

**ASSESSING FRAGILE SITES IN  
CARCINOGENIC ENVIRONMENTS:  
IS THIS AN ALERT SIGNAL?**

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A dissertation submitted to the Faculty of Science, University of the  
Witwatersrand, in fulfillment of the requirements for the degree of Master of  
Science.

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## DECLARATION

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I declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Annwyn Pamela Stafne

\_\_\_\_30th\_\_\_\_ day of \_\_\_\_March\_\_\_\_ 2005

## **ABSTRACT**

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Fragile sites are highly unstable regions of the genome, which have a tendency to form gaps and breaks in metaphase chromosomes under replication stress conditions. There are many common fragile sites in the human genome and exposure to carcinogens may affect several genes localised in fragile sites within a single cell, which could lead to activation of oncogenes and inactivation of tumour-suppressor genes simultaneously. FRA3B on chromosome 3 and FRA16D on chromosome 16 are the two most commonly expressed fragile sites and contain the *FHIT* and *WWOX* genes respectively. These genes are tumour suppressor genes and are inactivated in a number of different ways. Carcinogens found in cigarette smoke have been found to increase fragile site expression and could alter the integrity of these genes in active smokers.

Ten healthy non-smoking (control) individuals and twenty active smokers were recruited for the purpose of this study. Fluorescence *in situ* hybridisation was performed with probes spanning the *FHIT* gene and RT-PCR was performed to assess both *FHIT* and *WWOX* expression.

No significant difference in breaks at fragile sites was observed between controls and active smokers in the FISH experiments. In addition, no aberrant transcripts were detected for either *FHIT* or *WWOX* with RT-PCR.

Although the sampling group was limited and heterogeneous, no increase in the expression of breaks at fragile sites was seen in active smokers in the present study.

## DEDICATION

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I dedicate this to my parents:

To my mother Giuditta Stafne.

Thank-you for everything you have done for me.

To my father Leif Manuel Boyde Stafne

1949 – 2005

I will always remember you.

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<b>TABLE OF CONTENTS</b>	<b>Page</b>
DECLARATION	ii
ABSTRACT	iii
DEDICATION	iv
ACKNOWLEDGMENTS	v
TABLE OF CONTENTS	vi
LIST OF FIGURES	x
LIST OF TABLES	xii
<b>1. CHAPTER ONE - INTRODUCTION</b>	
<b>1.1. The Genetics of Cancer</b>	<b>1</b>
<b>1.2. Fragile Sites</b>	<b>3</b>
<b>1.3. The FRA3B Fragile site</b>	<b>4</b>
<b>1.4. The <i>FHIT</i> gene</b>	<b>6</b>
1.4.1. The t(3;8) Translocation	8
1.4.2. <i>FHIT</i> is a Tumour Suppressor gene	8
1.4.3. <i>FHIT</i> is a member of the Histidine Triad family	9
1.4.4. <i>FHIT</i> , Apoptosis and Cell Cycle control	10
<b>1.5. The FRA16D Fragile site</b>	<b>10</b>
<b>1.6. The <i>WWOX</i> gene</b>	<b>11</b>
<b>1.7. Possible Carcinogens affecting Fragile sites</b>	<b>13</b>
1.7.1. Culture conditions <i>in vitro</i>	13
1.7.2. Human Papillomavirus and FRA3B	13
1.7.3. Nutrition	14

1.7.4. The effects of Smoking	16
1.7.5. Maintenance of Integrity at Fragile sites	18
<b>1.8. Rationale of the Study</b>	<b>22</b>
<b>2. CHAPTER TWO – MATERIALS AND METHODS</b>	
<b>2.1. Specimens</b>	<b>24</b>
<b>2.2. Lymphocyte culture</b>	<b>24</b>
2.2.1. Slide preparation (Smokers and Non-smokers)	25
<b>2.3. Probe design</b>	<b>26</b>
2.3.1. Probe preparation	26
2.3.2. Preparation of glycerol stocks	28
2.3.3. DNA extraction from BAC clones using the Qiagen® Plasmid Purification midi kit	28
2.3.4. Agarose gel Electrophoresis	28
<b>2.4. DNA labelling</b>	<b>28</b>
2.4.1. Evaluation of labelled DNA	29
2.4.2. Enzyme inactivation of labelled probe	29
2.4.3. Repetitive sequence blocking with Cot 1 DNA and precipitation of labelled probe	29
<b>2.5. Hybridisation</b>	<b>30</b>
<b>2.6. Post Hybridisation washes</b>	<b>31</b>
<b>2.7. Counterstaining</b>	<b>31</b>
<b>2.8. Microscope analysis</b>	<b>31</b>
<b>2.9. Statistical analysis</b>	<b>32</b>

<b>2.10.</b>	<b>Expression analysis</b>	<b>32</b>
2.10.1.	Evaluation of extracted RNA	33
2.10.2.	RT-PCR	33
<b>2.11.</b>	<b>DNA studies</b>	<b>34</b>
<b>3.</b>	<b>CHAPTER THREE – RESULTS</b>	
<b>3.1.</b>	<b>Lymphocyte culture</b>	<b>35</b>
<b>3.2.</b>	<b>DNA extraction from BAC clones using the Qiagen®</b>	
<b>Plasmid Purification midi kit</b>		<b>35</b>
<b>3.3.</b>	<b>DNA labelling</b>	<b>35</b>
3.3.1.	Evaluation of labelled DNA	36
<b>3.4.</b>	<b>Hybridisation</b>	<b>36</b>
<b>3.5.</b>	<b>Microscope analysis</b>	<b>37</b>
<b>3.6.</b>	<b>Statistical analysis</b>	<b>47</b>
<b>3.7.</b>	<b>Expression analysis</b>	<b>50</b>
3.7.1.	Evaluation of extracted RNA	50
3.7.2.	RT-PCR	50
<b>4.</b>	<b>CHAPTER FOUR – DISCUSSION AND CONCLUSION</b>	
<b>4.1.</b>	<b>Discussion And Conclusion</b>	<b>54</b>
<b>4.2.</b>	<b>Future work</b>	<b>57</b>
	<b>APPENDIX A</b>	<b>59</b>
	<b>APPENDIX B</b>	<b>61</b>

<b>APPENDIX C</b>	<b>63</b>
<b>APPENDIX D</b>	<b>64</b>
<b>APPENDIX E</b>	<b>71</b>
<b>REFERENCES</b>	<b>74</b>

<b>LIST OF FIGURES</b>	<b>Page</b>
<b>Figure 2.1:</b> Schematic overview of Materials and Methods	23
<b>Figure 2.2:</b> Schematic representation of the <i>FHIT</i> gene	27
<b>Figure 3.1:</b> Evaluation of labelled DNA	36
<b>Figure 3.2:</b> Metaphase chromosomes of a control individual (C1) showing normal signals	38
<b>Figure 3.3:</b> Metaphase chromosomes of a smoking individual (SM16) showing normal signals	39
<b>Figure 3.4:</b> Metaphase chromosomes of a smoking individual (SM17) showing normal signals	40
<b>Figure 3.5:</b> Metaphase chromosomes of a smoking individual (SM20) showing normal signals	41
<b>Figure 3.6:</b> Metaphase chromosomes from a smoking individual (SM14) showing a full deletion of one set of signals	42
<b>Figure 3.7:</b> Metaphase chromosomes from a smoking individual (SM16) showing a split signal	43
<b>Figure 3.8:</b> Metaphase chromosomes from a smoking individual (SM19) showing missing red signals	44
<b>Figure 3.9:</b> Metaphase chromosomes from a smoking individual (SM4) showing red signals only	45
<b>Figure 3.10:</b> Graph of percentages of normal metaphases seen in each culture condition in smokers and non-smokers	49
<b>Figure 3.11:</b> RNA gel showing RNA extracted form active smokers and normal individuals	50

<b>Figure 3.12:</b> <i>GAPDH</i> RT-PCR results	51
<b>Figure 3.13:</b> <i>WWOX</i> RT-PCR results	52
<b>Figure 3.14:</b> <i>FHIT</i> RT-PCR results	53

<b>LIST OF TABLES</b>	<b>Page</b>
<b>Table 2.1:</b> Primer sets (forward and reverse) for <i>FHIT</i> , <i>WVOX</i> and <i>GAPDH</i>	33
<b>Table 3.1:</b> Test of normal distribution of the data	47
<b>Table 3.2:</b> Class level of information	48
<b>Table 3.3:</b> Analysis of variance	48
<b>Table A1:</b> Pkt /Year for each smoker	59
<b>Table A2:</b> Results of information collected from control as well as Smoking individuals	60

## **1. CHAPTER 1 - INTRODUCTION**

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### **1.1. The Genetics of Cancer**

Cancer is now recognised as a genetic disorder<sup>1</sup> at the cellular level that involves the mutation of certain genes. These genes can be activated in oncogenes or suppressed as a tumour suppressor gene, through the cell cycle. Activation or inactivation respectively, of these genes may cause uncontrolled cell division and tumour formation. Mutations in these genes can be transmitted through the germline and result in an increase susceptibility to cancer, or they can arise by somatic mutation<sup>2</sup>. Environmental factors and viruses are thought to contribute to genetic alterations that are necessary to transform normal cells into cancerous cells<sup>3,4</sup>.

Genomic alterations associated with cancer can involve small-scale changes such as a single nucleotide substitution or large-scale events that include chromosome rearrangement, chromosome loss or gain, or even the integration of viral genomes into chromosome sites. Large-scale genomic alterations are a common feature of cancer; the majority of human tumours are characterised by visible chromosomal changes<sup>4</sup>.

Mammalian cells have a number of safety mechanisms in place, which protect and in some cases prevent genes, which may have acquired oncogenic mutations, from developing into cancer. Cancer is a multi-step process, requiring a number of events / mutations in order for disease to develop. Therefore, only when several genes are affected does cancer develop. There are three classes of genes in which

oncogenic mutations can occur: Oncogenes, Tumour-suppressor genes and Stability genes.

1. Oncogenes: When oncogenes are mutated, it causes them to remain active in conditions where the wild-type gene is not. Activation of oncogenes is caused by: chromosomal translocations, gene amplifications or subtle intragenic mutations, which affect crucial areas in the gene that regulate the activity of the gene product.
2. Tumour-suppressor genes: These genes are affected through a different mechanism than that of oncogenes. Mutations of this class of genes, renders the gene product inactive or reduces the activity of the gene product substantially. Mutations in this class of genes can be one of the following: Missense mutations at residues in the gene that are responsible for its activity, mutations that result in a truncated protein, such as deletions or insertions of various sizes or epigenetic silencing by methylation of the 5' region.
3. Stability genes: These genes function in different ways when mutated. Mis-match repair, nucleotide excision repair and base excision repair genes fall into this class. They are responsible for repairing mistakes made during normal DNA replication, which is error-prone, and / or repairing breaks induced by exposure to exogenous mutagens. Genes in this class also control processes involved in mitotic recombination and chromosomal segregation, which involve large portions of chromosomes<sup>1,2,3</sup>.

## 1.2. Fragile Sites

Common fragile sites are highly unstable regions of the genome; they are present in every individual and are frequently involved in chromosome rearrangements in cancer. Features of common fragile sites are:

1. They are large regions, which have a tendency to form gaps and breaks across the entire region under replication stress conditions.
2. They contain a high percentage of nucleotides adenosine and thymidine.
3. Their sequences contain highly conserved regions.
4. They are recombinogenic and are induced by agents that retard DNA replication<sup>5,6,7</sup>.
5. They can be incompletely replicated, because their replication occurs later in the S phase. In specific culture conditions, this late-replicating genomic region might not be fully copied when the cell enters G2 and mitosis, leaving regions that appear as gaps in metaphase chromosomes<sup>8,9,10</sup>.

There are many common fragile sites in the human genome meaning that everyone has these weak links in their chromosomes, although there might be variations in the degrees of fragility among individuals. Carcinogen exposure may activate several fragile genes within a single cell, potentially this can result in activation of one or more oncogenes and inactivation one or more tumour-suppressor genes simultaneously. There is still much to learn about the induction of fragility, repair or mis-repair of damage, and the consequences to the genes in fragile regions. The two most commonly expressed fragile sites are FRA3B and FRA16D, which harbour potential tumour-suppressor genes<sup>11</sup>.

Fragile sites reveal cytogenetically detectable gaps after exposure of cells to specific reagents<sup>12, 13</sup> such as aphidicolin, (which inhibits the action of DNA polymerase  $\alpha$ , inducing gaps and breaks observed in the FRA3B region in normal metaphases) as well as folate deficient culture conditions<sup>14</sup>. Fragile sites can be divided into two types: (1) Rare fragile sites, which are observed in less than 5% of the population and contain trinucleotide repeat sequences<sup>15</sup>, and (2) common fragile sites, which appear to be present in all individuals with varying levels of expression. Common fragile sites have been demonstrated to have an elevated occurrence of sister chromatid exchanges, translocations and deletions in tumours, gene amplifications and *in vivo* tumour viral integration sites<sup>16</sup>.

### **1.3. The FRA3B Fragile Site**

FRA3B, the most frequently seen common fragile site encompasses the Fragile Histidine triad (*FHIT*) gene at 3p14.2<sup>17, 18</sup>.

Deletions of chromosome 3p have been noted frequently in many sporadic cancers and solid tumours including: lung, breast, kidney, cervical, and head and neck cancer<sup>17</sup>. Several reports have demonstrated that alterations in the genes on chromosome 3p may occur in preneoplastic lesions and set the stage for the development of malignant tumours<sup>19</sup>.

A papillomavirus insertion site and plasmid integration sites have also been mapped within the *FHIT* gene<sup>20</sup>. This provides direct support for the hypothesis that FRA3B, and likely other common fragile sites, may be "hot spots" for genetic

events, which may predispose individuals to cancer. Corbin *et al* (2002) suggest that the process of breakage and repair within FRA3B is an ongoing one. They further suggest that there is no single sequence within FRA3B that influences breakage or recombination within this region and report the presence of multiple “hot spots” within the *FHIT* / FRA3B locus<sup>21</sup>.

If fragile sites proved to be superimposed in location with oncogenes and tumour-suppressor genes, their remarkable proximity would enable them to be used as valuable markers in the search for alterations, which may alter these genes. FRA3B was previously defined as a large region of genomic instability covering approximately 500Kb. However, Fluorescence *in situ* hybridisation (FISH) and sequence-based analysis reveals that fragility at FRA3B extends over a 4Mb region containing five genes. In addition to *FHIT*, FRA3B also contains the *PTPRG* (Protein tyrosine phosphatase receptor type G), *HT021*, *CADPS* (Calcium dependant activator protein for secretion) and *SCA7* (Spinocerebellar ataxia 7) genes<sup>16</sup>.

The first three genes have complete loss of expression or aberrant gene expression in cervical cancer cell lines<sup>16</sup>. Gene expression analysis has revealed that the majority of the genes in FRA3B are down regulated in some cancers such as cervical cancer, whereas the surrounding genes do not demonstrate aberrant gene expression<sup>16</sup>. This indicates that the FRA3B region is a lot larger than previously thought. The active site of the fragility region is not always in the physical center of the fragile region. *FHIT* exons 4 and 5 have been defined as the center of the

FRA3B region, and remain the active site of FRA3B. The proximal end (centromeric) for FRA3B extends for 3Mb from the physical center, whereas the distal end (telomeric) extends approximately 700Kb from the physical centre<sup>16</sup>. In addition to *FHIT*, the *PTPRG* gene contained within the FRA3B region is centromeric to *FHIT* and is a putative tumour suppressor gene specifically thought to be involved in renal cell carcinoma<sup>16, 23, 24</sup>.

