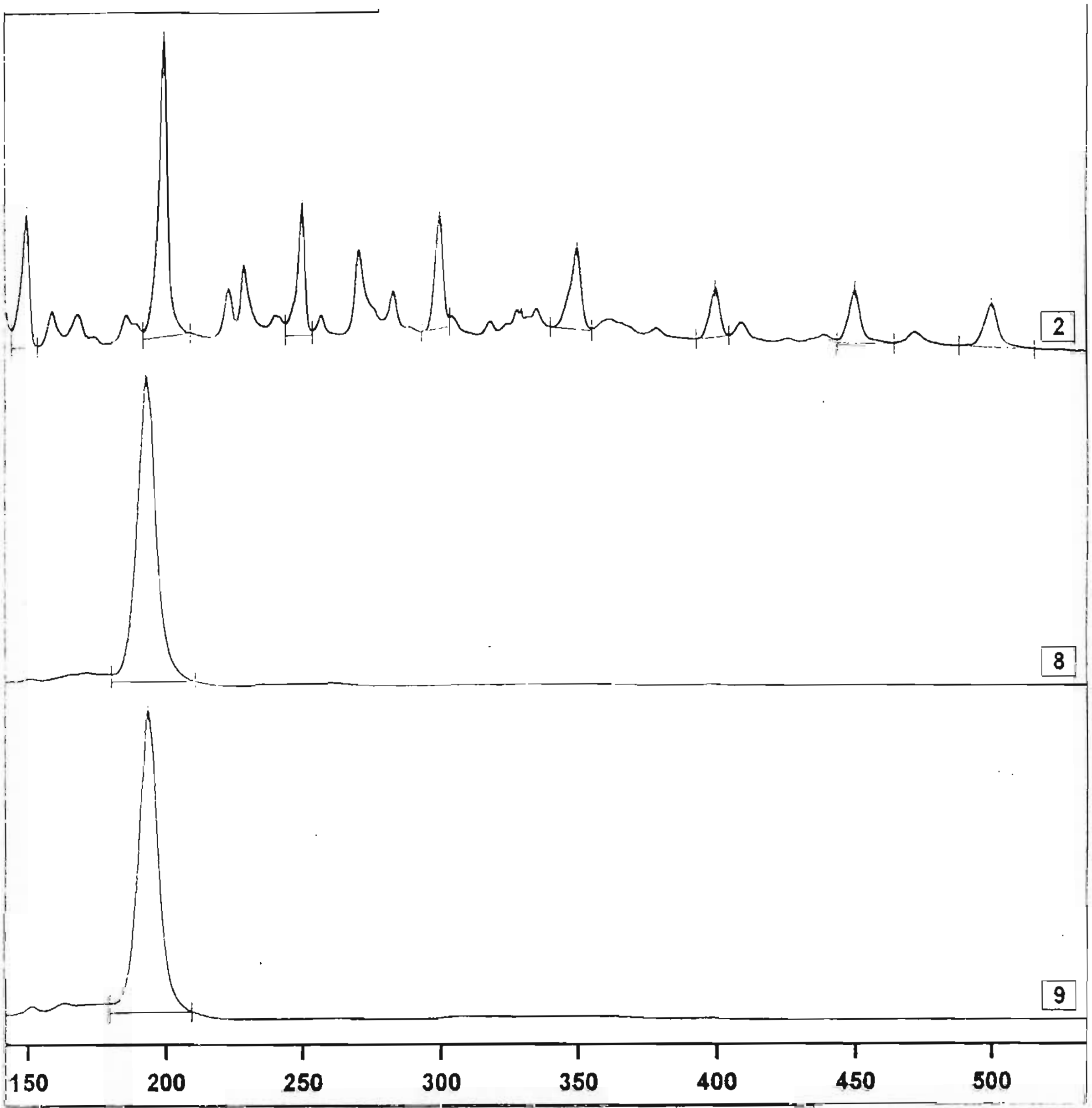
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
10	1	45:44	792.79	193.9	1
	2	46:54	515.1	202.5	0.64972
11	1	45:44	223.1	193.9	1
	2	46:55	177.64	202.6	0.79624

Appendix C - CRC >50: NAI - DCC



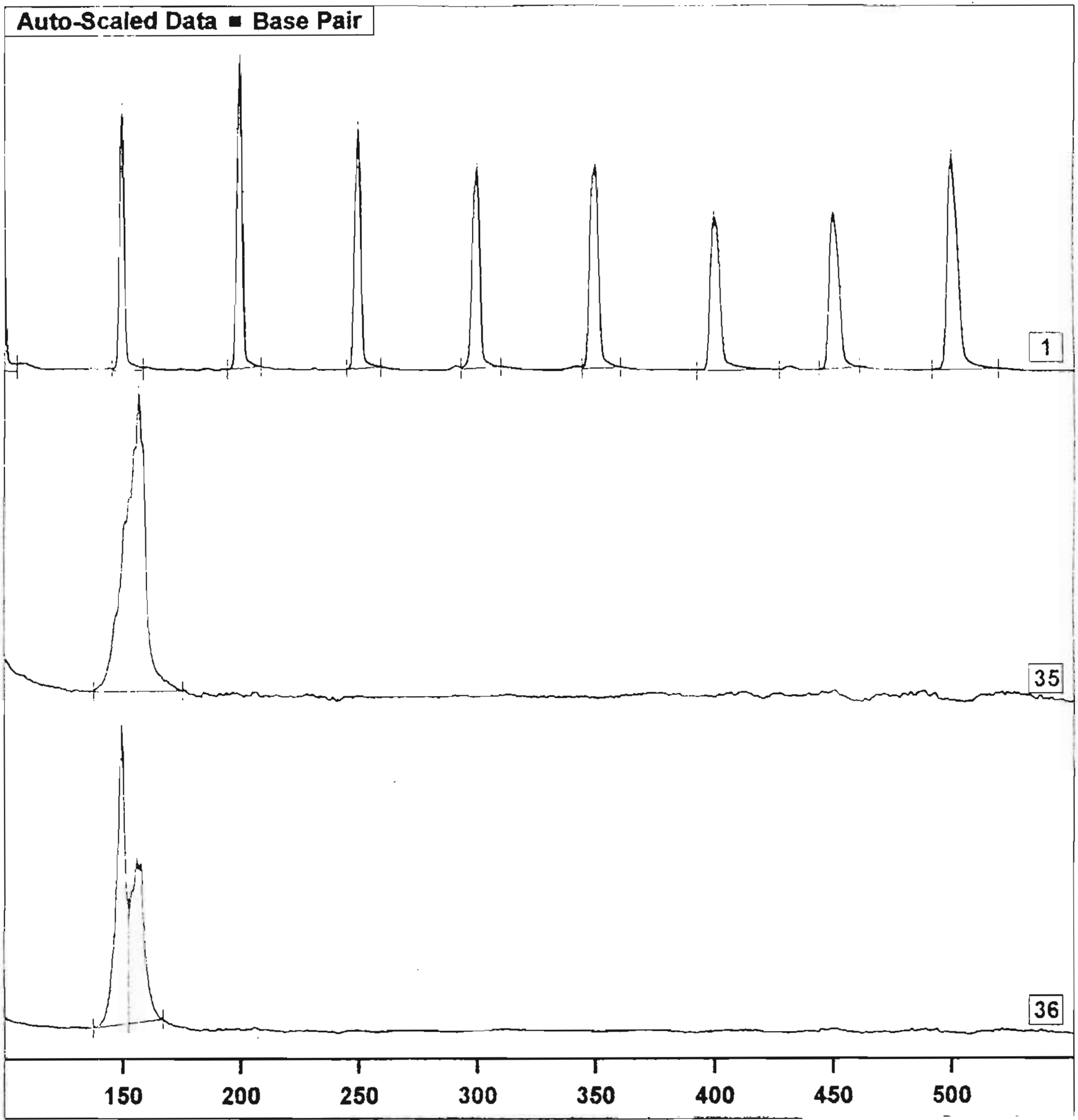
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:18	100.98	#50.0	0.41157
	2	24:20	83.328	#100.0	0.33963
	3	29:35	133.16	#150.0	0.54271
	4	35:13	178.84	#200.0	0.72892
	5	41:08	181.39	#250.0	0.7393
	6	47:14	171.56	#300.0	0.69923
	7	53:23	199.1	#350.0	0.81148
	8	59:32	172.98	#400.0	0.70505
	9	65:38	166.78	#450.0	0.67976
	10	71:32	245.35	#500.0	1
24	1	34:00	47.984	189.5	1
	2	35:04	28.609	198.7	0.59621
25	1	34:05	30.661	190.2	1

Appendix C - CRC >50: AI - DCC



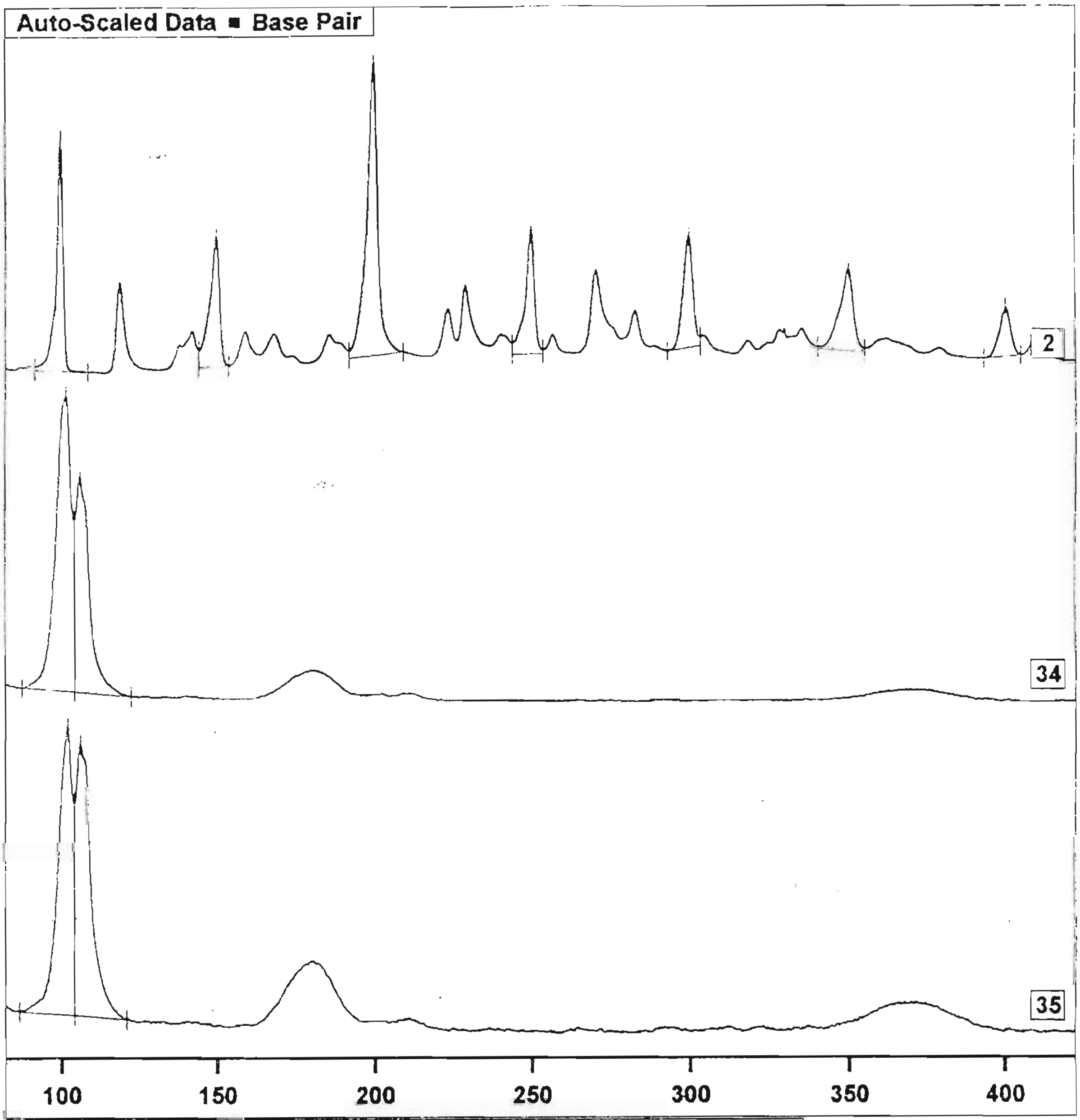
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
8	1	45:37	2312.4	193.0	1
9	1	45:41	1198.9	193.5	1

Appendix C - CRC >50: H- DCC



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:32	262.72	#50.0	0.56127
	2	19:40	185.55	#100.0	0.3964
	3	24:02	268.69	#150.0	0.57402
	4	28:40	363.36	#200.0	0.77627
	5	33:28	331.35	#250.0	0.70788
	6	38:20	346.92	#300.0	0.74115
	7	43:10	399.12	#350.0	0.85267
	8	47:52	339.98	#400.0	0.72634
	9	52:32	328.4	#450.0	0.70159
	10	57:02	468.08	#500.0	1
35	1	24:40	76.492	157.0	1
36	1	24:01	64.382	149.8	1
	2	24:36	53.274	156.3	0.82746

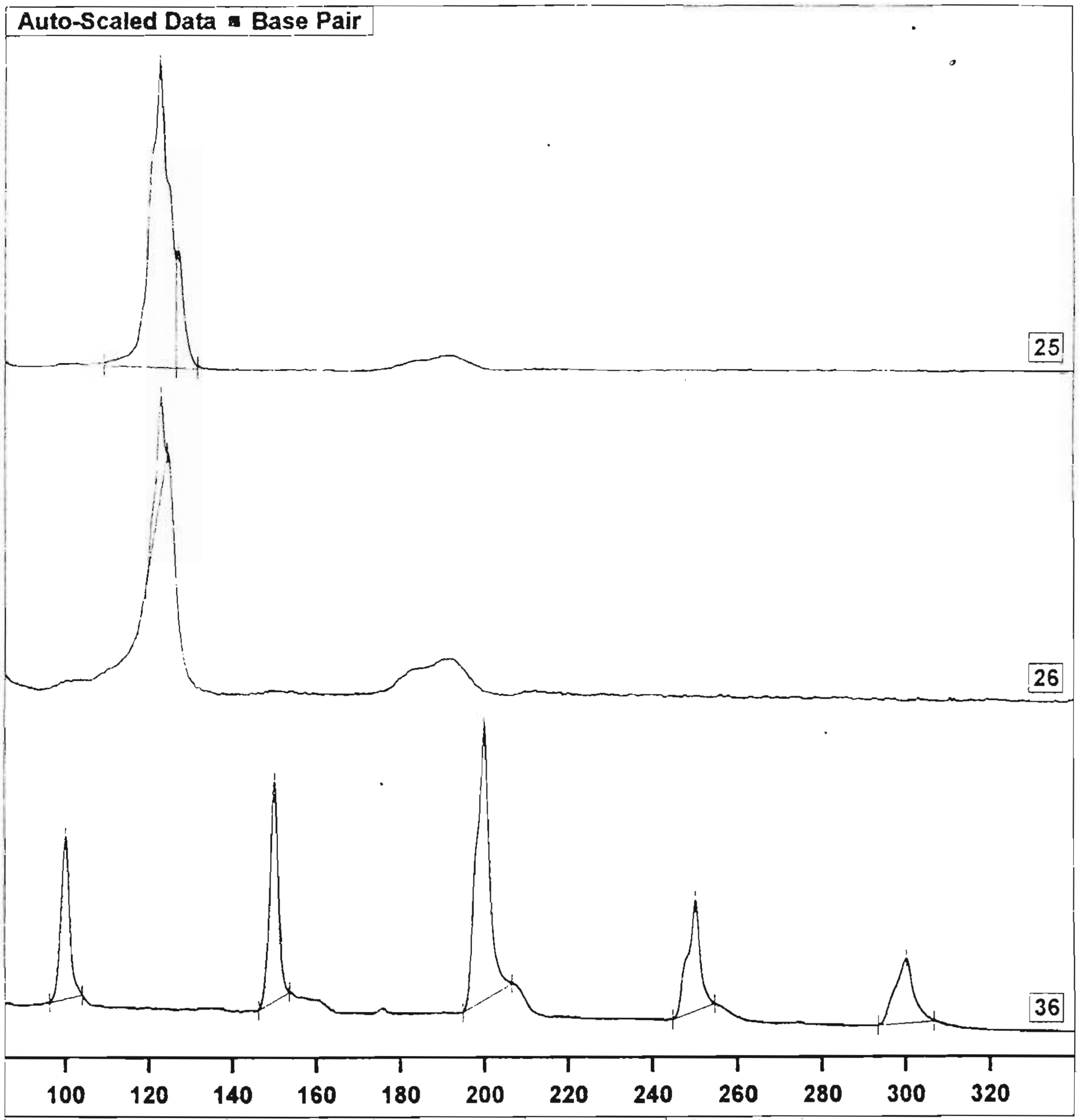
Appendix C - CRC >50: MSI - DCC



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
34	1	33:28	116.65	101.6	1
	2	34:05	80.669	106.1	0.69154
35	1	33:32	53.029	102.1	0.93891
	2	34:06	56.479	106.2	1

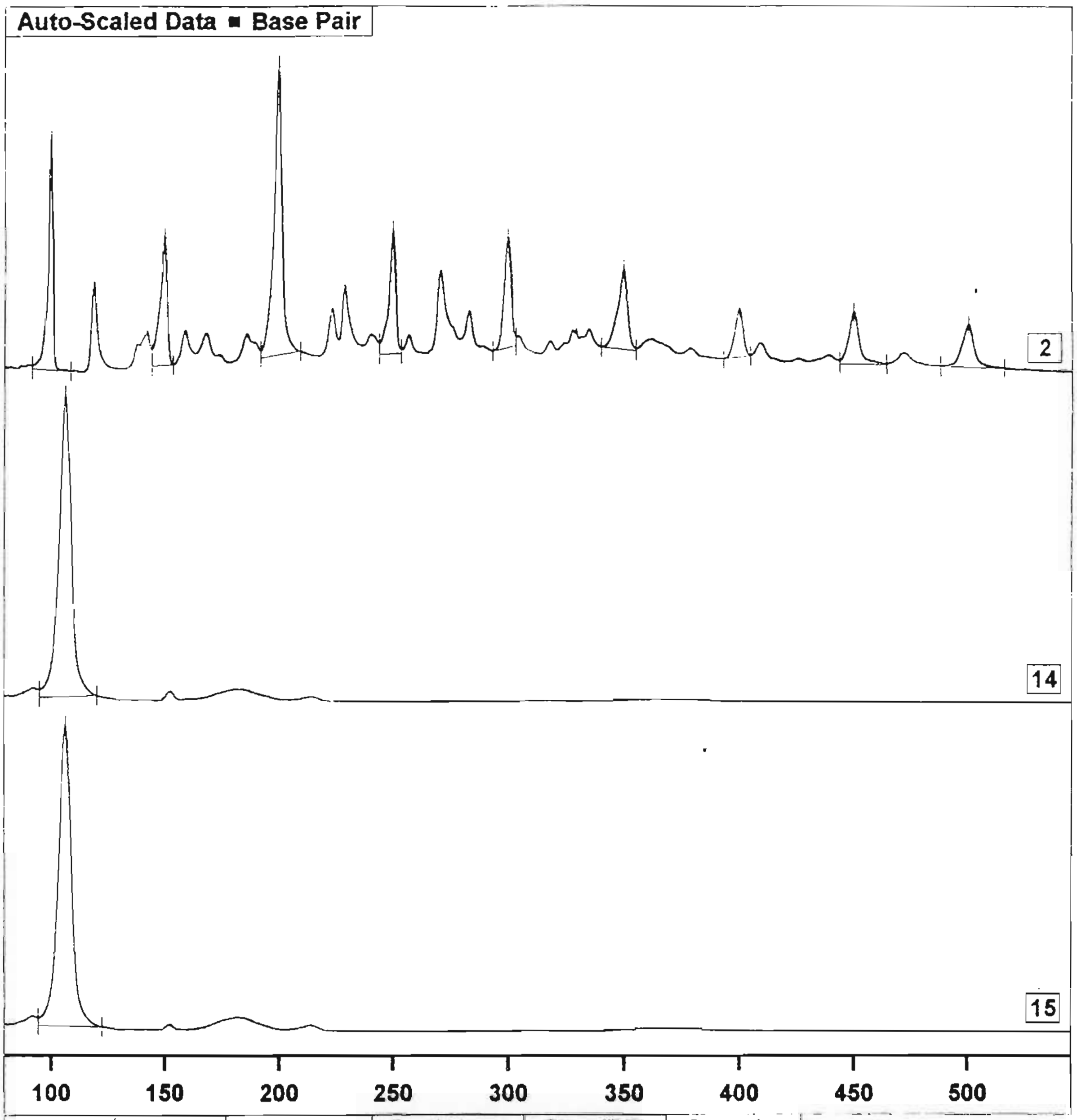
Appendix C - CRC >50: NAI -D18S34

05/Jul/98 09:42:08 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\CRC1311.ALF



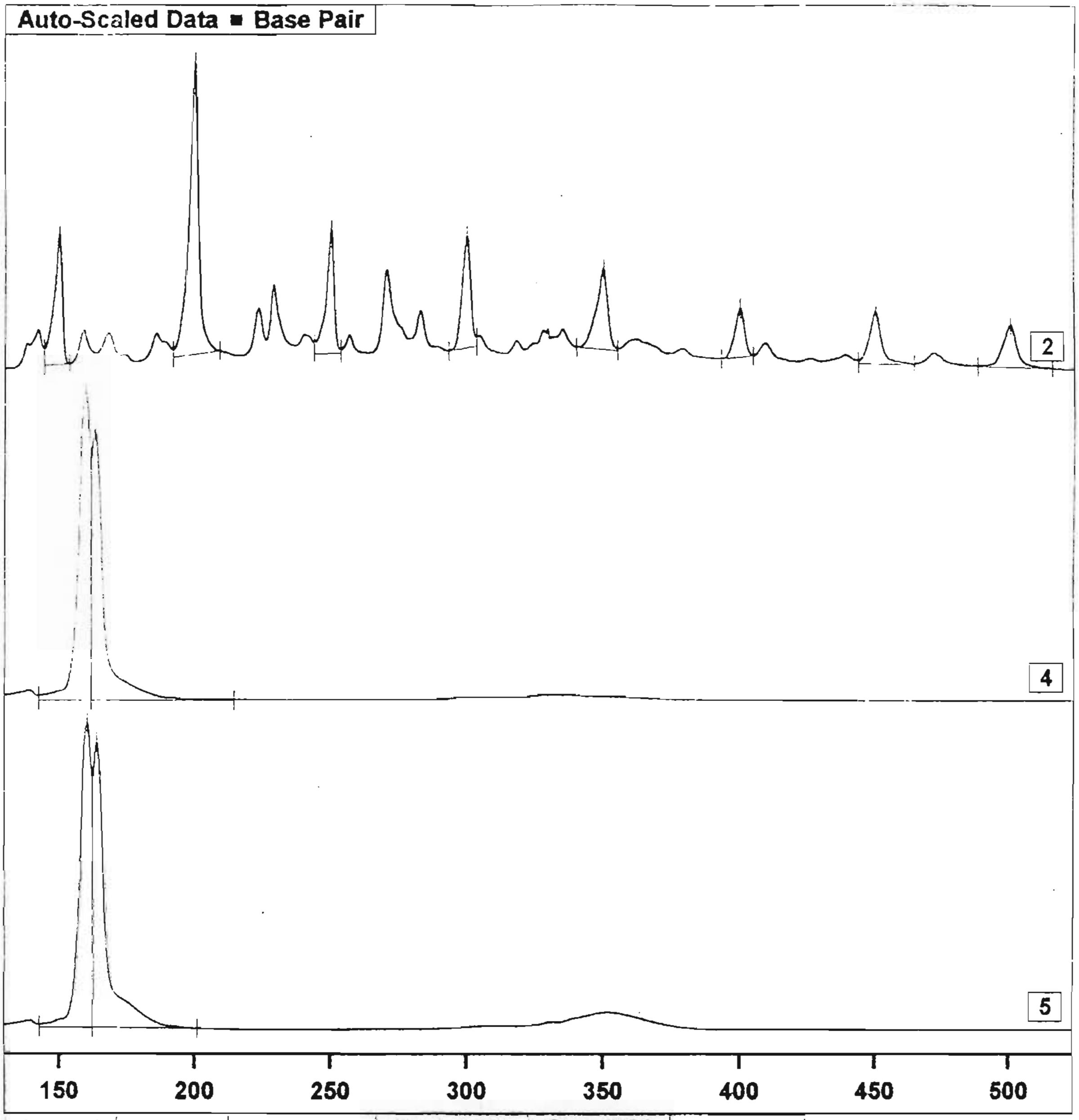
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
25	1	123:10	1294.1	123.9	1
	2	125:13	202.01	128.3	0.15611
26	1	123:14	62.105	123.9	1
36	1	90:24	458.39	#50.0	1
	2	112:45	107.53	#100.0	0.23459
	3	135:55	145.53	#150.0	0.31747
	4	160:42	307.02	#200.0	0.66977
	5	188:11	128.81	#250.0	0.28101
	6	216:37	108.57	#300.0	0.23685

