Cytogenetic analysis and development of FISH (Fluorescent In Situ Hybridisation) techniques to delineate deletions of chromosome 22 in Di George syndrome and related disorders

Marie McCluskey

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Cytogenetic analysis and development of FISH (fluorescent in situ hybridisation) techniques to delineate deletions of chromosome 22 in Di George syndrome and related disorders

By

Marie McCluskey  B. App. Sc.,

A Thesis Submitted in Partial Fulfilment of the Requirements for the award of Bachelor of Applied Science with Honours

at the Department of Human Biology, Edith Cowan University

Date of Submission: 31st October 1994.
Abstract

This honours project is based on the advancement of *in situ* hybridisation to metaphase chromosome spreads. The technique was carried out using cosmid probes which are specific for microdeleted regions on chromosome 22q11 associated with Di George syndrome (DGS) and related disorders. Disorders similar to DGS include partial Di George syndrome, III-IV pharyngeal pouch syndrome, velocardiofacial syndrome (VCFS), conotruncal facial anomaly and the CHARGE association. Recently the group of disorders with this microdeletion and comparable symptoms has been summarised by the acronym CATCh 22.

Large deletions, translocations, monosomies, and trisomies of chromosomes are apparent under the light microscope, when prepared as metaphase chromosome spreads for karyotyping. Subtle microdeletions, however, are seldom detected by this method, even when high resolution banding techniques are undertaken. A more suitable technique for the detection of such microdeletions is *in situ* hybridisation. *In situ* hybridisation (ISH) has successfully been employed to identify chromosomes, to detect chromosomal abnormalities, and to determine chromosomal locations of specific gene sequences. The aim of the present study is to apply *in situ* hybridisation to the detection of suspected microdeletions of chromosome 22q11. Fluorescent *in situ* hybridisation (FISH) and other forms of non-isotopic *in situ*
hybridisation (NISH) were carried out with cosmid probes specific for chromosome region 22q11, on peripheral blood samples of patients displaying DGS-like symptoms and controls. Patients suspected of having DiGeorge syndrome were surveyed by Dr. S. Worthington at Princess Margaret Hospital for Children (Western Australia) as part of a clinical study into familial congenital heart disease (CHD). All patients tested have CHD, and display some DGS-like symptoms. The advancement of the FISH technique will assist clinicians in the identification of patients with the CATCH 22 sequence, and will further serve to demonstrate the ability of a cosmid probe to detect a microdeletion associated with a clinical disorder. The effectiveness of the method will also be compared to results obtained by routine cytogenetic analysis. The study shows that although in situ hybridisation is technically difficult to establish, it holds great promise as a sensitive and specific diagnostic test and research tool.
Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.
Acknowledgments

I would like to thank all my supervisors for their help during this project. Prof. Alan Bittles brought a sense of enthusiasm and encouragement to the study which was greatly appreciated, his advice on the preparation of this manuscript was invaluable. I am also grateful to Dr. Ashleigh Murch, who not only allowed me unrestricted access to the facilities of the cytogenetics laboratory but also provided answers to numerous questions and, more importantly taught me how to locate responses for myself. Anne Mitchell over the past few years has acted as my mentor and academic adviser, her belief in my ability encouraged me to pursue this honours project and it is for this reason, along with her continued support that I am extremely grateful to her.

Due to the innovative nature of this project, collaboration was sought from many research groups. All the people contacted locally, interstate and overseas were more than generous with the donation of their time, resources, reagents, information and general good advice. Their altruism constantly amazed me, as William Shakespeare wrote they restored “my faith in human kindness”. If I neglect to directly mention any of the people involved in this collaboration I am truly sorry, please be assured that any exclusion is an oversight on my part and not intentional.

During the course of the year I spent time at a number of laboratories and was helped by the following people:
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Laing and Dr. Sue Fletcher were most generous in allowing me the use of a laboratory, and special thanks to Jo-Ann Stanton and Anthony Akkari whose advice and support made working within time constraints possible. I wish I was able to name the staff and students at ANRI individually, they all encouraged and supported this project and gave me a true sense of belonging and I thank them for this.

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Finally I would like to dedicate this thesis to my family and teachers who over the years have helped to change a curious child into an student with a desire for further education.
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<th>Description</th>
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<tr>
<td>α DIG FITC</td>
<td>Anti-digoxigenin fluorescein</td>
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<td>Biotin</td>
<td>Biotin-14-dUTP</td>
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<td>CATCH 22</td>
<td>The acronym which represents the clinical and genetic features of Di George syndrome (i.e., cardiac, abnormal facies, thymic hypoplasia, cleft palate, hypocalcaemia and chromosome 22).</td>
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<tr>
<td>CHD</td>
<td>Congenital heart disease</td>
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<td>eDNA</td>
<td>Complementary deoxyribose nucleic acid</td>
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<td>DGS</td>
<td>Di George syndrome</td>
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<td>DGCR</td>
<td>Di George critical region</td>
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<td>DIG</td>
<td>Digoxigenin-11-dUTP</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
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<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridisation</td>
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<td>FITC</td>
<td>Fluorescein-12-dUTP</td>
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<td>G-banding</td>
<td>Giemsa chromosome band staining</td>
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<td>HRP</td>
<td>Horse radish peroxidase</td>
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<td>HRS</td>
<td>High resolution banding studies</td>
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<td>IAA</td>
<td>Interrupted aortic arch</td>
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<td>λ-IGLC</td>
<td>Immunoglobulin light chain cluster</td>
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<td>ISH</td>
<td><em>In situ</em> hybridisation</td>
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<td>NISH</td>
<td>Non-isotopic <em>in situ</em> hybridisation</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PMH</td>
<td>Princess Margaret Hospital (WA)</td>
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<td>PRINS</td>
<td>Oligonucleotide-primed <em>in situ</em> synthesis</td>
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<td>Q-band</td>
<td>Quinacrine chromosome band staining</td>
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<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<td>TA</td>
<td>Truncus arteriosus</td>
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<td>TOF</td>
<td>Tetrology of Fallot</td>
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<tr>
<td>SHLS</td>
<td>State Health Laboratory Services</td>
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<td>UV</td>
<td>Ultra-violet</td>
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<td>VCFS</td>
<td>Velocardiofacial syndrome</td>
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I. Literature Review
1. Literature Review

1.1 Introduction

Di George syndrome (DGS) is a developmental field defect of the third and fourth brachial arches. It was first described by Angelo Di George (1968), and since that time numerous clinical, cytogenetic and molecular studies have been undertaken to identify the spectrum of associated defects (Greenberg et al., 1988a). The clinical features, which are diverse and vary in severity, include congenital heart disease (CHD) such as tetralogy of Fallot, interrupted aortic arch, ventricular septal defects and hypocalcaemia, facial abnormalities and a poor immune system due to the absence of the thymus. Most patients have evidence of CHD but some may present only with hypocalcaemic seizures (Conley et al., 1979). The first recorded familial cases of DGS were associated with an unbalanced chromosome 20:22 translocation, which resulted in monosomy 22q11 (De la Chapelle et al., 1981). The ability to detect monosomy 22q11 is questionable with routine cytogenetic analysis (Desmaze et al., 1993a). The microdeletion which is thought to be responsible for DGS, however, can be more readily detected by non-isotopic in situ hybridisation (NISH), particularly fluorescent in situ hybridisation (FISH) (Carey et al., 1992). In FISH the cosmid probe DNA is hybridised to its complementary sequence of chromosomal DNA, on the particular chromosome where that sequence is found. The cosmid probe which is used to detect the deletion associated with DGS corresponds to a
region on chromosome 22q11. In FISH the probe DNA is labelled with a fluorochrome to facilitate detection, and the chromosomes are visualised with an appropriate counterstain. If fluorescein, a yellow-green fluorochrome, is used to label the probe then propidium iodide is applied as the counterstain. The resultant red-stained metaphase chromosomes are then viewed under fluorescent microscopy, the hybridisation signal which highlights the chromosome-probe hybrid fluoresces as a bright yellow signal. In situ hybridisation has been used to detect the presence of many clinical disorders which localise to specific chromosome regions. This detection method plays an important role in a variety of research areas, including cytogenetics, tumour biology, gene amplification, gene mapping and also in prenatal diagnosis (Trask, 1991).

1.2. The history of Di George syndrome

DGS is named after Angelo Di George, an American clinician, who commenced research into congenital absence of the thymus in 1963 (Di George, 1968). However, he probably was not the first person to note the disorder. A journal article from as early as 1829 by H. Harrington described symptoms similar, if not identical, to those associated with DGS (Greenberg, 1993). Absence of thymic tissue is also often associated with loss of the parathyroid glands, or their ectopic placement, and has resulted in DGS-like symptoms (Lobdell, 1959). The major presenting symptoms which allow a provisional diagnosis of the disorder are CHD (De Ia Chapelle et al., 1981) and malformations of the palate. In DGS patients these are
accompanied by a grossly depressed cell mediated immunity caused by the absence of the thymus (Greenberg, 1993). During infancy hypocalcaemic seizures are caused by absence of parathyroids.

1.3. Clinical features of Di George Syndrome

Di George syndrome (DGS) consists of varying combinations and degrees of hypoparathyroidism, thymic hypoplasia with T-lymphocyte deficiency, congenital heart disease (CHD), and dysmorphic facies (Winter et al., 1984). Although the symptoms vary in severity, almost all cases result from a detectable deletion within chromosome 22q11 (Wilson, D. et al., 1993b). The array of clinical symptoms which make up DGS has been summarised by the acronym 'CATCH 22 syndrome'. This acronym represents the clinical features (Cardiac, Abnormal facies, Thymic hypoplasia, Cleft palate, and Hypocalcaemia) and chromosome 22 (Wilson, D. et al., 1993b). CATCH 22 also suggests the complexity which exists with regard to definition of the clinical features, and understanding the genetic ramifications of a deletion from a specific area on a particular chromosome (Hall, 1993).

DGS does not, as its title would suggest, have a set of symptoms which are common to all patients. For this reason DGS and other similar syndromes have been classed as 'contiguous gene syndromes' (Schmickel, 1986). Owing to these varying symptoms, it has been said of DGS that it probably is not a distinct syndrome of
single gene origin but rather an anomaly caused by a heterogeneous developmental field defect (Stevens et al., 1990).

Due to the variance of symptoms and their possible causes the disease has been given several names. The disorder associated with microdeletion 22q11 has not only been known as DGS, but also as III-IV pharyngeal pouch syndrome and partial Di George syndrome (Lischner et al., 1972). Reported cases of partial DGS include apparently balanced and unbalanced translocations. A balanced translocation t(2;22) (q14.1;q11.1) was observed in a girl with some, but not all, of the features of DGS. The same translocation was present in the girl’s mother and maternal aunt. The latter female had her fourth pregnancy aborted due to cardiac and other malformations which were detected by ultrasound. The karyotypes of individuals in the family show that this particular translocation can be associated with partial DGS, however the fetus which was aborted had a much more severe (complete) form of DGS (Augusseau et al., 1986).

I.4. DGS as a contiguous gene syndrome

The heterogeneity of symptoms within families is often associated with contiguous gene syndromes, which can be grouped into those with detectable chromosomal defects and those without any identifiable aberration at a cytogenetic level (Emanuel, 1988). One of the forms of DGS which has been shown to have a translocation at the cytogenetic level is partial DGS, a disorder that has been noted in many families. Greenberg et al., (1984) reported a family in which both siblings had DGS
phenotype. This phenotype was associated with an unbalanced 4;22 translocation in the younger child, which resulted in partial monosomy of chromosome 22q11. The older sibling was deceased and no cytogenetic analysis had been carried out. Autopsy revealed that the first sibling had Type I truncus arteriosus, thymic aplasia, and parathyroid hypoplasia, the second sibling also had Type I truncus arteriosus accompanied by dysmorphic features and T-cell abnormalities. The mother of both children had no history of heart murmur, seizures, or serious infections, although on physical examination she did have slightly dysmorphic facies and an identical translocation to her younger child. This study provides further evidence that, in some individuals, even when DGS is not obvious a microdeletion of 22q11 may be present (Greenberg et al., 1984).

DGS is also closely related to Shprintzen syndrome (also known as velo-cardiofacial syndrome, VCFS) (Wilson et al., 1992b), and the CHARGE association (Cyran et al., 1987). It has been surmised that VCFS is caused by a deletion of the same gene or set of genes as DGS (Scambler, 1993). It has also been proposed that not only do autosomal dominant DGS and VCFS have a common microdeletion, but they are in fact all cases of VCFS (Stevens et al., 1990).

1.5. Velocardiofacial syndrome (VCFS)

Velo-cardiofacial syndrome (VCFS) is a syndrome of multiple anomalies, including cleft palate, heart malformations, facial characteristics and learning disabilities. It
was described in a group of 12 unrelated children by Robert Shprintzen in 1978, although a familial case had been described previously (Strong, 1978). Since that time Shprintzen has examined over 100 affected individuals and has documented the phenotypes of all these cases (Goldberg et al., 1993). Shprintzen has proposed that most reported cases of familial DGS are in fact misdiagnosed VCFS (Shprintzen, 1994), a belief which may be justified given the phenotypic variability of VCFS. Like DGS, there is considerable phenotypic variability not only between unrelated patients but also within families (Holder et al., 1993). Molecular studies of VCFS patients have shown that like DGS they also display monosomy for a region of chromosome 22q11 (Kelly et al., 1993). Monosomy 22q11 was revealed in 18 patients with different probes specific for 22q11, using dosage analysis in search of monosomy at this locus. The patients tested in this study displayed variable phenotypes, although they had all been previously clinically diagnosed as having VCFS (Motzkin et al., 1993). Other studies have also noted extreme clinical variability in VCFS patients and Meinecke et al. (1986) concluded that VCFS is probably caused by a dominant gene with very variable expression. As a result of this variability, patients who do not exhibit obvious symptoms, such as cardiac defects, may not be correctly diagnosed. One clinical study into VCFS surveyed 38 children, all of whom were diagnosed as having the disorder, even though 42% of the children had no congenital heart defect. It must be noted, however, that no cytogenetic or molecular analysis was reported for this group. It has been suggested
therefore that lack of cardiac defect should not deter the clinician from making a diagnosis of VCFS (Lipson et al., 1991).

There is no dispute that VCFS and DGS share an aetiological connection. They both display evidence of monosomy 22q11 and have some common phenotypic features (Scambler et al., 1992). Due to the overlap in symptoms, some if not all cases of DGS may be misdiagnosed cases of VCFS (Goldberg et al., 1985). Some distinct clinical features which are specific to VCFS are not found in DGS (Driscoll et al., 1992b), also some researchers see DGS as a particular form of VCFS and not a separate disorder. There have been reported cases for example, of psychosis associated with VCFS which is not apparent in DGS (Dunham et al., 1992) (Shprintzen et al., 1992). Based on this evidence, DGS may be a particular form of VCFS, however many clinicians believe that it can be diagnosed separately from VCFS because of its particular combination of symptoms.

1.6. Possible causes of DGS

Although the title of this condition (or conditions) has been a matter of dispute (Lischner, 1972), it is widely agreed that the characteristic phenotype of DGS is the result of an abnormality in the formation of the III & IV pharyngeal pouches. An embryological disorder of this nature, due to inadequate neural crest contribution in development, is termed a neurocristopathy (Hong, 1991). When the DGS neurocristopathy was examined it was apparent that a number of events could occur
during early pregnancy to cause the condition. As well as chromosomal abnormalities, teratogens such as retinoic acid (a synthetic form of vitamin A found in acne treatments), have been linked to DGS (Lammer et al., 1985). It has also been suggested that maternal insulin dependent diabetes acts as a pathogenetic factor in the Di George anomaly. Two infants born to diabetic mothers had apparent DGS and renal agenesis. Both mothers and their infants had normal cytogenetic karyotypes. Molecular studies, using a probe which is believed to be specific for the gene region deleted in DGS, failed to detect a microdeletion of chromosome 22q11 in these patients (Wilson, T. et al., 1993).

DGS is referred to by many researchers as Di George anomaly rather than syndrome, the term anomaly being preferred because it infers the numerous suggested aetiologies associated with the disorder and stresses its origin as a developmental field defect (Opitz & Lewin, 1987). It should be noted that unless DGS and Di George anomaly are specifically distinguished from each other, essentially they can be regarded as the same condition.

