

**RESTRICTION ENDONUCLEASE BANDING OF HUMAN  
METAPHASE CHROMOSOMES**

by

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## **Abstract**

Restriction endonuclease banding is a recently introduced cytogenetic technique which reveals chromosomal polymorphisms at the centromeric region. These polymorphisms appear as variable sized structures and can be used in chromosome tracing analysis. In this project, metaphase chromosomes from normal subjects were banded using restriction endonuclease to establish the frequency of chromosomal polymorphism in the general population and also metaphase chromosomes were banded using restriction endonucleases in an attempt to trace the parental origin of embryonic chromosomes. The purpose of chromosome tracing analysis was to determine if uniparental disomy can be detected in early diploid spontaneous abortuses. The restriction endonuclease AluI was selected over RsaI, MboI, and DdeI, as it revealed reproducible polymorphisms at the centromeric regions of 20 out of 24 human chromosomes. The method of chromosome structure as standards was selected over the methods of linear measurements, surface area measurements, and polymorphism sizing, to quantify the size of the polymorphic region on each of the chromosomes. Using the chromosome structure as standards method, the frequencies of polymorphisms for each chromosome was calculated. This revealed that chromosomes 1, 6, 16, and Y were the most likely to be variable at the centromeric region. When parent to progeny chromosome tracing analysis was applied to metaphases treated with AluI restriction endonuclease, a visual method of tracing was superior to the method of chromosome structure as standards. Tracing analysis of AluI restriction endonuclease treated chromosomes revealed that 6% of chromosomes could be traced.

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To my wife,

Anita

# **I. Introduction**

## **A. Spontaneous Abortion**

### **1. Definition and Incidence**

Spontaneous abortion can be defined as the premature expulsion of the products of conception. Spontaneous abortions can be categorized into early spontaneous abortions if the loss occurs between 5 and 10 weeks of gestation and late spontaneous abortion if the loss occurs between 10 and 20 weeks of gestation (Dimmick and Kalousek, 1992).

It is estimated that 45% of all conceptuses fail to survive to term (Opitz, 1987). Loss before a pregnancy is clinically recognized (preclinical loss) accounts for the largest proportion of pregnancy losses and the rate of loss decreases in later gestational ages. The frequency of preclinical loss, as measured by levels of  $\beta$ -human chorionic gonadotropin in women 21 days after the last menses, was found to be 33% (50 out of 152) (Miller et al., 1980) and 31% (61 out of 198) (Wilcox et al., 1988). The estimated frequency of clinically recognized pregnancy loss is 15-20% (Jacobs and Hassold, 1987).

Morphological analysis of early spontaneously aborted specimens has revealed that 75% show growth disorganization, 5% have one or more localized malformations, and the remaining 20% are morphologically normal. Cytogenetic analysis of early spontaneously aborted specimens revealed normal diploid complement in 80% of morphologically normal embryos, 10% of embryos with malformations, and 50% of growth disorganized embryos (see figure 1).

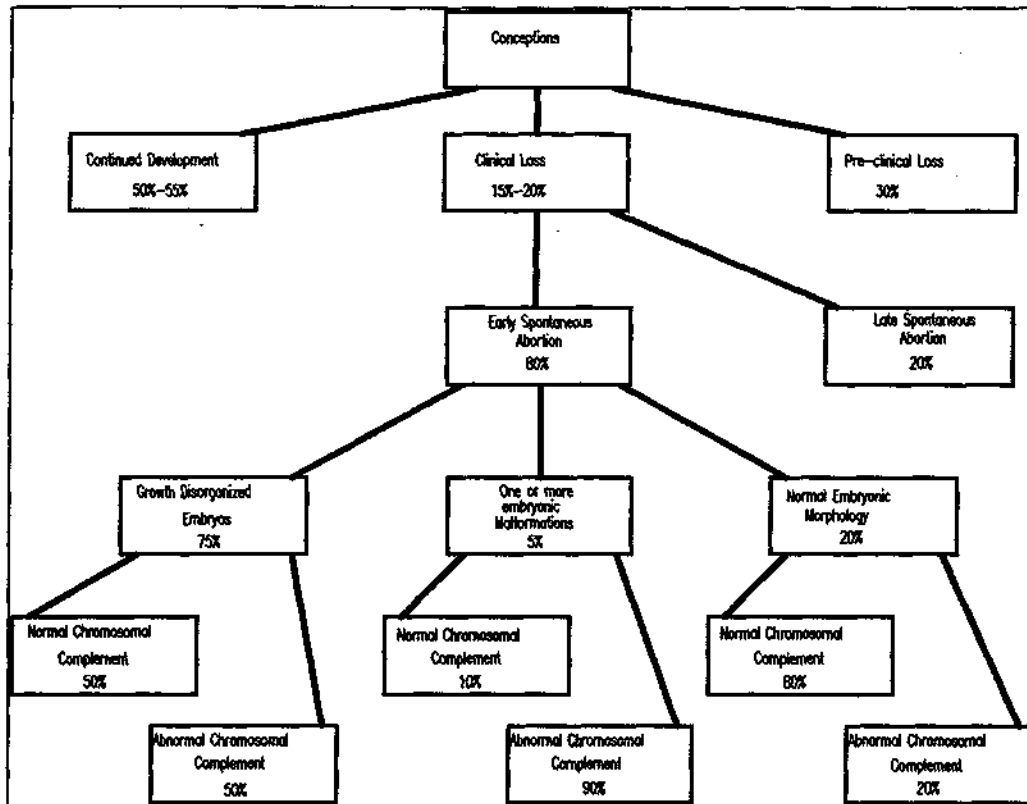


Figure 1 Diagram of the different types of early spontaneous abortions and the distribution of those with a normal versus an abnormal chromosomal complement.