Appendix C - CRC >50: AI - D18S34



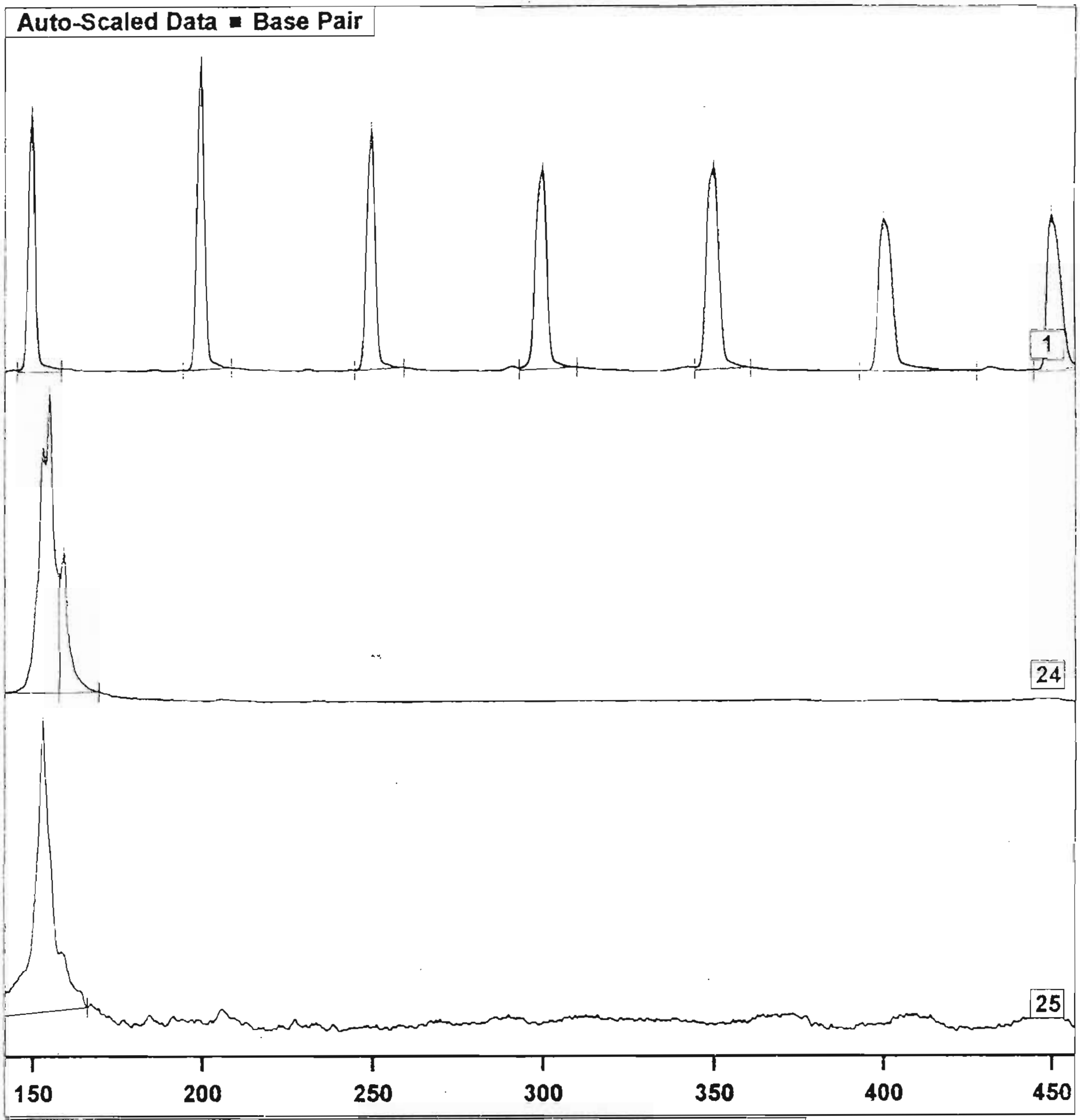
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
14	1	34:07	760.25	106.3	1
15	1	34:07	1259.6	106.3	1

Appendix C - CRC >50: H - D18S34



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:36	132.93	#50.0	0.2649
	2	33:15	220.86	#100.0	0.44014
	3	40:06	195.02	#150.0	0.38863
	4	46:31	501.81	#200.0	1
	5	54:04	200.54	#250.0	0.39964
	6	62:01	193.72	#300.0	0.38605
	7	70:15	197.84	#350.0	0.39426
	8	78:38	103.72	#400.0	0.2067
	9	87:06	143.98	#450.0	0.28692
	10	95:25	133.2	#500.0	0.26545
4	1	41:20	2282.4	159.6	1
	2	41:48	2004.9	163.2	0.87843
5	1	41:28	1560.3	160.6	0.98801
	2	41:54	1579.3	164.0	1

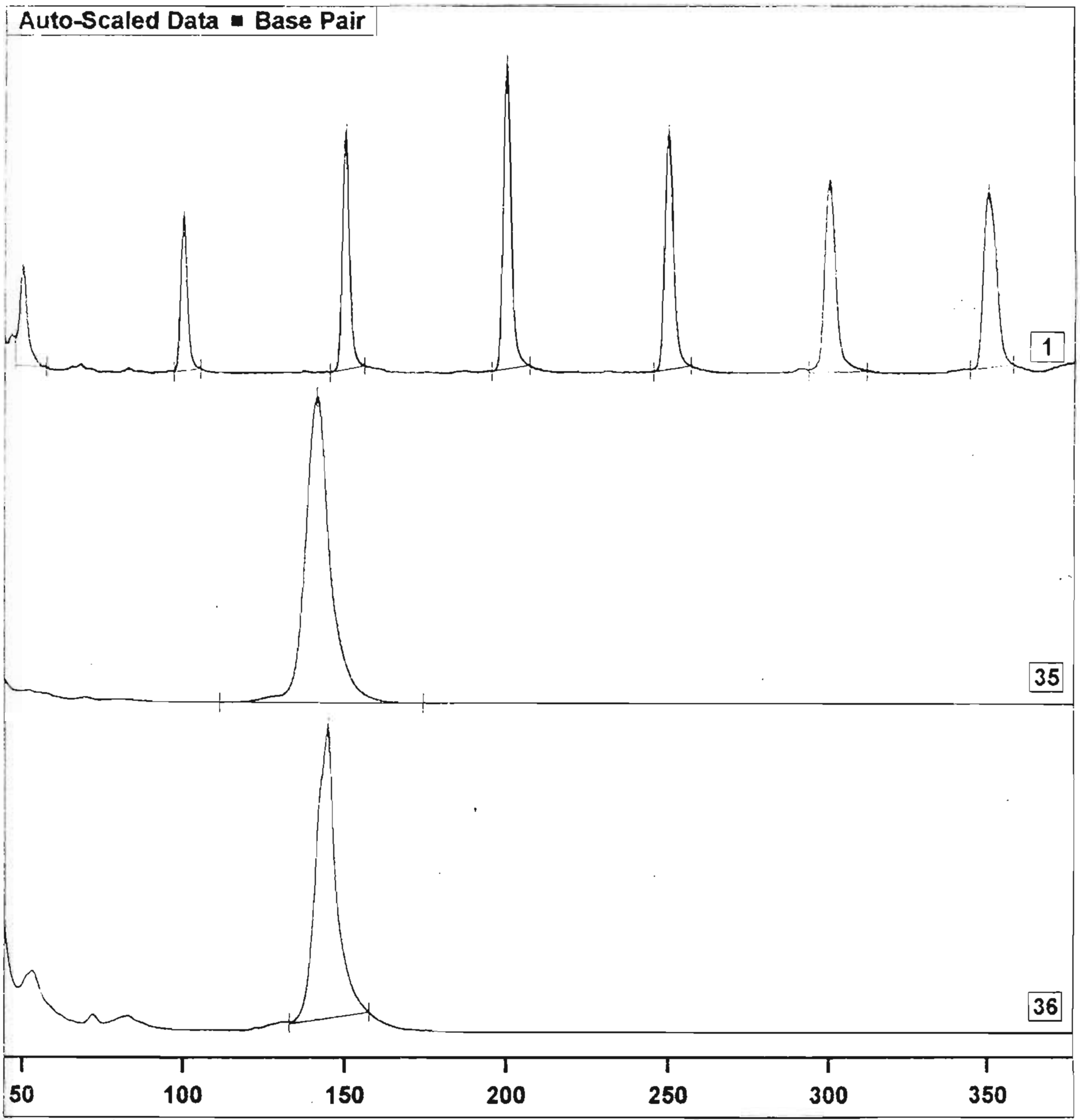
Appendix C - CRC >50: NAI - D18S58



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:32	262.72	#50.0	0.56127
	2	19:40	185.55	#100.0	0.3964
	3	24:02	268.69	#150.0	0.57402
	4	28:40	363.36	#200.0	0.77627
	5	33:28	331.35	#250.0	0.70788
	6	38:20	346.92	#300.0	0.74115
	7	43:10	399.12	#350.0	0.85267
	8	47:52	339.98	#400.0	0.72634
	9	52:32	328.4	#450.0	0.70159
	10	57:02	468.08	#500.0	1
24	1	24:31	120.57	155.3	1
	2	24:53	34.214	159.4	0.28376
25	1	24:20	23.748	153.3	1

Appendix C - CRC >50: AI - D18S58

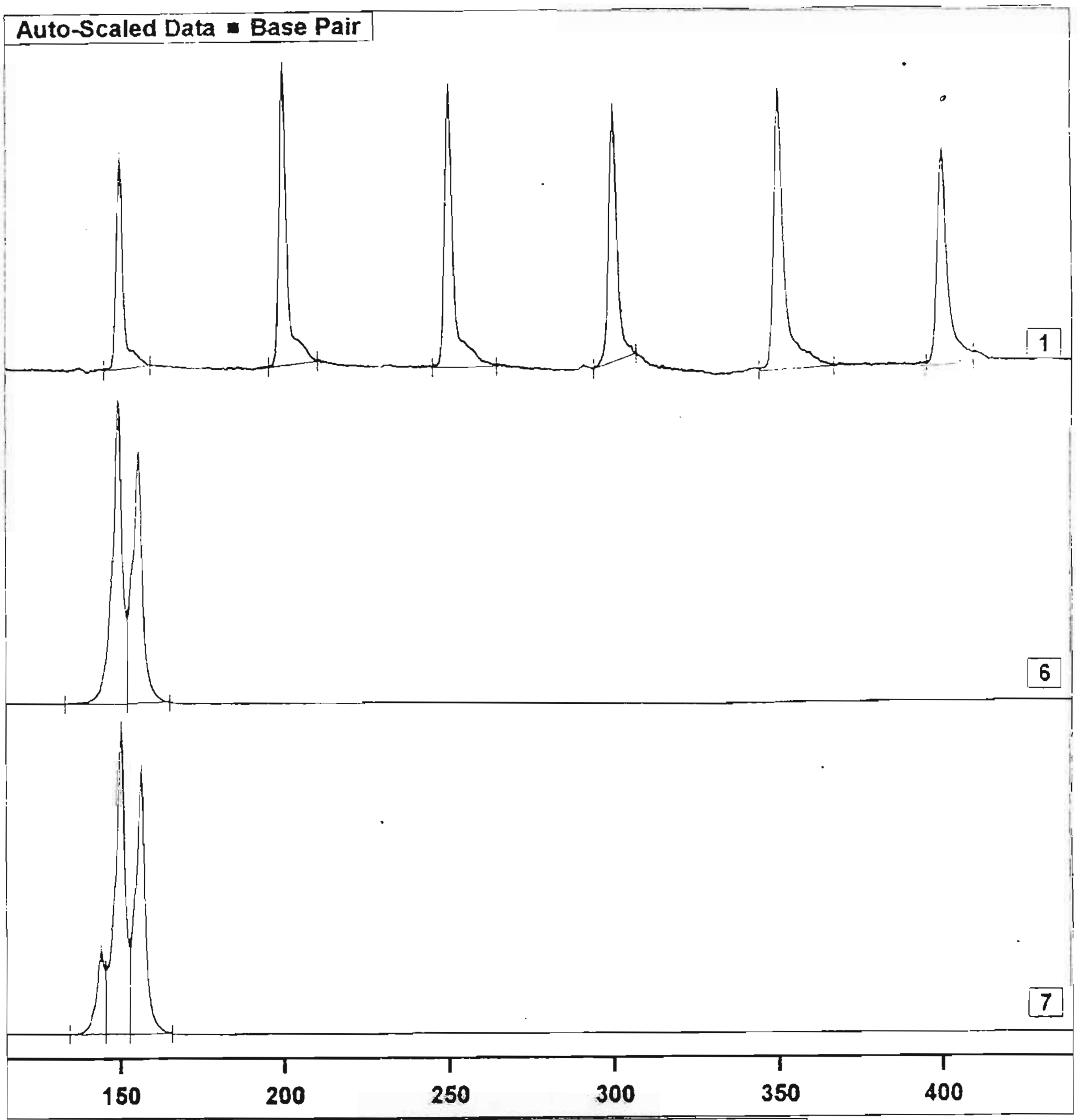
04/Jul/98 14:21:50 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\63MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	22:45	26.012	#50.0	0.30935
	2	28:43	31.341	#100.0	0.37273
	3	34:59	55.94	#150.0	0.66526
	4	41:41	83.839	#200.0	0.99706
	5	48:48	76.505	#250.0	0.90984
	6	56:10	83.758	#300.0	0.9961
	7	63:38	84.086	#350.0	1
35	1	33:52	3514.6	141.3	1
36	1	34:18	546.61	144.7	1

Appendix C - CRC >50: H - D18S58

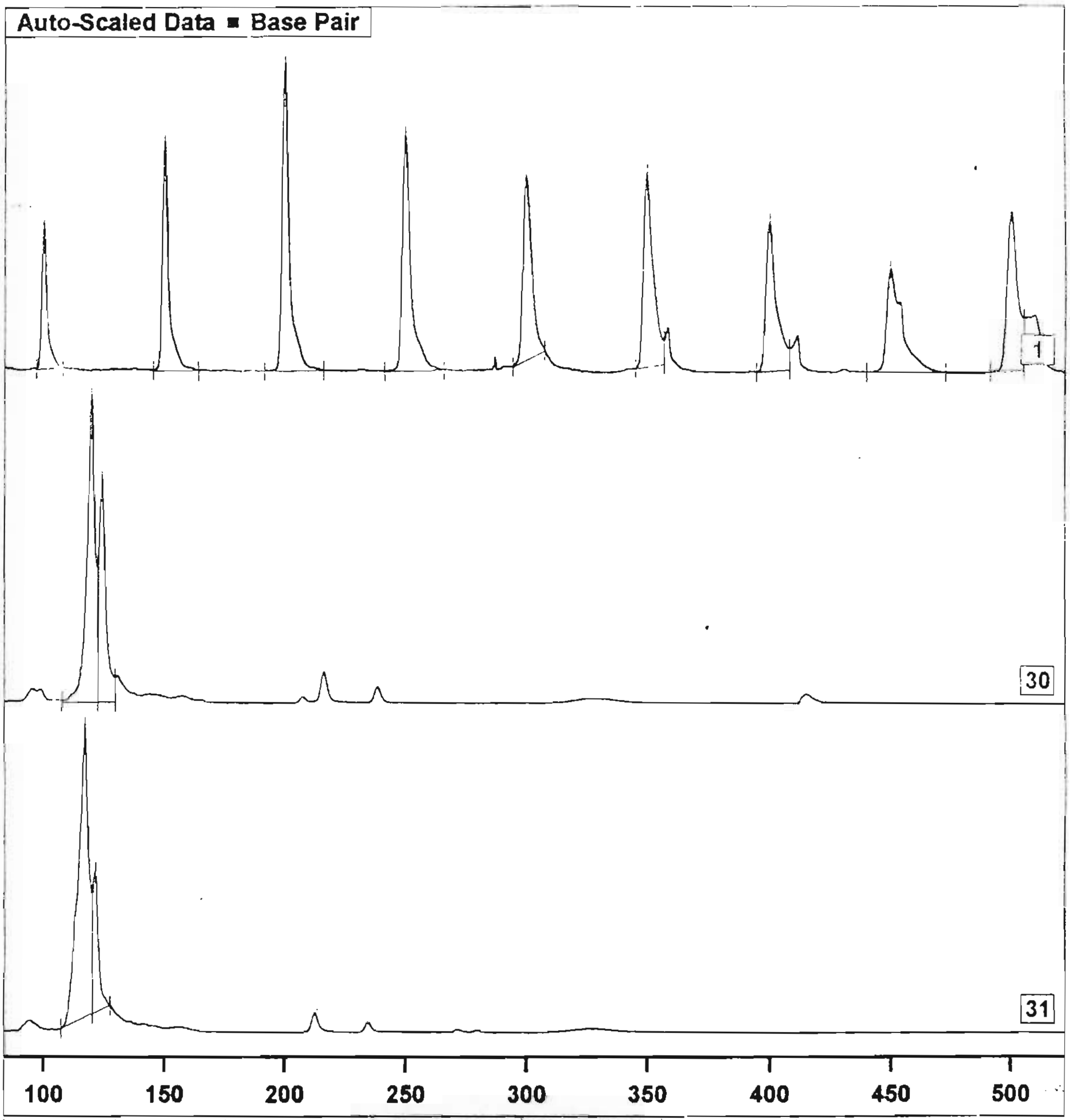
26/Jun/98 14:06:57 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\61MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	28:45	15.075	#100.0	0.23904
	2	35:05	25.953	#150.0	0.41151
	3	41:53	42.464	#200.0	0.67331
	4	49:07	46.961	#250.0	0.74462
	5	56:38	41.166	#300.0	0.65273
	6	64:19	59.523	#350.0	0.94381
	7	72:07	46.185	#400.0	0.73232
	8	79:57	44.125	#450.0	0.69964
	9	87:44	63.067	#500.0	1
6	1	35:00	211.06	149.4	1
	2	35:49	182.96	155.6	0.86686
7	1	34:17	84.455	143.9	0.22847
	2	35:06	369.65	150.1	1
	3	35:55	320.96	156.3	0.86829

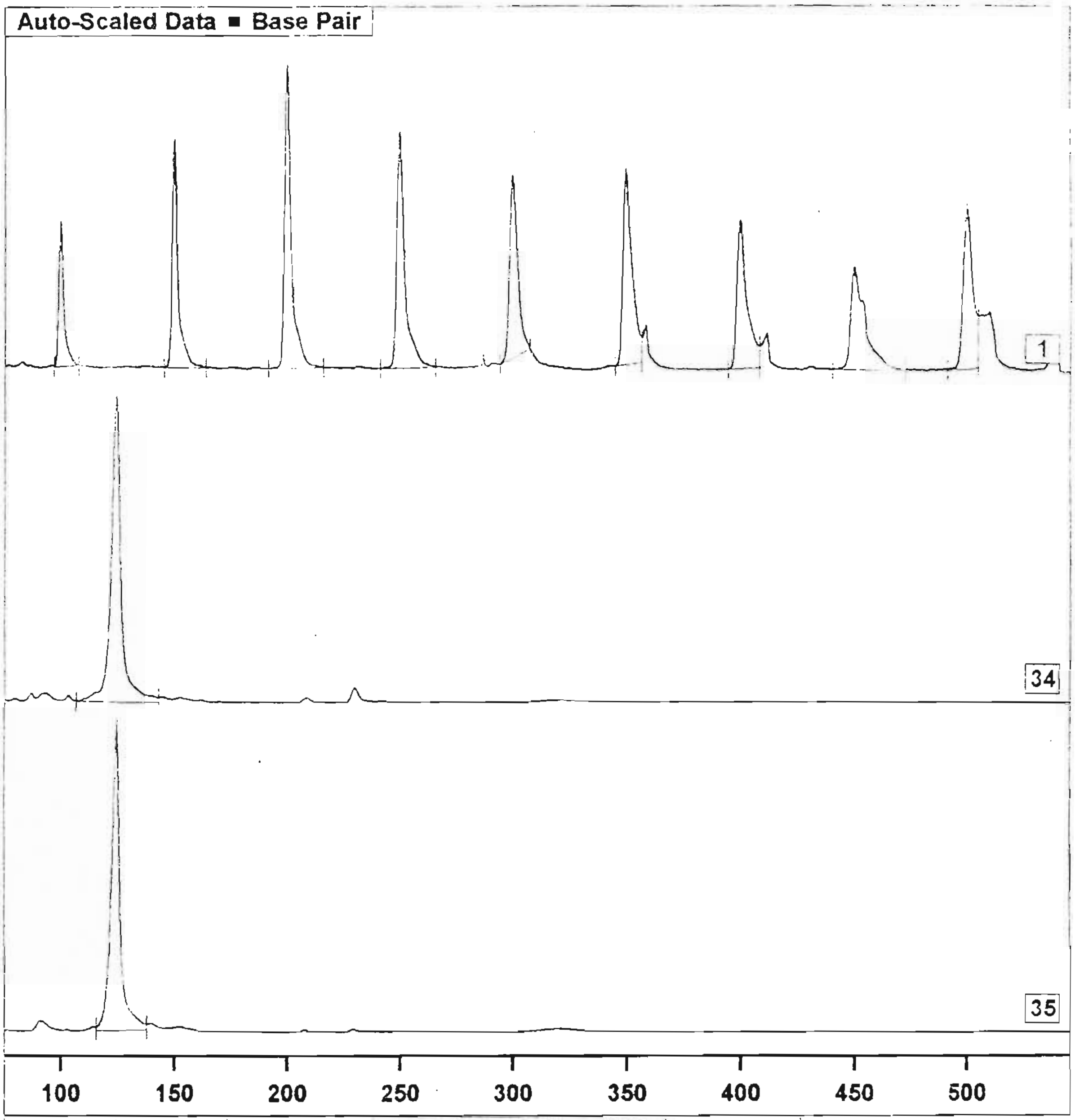
Appendix C - CRC >50: MSI - D18S58

03/Jul/98 14:33:27 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\93MSI98.ALF



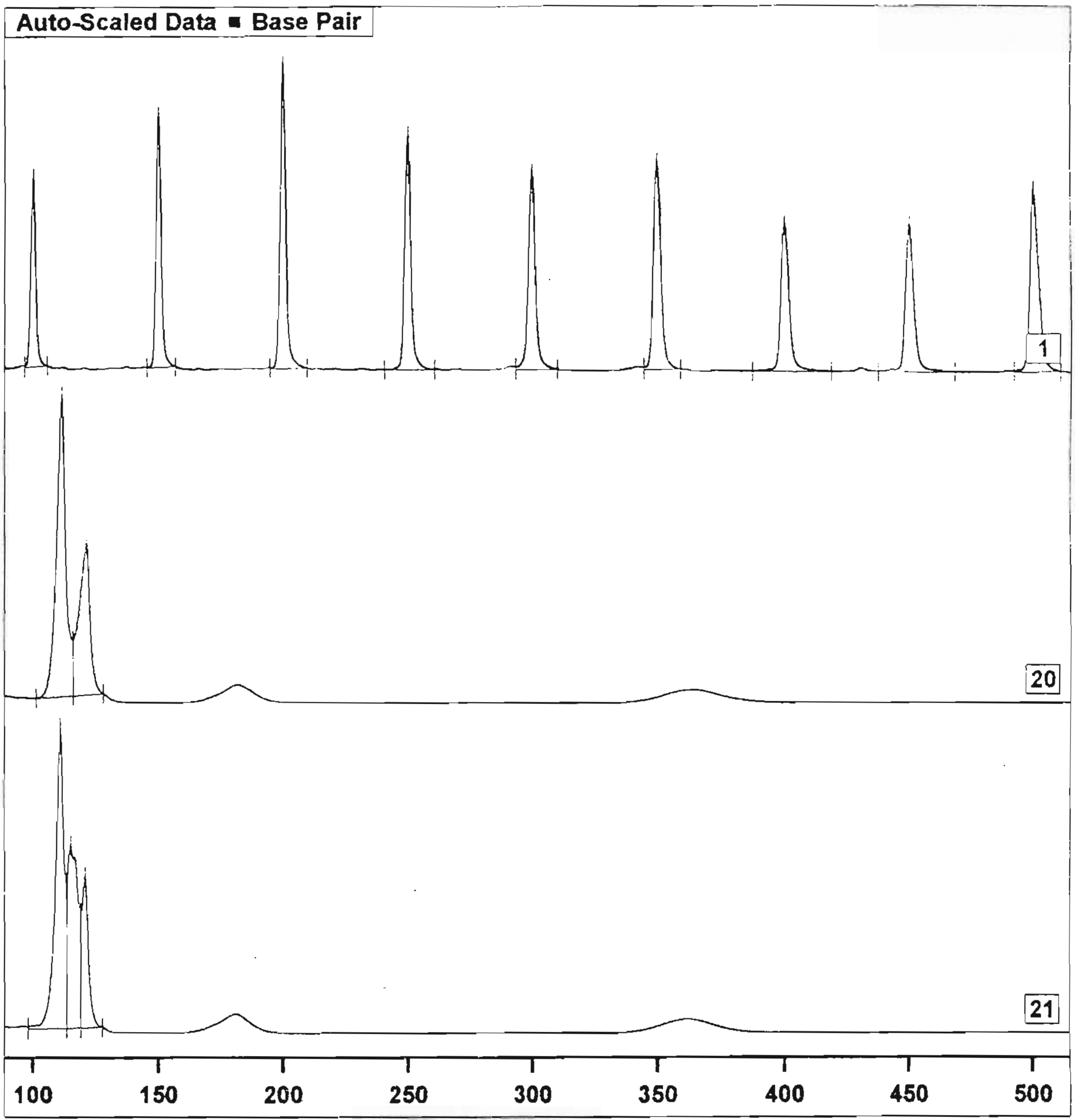
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:49	20.434	#50.0	0.23147
	2	20:16	28.371	#100.0	0.32138
	3	24:51	55.392	#150.0	0.62747
	4	29:42	88.278	#200.0	1
	5	34:44	81.725	#250.0	0.92576
	6	39:46	63.272	#300.0	0.71673
	7	44:46	73.335	#350.0	0.83072
	8	49:39	59.771	#400.0	0.67708
	9	54:25	59.002	#450.0	0.66837
	10	58:58	60.638	#500.0	0.6869
30	1	22:03	435.88	119.7	1
	2	22:27	282.99	124.1	0.64925
31	1	21:49	846.85	117.2	1
	2	22:13	225.17	121.6	0.26589