1.7. The variability of congenital heart defects (CHD) in DGS

Genetic defects are known to be a major cause of heart malformation (Wilson et al., 1991). In DGS and VCFS certain types of CHD are over-represented by comparison with their incidence in the general population (Worthington, 1994). Conotruncal cardiac defects are over-represented in both DGS and VCFS. The conotruncal
defects particular to DGS are interrupted aortic arch (type B) (IAA), truncus arteriosus (TA), and tetralogy of Fallot (TOF). TOF is also one of the most common forms of cardiac defect present in VCFS. Since DGS and VCFS have both been associated with microdeletions of chromosome 22q11, it was supposed that a deletion in the 22q11 region was also responsible for the types of cardiac defect common to these disorders. A study to substantiate this assumption was carried out on a group of patients with the tetralogy of Fallot defect, who had no other signs of either DGS or VCFS. A total of seventeen patients were tested for evidence of a microdeletion in chromosome 22q11 using restriction fragment length polymorphism (RFLP) and dosage analysis, five of the seventeen were shown to have a 22q11 deletion (Goldmuntz et al., 1993). As well as RFLP and dosage analysis to test for this deletion, Goldmuntz et al. (1993) also reported on the usefulness of FISH as a way to determine whether families with a history of CHD have the 22q11 microdeletion. Families who have a known deletion, even if unaccompanied by symptoms of DGS or VCFS, will be able to utilise clinical testing and genetic counselling to determine the risk of heritability of the deletion which accompanies CHD.

Other studies have provided similar evidence on the connection between isolated heart defects and the 22q11 microdeletion. DGS was diagnosed in an infant with IAA, hypoparathyroidism and low T-cell lymphocyte numbers. Two siblings had heart defects that are not common to DGS (a membranous ventricular septal defect,
and coarctation of the aorta) and neither sibling showed signs of DGS. Chromosome analysis of their mother, whose cardiovascular examination was normal, and her three offspring with heart defects all revealed a 22q11 interstitial deletion. The deletion was subsequently confirmed by molecular analysis (Wilson et al., 1991). This case promoted further study into deletions in nine families with recurrent outflow-tract heart defects. In five of the families, chromosome 22 deletions were detected in all of the living affected individuals, and they were also present in the clinically normal father of a further family in which there were three affected children. As a result of these studies it was proposed that deletions within band q11 of chromosome 22 are an important cause of some familial heart defects (Wilson et al., 1992a).

1.8 Prenatal detection of CHD
Congenital heart defects are often diagnosed prenatally by ultrasound, and it is normal to follow the detection of a CHD with prenatal karyotyping in order to determine the extent of chromosomal involvement. It has been revealed by such karyotyping that chromosome abnormalities are present in 15.4% of fetuses with an isolated cardiac malformation and in 42.7% with an additional malformation (Catherine et al., 1991). In children with DGS chromosome abnormalities can prove difficult to diagnose prenatally due to the failure of fetal blood to culture for karyotyping. This is because individuals with DGS have a reduced number of T-lymphocytes, and since lymphocytes are the cells cultured for karyotyping
insufficient numbers may be available to analyse for a karyotype. It has been proposed that if a fetal blood culture cannot be karyotyped when a CHD of the DGS-type is present, then fetal lymphocyte subpopulations should be examined in order to diagnose DGS (Catherine et al., 1991). Although it also has been suggested that, when a CHD is diagnosed prenatally, cytogenetic and molecular analysis of amniotic fluid cells or chorionic villi would be more sensitive and accurate than characterisation of fetal lymphocyte populations (Driscoll et al., 1991). It was subsequently confirmed that FISH of cultured amniocytes provided an efficient and direct method for the pre-natal detection of microdeletions including monosomy 22q11, which was detected in two separate pregnancies by this method (Driscoll et al., 1993).

Karyotyping of chorionic villi in a family with a known reciprocal balanced translocation involving chromosomes 9 and 22, at the regions q22 and q11.2 respectively, revealed an unbalanced karyotype in the fetus. This arose due to an adjacent-2 disjunction of the quadrivalent in the mother. The test was performed because a previous sibling had died at age three weeks due to cardiovascular complications. Karyotyping of this infant revealed the same unbalanced translocation as had previously been found in the fetus (Pivnick et al., 1990). Another case where an unbalanced translocation of chromosomes 9;22 was reported also resulted in symptoms of DGS (El-Fouly et al., 1991).
1.9 Prevalence of Di George syndrome

The number of children affected by DGS is not readily calculated. The prevalence has been estimated at 1 in 20,000, but this figure is probably an under-estimate based only on the ascertainment of severe cases (Scambler, 1993). If severe and mild cases are included then CHD seems to be quite a common disorder with, 1 in 100 live births resulting in some form of cardiac defect (Hoffman, 1990). In order to gain a meaningful insight into the prevalence of DGS, large groups of patients and their families will need to be assessed by clinical, cytogenetic and molecular examination. The results of such a study will provide not only useful information on the prevalence of DGS in the population, but also on the degree of variation in the severity of symptoms and their aetiology.

1.10 The genetic aetiology of DGS

The genetic aetiology of DGS has been attributed to a microdeletion of chromosome 22q11, in a region known as the Di George critical region (DGCR). When chromosome 22 is deleted in the defined region DGS results. The DGCR was first suggested by De la Chapelle and his colleagues (1981). In their study, a chromosome analysis of a large family was undertaken and some members of the family were shown to display an unbalanced chromosomal rearrangement leading to trisomy 20pter→20q11 and monosomy 22pter→22q11. The clinical features of trisomy 20p were already known and since they did not correspond to the features of
DGS (Francke, 1977), this trisomy was rejected as the cause of DGS. It was therefore suggested that DGS was due to a monosomy of the region 22pter→22q11 (De la Chapelle et al., 1981). This hypothesis was later verified by cytogenetic analysis of other DGS patients with interstitial deletions, del (22)(q11.21 q11.23) but no other apparent cytogenetic abnormality (Greenberg et al., 1988a).

On the basis of these findings it was hypothesised that the DGCR lies within 22q11 (Driscoll et al., 1992a). It has subsequently been shown cytogenetically (Wilson et al., 1992b), and by molecular methods (Driscoll et al., 1992a), that most DGS patients (approximately 90% as reviewed in Carey et al., 1992) have a detectable 22q11 microdeletion. This microdeletion is often the result of an unbalanced translocation. The specific breakpoint may be important in some cases, however the clinical features are probably more related to the activity of the alleles for which the patient is hemizygous (Faed et al., 1987). The modes of inheritance suggested for DGS, like that of its symptoms, have been varied. Cases reported to date have most often demonstrated an autosomal dominant pattern of inheritance, although autosomal recessive and X-linked chromosomal patterns have also been noted (Driscoll et al., 1992a). The first reports of autosomal dominant transmission of the Di George phenotype were made by De la Chapelle (1981), and they were subsequently verified by Kelley et al. (1982). The ambiguity which exists with regard to the inheritance of DGS is partly attributable to the difficulty in diagnosing a microdeletion. Although parents and siblings may have such a chromosomal
aberration, it is quite often missed even when high resolution cytogenetic analysis is undertaken (Keppen et al., 1988). In one family both siblings and their father had evidence of DGS. Their clinical features included hypocalcaemia, unusual facies, truncus arteriosus, impaired cell mediated immunity and the father had a relatively low number of T-lymphocytes. Chromosome banding studies nevertheless showed all family members to have normal karyotypes (Rohn et al., 1984).

Different chromosome regions have been linked to the phenotypic characteristics of DGS other than chromosome 22q11. These aberrations are summarised in Table I.1.

In spite of the aetiological heterogeneity of DGS, evidence suggests that the most common cause is monosomy 22q11 (Greenberg, 1993). It has been proposed, however, that the DGS gene would be a good model for gene defects which are likely to be affected by other genes or environmental influences, as a true polygenic or multifactorial disorder. Due to this possible heterogeneity it has been emphasised that other DGS loci so far suggested, such as 10p13, must not be neglected in future studies (Greenberg, 1993).

I.1. Cytogenetic analysis of chromosomes

Human cytogenetic studies are based on the analysis of karyotypes made from metaphase chromosome preparations. The earliest recorded attempt to study human chromosomes using cultures of leukocytes from peripheral blood was made by Chrustchoff and his co-workers in 1931 (Hamerton, 1971).
Table I.1: The less common chromosome aberrations associated with DGS

<table>
<thead>
<tr>
<th>Chromosome location</th>
<th>Reported by:-</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>isochromosome 18q, manifests as monosity 18p and trisomy 18q</td>
<td>van Essen et al. (1993)</td>
<td>Clinical signs of DGS with holoprosencephaly and streak ovaries</td>
</tr>
<tr>
<td>unbalanced paternal translocation t(18;22)</td>
<td>Bowen et al. (1986)</td>
<td>Clinical signs of DGS with absence of thymus but involved 22p not 22q</td>
</tr>
<tr>
<td>monosity 12p with trisomy 10q</td>
<td>Greenberg et al. (1988a)</td>
<td>Clinical signs of DGS</td>
</tr>
<tr>
<td>monosity 5p</td>
<td>Greenberg et al. (1988a)</td>
<td>Clinical signs of DGS</td>
</tr>
<tr>
<td>partial trisomy 1q</td>
<td>Greenberg et al. (1988a)</td>
<td>Clinical signs of DGS</td>
</tr>
<tr>
<td>monosity 17p13</td>
<td>Greenberg et al. (1988b)</td>
<td>Clinical signs of DGS</td>
</tr>
<tr>
<td>monosity 10p13</td>
<td>Greenberg et al. (1988a) &amp; Monaco et al. (1991)</td>
<td>This chromosomal rearrangement has been associated with DGS phenotype on more than one occasion</td>
</tr>
</tbody>
</table>
It was not, however, until 1956 that the chromosome number of humans was verified to be 46 (Tjio & Levan, 1956). Even then the available cytogenetic techniques were such that it was impossible to clearly distinguish individual chromosomes within a particular group. Human metaphase chromosomes can be organised after simple staining into seven groups (A-G) according to their relative size and centromeric position. Chromosome identification was altered by the advent of quinacrine mustard staining (Q-banding) (Caspersson et al., 1968), which allowed each human chromosome pair to be individually recognised. In order to visualise Q-banded chromosomes a fluorescent microscope is required. Shortly after the invention of Q-banding another type of banding technique was discovered, giemsa staining with trypsin (G-banding), and this type of banding is the preferred method of staining in most laboratories. Although, G-banding and Q-banding give similar quality karyotypes, G-banding is more common because it is permanent and can be detected with a standard light microscope (Hamerton, 1971).

1.12. Production of metaphase chromosome preparations

Chromosomes analysed by karyotyping are harvested from cultured cells, with a droplet of cultured cell suspension fixed on to a microscope slide as a metaphase spread. Metaphase spreads are prepared from various specimens such as, peripheral blood, amniocytes, chorionic villi, or any tissue or cell line which is actively dividing (Verma and Babu, 1989). The specimens are cultured and treated so that the cell cycle is arrested at the metaphase stage of mitosis. The cells are then treated with a
hypotonic solution (Emery, 1979). This ensures that on dropping the suspension on to a microscope slide the chromosomes in a high proportion of the nuclei will spread. The nuclei disperse in such a manner that the chromosomes from each nucleus remain distinct from those in adjacent nuclei on the slide. The subsequent application of a fixative ensures that the cells adhere to the slide. When the metaphase spreads have been fixed to the slides they can then be banded.

As previously mentioned, DGS was found to be linked most often with an interstitial deletion of 22q11 (Greenberg et al., 1988a). Due to the small size of chromosome 22, standard synchronised G-banded metaphase spreads do not clearly define band q11 and for this reason it is necessary to produce high resolution preparations in order to see a microdeletion in this region. This is achieved by decreasing the time that the culture is exposed to colcemid. Colcemid, when added to chromosomes, affects spindle formation and the chromosomes remain at the metaphase stage of the cell cycle. When the chromosomes are exposed to colcemid for a relatively long time they begin to constrict and fewer bands can be defined when stained. By reducing colcemid exposure time the chromosomes appear to be longer and more bands are produced, which results in suitably high resolution chromosomes (i.e., at least 850 bands/haploid set) (Standing Committee on Human Cytogenetic Nomenclature, 1985). It has been demonstrated that, even when metaphase spreads are prepared at this high resolution, it may still not be possible to locate microdeletions cytogenetically (Carey et al., 1992). If molecular methods are not
available, however, it has been suggested that high resolution studies of suspected microdeletions are justified, although the number of patients shown to have an interstitial deletion by high resolution karyotype may be low (Mascarello et al., 1989).

1.13. Molecular analysis of the Di George critical region (DGCR)

Only large interstitial deletions of chromosome 22 can be observed cytogenetically with high resolution karyotypes (Greenberg et al., 1988a), and so deletions of less than 3−5 × 10⁶ base pairs are not reliably detectable using cytogenetic analysis. It is therefore necessary to employ techniques such as in situ hybridisation (ISH) to detect microdeletion 22q11. In one study a series of ten patients affected by DGS were screened by high resolution banding and FISH of a cosmid probe. All ten patients had a microdeletion of 22q11 by in situ hybridisation. However, only two of the ten were suspected of having this deletion by high resolution banding (Desmaze et al., 1993a).

1.14. In situ hybridisation (ISH)

In situ hybridisation is based on the double-stranded nature of DNA. When denatured the two strands of DNA separate and then quickly rejoin (Leversha, 1993). The principle by which ISH works is to incorporate a DNA probe during the renaturation process into the chromosomal DNA of an intact chromosome. A DNA hybrid is then formed between a single strand of the probe DNA and its
complementary single strand of chromosomal DNA as they reanneal. The probe is labelled so that when it is joined to the chromosomal DNA and forms a hybrid, a signal of this region can be detected.

Initially, probes were labelled isotopically and detected by autoradiography. The first isotopic in situ hybridisation was based on the early work of Gall and Pardue in 1969, however this technique is technically difficult and potentially hazardous due to radioactivity. It is also much slower than non-isotopic in situ hybridisation (NISH), as signals can take 2-3 weeks to be detected. Isotopic in situ hybridisation has largely been superseded by NISH (Lichter & Ward, 1990). In NISH, hybridisation usually takes place overnight and the probe is detected by a reporter molecule bound to a histochemical stain aggregate sufficiently large to permit visualisation. Enzymes such as horse radish peroxidase will catalyse the precipitation of deposits which can be seen with a light microscope (Leversha, 1993). Alternatively, fluorochromes can be linked to the probe via a reporter molecule that, after hybridisation, binds to fluorescent affinity reagents. Probes may also be directly conjugated with fluorescent molecules, the most common of which are fluorescein isothiocyanate, rhodamine and Texas Red (Trask, 1991). Fluorescent labels have the added advantage that chromosomes can be simultaneously Q-banded as well as have probes hybridised to them.
1.15. FISH and the detection of DGS

FISH has been successfully used to detect DGS by several research groups. Carey et al. (1992) were able to demonstrate that 21 out of 22 patients suspected of having DGS were hemizygous for 22q11. When this result was combined with one of their earlier studies, 33 out of 35 DGS patients had chromosome 22q11 deletions detectable by DNA probes (Carey et al., 1992). A similar study also using a cosmid probe showed 10 out of 10 patients to have a detectable 22q11 deletion, whereas only two of the ten were diagnosed cytogenetically (Desmaze et al., 1993a). A second study by the same researchers describes the relative ordering by FISH of cosmid loci and translocation breakpoints in the Di George syndrome critical region (DGCR) of chromosome 22 (Desmaze et al., 1993b). Such studies aim to define the smallest region which can be lost and still result in DGS. As well as providing a diagnostic tool for DGS patients, FISH can also be utilised for prenatal diagnosis and genetic counselling (Driscoll et al., 1993). The use of a cosmid probe in FISH has also enabled researchers to show that similar conditions may in fact be related to DGS. Burn et al., (1993) showed that a cosmid probe could delineate a 22q11 deletion in the conotruncal anomaly facial syndrome.

1.16. In situ hybridization as a research tool

ISH can be used to find the chromosomal location of DNA probes. A probe for the λ immunoglobulin light chain cluster (λ IGLC) constant region was hybridised to
chromosome preparations of two patients with DGS-related translocations. The results showed that the gene in question, λ IGLC, was not involved in DGS (Cannizzaro and Emanuel, 1985). Although this study did not show the location of the DGS gene or genes to be within the λ IGLC region, it effectively eliminated this area from the search for such a region in 22q11. There have been many similar studies undertaken with respect to the physical mapping of the DGCR, which was narrowed down to chromosome 22q11.21–q11.23 by Fibison et al. (1990). Numerous DNA markers which are deleted in patients with DGS have been mapped to the 22q11-pter region (Carey et al., 1990), and these markers provide a basis for adding fine detail to the proximal map of 22q11.