## 2. Factors Contributing to Clinical Losses

50% of clinically recognized pregnancy losses are due to cytogenetic errors of which 53% are autosomal trisomies, 19% are monosomy X, 17% are triploidy, 6% are tetraploidy, and 5% are other chromosomal abnormalities (Simpson and Bombard, 1987; Simpson, 1990). Other factors such as maternal illness, abnormalities of the uterine environment, endocrine conditions, immunologic conditions, and teratogenic effects such as cigarette smoking and alcohol consumption have also been implicated in causing early abortions. (Simpson, 1990). The exact contribution of each of these above mentioned factors to the etiology of pregnancy loss is unknown.

## 3. Current Knowledge Regarding Early Diploid Abortuses

Although the loss of some early morphologically normal diploid abortuses may be explained by maternal factors such as abnormal endocrine control of pregnancy, a

proportion of these abortuses are likely to abort due to genetic imbalances. Lethality of diploid conceptuses due to uniparental disomy is suggestive based on experiments in mouse (Cattanach, 1986). Warburton (1988) estimated that 1/30000 (0.003%) conceptions might be uniparental isodisomy when a union between a nullisomic and a disomic gamete occurs. In this thesis a method that is able to detect uniparental disomy in diploid abortuses is studied.

## **B. Uniparental Disomy**

### **1. Definition**

A chromosomally normal zygote contains two haploid sets of chromosomes; one set from each parent. A haploid set consists of 22 autosomes and 1 sex chromosome. Together the haploid sets make a diploid complement of 44 autosomes and 2 sex chromosomes. A conceptus with uniparental disomy of an autosome would have 23 autosomes and 1 sex chromosome from one parent and 21 autosomes and 1 sex chromosome from the other parent. Although the conceptus contains 46 chromosomes and can be referred to as a diploid, an unequal number of chromosomes are inherited from both parents. This conceptus has an uniparental disomy. Therefore, uniparental disomy can be defined as the condition of having both members of a pair of homologous chromosomes from one parent in a disomic cell line (Engel, 1980).

### **2. Types of Uniparental Disomy**

Isodisomy and heterodisomy are subsets of uniparental disomy. Uniparental isodisomy is when both members of the chromosome pair from a parent are identical at the nucleotide level (Engel, 1980; Warburton, 1988; Spence et al., 1988; Hall, 1990). Uniparental heterodisomy is when both members of the pair are not identical and yet they originate from one parent (Hall, 1990)(see figure 2).