Appendix C - CRC >50: AI - D3S659



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:49	20.434	#50.0	0.23147
	2	20:16	28.371	#100.0	0.32138
	3	24:51	55.392	#150.0	0.62747
	4	29:42	88.278	#200.0	1
	5	34:44	81.725	#250.0	0.92576
	6	39:46	63.272	#300.0	0.71673
	7	44:46	73.335	#350.0	0.83072
	8	49:39	59.771	#400.0	0.67708
	9	54:25	59.002	#450.0	0.66837
	10	58:58	60.638	#500.0	0.6869
34	1	22:30	1225.2	124.7	1
35	1	22:30	757.23	124.7	1

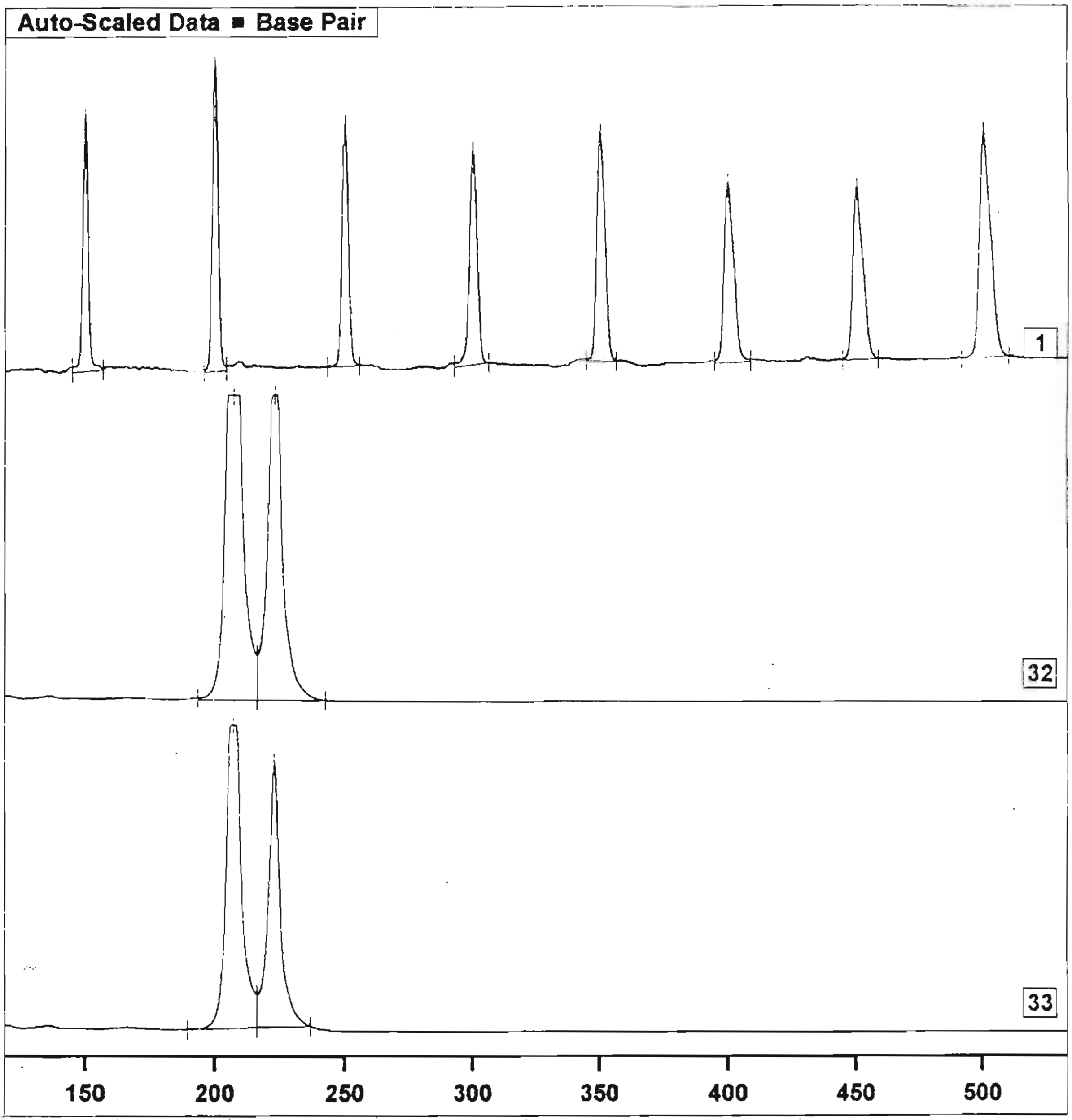
Appendix C - CRC >50: H - D3S659



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	25:59	254.46	#50.0	0.38421
	2	31:54	292.75	#100.0	0.44203
	3	38:29	463.44	#150.0	0.69975
	4	45:45	656.55	#200.0	0.99134
	5	53:31	580.05	#250.0	0.87583
	6	61:34	582.39	#300.0	0.87936
	7	69:47	635.92	#350.0	0.96019
	8	78:08	518.11	#400.0	0.7823
	9	86:33	532.31	#450.0	0.80375
	10	94:51	662.29	#500.0	1
20	1	33:20	1487.8	111.3	1
	2	34:36	936.02	121.2	0.62912
21	1	33:16	1254.2	110.8	1
	2	33:47	933.38	114.9	0.74422
	3	34:32	499.83	120.6	0.39853

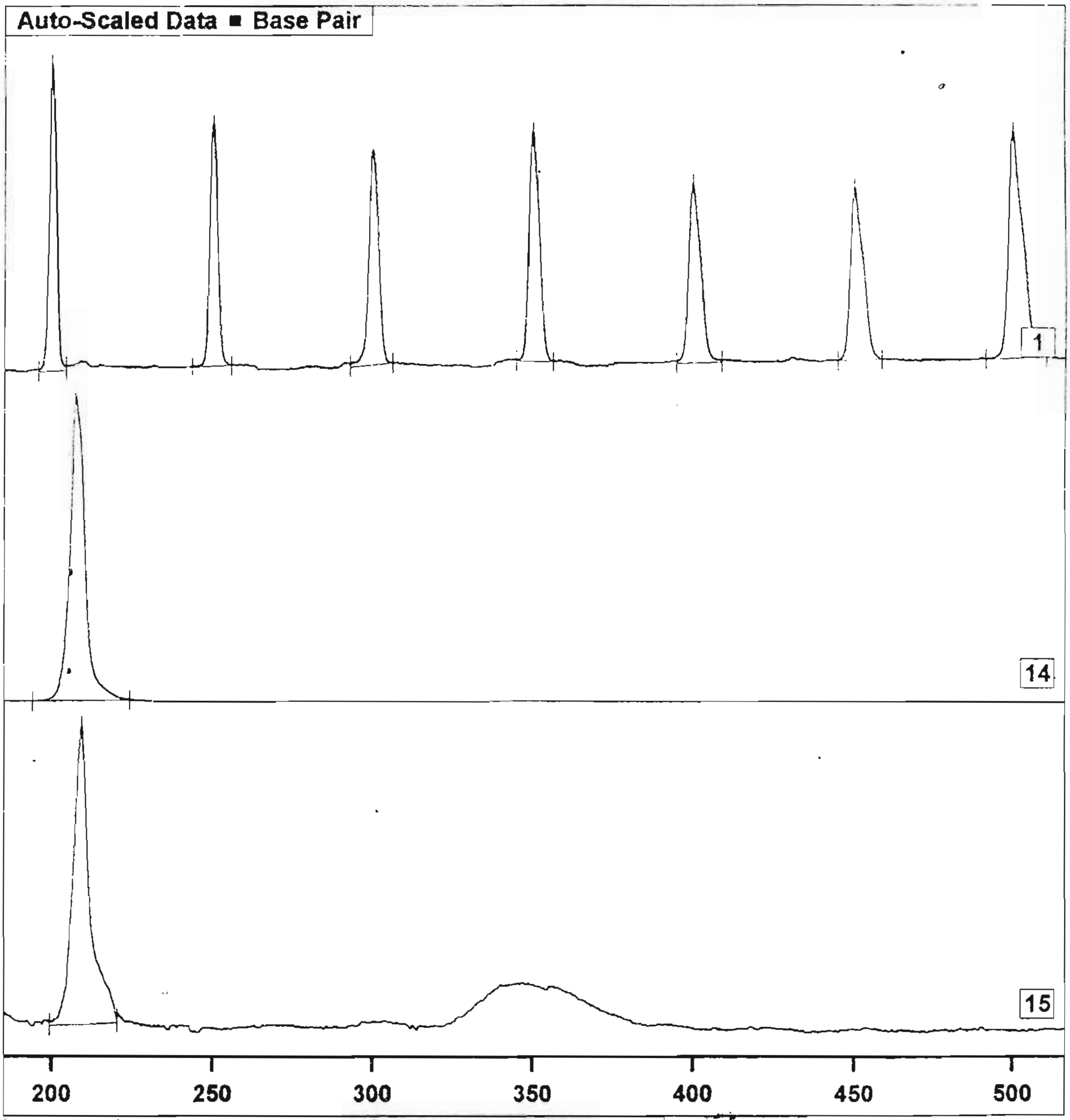
Appendix C - CRC >50: MSI - D3S659

03/Jul/98 15:13:07 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\106MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	20:12	12.879	#50.0	0.2036
	2	25:05	17.109	#100.0	0.27046
	3	30:12	28.422	#150.0	0.4493
	4	35:39	39.118	#200.0	0.61839
	5	41:26	36.654	#250.0	0.57943
	6	47:25	40.564	#300.0	0.64124
	7	53:32	47.216	#350.0	0.74641
	8	59:46	42.678	#400.0	0.67467
	9	66:01	43.775	#450.0	0.69201
	10	72:15	63.258	#500.0	1
32	1	36:28	6031.5	207.2	1
	2	38:17	4915.4	223.1	0.81496
33	1	36:28	4807.5	207.2	1
	2	38:16	3240.7	222.9	0.67409

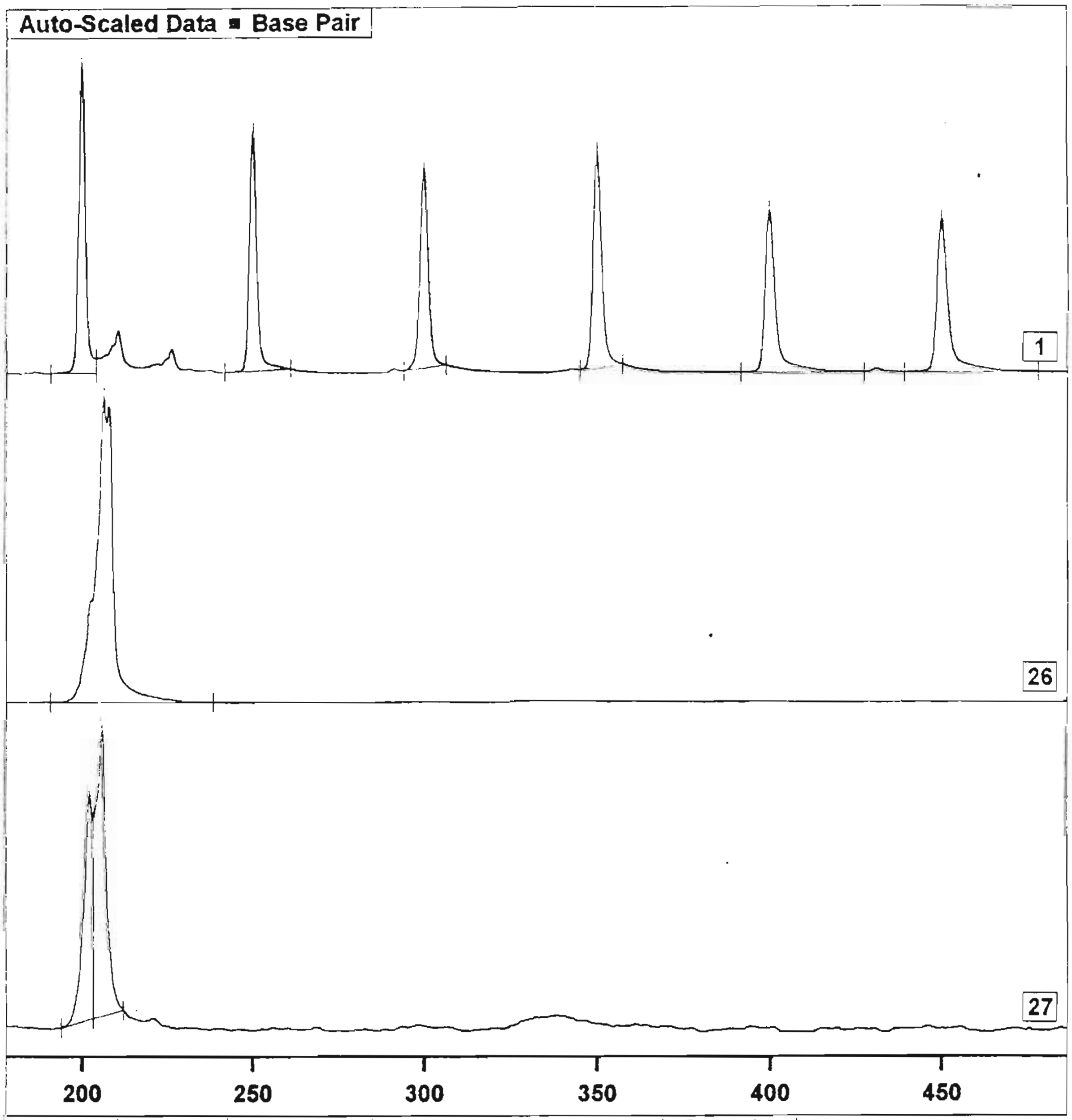
Appendix C - CRC >50: NAI - D2S123



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	20:12	12.879	#50.0	0.2036
	2	25:05	17.109	#100.0	0.27046
	3	30:12	28.422	#150.0	0.4493
	4	35:39	39.118	#200.0	0.61839
	5	41:26	36.654	#250.0	0.57943
	6	47:25	40.564	#300.0	0.64124
	7	53:32	47.216	#350.0	0.74641
	8	59:46	42.678	#400.0	0.67467
	9	66:01	43.775	#450.0	0.69201
	10	72:15	63.258	#500.0	1
14	1	36:29	1977.4	207.4	1
15	1	36:42	56.296	209.3	1

Appendix C - CRC >50: H - D2S123

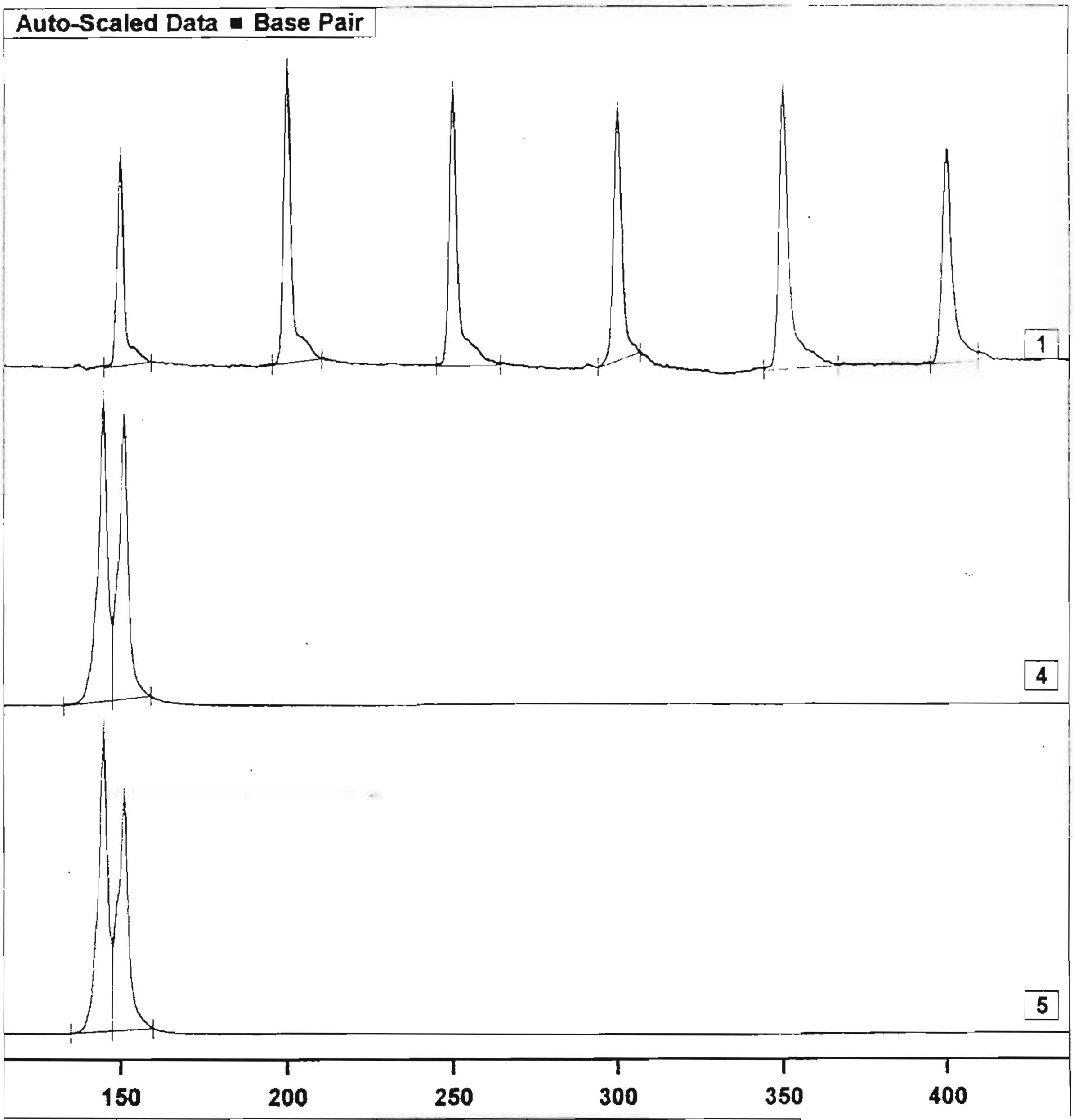
26/Jun/98 14:40:59 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\19MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
26	1	35:37	714.79	206.3	1
27	1	35:09	40.273	202.2	0.65774
	2	35:34	61.229	205.9	1

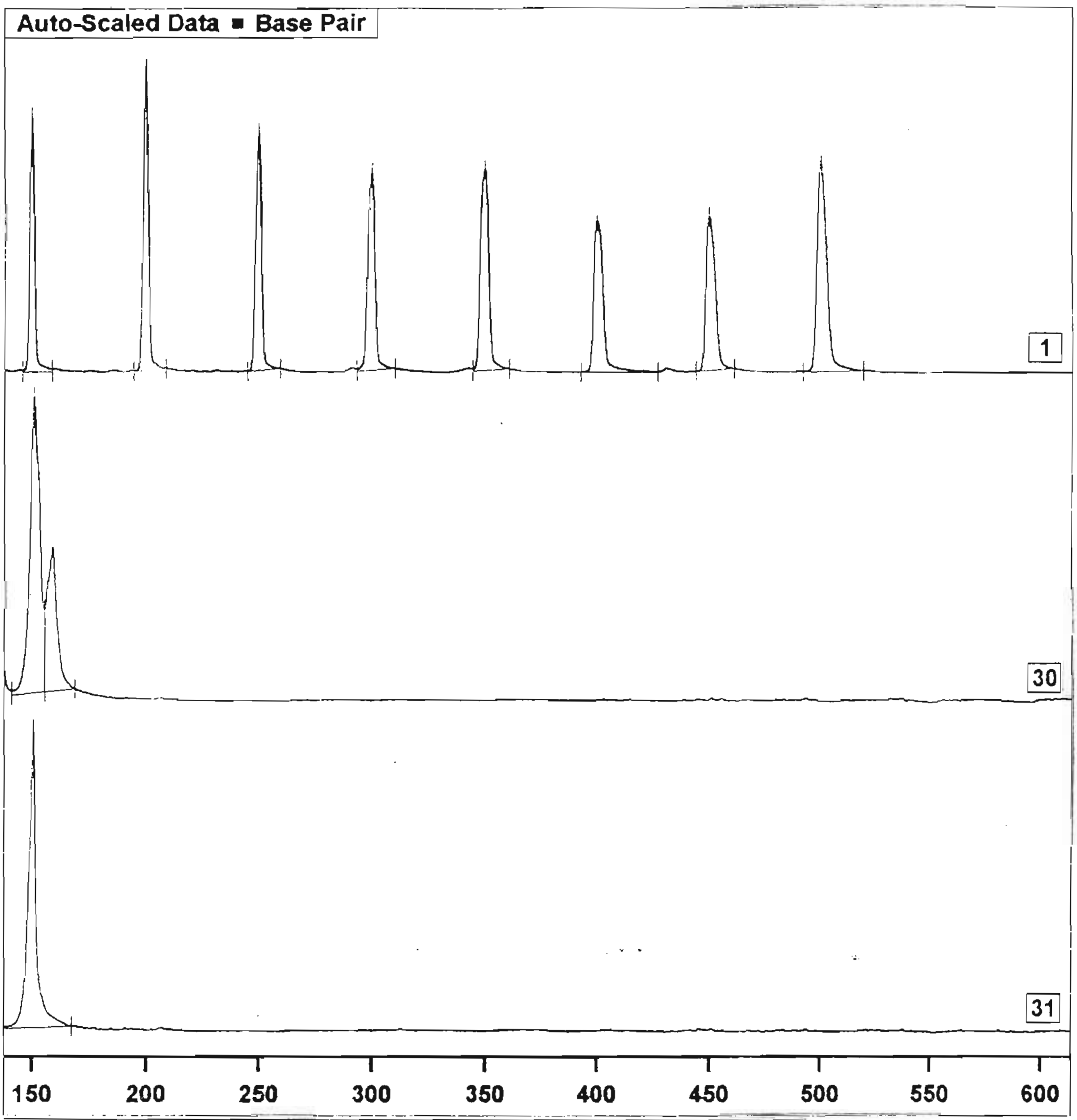
Appendix C - CRC >50: MSI - D2S123

26/Jun/98 14:14:55 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\61MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	28:45	15.075	#100.0	0.23904
	2	35:05	25.953	#150.0	0.41151
	3	41:53	42.464	#200.0	0.67331
	4	49:07	46.961	#250.0	0.74462
	5	56:38	41.166	#300.0	0.65273
	6	64:19	59.523	#350.0	0.94381
	7	72:07	46.185	#400.0	0.73232
	8	79:57	44.125	#450.0	0.69964
	9	87:44	63.067	#500.0	1
4	1	34:25	107.17	144.9	1
	2	35:14	106.52	151.1	0.99397
5	1	34:25	200.67	144.9	1
	2	35:14	182.9	151.1	0.91144