Other anonymous DNA markers for the DGCR have since been mapped using hybrids from patients with DGS (Sharkey et al., 1992). One such study (Budarf et al., 1992) indicated that a gene may have been isolated from the DGCR, although the complete sequence, function and development expression pattern of the gene have not yet been determined. One of the most recent cosmid markers, cHKAD-26 (Kurahashi et al., 1994) to be assigned to the DGCR was from a genomic cosmid library constructed from a Chinese hamster/human hybrid, this marker may prove useful in the clinical diagnosis of DGS and VCFS. Another 108 cosmid markers were regionally assigned to human chromosome 22 in the search for the DGCR probe. It is envisaged that these will be used for further investigation in the
construction of long range physical maps and for localising genetic alterations on the chromosome (Kurahashi et al., 1994).

1.17. The clinical use of *in situ* hybridisation

*In situ* hybridisation can be used as a clinical diagnostic tool not only for DGS and VCFS but also for many chromosomal abnormalities. As well as the ISH of metaphase chromosomes, interphase nuclei analysis has also been suggested. This method is a rapid and efficient tool in the identification of trisomy 21 (Lichter et al., 1988). ‘Chromosome painting’ has been recommended as a diagnostic adjunct to classical metaphase analysis (Hulten et al., 1991). FISH is now used in many cytogenetic laboratories as a diagnostic tool and probes for a large number of chromosomal abnormalities are commercially available.

One of the most recent molecular methods in the detection of specific chromosome regions is the ‘PRINS’ technique. It enables rapid chromosome identification by oligonucleotide-primed *in situ* DNA synthesis. Labelled nucleotides are incorporated directly into the DNA of cytological preparations. The primary advantage of PRINS over ISH is that detection takes just two hours, as compared to an overnight reaction. For this reason the PRINS technique may well become the preferred method in the future (Gosden and Lawson, 1994).
II. Materials
## II. Materials

### II.1. List of chemicals used

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid (96%)</td>
<td>Merck</td>
</tr>
<tr>
<td>Acetic Acid Glacial</td>
<td>Rhone Poulenc</td>
</tr>
<tr>
<td>Agarose (DNA grade)</td>
<td>Laboratory Products</td>
</tr>
<tr>
<td>Aluminium Sulphate</td>
<td>Sigma</td>
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<tr>
<td>Anti-digoxigenin-fluorescein, Fab fragments</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-digoxigenin-POD, Fab fragments</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Anti-fluorescein-POD, Fab fragments</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
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<td>Promega</td>
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<td>Bacto-tryptone</td>
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<tr>
<td>Bacto-yeast extract</td>
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<td>Boehringer Mannheim</td>
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<td>Benzyl Penicillin (600mg/vial)</td>
<td>CSL (Commonwealth Serum Laboratories)</td>
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<tr>
<td>Bovine Serum Albumin (lyophilized powder)</td>
<td>Sigma</td>
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<td>Chemical Name</td>
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<td>------------------------</td>
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<tr>
<td>Bromophenol blue</td>
<td>Sigma</td>
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<td>Caesium Chloride (dihydrate)</td>
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<td>Life Technologies</td>
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<tr>
<td>CuSO₄</td>
<td>Sigma</td>
</tr>
<tr>
<td>DABCO®</td>
<td>Aldrich</td>
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<tr>
<td>2'-Deoxycytidine</td>
<td>Sigma</td>
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<td>DePeX mounting medium 'Gurr®'</td>
<td>Analar® BDH</td>
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<tr>
<td>3,3'-Diaminobenzidine (powder)</td>
<td>Sigma</td>
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<td>3,3'-Diaminobenzidine Sigma fast™(tablets)</td>
<td>Sigma</td>
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<tr>
<td>DIG DNA labelling and detection Kit-(digoxigenin-11-dUTP)</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Dimethyl formamide</td>
<td>Analar® BDH</td>
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<td>DNA (from herring sperm)</td>
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<tr>
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<td>Life Technologies</td>
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<tr>
<td>Fluorogreen (fluorescein-11-dUTP)</td>
<td>Amersham</td>
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</tbody>
</table>
Formamide (redistilled)
Giemsa’s Azur-cosin-methylene blue
Glucose
Haematoxylin
HCl
Heparin (1000 units/ml)

Hind III
Human COT-1™ DNA (Gibco BRL)
Hybond N+
Hydrogen Peroxide solution (~30%)  
8-Hydroxyquinoline
iso-amyl alcohol (Univar)
Kanamycin monosulphate
K₂HPO₄
L-glutamine (Gibco BRL)
Lithium Chloride
Lysozyme
2-mercaptoethanol (also β-mercaptoethanol)
Methanol
Mixed bed ion exchange resin
NaCl

Progen Industries Ltd
Merek
AnalaR® BDH
Sigma
AnalaR® BDH
CSL (Commonwealth Serum Laboratories)
Promega
Life Technologies
Amersham
AnalaR® BDH
AnalaR® BDH
Ajax Chemicals
Sigma
AnalaR® BDH
Life Technologies
Sigma
Sigma
Bio-Rad
AnalaR® BDH
Bio-Rad
AnalaR® BDH
Na₂HPO₄
NaOH
Nick Translation kit™
N-laurylsarcosine blocking reagent
OptiMEM® 1 (reduced serum medium)
Phenol
Phytohaemagglutinin P
Polaroid T667 film
Potassium Acetate
Propan-2-ol (Isopropanol)
Propidium Iodide (95-98%)
Proteinase K
Restriction enzyme 10x buffer (B, C & H)
RNase A
Streptavidin-fluorescein
Streptomycin Sulphate
Sodium Citrate
Sodium Dodecyl Sulphate [SDS]
Sodium Iodate
Thymidine
TRIZMA® base
TWEEN®20 [Poly(oxyethylene)_₇-sorbitan monolaurate]
Westar cement

Whatman® Filter paper #1

Xylene Cyanole FF (Acid blue)

Jacksons, Subiaco, WA

Selby

Sigma
II.2. Reagents in use

**Anti-fade mounting media**

Tris·Cl 20 mM pH 7.5 1 part

Glycerol (containing DABCO 2%) 9 parts

To make glycerol/DABCO:

Dissolve 360mg of DABCO into 18ml of glycerol at 70°C for 5 minutes.

To make antifade:

Add 2ml of Tris·Cl 20 mM (pH 7.5) to 18ml of glycerol/DABCO mixture.

Mix on wheel for 2 hours, then store at 4°C.

(Laboratory Manual, Dept. of Cytogenetics and Molecular Genetics, Adelaide Children's Hospital, SA. -FISH technique)

**Buffer #1 (For Minipreparations of DNA)**

50mM glucose

25mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

The above solution is prepared in batches of 500ml, it is then autoclaved for 15 minutes at 10lb/sq.in. on liquid cycle, and stored at 4°C.

(Sambrook et al., 1989, p. B.22)
**Buffer #2** (For Minipreparations of DNA)

0.2M NaOH (freshly diluted from 10 M stock)

1% SDS

Buffer stored at room temperature

(Sambrook *et al.*, 1989, p. B.22)

**Buffer #3** (For Minipreparations of DNA)

5 M potassium acetate  60ml

glacial acetic acid  11.5ml

H₂O (double distilled)  28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

(Sambrook *et al.*, 1989, p. B.22)

**Deionised Formamide**

Formamide (redistilled)  500ml

Mixed-bed ion exchange resin  25g

Place formamide and resin in a dark bottle on a magnetic stirrer for at least one hour.

After this time filter formamide twice through Whatman filter paper #1 and store in aliquots of 50ml at -20°C until needed. [NB. formamide is deionised when there is no yellow colour in it and some of the resin remains blue.]

(Sambrook *et al.*, 1989, p.1.102)
**0.5 M EDTA (pH 8.0)**

Disodium ethylenediaminetetraacetic acid (EDTA) 186.1g  
H₂O (double distilled) 800ml

Add water to EDTA and stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (EDTA will not go into solution until pH is adjusted). Dispense solution into 100ml aliquots and sterilise by autoclaving (15 minutes at 10lb/sq.in. on liquid cycle).

(Sambrook *et al.*, 1989, p.B.11)

**Ethidium Bromide**

Ethidium Bromide 5.0g  
Double distilled water 500ml

Add ethidium bromide to water and stir on magnetic stirrer for several hours until the dye had dissolved. Store at room temperature in a dark bottle. NB: Gloves and mask should be worn when handling ethidium bromide.


**Gill’s Haematoxylin (1.5 x strength).**

To make 1.5L:

Ethylene glycol (ethanediol) 375ml  
Haematoxylin 4.5g  
Sodium Iodate 0.45g
Aluminium Sulphate 39.6g
Glacial Acetic Acid 30ml
Double-distilled water 1080ml

All of the reagents are mixed for at least 1 hour before the glacial acetic acid is added. This solution is stored at room temperature.

(Laboratory manual of Histopathology Department at the University of Western Australia, Department of Pathology).

Hybridisation buffer (to check the labelling of probe).

Dilution of the following reagents is made into 5xSSC

N-laurylsarcosine Blocking reagent 5% w/v
(Böhringer Mannheim)

Sodium salt 0.1% w/v

SDS 0.2% w/v

This solution is cloudy when made and it is stored at 4°C.

(Karen Smith, University Department of Pathology UWA. Personal correspondence).

"Kevit’s" Hybridisation Mix.

To make 100ml:

Dextran sulphate (10%) 10g
20xSSC 10ml
Formamide-deionised (FA) 50ml
Mix FA, SSC, and ddH₂O, pH to 7.0. Add dextran sulphate and stir at room temperature for 1-2 hours. Remove from stirrer and add TWEEN®20, roll to mix. Aliquot useful lots into Eppendorf tubes (eg. 100µl) and store at -20°C. Discard unused portion once thawed.

(Laboratory Manual, Dept. of Cytogenetics and Molecular Genetics, Adelaide Children's Hospital, SA. -FISH technique)

**LB Agarose**

First prepare LB liquid media and then add agarose.

**LB Liquid Medium (Luria-Bertani Medium):**

Per litre:

To 950ml of double distilled water (ddH₂O), add:

- Bacto-tryptone 10g
- Bacto-yeast extract 5g
- NaCl 10g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH (~ 0.2ml). Make up to a final volume of 1 litre with dd H₂O. Sterilize by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle.

To prepare LB agarose make LB medium from the recipe above. Just prior to autoclaving add one of the following:
15g/L of agarose for plates or 7g/L for top agar.

To sterilize autoclave as above. Before pouring plates allow agarose to cool to ~50°C add any heat labile substances at this temperature (eg. antibiotics).

(Sambrook et al., 1989, p. A.1 & A.4)

**Lithium Chloride (LiCl) for random primed labelling reaction**

4M LiCl solution is used in the random primed method, however

3M sodium acetate or

3M sodium chloride may be substituted for LiCl.

(Boehringer Mannheim, The DIG system user's guide for filter hybridisation, p.7)

**Loading buffer (for electrophoresis gels)**

6x Buffer type III  
0.25% bromophenol blue

0.25% xylene cyanole FF

30% glycerol in water

This buffer is stored at 4°C.

(Sambrook et al., 1989, p.6.12)

**Opti-MEM (Blood culture medium)**

To 100ml of OptiMEM® 1 add by sterile technique:-

L-glutamine 1.0ml

Penicillin and Streptomycin 1.0ml
Heparin (1000U/ml) 1.0ml
Phytohaemagglutinin 1.25ml
Fetal Bovine serum 5.0ml

Store medium at 4°C, after the above reagents are added to OptiMEM discard unused portion within 2 weeks.

(Cytogenetics Laboratory manual, p.64. SHLS, WA).

**Phosphate Buffered Saline (PBS)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24g</td>
</tr>
</tbody>
</table>

Dissolve the above in 800ml of double distilled water (ddH₂O). Adjust the pH to 7.4 with HCl and add ddH₂O to make a final volume of 1 litre. Sterilise by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle, then store at room temperature.


**Phenol (equilibrated)**

Before use phenol must be equilibrated to a pH >7.8.

Phenol prior to equilibration should be stored at -20°C. To equilibrate:

1. Remove phenol from freezer, allow it to warm to room temperature and melt it at 68°C.
2. Add hydroxyquinoline at a final concentration of 0.1%, this gives the phenol a yellow tinge and allows rapid identification of the organic phase.

3. To the melted phenol add an equal volume of buffer [0.5M TrisCl pH 8.0 at room temperature]. Stir the mixture on a magnetic stirrer for 15 minutes. Allow the two phases to separate and aspirate as much as possible of the upper aqueous phase.

4. Repeat step 3, as many times as is required to get phenol to a pH of >7.8 (measure pH with pH paper).

5. After phenol is equilibrated and final aqueous layer is removed, add 0.1 volume of 0.1M TrisCl (pH 8.0) containing 0.2% β-mercaptoethanol. Phenol solution is stored in this form under 100mM TrisCl (pH 8.0) in a dark bottle at 4°C for up to 1 month.

(Sambrook et al., 1989, p. B.4).

**Phenol:Chloroform:isoamyl alcohol (25:24:1)**

Phenol is equilibrated as above and added in equal parts to chloroform:isoamyl alcohol (24:1). Neither chloroform nor isoamyl alcohol require treatment before use. The phenol:chloroform:isoamyl alcohol mixture is stored under 100mM TrisCl (pH 8.0) in a dark bottle at 4°C for up to 1 month.

(Sambrook et al., 1989, p. B.5).
RNase A

Sodium acetate 0.01M (pH 5.2)

Tris·Cl 1M

Dissolve RNase A to a concentration of 10mg/ml in sodium acetate. Heat to 100°C for 15 minutes, allow to cool and adjust the pH by adding 0.1 volumes of 1m Tris·Cl (pH 7.4). Dispense into 500μl aliquots and store at -20°C. For RNase slide preparation prior to *in situ* hybridisation dilute RNase A to a concentration of 100μg/ml with sterile water or 2xSSC.


Scott’s tap water substitute.

To make 2 litres:

- Sodium bicarbonate: 7.0g
- Magnesium Sulphate: 40g
- Crystal thymol: 3-6 crystals
- Tap water: approximately 2 litres

All reagents are mixed in 1.8 litres of tap water until completely dissolved, then the final volume of the solution is made up to 2 litres.

(Laboratory manual of Histopathology Department at the University of Western Australia, Department of Pathology).
**Sodium dodecyl sulphate (SDS)**

SDS (electrophoresis grade) 100g

Double distilled water (ddH₂O) 1 litre

Dissolve SDS in 900ml of ddH₂O. Heat to 68°C to assist dissolution and adjust pH to 7.2 by adding a few drops of c.HCl, make up to a final volume of 1 litre and store at room temperature.

(Sambrook et al., 1989, p. B.13).

**STE buffer**

NaCl (0.1 M)

TrisCl (10M, pH 8.0)

EDTA (1mM, pH 8.0)

Mix NaCl, TrisCl, and EDTA solutions of the above concentrations to final pH of 8.0. Sterilise by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle. Store at room temperature.

(Sambrook et al., 1989, p. B.13).

**20X SSC**

NaCl 175.3g

Sodium Citrate 88.2g

Double distilled water (ddH₂O) ~1 litre
Dissolve NaCl and sodium citrate in 800ml of ddH$_2$O and adjust pH to 7.0 with 10M NaOH, make up to a final volume of 1 litre with water. Sterilise by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle. Store at room temperature.

(Sambrook et al., 1989, p. B.13).

**TAE buffer**

50x stock solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>242g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>57.1ml</td>
</tr>
<tr>
<td>0.5M EDTA (pH 8.0)</td>
<td>100ml</td>
</tr>
</tbody>
</table>

1x buffer contains 0.04M tris acetate and 0.001M EDTA

(Sambrook et al., 1989, p. B.23)

**Tris-buffered saline (TBS)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2g</td>
</tr>
<tr>
<td>Tris base</td>
<td>3.0g</td>
</tr>
</tbody>
</table>

Dissolve the above into 800ml of double distilled water (ddH$_2$O), and adjust pH to 7.4 with HCl. When desired pH is achieved make up to a final volume of 1 litre with ddH$_2$O. Sterilise by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle. Store at room temperature.