The *PTPRG* gene is a receptor phosphatase<sup>23</sup> and the gene product is a protein tyrosine phosphatase gamma. The protein undergoes N-linked glycosylation and constitutive phosphorylation of serine residues<sup>25</sup>. The *FHIT* and *PTPRG* genes are commonly down regulated in cancer and *HTO21*, a slightly smaller gene in comparison, has also shown marked inactivation in cancer. It therefore appears that “large” genes are often targeted in the FRA3B region<sup>16</sup>.

#### **1.4. The *FHIT* Gene**

The Fragile Histidine triad (*FHIT*) gene was cloned in 1996 by Ohta *et al*<sup>12</sup>. It is located at chromosome 3p14.2 and is involved in many different types of cancer<sup>26</sup>. Since its discovery, more than 300 articles concerning the *FHIT* gene and/or protein have been published. They raise controversy regarding the functional role of *FHIT* in cancer. The gene lies within the most active chromosome fragile site in humans, FRA3B, and is inactivated through deletions or promoter methylation in cancers of many tissues<sup>27</sup>.

The *FHIT* gene, spanning at least 1-2Mb is composed of 10 exons, three of which are untranslated<sup>28</sup> and five are protein – coding. It encodes a small mRNA (1.1kb) and a small protein. Breakpoints at 3p14.2, usually interrupt the third, fourth or

fifth introns of the *FHIT* gene, inactivating one of the two *FHIT* alleles. *FHIT* exon five, which is near the heart of the FRA3B region, is the most consistently deleted of the protein - coding exons<sup>29</sup>. Abnormal transcripts which lack exon 5, contain an in-frame ATG start codon elsewhere, therefore FHIT protein encoded in these cases is unlikely to have functional properties<sup>28</sup>. The *FHIT* loci is often involved in Loss of Heterozygosity (LOH) in human tumours and is involved in a t(3;8)(p14.2;q24) chromosome translocation, demonstrated in a family with hereditary renal cell carcinoma (RCC). Alterations in the *FHIT* gene may occur either early or late in the development of cancers. The latter is possibly associated with progression to more aggressive neoplasias<sup>30</sup>. More over, down-regulation of FHIT protein expression is associated with highly proliferative and large tumours<sup>31</sup>.

The fact that *FHIT* is located within a fragile site, suggests that this gene may be susceptible to rearrangements induced by a variety of mutagens and carcinogens known to affect fragile sites. It also suggests that the degree of chromosome fragility at this site may contribute to the degree of cancer susceptibility<sup>30</sup>. The mechanism of mutation remains unclear. Sequence analysis of the FRA3B/*FHIT* locus showed the locus to be prone to homologous recombination between long, interdispersed nuclear element sequences<sup>32</sup>. Campiglio *et al* (1999) suggest that an insertion even outside the coding region can abolish FHIT protein synthesis<sup>31</sup>.

The *FHIT* gene is expressed at low levels in most adult tissues but is absent or undetectable in some tumour cell lines with deletions. Another feature of *FHIT* is the virtual absence of point mutations<sup>12</sup>. Croce *et al* (1999) found that the

complete absence of FHIT protein was more common than p53 mutation in lung cancers<sup>30</sup>. However, Gemmill *et al* (1998) had previously reported that p53 alterations appear to be a prerequisite<sup>33</sup> for loss of *FHIT* expression. Further work in this area is needed to obtain conclusive results.

#### 1.4.1. The t(3;8) Translocation

The 3;8 chromosomal translocation, t(3;8)(p14.2;q24.1), was described in a family with classical features of hereditary RCC<sup>34</sup>. The family had an unusual cluster of renal cancers that occurred in young adults and usually affected both kidneys with several tumours<sup>35</sup>. The t(3;8) break falls between untranslated 5' exons 3 and 4<sup>7</sup>. Frequent 3p loss of heterozygosity in sporadic RCC lead to the initial assumption that the *FHIT* gene was the causative gene in the hereditary t(3;8) family. It has now been shown that the 3;8 translocation fuses *FHIT* to an alternative partner gene on chromosome 8, the *patched* - related gene, TRC8<sup>34</sup>.

#### 1.4.2. *FHIT* is A Tumour Suppressor Gene

*FHIT* is as a tumour suppressor gene<sup>30, 36</sup>. Findings that support this fact include: (a) A t(3;8)(p14.2;q24) translocation breakpoint in a familial RCC within the *FHIT* locus, which inactivates *FHIT* expression; (b) Homozygous deletions of *FHIT* (some of which encompass exons) in several cancer cell lines and primary tumours; and (c) Aberrant *FHIT* transcripts and absent or reduced FHIT protein expression in several types of epithelial cancers<sup>37</sup>. *FHIT* is partially or entirely lost in most human cancers, suggesting a tumour-suppressor function. Inactivation of only one *FHIT* allele suggests that a one-hit mechanism may be operative<sup>35</sup> as will

be discussed below. This demonstrates a dominant negative effect. *FHIT* inactivation provides a selective advantage for clonal expansion *in vitro*<sup>26</sup>.

Functional studies showed that replacement of *FHIT* in some cancer cells that lacked expression of the endogenous gene suppresses tumourigenicity, demonstrating that *FHIT* behaves as a tumour suppressor<sup>38</sup>. This was confirmed in knockout mice<sup>39</sup>. Over-expression of exogenous *FHIT* by infection or transfection caused apoptosis of the *FHIT* over-expressing cancer cells. The signal pathways through which *FHIT* exerts its suppressive effect are not fully known. However, experimentally it has been shown that tumour development can be inhibited through recombinant viral *FHIT* gene delivery, which opens the way to therapeutic applications<sup>38</sup>. Ishii *et al* (2004) show that viral vector-mediated *FHIT* gene transfer to *Fhit*-deficient mice not only prevents but reverses the carcinogen-induced tumour development *in vivo*, in accordance with the oncosuppressive properties of Fhit protein. The strong pro-apoptotic activity following *FHIT* transfection of cancer cells strengthens the case for further exploration of *FHIT* gene therapy in cancer prevention and treatment<sup>39</sup>.

#### 1.4.3. *FHIT* is A Member Of The Histidine Triad Family

The *FHIT* gene is a member of the histidine triad super family of enzymes and is a dinucleoside 5',5''-P<sup>1</sup>,P<sup>3</sup>-triphosphate (Ap3A) hydrolase<sup>40</sup>. It encodes a cytoplasmic M<sub>r</sub> 16,800 protein with diadenosine triphosphate hydrolase activity<sup>31</sup>. The *in vivo* function of this enzyme, which produces ADP and AMP *in vitro* from the diadenosine substrate, is not known<sup>29</sup>. Site - directed mutagenesis of *FHIT*

demonstrated that all four conserved histidines are required for full activity, and the central histidine of the triad is absolutely essential for Ap3A hydrolase activity. FHIT is the first HIT protein in which the histidine residues have been demonstrated by mutagenesis to be critical for function<sup>40</sup>. However, Lee *et al* (2001) reported that *FHIT* tumour-suppressive function appears to be independent of its hydrolase activity<sup>41</sup>. This study contradicts what the majority of literature has reported and more recently Roz *et al* (2002) found that the tumour-suppressive function of *FHIT* is in fact dependant on its hydrolase activity<sup>42</sup>.

#### 1.4.4. *FHIT*, Apoptosis And Cell Cycle Control

At the cellular level, *FHIT* has been shown to induce apoptosis and retard tumour cell proliferation *in vitro* and *in vivo*<sup>32</sup>. Activation of caspase 8 has been associated with *FHIT* - mediated apoptosis<sup>42</sup>. Restoration of *FHIT* expression also suppresses tumourigenicity. This demonstrates the oncosuppressive properties and strong pro apoptotic activity of the FHIT protein in cancer<sup>42, 43</sup>.

A two - fold increase in Bak protein levels and an increased level of p21 protein, paralleled by an up - regulation of p21 transcripts was found in *FHIT* expressing clones. Onset of *FHIT* - induced apoptosis could also involve Bak up – regulation. Roz *et al* (2002) reported that apoptosis in human cultured cells is associated with a decrease of free Ap3A levels<sup>42</sup>.

### 1.5. The FRA16D Fragile Site

FRA16D and FRA3B are the most frequently expressed of the more than 80 described common chromosomal fragile sites<sup>44</sup>. Genetic alterations at FRA16D

are associated with exposure to mutagens and environmental carcinogens<sup>45</sup> and FRA16D is an aphidicolin inducible fragile site<sup>46</sup>. Chromosome 16q has been implicated as the site of a tumour suppressor gene in a large number of cancers because high frequencies of LOH of microsatellite markers have been detected in many different tumours<sup>45</sup>. The region 16q23.2 – 24.1 is recognised as the common fragile site FRA16D<sup>44</sup>. It contains the *WWOX* gene and demonstrates DNA loss<sup>47</sup> as well as a high incidence of LOH<sup>47, 48</sup>.

### **1.6. The *WWOX* Gene**

The *WWOX* gene spans more than 1Mb<sup>48</sup> and occupies most of the FRA16D region<sup>43</sup>. *WWOX* is a candidate tumour suppressor gene<sup>44, 45</sup>. Reid *et al* (2000) cloned the same gene and called it *FOR* and reported the existence of alternative mRNA 3'-end-spliced variants<sup>49</sup>.

The *WWOX* gene was named so because it contains two WW domains coupled to a region with high homology to the short-chain dehydrogenase / reductase family of enzymes<sup>48, 50</sup>. The gene is composed of nine exons. The open reading frame (ORF) is 1245bp and it encodes a 414 amino acid protein. The protein is approximately 46kDa<sup>50</sup>. Of the nine exons, exons 5 – 8 and 6 – 8 show consistent deletions<sup>44</sup>. Exons 6 – 8 encode the major portion of the enzymatic *WWOX* domain<sup>45</sup>.

The mouse homologue *Wox1* was cloned and shown to associate with p53 and to be an essential mediator of Tnf- $\alpha$ -induced apoptosis<sup>48</sup>. It could therefore potentially play a role in apoptosis<sup>44</sup>.

*WWOX* is homozygously deleted in several tumour types<sup>48</sup>. *WWOX* demonstrates alternative splicing, Paige *et al* (2001) found additional transcripts lacking exons 6 – 8 which could be cancer associated<sup>48</sup>. Point mutations in *WWOX* are extremely rare but biallelic deletions are common. Yendamuri *et al* (2003) demonstrated frame shift deletions in the open reading frame (ORF)<sup>51</sup> in amplification products from aberrant *WWOX* transcripts in tumour cell lines.

Ishii *et al* (2004) tested transcriptional regulation of *FHIT* and *WWOX* and found that *E2F-1* over expression resulted in an increase of the above-mentioned gene products, concurrent with apoptosis induction<sup>52</sup>. Since the tumour suppressor RB1-E2F1 pathway is altered early in carcinogenesis and *E2F-1* is a crucial factor for cell proliferation and apoptosis induction, Ishii *et al* (2004) suggest that *E2F-1* over expression can play a role in suppression of tumours, at least in part through transcriptional regulation of *FHIT* and in direct activation of *WWOX*<sup>52</sup>.

Ishii *et al* (2003) found that aberrant *FHIT* reverse transcription-PCR products correlated significantly with the occurrence of *WWOX* alterations. They concluded that *WWOX* expression is frequently altered or absent in haematopoietic disorders, often in association with *FHIT* alterations, and that alterations of these fragile

genes may result not only from genomic deletions but also from epigenetic modifications associated with expression of fragility<sup>53</sup>.

## **1.7. Possible Carcinogens Affecting Fragile Sites**

### **1.7.1. Culture Conditions *In Vitro***

A number of studies provide evidence that alteration in DNA replication provokes the expression of aphidicolin-inducible fragile sites; however the exact mechanism remains unresolved. The inhibition of DNA polymerase  $\alpha$  and  $\delta$  may induce fragile sites by delaying DNA replication further, in regions that are already late replicating or slow to replicate. Failure to complete replication of FRA3B sequences before the condensation of chromatin into metaphase chromosomes may give rise to the chromosomal breaks and gaps characteristic of fragile sites<sup>9, 10, 54</sup>. Total and site-specific damage appears to be dose dependant and cumulative when folic acid levels are decreased in aphidicolin stimulated cultures<sup>55</sup>.

It is also possible that stalled replication forks are converted into double-stranded breaks within FRA3B, which may lead to aberrant recombination repair and result in genetic instability<sup>54</sup>. Aphidicolin represents an important tool for detection of fragile sites on human chromosomes through mechanisms of DNA polymerase alpha inhibition<sup>55</sup>.

### **1.7.2. Human Papillomavirus And FRA3B**

Cytogenetic studies have suggested a correlation between common chromosomal fragile sites and the integration sites of Human papillomavirus (HPV). HPV, a

DNA virus, affects both the nucleus and the cytoplasm of the infected cells with specific changes. HPV DNA may occur within epithelial cell nuclei as either unintegrated or integrated. Nuclear changes tend to be more pronounced in cases where the HPV DNA is integrated into the epithelial cell nuclear DNA<sup>38</sup>. Previously, an HPV16 viral integration site was mapped between exons 4 and 5 of the *FHIT* gene, which coincide with the active site and physical centre of FRA3B. Recently an additional HPV16 cervical integration site was mapped within the region of FRA3B fragility<sup>16</sup>. The new HPV16 integration site, which was mapped, is located 2Mb centromeric to the previously defined FRA3B region of instability. The integration site falls between the *CADPS* and *SCA7* genes. Due to the fragility of the FRA3B region, integration of HPV sequences into this region could disrupt any of the 5 genes, specifically *FHIT*, *PTPRG* or *HT021*, which could have relevance to the malignant transformation of these cells<sup>16</sup>. This could be sufficient to inactivate these genes and initiate the development of cancer; however, studies are needed to support this hypothesis.

### 1.7.3. Nutrition

Diet is thought to be one of the most important contributing factors to cancer risk and the contribution of diet to cancer is linked to genetic factors. Cancer is a disease of genes and there is overwhelming evidence that environment and lifestyle are the predominant cause of somatic genetic alterations that lead to most sporadic cancers<sup>56</sup>.

Vitamins are organic compounds that cannot be synthesized by humans and therefore must be ingested to prevent metabolic disorders. Although vitamin deficiency is encountered infrequently in developed countries, inadequate intake or subtle deficiencies in several vitamins are risk factors for chronic disease such as cancer<sup>57</sup>.

Folate (Folic acid) is a water-soluble B vitamin that is necessary in forming coenzymes for purine and pyrimidine synthesis<sup>57</sup>. Folate is necessary for the production and maintenance of new cells. This is especially important during periods of rapid cell division and growth such as infancy and pregnancy<sup>58</sup>. Folate deficiency is generally caused by poor intake or alcoholism and Baron *et al* (1998) suggest that alcohol drinking and cigarette smoking may impair the biologic actions of folate<sup>59</sup>. The role of folate in chronic health disease and cancer has recently been investigated<sup>57</sup>.

Some evidence associates low blood levels of folate with a greater risk of cancer. Folate is involved in the synthesis, repair, and functioning of DNA and a deficiency of folate may result in DNA damage. Studies have associated diets low in folate with increased risk of breast, pancreatic, and colon cancer<sup>58</sup>. Findings from a study suggested that long-term folic acid supplementation (for 15 years) was associated with a decreased risk of colon cancer in women aged 55 to 69 years of age. Researchers are continuing to investigate whether enhanced folate intake from foods or folic acid supplements may reduce the risk of cancer<sup>58</sup>.