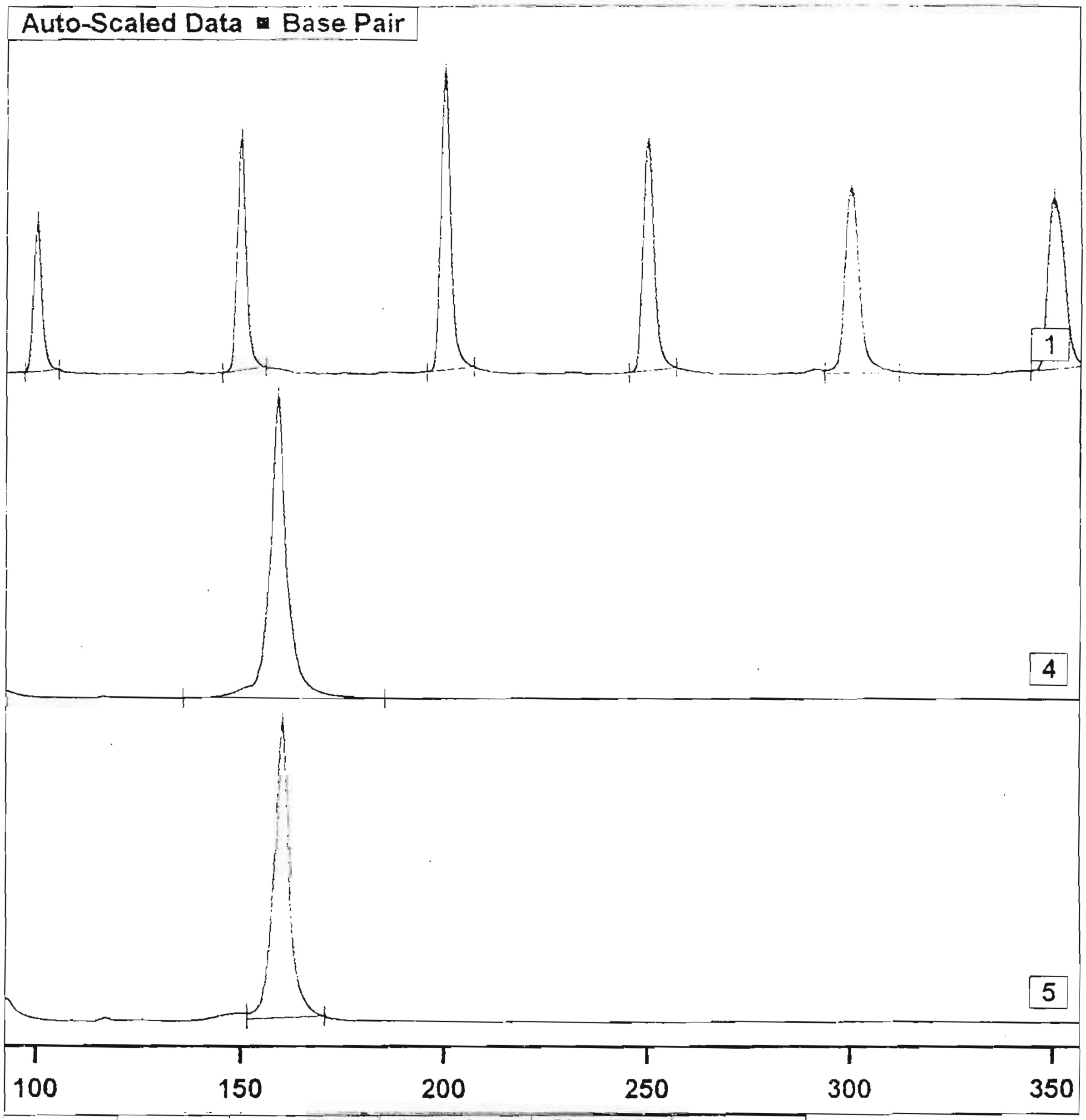
Appendix C - CRC >50: NAI - D3S1255



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:32	262.72	#50.0	0.56127
	2	19:40	185.55	#100.0	0.3964
	3	24:02	268.69	#150.0	0.57402
	4	28:40	363.36	#200.0	0.77627
	5	33:28	331.35	#250.0	0.70788
	6	38:20	346.92	#300.0	0.74115
	7	43:10	399.12	#350.0	0.85267
	8	47:52	339.98	#400.0	0.72634
	9	52:32	328.4	#450.0	0.70159
	10	57:02	468.08	#500.0	1
30	1	24:07	121.97	150.9	1
	2	24:51	60.019	159.0	0.49208
31	1	24:06	71.845	150.7	1

Appendix C - CRC >50: AI - D3S1255

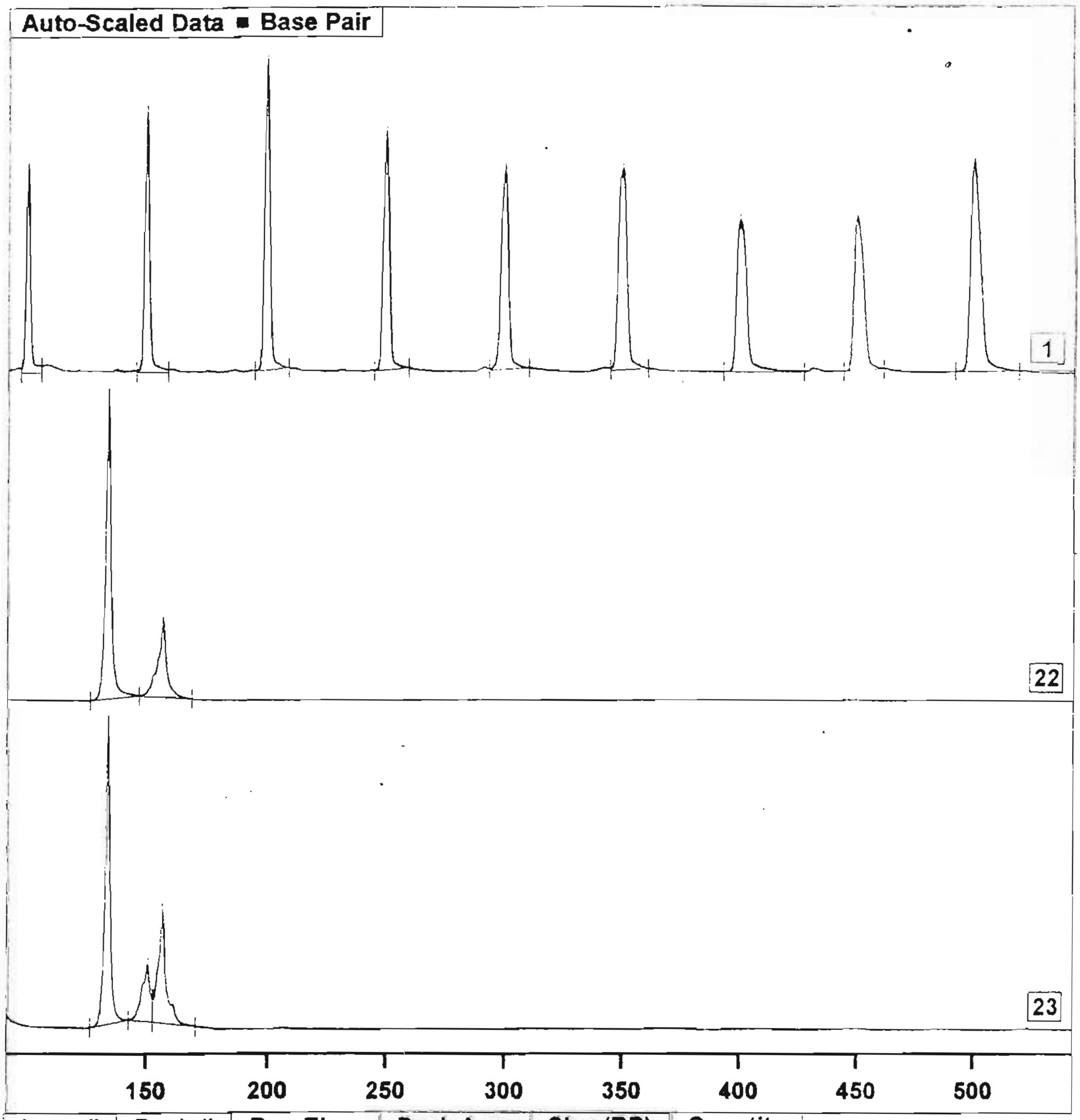
01/Apr/98 09:56:11 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\63MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	22:45	26.012	#50.0	0.30935
	2	28:43	31.341	#100.0	0.37273
	3	34:59	55.94	#150.0	0.66526
	4	41:41	83.839	#200.0	0.99706
	5	48:48	76.505	#250.0	0.90984
	6	56:10	83.758	#300.0	0.9961
	7	63:38	84.086	#350.0	1
4	1	36:10	790.6	159.1	1
5	1	36:19	235.6	160.2	1

Appendix C - CRC >50: H - D3S1255

04/Jul/98 13:21:46 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\77MSI98.ALF

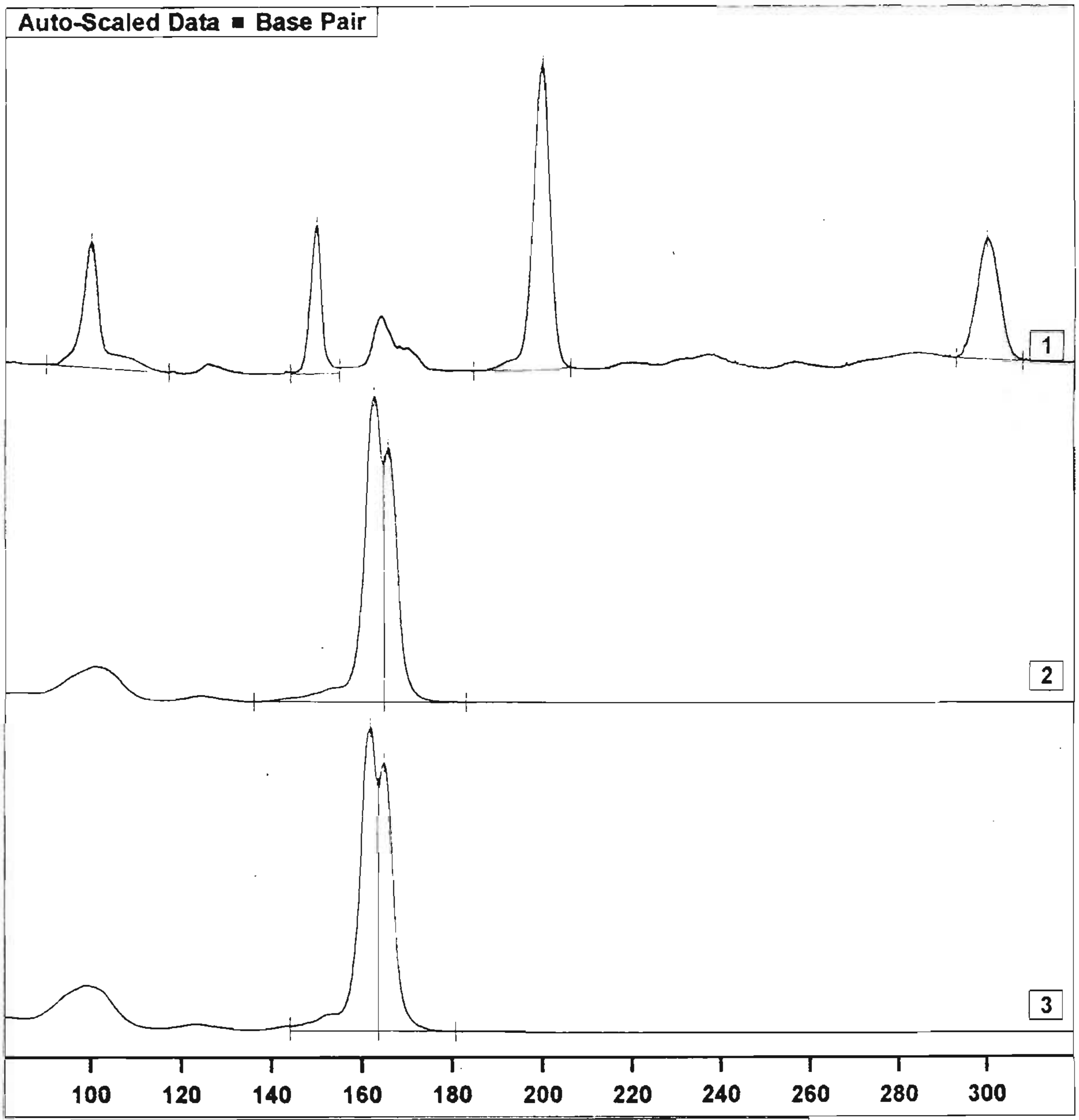


Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:32	262.72	#50.0	0.56127
	2	19:40	185.55	#100.0	0.3964
	3	24:02	268.69	#150.0	0.57402
	4	28:40	363.36	#200.0	0.77627
	5	33:28	331.35	#250.0	0.70788
	6	38:20	346.92	#300.0	0.74115
	7	43:10	399.12	#350.0	0.85267
	8	47:52	339.98	#400.0	0.72634
	9	52:32	328.4	#450.0	0.70159
	10	57:02	468.08	#500.0	1
22	1	22:37	424.16	134.1	1
	2	24:40	173.63	157.0	0.40934
23	1	22:37	112.35	134.1	1
	2	24:07	32.197	150.9	0.28659
	3	24:40	65.403	157.0	0.58215

Appendix C - CRC >50: MSI - D3S1255

APPENDIX D

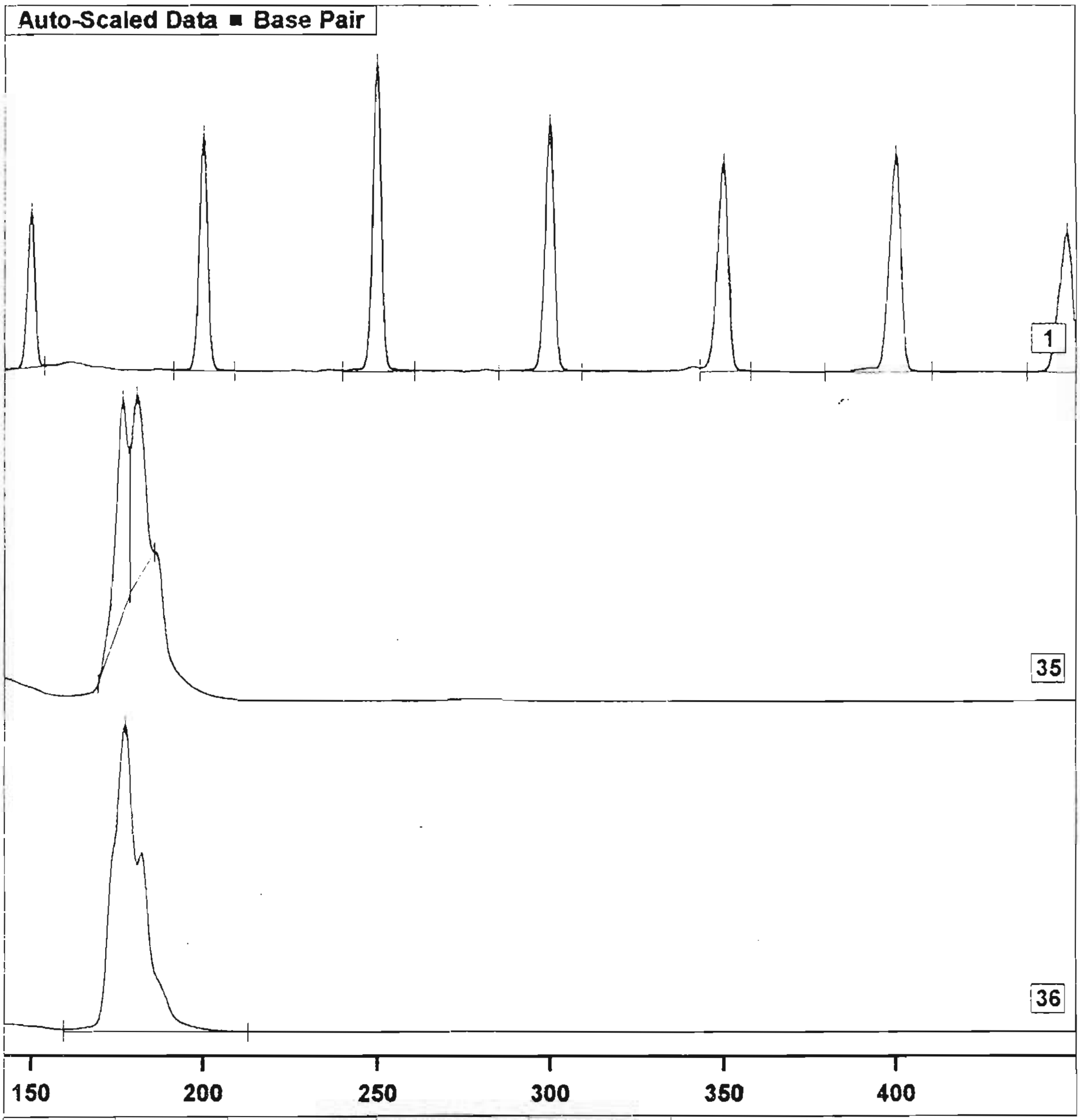
05/Jul/98 10:31:16 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\CRC711.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	32:25	228.27	#50.0	0.52794
	2	36:48	87.529	#100.0	0.20243
	3	43:10	91.455	#150.0	0.21151
	4	52:16	432.39	#200.0	1
	5	78:47	232.7	#300.0	0.53817
2	1	45:12	1990	162.8	1
	2	45:43	1228.5	165.9	0.61731
3	1	45:03	1668.1	161.9	1
	2	45:33	1203.7	164.9	0.72156

Appendix D - OESOPHAGEAL CANCER: NAI - DCC

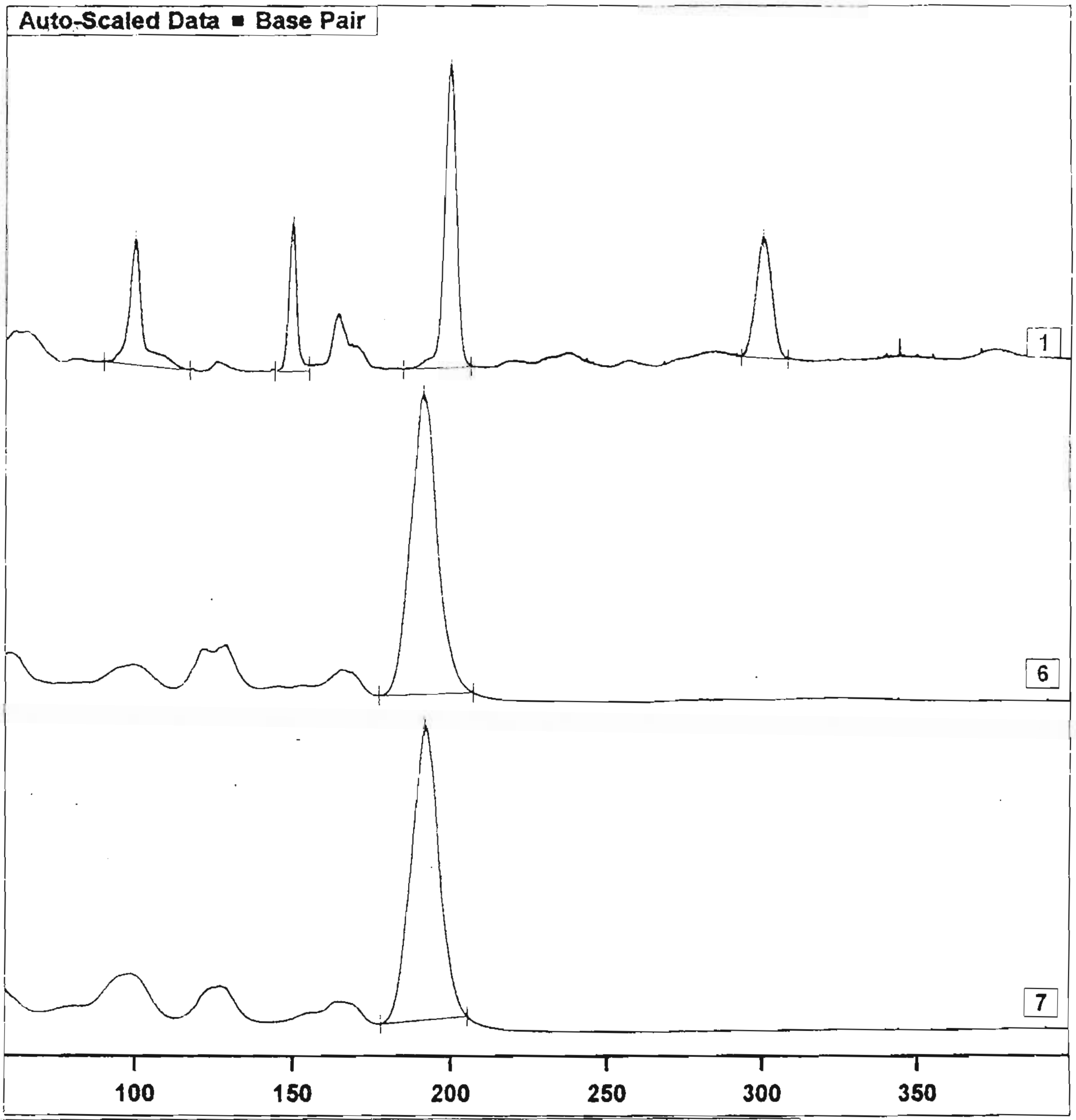
07/Jul/98 11:56:36 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\142MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	15:18	476.1	#50.0	1
	2	17:11	138.35	#100.0	0.29059
	3	20:36	113.4	#150.0	0.23818
	4	24:03	190.05	#200.0	0.39919
	5	27:42	280.19	#250.0	0.58851
	6	31:32	250.46	#300.0	0.52606
	7	35:29	252.27	#350.0	0.52987
	8	39:31	299.06	#400.0	0.62816
	9	43:38	221.01	#450.0	0.46421
	10	47:47	230.04	#500.0	0.48317
35	1	22:27	321.86	176.8	1
	2	22:44	291.54	180.9	0.90579
36	1	22:30	3521.1	177.5	1

Appendix D - OESOPHAGEAL CANCER: AI - DCC

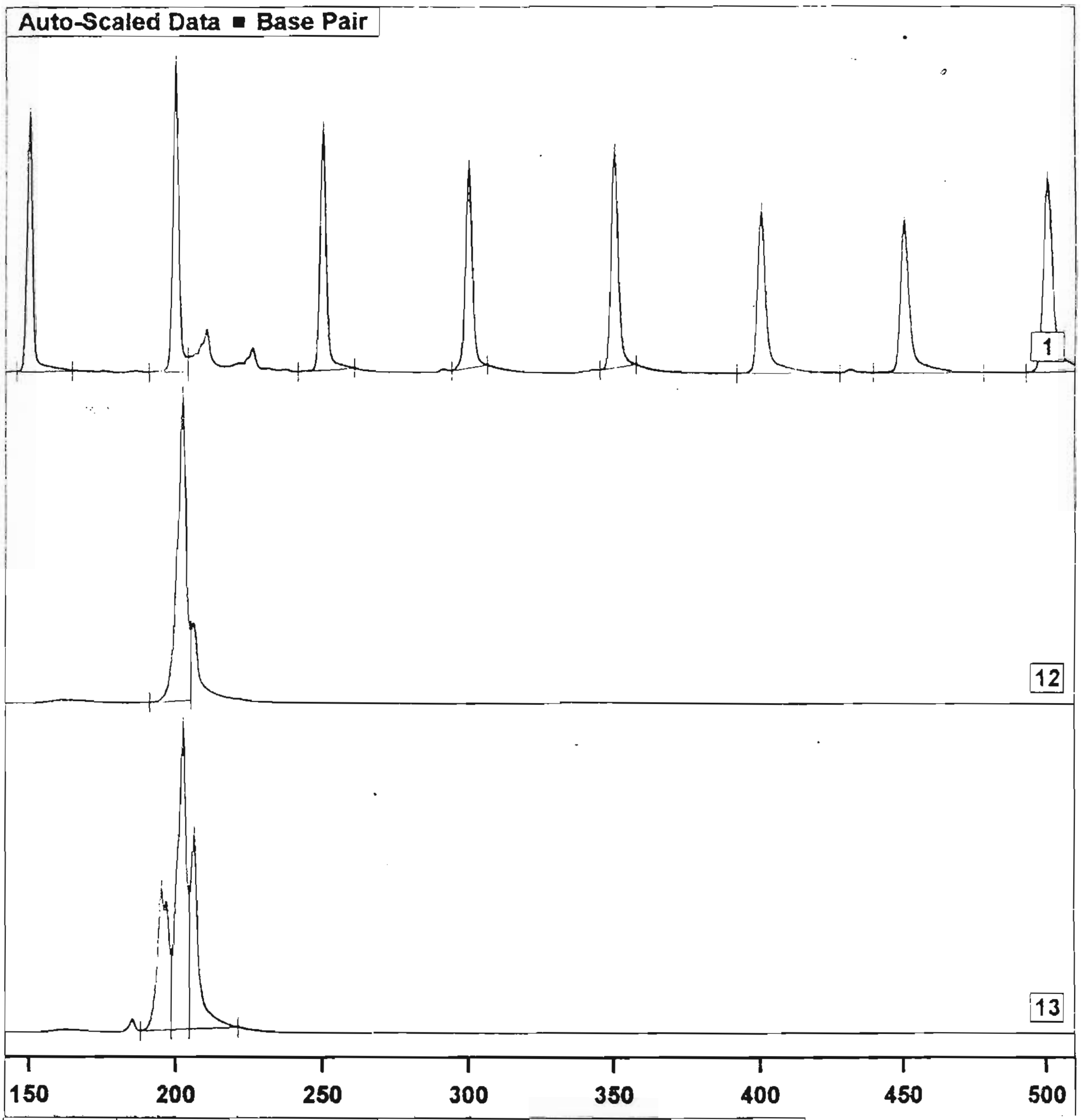
05/Jul/98 10:43:34 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\CRC711.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	32:25	228.27	#50.0	0.52794
	2	36:48	87.529	#100.0	0.20243
	3	43:10	91.455	#150.0	0.21151
	4	52:16	432.39	#200.0	1
	5	78:47	232.7	#300.0	0.53817
6	1	50:26	6262.3	191.3	1
7	1	50:34	3537.9	191.9	1