(Sambrook et al., 1989, p. B.14).
**TE Buffer (pH 7.4)**

10mM Tris Cl pH 7.4

1mM EDTA pH 8.0

This buffer is stored at room temperature.


**Tris-HCl buffer**

Tris base 121.1g

Double distilled water (ddH2O) ≈1L (less due to addition of HCl)

Add Tris base to 800ml of water and adjust pH by adding concentrated HCl:-

<table>
<thead>
<tr>
<th>pH</th>
<th>c.HCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.4</td>
<td>70ml</td>
</tr>
<tr>
<td>7.6</td>
<td>60ml</td>
</tr>
<tr>
<td>8.0</td>
<td>42ml</td>
</tr>
</tbody>
</table>

Make final adjustments to pH when solution is at room temperature, make up to final volume of 1 litre. Sterilise by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle. Store at room temperature. [NB the pH of Tris solutions is temperature-dependent and decreases approximately 0.03 pH units for each 1°C increase in temperature].

2xYT medium

Per Litre:

To 900ml of double distilled (ddH₂O), add:

- Bacto-tryptone 16g
- Bacto-yeast extract 10g
- NaCl 5g

Shake until the solutes have dissolved. Adjust the pH to 7.0 with 5 N NaOH. Make up to a final volume of 1 litre with dd H₂O. Sterilize by autoclaving for 20 minutes at 15lb/sq.in. on liquid cycle.

2xYT Agarose

To prepare 2xYT agarose make 2x YT medium from the recipe above. Just prior to autoclaving add one of the following:

- 15g/L of agarose for plates or 7g/L for top agar.

To sterilize autoclave as above. Before pouring plates allow agarose to cool to ~ 50°C add any heat labile substances at this temperature (e.g. antibiotics).

(Sambrook et al., 1989, p. A.3 & A.4)
III. Methods
III. Methods

III.1. Preparation of a cosmjd for labelling

III.1.i). Chromosome 22 specific cosmjd probes

Two different cosmjd probes were used for *in situ* hybridisation. The first, cosmjd C179, was obtained from Prof. J Burn of the Department of Human Genetics at the University of Newcastle upon Tyne, U.K. It is a unique copy probe for a single repeat region of the genome which corresponds to chromosome 22q11. This region is believed to be deleted in affected individuals with DGS. The other cosmjd probe used was D22S75, and it too is a marker within the DGCR. The probe was purchased from ONCOR®, Gaithersburg, USA. The commercial probe is prepared for *in situ* hybridisation prior to purchase. It is labelled with digoxigenin and packaged in a hybridisation solution as single stranded DNA. This means that it requires no labelling or denaturation before hybridisation, unlike the donated probe.

III.1.ii). The preparation of non-commercial cosmjd probes

Cosmid C179 arrived from the U.K. in a kanamycin-resistant strain of enterobacteria, as a stab culture. (Despite repeated queries to the donor laboratory for further details, information on the provenance of the probe and the host organism were not forthcoming). To isolate the cosmjd probe from the stab for use in an *in situ* hybridisation experiment, the bacteria was streaked on to an agar plate, containing
kanamycin, and allowed to grow. A single colony was then amplified in the appropriate medium. Once amplified, the cosmid was extracted from the bacteria by alkaline lysis and purified. Cosmid C179 was isolated and purified using this method by Dr. E. Edkins at the Molecular Biology Department of Princess Margaret Hospital for Children (WA). After the probe DNA was extracted and purified, it was ready for in situ hybridisation with metaphase chromosome spreads. A small-scale preparation (termed a mini-preparation, Sambrook et al., 1989 p.125) was first attempted, to ensure presence of the desired cosmid in the bacteria. When this had been confirmed, a large scale version was performed.

III.1.iii). Isolation of cosmid DNA from bacteria (Small-scale preparation of C179)

**DAY 1:**

A loop of cosmid C179 was first streaked on to a 2xYT agarose plate by sterile technique. The agarose contained kanamycin at a concentration of 10µg/ml. To pour the plate, 2xYT agarose (see 'II.2. Reagents in use') was autoclaved and allowed to cool in an incubator to around 50°C. This temperature is sufficiently cool to permit the addition of antibiotic to the agar without altering the molecular structure of the antibiotic and destroying its effect. After the agarose had been mixed with the kanamycin, the plates are poured and left to set. A loop of the kanamycin-resistant bacteria which contains C179 could then be removed from the stab culture and streaked onto a 2xYT agarose plate. The plate was then inverted and incubated at 37°C overnight.
**III.I.iv. Amplification of cosmid DNA**

**DAY 2:**

To culture the bacteria containing C179, a single colony of C179 was transferred from the agarose plate into 2ml of 2xYT medium containing kanamycin (10µg/ml) in a loosely capped 15ml tube. The tube was again incubated at 37°C overnight with vigorous shaking.

**DAY 3:**

An aliquant of culture (1.5ml) was pipetted into a microcentrifuge tube with any surplus culture stored at 4°C. The tube was centrifuged at 12 000g for 30 seconds at 4°C in a microfuge. The supernatant was removed from the tube by aspiration and inverted on to a paper towel for 2 minutes, this ensured that the bacterial pellet was as dry as possible. The pellet was resuspended by vigorous vortexing in 100µl of ice cold Buffer #1 (see 'II.2. Reagents in use' for Buffers #1, #2 & #3). Freshly prepared Buffer #2 (200µl) was added, the cap on tube replaced tightly, and the contents mixed by rapidly inverting the tube 4-5 times. The tube was then placed on ice and 150µl of ice cold Buffer #3 added. The tube was vortexed gently for 10 seconds to disperse Buffer #3 through the viscous lysate, and placed back on ice for 3-5 minutes. After this interval the tube was centrifuged at 12 000g for 5 minutes at 4°C in a microfuge and the supernatant transferred to a fresh tube.
III.I.v). Precipitation of cosmid DNA

The DNA was precipitated with 2 volumes of ethanol at room temperature and the tube mixed by vortexing. The mixture was allowed to stand at room temperature for 2 minutes, and the tube then centrifuged at 12,000g for 5 minutes at 4°C in a microfuge. The supernatant was removed by gentle aspiration and the tube allowed to stand in an inverted position on a paper towel to allow all of the fluid to drain away. The DNA pellet was rinsed with 1ml of 70% ethanol at 4°C and the supernatant removed as before without disturbing the pellet. The pellet was allowed to dry at room temperature for 10 minutes and then redissolved in 50μl of TE buffer (pH 8.0) containing 20μg/ml of DNAase free RNase A. After vortexing briefly the DNA was stored at -20°C.

III.I.vi). Determination of the concentration and purity of cosmid DNA by the absorbance method

To measure the absorbance of the DNA sample at a wavelength of 260nm and 280nm, a 1:2 000 dilution of the DNA sample was made in sterile water. The spectrophotometer was blanked with sterile water at both wavelengths and the absorbance of the sample measured. The concentration of the DNA in the sample could then be calculated with the use of a software packaged linked to the spectrophotometer.
III.1(vii). Verification of presence of cosmid DNA

A sample of cosmid C179 DNA which had been extracted from the bacteria by mini-preparation, was run on an agarose mini-gel to verify the presence of insert DNA and to determine if any contamination was present. An agarose gel (0.8% in TAE buffer) was poured on to a mini-gel tray, a small toothed comb inserted and the gel allowed to set for 10 minutes. When set the comb was removed and the DNA sample to be tested was loaded into one of the wells along with markers of known size in the adjacent wells. The gel was run at a constant 100 Volts for 1 hour. It was then removed from the gel tray and washed in ethidium bromide with agitation for 10 minutes. The gel was then visualised on a fluorescent (UV) light box, photographed and the photograph then analysed, the distance that the DNA travelled through the gel is indicative of its fragment size when compared to the size of the known marker (in this case λ Pst I). The un-cut probe DNA purity could be estimated by comparing the number of bands which appear in the probe DNA lane to those of a known pure control sample photograph. This comparison was usually made between the photographs of the control and the tested DNA.

III.1(viii). Large scale preparation of cosmid DNA

When the cosmid DNA had been isolated by mini-preparation, a large scale or what is termed a maxi-preparation was set up. This preparation is a modified version of the alkaline lysis mini-preparation method (Felliciello and Chinali, 1993), and it yields a greater volume of probe DNA. The protocol for this procedure was;
DAY 1:
A single colony of kanamycin-resistant bacteria containing C179, was transferred into 500ml of 2xYT media (10μg/ml kanamycin) in a 2 litre capacity conical flask. The flask was incubated overnight at 37°C with vigorous shaking.

DAY 2:
The contents of the flask were transferred into 2 centrifuge bottles (250ml) and spun at 4000g in a Sorvall GS3 rotor for 15 minutes at 4°C. The supernatant was discarded and the bottles allowed to drain in an inverted position on paper towels. The bacterial pellets in each bottle were then resuspended in 50ml of ice-cold STE buffer. After mixing the bottles were centrifuged at 4000g in a Sorvall GS3 rotor for 15 minutes at 4°C. The supernatant was discarded and 9ml of ice-cold buffer #1 added, the bottles were then stood at room temperature for 5 minutes before 20ml of freshly prepared buffer #2 was added. The caps on the bottles were replaced tightly, and they were then inverted several times. Ice-cold buffer #3 (10ml) was added to the bottles, they were shaken vigorously, and stood on ice for 10 minutes. The resulting lysate was centrifuged at 4000g for 15 minutes at 4°C. After this interval the supernatant from both bottles was filtered through 4 layers of cheesecloth into a single Oak Ridge tube and centrifuged at 5000g for 15 minutes at room temperature. The supernatant was carefully decanted from the tube and the pellet and the walls of the tube were rinsed in 70% ethanol at room temperature. All ethanol was drained from the tube by inversion for several minutes on to a paper towel, after which time the pellet was dissolved in 3ml of TE buffer (pH 8.0).
The C179 cosmid DNA was then purified by equilibrium centrifugation in CsCl-ethidium bromide gradients. After isolation, the purity and concentration of the probe were tested by the same methods as described in the small-scale preparation of probe DNA.

III.2. Labelling of cosmid DNA

III.2.i). Methods of labelling cosmid DNA

There are three different methods of labelling cosmid DNA for use with in situ hybridisation. These are the direct, nick translation, and random priming methods. Each is outlined below:-

III.2.i.a. Fluorescein direct-labelling method

This method labelled the DNA directly, i.e., biotin or digoxigenin were not required. The fluorescein was incorporated into the cosmid DNA, rather than attached to a hapten or reporter molecule. The DNA was diluted in sterile water to the desired concentration. Then, using a nick translation kit, the fluorescein was incorporated into the cosmid DNA. The tubes from the nick translation kit were thawed and then placed on ice. It should be noted that Klenow enzyme must be kept at -20°C, to protect its activity. Once the rest of the kit had thawed, nucleotides and water were added to the DNA according to the following protocol:

Cosmid C179 (1μl) at a concentration of 100 ng/μl was placed into a clean microcentrifuge tube, to which the following were added:
10µl dATP, 10µl dCTP, 10µl dGTP, 2µl dTTP, 1µl of Fluorogreen (always kept in dark) (Amersham), 11µl of sterile water (to make up to a final volume of 50µl) and 5µl of enzyme directly from the freezer. The tube was then centrifuged in a microfuge at 12 000g for 20 seconds. The tube was incubated for 4 hours at 15°C and the reaction stopped by the addition of 5µl of 0.2 M EDTA. The labelled probe was then pipetted into 10µl aliquots and stored in the dark at -20°C.

III.2.i.b. Nick translation

An aliquot of extracted C179 was sent to the Department of Cytogenetics and Molecular Genetics at the Adelaide Women’s and Children’s Hospital. This sample was labelled by nick-translation using the method developed by David Ward and co-workers at Yale University (Langer et al., 1981), as described below:

Labelling reaction for nick-translation:

The following reagents were mixed in an Eppendorf tube, 5µl of 10x concentration nick-translation buffer (0.5M Tris-HCl; pH 7.8, 50mM MgCl₂, 0.5mg/ml BSA, nuclease free), 5µl of 100mM Dithiothreitol (DTT), 4µl of deoxynucleotide triphosphates (0.5mM dATP, dGTP, dCTP, each), 1µl of 0.5mM dTTP, 2µl of 1mM Biotin-16-dUTP (Fluorescein or digoxigenin could alternatively be used), 1µg of C179 probe, 1µl of DNAase I (1 000 times diluted from 1mg/ml stock) and 10 units (1µl) of DNA polymerase I. The mixture was made up to a final volume of 50 µl with sterile water and after mixing was incubated for 2 hours at 15°C. The reaction was stopped by the addition of 5µl 0.5M EDTA (pH 7.4). The DNA was then purified by Sephadex G-50 chromatography using a pasteur pipette column,
equilibrated with 10 mM Tris-HCl, 1 mM EDTA and previously flushed with 0.1% SDS (to prevent non-specific binding). The high molecular weight DNA eluted in the second 600 µl; this fraction was collected and stored at -20°C.

III.2.ii. Random priming method of labelling DNA

This third method was carried out on a portion of the extracted cosmid probe. In each reaction between 3 µg to 10 ng of DNA could be labelled. The DNA concentration of C179 was 0.1 µg/µl and 10 µl was used i.e., in this instance 1 µg of DNA was labelled. The DNA was first denatured in an Eppendorf tube with a hole pricked in its lid, by heating it in a boiling water bath (100°C) for 10 minutes. It was removed from the water bath and immediately placed on ice for 5 minutes.

The following reagents were then added to the tube on ice; 2 µl of hexanucleotide mixture, 2 µl of dNTP mixture, 5 µl of nuclease-free water (to make up to a volume of 19 µl) and 1 µl of Klenow enzyme, which resulted in a total tube volume of 20 µl. It was ensured that the cap was firmly fastened on to the tube, the tube was briefly vortexed and then centrifuged in a microfuge at 12 000g (≈ 10-20 seconds). The tube was incubated overnight in a waterbath at 37°C. The next day the reaction was stopped by the addition of 2 µl of 0.2 M EDTA (pH 8.0). The labelled DNA was precipitated with 2 µl of 4 M LiCl and 60 µl of pre-chilled absolute ethanol (-20°C). The tube was well mixed and left for 1 hour at -70°C, then centrifuged for 5 minutes at 12 000g (room temperature) and the supernatant removed. The pellet was washed with cold (-20°C) 70% ethanol, and dried under a vacuum to zero volume. The DNA was dissolved in 50 µl of TE buffer (pH 8.0) at 37°C and then stored at -20°C.
III.2.ii). The reporter molecules used to label cosmid probe

As previously stated (p20), probes need to be labelled either directly or with a reporter molecule prior to use for in situ hybridisation reactions. To directly label C179 Fluorogreen® (fluorescein-12-dUTP) from Amersham was used, the probe was labelled in accordance with the manufacturer’s recommendations using a nick-translation kit. This type of kit was also used to label C179 with biotin-16-dUTP. Biotin acted as a reporter molecule (or hapten), it was incorporated into the probe DNA and detected by affinity cytochemistry. In the case of biotin-labelled probes, avidin or streptavidin had a high binding capacity to biotin and were used to detect its presence (Langer et al., 1981). The second type of hapten used was digoxigenin-11-dUTP (DIG), C179 was labelled with this molecule by the random prime method. Hybridised DIG probes are detected by high affinity anti-digoxigenin antibodies which are conjugated to enzymes.

III.2.iii). Confirmation of probe labelling

To confirm that the probe had been labelled, a 5 μl aliquot was hybridised to a piece of nylon membrane and detected by the antibody to the label conjugated to an enzyme; C179 labelling with DIG was confirmed by this method:
DAY 1:

Unlabelled C179 (2μl) was pipetted into an Eppendorf tube and 2μl of unlabelled salmon sperm DNA into another. The contents of both tubes were denatured by boiling in a waterbath at 100°C for 10 minutes, then immediately removed from the waterbath and placed on ice. A piece of nylon membrane (Amersham Hybond N+), was cut to approximately 2.5 x 5.0 cm and a small nick removed from one corner of the membrane, below which a cross was drawn with a pencil (see figure IV.1). This is to enable the DNA spots which are loaded on to the membrane to be distinguished from one another. Two aliquots of C179 were loaded onto the membrane (1μl each) along with two lots of salmon sperm DNA, so that there were 4 separate dots of DNA. The position of each dot of DNA relative to one another was recorded. The DNA was then bound to the membrane by heating at 62°C for 1 hour. DIG-labelled C179 (5μl) was pipetted into a microfuge tube, denatured by heating to 100°C in a boiling water bath for 10 minutes, and immediately placed on ice. The membrane was put into a hybridisation bag with 1-2 ml of hybridisation buffer (see II.2. Reagents in use), containing 5μl of denatured DIG-labelled C179. The bag was sealed making sure that there were no air bubbles, and the membrane hybridised overnight in a water bath set to 68°C.