#### 1.7.4. The Effects Of Smoking

Lung cancer is the most common cause of death in both men and woman. It is responsible for more cancer-related deaths than colorectal cancer, breast cancer and prostate cancer combined. The risk of lung cancer increases with the duration of smoking and the number of cigarettes smoked daily<sup>60</sup>. Approximately 8% of all adult deaths in South Africa (more than 20 000 deaths a year) are caused by smoking<sup>61</sup>. Internationally, smoking prevalence is higher among males than females. In South Africa, Coloured people have the highest smoking prevalence followed by Whites and Indians, with the lowest prevalence among the black population<sup>62</sup>.

It is estimated that between 10 and 20 genetic events are required for lung tumourigenesis<sup>63</sup>. Epidemiological data have strongly indicated that cigarette smoking is linked to the development of lung cancer. However, little is known of the molecular targets of carcinogens contained in tobacco smoke<sup>64</sup>. However, Pylkkanen *et al* (2002) suggest that *FHIT* allele losses could be the outcome of tobacco-induced mutagenesis<sup>65</sup>. *FHIT* loss was as frequent as abnormalities of expression of p53, RB and p16<sup>66</sup>, with p16 being inactivated not only by genetic alterations but also by transcriptional silencing due to hypermethylation<sup>67</sup>. Hirao *et al* (2001) stated that there is an association between an early age of onset of smoking and tobacco smoke-induced DNA damage<sup>68</sup>.

Smoking may induce *FHIT* alterations<sup>69, 70</sup>. Many studies have reported altered *FHIT* in pre-cancerous lesions and in environmental carcinogen-related cancers,

such as those of the lung and oesophagus. This suggests that *FHIT* alterations may be an early event in carcinogenesis and that pre-disposing genetic changes have occurred even in normal-appearing epithelium in cases heavily exposed to environmental carcinogens<sup>69, 71, 72, 73</sup>.

Stein *et al* (2002) demonstrated that active smokers exhibit a significantly higher frequency of fragile site expression, including FRA3B, compared to that of non-smokers and patients diagnosed with small cell lung cancer (SCLC) that have stopped smoking<sup>5, 74</sup>. This suggests that active tobacco exposure increases chromosome fragile site expression and strengthens the case for *FHIT* involvement in the multistage development of lung cancer<sup>74, 75</sup>.

Yendamuri *et al* (2003) demonstrated that the *WWOX* gene located at FRA16D is altered by deletion and/or aberrant expression in non-small cell lung cancer (NSCLC)<sup>51</sup>.

Smoking is commonly accepted as a major cause of lung cancer. Recent studies have suggested that specific alterations, such as deletions, especially 3p, in lung cancer occur in pre-malignant clones long before the appearance of evident malignancies. Furthermore, these changes may persist for many years after smoking termination<sup>68</sup>. Many genetic alterations have been described in lung cancer, but their association with exposure to tobacco and other lung carcinogens has not been well studied. Weincke *et al* (1999) reported recently that in former smokers, age at smoking initiation was inversely associated with increasing

(polynuclear aromatic hydrocarbon - PAH) PAH<sup>3</sup>-DNA adduct (DNA adduct – compound e.g. carcinogen, drawn to DNA) levels in normal lung tissue<sup>76</sup>. This suggests that smoking during adolescence may produce physiological alterations that lead to increased DNA adduct presence<sup>67</sup>. The prevalent type of pre-malignant mutation in a diverse group of human cancers, including lung cancer, is alteration of the TP53 gene<sup>77</sup>. TP53 is an inducer of apoptosis, and is related to DNA adducts of benzo(*a*)pyrene from cigarette smoking<sup>77</sup>. These DNA adducts interfere with DNA replication and repair<sup>28</sup>. The chemical compounds associated with smoking are present in the peripheral circulation of heavy smokers and there is a reduction in the level of these substances over time after the individual stops smoking. Given that carcinogenesis is a multi-step process, even after the individual stops smoking and fragile site expression returns to normal levels, the risk for developing lung cancer continues for many years<sup>74</sup>.

It would be of great interest to investigate whether the FRA3B and FRA16D regions are altered in active smokers as shown in Stein *et al* (2002) study, and to further assess *FHIT* and *WWOX* expression since these genes are located in fragile sites<sup>74</sup>. If this is the case these gene alterations could create a risk for the development of lung cancer.

#### 1.7.5. Maintenance Of Integrity At Fragile Sites

Several studies have suggested that the activity of repair proteins is important in maintaining the integrity of common fragile regions. A number of familial cancer

syndromes are due to germ-line mutations in genes required for repair of various forms of DNA damage, such as mismatches and double-strand break repair<sup>78</sup>.

DNA damage checkpoints are feedback mechanisms that sense the physical state of the genome. Should the integrity be compromised, checkpoint proteins signal these events and thereby prevent initiation of the next cell cycle phase<sup>79</sup>.

The *FHIT* gene may be a target of damage in some mismatch repair-deficient tumours, leading to FHIT protein loss and clonal expansion of FHIT-negative cells. There is in fact a microsatellite marker in intron 5 causing the *FHIT* gene to be a potential target of this kind of damage. Genes at fragile sites are frequently involved in tumorigenesis of repair deficient cancers<sup>78</sup>. To further demonstrate this Turner *et al* (2002) analysed the frequency of aphidicolin induced chromosome gaps and breaks in *PMS2*-, *BRCA1*-, *MSH2*-, *MLH1*-, *FHIT*- and *TP53*-deficient cell lines<sup>78</sup>. Each of these repair-deficient cell lines showed elevated expression of gaps and breaks, which is consistent with the proposal that proteins involved in mismatch and double-strand break repair are important in maintaining the integrity of common fragile sites<sup>78</sup>.

Mori *et al* (2001) investigated the relationship between mismatch repair protein Msh2 and *FHIT* expression<sup>80</sup>. They found that altered expression of Msh2 correlated with loss of FHIT protein. They concluded that loss of Msh2 leads to enhanced damage and may be important in maintaining the integrity of the common fragile locus around the *FHIT* gene<sup>80</sup>.

An important regulator of DNA damage checkpoints is the Ataxia-Telangiectasia and Rad3-Related gene (*atr*)<sup>81</sup>. *atr* is an unconventional protein kinase that phosphorylates and activates signal transduction pathways that ultimately interface with Cdk / Cyclin machinery<sup>79, 82</sup>. *atr* is critical for the maintenance of common fragile site stability<sup>80</sup>.

The *atr* protein responds to stalled DNA replication by activating Chk1, a conventional protein kinase that interfaces with the normal synchronous regulators of Cdc2. These regulators control Cdc2 by modulating phosphorylation of two amino acid residues (T14 and T15), phosphorylation of which inhibits Cdc2's kinase activity and prevents entrance into M phase<sup>83</sup>. Thus the *atr*-dependent pathway is a regulator of S to M phase transition when DNA replication stalls. It has also been shown that when *atr* is deleted, DNA replication inhibitors prevent entry into mitosis. This suggests that there must be another mode of CyclinB / Cdc2 inhibition that operates independently of *atr*<sup>83</sup>.

The expression of common fragile sites increases when *atr* function is lost – even in the absence of aphidicolin. Absence of *atr* function together with doses of aphidicolin increases fragile site expression 10 – 20 fold. *atr* functions in two ways:

1. It prevents cell cycle progression until normal DNA replication is completed.
2. *atr* stabilises stalled replication forks<sup>82</sup>.

Downstream targets of *atr* include: CHK1, BRCA1, p53 and H2AX<sup>81</sup>.

Thus, there are many factors contributing to fragile site expression and thereby influencing the genes located at these regions.

### **1.8. Rationale Of The Study**

The increased expression of fragile site loci may affect the integrity of tumour-suppressor genes at these sites in heavy smokers. Folate deficiency may increase the expression of fragile sites, which may further enhance the effects on genes in heavy smokers.

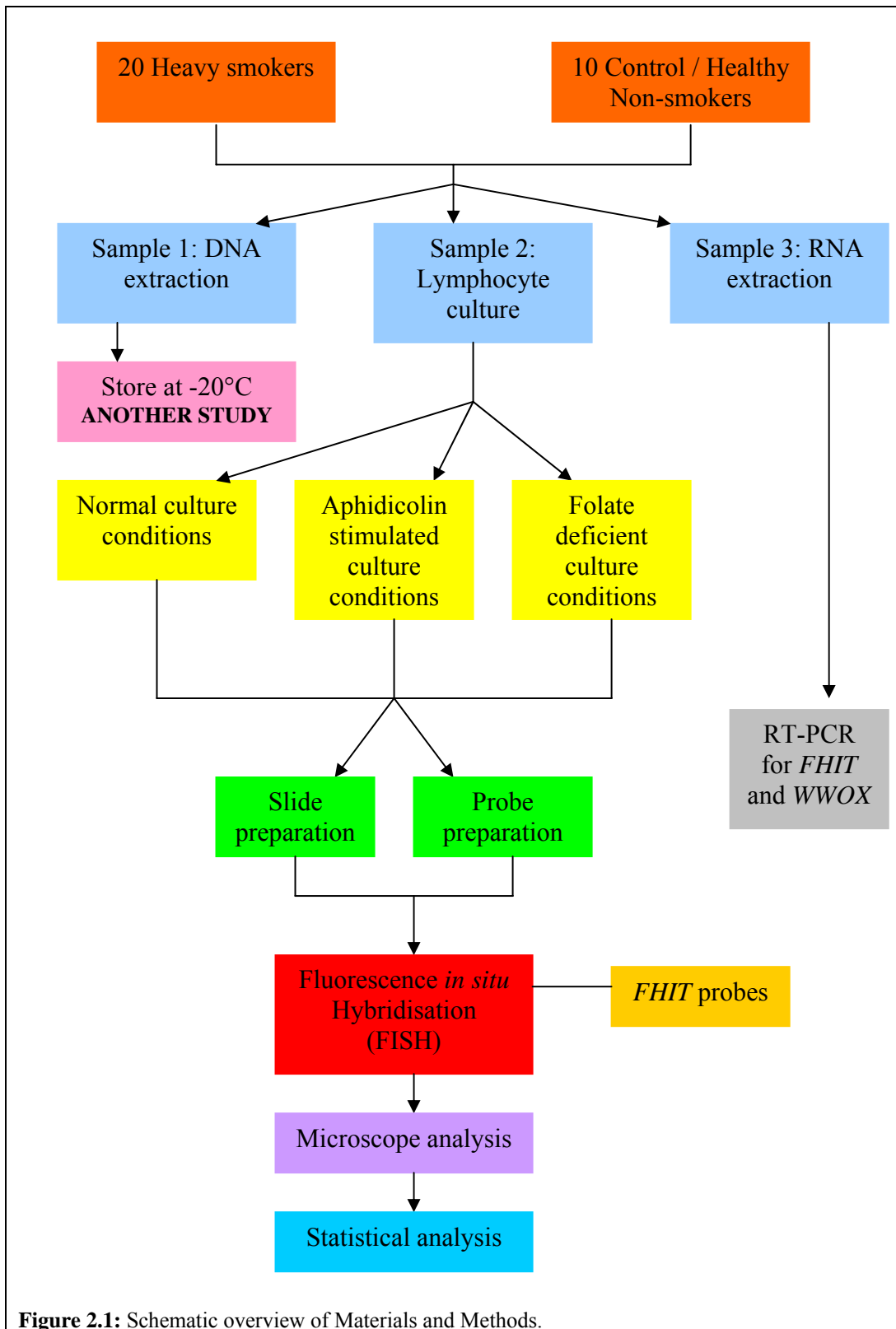
The aims of this study are therefore:

1. To assess whether the FRA3B loci integrity is affected in peripheral blood lymphocytes of smokers under 3 sets of culture conditions:
  - a. Normal cultures
  - b. Aphidicolin stimulated cultures
  - c. Folate deficient cultures

This will be done using FISH methodology.

2. To assess the expression of *FHIT*, located at FRA3B and *WWOX*, located at FRA16 in active smokers using RT-PCR.

## 2. CHAPTER 2 - MATERIALS AND METHODS



**Figure 2.1:** Schematic overview of Materials and Methods.

## 2.1. Specimens

This was a prospective study. Peripheral blood specimens were collected from twenty smokers (SM) and ten non-smoking control individuals (C). Length of time smoking by smokers ranged from 2.5 Pkt / Year (Packet / Year) to 45 Pkt / Year (Appendix A). The samples were collected over a period of 6 months. This study was performed in the Somatic Cell Genetics Unit, University of the Witwatersrand – Medical School. Ethical clearance was obtained for this study. Ethical clearance number M03-09-43. Informed consent was obtained from all participants.

## 2.2. Lymphocyte culture

Lymphocytes were cultured from the peripheral blood of both the Smokers and Non-smokers for 96 hours in 3 different conditions.

- 1) RPMI 1640 (Appendix B) with 10% foetal bovine serum (Appendix B) (**N cultures**),
- 2) RPMI 1640 (Appendix B) with 10% foetal bovine serum (Appendix B) and 0.4 $\mu$ M aphidicolin (Appendix B) added 24 hours before harvest (**A cultures**). Aphidicolin is an inhibitor of DNA polymerase  $\alpha$  and will induce gaps and breaks in metaphase chromosomes.
- 3) Folic acid-deficient RPMI 1640 (Appendix B) with 10% foetal bovine serum (Appendix B) (**F<sup>-</sup> cultures**).

All media were supplemented with 4mM penicillin / streptomycin (Appendix B) and 1 $\mu$ g / ml Phytohaemagglutinin (PHA) (Appendix B). Cultures were synchronised with 2 X 10<sup>-5</sup> M Amethopterin (MXT) (Appendix B) for 16 hours

prior to exposure to  $10^{-3}$  M Thymidine (Appendix B) and 4 and a half hours after to colcemid (Appendix B). This was done in order to yield a maximum number of metaphases per culture.

Cells were harvested by standard protocol using a 0.075 M KCl (Appendix B) hypotonic solution with an incubation time of 20 minutes, allowing for swelling of nuclei, followed by acetic methanol [1:3] fixation (Appendix B).

#### 2.2.1. Slide preparation (Smokers and Non-smokers)

Once the cultures were harvested, each culture was placed into a 1,5ml eppendorf tube and labelled accordingly. Fix changes were done until the supernatant ran clean. Enough fixative was then removed so as to concentrate each pellet. Cultures were stored at  $-20^{\circ}\text{C}$  until slides could be prepared. Slides were prepared for each N, A and F<sup>-</sup> culture for each individual.

Slides were cleaned in methanol (Appendix B) and then rinsed in distilled water and labelled accordingly. Distilled water prevents the formation of a hazy film on the slides. Metaphase slides were prepared by dropping the cell suspension onto clean slides prior to steaming. Each slide was viewed under a phase contrast microscope (Olympus CK30) to assess the spreading of metaphases on the slide. Slides were then dehydrated through 70% - 100% ethanol (Appendix B) for 3 minutes each. Slides were stored at  $-70^{\circ}\text{C}$  until use.