Appendix D - OESOPHAGEAL CANCER: H- DCC

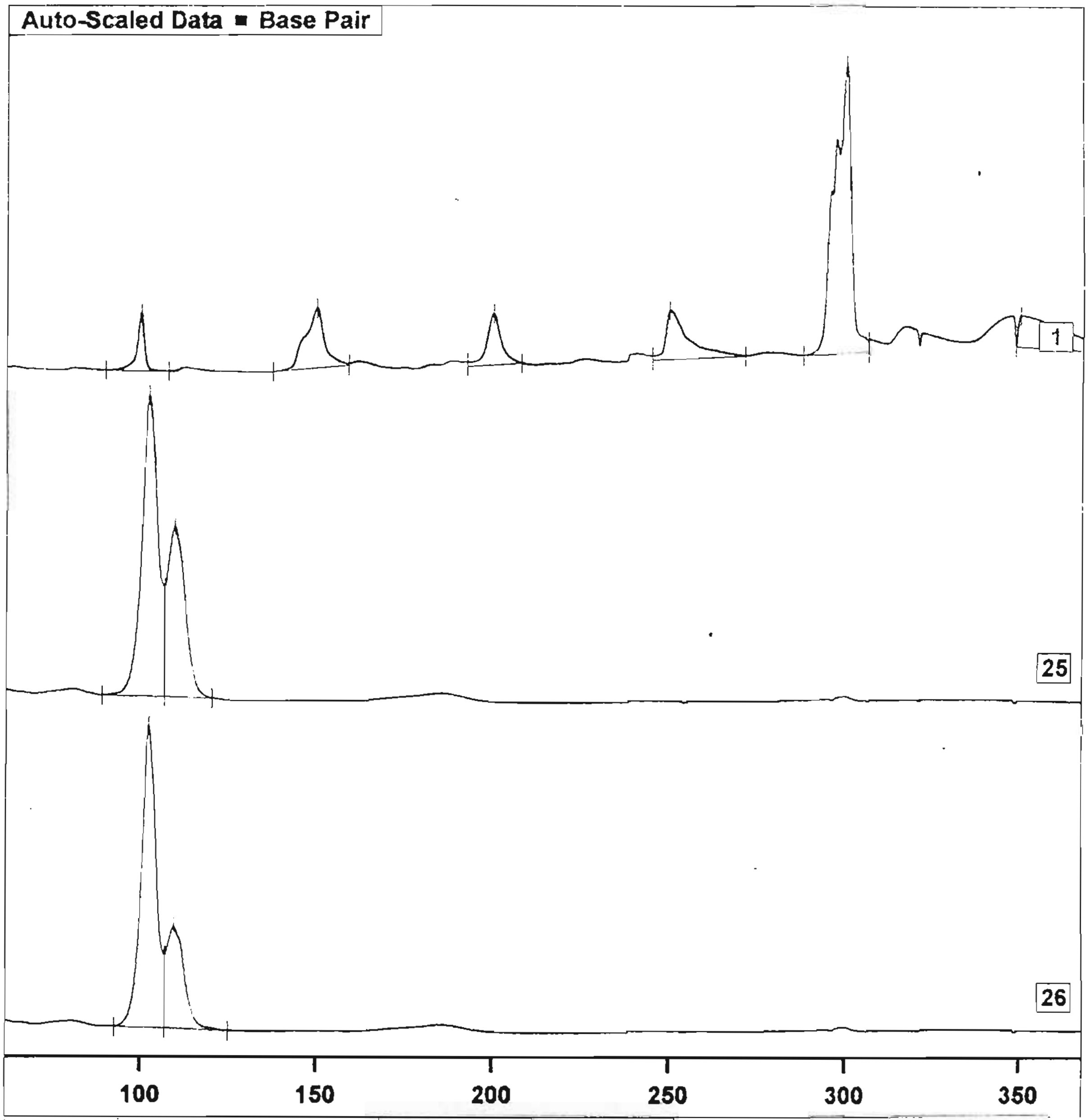
26/Jun/98 14:59:12 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\19MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
12	1	35:11	972.04	202.5	1
13	1	34:21	716.38	195.1	0.56402
	2	35:12	1270.2	202.6	1
	3	35:38	733.93	206.4	0.57783

Appendix D - OESOPHAGEAL CANCER: MSI - DCC

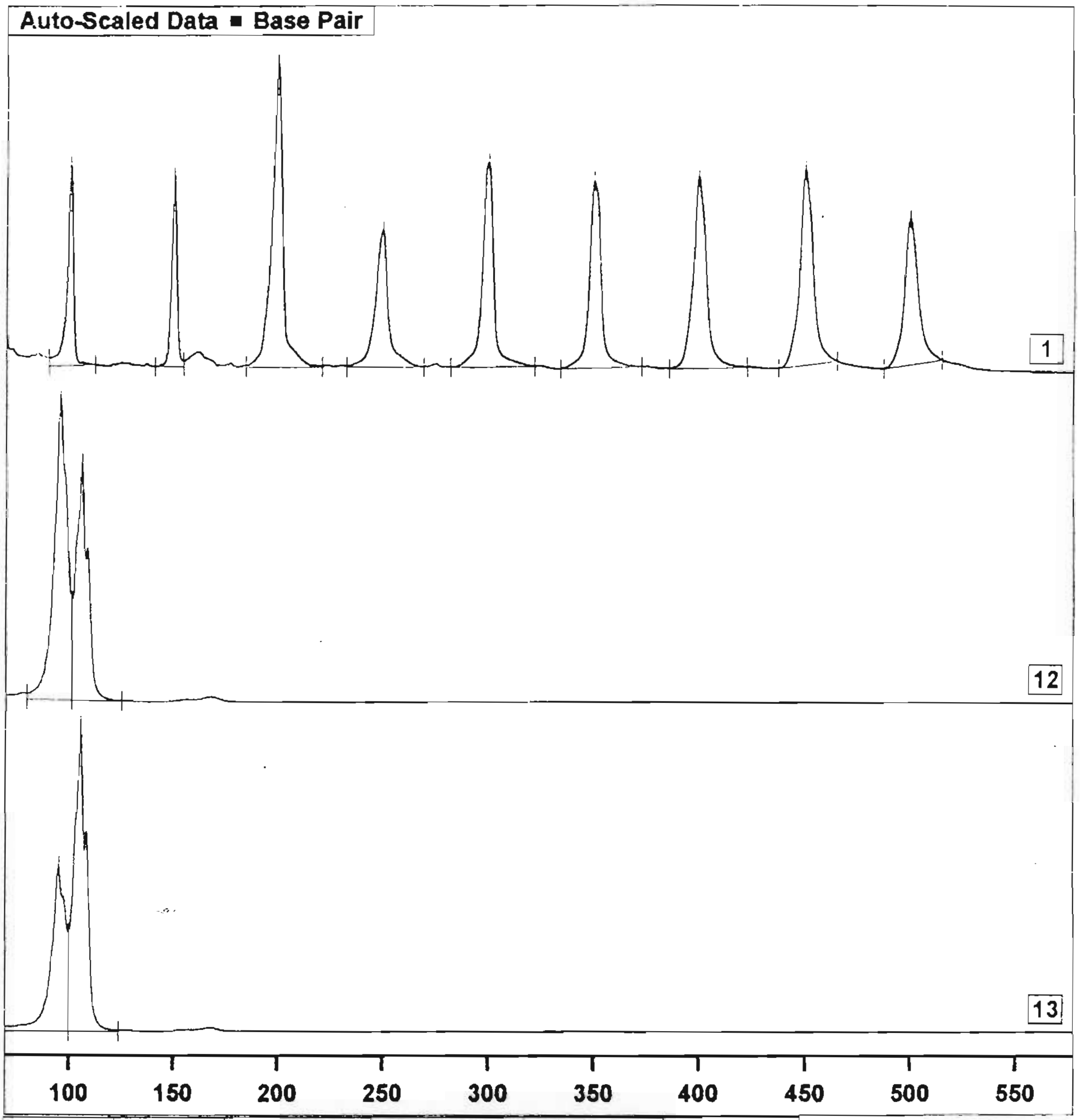
04/Jul/98 09:59:01 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI30.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	42:27	478.23	#50.0	0.37563
	2	47:54	155.54	#100.0	0.12217
	3	55:25	459.54	#150.0	0.36096
	4	63:47	337.67	#200.0	0.26523
	5	73:11	313.35	#250.0	0.24613
	6	77:54	1273.1	#300.0	1
	7	83:27	422.9	#350.0	0.33217
	8	88:04	425.36	#400.0	0.33411
25	1	48:15	931.12	102.3	1
	2	49:21	614.1	109.6	0.65953
26	1	48:15	817.09	102.3	1
	2	49:21	344.21	109.6	0.42126

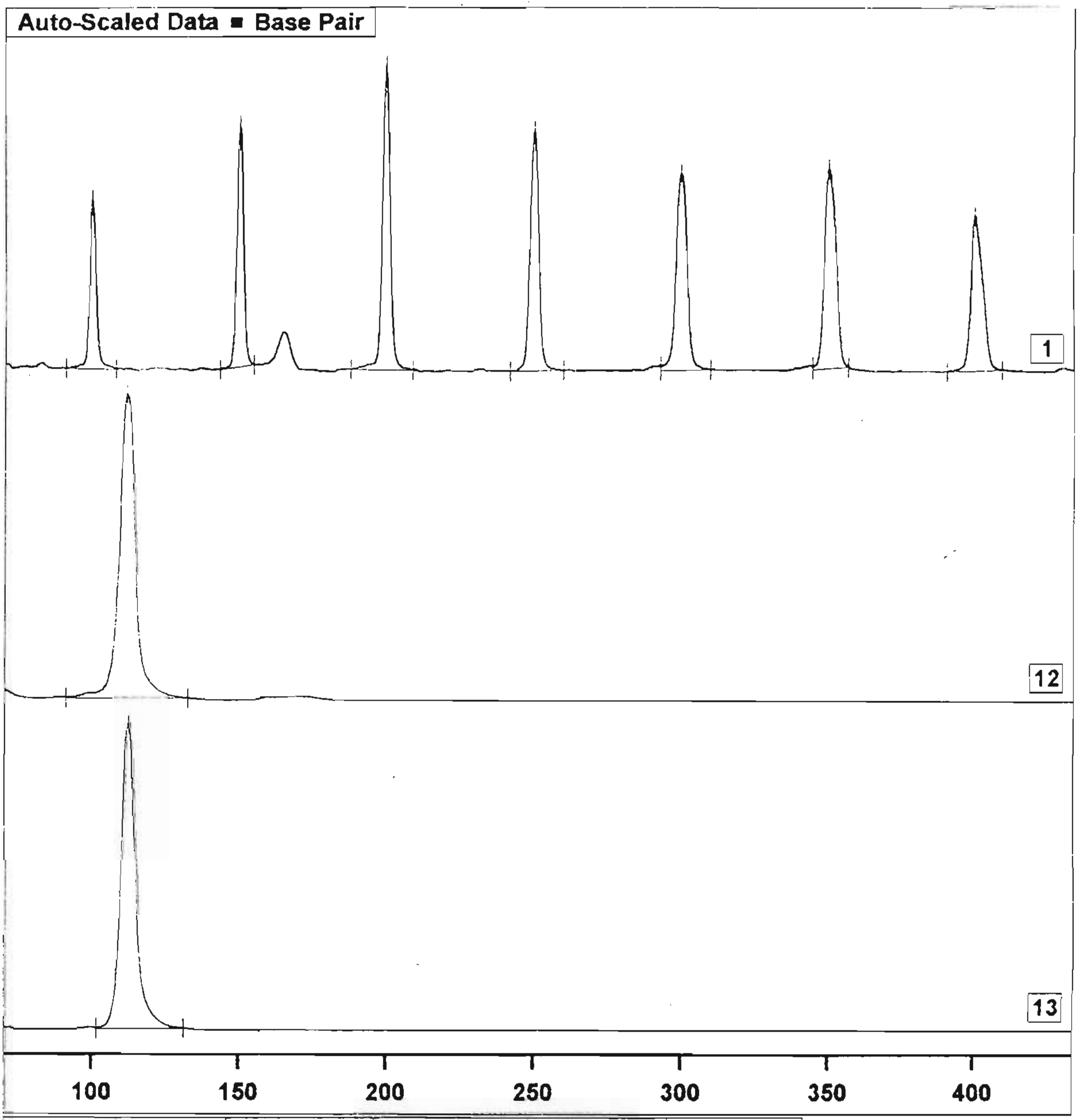
Appendix D - OESOPHAGEAL CANCER: NAI -D18S34

05/Jul/98 17:25:31 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI28.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	21:32	176.11	#50.0	0.30151
	2	24:53	152	#100.0	0.26024
	3	29:17	172.57	#150.0	0.29544
	4	35:03	584.09	#200.0	1
	5	40:39	309.4	#250.0	0.52971
	6	46:12	396.18	#300.0	0.67828
	7	51:57	404.75	#350.0	0.69296
	8	57:55	462.38	#400.0	0.79162
	9	63:55	473.05	#450.0	0.80988
	10	69:51	372.16	#500.0	0.63716
12	1	24:33	2631.5	95.5	1
	2	25:20	1976.1	105.9	0.75096
13	1	24:31	1740.1	95.1	0.59166
	2	25:18	2941	105.4	1

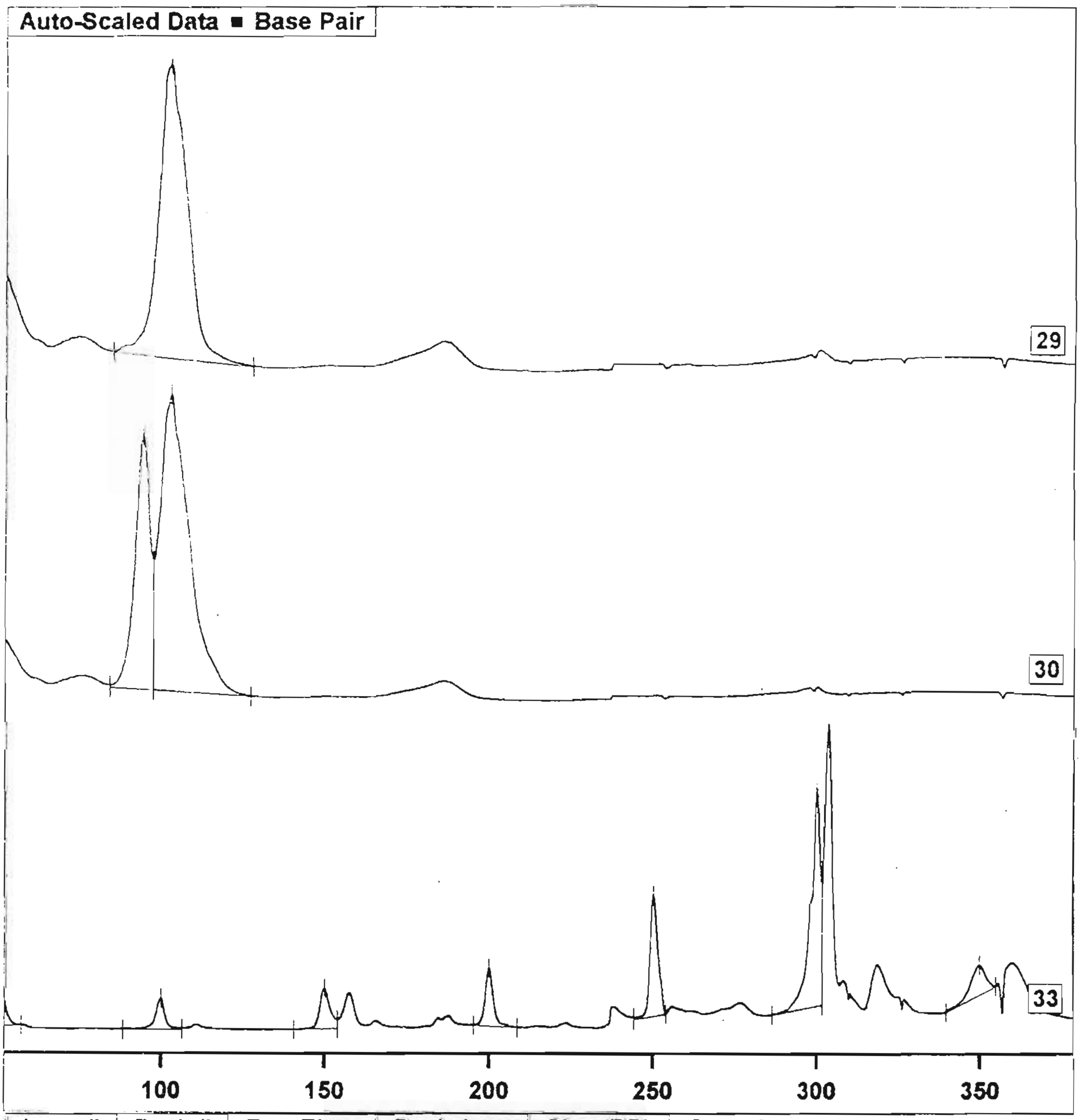
Appendix D - OESOPHAGEAL CANCER: AI - D18S34



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	18:47	11.709	#50.0	0.21443
	2	23:26	19.237	#100.0	0.35229
	3	28:20	29.938	#150.0	0.54826
	4	33:37	47.619	#200.0	0.87204
	5	39:14	44.636	#250.0	0.81743
	6	45:02	45.948	#300.0	0.84144
	7	50:56	49.345	#350.0	0.90366
	8	56:57	41.327	#400.0	0.75682
	9	63:01	41.675	#450.0	0.7632
	10	69:01	54.606	#500.0	1
12	1	24:33	781.13	111.7	1
13	1	24:37	3102.5	112.4	1

Appendix D - OESOPHAGEAL CANCER: H - D18S34

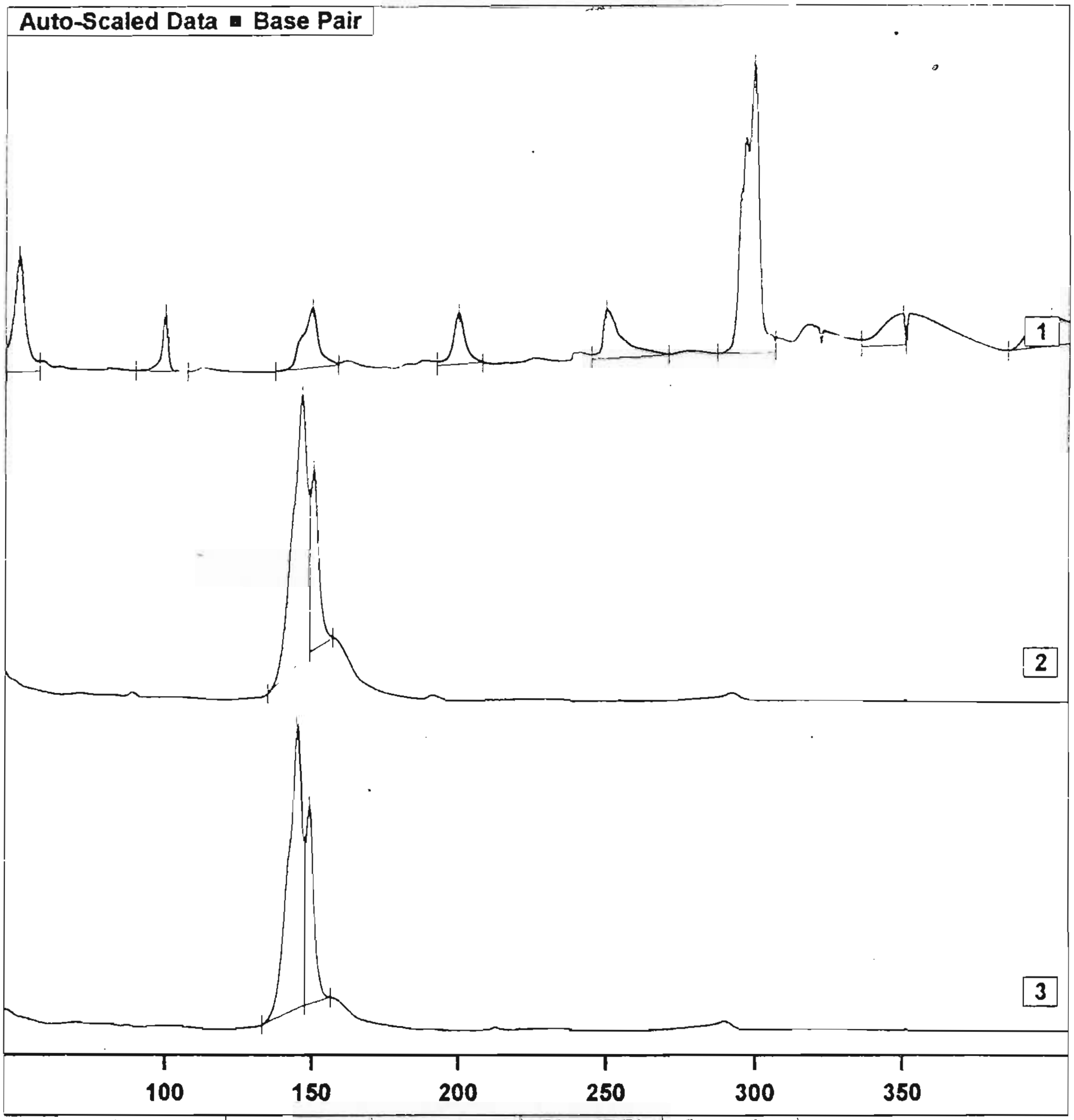
04/Jul/98 09:35:23 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI30.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
29	1	48:47	459.18	102.7	1
30	1	47:48	237.94	94.2	0.4282
	2	48:49	555.68	102.9	1
33	1	42:42	159.93	#50.0	0.37654
	2	48:28	67.962	#100.0	0.16001
	3	54:59	121.67	#150.0	0.28647
	4	64:26	186.22	#200.0	0.43843
	5	73:15	221.55	#250.0	0.52162
	6	77:59	421.34	#300.0	0.99202
	7	83:37	183.13	#350.0	0.43117
	8	87:32	424.73	#400.0	1
	9	92:33	299.28	#450.0	0.70464
	10	98:04	103.18	#500.0	0.24294