DAY 2:

The membrane was removed from the hybridisation bag and washed with agitation as follows:
2xSSC; 0.1% SDS for 2x5 minutes at room temperature, then 0.1xSSC; 0.1% SDS for 15 minutes at room temperature. The hybridised DNA on the membrane was then detected by the following protocol:

The membrane was washed briefly (2 minutes) in buffer #1 (from Boehringer Mannheim labelling kit; containing 100mM maleic acid, 150mM NaCl, pH 7.5) with agitation. It was then incubated in buffer #2 (also from the kit; 1% blocking reagent in buffer #1) for 30 minutes. The membrane was washed for a second time in buffer #1 for 2 minutes with agitation. The antibody conjugate (vial 8 from kit; anti-DIG alkaline phosphatase) was diluted to a concentration of 1:5000 in buffer #1 i.e., 4μl of anti-DIG alkaline phosphatase is mixed with 20 ml of buffer #1. The membrane was incubated with the diluted antibody conjugate at room temperature for 30 minutes. It was then washed twice in buffer #1 at room temperature for 15 minutes each with agitation to remove unbound antibody. After washing, the membrane was equilibrated for 2 minutes in 20 ml of buffer #3 (from kit; 100mM Tris-HCl, pH 9.5; 100 mM NaCl, 50 mM MgCl₂). The following solutions were mixed to make the colour solution just prior to use; 10 ml of buffer #3, 45μl of NBT solution (vial 9 from kit which contained 75mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide), 35μl of X-phosphate solution (vial 10 from kit which contains 50mg/ml 5-bromo-4-chloro-3-indoly l phosphate, toludinium salt in 100% dimethylformamide). The membrane was incubated with 10 ml of colour solution whilst sealed in a darkened box. The colour precipitate which demarcates the hybridisation areas on the membrane, usually formed within 10-30 minutes and
continued forming for 16 hours. When the required intensity had formed the reaction was stopped by washing the membrane in approximately 10 ml of TE buffer at pH 8.0 (buffer # 4 in kit). A photocopy or photograph of the membrane was taken to create a permanent result, the membrane was then dried and sealed in a plastic bag. The colour of the precipitate faded upon drying but it could be recovered by wetting the membrane in buffer # 4.

III.3. Preparation of metaphase chromosome spreads

III.3.i). Collection of peripheral whole blood

Peripheral whole blood (1-2ml) was taken by veno-puncture from each patient, then placed into sterile 5ml vials each containing 0.1ml of heparin (1000 units/ml) and the specimens sent to the Cytogenetics Department at State Health Laboratory Services. Heparin was used because it is an efficient anticoagulant which did not interfere with lymphocyte culture.

III.3.ii). Culturing of blood, synchronisation of cell cycle and arrest at metaphase

Throughout the whole of the culturing process, specimens were handled by sterile technique. The tubes were first ‘set-up’, i.e., 3 sterile tubes (10 ml capacity) of blood were set up on each patient, 2 were processed as synchronised cultures and 1 as a high resolution culture. All 3 of the tubes received 0.25 ml of whole blood and 5.0ml of Opti-MEM supplemented medium, the caps were put on the tubes loosely
and they were incubated at 37°C in a CO₂ enriched environment for 48 hours. After which the tubes were removed from the incubator and 100μl of thymidine, was added by sterile technique to block DNA synthesis. The tubes were returned to the incubator with the same conditions as before for a further 17 hours. After this time 100μl of deoxycytidine was added to release the block on DNA synthesis and the cells in culture were allowed to resume the cell cycle in the incubator at 37°C. Colcemid (50μl) was added after 4 1/2 hours to the 2 tubes which were to be used for synchronised karyotyping and in situ hybridisation, they were then incubated for a further 30 minutes prior to the harvesting the cells. The tube which was to be used for high resolution (HRS) karyotyping remained in the incubator for 4 hours and 50 minutes before it too received 50μl of colcemid. It was then returned to the incubator for 10 minutes and the cells were harvested at the same time as those of the synchronised cultures.

III.3.iii). Harvesting of lymphocytes and fixation of cell suspension

The following reagents were needed for harvesting blood cultures:-

KCl (0.057M) warmed to 37°C in a water bath; allow 5ml of KCl per tube.

Acetic acid (4%) at room temperature; allow 5ml per tube.

Fixative (3 parts methanol to 1 part acetic acid) prepared fresh; allow 5ml/wash.

Procedure:

Working in a biohazard hood, the blood culture was transferred to a centrifuge tube (10ml) and the cap was screwed on tightly. The tubes were then centrifuged at 1500
rpm for 5 minutes, the supernatant was removed and the pellet of lymphocytes resuspended. A hypotonic solution was added to the tube (5ml of KCl per tube at 37°C) and mixed, the tube was then centrifuged at 1500 rpm for 5 minutes to lyse the erythrocytes. The tube was carefully removed from the centrifuge, the supernatant above the buffy coat removed and the pellet resuspended. Acetic acid (5ml of 4%) was added to the tube with mixing and the tubes centrifuged at 1500 rpm for 5 minutes. All but 0.5ml of supernatant was removed and the pellet was resuspended in the excess supernatant. The pellet was washed in 5ml of fresh fixative ensuring that the tube was well mixed, then centrifuged at 1500 rpm for 5 minutes. The supernatant was then carefully removed without disturbing the pellet. A further 5 minute wash in fixative followed this step and the tube was centrifuged as before. The supernatant was removed from the tube (with approximately 0.2ml left in the tube) and the pellet resuspended. The cell suspension was then ready to drop as metaphase spreads.

III.3.iv) Dropping and fixation on to microscope slides of cell preparation

With a thin tipped 1ml disposable pipette, fresh fixative was added to the cell suspension drop by drop until the suspension cleared to a very faintly opaque consistency. A microscope slide was placed flat on the bench top and, using a fresh pipette, from a height of approximately 20cm 1 drop of cell suspension was dropped on to the slide. (All microscope slides were pre-cleaned by soaking in methanol for at least 30 minutes and allowed to air dry under a dust cover). Just before the drop
dried it was covered with a drop of fresh fixative and allowed to dry before the slide was moved, (as the drop of methanol/acetic acid fix spread across the slide the sides were carefully wiped with a tissue to prevent any back-flow of fix interfering with chromosome fixation).

III.3.v). Examination by phase contrast microscopy

When the slide was dry it was examined by phase contrast microscopy. Metaphase chromosomes should be clearly visible. Chromosomes were judged to be well spread when there were at least 20-30 clear metaphases viewed under the microscope. Good preparations should be dark grey in colour with a minimal amount of cytoplasm visible; if the chromosomes were pale it was a sign that they were too dry and if they were shiny and black they were said to be refractile. Both of these variant forms would be detrimental for in situ hybridisation or Giemsa banding. To prevent chromosomes from being too dry they were dropped onto a microscope slide which was placed on top of a paper towel that had been pre-warmed by soaking in hot water. Refractile chromosome preparations could be dropped in a fume hood next to a lighted Bunsen burner in order to optimise their banding potential.

III.3.vi). Storage of slides and surplus suspension

If the slides were not immediately required for dropping, the tubes containing the harvested cell suspension were filled to the brim with fresh fixative and stored at 4°C for up to 3 months. Alternatively, only 5ml of fresh fixative was added and they
were stored at -20°C for up to 1 year. After storage the tubes were twice centrifuged as before and the supernatant removed, the pellet was then washed both times in 5ml of fresh fix before dropping.

III.4. In situ hybridisation

III.4.i). Protocols for in situ hybridisation

A number of different protocols were used in order to produce a suitable method for in situ hybridisation which would be successful with the C179 probe. The protocols were modified for the various labelling techniques that were carried out. In the following pages these protocols are outlined and the specific variations made to each for C179 cosmid probe in situ hybridisation will be expanded upon in the Discussion section of the thesis.

III.4.i).a. Protocol #1 direct method

The first attempt at in situ hybridisation was using C179 which had been labelled directly with Fluorogreen® (Amersham). It is a modification of the in situ hybridisation protocol from the Department of Cytogenetics and Molecular Genetics at Adelaide Women's and Children's Hospital and the protocol supplied with the fluorochrome by Amersham.

**DAY 1:**

1. A Coplin jar (with lid) containing 50ml of 70% deionised formamide/2xSSC (pH 7.0) was left to heat in a water bath set at 70°C.
2. RNase A (200μl of 0.1mg/ml) was pipetted on to each slide covered with a glass coverslip (22 x 50mm). The slides were incubated for 1 hour at 37°C in a sealed plastic container with a 2xSSC saturated environment.

3. At the end of the hour, the coverslips were removed from the slides and the slides washed as follows:

   The slides were washed for 4 x 1 minute in 2xSSC at room temperature with agitation, using fresh 2xSSC each wash. The slides were dehydrated through a series of alcohols (70% ethanol, 95% ethanol) for 1 minute in each, then rinsed quickly in 1-2ml of 100% ethanol. The slides were then stood upright and allowed to dry at room temperature. When dry they could remain at this stage for several hours.

4. In order to hybridise probe DNA to chromosomal DNA both were denatured so that they were single-stranded. The probe DNA was denatured first, with 100ng of probe DNA used per slide. C179 with Fluorogreen had a concentration of 100ng/μl, therefore 1μl of C179 per slide was pipetted into a small (600μl capacity) microfuge tube along with driver DNA. The driver DNA used in this instance was autoclaved salmon sperm DNA; a ratio of 75:1 driver:cosmid DNA was used. The driver DNA and the probe DNA were then desiccated to zero volume in a “speedivac”. (It should be noted that fluorescein is light-sensitive and as such should be exposed to only the minimal amount of light). When this reduction in volume had been achieved 10μl per slide of Kevil’s hybridisation mix was added to the tube. The tube was quickly vortexed and then centrifuged at 12000g for ~20 seconds in a microfuge. The DNA was then be denatured by heating for 5 minutes in a thermal cycler, (various
temperatures, 70°C or 94°C, were tested using this method). The tube was removed from the machine at the end of the cycle and placed immediately on ice for 1 minute. The probe was then allowed to pre-reassociate for 30 minutes at 37°C. The chromosomal DNA on the slides was then denatured to correspond to the completion of the 30 minute incubation period. To denature the slides they were placed for 3 minutes in the 70% formamide/2xSSC which had been pre-warmed to 70°C. While slides were in the Coplin jar the tube was vortexed and quickly centrifuged at 12000g for ≈ 20 seconds in a microfuge. The slides were removed from the Coplin jar and, without allowing them to dry, 10µl of the tube contents was applied to the centre of each slide. The slides were then covered immediately with a 22 x 22mm glass coverslip which was secured with a spot of clear nail varnish. The slides were put into a sealed plastic container with a 2xSSC saturated atmosphere and hybridised overnight (16 hours) at 37°C.

DAY 2:

1. Two Coplin jars containing 50ml aliquots of 50% formamide/2xSSC were heated in a waterbath to 42°C. When this temperature was attained, the sealed container with slides in it was removed from the incubator. The slides were taken out of the container and the coverslips removed. The spot of nail varnish which secured the coverslips should also slide off, if it did not the edge of a scalpel blade was used to chip the varnish off the slide and the coverslip then removed.

2. After the coverslips had been removed the slides were washed with agitation as follows:
Twice for 10 minutes in 50% formamide/2xSSC at 42°C, twice for 5 minutes in 2xSSC at room temperature and once for 5 minutes in 1xSSC at room temperature.

3. The slides were then rinsed twice for 2 minutes in PBS at room temperature and drained briefly but not allowed to dry.

4. The chromosomes were counter-stained with the application of 20μl of anti-fade solution with propidium iodide to each slide.

5. The slide was then covered with a 22 x 50mm coverslip, and at this stage the slide was ready to view under a fluorescent microscope at the appropriate wavelength. NB slides were kept in the dark at 4°C, as colour of signal fades significantly within 48 hours if exposed at room temperature.

III.4.(b). Protocol #2 in situ hybridisation of CI79 labelled with Biotin-16-dUTP

This labelled probe was received from the Department of Cytogenetics and Molecular Genetics at the Adelaide Women’s and Children’s Hospital. The protocol used was the one supplied by that Department.

DAY 1:

1. Steps 1-3 from day 1 protocol #1 were repeated.

2. Again the probe DNA was denatured prior to the denaturation of chromosomal DNA. The optimal ratio of driver:probe DNA was determined by the Adelaide laboratory to be 400:1. To accomplish this ratio 20ng of probe and 8000ng of driver COT-1 DNA (COT-1) per slide were pipetted into a small microfuge tube and co-desiccated to zero volume in a “speedivac” as before.
3. Kevil's hybridisation mixture (10μl/slide) was added to the tube, which was then vortexed and quickly spun in a microfuge as in step 4 protocol #1 (DAY 1).

4. The tube contents were denatured by placing the tube in a polystyrene foam surround and floating it in a water bath at 70°C for 5 minutes. It was then immediately placed on ice for 1 minute, after which time the tube was incubated at 37°C for 40-90 minutes. The chromosomal DNA was denatured on the slides in 70% formamide/2xSSC as in step 4 protocol #1 (DAY 1) and 10μl of denatured probe was applied to each slide and covered as before. The slides were again hybridised overnight at 37°C in a 2xSSC saturated atmosphere.

**DAY 2:**

1. The slides were removed from the sealed container and steps 1-2 from protocol #1 (DAY 2) were repeated.

2. This probe was labelled with biotin and it was therefore necessary to incubate the slides with an antibody conjugated to fluorescein or an enzyme to detect hybridisation signal in this protocol. In this case streptavidin-FITC was used; streptavidin has a high binding affinity to biotin and it was conjugated to fluorescein.

3. Before the streptavidin-FITC was added, the slides were first treated at room temperature as follows: The slides were rinsed for 3 minutes in 4xSSC/0.05%Tween 20® in a Coplin jar at room temperature. The slides were pre-incubated in 4xSSC/1% BSA (100μl per slide under lab film) for 10 minutes (2xSSC saturated atmosphere).
4. The slides were kept in the dark as soon as the fluorescein conjugate was applied and they were not allowed to dry out. Drying was prevented by keeping slides in a 2xSSC saturated atmosphere during incubation periods. The streptavidin-FITC was made up to a dilution of 1:250 in PBS. 60μl per slide (under lab film) was applied to each slide, and the slides incubated at room temperature for 20 minutes.

5. The slides were washed and rinsed as follows:
Twice for 5 minutes in 4xSSC/0.05% Tween 20® (100μl per slide under lab film), then once for 2 minutes in 2xSSC and finally twice for 2 minutes in PBS.

6. The slides were then counter-stained and covered with coverslips as in steps 4 & 5, protocol #1 (DAY 2).

III.4.i.e. Protocol # 3 in situ hybridisation of C179 labelled with Digoxigenin-11-dUTP
A number of detection methods were attempted with the C179 probe that was labelled with digoxigenin (DIG). These include fluorescent in situ hybridisation (FISH) using an antibody conjugated to FITC, and non-isotopic in situ hybridisation without the use of fluorescence. The first part of the protocol for both types of detection was the same.

DAY 1:

1. Steps 2-3 from day 1 protocol #1 were repeated to RNase slides and the slides rinsed.
2. COT-1 driver DNA and probe DNA were pipetted into a small microfuge tube and co-desiccated as before to zero volume. A number of driver:probe ratios were tried, they ranged from 16:1 to 500:1.

3. Kevit’s hybridisation mixture (10μl of per slide) was added to the tube and the tube was then quickly vortexed and centrifuged as before.

4. The tube contents were denatured by floating it in a 70°C waterbath for 5 minutes, it was then placed on ice for 1 minute to ensure that the DNA remained single-stranded.

5. The probe DNA pre-reassociated by leaving it to stand at 37°C for 10-90 minutes.

6. At the end of this time the tube was vortexed and centrifuged as before and 10μl of probe mixture was applied to each slide.

7. The mixture was covered with a 22 x 22mm glass coverslip and all the edges of the coverslip and slide were sealed with rubber cement.