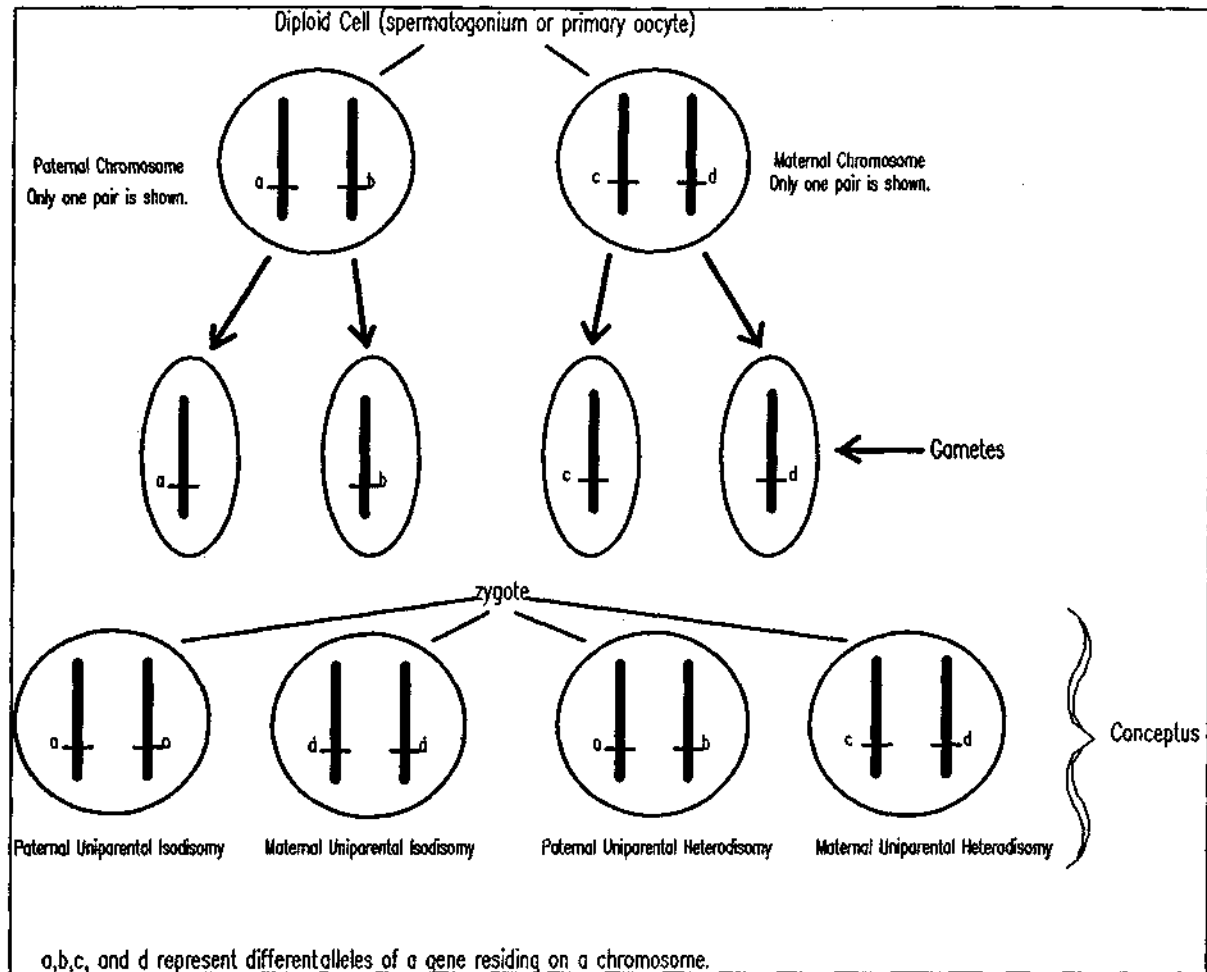


Figure 2. Types of uniparental disomy.

### 3. Models for the Origin of Uniparental Disomy

A modification of the models presented by Spence et al. (1988) demonstrate how uniparental disomy may arise are shown in Figure 3.

1) A non-disjunction event may take place in both the oocyte and the sperm producing one nullisomic (hypohaploid) and one disomic (hyperhaploid) gamete for a specific chromosome. At conception, the zygote will have a chromosomal constitution of 46,XX or 46,XY.

2) Through a postzygotic mitotic nondisjunction, a trisomic zygote may lose one of its trisomic chromosomes. This would result in a tetrasomic cell and diploid cell. The

tetrasomic cell is unlikely to survive. The diploid cell may have uniparental disomy or be normal depending on the trisomic chromosome that is lost.

3) A monosomic zygote could undergo a postzygotic mitotic nondisjunction which would result in a nullisomic cell and a diploid cell. The double nullisomic progenitor is unlikely to survive. The diploid cell would be uniparental isodisomy.

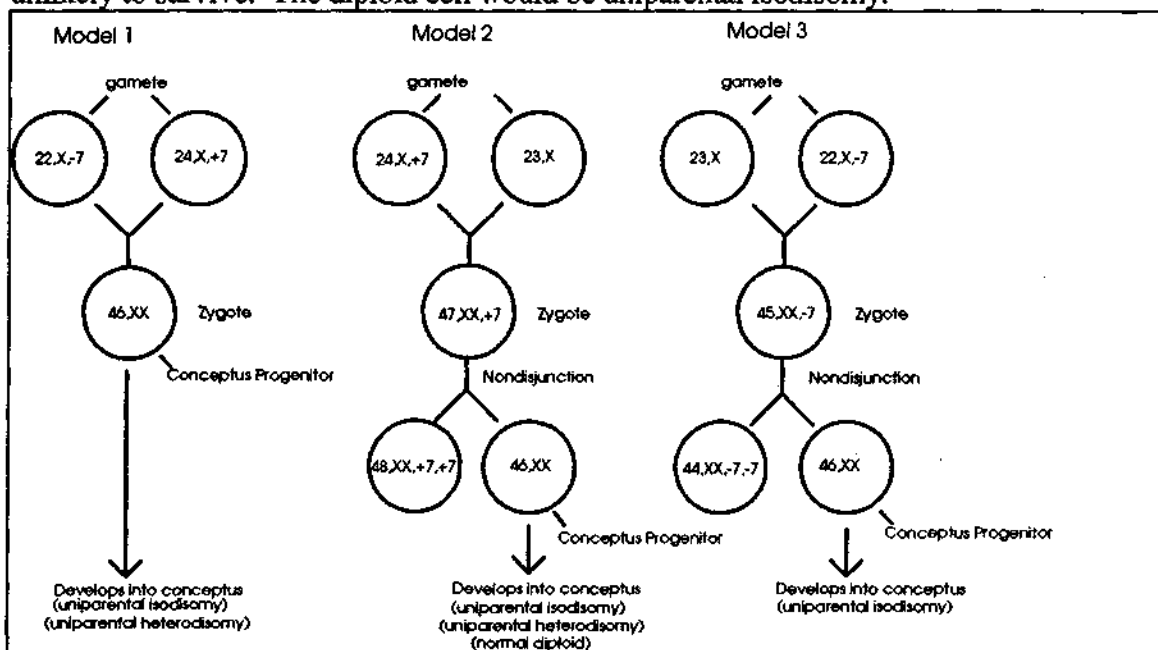


Figure 3. Uniparental disomy formation models.

#### 4. Frequency of Uniparental Disomy in Humans

The frequency of uniparental disomy in humans is not known. Warburton (1988) estimated that  $1/30000$  (0.003%) conceptions might be uniparental isodisomy when a union between a nullisomic and a disomic gamete occurs. This estimate is based on a frequency of 1% for each of nullisomy and disomy in sperms and of 12% for each of nullisomy and disomy in oocytes. Using more recent data reported by Martin et al. (1991), the frequency of uniparental disomy from the union of a nullisomic and a disomic gamete (model 1 in Fig. 3) can be calculated (see table 1 and 2). The frequency of trisomy and monosomy conceptuses formed as shown previously in models 2 and 3 of



figure 3, can also be calculated using the data reported by Martin et al. (1991) (see table 1).