Appendix D - OESOPHAGEAL CANCER: MSI -D18S34

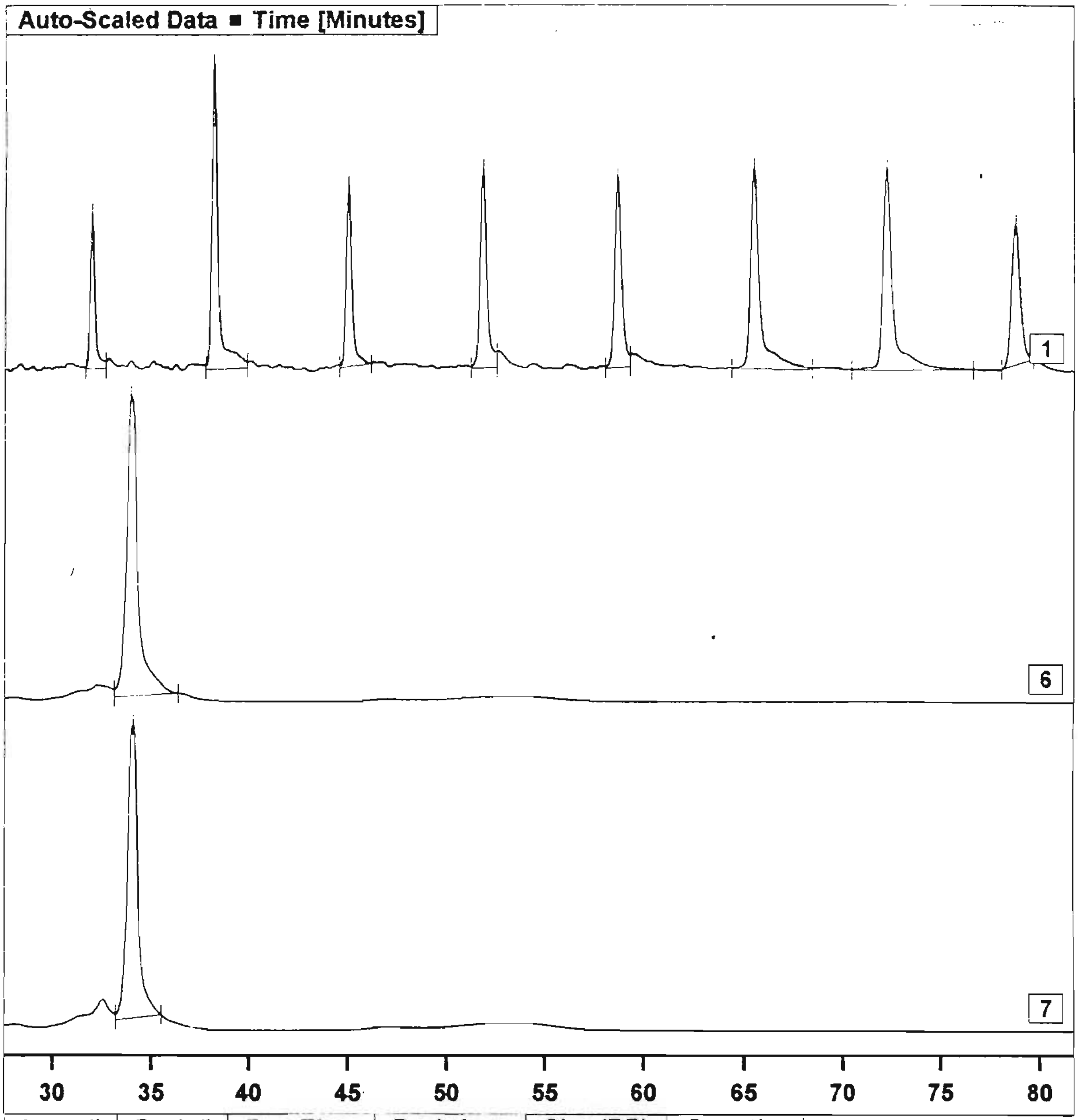
04/Jul/98 09:09:33 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\OMSI30.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	42:27	478.23	#50.0	0.37563
	2	47:54	155.54	#100.0	0.12217
	3	55:25	459.54	#150.0	0.36096
	4	63:47	337.67	#200.0	0.26523
	5	73:11	313.35	#250.0	0.24613
	6	77:54	1273.1	#300.0	1
	7	83:27	422.9	#350.0	0.33217
	8	88:04	425.36	#400.0	0.33411
2	1	54:54	2213.1	146.6	1
	2	55:31	903.48	150.6	0.40825
3	1	54:42	1607.9	145.2	1
	2	55:18	709.05	149.2	0.44097

Appendix D - OESOPHAGEAL CANCER: NAI - D18S58

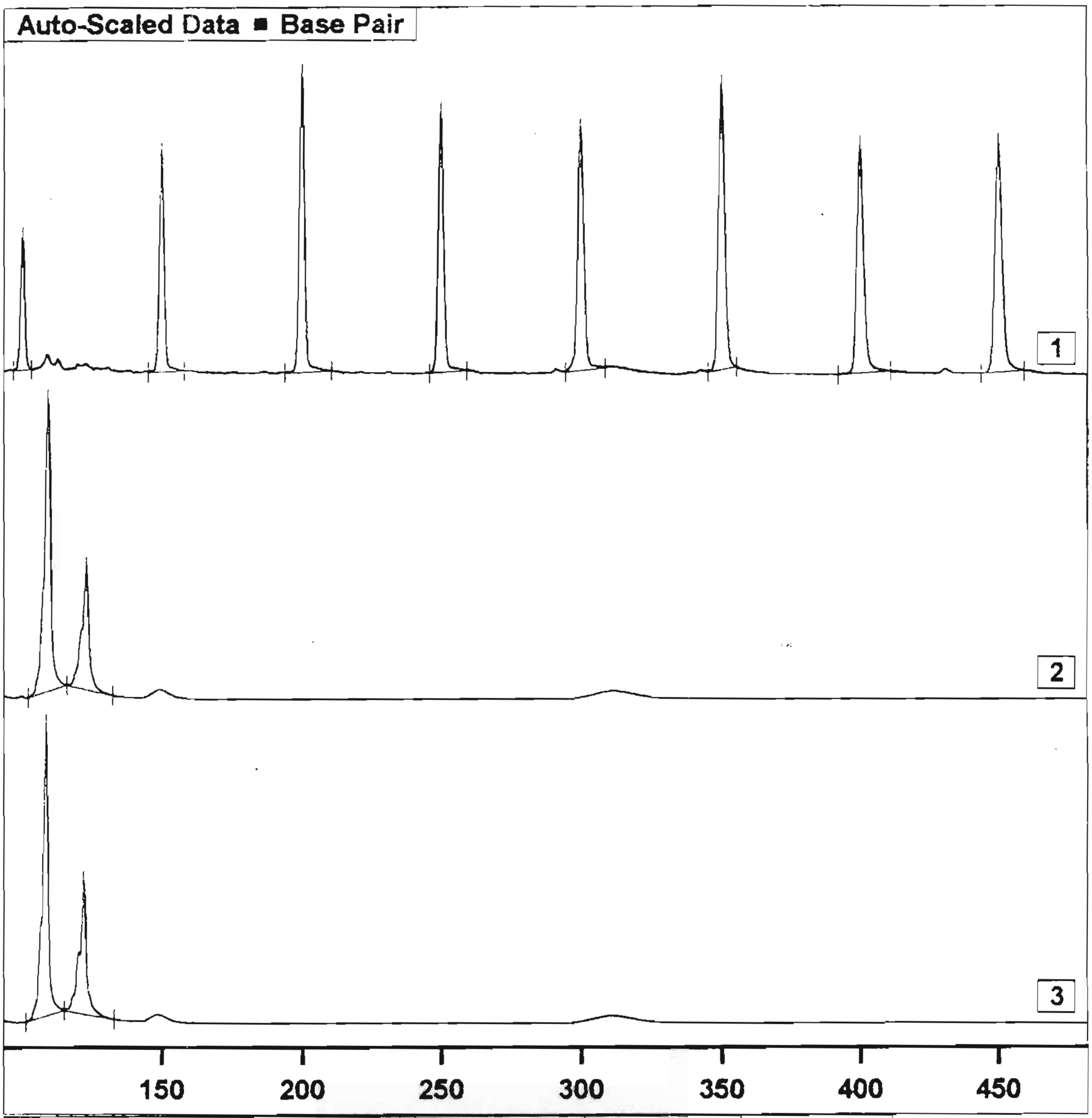
04/Jul/98 19:58:59 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\12MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	20:28	312.39	#50.0	0.52801
	2	25:59	102.99	#100.0	0.17407
	3	31:56	209.92	#150.0	0.35481
	4	38:11	574.34	#200.0	0.97077
	5	45:00	301.12	#250.0	0.50896
	6	51:50	384.75	#300.0	0.65032
	7	58:41	385.25	#350.0	0.65116
	8	65:29	549.48	#400.0	0.92875
	9	72:11	591.64	#450.0	1
	10	78:43	343.66	#500.0	0.58086
6	1	33:56	1268.4	166.4	1
7	1	34:03	541.43	167.3	1

Appendix D - OESOPHAGEAL CANCER: H - D18S58

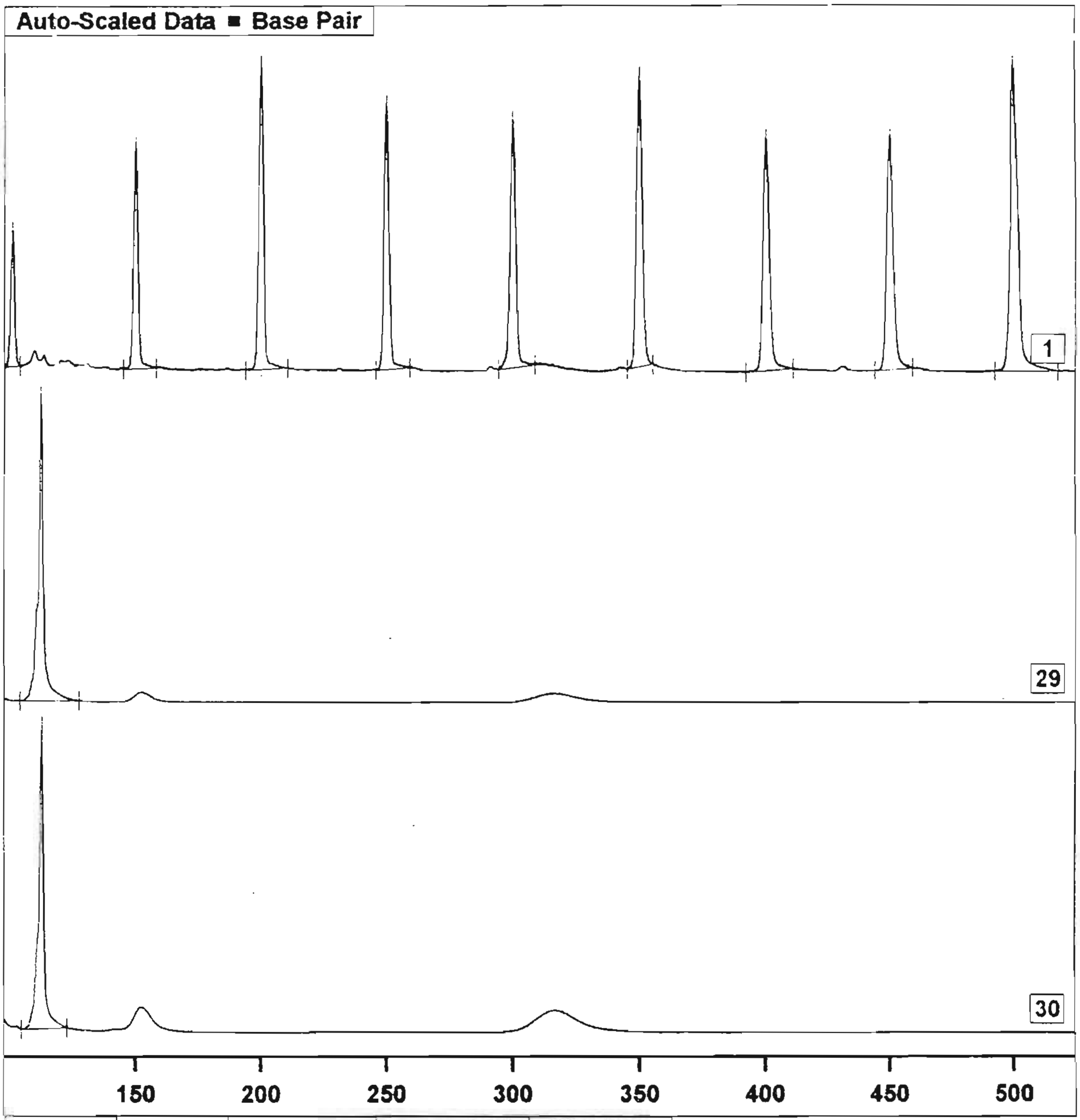
22/Apr/98 10:56:00 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\87MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	17:07	6.743	#50.0	0.086525
	2	21:46	16.338	#100.0	0.20964
	3	26:45	32.788	#150.0	0.42073
	4	32:00	50.289	#200.0	0.6453
	5	37:30	47.242	#250.0	0.6062
	6	43:04	49.966	#300.0	0.64116
	7	48:39	58.086	#350.0	0.74535
	8	54:10	51.119	#400.0	0.65596
	9	59:35	53.631	#450.0	0.68819
	10	64:51	77.931	#500.0	1
2	1	22:40	1533.7	109.3	1
	2	24:01	741.75	122.9	0.48364
3	1	22:36	1386.5	108.6	1
	2	23:55	771.62	121.9	0.55652

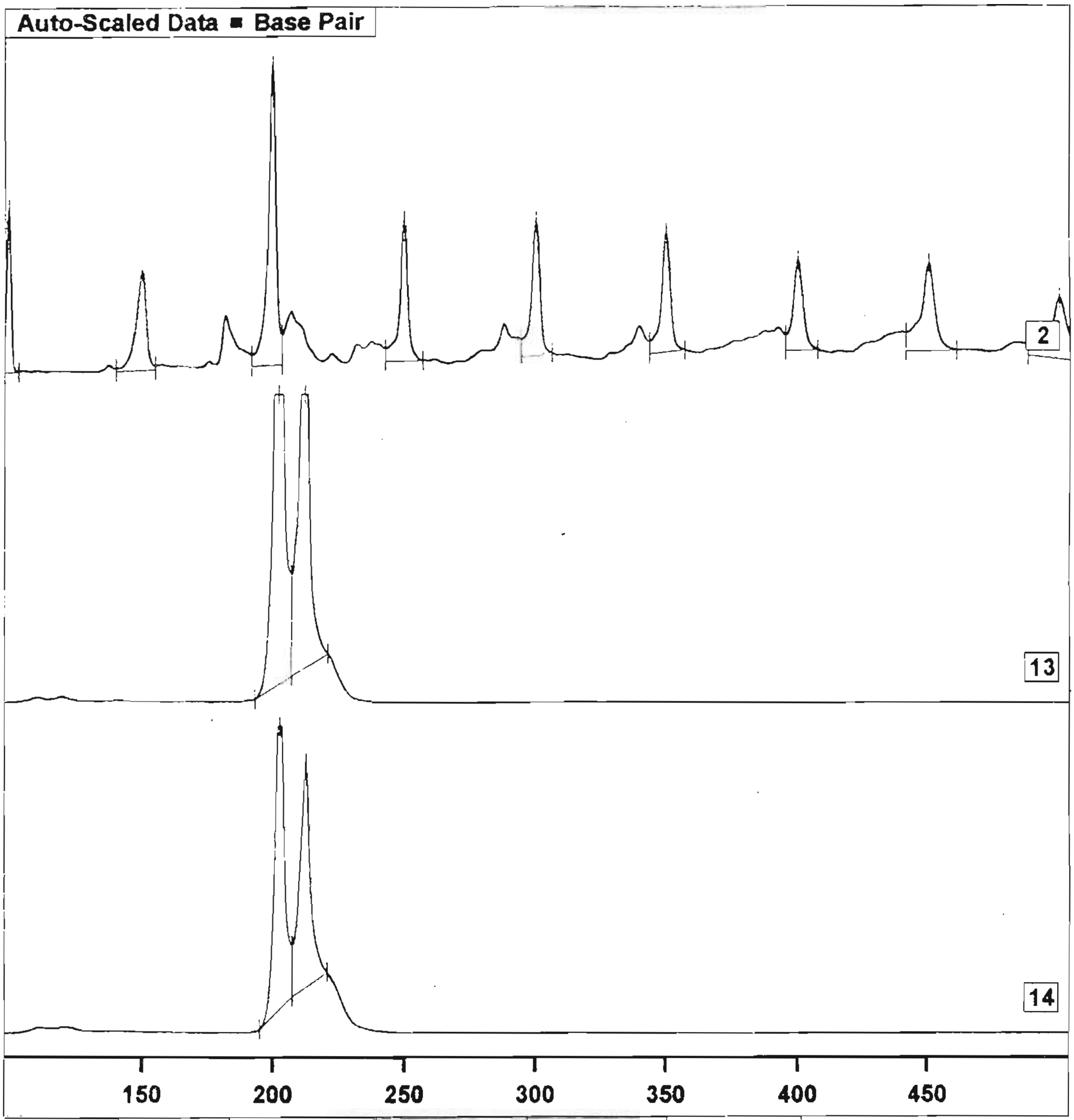
Appendix D - OESOPHAGEAL CANCER: NAI - D3S659

24/Jun/98 15:52:21 Pharmacia DNA Fragment Manager V1.2 Page 1
F:\USR\STAFF\PHYSIOLO\NAIDOOR\D3S1255\87MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	17:07	6.743	#50.0	0.086525
	2	21:46	16.338	#100.0	0.20964
	3	26:45	32.788	#150.0	0.42073
	4	32:00	50.289	#200.0	0.6453
	5	37:30	47.242	#250.0	0.6062
	6	43:04	49.966	#300.0	0.64116
	7	48:39	58.086	#350.0	0.74535
	8	54:10	51.119	#400.0	0.65596
	9	59:35	53.631	#450.0	0.68819
	10	64:51	77.931	#500.0	1
29	1	22:55	842.09	111.8	1
30	1	22:57	223.21	112.2	1

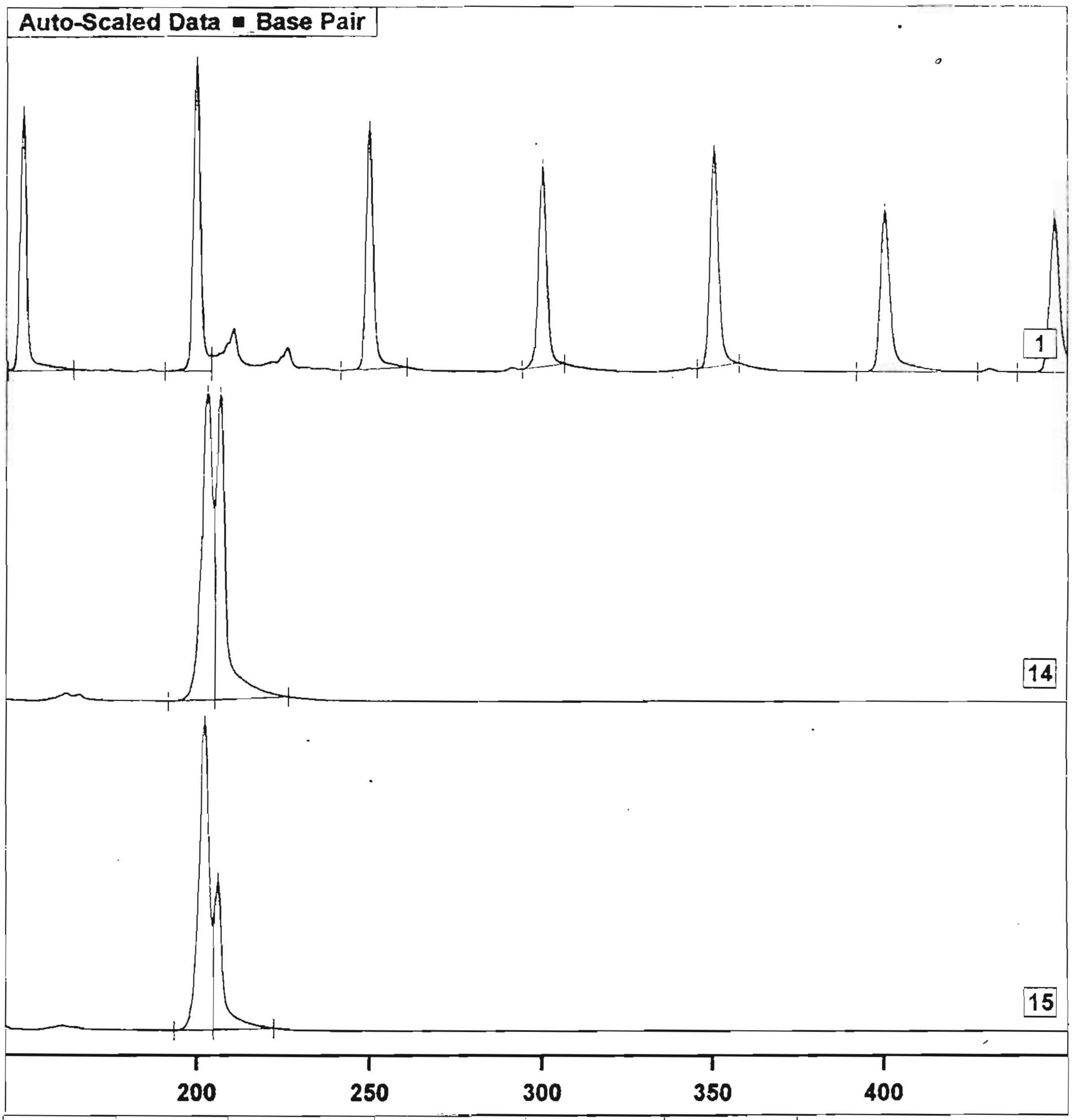
Appendix D - OESOPHAGEAL CANCER: H - D3S659



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:52	178.23	#50.0	0.38253
	2	36:08	162.79	#100.0	0.34939
	3	43:58	199.18	#150.0	0.42749
	4	51:12	465.94	#200.0	1
	5	59:26	249.64	#250.0	0.53579
	6	68:04	251.3	#300.0	0.53934
	7	76:56	251.12	#350.0	0.53897
	8	85:57	208.39	#400.0	0.44726
	9	95:04	292.77	#450.0	0.62835
	10	104:05	315.51	#500.0	0.67715
13	1	51:36	5887.7	202.4	1
	2	53:13	5203.8	212.2	0.88385
14	1	51:41	4195.4	202.9	1
	2	53:18	3492.7	212.8	0.83251

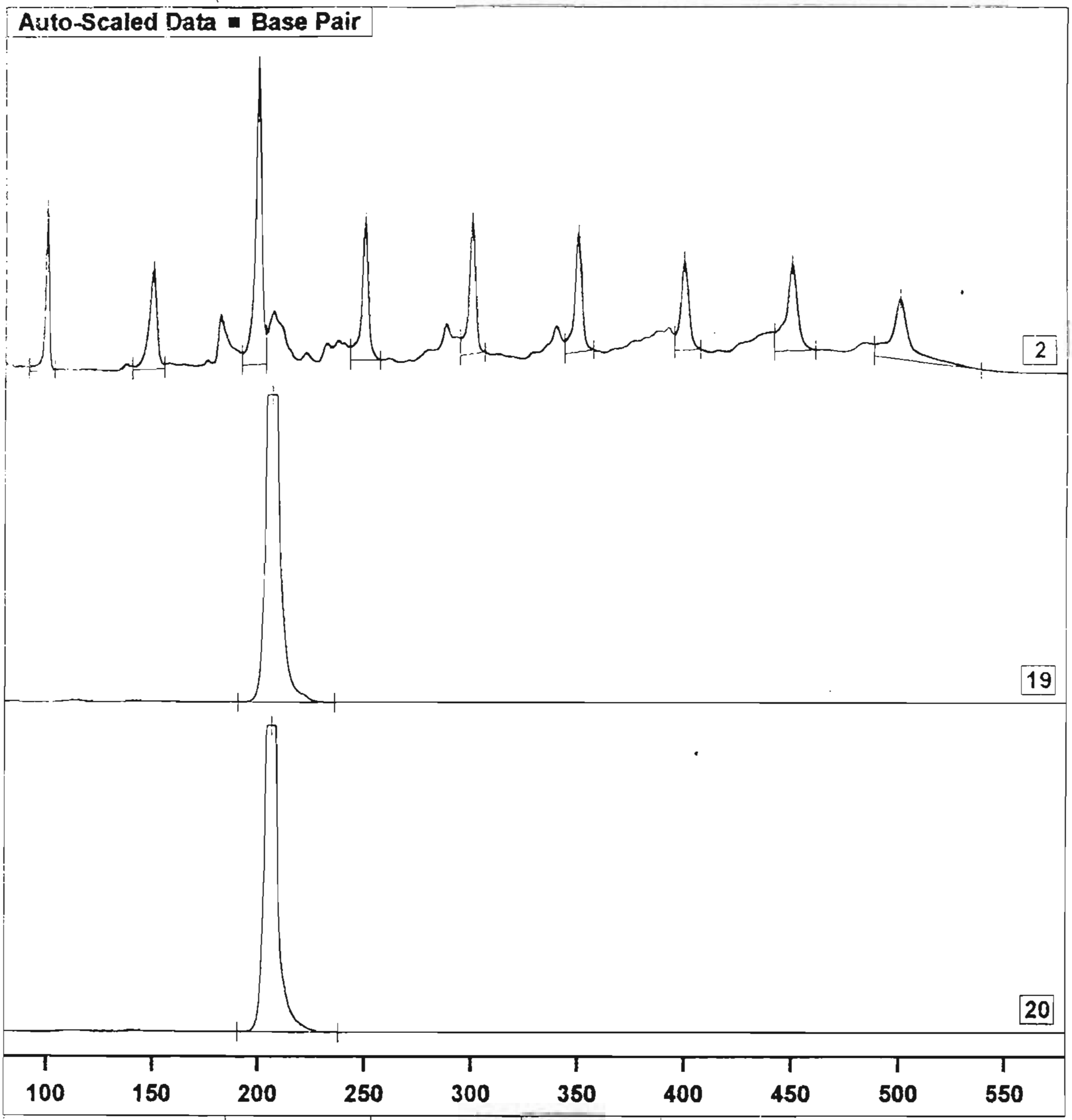
Appendix D - OESOPHAGEAL CANCER: NAI - D2S123