8. The rubber cement was allowed to dry for 5-15 minutes, and the chromosomal DNA was then denatured by heating the slide on the upper surface of a metal plate in a waterbath at 70-75°C for 10 minutes. (The metal plate was positioned on the surface of the water and prevented the slide from becoming immersed).

9. The slide was removed from the waterbath and incubated overnight at 37°C in a 2xSSC saturated environment.

**DAY 2:**

1. Rubber cement was removed from around the coverslip with forceps and the coverslip was then allowed to slide off the slide as it was washed.
2. Slides were washed at 37°C with agitation as follows:

Twice for 6 minutes in 2xSSC, twice for 7 minutes in 1xSSC and then once for 8 minutes in 0.1xSSC.

3. After washes the detection method of choice was followed.

III.4.i).c.(a). Anti-digoxigenin horse radish peroxidase (POD) or (α-DIG HRP)
detection

1. Each slide received 7.5 units of α-DIG HRP in TBS to a total volume of 70μl per slide, (3.5μl of α-DIG HRP in 66.5μl of TBS). The slides were incubated in this solution for 1 hour at 37°C, in a 2xSSC saturated environment.

2. The slides were then washed twice for 5 minutes in TBS.

3. To detect the horse radish peroxidase a colour solution was applied to the slides. The colour solution was made from the following reagents; 6.0mg of diaminobenzidine solution (DAB), 10ml of TBS and 10μl of H₂O₂ (30%). It was mixed and then filtered through a sheet of fluted filter paper on to the slides. The solution was never prepared in advance because it is extremely heat and light sensitive, any surplus was discarded. The colour solution was left on the slides for 10 minutes and then removed by three 1 minute washes with agitation in TBS at room temperature.

4. The slides were most often counter-stained in 3% Giemsa solution for 10-15 minutes. Alternatively, haematoxylin followed by Scott's tap water was used as a counterstain (see II.2. Reagents in use).
5. Each slide was air-dried and mounted in DePeX mounting media, then covered with a glass 22 x 50mm coverslip. When the mounting media was dry slides were viewed under a light microscope.

III.4.i).c.(b). Anti-digoxigenin fluorescein (α-DIG FITC)

1. After the slides had been washed as in step 3 (DAY 2) they were rinsed for 3 minutes in 4xSSC/0.05%Tween 20® in a Coplin jar at room temperature. The slides were then pre-incubated (in a 2xSSC saturated atmosphere) for 10 minutes in 4xSSC/1% BSA (100μl per slide under lab film).

2. The slides were kept in the dark from this step on. The antibody used for detection, α-DIG FITC, was made up to a dilution of 1:250 in PBS and applied to the slides (60μl per slide under lab film), they were then incubated for 20 minutes in a 2xSSC atmosphere at room temperature.

3. The slides were washed and rinsed as follows:

   Twice washed with agitation for 5 minutes in 4xSSC/0.05%Tween 20® (100μl per slide under lab film), then rinsed once in 2xSSC for 2 minutes and twice in PBS for 2 minutes.

4. The slides were counter-stained and coverslips applied as in steps 4 & 5, protocol #1 (DAY 2).

III.4.i).d. In situ hybridisation of Oncor probe labelled with DIG-11-dUTP

This probe was hybridised in accordance with manufacturer’s recommendations.
**DAY 1:**

1. The slides were pre-treated in 2xSSC for 30 minutes at 37°C. The slides were then dehydrated through a series of ethanols (70%, 80%, and 100%) at room temperature for 2 minutes each, after which they were stood upright and allowed to dry.

2. The slides were denatured in 70% formamide/2xSSC at 70°C for 2 minutes then dehydrated through a series of ethanols (70%, 80%, and 100%) at -20°C for 2 minutes each. Again the slides were stood upright and allowed to dry.

3. The probe was pre-warmed at 37°C for 5 minutes, it was imperative that this probe was not heat denatured.

4. The probe which was mixed with a hybridisation solution prior to purchase, was applied (10µl to each slide) and covered with a 22 x 22mm glass coverslip which was then sealed with rubber cement.

5. The slides were hybridised overnight (16 hours), in a humidified chamber at 37°C.

**DAY 2:**

1. The slides were washed in 50% formamide/2xSSC (pH 7.0) at 43°C for 15 minutes, followed by an 8 minute wash in 2xSSC at 37°C. The slides were then transferred to PBS at room temperature where detection could proceed.

2. Full strength anti-digoxigenin FITC was applied to slides (60µl per slide) and they were incubated in a dark humidified chamber at 37°C for 5-30 minutes.

3. Slides were then rinsed three times for 1 minute in PBS at room temperature.
4. The chromosomes were counter-stained in antifade mounting medium with propidium iodide, at a final concentration of 0.3 μg/ml.

III.4.ii). Amplification steps for *in situ* hybridisation

All of the hybridisation protocols #2-4 can make use of amplification steps in order to amplify hybridisation signal. Instead of just one antibody being used, 2 or more steps were carried out. For example, rather than using just anti-digoxigenin horse radish peroxidase for a 1-step detection, anti-digoxigenin fluorescein followed by anti-fluorescein horseradish peroxidase could be used. This amplification step can increase the intensity of hybridisation signal.

III.4.iii). Enhancement of DAB

Hybridisation signal detected by the DAB colour reaction can also be intensified by the use of metals. At the end of the colour reaction and before washing in TBS, slides were rinsed in 2% CuSO_4(aq) at room temperature for 2 minutes. This increased the precipitation signal of DAB.
III.5. **Microscopy**

III.5.i). **Fluorescent microscope for fluorescein/PI**

To view slides counterstained with propidium iodide a Leitz fluorescent microscope was used at a wavelength of 595nm, this wavelength allowed the simultaneous visualisation of both propidium iodide and fluorescein-12-dUTP.

III.5.ii). **Confocal scanning microscope**

Slides which were stained by fluorescent reagents could be viewed under a confocal scanning microscope. This microscope was more sensitive than a standard fluorescent one and it can therefore detect hybridisation signal with greater clarity. The microscope used was at the Electron Microscopy Centre at the University of Western Australia and was operated by Dr. Ulrich Seydel.

III.5.iii). **Light microscope for HRP/DAB/Giemsa**

Slides counter-stained with Giemsa were viewed under a Leitz light microscope at high power with oil immersion.

III.5.iv). **Genevision used to produce images of light microscope slides**

A light microscope at the Cytogenetics Department of SHLS (WA) is connected via a video camera to a software package called "Genevision". This package allowed images from microscope slides at high power to be transferred on to a computer.
screen so that karyotyping could be done. Print outs from "Genevision" of hybridised metaphase spreads are shown in the results section.
IV. Results
IV. Results

IV.1. Isolation of C179 from bacteria

IV.1.i) Isolation of C179 DNA from bacteria and determination of the size

As previously stated (p44), cosmid C179 arrived from the U.K. as an agar stab culture, since no information was provided on the species or strain of the bacterium it was treated as *Escherichia coli* (*E. coli*) one of the more common types of bacteria used for plasmid and cosmid transfection. The Mini-preparation of C179 produced a visible pellet of DNA and the cosmid DNA thus isolated by this method was run on a mini-gel against markers of known size. It was determined that the DNA isolated was around 40kb in size when compared to markers of known molecular weight.

IV.1.ii) Maxi-preparation of cosmid C179

After C179 was successfully isolated from a small scale preparation, a maxi-preparation was set up. The maxi-preparation yielded 16 Eppendorf tubes of DNA at various w/v concentrations. Half of the tubes were sent to the Department of Cytogenetics and Molecular Genetics at Adelaide Women's and Children's Hospital, the remainder were retained to be labelled and used in *in situ* hybridisation reactions.
IV.2. Probe labelling

IV.2.i. Concentration of cosmid DNA

In order to label the probe the DNA concentration must first be determined, this was done by the absorbance method outlined in the Methods section. When a sample of C179 was tested by this method the following results were obtained.

Table IV.1  Concentration and purity of cosmid DNA

<table>
<thead>
<tr>
<th>ABSORBANCE RATIO MODE</th>
<th>DNA PURITY CHECK FACTOR 50</th>
</tr>
</thead>
<tbody>
<tr>
<td>wavelength</td>
<td>260nm</td>
</tr>
<tr>
<td>ref</td>
<td>0.000A</td>
</tr>
<tr>
<td>cell 1</td>
<td>0.055A</td>
</tr>
</tbody>
</table>

After a 1 in 2000 dilution the isolated cosmid DNA had a concentration of 2.75µg/ml; this figure was used in order to calculate the volume of DNA required for labelling reactions.
IV.2.ii). Labelling of C179 with digoxigenin by the random primed method

A number of aliquots of DNA were labelled during the course of this research, and all were first tested to determine DNA concentration and purity as described above. The volume of DNA in solution which corresponded to 1µg was diluted to a final volume of 10µl in ddH₂O for each labelling reaction. Labelling of DNA with digoxigenin was confirmed as previously described (p54), using a nylon membrane with alkaline phosphatase to detect hybridisation. Figure IV.1 shows an example of one such membrane. The dots represent two areas of hybridisation with the labelled cosmid DNA, they appeared after 10 minutes incubation and the reaction was stopped after 40 minutes (the signal remained visible on the membrane for a further 4 months).

IV.2.iii). Confirmation that the isolated probe was C179

In addition to the request for information as to the nature of the bacterium supplied, the Newcastle (U.K.) group were also asked to provide a cosmid map and restriction digest information. To ensure that the DNA isolated was cosmid C179 (and did not result from contamination with another cosmid or plasmid), a restriction digest of the cosmid was performed with 3 of the more common restriction enzymes, Bam HI, Eco RI, and Hind III. The resulting mini-gel which consists of lanes 1-8 running from left to right is produced in figure IV.2. It shows that the uncut C179 in lane 3 has three bands representing the three possible forms of DNA, these are:-
(i) Double stranded circular DNA which has only one of the strands nicked, i.e., it travels slowly through the gel as a large circle of DNA.

(ii) Double stranded circular DNA which has both strands nicked and moves through the gel more quickly as a linear strand of DNA.

(iii) Double stranded circular DNA that has not been nicked, this form of DNA tends to constrict into a spherical form and migrates through agarose gels faster than either of the nicked DNA forms.

Lanes 4-6 show the restriction digest of C179 with Bam HI, Eco RI & Hind III respectively. Lane 8 contains a DNA marker (λ-Pst 1) of known size. The results of a similar experiment were recently received from the U.K. and are shown in figure IV.3 as a southern blot of C179 digested by Hind III. Although this digest cannot be directly compared to those shown in figure IV.2, the size of the bands which result from the C179 Hind III lane were of equivalent size when compared to IV.3. The photograph (IV.3) was supplied by the laboratory which donated the probe, The Department of Human Genetics at the University of Newcastle Upon Tyne (UK).
Figure IV. 1:
Membrane to check labelling of C179 probe with digoxigenin, dots represent areas of hybridisation with labelled probe.
Figure IV.2:

Restriction digest of C179 cut with the restriction enzymes, Bam HI, Eco RI and Hind III. Gel lanes are loaded as follows:

<table>
<thead>
<tr>
<th>LANE CONTENTS &amp; NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>nil    nil    uncut    C179   → C179   C179   nil   λ-Pst 1</td>
</tr>
<tr>
<td>with        with    with   with   Bam HI  Eco RI  Hind III</td>
</tr>
<tr>
<td>1        2        3        4        5        6        7        8</td>
</tr>
</tbody>
</table>
Figure IV.3:

Southern blot from the U.K. of C179 digested with Hind III as marked.
IV.3. Preparation of metaphase chromosome spreads

IV.3.i). Chromosome spread production and quality

Metaphase chromosome spreads were processed at the Cytogenetics Department of SHLS. Although there was insufficient time during this project to perform *in situ* hybridisation on all of them, over 100 specimens were cultured and processed. All specimens have been karyotyped from synchronised cultures, and many high resolution karyotypes have also been performed by this department. The specimens set up yielded high quality metaphase chromosomes which banded successfully.

IV.3.ii). Results of cytogenetic karyotyping of DGS patients

As was expected, few of the suspected DGS patients showed a microdeletion of chromosome 22q11 cytogenetically (Carey *et al.*, 1992), however to date two patients in the study show monosomy 22q11 from high resolution studies. The resulting metaphase spread and karyotype of the synchronised culture and the metaphase spread and ideogram of the high resolution culture is shown from one of these patients in figures IV.4-IV.7. No apparent aberration is observed from the synchronised culture but monosomy 22q11 is detected on the high resolution slide (see figures IV.6, arrows indicate both 22 chromosomes and Figure IV.7 which shows a partial karyotype of the chromosomes). The work on these karyotypes was performed by the Cytogenetics Department at SHLS.
Figure IV.3:

Metaphase spread of synchronised peripheral whole blood culture.
Figure IV.5:

Karyotype of synchronised peripheral whole blood culture, shown in figure IV.4.
Figure IV.6:

Metaphase spread of high resolution (HRS) peripheral whole blood culture.

46,XY,del(22)(q11.2)
Figure IV.7:

High resolution partial karyotype of patient displaying monosomy 22q11, with accompanying ideogram of chromosome 22.
IV.4. *In situ* hybridisation

**IV.4.i. In situ hybridisation with Fluorogreen labelling**

All attempts at hybridisation using this method failed to show hybridisation signal. The metaphase chromosomes stained well with propidium iodide and were clearly visible under the fluorescent microscope at $\lambda = 595\text{nm}$. There also was no background signal detected by this method. Fluorogreen from the same batch had been used to label a probe by this method at the Department of Haematology at Royal Perth Hospital, but this probe labelling was also unsuccessful. It was determined therefore that this method would not be pursued further, both because no new Fluorogreen was available and the random primed method with digoxigenin labelling was proving to be reliable.

**IV.4.ii. In situ hybridisation with biotin labelling**

As previously noted, a sample of C179 was sent to the Department of Cytogenetics and Molecular Biology at the Adelaide Women's and Children's Hospital. This sample was sent to the laboratory primarily to verify that the cosmid isolated in Perth was C179, and that it did in fact hybridise to chromosome 22q11. The reason for the choice of this particular laboratory was it’s past success in using a cosmid probe for chromosome 22q11 donated by the Newcastle group (U.K.), and it also has several years experience of *in situ* hybridisation. In Adelaide it was confirmed by fluorescent *in situ* hybridisation (FISH) that C179 cosmid hybridised to chromosome
22q11. This confirmation, and the results of the restriction digest and southern blot, suggest that the correct cosmid was isolated by maxi-preparation. A sample of biotinylated C179 probe was returned from Adelaide for use in the present study. Several attempts were made to undertake FISH with this probe using a slightly modified version of the Adelaide protocol, however none of the metaphase chromosome spreads showed hybridisation signal, although fluorescent background signal was apparent on many of the slides.