Chromosome Group	Number of Chromosomes in Group	Hyperhaploid sperm (n=3259)	Hypohaploid sperm (n=3259)	Hyperhaploid oocyte (n=772)	Hypohaploid oocyte (n=772)	Paternal duplication /Maternal deficiency	Maternal duplication /Paternal deficiency
A	3	0.00399	0.00552	0.01036	0.00777	$1.0334 \times 10^{-5}$	$1.9062 \times 10^{-5}$
B	2	0.00245	0.00644	0.01036	0.00907	$1.1111 \times 10^{-5}$	$3.3359 \times 10^{-5}$
C	7	0.00338	0.03160	0.00259	0.04016	$1.9392 \times 10^{-5}$	$1.1692 \times 10^{-5}$
D	3	0.00276	0.01872	0.02332	0.05181	$4.7665 \times 10^{-5}$	$1.4552 \times 10^{-4}$
E	2	0.00307	0.02762	0.01036	0.03109	$3.1815 \times 10^{-5}$	$9.5381 \times 10^{-5}$
F	2	0.00123	0.01596	0.00259	0.02073	$1.2749 \times 10^{-5}$	$2.0668 \times 10^{-5}$
G	2	0.00583	0.02915	0.04016	0.04922	$1.4348 \times 10^{-4}$	$5.8533 \times 10^{-4}$
X or Y	2	0.00368	0.01043	0.00518	0.00518	$9.5312 \times 10^{-6}$	$2.7014 \times 10^{-5}$
Total	23	0.02639	0.14422	0.10492	0.21503	$2.9124 \times 10^{-4}$	$9.3803 \times 10^{-4}$

Table 1. Frequencies of aneuploid sperm, aneuploid oocytes, and calculated estimates of uniparental disomy frequencies in zygotes in humans. Aneuploid gamete data from Martin et al. (1991). Duplication/deficiency values were calculated under the assumption that all chromosomes from each group are equally likely to have undergone nondisjunction which would lead to a hyperhaploid or hypohaploid gamete. For example, chromosome group A is composed of 3 chromosomes, #1, #2, and #3. Paternal duplication/Maternal deficiency is calculated as follows:

$$(0.00399/3) * (0.00777/3) * 3 = 1.0334 \times 10^{-5}$$

Uniparental Chromosomal Disomy:	
Paternal duplication/Maternal deficiency	$2.9124 \times 10^{-4}$
Maternal duplication/Paternal deficiency	$9.3803 \times 10^{-4}$
Monosomy:	
Zero paternal copy/One maternal copy	0.09808
Zero maternal copy/One paternal copy	0.17834
Trisomy:	
Two paternal copies/One maternal copy	0.01795
Two maternal copies/One paternal copy	0.08702

Table 2. Estimates of zygotes that show uniparental disomy, trisomy, and monosomy. Estimates are based on data from Martin et al. (1991).

Uniparental chromosomal disomy (duplication/deficiency) were calculated by summing duplication/deficiency of chromosome groups.

Monosomy probabilities were calculated by multiplying the probability of a hypohaploid gamete with the probability of conceiving with a haploid gamete. e.g.

$$\text{Zero paternal copy/One maternal copy} = 0.14422 * (1 - 0.10492 - 0.21503) = 0.09808.$$

Trisomy probabilities were calculated by multiplying the probability of a hyperhaploid gamete with the probability of conceiving with a haploid gamete. e.g.

$$\text{Two paternal copies/One maternal copy} = 0.02639 * (1 - 0.10492 - 0.21503) = 0.01795$$

Model 1 of figure 3 demonstrates the formation of a uniparental disomic zygote through the union of a gamete that is hypohaploid for some chromosome and a gamete

that is hyperhaploid for the same chromosome. The incidence of such an event occurring can be estimated at 0.12%.

Model 2 of figure 3 demonstrates the formation of a diploid conceptus through the loss of an extranumerary chromosome in a trisomic zygote. The formation of a trisomic zygote, through the union of a hyperhaploid gamete and a haploid gamete, can be estimated at 10.5%. The frequency of the nondisjunction event leading to a uniparental disomy conceptus to be formed is not known. The frequency of 2% of confined placental mosaicism in 9-11 weeks gestational age abortuses suggests that postzygotic mitotic errors are common (Kalousek et al., 1991).

Model 3 of figure 3 demonstrates the formation of a uniparental disomic conceptus through a nondisjunction event occurring in the monosomic zygote. The formation of a monosomic zygote, through the union of a hypohaploid gamete and a haploid gamete, can be estimated at 27.6%. The actual incidence of a nondisjunction occurring in a monosomic zygote leading to a uniparental isodisomy condition is not known.

## **5. Detection of Uniparental Disomy**

Uniparental disomy can be detected using two different approaches: DNA analysis and cytogenetic analysis. Molecular biology techniques are available to identify DNA sequence variation between chromosomes which can be used to identify uniparental disomy and distinguish between uniparental isodisomy and uniparental heterodisomy. The cytogenetic approach of detecting uniparental disomy is based on tracing the parental origin of chromosomes by using chromosomal polymorphisms as markers. Since chromosomal polymorphisms can be structurally variable between individuals, these variations in chromosome structure are useful in tracing the segregation of chromosomes from parent to progeny.