26/Jun/98 14:55:30 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\19MSI98.ALF



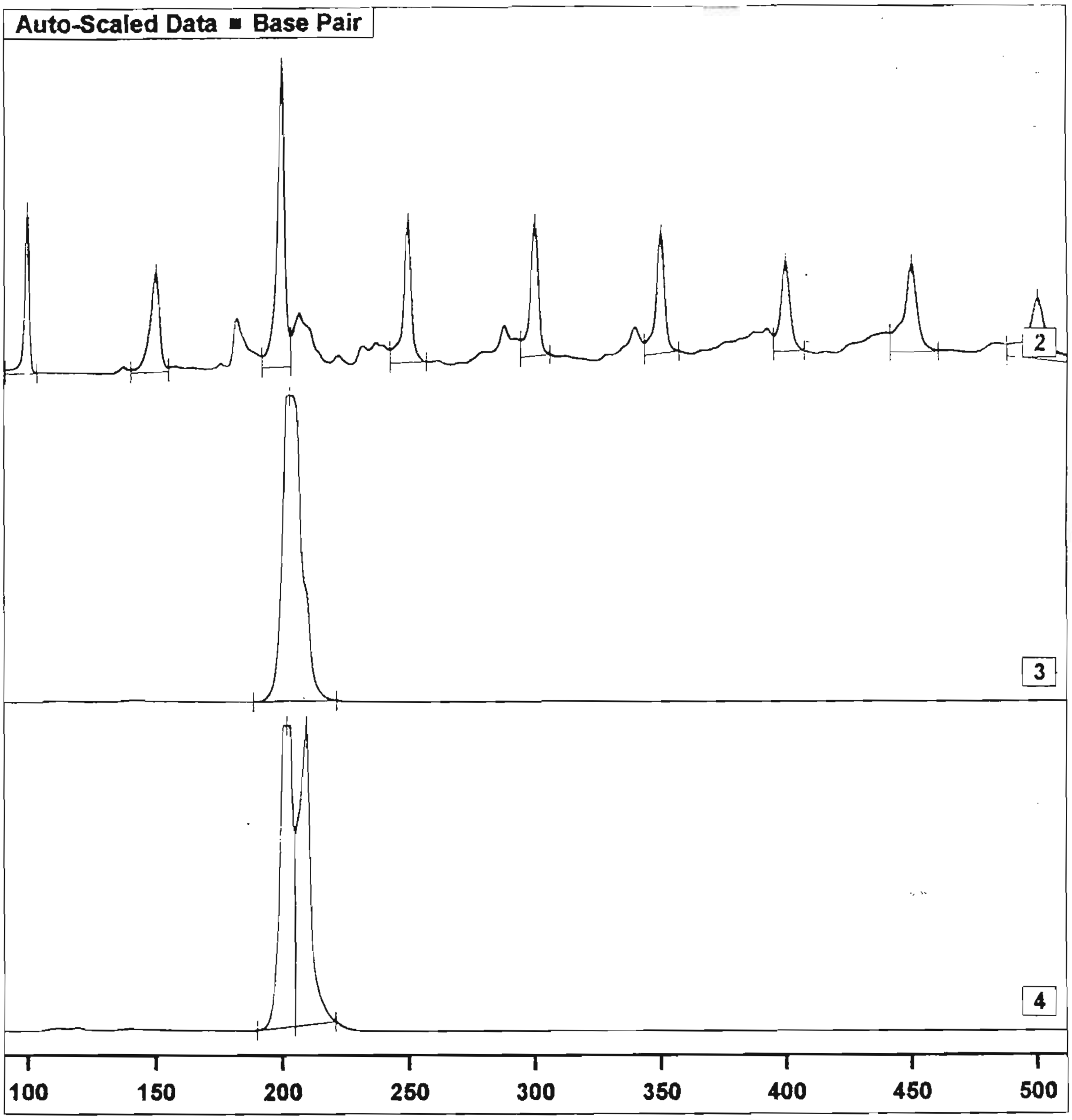
Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	19:42	268.54	#50.0	0.27374
	2	24:19	398.96	#100.0	0.40669
	3	29:24	681.27	#150.0	0.69446
	4	34:54	890.36	#200.0	0.9076
	5	40:42	768.13	#250.0	0.78301
	6	46:39	708.73	#300.0	0.72246
	7	52:41	807.31	#350.0	0.82294
	8	58:44	760.84	#400.0	0.77558
	9	64:42	757.35	#450.0	0.77202
	10	70:32	981	#500.0	1
14	1	35:16	2486	203.2	1
	2	35:41	2305.2	206.9	0.92727
15	1	35:10	2331	202.3	1
	2	35:36	1023.5	206.1	0.43908

Appendix D - OESOPHAGEAL CANCER: AI - D2S123



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:52	178.23	#50.0	0.38253
	2	36:08	162.79	#100.0	0.34939
	3	43:58	199.18	#150.0	0.42749
	4	51:12	465.94	#200.0	1
	5	59:26	249.64	#250.0	0.53579
	6	68:04	251.3	#300.0	0.53934
	7	76:56	251.12	#350.0	0.53897
	8	85:57	208.39	#400.0	0.44726
	9	95:04	292.77	#450.0	0.62835
	10	104:05	315.51	#500.0	0.67715
19	1	52:17	8555.8	206.6	1
20	1	52:15	8014.8	206.4	1

Appendix D - OESOPHAGEAL CANCER: H - D2S123

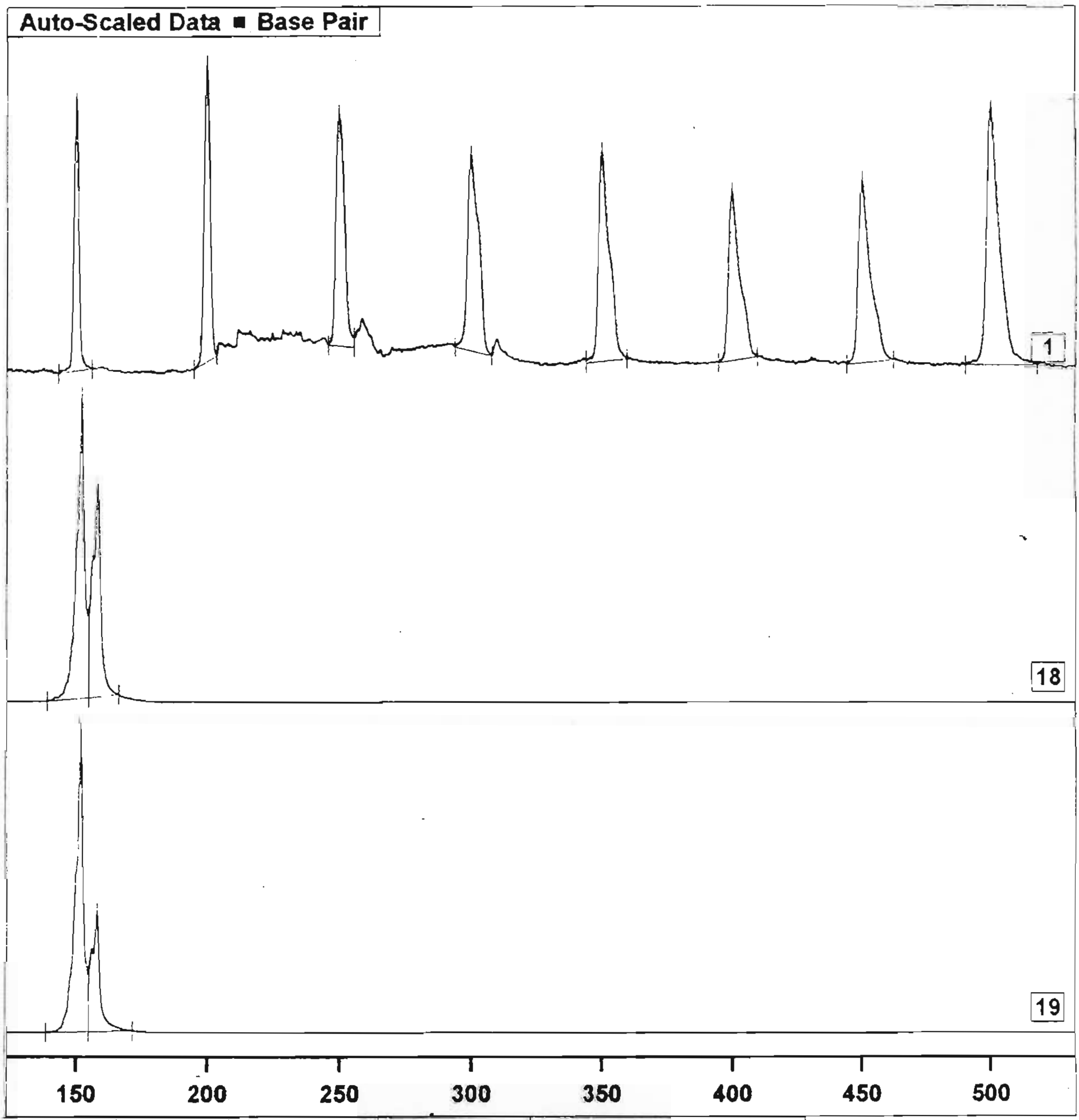


Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
2	1	28:52	178.23	#50.0	0.38253
	2	36:08	162.79	#100.0	0.34939
	3	43:58	199.18	#150.0	0.42749
	4	51:12	465.94	#200.0	1
	5	59:26	249.64	#250.0	0.53579
	6	68:04	251.3	#300.0	0.53934
	7	76:56	251.12	#350.0	0.53897
	8	85:57	208.39	#400.0	0.44726
	9	95:04	292.77	#450.0	0.62835
	10	104:05	315.51	#500.0	0.67715
3	1	51:43	8850.1	203.1	1
4	1	51:30	5712.4	201.8	1
	2	52:46	5529.7	209.5	0.96803

Appendix D - OESOPHAGEAL CANCER: MSI - D2S123

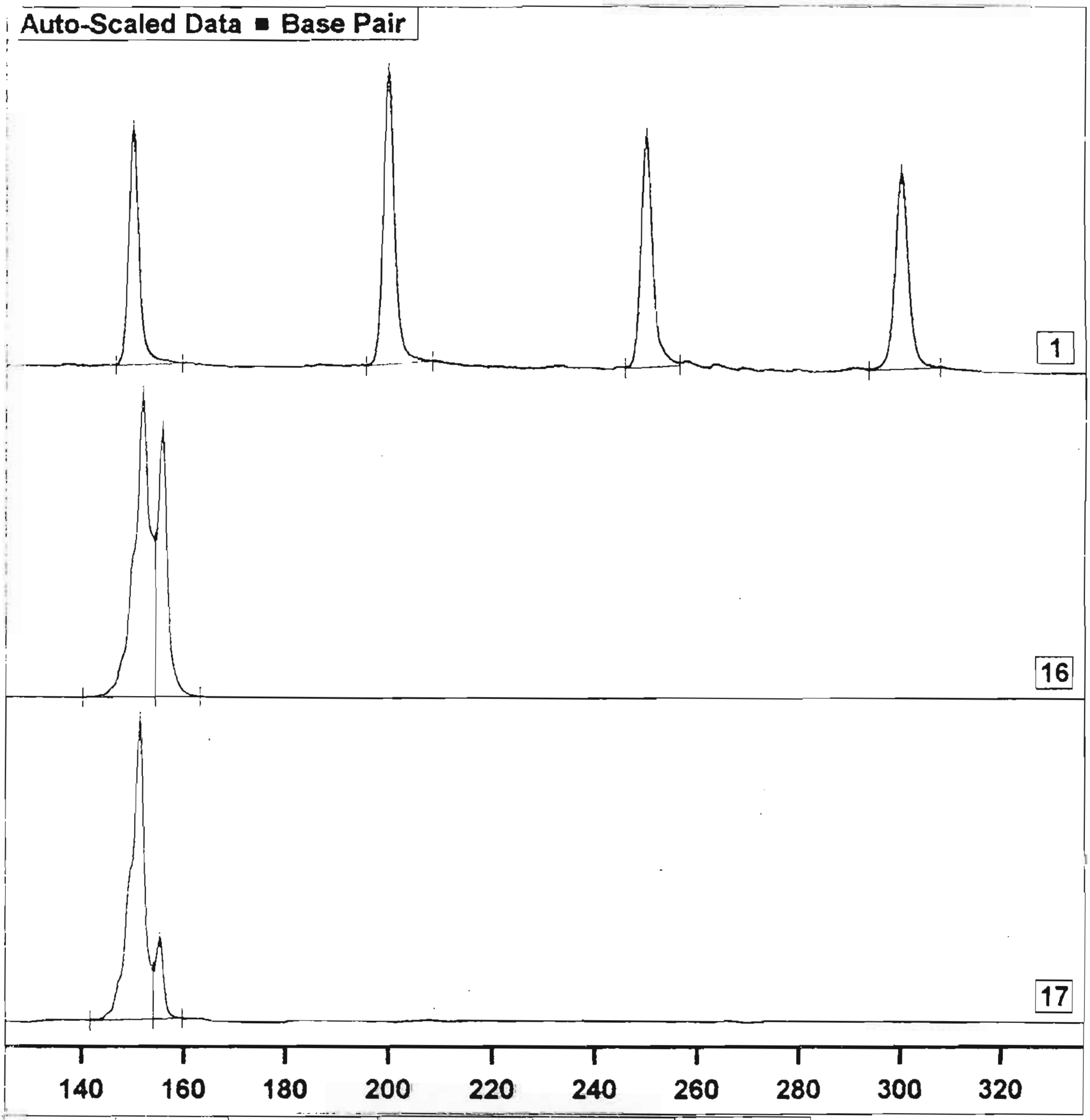
RATIO = 0,6

04/Jul/98 12:48:13　　Pharmacia DNA Fragment Manager V1.2　　Page 1
A:\85MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	22:21	7.1361	#100.0	0.17803
	2	26:54	14.191	#150.0	0.35403
	3	32:09	20.957	#200.0	0.52282
	4	37:47	24.545	#250.0	0.61234
	5	43:34	26.396	#300.0	0.65851
	6	49:20	27.944	#350.0	0.69715
	7	55:00	23.2	#400.0	0.57878
	8	60:29	26.302	#450.0	0.65617
	9	65:55	40.084	#500.0	1
18	1	27:07	331.21	152.2	1
	2	27:44	238.13	158.4	0.71895
19	1	27:05	573.96	151.9	1
	2	27:43	251.23	158.2	0.43771

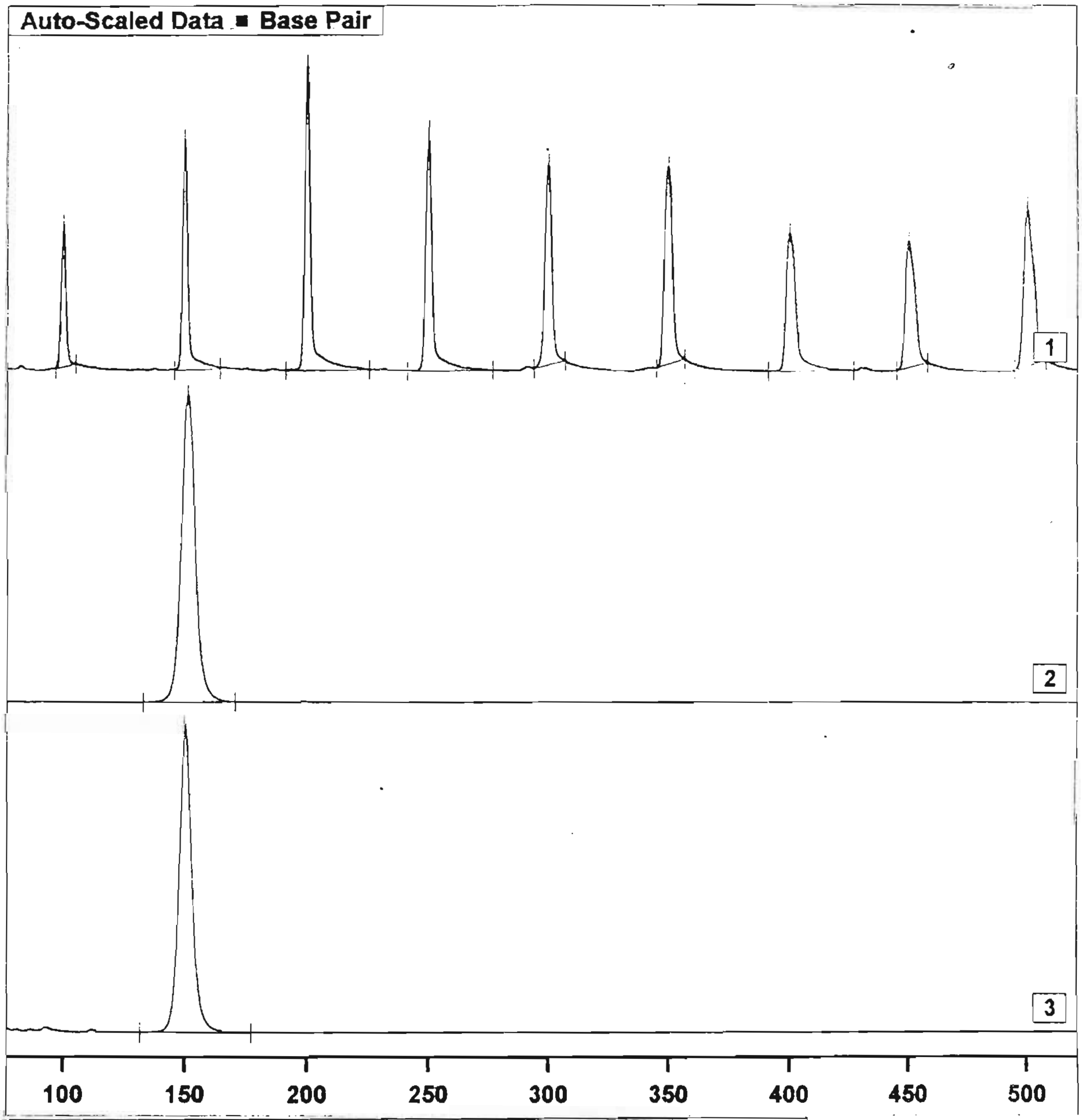
Appendix D - OESOPHAGEAL CANCER: NAI - D3S1255



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	30:13	24.04	#50.0	0.24973
	2	37:19	33.308	#100.0	0.34602
	3	45:14	50.451	#150.0	0.5241
	4	53:52	72.38	#200.0	0.7519
	5	63:16	65.717	#250.0	0.68269
	6	73:11	64.907	#300.0	0.67428
	7	83:31	78.519	#350.0	0.81568
	8	94:10	64.089	#400.0	0.66578
	9	105:01	68.066	#450.0	0.70709
	10	115:54	96.262	#500.0	1
16	1	45:33	1015.7	151.9	1
	2	46:12	580.01	155.8	0.57106
17	1	45:28	1443.8	151.4	1
	2	46:07	242.77	155.3	0.16815

Appendix D - OESOPHAGEAL CANCER: AI - D3S1255

04/Jul/98 19:14:03 Pharmacia DNA Fragment Manager V1.2 Page 1
A:\48MSI98.ALF



Lane #	Peak #	Run Time	Peak Area	Size (BP)	Quantity
1	1	24:52	192.43	#50.0	0.23095
	2	30:52	251.34	#100.0	0.30165
	3	37:09	523.77	#150.0	0.62861
	4	43:54	833.23	#200.0	1
	5	51:05	756.16	#250.0	0.9075
	6	58:33	670.28	#300.0	0.80444
	7	66:12	750.91	#350.0	0.9012
	8	73:56	708.02	#400.0	0.84972
	9	81:47	605.59	#450.0	0.7268
	10	89:35	791.05	#500.0	0.94938
2	1	37:20	2749.6	151.4	1
3	1	37:12	1107.6	150.4	1

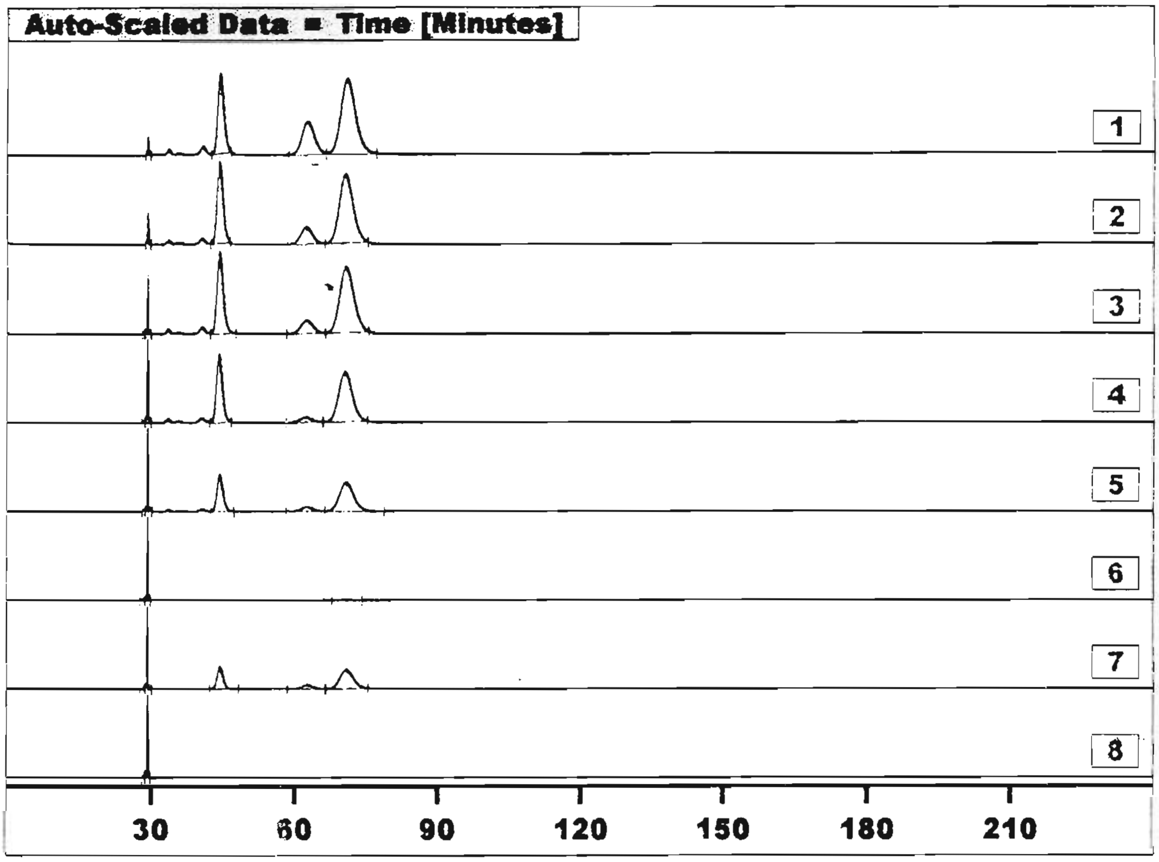
Appendix D - OESOPHAGEAL CANCER: H - D3S1255

APPENDIX E

PCR optimisation Reaction - D2S123

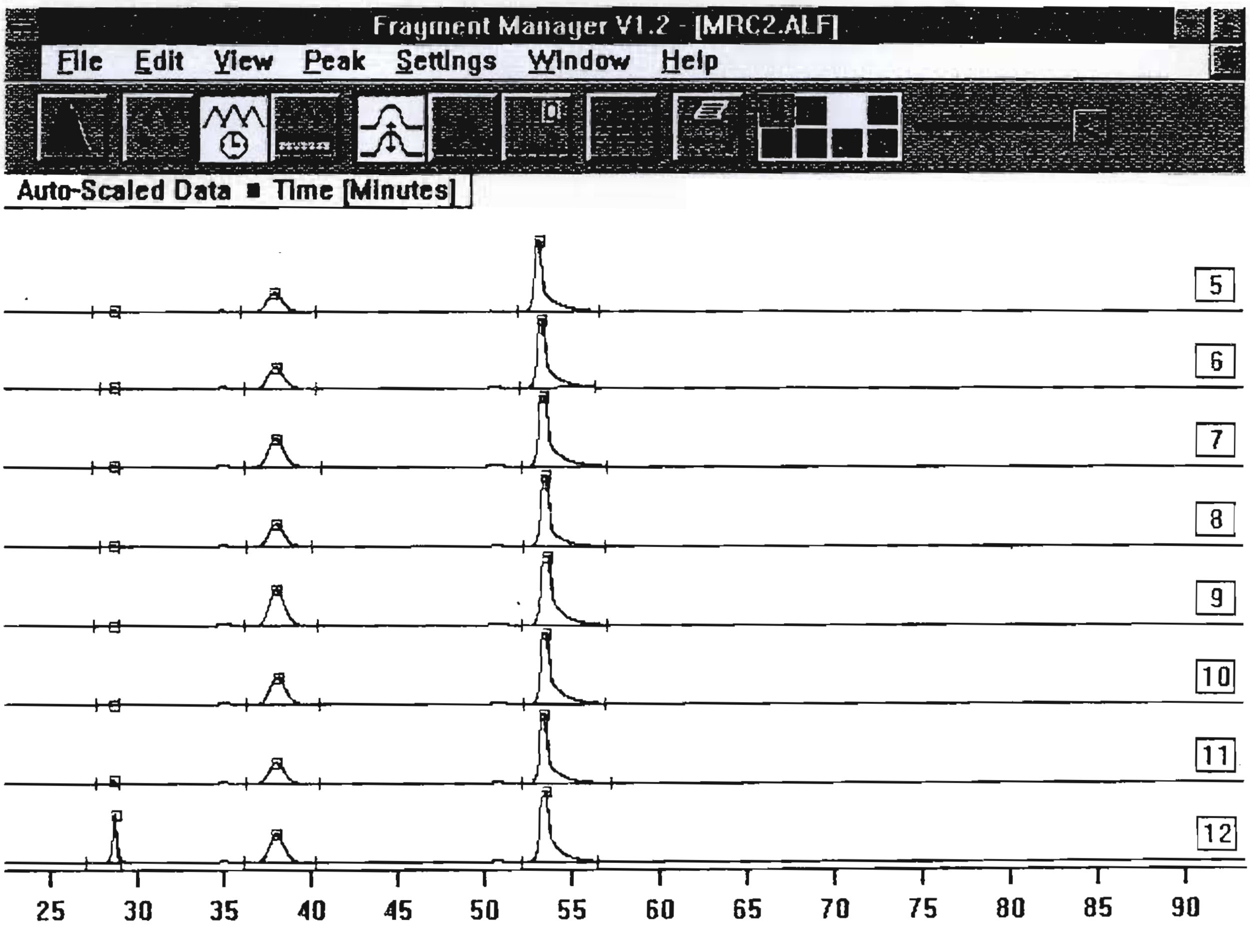
Tube #	Primer 1 labelled	Primer 2 unlabelled	10x buffer with 1.5mM $MgCl_2$	dNTPs	water	DNA	Taq
1	0.5 µl	0.5 µl	1 µl	1 µl	5.9 µl	1 µl	0.15 µl
2	1.0	1.,0	1	1	4.9	1	0.15
3	1.5	1.5	1	1	3.5	1	0.15
4	2.0	2.0	1	1	3.0	1	0.15
5	2.5	2.5	1	1	2.0	1	0.15
6	0	1	1	1	5.9	1	0.15
7	1	0	1	1	5.9	1	0.15
8	0	0	1	1	7.0	1	0.15

24/Nov/98 11:29:45 **Page 1**
C:\AMDATA\MRC1.ALF



PCR optimisation Reaction - D2S123
using different $MgCl_2$ concentrations (Total reaction volume - 25 μl).