### 2.3. Probe design

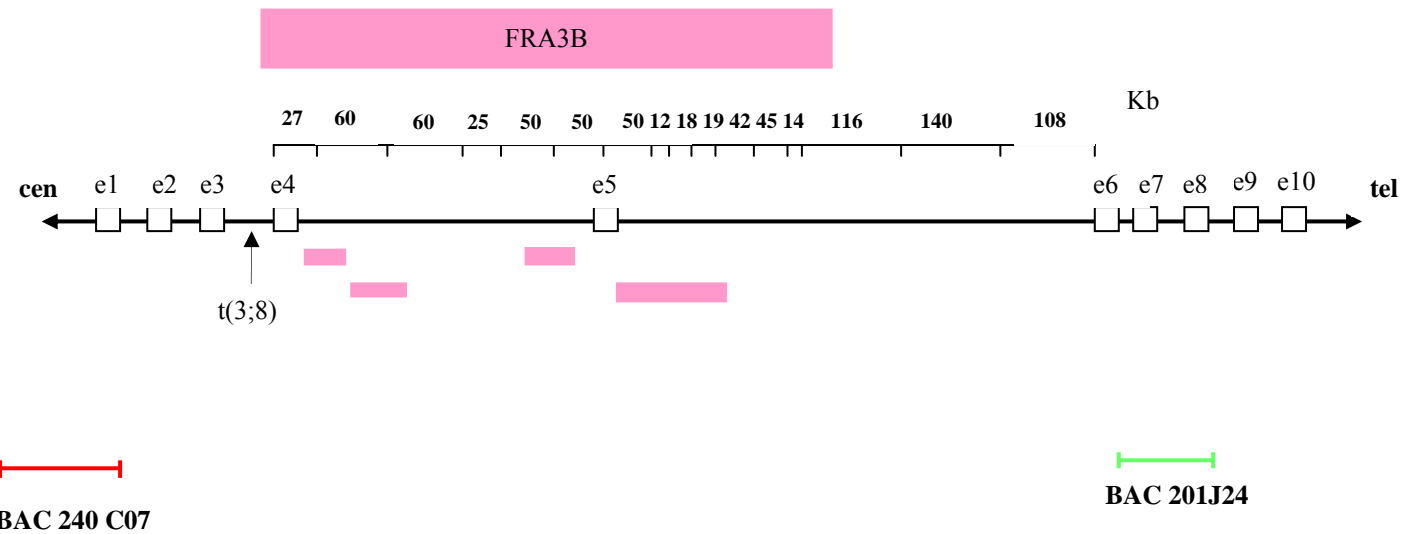
The probes used in this study were **BAC 201 J24**, which is telomeric to FRA3B / *FHIT* and **BAC 240 C07**, which is centromeric to FRA3B / *FHIT*. These probes flank the FRA3B fragile site, which contains the *FHIT* gene. Refer to **Figure 2**. The probes are situated approximately 100Kb apart and would therefore detect those rearrangements disrupting *FHIT* gene loci. They would not detect small deletions in between.

#### 2.3.1. Probe preparation

Bacterial artificial chromosomes (BACs) (Appendix C) were obtained from a previous project.

BACs are Bacterial artificial chromosomes, which can take a human DNA insert of 100 – 500Kb in size. BACs were propagated through culture of *E. coli* in LB Broth medium (Appendix C) at 37°C.

Approximately 200ml of LB Broth was poured into autoclaved flasks. 160µl of Chloramphenicol (Appendix C) was added to the flasks and mixed well. Chloramphenicol allows for the selective growth of our choice of BACs. Approximately 5ml of this medium was poured into separate labelled Nunc tubes, which were inoculated with its labelled BAC. The Nunc tubes were then placed into an orbital incubator (Gallenkamp) at 200rpm at 37°C overnight. The cells were grown until they reached mid-log phase. The cells from the Nunc tubes were then transferred into the flasks containing the remaining LB broth and antibiotic. The cells were grown at 37°C overnight in the orbital incubator (Gallenkamp) at 200rpm.



**Figure 2.2:** Schematic representation of the FHIT gene. The 10 exons are shown and the position of the FRA3B fragile site is indicated. The short pink lines represent regions of highest fragility within FRA3B. The t(3;8) translocation found in familial RCC lies between exons 3 and 4. The position of the probes we obtained are shown. We used BAC 201J24 and BAC 240 C07. These probes are at least 100Kb apart and would allow for the detection of breaks located anywhere within the region between the two probes.

### 2.3.2. Preparation of Glycerol Stocks

850µl of cells in mid-log phase were aliquoted into separately labelled 2ml Cryo tubes containing 150µl of autoclaved glycerol (Appendix C). The tubes were mixed by vortexing well and then stored at -70°C until required. Preparation of glycerol stocks is performed under strict aseptic conditions.

### 2.3.3. DNA extraction from BAC clones using the Qiagen® Plasmid Purification midi kit

BAC DNA was extracted using the Qiagen® Plasmid purification midi kit, according to the manufacturer's specifications.

### 2.3.4. Agarose gel electrophoresis

The concentration of the extracted BAC DNA was determined by comparing the samples to a serial dilution of  $\lambda$  – DNA (Appendix D) on a 2% agarose gel (Appendix D).

## **2.4. DNA labelling**

BAC 240 C07 and BAC 201 J24 were fluorescently labelled with SpectrumOrange™-dUTP (Appendix D) and SpectrumGreen™-dUTP (Appendix D) respectively. The labelling reactions with a total volume of 100µl, contained 10µl of 0.1M  $\beta$  - mercaptoethanol (Appendix D), 10µl of 10X nick translation buffer (Appendix D), 3µl of DNA Polymerase 1 (Appendix D), 1µl of DNase 1 (Appendix D), 2µg of BAC DNA and 8µl of SpectrumOrange™ / SpectrumGreen™ nucleotide stock. The DNase 1 concentration also had to be tested and optimised for each BAC in order to obtain

probe molecules between 200 – 500bp in size, which would give good hybridisation efficiency.

The nucleotide stock comprised 0.5mM each of dATP, dCTP, dGTP, 0.25mM dTTP and 0.25mM SpectrumOrange™ / SpectrumGreen™. The dTTP and SpectrumOrange™ or SpectrumGreen™ were prepared in 50:50 ratio to prevent steric hindrance caused by the large molecular structure of the SpectrumOrange™-dUTP / SpectrumGreen™-dUTP. The labeling reaction was performed in an Eppendorf Master cycler for 2 hours at 15°C.

#### 2.4.1. Evaluation of labelled DNA

The size of the labelled probes were assessed by denaturing an aliquot of each probe at 96°C for 3 minutes prior to loading on a 2% agarose gel (Appendix D). If the probe fragment sizes exceeded 500bp, a further DNase 1 Digestion was performed. Over-digested probes were discarded.

#### 2.4.2. Enzyme inactivation of labelled probe

3µl of 0.5M EDTA (Appendix D) and 1µl of 10% SDS (Appendix D) were added to each labelled probe. Probes were then incubated at 65°C for 15 minutes to inactivate them and prevent further digestion by DNase 1.

#### 2.4.3. Repetitive sequence blocking with Cot 1 Human DNA and precipitation of labelled probe

Inactivated probe was purified in order to separate out labelled DNA from unincorporated nucleotides. 1µg / 1µl of Cot 1 DNA (Appendix D) was added to the

labelled probe in an eppendorf tube. 20µg of Cot 1 DNA was used, which is ten fold more than that of the probe, and blocks any repetitive sequences and prevents non-specific hybridisation of the probe. One-tenth Sodium acetate (Appendix D) was added together with two-and-a-half times the volume of 100% ethanol (Appendix D).

The eppendorf tubes were then placed at -70°C for 30 minutes prior to centrifugation at 13 000rpm for 30 minutes at 4°C. The supernatant was then removed and 300µl of 70% ethanol (Appendix D) was added to each tube and centrifuged at 13 000rpm for 10 minutes at 4°C. The supernatant was discarded and the eppendorf containing the DNA pellet was left to air-dry for approximately 10 minutes. Each DNA pellet was then reconstituted in 60µl (which contains approximately 2µg of BAC DNA) of Hybridisation buffer (Appendix D).

## **2.5. Hybridisation**

10µl of each probe (BAC 201 J24 and BAC 240 C07) was added to an eppendorf tube. The probe was then denatured at 96°C for 10 minutes and then pre-annealed at 37°C for 30 minutes in an Eppendorf Master cycler and then kept at 4°C until the slides were ready. The slides were denatured in denaturing buffer (Appendix D) for 5 minutes at 76°C. The formamide in the denaturing solution allows for a lower denaturation temperature when denaturing the slides to preserve cell morphology. The slides were then dehydrated by passing them through an alcohol series of 70 – 90 - 100% ice-cold ethanol (Appendix D) for 5 minutes each. Slides were air dried before placing the probe on a designated area on the slide and covered by a cover slip. The cover slip was cemented down with glue (Appendix D) to prevent dehydration and allowed to dry. The slides were placed in a damp incubator at 37°C overnight

## **2.6. Post Hybridisation washes**

The slides were washed in 3 sets of 50% formamide at 42°C for 10 minutes each. The slides were then washed in 2X SSC for 10 minutes and then Tween for 5 minutes, both at 42°C.

## **2.7. Counterstaining**

Slides were placed in DAPI (Appendix D) solution for 15 minutes at room temperature. Slides were then washed for 5 minutes in Tween (Appendix D). A drop of anti-fade solution (Appendix D) was placed onto each slide before a cover slip was applied. The cover slip was cemented down with glue (Appendix D).

## **2.8. Microscope analysis**

The slides were screened under a fluorescence microscope. The fluorescence microscope has three filters, which allow visualisation of the three fluorescent signals, red, green and blue. The red filter allows visualisation of the SpectrumOrange™, the green filter allows visualisation of SpectrumGreen™ and the DAPI filter allows DAPI to be visualised. DAPI is a counter stain and stains all nuclear material blue.

The probes chosen namely BAC 201 J24 (telomeric) and BAC 240 C07 (centromeric) flank the FRA3B fragile site. When the probes bind to their target site on the chromosome they can be visualised as red (in the case of BAC 240 C07) signals or green (in the case of BAC 201 J24) signals. In some instances for example when the chromosomes are very condensed the probes may bind very close to each other and then fluoresce yellow – this is a normal signal as well. When a break occurs in the

region between the probes, the red and green signals are visualised far apart from each other and therefore these probes are known as break apart probes.

50 metaphases / slide were analysed. Images were acquired using the Genus computer programme (Applied Imaging).

## **2.9. Statistical analysis**

The Analysis of Variance (ANOVA) technique was used to determine the differences between the effects of **smoking** and **non-smoking** on each specimen **N**, **A**, and **F**. Since the ANOVA technique is based on the assumption of normal distribution of the data a non-parametric test of normal distribution of the data was also performed.

The null hypothesis is therefore:

$$\mathbf{H}_0: \mu_1 = \mu_2 = \mu_3 = \mu_4 = \mu_5$$

Where  $\mu_1$  refers to for e.g. smoker specimen N or non-smoker specimen N

The variables under consideration are said to be significantly different at the 5% level of significance if the p-value / Pr > F value is less than 0,05 otherwise; the variables are not significantly different.

## **2.10. Expression analysis**

RNA was extracted from each sample using the QIAamp® RNA Blood Mini Kit as per manufacturers instructions.

### 2.10.1. Evaluation of extracted RNA

Extracted RNA was run on a denaturing gel (Appendix D) to evaluate whether RNA was intact after extraction. Extracted RNA was labelled and stored at -70°C until use.

### 2.10.2. RT-PCR

RT-PCR was performed on each sample using the QIAGEN® OneStep RT-PCR kit as per manufacturers instructions using primer sets, designed in our laboratory, for *GAPDH*, *FHIT* and *WVOX*. (Table 2.1) *GAPDH* is amplified as a control.

**Table 2.1:** Primer sets (forward and reverse) for *FHIT*, *WVOX* and *GAPDH*.

<b>GENE</b>	<b>PRIMERS</b>	<b>PRIMER BINDING</b>	<b>MANUFACTURER</b>
<i>FHIT</i> (987bp)	<b>F 5' – 3'</b> CTTTTGGCCCTCTGTCCCG <b>R 5' – 3'</b> TGCCTGTCTGAGCCGTTTAGGTC	Exon 1 Exon 10	MWG Biotech (F) Genosys (R)
<i>WVOX</i> (983bp)	<b>F 5' – 3'</b> GAGTTCCTGAGCGAGTGGA <b>R 5' – 3'</b> GCTCGTTGGAGAAGAGGAT	Exon 1 Exon 9	Inqaba Biotec
<i>GAPDH</i> (589bp)	<b>F 5' – 3'</b> CCCTTCATTGACCTCAACTACATG <b>R 5' – 3'</b> CATGCCAGTGAGCTCCCGTTCAG		Inqaba Biotec

Reactions were performed on an Eppendorf Master Cycler as follows:

1. *FHIT*: 50°C for 30 minutes (reverse transcription step), initial denaturation of 95°C for 15 minutes then 35 cycles of amplification: 95°C for 1 minute (denature), 62,5°C for 1 minute (annealing) and 68°C for 3 minutes (extension). The final extension was at 68°C for 7 minutes. All PCR products were stored at -20°C until being run on a 2% agarose gel (Appendix D).

2. *WVOX*: 50°C for 30 minutes (reverse transcription step), initial denaturation of 94°C for 15 minutes then 30 cycles of amplification: 94°C for 1 minute (denature), 66°C for 1 minute (annealing) and 72°C for 1 minute (extension). The final extension was at 72°C for 7 minutes. All PCR products were stored at -20°C until being run on a 2% agarose gel (Appendix D).
3. *GAPDH*: 50°C for 30 minutes (reverse transcription step), initial denaturation of 94°C for 15 minutes then 30 cycles of amplification: 94°C for 1 minute (denature), 56°C for 1 minute (annealing) and 72°C for 1 minute (extension). The final extension was at 72°C for 7 minutes. All PCR products were stored at -20°C until being run on a 2% agarose gel (Appendix D).

### **2.11. DNA studies**

A third tube of blood was drawn from each individual and batch stored at 4°C for future DNA studies.

### **3. CHAPTER 3 - RESULTS**

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#### **3.1. Lymphocyte cultures**

All cultures grew well with the exception of Control 6, which failed to yield any metaphases. This sample will therefore be excluded from the study. It was noted that out of the three sets of culture conditions:

1. Normal cultures grew the best
2. Aphidicolin cultures grew well, however the chromosomes did not look as healthy as the Normal cultures, which was to be expected.
3. Folate deficient cultures grew generally well in most cases, yielding far fewer metaphases than the Normal and Aphidicolin cultures, which was also to be expected.