## **6. Significance of Uniparental Disomy**

Maternal and paternal chromosomes undergo modification during early embryonic development and are not functionally equivalent (Hall, 1990). For normal development both maternal and paternal copies of each chromosome are required. Crouse (1960) introduced the term 'imprinting' to characterize a modification of chromosomes which described the behavior of the maternal and paternal autosomes and X-chromosomes in the early stages of development in *Sciara*. The term imprinting has also been used in context with the heterochromatic condensation and genetic inactivation of paternal chromosomes in mealy bugs (Brown and Nelson-Rees, 1961) and in the paternal X-chromosome inactivation within the extraembryonic membrane in mouse (Takagi, 1983). In this thesis, the term imprinting is used in the same context as defined by Hall (1990) and refers to the 'differential expression of genetic material, at either a chromosomal or allelic level, depending on whether the genetic material has come from the male or female parent'.

### **a. Uniparental Disomy in Mouse**

It has been shown in experiments with mice that uniparental disomy of specific chromosomes affects prenatal and postnatal development. Intercrosses between mice carrying Robertsonian translocations can lead to chromosomally balanced progeny having a pair of homologous chromosomes inherited from only one parent (Cattanach, 1986). Uniparental disomy for either maternal or paternal chromosomes 1, 3, 4, 5, 9, 13, 14, and 15 are tolerated in mice (Cattanach, 1986; Miller, 1987). Disomic lethality due to imprinting has been observed for maternal uniparental disomy, that is maternal duplication and paternal deficiency, of chromosome 6, a segment of chromosome 7, and the distal region of chromosomes 2 and 8. Disomic lethality has been observed for paternal uniparental disomy of the proximal portion of chromosome 17 (Cattanach, 1986). Maternal and paternal uniparental disomy for chromosome 11 has been associated with disproportionate body size. Maternally derived disomics are smaller than their litter mates and paternally derived disomics are consistently larger. The two types of animals are

otherwise normal. Crosses performed between the disomics and normal mice have demonstrated that the proximal region of chromosome 11 is imprinted and is associated with this size phenomenon (Cattanach and Kirk, 1985). The distal portion of chromosome 2 is responsible for flat sided, arch backed, hypokinetic newborn when progenitors have maternal duplication/paternal deficiency. Paternal duplication/maternal deficiency of the distal portion of chromosome 2 gave rise to the opposite phenotype: short, square bodied, broad flat backs, and hyperkinetic newborns (Cattanach, 1986). These studies demonstrate that imprinting of specific regions of both maternal and paternal chromosomes are required for the normal development of the mouse conceptus and that maternal and paternal chromosomal segments are not always functionally equivalent.

#### **b. Uniparental Disomy in Humans**

It is known that uniparental disomy for maternal chromosome 15 results in Prader-Willi syndrome while a paternal disomy can lead to Angelman syndrome (Hall, 1990). Two patients with cystic fibrosis and short stature were diagnosed as having uniparental disomy for chromosome 7 (Spence et al., 1988; Voss et al., 1988). Numerous cDNA markers to restriction fragment length polymorphisms along the entire length of the chromosome revealed both chromosome 7 originated from the mother in both patients. The phenotypic expression of the cystic fibrosis gene was likely due to the genes being homozygous recessive; however, imprinting of all or part of chromosome 7 may have been responsible for the short stature seen in both patients (Spence et al., 1988; Voss et al., 1988). Although there is only a limited number of cases with uniparental disomy described in the literature, by studying patients categorized as having sporadic syndromes of unknown etiology more cases of uniparental disomy could be discovered.

The effects of uniparental disomy in humans and mice have been documented. In mice, uniparental disomy of specific chromosomes or chromosome regions have resulted in lethality of the conceptus. Studies involving the association of uniparental disomy and early diploid spontaneous abortions in humans have not been performed.

## **C. Chromosomal Polymorphisms**

### **1. Definition**

Chromosomal polymorphisms can be defined as normal variation in chromosome structure having no effect on phenotype. Polymorphic regions can vary in size, position, and in staining properties (Therman, 1986). Chromosomal polymorphisms are made visible after specific treatment. Commonly used treatments for revealing these variable areas of the chromosomes are: C-banding, G-11 banding, Q-banding, and restriction endonuclease banding.

### **2. Traditional Techniques in Revealing Chromosomal Polymorphism**

#### **a. Q-banding**

Chromosomes treated with Quinacrine mustard or quinacrine show distinctive fluorescent banding under ultraviolet light. Quinacrine causes increased fluorescence in adenine-thymidine rich heterochromatic regions of DNA and quenches fluorescence in the guanine-cytosine rich euchromatic regions (Welsblum and deHaseth, 1972). Q-banding reveals polymorphisms of the satellites on the acrocentric chromosomes and the centromeric region of chromosomes 3 and 4 (Babu and Verma, 1987).

#### **b. C-banding**

Methanol/acetic acid fixed chromosomes are treated with basic solutions such as barium hydroxide and treated with SSC (sodium citrate/sodium chloride). The SSC treatment is thought to break apart the DNA causing 60% of the total DNA being lost from the chromosome. The loss occurs preferentially in the non-C-banded regions and the DNA is preserved in the centromeric region of all chromosomes. This region is also known as the C-banded region. C-banded chromosomes are differentially stained between the centromeric region and the surrounding chromosome arms (Sumner, 1972; Babu and Verma, 1986; Verma et al., 1978). Since C-banding does not band the chromosome arms, identification of individual chromosomes is not possible.

### **c. G-11 Banding**

G-11 bands are obtained by treating chromosomes in a Giemsa solution adjusted to pH 11 (Babu and Verma, 1987). G-11 banding results in a residual G-banding like pattern on chromosome arms and chromosomes 1, 5, 7, 9, 10, 13, 14, 15, 17, 20, 21, and 22 give a C-like banding profile.