Tube #	Primer 1 labelled	Primer 2 unlabelled	10x buffer - $MgCl_2$	$MgCl_2$	dNTPs	water	DNA	Taq
1	0.5 μl	0.5 μl	2.5 μl	1.0	0.5 μl	18.85 μl	1 μl	0.15 μl
2	0.5	0.5	2.5	1.25	0.5	18.6	1	0.15
3	0.5	0.5	2.5	1.5	0.5	18.35	1	0.15
4	0.5	0.5	2.5	1.75	0.5	18.10	1	0.15
5	0.5	0.5	2.5	2	0.5	16.85	1	0.15
6	0.5	0.5	2.5	2.5	0.5	16.35	1	0.15
7	0.5	0.5	2.5	5	0.5	13.85	1	0.15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File Edit View Peak Settings Window Help
Auto-Scaled Data ■ Time [Minutes]
5
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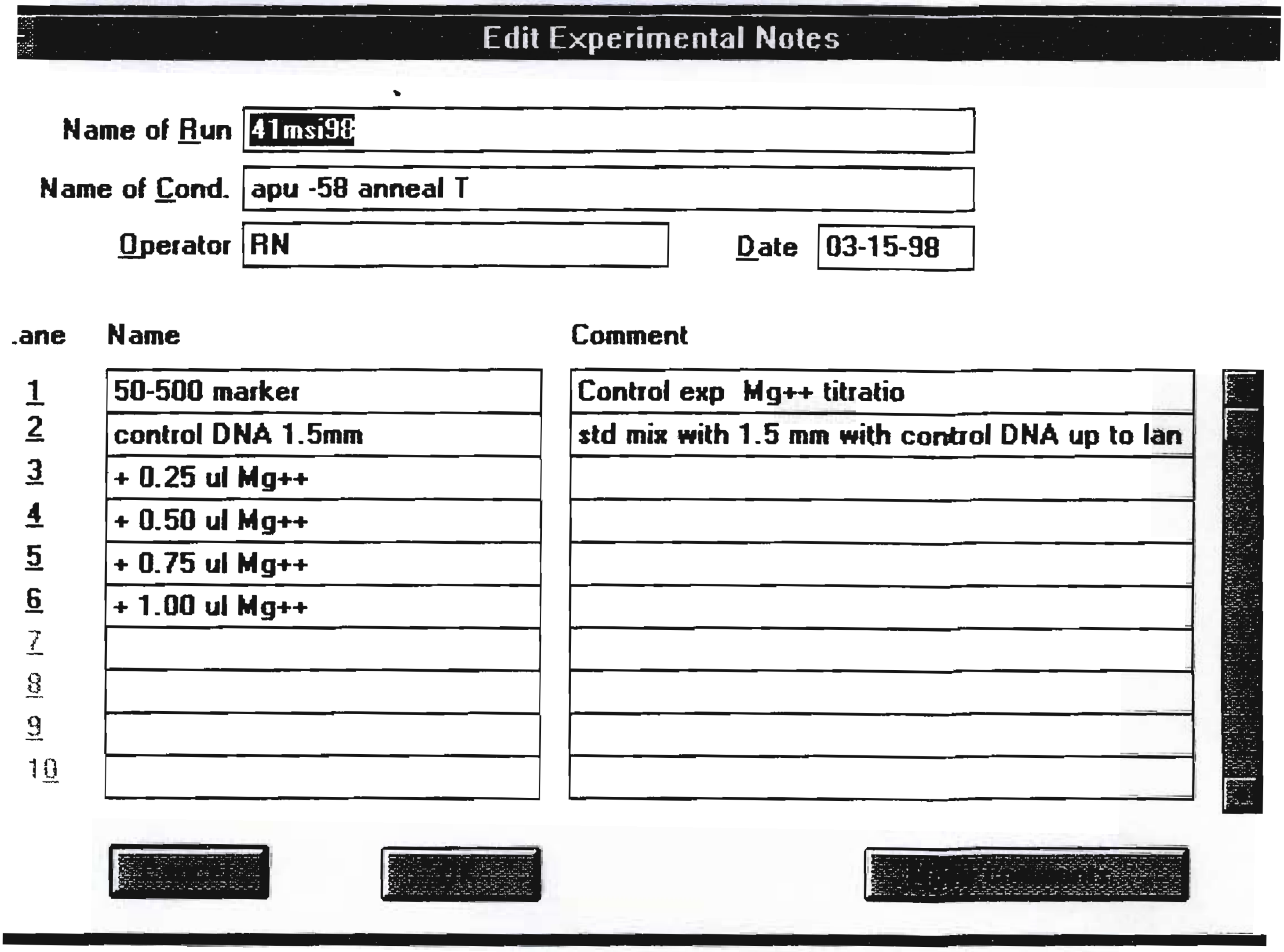
25 30 35 40 45 50 55 60 65 70 75 80 85 90

$MgCl_2$ Titration for marker D3S1255



Name of Run: 41msi98

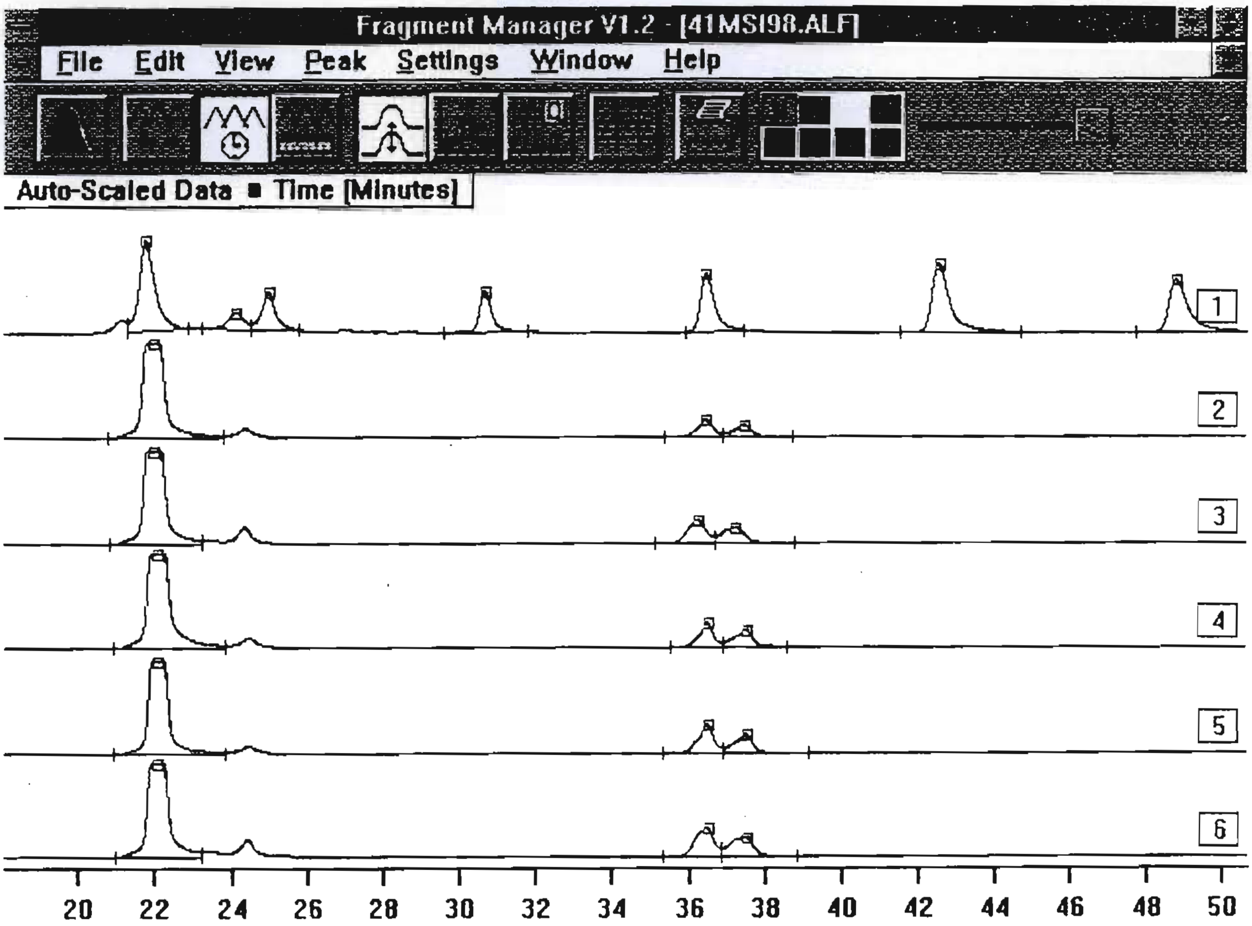
Name of Cond.: apu -58 anneal T

Operator: RN

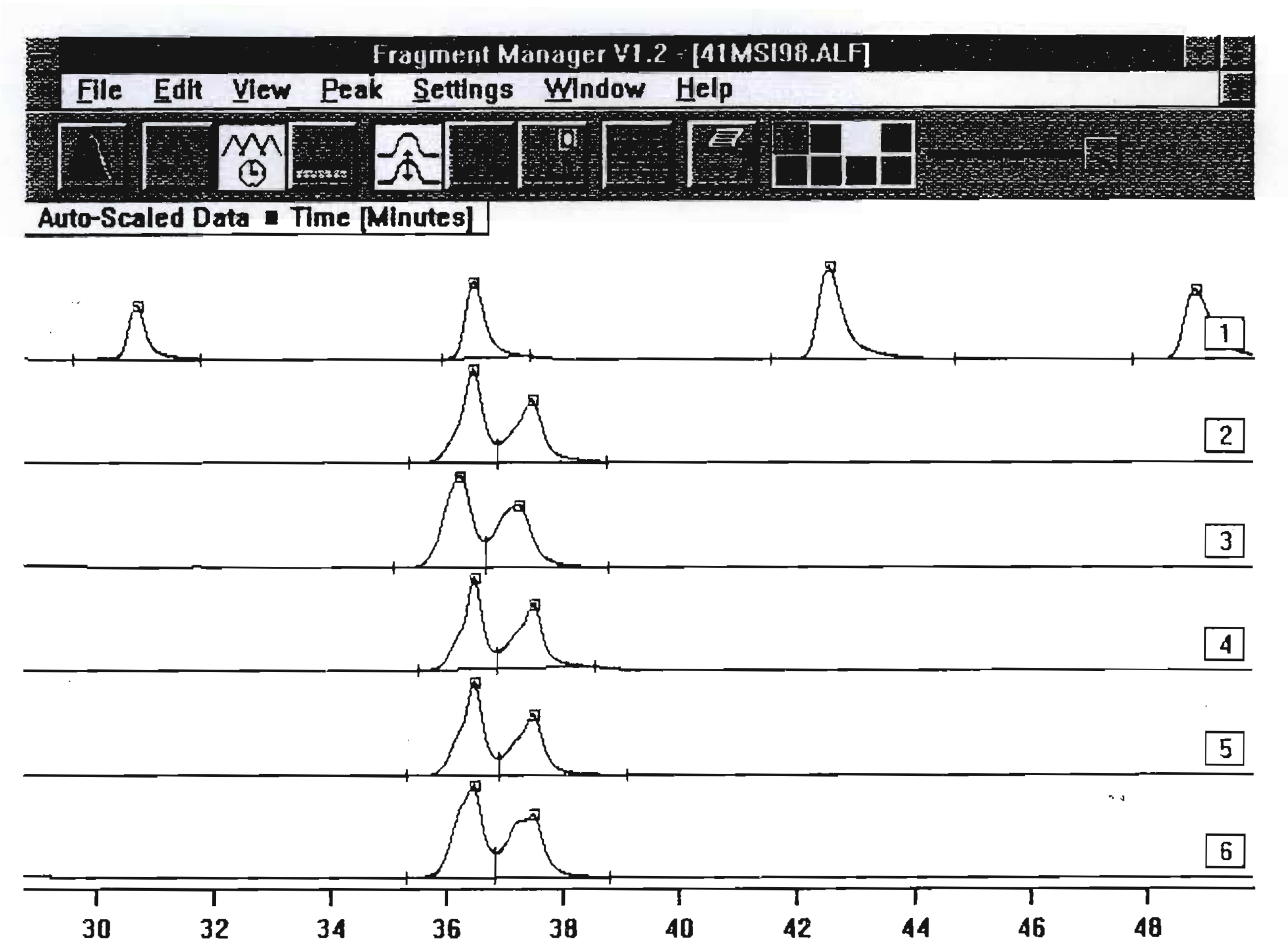
Date: 03-15-98

.ane	Name	Comment
1	50-500 marker	Control exp Mg++ titratio
2	control DNA 1.5mm	std mix with 1.5 mm with control DNA up to lan
3	+ 0.25 ul Mg++	
4	+ 0.50 ul Mg++	
5	+ 0.75 ul Mg++	
6	+ 1.00 ul Mg++	
7		
8		
9		
10		

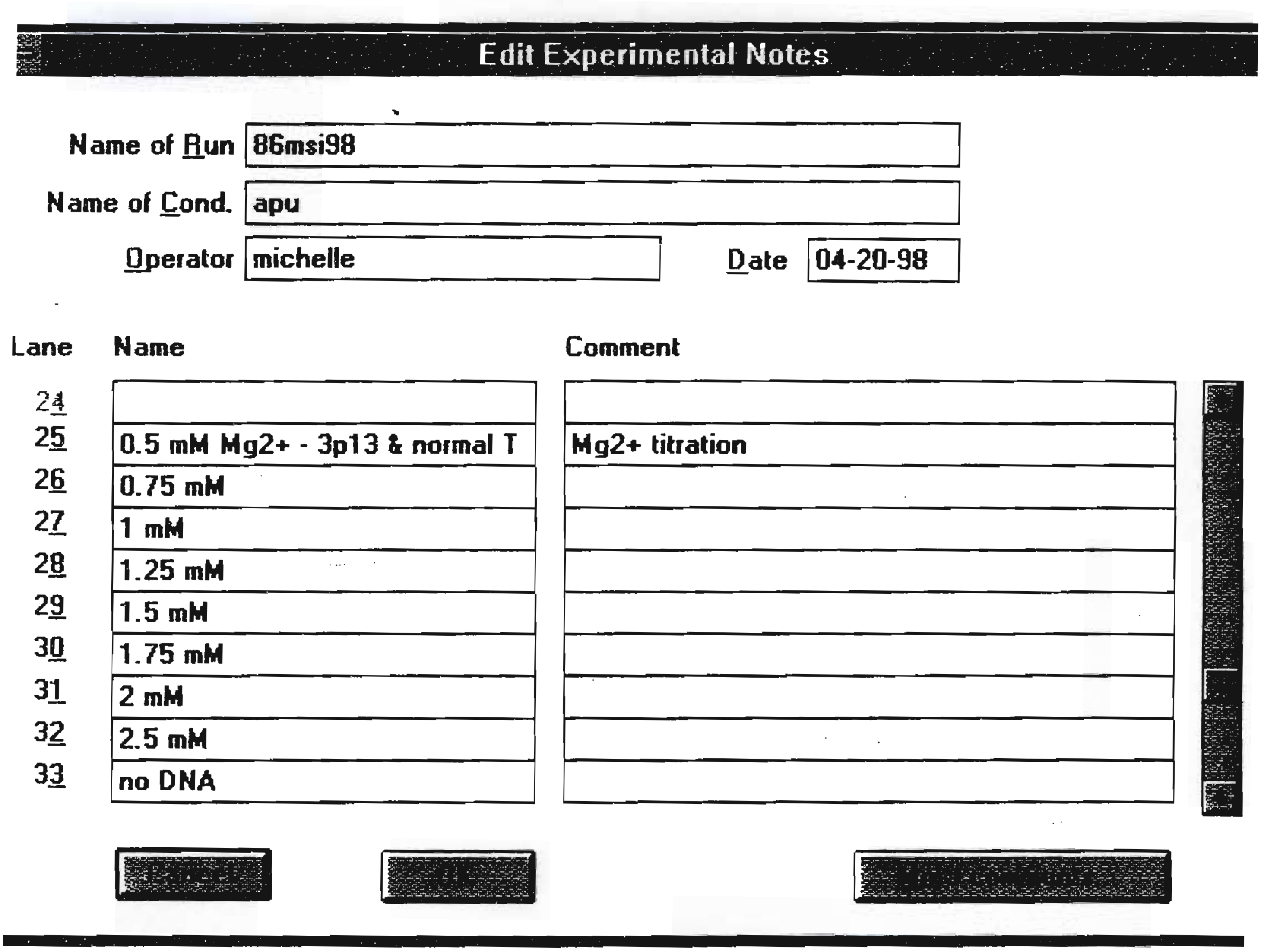
MgCl$_2$ Titration for marker D3S1255



$MgCl_2$ Titration for marker D3S1255



$MgCl_2$ Titration for marker D3S659 - (3p13)



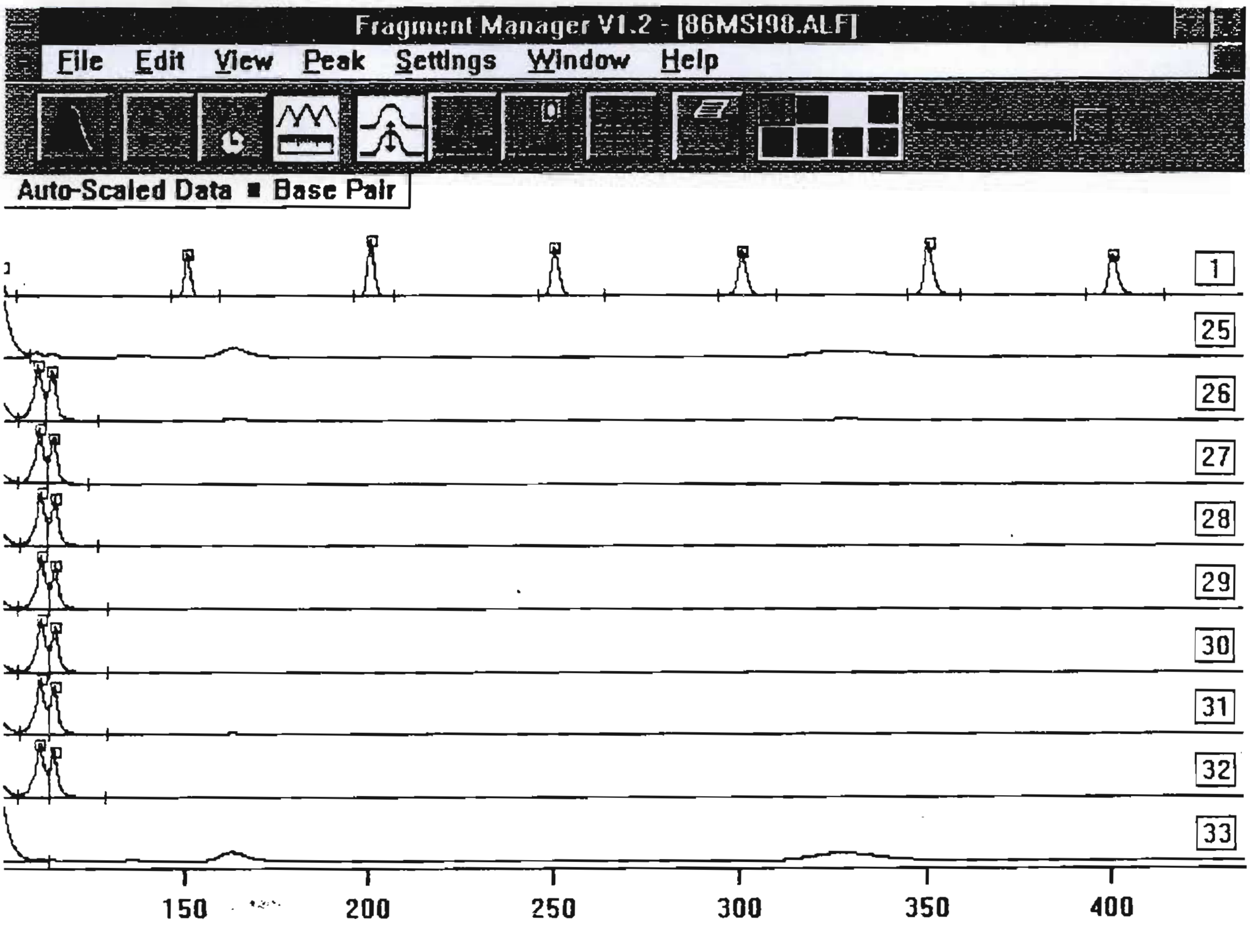
Name of Run: 86msi98

Name of Cond.: apu

Operator: michelle

Date: 04-20-98

Lane	Name	Comment
24		
25	0.5 mM Mg2+ - 3p13 & normal T	Mg2+ titration
26	0.75 mM	
27	1 mM	
28	1.25 mM	
29	1.5 mM	
30	1.75 mM	
31	2 mM	
32	2.5 mM	
33	no DNA	


Fragment Manager V1.2 - [86MSI98.ALF]
File
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Peak
Settings
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Help
Auto-Scaled Data ■ Base Pair
1
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26
27
28
29
30
31
32
33
150
200
250
300
350
400

APPENDIX F

APPENDIX F

Statistical Analysis

The Chi Squared test using the 2 x 2 tables and the Yates' correction were used. Only p values < 0.05 were deemed significant. The statistical analysis was calculated using the following formula: $X^2 = \Sigma \{(0-E)^2/E\}$, where 0 is the observed value and E is the expected value.

2p:

versus age: $p > 0.10$
versus gender: $p > 0.10$
versus grade: $p > 0.20$
versus stage: $p > 0.30$

3p:

versus age: $p > 0.1$
versus gender: $p > 0.1$
versus grade: $p > 0.1$
versus stage: $p > 0.1$

18q:

versus age: $p > 0.1$
versus gender: $p > 0.1$
versus grade: $p > 0.1$
versus grade: $p > 0.1$