#### **3.2. DNA extraction from BAC clones using the Qiagen® Plasmid Purification Midi kit**

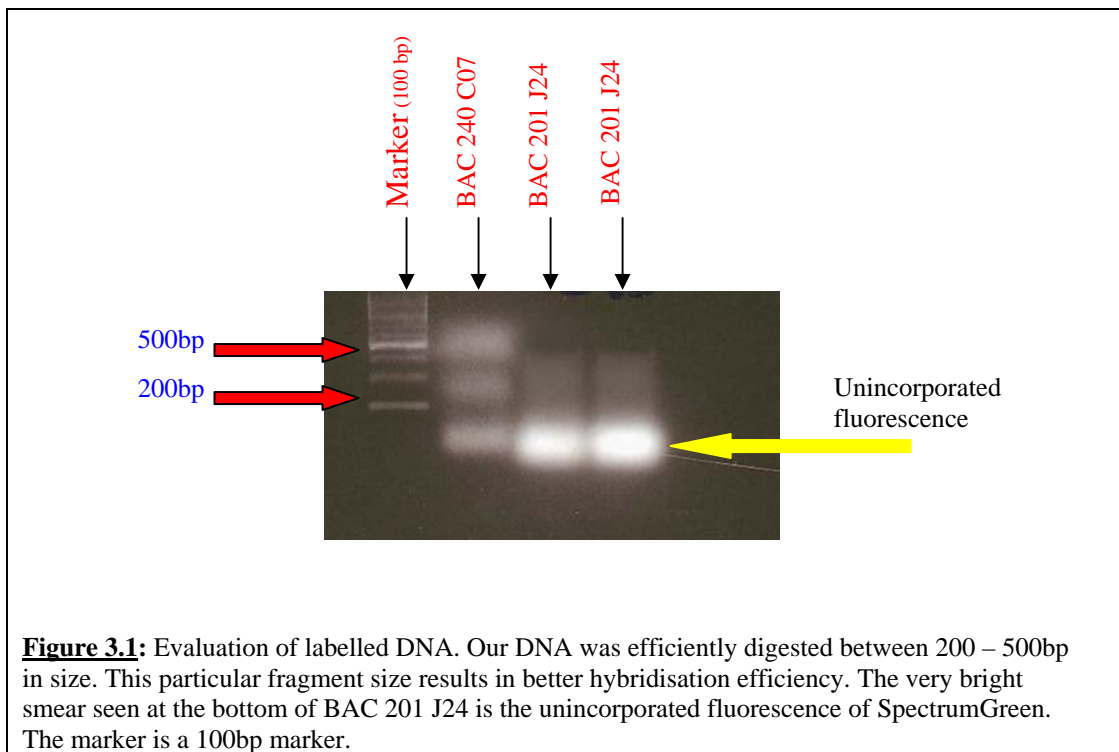
Good concentrations of BAC DNA, namely BAC 201 J24 and BAC 240 C07, were yielded using this kit.

#### **3.3. DNA labelling**

To label our DNA we had to optimise the Nick Translation method of DNA labelling, using SpectrumOrange™-dUTP and SpectrumGreen™-dUTP which directly fluorescently labels BAC DNA. A 50:50 ratio of dTTP and SpectrumOrange™ / SpectrumGreen™ prevented steric hindrance caused by the large molecular structure of the SpectrumOrange™-dUTP / SpectrumGreen™-dUTP. All reactions were performed in an Eppendorf Mastercycler, which kept the

temperature at a constant 15°C and we therefore obtained good concentrations of labelled DNA, which were digested efficiently.

### 3.3.1. Evaluation of labelled DNA



**Figure 3.1:** Evaluation of labelled DNA. Our DNA was efficiently digested between 200 – 500bp in size. This particular fragment size results in better hybridisation efficiency. The very bright smear seen at the bottom of BAC 201 J24 is the unincorporated fluorescence of SpectrumGreen. The marker is a 100bp marker.

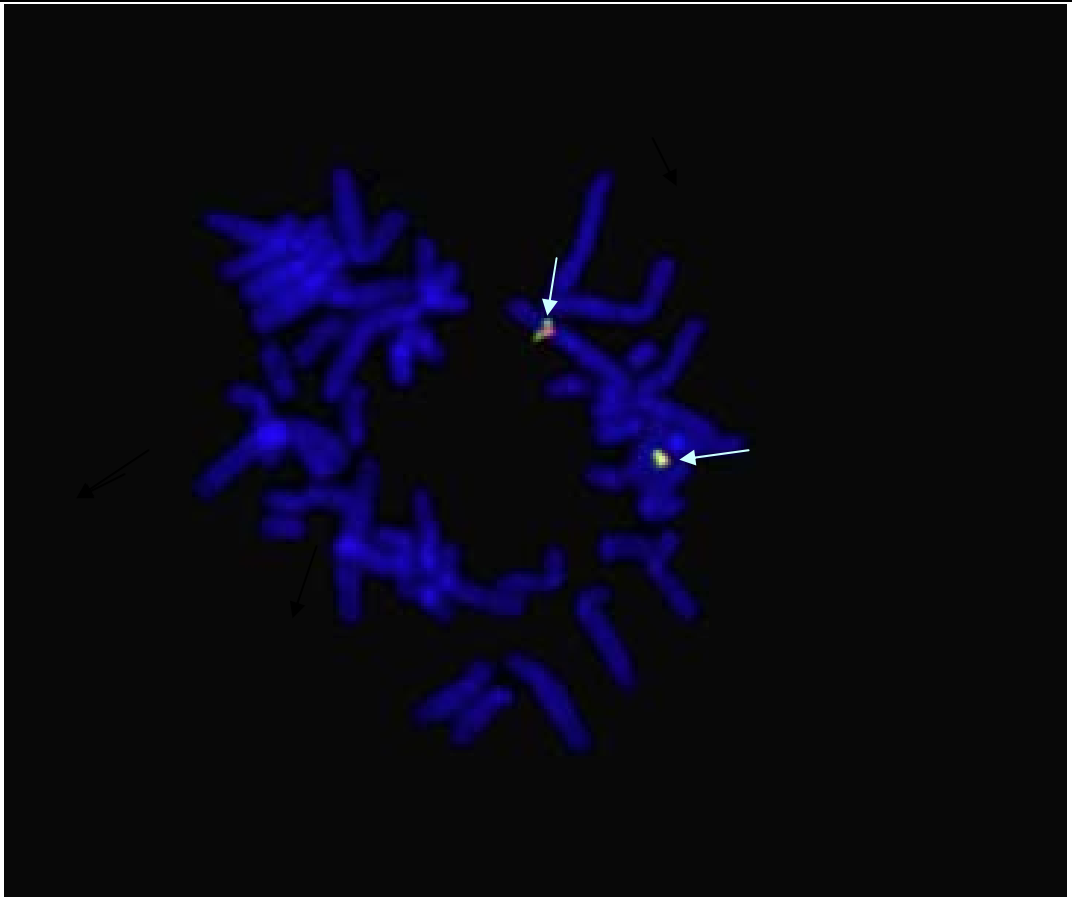
BAC 201 J24 was labelled with SpectrumGreen™-dUTP and BAC 240 C07 was labelled with SpectrumOrange™-dUTP.

### 3.4. Hybridisation

Hybridisation conditions had been previously optimised. Since directly labelled probe was hybridised to the slides there was no need for Immunodetection. The directly labelled probes hybridised well to all slides.

### **3.5. Microscope analysis**

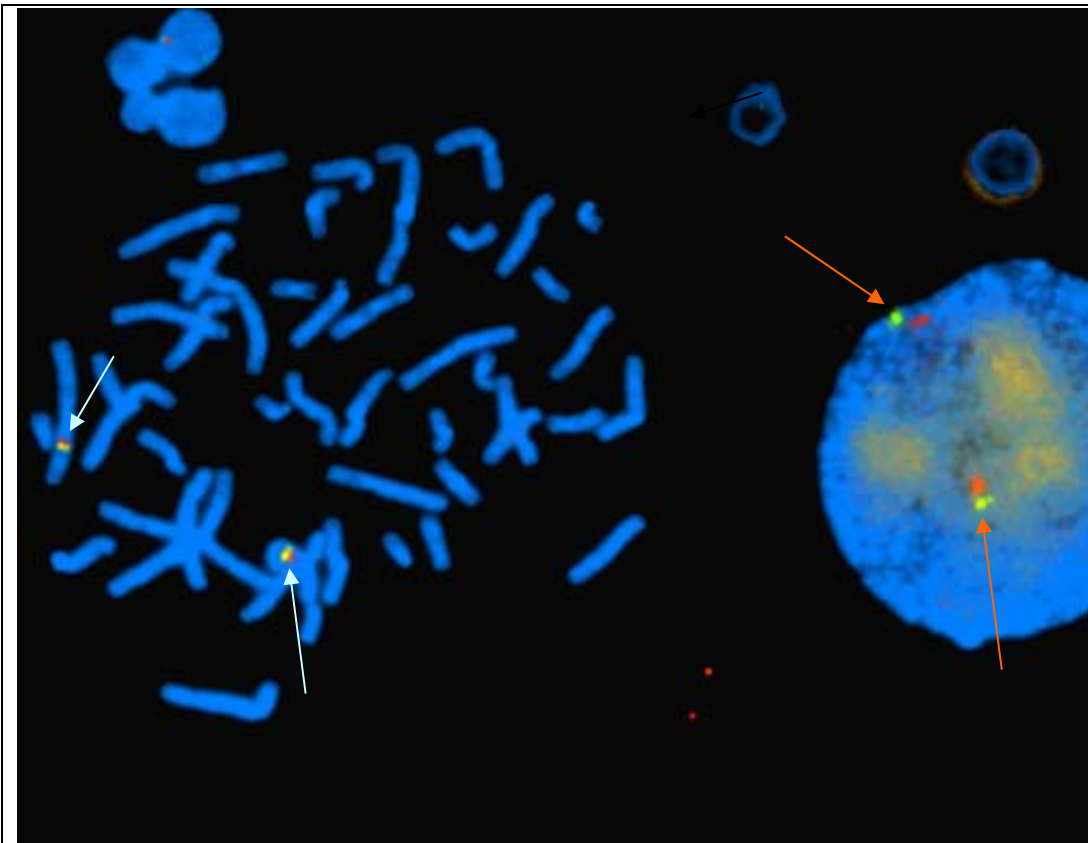
All slides were screened under a fluorescence microscope. 50 metaphases / slide were analysed and images were acquired using the Genus computer program (Applied Imaging). Refer to Figures 3.2 to 3.9 for results of FISH experiments.



**Figure 3.2:** Metaphase chromosomes of a control individual (C1) showing normal signals - from an **Aphidicolin** stimulated culture. The red and green signals (white arrows) are very close if not superimposed, which is seen as a yellow signal. This is due to the fact that the chromosomes are condensed and thicker when in metaphase. This is a normal signal.

BAC 240 C07 – Red signals

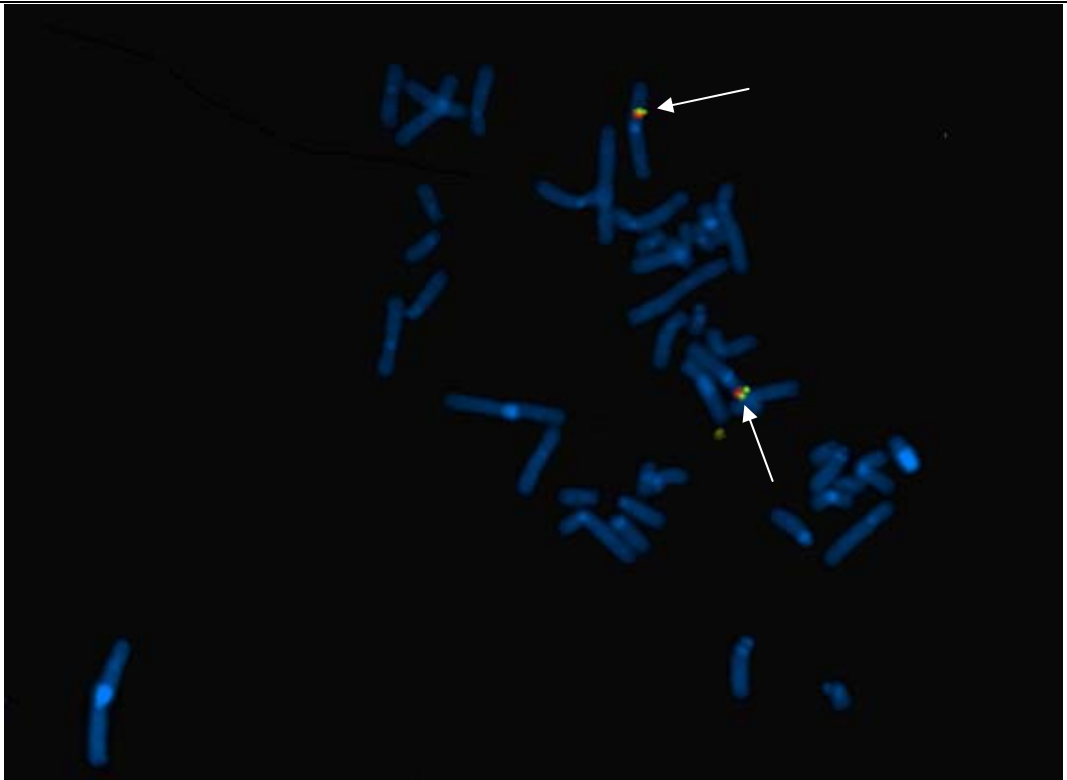
BAC 201 J24 – Green signals



**Figure 3.3:** Metaphase chromosomes of a smoking individual (SM16) showing normal signals - from an **Aphidicolin** stimulated culture. The red and green signals are very close if not superimposed, which is seen as a yellow signal (white arrows). This is a normal metaphase from an **Aphidicolin** stimulated culture from a smoking individual. The interphase cell on the right also has normal signals (orange arrows).

BAC 240 C07 – Red signals

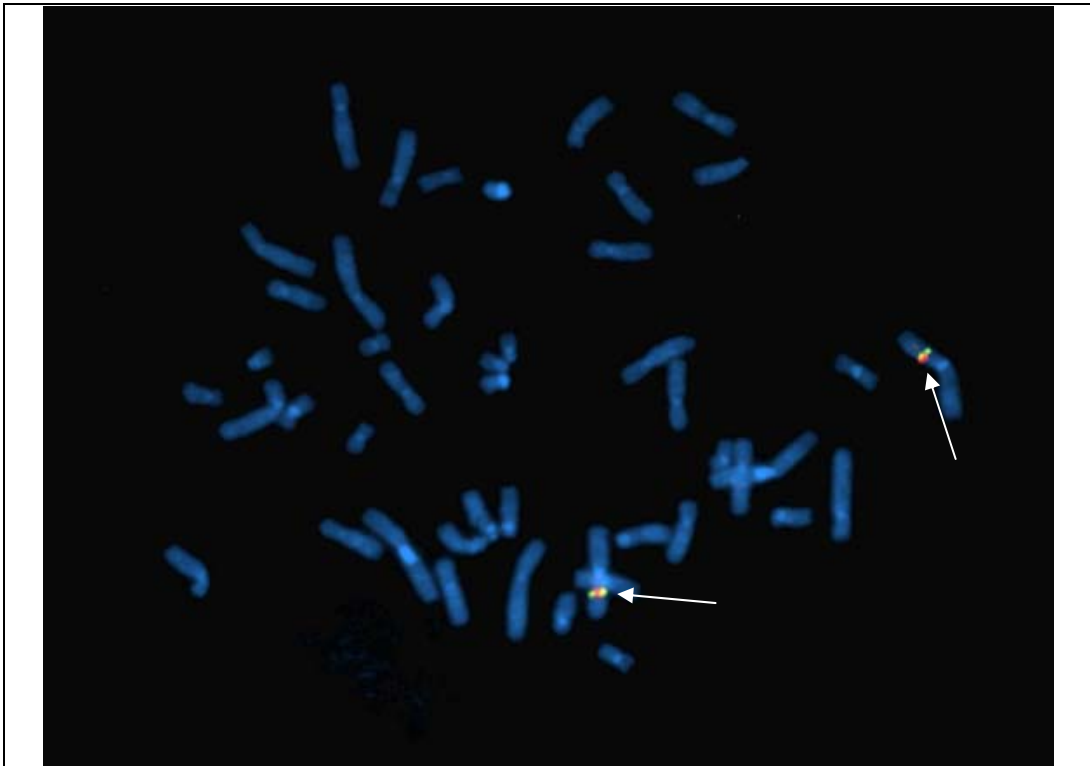
BAC 201 J24 – Green signals



**Figure 3.4:** Metaphase chromosomes of a smoking individual (SM17) showing normal signals - from a **Normal** culture. The red and green signals can each be seen and are very close together (white arrows). This is a normal signal.