### **3. Restriction Endonuclease Banding**

Although the various techniques discussed above expose chromosomal polymorphic sites, none of these techniques will concurrently reveal both polymorphic sites and allow for the identification of the majority of human chromosomes. Unlike these traditional methods, restriction endonuclease banding can reveal both polymorphic sites and banding that is necessary for chromosome identification.

Restriction endonucleases or restriction enzymes cleave double stranded DNA at specific recognition sites. Acetic acid/methanol fixed metaphase chromosomes exposed to restriction enzymes result in differential staining consisting of C-banding and/or G-banding depending on the enzyme used (Jones, 1977; Miller et al., 1983; Bianchi et al., 1984). Chromosomes treated with the restriction enzyme HaeIII and stained with Giemsa give a G-banded pattern (see Fig. 4) while restriction enzyme AluI gives a C-like banding pattern with a faint G-like banding pattern (see Fig. 5).

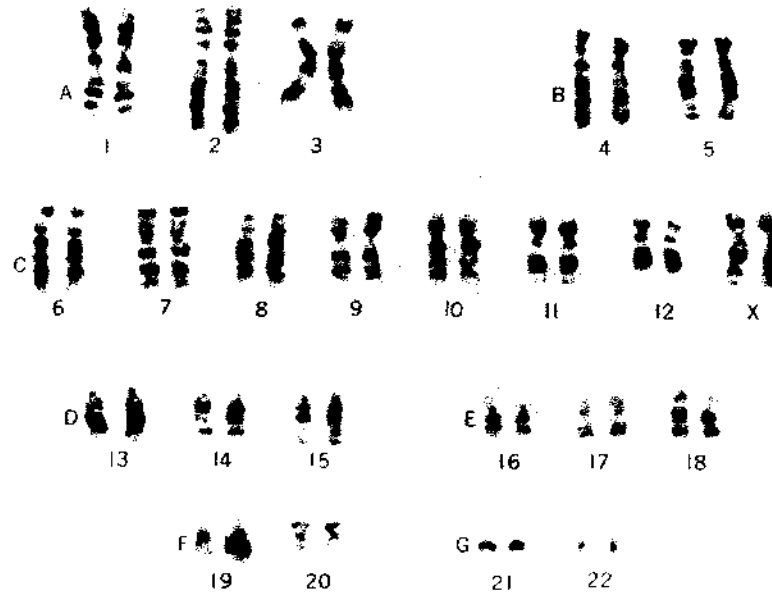


Figure 4. G-banded chromosomes.



Figure 5. AluI digested chromosomes. Arrows indicate G-like bands, C-like bands, and gaps.

#### a. Mechanism of Restriction Endonuclease Banding

Accessible DNA in the metaphase chromosomes is cleaved at specific sites by the restriction endonucleases. The cleaved DNA is then free to diffuse out of the chromosome structure. Two theories on the mechanism of banding using restriction

enzymes have been proposed. The fundamental difference between the two theories is related to the quantity of DNA accessible by the enzyme.

i) Miller et al. (1983) proposed that DNA in all regions of chromosomes are equally accessible to restriction endonucleases. They suggested that a region with a low frequency of restriction enzyme sites will result in high stain uptake compared to regions with a high frequency of restriction enzyme sites.

ii) Mezzanotte et al. (1983a) proposed that the structural organization of the chromatin and not the prevalence of restriction enzyme sites was responsible for banding patterns. Coiling of the DNA may render the enzyme inaccessible to the restriction sites and the coiling may also inhibit the DNA from eventually diffusing away from the chromosome once it is cleaved (Peretti et al., 1990).

G-like bands in chromosomes are thought to result by the minimal cleaving of DNA by restriction endonucleases. When DNA specific stains such as Feulgen or ethidium stain were used subsequent to digestion with EcoRI, HpaII, or HindIII, no visible banding pattern was produced and the chromosomes were stained dark similar to untreated controls. However, staining with Giemsa after enzyme treatment resulted in G-like bands (Mezzanotte et al., 1985). This G-banding pattern is thought to be due to staining of the protein component of the chromosome. Enzymes that normally leave a C-banding pattern, such as AluI or MboI, can also result in G-like banding if the enzyme digestion time is decreased (Babu and Verma, 1986; Luke and Verma, 1991). This simple experiment implies two facts. First, few exposed restriction endonuclease recognition sites for EcoRI, HpaII, HaeIII, and HindII are present and second, DNA thus remains fixed in the chromosome. The protein component was held in place by some strands of DNA. Once the DNA was cut, the protein had shifted into a new position on the chromosome.

C-like banding is proposed to occur when the quantity of DNA cleaved is increased over the amount in G-like banding (Miller et al., 1983). Fragments of 1000 base



pairs or longer remain fixed in the chromatin and are subsequently stained with Giemsa or Feulgen (Mezzanotte et al., 1985). These fragments are found close to the juxtacentromeric region of the chromosome, hence, the chromosome appears C-like banded (see Fig. 5). Restriction enzymes responsible for this type of banding include AluI, DdeI, RsaI, MboI, and HinfI (Miller et al., 1983; Gosalvez et al., 1990).

Gap banding, that is a gap in the polymorphic region is thought to occur when restriction sites are 50 to 70 base-pairs apart. These smaller fragments diffuse out of the chromosome. A gap appears as an unstained or faintly stained area near the juxtacentromeric region (see Fig. 5).