IV.4.iii). *In situ* hybridisation with digoxigenin labelling

IV.4.iii).a. Horse radish peroxidase and DAB conjugated to DIG

Due to a lack of success with FISH using C179 labelled with Fluorogreen and biotin, an alternative label and detection system was investigated. The C179 probe was labelled with digoxigenin by the random primed method and tested for correct labelling. When it was confirmed that this probe was labelled with digoxigenin, *in situ* hybridisation was undertaken. Horse radish peroxidase was used instead of fluorescein to detect the reporter molecule, and hybridisation signal was then visualised with diaminobenzidine (DAB). Several hybridisation experiments using this method resulted in apparent hybridisation of C179 to a G-group chromosome, presumably chromosome 22. A summary of some of these results are outlined in table IV.2 which is accompanied by figures IV.8-IV.20. Double arrows indicate regions of apparent hybridisation on G-group chromosomes, and single arrows point to the other G-group chromosomes where no signal is visible. There are no arrows
on metaphase spreads which show excessive non-specific signal. Unless indicated,
patients all display phenotypes consistent with DGS.
Table IV.2. **Summary of in situ** hybridisation results

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>fig #</th>
<th>Hybridisation</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>34924M AP</td>
<td>8</td>
<td>✔ on 1 G-chromosome marked by double arrow</td>
<td>Signal was strong some background visible</td>
</tr>
<tr>
<td>36153M TC</td>
<td>9</td>
<td>✔ on 1 G-chromosome marked by double arrow</td>
<td>Signal visible under microscope - dark on print</td>
</tr>
<tr>
<td>36154M RO</td>
<td>10</td>
<td>✔ on 1 G-chromosome marked by double arrow</td>
<td>Poor chromosome morphology</td>
</tr>
<tr>
<td>35181M LS</td>
<td>11</td>
<td>✔ on 1 G-chromosome marked by double arrow</td>
<td>Mother of patient in figs IV.4-IV.7; only subtle signs of DGS</td>
</tr>
<tr>
<td>36019M NP</td>
<td>12</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Signal and chromosomes pale</td>
</tr>
<tr>
<td>35976M SC</td>
<td>13</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Chromosomes pale but signal present</td>
</tr>
<tr>
<td>36055M IH</td>
<td>14</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Morphology of chromosomes poor</td>
</tr>
<tr>
<td>35990M AB</td>
<td>15</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Signal and chromosomes pale</td>
</tr>
<tr>
<td>44444M (control)</td>
<td>16</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Signal and chromosomes pale</td>
</tr>
<tr>
<td>36159 M AA</td>
<td>17</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Signal was visible under microscope - dark on print</td>
</tr>
<tr>
<td>35945M CA</td>
<td>18</td>
<td>✔ on 2 G-chromosomes marked by double arrows</td>
<td>Chromosomes dark but signal present</td>
</tr>
<tr>
<td>36225M JP</td>
<td>19</td>
<td>No signal visible</td>
<td>4X normal strength DAB</td>
</tr>
<tr>
<td>35506M NT</td>
<td>20</td>
<td>Excessive signal on all chromosomes</td>
<td>Impossible to comment on G-group chromosomes</td>
</tr>
</tbody>
</table>
Figure IV.8:
Metaphase spread of patient 34924M AP, shows hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 1 G-group chromosome (marked with double arrows).
Figure IV.9:

Metaphase spread of patient 36153M TC, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 1 G-group chromosome (marked with double arrows). Counter-staining is dark, but signal was visible when viewed under the microscope.
Figure IV.10:

Metaphase spread of patient 36154M RO, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 1 G-group chromosome (marked with double arrows). Counter-staining is dark and chromosome morphology is poor, however signal is clearly visible.
Figure IV.11:

Metaphase spread of patient 35181M LS, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 1 G-group chromosome (marked with double arrows). This culture was prepared from the mother of patient 30604 LS, who was shown to have monosomy 22q11 by HRS karyotyping.
Figure IV.12:
Metaphase spread of patient 36019M NP, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal and chromosomes were pale due to shorter exposure time to counter-stain.
Figure IV.13:
Metaphase spread of patient 35976M SC, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal and chromosomes were pale due to shorter exposure time to counter-stain.
Figure IV.14:
Metaphase spread of patient 36055M IH, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal and chromosomes were pale and chromosome morphology was poor, this was probably caused by washing slides with too high a stringency.
Figure IV.15:

Metaphase spread of patient 35990M AB, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal was pale but visible when viewed under the light microscope; hybridisation signal was also detected on 2 chromosomes from this patient with the confocal microscope.

09/30/94 35990M ME1 35990M AB BL MM 1 109.0 48.3
Figure IV.16:

Metaphase spread of control patient 44444M, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Non-specific hybridisation signal was also seen on some chromosomes, this can be reduced by increasing stringency of washes and by altering probe:driver ratio.
Figure IV.17:

Metaphase spread of patient 36159M AA, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal is distinctly visible on both chromatids of the two chromosomes marked.
Figure IV.18:

Metaphase spread of patient 35945M CA, hybridisation signal detected with the use of anti-DIG HRP and visualised with DAB, on 2 G-group chromosomes (marked with double arrows). Hybridisation signal is dark on both chromosomes.
Figure IV.19:

Metaphase spread of patient 36225M JP, no hybridisation signal was detected on the chromosomes. The strength of DAB was increased in order to increase hybridisation signal intensity, however this change darkened all chromosomes and distorted their morphology.
Figure IV.20:

Metaphase spread of patient 35506M NT, a high degree of non-specific hybridisation signal was detected on all chromosomes. This amount of signal was due to a reduction in the driver:probe ratio and means that any signal detected on the G-group chromosomes is meaningless.
IV.4.iii).b. Fluorescein conjugated to DIG.

Fluorescent *in situ* hybridisation was also performed on C179 labelled with digoxigenin. No hybridisation signal was detected using either 1- or 2-step amplification methods by fluorescent microscopy, however a signal was apparent on one metaphase when scanned by a confocal microscope (see figure IV.21). Other metaphase spreads with obvious background signal present when viewed by this method revealed large amounts of cytoplasm covering chromosomes (see figures IV.22-IV.25). It can be presumed that excessive cytoplasm prevents the hybridisation of probe DNA to chromosomal DNA by acting as a physical barrier. Signal was also detected from interphase nuclei by this method (see figure IV.26), although it was not determined if this was hybridisation signal or background signal. All hybridisation and background signals on figures IV.21-IV.26 are indicated by arrows.
Figure IV.21 and IV.22:

IV.21. Shows metaphase spread with apparent signal on 2 chromosomes.

IV.22. Non-specific signal shown.
Figure IV.23 and IV.24:

IV.23. Shows metaphase spread with surrounding background signal.

IV.24. Bright background signal shown.
Figure IV.25 and IV.26:

IV.25. Shows metaphase spread and surrounding interphase nuclei with signal.

IV.26. Background signal outside interphase nuclei with pale signal inside 1 nucleus.
IV.4.iv). *In situ* hybridisation with D22S75 Oncor probe

D22S75 was purchased from Oncor, with sufficient probe supplied to hybridise 20 slides. Using this probe fluorescent *in situ* hybridisation was carried out on three slides but since a hybridisation signal was not detected on any of these slides, and due to the expense of the cosmid probe (over $30 for each slide), it was decided that a more detailed protocol probably was required. After discussion with the manufacturer, two Australian research groups who have used this probe have agreed to supply their successful modified protocols, and on receipt of these protocols sufficient probe remains to process a further 17 slides.
V. Discussion
V. Discussion

V.1. Introduction

The detection of genetic disorders by the use of *in situ* hybridisation has been shown by numerous researchers to be a most effective method to confirm clinical diagnoses (Carey *et al.*, 1992). Although initially this technique has proved difficult to execute, once the requisite experimental conditions are optimised routine diagnosis is possible. Individual probes appear to have distinct optimal conditions for *in situ* hybridisation which further complicates establishment of the technique. This is particularly the case for unique sequence probes, such as those used in the present study, which require highly specific experimental conditions in order to visualise signal without an excessive amount of non-specific signal. In the following discussion, aspects of *in situ* hybridisation which are necessary to successfully expedite its use will be addressed.

V.2. Probe preparation

V.2.i. The preparation of an agar stab culture

Plasmids are small circular molecules of double-stranded DNA that occur naturally in both bacteria and yeast; they replicate as independent units as the host cell proliferates. This self-replication is exploited in molecular cloning (Alberts *et al.*, 1983). Cosmids in their simplest form are modified plasmids that carry a copy of the DNA (cos) sequences required for packaging DNA into bacteriophage λ particles.
Cosmids carry an origin of replication and drug resistance markers so they can be introduced into bacterial vectors by standard transformation procedures and propagated as plasmids (Sambrook et al., 1989, p. 3.5). The DGCR probe used in this study is a cosmid probe (C179), and it has an insert of DNA which is complementary to part of the DGCR. In order to clone this insert sequence the cosmid was transfected into a bacterial vector in which it could replicate. Cosmid probe C179 was received in a kanamycin-resistant bacterium as an agar stab culture from Professor J Burns group in the UK. In addition to this probe, similar cosmid probes from the U.K. group have been isolated from chromosome 22 libraries and digested with the restriction enzyme Hind III, with fragments then shotgunned into a Hind III digested vector to form cosmids (Wadey et al., 1993). A photograph of C179 cut with Hind III was received from Burns et al. (figure V.3). In order to label the cosmid for in situ hybridisation it must first be amplified and then isolated from the bacterial host.

V.2.ii) Yield of cosmid DNA

Experimental conditions effect both the yield and purity of cosmid DNA prepared by alkaline lysis (Sambrook et al., 1989, p 1.25). To ensure a high yield of DNA, special care must be taken to remove all supernatant from the bacterial pellet and nucleic acid precipitate phases. Attention should also be paid to the particular temperature required at different stages of the reaction, e.g., if nucleic acids are centrifuged at 4°C instead of room temperature, the sodium salt present in solution
may precipitate and become pelleted, thus causing contamination of the resultant DNA (Sambrook et al., 1989, p 1.39).

V.2.iii. The concentration of the isolated DNA

The concentration (w/v) of the isolated DNA must be measured so that a known quantity of DNA can be labelled for in situ hybridisation. The concentration of DNA measured using the absorbence method will highlight experimental contaminants such as salts by yielding an abnormal value. This method will not indicate the presence of contaminant DNA or the isolation of the incorrect cosmid, because it is unable to distinguish between different forms of DNA, or protein. For this reason it was necessary to perform a restriction digest on isolated C179, the results of which can be compared to the restriction digest of a pure sample of the probe. This method allows confirmation that the correct cosmid was isolated and indicates its level of purity. If contaminant DNA is present, the restriction digest banding pattern will differ from that given by the pure probe. If this digest is not undertaken and contaminant DNA is labelled, hybridisation signals can occur on unexpected chromosomes giving confusing results and high levels of non-specific hybridisation.

V.3. Probe labelling

When it was confirmed that C179 had been isolated, probe labelling was carried out using three different methods. These are summarised below:
V.3.i) Direct labelling

This probe labelling technique was attempted with Fluorogreen™ (Amersham) a fluorescein-12-dUTP conjugate which can be attached directly on to either a plasmid, cosmid or YAC probe, by nick translation. The FISH carried out with directly labelled C179 was unsuccessful, with no hybridisation or background signal detected. The absence of signal could be due to a number of reasons, including failure of the nick translation reaction to incorporate fluorescent nucleotide into the probe. However, without checking the probe labelling it cannot be assumed that Fluorogreen was conjugated to C179. If labelling of the probe was achieved and hybridisation did occur, it still may be difficult to detect signal. Directly labelled probes require no amplification steps to detect hybridisation signal in FISH, and this lack of amplification may be the reason why signal was not detected using probe labelled by this method.

V.3.ii) Biotin labelling

Biotin labelling of C179 was carried out by the Adelaide research group using the nick translation method. Probe labelling was not checked during the course of this study because the biotinylated probe was used to successfully delineate chromosome 22q11 in Adelaide by FISH. Although hybridisation signal was not visible when this probe was used in Perth, background signal was detected on some metaphases. This tends to suggest that the probe was labelled and that it was either a deficiency in
hybridisation or detection of hybridisation during another stage in the FISH reaction that prevented visualisation of the signal.

V.3.iii). DIG labelling

C179 probe was labelled with digoxigenin-11-dUTP using the random primed method. This form of labelling was confirmed by detecting the hybridised probe on a nylon membrane with the enzyme alkaline phosphatase. Digoxigenin-11-dUTP (DIG) labelling proved to be very successful and it was straightforward to perform the labelling of the probe and to check this labelling. Hybridisation signal from ISH experiments was detected in each of the several batches of probe labelled by this method.

V.4. Slide preparation

Slide age and condition are among the numerous factors which effect the overall quality of chromosome metaphase spreads. Only good quality slides are used at the Cytogenetics Department at SHLS, each batch of slides is dated on arrival at the laboratory and those which are not used within six months of receipt are discarded. Even though the slides are clean when purchased, they are further cleaned by soaking in methanol, which ensures that when the cell suspension is dropped on to the slide it spreads evenly and is not affected by contaminant debris either on the slide or arising from the cell preparation. Excessive amounts of cytoplasm can be a particular problem encountered in the production of metaphase spreads, and many innovative
techniques have been employed in order to reduce the amount of cytoplasm around cells. The method which seemed most efficient in decreasing unwanted cytoplasm was recommended by the Cytogenetics and Molecular Genetics Department at Adelaide Women's and Children's Hospital (E. Baker, personal correspondence). She suggested that, after cleaning, slides should be soaked in absolute ethanol at room temperature and then dipped into luke warm distilled water before the cell suspension is dropped on to the wet slides held at an angle of approximately 45° over a water bath at 50°C. This technique proved most helpful when fixed cell suspension had been stored at 4°C or -20°C for extended periods after harvesting of the cells.

Slides used for in situ hybridisation require different conditions prior to use to those dropped for synchronised karyotyping with G-banding. Slides are not baked at 60°C overnight to artificially age chromosomes, instead those dropped for in situ hybridisation reactions are stored at room temperature, because baking slides can decrease the hybridisation signal. It has also been noted that slides which are stored in dry conditions (in a desiccator) at room temperature produce a better hybridisation signal (Lisa Robson, Cytogenetics Dept. Camperdown Children's Hospital NSW, personal correspondence). Slides were usually employed in this study 1-5 days after dropping, however in most instances it is recommended that slides be used within a 30 day period after dropping. If the cell suspension is not dropped immediately after processing it can be stored at 4°C or -20°C. Unfortunately it was observed that hybridisation may be hindered by increased amounts of cytoplasm surrounding those
cells which have been stored in this way. Although there are techniques to reduce this problem, it is advisable to perform ISH on cells as soon as possible after they are processed.

V.5. In situ hybridisation

The variation of in situ hybridisation protocols found in this study, and from laboratory to laboratory, reflects the common difficulty in establishing the technique. Even though the use of ISH is becoming more widespread in molecular biology, as Stahl & Baskin (1993) point out ISH is essentially a histochemical method and, as such, not only requires ‘getting it to work’ but also verifying that ‘it is working properly’. Concerns of signal specificity, sensitivity, resolution and controls must be addressed in order to use ISH as a diagnostic test. Although some degree of success was accomplished with respect to the use of in situ hybridisation in the present study, further detailed investigation is needed in the optimisation of the procedure. Specific hybridisation signal detection and the reduction of background signal must be improved to make ISH a viable diagnostic test in the detection of DGS and related microdeletions. However some evidence from this study suggests that ISH will provide a valuable test for DGS. For example, figure IV.11 shows a mother with an apparent 22q11 deletion, detected by ISH; subsequently her son was also shown to have this deletion by HRS karyotype analysis (see figures IV.4-IV.7). An infant who was shown to have a microdeletion of chromosome 22q11 with ISH, using a DIG-
labelled Cl79, has since died as the result of complications due to VSD cardiac
disease, post mortem examination revealed classic symptoms of DGS.

The use of a digoxigenin labelled probe visualised by anti-digoxigenin HRP and
DAB allowed the use of a light microscope rather than a fluorescent instrument.
This form of detection is preferable to fluorescent microscopy because the signal on
slides is permanent. Thus, they can be hybridised immediately after the cell
suspension is processed (which reduces interference from cytoplasm and
concomitantly increases signal intensity) and then stored for analysis at a later time.
One of the major disadvantages with this detection method is, however, that after
hybridisation when chromosomes are counterstained and mounted they cannot be
banded to identify individual chromosomes. This can be overcome by the use of
another probe which is specific to the chromosome of interest, e.g., towards the end
of the study a stab culture containing a probe which is specific for the telomeric
region of chromosome 22 was made available by the Newcastle upon Tyne group.
The probe was prepared from a kanamycin-resistant bacterium as previously
described and run on a mini-gel to assess the purity of the isolated DNA (see
Appendix I. figure VII.1). The probe has been stored at this stage and with further
purification and labelling will be available for future use as another control.
Conversely, FISH has the advantage over other forms of non-isotopic in situ
hybridisation because it allows the Q-banding of hybridised chromosomes which
enables their rapid identification. Unfortunately, it is not however as simple to detect
a FISH signal using a fluorescent microscope as it is to detect DAB signal on the light microscope. For this reason, many investigators use confocal laser scanning microscopy or cooled CCD cameras in order to detect signal from FISH and identify individual chromosomes (Stahl and Baskin, 1993).

V.6. Conclusions and future investigations

The purpose of the present study was to explore the various possible techniques for the detection of hybridisation signal in non-isotopic in situ hybridisation, and to compare them with cytogenetic techniques using a probe specific for chromosome 22q11. Variability exists at every stage of the ISH reaction. Investigators must deduce what type of isolation, labelling, hybridisation and detection protocols are appropriate for use with each specific probe. Not only must hybridisation signal be maximised and background signal minimised, but chromosome morphology has to be retained in order to identify which chromosomes actually have signal. This factor conveys both advantages and disadvantages. For example, when the stringency of the washing solutions is increased by raising the formamide concentration, or by increasing the temperature and decreasing the concentration of SSC, non-specific signal is reduced. However, changes of this nature in the protocol also have an adverse effect on chromosome morphology. Although all ISH protocols are based on the same principles, it is only by investigative trial and error that optimal conditions for ISH with specific probes and reagents can be achieved. During the
course of this study several protocols were tried with two probes in order to achieve hybridisation signal on metaphase chromosome spreads.