BAC 240 C07 – Red signals

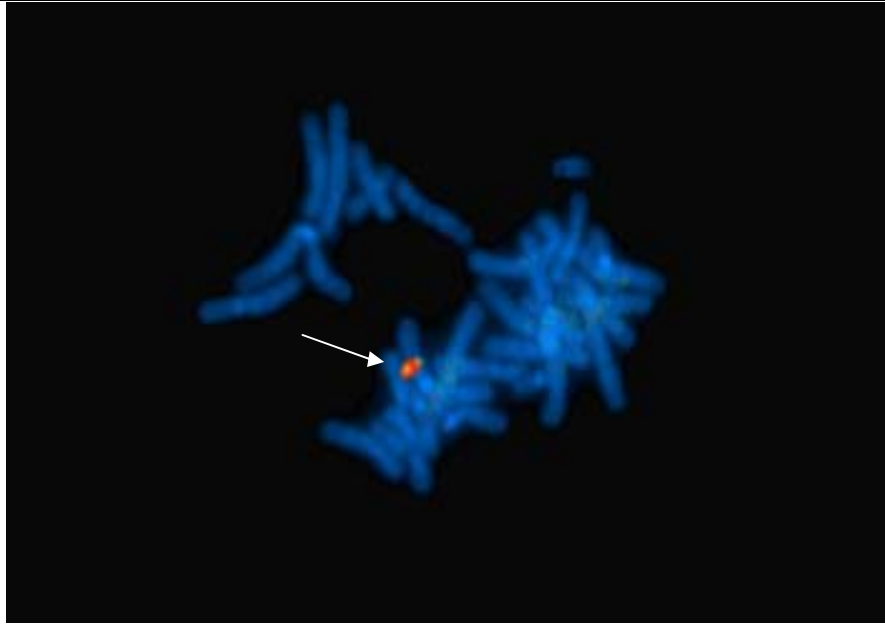
BAC 201 J24 – Green signals



**Figure 3.5:** Metaphase chromosomes of a smoking individual (SM20) showing normal signals - from a **Normal** culture. The red and green signals are very close if not superimposed, which is seen as a yellow signal (white arrows). This is due to the fact that the chromosomes are condensed and thicker when in metaphase. This is a normal signal.

BAC 240 C07 – Red signals

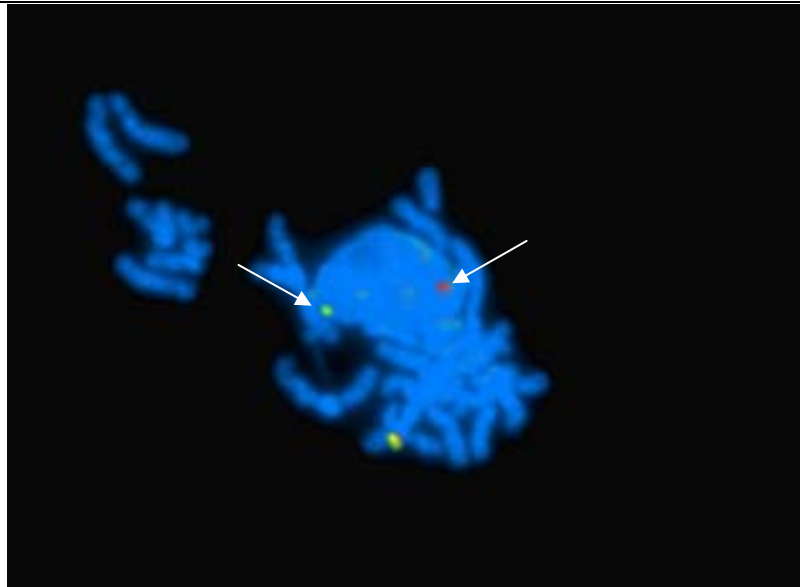
BAC 201 J24 – Green signals



**Figure 3.6:** Metaphase chromosomes from a smoking individual (SM14) showing a full deletion of one set of signals - from a **Normal** culture. There is only one set of signals present (white arrow). The red and green signals are very close together indicating that the signals present are normal; however, the other set is missing. We cannot ascertain whether, in the missing signal, that the portion to which the probe binds is absent or whether a full copy of chromosome 3 is deleted.

BAC 240 C07 – Red signals

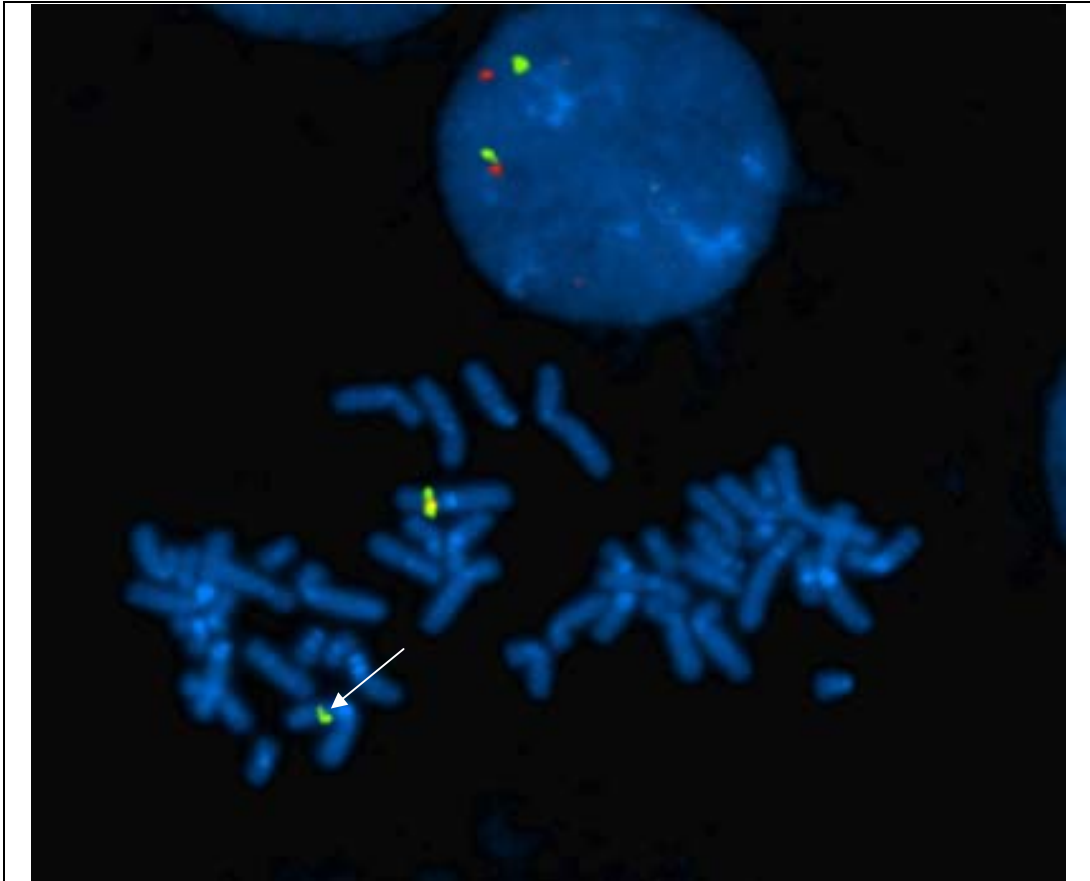
BAC 201 J24 – Green signals



**Figure 3.7:** Metaphase chromosomes from a smoking individual (SM16) showing a split signal - from a **Normal** culture. Both sets of signals are present, however, one set is split apart (white arrows). This indicates that there is a break somewhere in the region between where the probes have bound.

BAC 240 C07 – Red signals

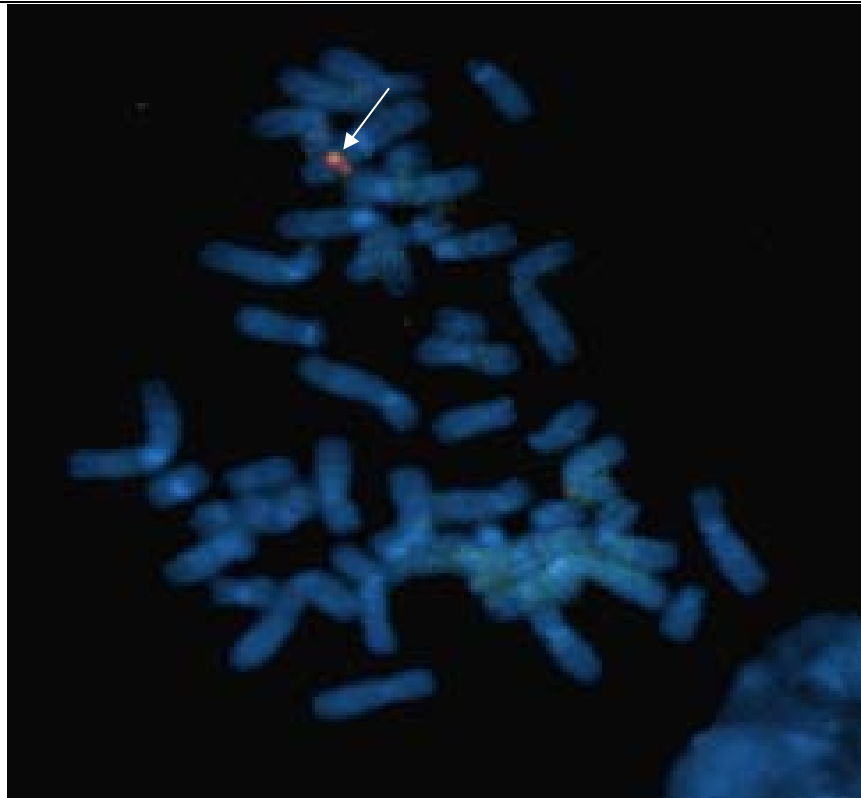
BAC 201 J24 – Green signals



**Figure 3.8:** Metaphase chromosomes from a smoking individual (SM19) showing a missing red signal - from an **Aphidicolin** stimulated culture. One set of signals is normal and the other set has only green signals (white arrow). The red signal is missing and could be due to deletion of that area on the chromosome.

BAC 240 C07 – Red signals

BAC 201 J24 – Green signals



**Figure 3.9:** Metaphase chromosomes from a smoking individual (SM4) showing one red signal only - from a **Normal** culture. Only one red signal is present (white arrow). The other red and both other green signals are absent.

BAC 240 C07 – Red signals

BAC 201 J24 – Green signals

Break apart probes are designed to detect breaks / split signals occurring between the probes (explained in materials and methods 2.8 Microscope analysis). We did not in general find many split signals. However, we did find more signals falling into the “Other” category (Appendix E). The most prominent being a higher incidence of a full deletion of one set of probes in active smokers especially in A and F<sup>-</sup> cultures, with F<sup>-</sup> cultures showing the most deletions (data not shown).

### 3.6. Statistical analysis

Results of the microscope analysis (Appendix E) were statistically analysed using the ANOVA technique. **Table 3.1** displays the results of the test of normal distribution of the data (for aberrations at *FHIT* loci – see Appendix E) for each of the three culture conditions separately for smokers and non-smokers. When this technique is applied, the assumption of a normal distribution is rejected at the 5% level of significance if the p-value is less than 0,05.

**Table 3.1:** Test of normal distribution of the data.

Smoking	Culture condition	n	Mean	STD	Skewness	Median	P Value
Non Smoker	A	9	5.11	4.91031	1.38	4	0.14102
	F	9	5.33	6.63325	2.35	4	0.00139
	N	9	5.78	3.07318	0.24	6	0.30762
Smoker	A	20	5.70	3.85391	0.09	6	0.10789
	F	20	5.20	5.04297	0.99	4	0.02446
	N	20	4.60	2.25715	-0.174	4	0.11734

In **Table 3.1** we can see that the assumption of normality for this data is only rejected for culture F, irrespective of whether the patient was a smoker or not. (The p-value being less than 0,05 for F smoker and F non-smoker). Since non-normality was detected for only one culture condition, the data was roughly assumed to be normally distributed overall, and the analysis of variance was applied.

**Table 3.2** and **3.3** show the results of the analysis of variance. **Table 3.3** shows that in general none of the two factors (smoking and culture condition) had a significant effect (Pr>F value is not less than 0,05 for smokers and non-smokers). Therefore there is no significant difference at the 5% level between smokers and non-smokers nor is there any significant difference between the culture conditions (N, A and F) at the 5% level.

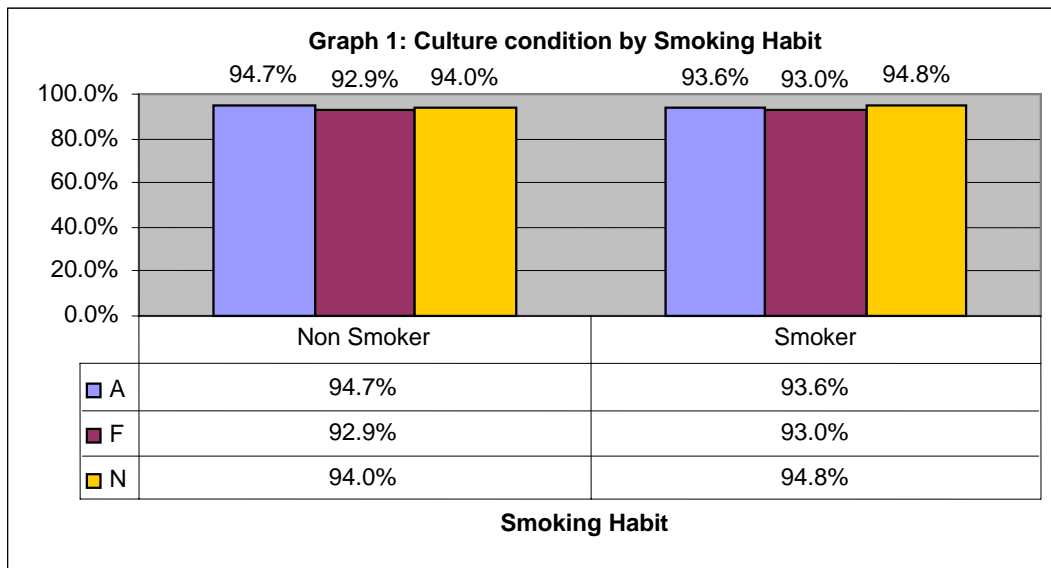
**Table 3.2:** Class level of information.