Peretti et al. (1990) reported that the characteristic C-like banding of chromosomes treated with a restriction endonuclease were not solely due to the cleaving properties of the enzyme. Restriction enzyme digested unfixed (methanol/acetic acid fixation not used) chromosomes that were stained with Giemsa were found to be uniformly lightly stained which implies that the chromosomes are fully digested. Methanol fixed chromosomes treated with restriction enzymes, (methanol is thought to preserve chromatin structure) were also uniformly lightly stained and thus the chromosomes were fully digested. However, methanol/acetic acid fixed chromosomes treated with restriction enzymes, in which the fixation step is known to alter the structure of the chromatin by extracting proteins, were C-banded (Peretti et al., 1990).

The evidence to date suggests that restriction endonuclease banding depends primarily on the method of preparation of the chromosomes and the subsequent banding patterns are dependent on the specific restriction enzyme used.

#### **b. Centromeric Region Staining Revealed by Restriction Endonuclease Banding**

In general, the C-like banding patterns induced by restriction endonucleases are a portion of the corresponding C-bands induced by standard C-banding methods (Babu and Verma, 1990; Babu and Verma, 1988; Babu and Verma, 1980). Suggestions have been made that C-like bands revealed by restriction endonuclease have increased variability over

traditional C-banded chromosomes (Babu and Verma, 1986b; Babu and Verma, 1986c). Interindividual banding polymorphisms have been reported for a variety of restriction endonucleases (Bianchi et al., 1985). These chromosomal polymorphic differences can be used as markers for the purpose of chromosome tracing analysis.

#### **D. Chromosomal Tracing Analysis**

The stability of specific alleles or variants in polymorphic regions through multiple generations is important in performing tracing analysis. It is established that satellite and C-band variants are stably inherited. This topic is discussed extensively by Magenis et al. (1977). Numerous studies involving the tracing of specific chromosomes from parent to progeny have been performed using C-banded chromosomes (Balicek et al., 1978; Babu and Verma, 1986b). Polymorphic variations revealed using restriction endonuclease banding are also found to be stably inherited (Luke and Verma, 1991; Babu and Verma, 1987; Babu and Verma, 1986b). Relatively few cases of unexpected segregation of polymorphic markers have been reported. The few that have been reported can be explained by mismatched paternity or meiotic crossing over (Craig-Holmes et al., 1975).

#### **E. Detection of Uniparental Disomy Using Chromosomal Markers**

The detection of uniparental disomy in spontaneous abortion specimens involves a method capable of tracing a set of homologous chromosomes to one parent. Tracing can be accomplished using C-like banded polymorphic region as chromosomal markers revealed by restriction endonuclease banding. Figure 6 illustrates the polymorphic chromosomes of a conceptus with uniparental isodisomy and heterodisomy. It also shows how the chromosomes from the parent segregated to the conceptus. Note that comparison of the parental chromosomes and the chromosomes of the conceptus reveal that the homologous chromosomes were unambiguously inherited from one parent.

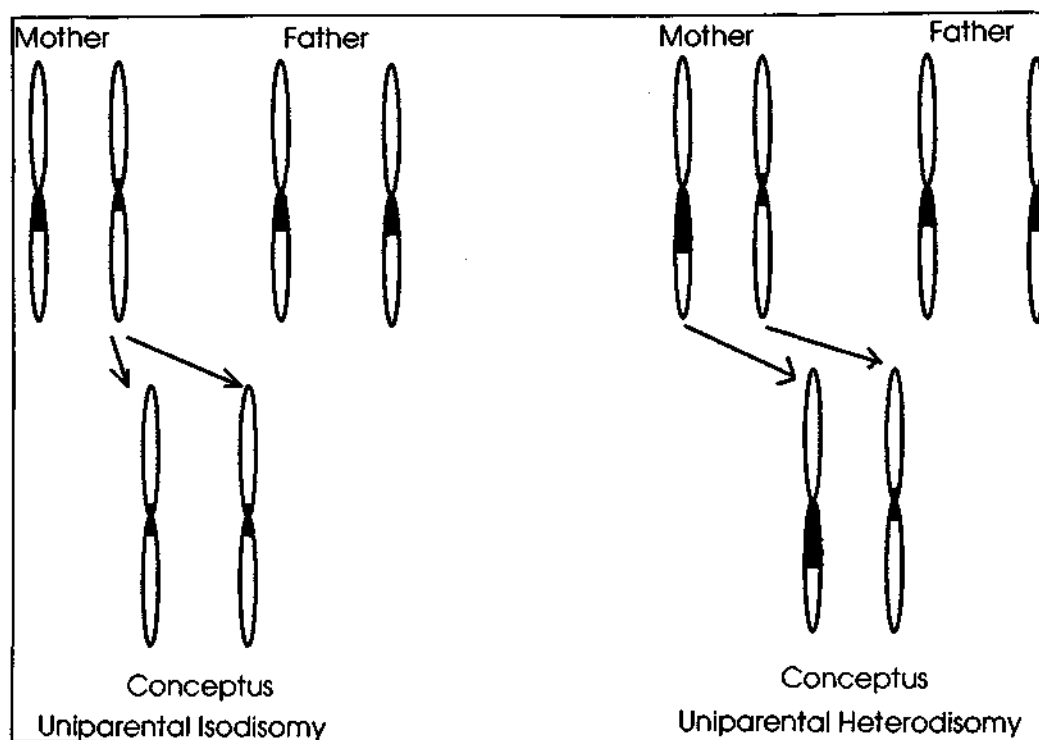


Figure 6. Identification of uniparental isodisomy and uniparental heterodisomy conceptus.