It is envisaged that the information obtained in this study regarding the degree of success achieved with each of the experimental protocols tested will help future researchers who wish to use the probe. Over 100 patients were examined and bled as part of this study at Princess Margaret Hospital for Children (WA). All of these samples have been cultured and processed at the Cytogenetics Department of SHLS, and are now ready for ISH studies. An amniotic fluid sample and fetal tissue samples were also processed, then cryogenically treated and stored at -70°C for future investigation into the use of ISH for DGS. Results of these continuing studies will further advance our understanding of disorders such as DGS which display marked genetic heterogeneity, and as a result the counselling of affected individuals and their families should improve.
VI. References
VI. References


VII. Appendices
Appendix I

Figure VII.1: Mini-gel resulting from maxi-preparation of cosmid probe specific for telomeric region of chromosome 22:

Only lanes whose wells were loaded are numbered. Lane 1 is at the left hand side of the gel. Lanes 1-6 all contain 1μl of cosmid probe and 1μl of loading buffer, lane 7 contains 10μl of cosmid probe and 2μl of loading buffer and lane 8 was loaded with λ-PstI. With further purification and labelling this probe could be used to identify chromosome 22.
Appendix II

The clinical research proposal for this study by Dr. Sharon Worthington, Princess Margaret Hospital for Children, WA.
TITLE: THE CLINICAL AND MOLECULAR DNA STUDY OF FAMILIAL CONGENITAL HEART DISEASE

AIMS:
Main aim: To investigate chromosome 22q11 in patients and their families with CATCH 22 disorders.

Specific aims:

1. To explore in detail the clinical spectrum of the individual syndromes embraced by the acronym CATCH 22.
2. To explore the inheritance pattern of these disorders.
3. To determine the prevalence of these conditions in the West Australian population.
4. To provide more accurate data about the natural history of these disorders upon which to base genetic counselling for a specific group of heart defects.

BACKGROUND

The incidence of congenital heart disease (CHD) is approximately one percent of live births(1). Recent advances in cardiac surgery have resulted in long term survival of patients with complex CHD into adulthood. As a consequence there has been increased interest into the genetics of CHD. For the patients, their parents and extended families the recurrence risks and the possibility of prenatal diagnosis are important issues.

Recurrence risks for CHD depend upon the mode of inheritance. Chromosomal abnormalities occur in twelve percent of CHD whereas three percent of CHD is due to classical Mendelian gene effects with recurrence risks of up to 50 percent(1). In most types of CHD however, the inheritance pattern is complex and is assumed to be of the multifactorial type with recurrence risks of 3-5 percent depending on the type of lesion(1). This model assumes that the cause of the defect is due to the effects of several genes which are modulated by environmental factors, but it does not adequately explain the higher risks for some families of CHD or the increased risk for specific anatomic defects such as obstructive lesions of the left side of the heart(2).
CHD may be associated with extracardiac manifestations as part of an identifiable malformation syndrome. In 1981 an association was noted between abnormalities of chromosome 22 and the DiGeorge Sequence (DGS) (3). Subsequent investigations have shown deletions of 22q11 at the DiGeorge Critical region (DGCR) in 83 percent of cases of DGS (4). It has been postulated that these genes may play a role in neural crest cell migration which may be critical to the development of the normal conotruncus (5) with deletions resulting in CHD. This theory is supported by evidence of teratogenic action of tretinoin which can produce very similar fetal defects (6).

Submicroscopic deletions of chromosome 22q11 have been associated with other syndromes such as Velo-Cardio-Facial Syndrome (VCFS) in 65-70 percent of cases (4), Conotruncal Anomaly Face Syndrome (CTFA) in 10-30 percent of cases (7), and the CHARGE association. These syndromes share common features (see table 1) and the acronym CATCH 22 (Cardiac, Abnormal facies, Thymic hypoplasia, Cleft palate and Hypocalcemia) has been coined to encompass this group of disorders associated with deletions of chromosome 22q11. Nonsyndromal CHD has also been associated with deletions of chromosome 22q11. Patients with conotruncal defects, a cardiac defect which is overrepresented in the CATCH 22 disorders, have deletions of chromosome 22q11 in 29 percent of cases (9). Deletions of chromosome 22q11 also occur in familial CHD (10).

Recurrence risks for these disorders vary from one to fifty percent and is dependent upon the inherited pattern, gene deletions or other chromosomal abnormality and other causes such as drugs. Counselling for patients with DGS and VCFS where a chromosome 22q11 deletion is present in both the child and one of the parents is for an autosomal dominant inheritance with a recurrence risk of 50 percent. The risk is negligible if the parents are definitely unaffected. Genetic counselling of these disorders is limited by difficulties in diagnosis and lack of knowledge of the mode of inheritance of these disorders. Identification of patients with the CHARGE association and VCFS can be difficult because of the variability of these conditions and they are thus commonly underdiagnosed (11). Cases reported in the literature thus far have come from only a few centres and have been identified through craniofacial centres or genetic units.

In Western Australia there is a centralised service for the treatment of cardiac defects which would be ideal to study the population for the prevalence and natural course of these disorders. In the proposed study we intend to identify families with the CATCH 22 disorders in WA from the Birth Defects Registry and from the cardiology department and investigate chromosome 22q11.
MECHANISMS FOR PHENOTYPE-GENOTYPE CORRELATIONS

The mechanism of inheritance of these disorders has not been established. There is a wide spectrum of clinical manifestations with the potential for more severe birth defects occurring with future generations(9). Numerous mechanisms have been postulated to explain these manifestations. Schmickel suggests that DGS is a contiguous gene syndrome with hemizygosity for several genes resulting from the loss of multiple single genes in a small deletion(13). Contiguous genes are genes which are physically adjacent on a chromosome but are otherwise unrelated. A broad spectrum of clinical abnormalities occur due to the wide range of molecular defects caused by the loss of these unrelated genes. This concept can explain interfamilial differences but does not adequately explain differences in expression of these disorders between family members.

The interfamilial differences could be due to parental mosaicism with no mosaicism in the more severely affected child or an expanding mutation increasing in severity with each generation(4). The apparent increase in severity with a subsequent generation may be a result of selection bias of previous studies as they have not included children with the less severe manifestations of these disorders. Epigenic phenomena, that is genes at separate loci or environmental factors could modify the effects of those genes deleted resulting in interfamilial variability(10). The effects of maternal transmission and parental origin of deletions are other mechanisms that have not been adequately explored in these disorders.

The proposed population based project will explore the clinical spectrum and the genetics of these disorders to establish more accurate genetic counselling and thus more meaningful prenatal diagnosis.

LABORATORY METHODS

Deletions of chromosome 22q11 have been defined utilizing a number of molecular genetic techniques. Cytogenetic studies reveal chromosome abnormalities in only 15-20% of DGS cases. Molecular investigation with gene dosage analysis is a much more sensitive technique but is very time consuming(14). Gene dose analysis involves using densitometry to quantitatively analyse Southern blots to determine the number of copies of a locus(identified by probes specific for that locus) that are present. Homozygosity occurs if there are two copies of the locus(these are unaffected individuals) and hemizygosity if only one copy is present(affected individuals).
Fluorescence in situ hybridization (FISH) has been shown to be a more efficient and direct method for detection of 22q11 deletions in subjects with CATCH 22 disorders (14, 15). In situ hybridization is a technique used to identify the chromosomal location of genes directly by hybridization of genomic or complementary DNA in situ to the DNA of a cytological preparation. This technique is currently being used to more accurately define the limits of the DCGR. For diagnostic purposes a probe specific for the areas of interest is labelled with an antibody and hybridised to its matching DNA sequence on a cytological preparation from the patient. The preparation is counterstained with a dye which fluoresces and this is viewed using a fluorescence microscope. If a hybridisation signal is seen on only one of the chromosomes 22 this is consistent with gene deletions in the region recognised by the probe.

**PROTOCOL**

1. **STUDY POPULATION**

Subjects with VCFS, DGS, CTFA, the CHARGE association and those with cardiac defects associated with facial dysmorphism or palatal abnormalities will be identified from the Birth Defects Registry of WA. Further information concerning these patients will be obtained from medical records at Princess Margaret Hospital (PMH), the Cardiology and Genetic Departments at PMH.

Subjects with familial CHD or specific cardiac defects of obstructive lesions of the left and right side of the heart will be identified from the Cardiology Department Records.

2. **DATA SHEET**

A request to access information from the Birth Defects Registry has been submitted. The project has been approved by the cardiologists at PMH. It is assumed that most of the patients will have consulted the Geneticists or Cardiologists at PMH. Where this is not the case, their consultant will be contacted by letter (see letter 1) and a request for the patient's inclusion in the trial will be made.

3. **RECRUITMENT TO STUDY**

Parents will be sent information as an introduction to the trial by mail (see letter 2). They will then be approached by telephone and arrangements for an interview will be made. Initial work will involve examination of the proband for confirmation of diagnosis of the aforementioned syndromes. An information sheet (see attached) regarding the study will be given to the parents at the interview and consent (see attached) for inclusion in the study obtained. Diagnosis of the individual syndromes will be made according to accepted criteria (see table 1).
4. FURTHER CLINICAL DATA

For the families who fulfill the inclusion criteria, an assessment of their pedigrees will occur during the interview. Further examination of other affected family members may be requested. Information concerning possible intrauterine infections, alcohol intake, and medications that may have been taken during the pregnancy will also be obtained.

5. TESTING FOR 22q11 MICRODELETIONS

Blood samples will be obtained from the subjects and members of the families for DNA analysis. A FISH assay will be used to detect deletions of chromosome 22q11. Laboratory work will be done at the Cytogenetic State Health Laboratory.

6. CONFIDENTIALITY AND SECURITY

Information regarding the patients involved will be stored in the genetics Department at PMH in a locked room as is routine for all patients seen at PMH genetics clinics.

7. LIMITATIONS OF STUDY

There is a wide spectrum of clinical manifestations of these CATCH 22 disorders and it will not be possible to include the milder forms, for example isolated learning disorders. Parents or siblings of the proband however, may have milder forms of these disorders and will give an indication of their spectrum.

In this study only the immediate family will be tested for deletions of chromosome 22q11. Extended family members who wish further information or investigation will be referred to the Genetics outpatient clinic.

In summary, this study will explore the clinical and genetic aspects of the CATCH 22 disorders to gain information on their prevalence in the community, the relationship between the clinical course of the disorder and the gene deletion/s on chromosome 22 and the inheritance pattern of these disorders. This information will allow more accurate genetic counselling for families in the future.
REFERENCES


### TABLE 1: CLINICAL FEATURES OF SYNDROMES ASSOCIATED WITH DELETIONS OF CHROMOSOME 22q11

<table>
<thead>
<tr>
<th></th>
<th>CARDIAC DEFECT</th>
<th>FACIAL ABNORMALLY</th>
<th>PALATAL</th>
<th>STATURE</th>
<th>ENDOCRINE</th>
<th>PSYCHIATRIC</th>
<th>IMMUNE</th>
<th>DEAFNESS</th>
<th>OTHER</th>
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<tr>
<td>DGS (8,13)</td>
<td>IAA</td>
<td>ears low set, circular</td>
<td>cleft</td>
<td>short</td>
<td>normal to delayed</td>
<td>present</td>
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<tr>
<td></td>
<td>PA/VSD</td>
<td>narrow palpebral fissures</td>
<td>high arched</td>
<td></td>
<td>hypothyroidism</td>
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<tr>
<td></td>
<td>TOF</td>
<td>prominent nasal root</td>
<td>velopharyngeal</td>
<td></td>
<td>incompetency</td>
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<td></td>
<td>DORV</td>
<td>small mouth</td>
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<tr>
<td></td>
<td>TA</td>
<td>short philtrum</td>
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<tr>
<td>VCFS (11,16)</td>
<td>VSD</td>
<td>ears prominent, circular</td>
<td>cleft</td>
<td>short</td>
<td>hypothyroidism</td>
<td>learning difficulties</td>
<td>abnormal T cell</td>
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<td>broad nasal root, long nose</td>
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<td>incompetency</td>
<td>ADDH</td>
<td>function can occur</td>
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<td>psychosis</td>
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<td></td>
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<td>CTAF (7)</td>
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<td>low set ears</td>
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<td>nasal speech</td>
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<td>short</td>
<td>mental retardation</td>
<td>present</td>
<td>-</td>
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**Abbreviations:**
- DGS = DiGeorge Sequence
- VCFS = Velocardiofacial Syndrome
- CTAF = Conotruncal Anomaly Face Syndrome
- IAA = interrupted Aortic Arch
- DORV = Double Outlet Right Ventricle
- PA = Pulmonary Atresia
- VSD = Ventricular Septal Defect
- TOF = Tetralogy of Fallot
- TA = Truncus Arteriosus
- PDA = Patent Ductus Arteriosus
- ADDH = Attention Deficit Disorder and Hyperactivity
HEART DEFECTS IN FAMILIES AND THEIR GENE ABNORMALITIES

INFORMATION SHEET FOR PARENTS

Many children are born with heart defects which vary in severity. Some complex defects cause death, others may require major heart surgery, and some are minor requiring no treatment. Causes of heart defects include infections and drugs taken during the pregnancy and heredity, but more frequently the cause is unknown.

At Princess Margaret Hospital the Cardiology and Genetic Departments are looking more closely at heart defects that may have a genetic cause. Specific heart defects that may be associated with other birth defects and may run in the family are known to occur when genes are missing on human chromosome 22.

We intend to look at all children in Western Australia with these heart defects to see if there are other minor birth defects that may not be obvious and assess their families for heart and birth defects. The study will determine how common these conditions are in W.A. and give us a better understanding of how they may possibly be passed on to other family members. The information obtained could be of great benefit to you and your family.

If you agree to participate in the study, it will involve your family and children being seen in a genetic clinic at the hospital, assessing the family tree and an examination to look for other minor birth defects. It will also require the taking of a blood sample from your child and possibly other family members to look at chromosome 22 for missing genes.

All information about an individual family and their test results will be confidential. Refusal to participate in the study will in no way jeopardise your child's medical management.

Any queries should be directed to Dr S Worthington at the genetics department at PMH, telephone number 340 8354.

SHARRON WORTHINGTON
Paediatric Registrar
Approximately one out of every 100 children is born with an abnormality of the heart. There are many different types of heart defects with complex heart defects resulting in death of a child at a young age and other heart problems that may be sufficiently minor not to interfere with the child's lifestyle. Many of the major heart defects are now correctable by heart surgery. Therefore there are now many adults who have had heart defects and who are contemplating families. The risks to their children and relatives of inheriting heart defects are important issues.

Some heart defects recur in families and are sometimes associated with an abnormality of chromosome 22. Specifically, there are genes that are missing from part of this chromosome. These heart defects may be quite complex and in some cases result in death of the child can also be associated with other birth defects as part of a syndrome.

In this study, children with heart defects and some syndromes will be identified from the Birth Defects Registry and the Cardiology Department at Princess Margaret Hospital. The children and their families will be notified about the study by mail. They will then be approached by telephone to arrange an interview, during which further information about heart defects and other disorders that may be present in the family will be sought. Family members may need to be examined to see if they have subtle features of these disorders. An information sheet will be given to the parents at the interview and if they agree to their child entering the study a they will be asked to sign a standard consent form. A blood sample will be taken from the children and possibly other members of the family to look for missing genes on chromosome 22.

The study will identify a group of children with specific heart defects which are associated with missing genes on chromosome 22. Children and parents with this chromosome abnormality have a high risk of giving birth to children with heart defects which may be even more severe than their own. The information gained from the study will enable doctors to give more accurate advice to people about these disorders when they are contemplating families. Questions such as how severely the child may be affected, the type of heart lesions that may occur, other physical abnormalities the child could have, how disabled the child may be, and the likelihood of having a normal versus an affected child may be answered. It may be possible to prevent these defects by looking for this gene abnormality in the baby during pregnancy.

"Risks" to the subjects and their families are the potential for discovery of physical abnormalities or diagnosis of a syndrome not previously recognised. Appropriate genetic counselling will be arranged if this occurs.

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