<b>Class</b>	<b>Levels</b>	<b>Values</b>
<b>Smoking</b>	2	Non-Smoker; Smoker
<b>Culture condition</b>	3	A; F; N

**Table 3.3:** Analysis of Variance

<b>Source</b>	<b>DF</b>	<b>Sum of Squares</b>	<b>Mean Square</b>	<b>F Value</b>	<b>Pr &gt; F</b>
<b>Model</b>	3	5.492976	1.830992	0.10	0.9588
<b>Error</b>	83	1492.438059	17.981181		
<b>Corrected Total</b>	86	1497.931034			

<b>Source</b>	<b>DF</b>	<b>Type I SS</b>	<b>Mean Square</b>	<b>F Value</b>	<b>Pr &gt; F</b>
<b>Smoking</b>	1	1.07918263	1.07918263	0.06	0.8071
<b>Culture condition</b>	2	4.41379310	2.20689655	0.12	0.8847



**Figure 3.10:** Graph of percentages of normal metaphases seen in each culture condition in smokers and non-smokers.

**Figure 3.10** shows the numbers / percentages of normal metaphases in cultures N, A and F among smokers and non-smokers. The remaining percentages are therefore made up of split signals and / or other aberrant signals (Appendix E). As seen in the graph the incidences between the two groups are roughly equal, that is there is no significant difference between each group. We had expected to see lower levels of normal metaphases in each N, A and F in smokers. By increasing the sample size and selecting for heavier smokers one may find a significant difference between the two. This remains future work.

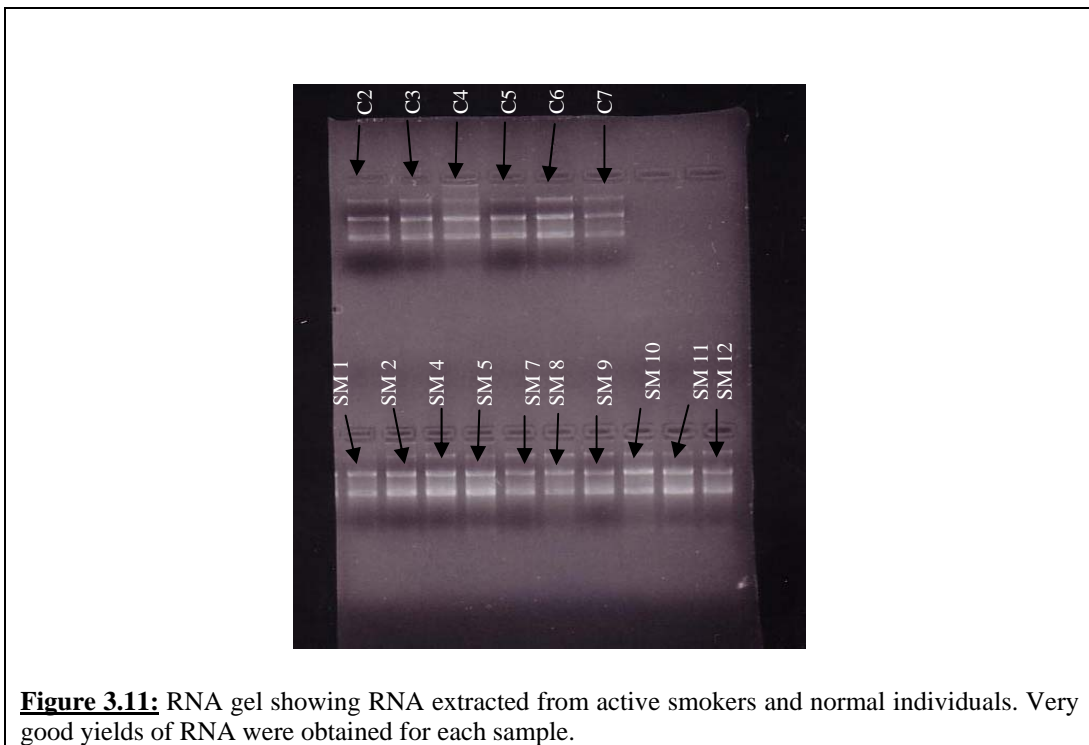
Using the combination of BAC 201 J24 and BAC 240 C07 we did not find a significant difference between active smokers and non-smokers (controls) and the different culture conditions, namely N, A and F-.

### 3.7. Expression analysis

The QIAamp® RNA Blood Mini Kit was used to extract RNA from each sample and very good yields were obtained using the kit.

#### 3.7.1. Evaluation of Extracted RNA

Extracted RNA was run on a denaturing gel and looked as follows:

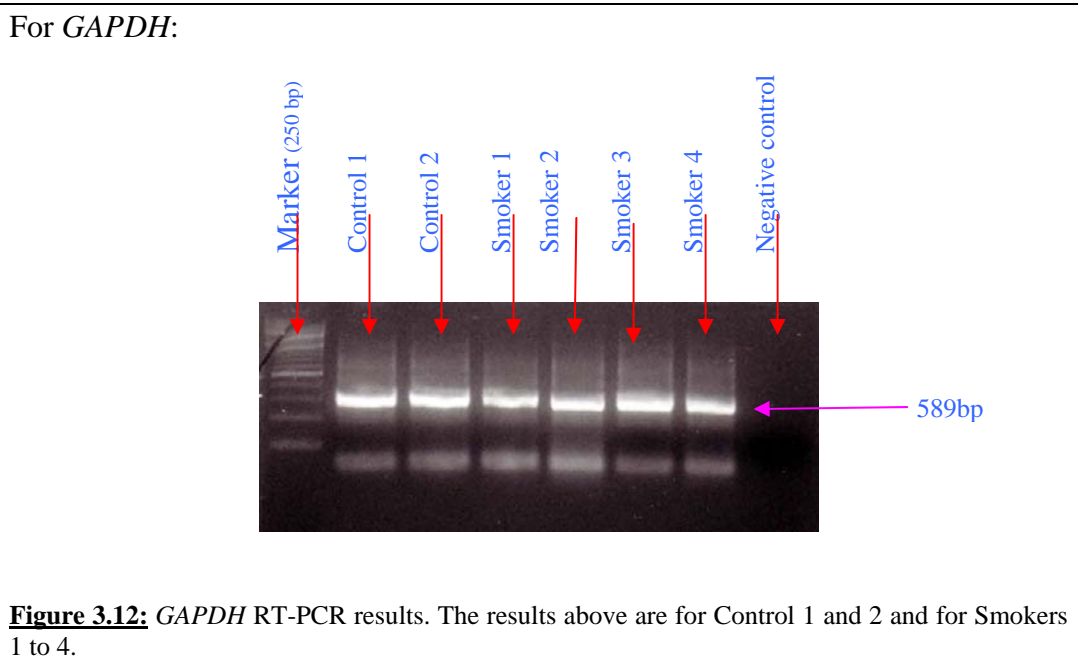


**Figure 3.11:** RNA gel showing RNA extracted from active smokers and normal individuals. Very good yields of RNA were obtained for each sample.

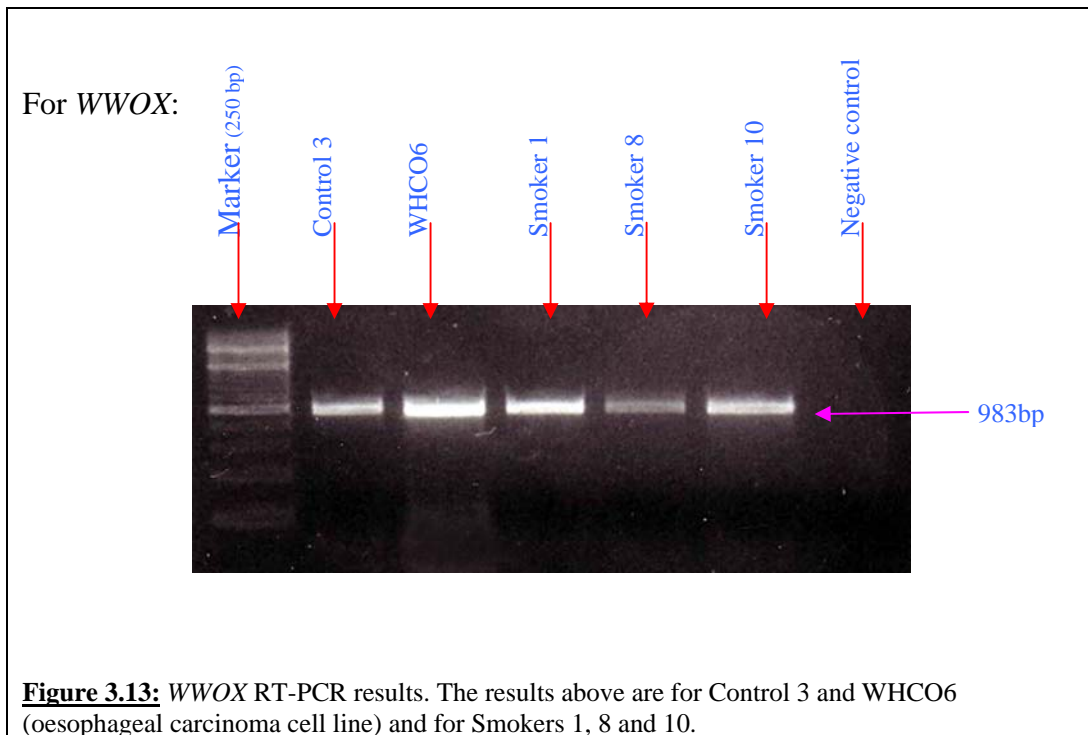
#### 3.7.2. RT-PCR

The QIAGEN® OneStep RT-PCR kit was used to perform the RT-PCR reactions. We looked at the expression of *FHIT* and *WWOX*, with *GAPDH* (which is constitutively expressed) being our control.

The results of our PCR's were as follows:

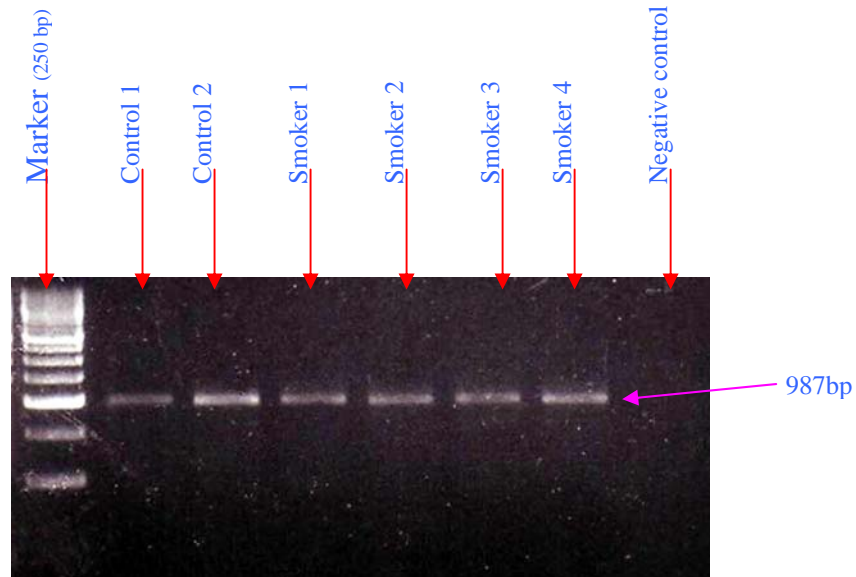


The intensity of the bands is very high indicating that *GAPDH* is expressed in high amounts. The results shown above were the same for every sample (Product band at 589bp).



WHCO6 was included as a positive control in this case. No aberrant transcripts were observed and the results shown above were the same for every sample (Product band at 983bp). The intensity of these bands is not as high as those seen in *GAPDH*, indicating that *WWOX* is not as highly expressed as *GAPDH*. One cannot say from the results above whether for e.g. the expression of *WWOX* in smoker 8 is lower than that of smoker 10. One would need to perform a quantitative PCR in order to determine this.

For *FHIT*:



**Figure 3.14:** *FHIT* RT-PCR results. The results above are for Control 1 and 2 and for Smokers 1 to 4.

No aberrant transcripts were observed and the results shown above were the same for every sample (Product band at 987bp). The intensity of these bands is not as high as those seen in *GAPDH*, indicating that *FHIT* is not as highly expressed as *GAPDH*. In addition, the expression of *FHIT* is lower than that of *WWOX*. One cannot say from the results above whether for e.g. the expression of *FHIT* in control 1 is lower than that of control 2. One would need to perform semi-quantitative PCR in order to determine this.

## **4. CHAPTER 4 – DISCUSSION AND CONCLUSION**

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### **4.1. Discussion And Conclusion**

Considerable evidence suggests that cancer has a congenital or acquired genetic basis, as evidenced by the development of genomic alterations, such as deletions, translocations, mutations, and / or methylation in critical genes for homeostasis and cellular functions (including cell cycle control, survival, and DNA replication). Genomic alterations that inactivate tumour suppressor genes or activate oncogenes promote tumour growth. Current studies of the molecular basis of cancer provide a strong rationale to consider gene therapy approaches to cancer as a method to target genes at play in cancer<sup>84</sup>.

The characterisation of a chromosome translocation associated with familial renal cancer, the discovery of aphidicolin-inducible constitutive fragile sites and the observation that these fragile sites coincide with sites of frequent, specific cancer-associated chromosome rearrangements have all contributed greatly to our understanding of the possible role of common fragile sites in the development of cancer. There is still much to learn about how these conserved weak links contribute to chromosome structure, evolution and tumourigenesis<sup>35</sup>.

The integrity of fragile sites within tobacco associated carcinogen exposure was investigated using FISH in active smokers and control individuals. FISH is a method of *in situ* hybridisation that utilises probes labelled with a fluorescent tag, causing the site of hybridisation to fluoresce when viewed under the fluorescent microscope<sup>4</sup>. FISH was developed in the 1980s and represented a major advance in

chromosome analysis. The method is highly sensitive and specific and the speed at which the assays can be performed has made FISH a powerful tool with a number of different applications. A variety of strategies have been developed for the detection of recurring translocations in interphase cells that take advantage of the ability to detect hybridised probes using multiple fluorochromes<sup>85</sup>.

In the present study FISH was used as a flag – the rationale to use FISH was to detect rearrangements and breaks at the *FHIT* and *WWOX* loci on a cell-to-cell basis. This could then be used as a flag: to reflect on the percentage of cells whose fragile site loci are affected and could in turn impair gene expression within these loci. It was therefore predicted that FISH would detect the number of aberrations and RT-PCR was used in conjunction to assess the expression of these genes.

However, and since no significant differences could be surfaced in this study, there is a strong need to compliment the present study with both additional pre-selected heavy smokers as well as semi-quantitative Real time RT-PCR analysis.

We used the BAC combination of BAC 201 J24, directly labelled with SpectrumGreen™, and BAC 240 C07 directly labelled with SpectrumOrange™ for detection. These two probes are on either side of the *FHIT* / *FRA3B* region and are approximately 100Kb apart, which would allow for the detection of breaks or a break-apart anywhere in the 100Kb region between the two. **Figure 2.1.**

The limitations of this probe system are that small internal deletions in between the two BACs would not be detected. Breaks of chromatids occurring between the two BACs but not separating them are also difficult to visualise.

Within these limitations and using this combination of probes, we did not find a significant difference between active smokers and non-smokers (controls) in the different culture conditions, namely N, A and F<sup>-</sup>. We did however; find a higher incidence of a full deletion of one set of probes in active smokers especially in A and F<sup>-</sup> cultures with F<sup>-</sup> cultures showing the most deletions (data not shown). Since these observations are not statistically significant the study will be reinforced by completing the study with at least ten additional heavy smokers.

The ANOVA test was used to statistically analyse the data obtained in this study. This test analyses the differences between variances namely active smoker and non-smoker and culture conditions N, A and F<sup>-</sup>. These findings differ from those reported by Stein *et al* (2002). This could be due to the following:

- The sample size was insufficient. Only 10 non-smokers (controls) and 20 active smokers were analysed. Stein *et al* (2002) had 33 subjects in all, including 12 smokers, 11 non-smokers and 10 patients (newly diagnosed small cell lung cancer patients who had discontinued smoking 3 weeks to 25 years). The larger the sample size the more significant the results shall be. Analysis of more samples from active smokers may clarify this.
- Selection of heavy smokers needs to be more stringent. Light, moderate and heavy active smokers were included in this study. Analysis of more

heavy smokers (>20 Pkt / Year) should also make the study more significant.

We are currently recruiting additional active heavy smokers (minimum 5 Pkt / Year), which should make the study more statistically significant.

The full deletion of one set of probes mentioned earlier could result from that portion of chromosome 3 being deleted or the entire chromosome 3 having been deleted. One way to assess this in the future would be to perform the FISH experiments using an additional Cep3 control probe. Cep3 binds to the centromere of chromosome 3 and this would therefore allow you to visualise whether a complete chromosome 3 is missing or just a portion of it.

RT-PCR was performed on every sample using primers for *FHIT*, *WWOX* and *GAPDH*, (which is a control as it is constitutively expressed). Normal transcripts were found in both *FHIT* and *WWOX*. However, their expression is lower than that of *GAPDH*, with *FHIT* showing the lowest expression. Refer to **Figures 3.11, 3.12 and 3.13**. Semi-quantitative RT-PCR would clarify this observation. Specifically, Real time RT-PCR remains one of our future additions to this project.

#### **4.2. Future Work**

Additional work to be carried out after submission of this dissertation is as follows:

- ✚ Additional active smokers shall be recruited and FISH performed on each new specimen in order to increase the sample size, (specifically by ten).
- ✚ Only heavy (15 – 20 cigarettes / day) active smokers shall be recruited.
- ✚ Metaphases from active smokers and non-smokers (controls) shall be stained with Wrights stain and analysed under light microscopy for metaphase breaks, gaps and telomere association. This should help shed more light as to what is happening under each culture condition for active smokers and non-smokers (controls).
- ✚ Semi-quantitative Real time RT-PCR shall be performed on each sample for *FHIT* and *WWOX* expression. Traditional PCR is not sensitive enough to detect low-abundance transcripts.



**Table A2:** Results of information collected from Control as well as Smoking individuals. Culture Control C6 failed to grow and was therefore excluded.

Light smoker: 1 – 5 cigarettes per day.

Moderate: 5 – 15 cigarettes per day.

Heavy smoker: 15 or more cigarettes per day.

<b>Smoker</b>	<b>Age</b>	<b>Length of time smoking</b>	<b>Light/Moderate/Heavy</b>		
C1	49	NA	NA		
C2	29	NA	NA		
C3	40	NA	NA		
C4	32	NA	NA		
C5	33	NA	NA		
<b>C6</b>	<b>Failed</b>				
C7	29	NA	NA		
C8	29	NA	NA		
C9	29	NA	NA		
C10	32	NA	NA		
SM1	56	30 years	Heavy		
SM2	38	15 years	Heavy		
SM3	58	40 years	Light		
SM4	55	15 years	Heavy		
SM5	50	15 years	Heavy		
SM6	43	20 years	Moderate		
SM7	37	12 years	Moderate		
SM8	46	20 years	Heavy		
SM9	31	10 years	Light		
SM10	55	20 years	Light		
SM11	32	5 years	Heavy		
SM12	28	15 years	Heavy		
SM13	38	7 years	Moderate		
SM14	48	24 years	Moderate		
SM15	64	45 years	Heavy		
SM16	32	10 years	Heavy		
SM17	33	16 years	Heavy		
SM18	29	10 years	Moderate		
SM19	48	16 years	Moderate		
SM20	40	13 years	Heavy		

## APPENDIX B

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**RPMI 1640** (Sigma®)

**Folate deficient RPMI 1640** (Sigma®)

**10% Foetal bovine serum** (Gibco BRL)

500ml Foetal bovine serum (Gibco BRL)

Deactivated for 2 hours at 60°C

Aliquoted into 50ml Nunc tubes.

50ml added to 450ml RPMI medium

**Aphidicolin** (Sigma®)

**Penicillin / Streptomycin** (Gibco BRL)

100ml Penicillin / Streptomycin (Gibco BRL)

Aliquoted into 5ml tubes

5ml added to 450ml RPMI medium

**Phytohaemagglutinin (PHA)** (Sigma®)

1mg per ml of Sabax water

Stable for 1 month at 4°C and 6 months at -20°C

**Amethopterin (MXT)** (Sigma®)

0.1ml of 2.5mg/ml MXT stock solution

9.9ml of sterile water

Add 1ml of this solution to 9ml of sterile water

(Final concentration is 0.25µg / 5ml culture)

**Thymidine** (Sigma®)

Stock: 4.8mg in 10ml sterile medium – lasts one week.

Make a 100X dilution of stock

For 20 cultures – 1ml of stock in 99ml of medium

**Colcemid** (Gibco BRL)

10µg / ml stock solution

**0.075 M KCl** (Saarchem)

2.8g KCL

500ml distilled water

Incubate at 37°C

**Fixative**

3 parts Methanol (BDH)

1 part Glacial Acetic acid (Merck®)

Keep ice-cold

Slides are cleaned in Methanol (BDH) and then rinsed in distilled water.

Slides are dehydrated in Ethanol (BDH) once the sample has been applied to the slide.

## **APPENDIX C**

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BACs were originally received from genomic library resources.

BAC clones obtained:

BAC 201 J24

BAC 240 C07

### **LB Top agar – BAC growth medium**

10g Bacto® tryptone (Difco)

5g Bacto® yeast extract (Difco)

5g NaCl (SMM Chemicals)

Fill up to 1 litre with distilled water

Autoclave

**Chloramphenicol** (Sigma®) 20µg / ml

### **GLYCEROL SOLUTION (For freezing and storage of BAC cultures)**

- 65% Glycerol (vol / vol) (Merck®)
- 0.1M MgSO (Merck®)
- 0.025M Tris.Cl, pH 8 (Merck®)

### **Preparation of frozen stock cultures**

- Add 850µl of mid-log phase culture to 150µl of glycerol solution (autoclaved) in a Cryo tube
- Mix well by vortexing
- Store at -70°C

## **APPENDIX D**

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### **BUFFERS AND SOLUTIONS**

#### **BUFFERS FOR GEL ELECTROPHORESIS**

##### **2% Agarose gel**

- 2g Agarose (Gibco BRL)
- 100ml 1X TAE buffer

**Ethidium bromide** (Boehringer Mannheim®)

##### **50X Stock solution of TAE buffer**

- 242g Tris base (Merck®)
- 57.1ml Glacial acetic acid (Merck®)
- 18.6g EDTA (Boehringer Mannheim®)
- Adjust volume to 1 litre with distilled water
- Autoclave

##### **Preparing 2 litres of 1X TAE buffer from 50X stock solution**

- 40ml of 50X Stock solution TAE buffer
- 1960ml distilled water
- Autoclave

**λ-DNA** (Sigma®) 250ng / μl which we diluted to 50ng and 25ng / μl in order to check extracted DNA concentrations.

## **BUFFERS FOR NICK TRANSLATION METHOD OF DNA LABELLING**

### **SpectrumOrange™-dUTP (Vysis)**

- 50nmol
- Add 50µl dH<sub>2</sub>O to give a final concentration of 1mM.

### **SpectrumGreen™-dUTP (Vysis)**

- 50nmol
- Add 50µl dH<sub>2</sub>O to give a final concentration of 1mM.

### **10x nucleotide stock – SpectrumOrange™-dUTP**

- 0.5mM dATP (Boehringer Mannheim®)
- 0.5mM dGTP (Boehringer Mannheim®)
- 0.5mM dCTP (Boehringer Mannheim®)
- 0.25mM dTTP (Boehringer Mannheim®)
- 0.25mM SpectrumOrange-dUTP (Vysis)

### **10x nucleotide stock – SpectrumGreen™-dUTP**

- 0.5mM dATP (Boehringer Mannheim®)
- 0.5mM dGTP (Boehringer Mannheim®)
- 0.5mM dCTP (Boehringer Mannheim®)
- 0.25mM dTTP (Boehringer Mannheim®)
- 0.25mM SpectrumGreen-dUTP (Vysis)

### **Nick translation buffer (10x)**

- 0,5M Tris-HCl pH8 (Merck®)
- 50mM MgCl (Promega)
- 0,5mg/ml BSA (Boehringer Mannheim®)

### **0.1M $\beta$ -mercaptoethanol**

- 0.1ml  $\beta$ -mercaptoethanol (BDH)
- 14,4ml double-distilled water

### **DNase 1** (Boehringer Mannheim®)

- 3mg DNase 1
- 0.5ml 0.3M NaCl
- 0.5ml Glycerol
- Store at -20°C
- 0.4 $\mu$ l DNase 1 diluted in 1000 $\mu$ l Sabax water for BAC 201 J24 and 0.48 $\mu$ l DNase 1 diluted in 1000 $\mu$ l Sabax water for BAC 240 C07. 1 $\mu$ l of each is used for each labelling reaction.

### **Polymerase** 9 units per $\mu$ l (Promega)

## **BUFFERS FOR ENZYME INACTIVATION OF OUR LABELLED PROBE**

0.5M EDTA - 3 $\mu$ l (Boehringer Mannheim®)

10% SDS - 1 $\mu$ l (BDH)

For each probe.

## **BUFFERS FOR REPETITIVE SEQUENCE BLOCKING AND PRECIPITATION WITH COT 1 DNA**

**COT 1** 1 $\mu$ g / 1 $\mu$ l (Boehringer Mannheim®)

### **3M Sodium Acetate**

- 3M Sodium Acetate (Merck®)
- Adjust pH to 4.6 with 3M Glacial Acetic Acid (Merck®)
- Autoclave and store at room temperature

**100% Ethanol** (BDH) Two and a half times the volume is added to the probe.

**70% Ethanol** (BDH) Approximately 300µl is added.

### **Hybridisation Buffer**

- 50% De-ionised Formamide
- 2X SSC
- 10% Dextran Sulphate
- 50mM Sodium Dihydrogen Orthophosphate (Univar®)
- pH to 7 with Disodium Hydrogen Orthophosphate (Univar®)

### **For 25ml of Hybridisation buffer**

- 12ml De-ionised Formamide
- 2.5ml of 20X SCC
- 2.5g Dextran sulphate
- 0.195g Sodium Dihydrogen Orthophosphate (Univar®)
- pH to 7 with Disodium Hydrogen Orthophosphate (Univar®)

## **BUFFERS USED IN HYBRIDISATION OF PROBES TO SLIDES**

### **De-ionised Formamide**

- 1 spatula full Analytical grade mixed bed resin (Bio Rad) for every 100ml formamide (Univar®)
- Place on stirrer for 2 hrs

- Filter with Whatman No1 filter paper
- Store at 4°C

**Denaturing buffer** (For 50ml)

- 35ml De-ionised Formamide
- 5ml Phosphate buffer
- 5ml 20X SSC
- 5ml Distilled water
- pH to 7 with concentrated HCl

**Phosphate buffer**

**Solution A:**

- 4.54g  $\text{KH}_2\text{PO}_4$  pH 4.51
- Adjust volume to 500ml with Distilled water

**Solution B:**

- 5.94g  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  pH 8.97
- Adjust volume to 500ml with Distilled water
- Combine the 2 solutions and come to a final pH of 7.00.
- Autoclave and store at room temperature.

**20X SSC**

- 3M NaCl (SMM Chemicals)
- 0.3M Sodium Citrate (SMM Chemicals)
- pH to 7 with HCl
- Autoclave

### **2X SSC**

- 50ml of 20X SSC
- 500ml of Distilled water

### **100% Ethanol** (BDH)

### **90% Ethanol** (BDH)

### **70% Ethanol** (BDH)

### **Glue** (Pax rubber cement)

Used to seal slides.

## **BUFFERS USED IN WASHING OF HYBRIDISED SLIDES**

### **50% Formamide**

- 20ml 20X SSC
- 80ml Distilled water
- 100ml Formamide (Univar®)
- pH to 7 with concentrated HCL

### **2X SSC**

- 50ml of 20X SSC
- 500ml of Distilled water

### **DAPI**

- 100µl DAPI (Sigma®)
- 100ml 2X SSC

### **Tween**

- 50ml 2X SSC
- 25µl Tween
- Mix well

### **Vector Shield** (Vector Laboratories Inc.)

#### **10X FA gel buffer (Denaturing gel for RNA analysis)**

- 200mM 3-[N-morpholino]propanesulfonic acid, free acid
- 50mM Sodium acetate
- 10mM EDTA
- pH to 7 with Sodium hydroxide

#### **1X FA running buffer**

- 100ml 10X FA gel buffer
- 20ml 37% Formaldehyde
- 880ml RNase free water

### **RNA gel**

- 0,51g Agarose
- 50ml 1X FA running buffer
- 0,54ml 37% Formaldehyde (add after boiling agarose and FA running buffer)
- 0,5µl Ethidium bromide

Once the gel has set, place into gel tank in FA running buffer for 30 minutes, before loading and running RNA gel.

## APPENDIX E

Specimen	Culture	Split signal	Other	Specimen	Culture	Split signal	Other*
C1	N	0%	6%	SM1	N	6%	6%
	A	0%	16%		A	2%	8%
	F	0%	22%		F	2%	18%
				SM2	N	0%	6%
					A	0%	12%
					F	0%	12%
C3	N	2%	4%	SM3	N	0%	6%
	A	2%	4%		A	0%	6%
	F	2%	4%		F	0%	12%
				SM4	N	0%	2%
					A	0%	0%
					F	0%	4%
C5	N	0%	2%	SM5	N	0%	6%
	A	0%	8%		A	2%	2%
	F	0%	6%		F	0%	10%
				SM6	N	0%	8%
					A	0%	12%
					F	0%	6%
C8	N	0%	4%	SM7	N	0%	4%
	A	0%	6%		A	0%	2%
	F	2%	4%		F	0%	4%
				SM8	N	0%	8%
					A	0%	6%
					F	6%	0%

C4	N	0%	2%	SM9	N	4%	8%
	A	0%	2%		A	2%	2%
	F	6%	4%		F	4%	2%
				SM10	N	0%	0%
					A	4%	0%
					F	4%	2%
C2	N	0%	10%	SM11	N	0%	2%
	A	0%	4%		A	2%	2%
	F	2%	6%		F	2%	0%
				SM12	N	0%	4%
					A	0%	6%
					F	6%	8%
C9	N	0%	6%	SM13	N	0%	6%
	A	0%	0%		A	0%	4%
	F	0%	0%		F	4%	0%
				SM14	N	0%	4%
					A	2%	8%
					F	2%	0%
C7	N	0%	8%	SM15	N	0%	6%
	A	0%	0%		A	0%	8%
	F	2%	2%		F	2%	4%
				SM16	N	2%	2%
					A	0%	8%
					F	4%	4%
				SM17	N	0%	4%
					A	0%	4%

					F	0%	6%
C10	N	0%	10%	SM18	N	0%	4%
	A	0%	6%		A	0%	10%
	F	2%	0%		F	0%	0%
				SM19	N	0%	2%
					A	0%	10%
					F	0%	10%
				SM20	N	0%	4%
					A	0%	2%
					F	0%	2%

**\*Other:**

1 Full deletion  
 No signals  
 Green only  
 Red only  
 1 Red deleted  
 1 Green deleted

Extra red  
 Extra green  
 Extra signal (i.e. green and red superimposed)  
 Missing red  
 Missing green

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