## F. Hypothesis and Strategy

The objective of this thesis was to develop the cytogenetic technique that could be used to demonstrate the presence of uniparental disomy in early spontaneously aborted diploid embryos with abnormal phenotypes. Over 40% of early spontaneously aborted conceptuses show apparently normal diploid chromosome complements. Although the karyotypes of these abortuses are normal the majority of these abortuses have an abnormal phenotype. It has been suggested that a diploid conceptus may not develop to term because it may have uniparental disomy for a specific chromosomal pair.

The specific aim of this study was to provide a technique which can detect uniparental disomy in early spontaneously aborted specimens by using chromosomal polymorphic markers revealed by restriction endonuclease banding. The study was carried out in five sections:

- 1) Evaluate the chromosomal polymorphisms revealed when chromosomes are treated with the restriction endonuclease AluI, RsaI, MboI, and DdeI.
- 2) Select one enzyme that appears most promising in finding uniparental disomy based on reproducibility and reliability of the enzyme and its ability to reveal polymorphisms on most chromosomes.
- 3) Evaluate four methods of quantifying the polymorphic regions produced by the selected enzyme and identify the method best suited for chromosomal tracing analysis.
- 4) Determine the variability of the polymorphic region in a normal population.
- 5) Evaluate chromosomal tracing analysis using the selected enzyme and quantification method

Achieving the above would allow to use the technique of restriction endonuclease banding for the search for uniparental disomy in diploid spontaneously aborted specimens.

## **II. Materials and Methods**

### **A. Specimen Selection and Collection**

Forty peripheral human blood samples from adult female and male patients referred to the cytogenetic laboratory at Vancouver General Hospital (VGH) and peripheral human blood from 15 parents (6 fathers and 9 mothers) of spontaneously aborted specimens were collected by a venous puncture into a sodium heparin treated Vacutainer tube. The forty samples collected from VGH were used as normal controls.

Fibroblast cultures were established from 11 growth disorganized embryos or from pregnancies diagnosed on ultrasound as having an empty gestational sac. Of these 11 samples, both parental blood samples were obtained from 2 of these specimens. Fibroblast cultures were also established from 8 triploid conceptuses. Of these 8 specimens, both parental blood samples were obtained in 3 of these specimens.

### **B. Fibroblast Cultures**

#### **1. Preparation of Tissue for Culture**

A piece of amnion and chorion approximately 2 cm<sup>2</sup> or a 1 g segment of chorionic villi was collected from each conceptus. To reduce the possibility of maternal contamination, the tissue selected was amnion, chorion, or chorionic villi in descending order of preference. In addition, approximately 1.5 g of decidua representing maternal tissue was collected from each specimen.

The tissues were cleaned separately in sterile isotonic saline (0.9% NaCl, BDH Chemicals) containing antibiotics/antimycotic (2% PSF composed of 10000 Units/ml Penicillin, 10000 µg/ml Streptomycin, 25 µg/ml Fungizone in 0.85% saline, Gibco) under aseptic conditions with the aid of a dissecting microscope. For all tissues, blood clots were removed. In addition chorionic villi attached to chorion or decidua were removed. All tissues were then rinsed in three changes of sterile saline.

## **2. Culturing and Maintenance of Cultures**

Amnion, chorion, chorionic villi, and decidua were cultured to propagate fibroblasts. Both tissue setup and trypsinizing procedures were performed under sterile conditions in a laminar flow hood.

The tissue sample was mechanically dissociated with scissors to produce tissue fragments of 1 mm<sup>2</sup>. The tissue was then used to "seed" three 60 mm culture dishes (Nunclon, Gibco) using culture medium (Minimal Essential Medium (Gibco), 30% by volume of Fetal Bovine Serum (Gibco), 1% by volume of L-glutamine concentrate (Gibco), and 1% by volume PSF (Gibco) (will be referred to as the 'medium'). The dishes were incubated in a incubator at 37° Celsius, 90% humidity, and 5% CO<sub>2</sub> (will be referred to as the 'incubator'). Additional medium was added after tissue fragments had attached to the dish. Routine maintenance of the cultures was performed by discarding the spent medium and replacing it with 3 ml of medium (20° C.) every 3rd or 4th day.

Trypsinizing of a culture was performed when fibrocytes occupied 50%-100% of the available surface area of the culture dish. The dish was rinsed with Hanks Balanced Salt Solution, HBSS (Gibco) prior to the cells being exposed to Trypsin/EDTA (0.05% Trypsin, 0.53mM EDTA•4Na in HBBS). When 90% of cells had detached from the culture dish (approximately 5 minutes), 3 ml of medium at 20° C. was added to inhibit the action of the trypsin. The resultant cell suspension was used to seed three new 60 mm culture dishes and the dishes were placed in the incubator.

## **3. Harvesting of Cultures**

Culture dishes were evaluated for the presence of mitotic cells using an inverted microscope (Nikon). When approximately 5% of cells appeared "rounded up" the culture dish was harvested. 0.1 ml of colcemid solution (10 µg /ml of colcemid in HBBS) was added into each dish and incubated for 25-30 minutes. The medium was replaced with 9 ml of 37° C. 1% sodium citrate (BDH Chemical) for 30 minutes at room temperature. 1

ml of 3:1 fixative (3 parts methanol (Baker analyzed, New Jersey): 1 part glacial acetic acid (BDH Chemical)) was added for 5 minutes. The solution was removed until approximately 6 ml remained. Immediately 2 ml of 3:1 fixative was added for 5 minutes. The remaining solution was removed and 9 ml of 3:1 fixative was added for a minimum of 5 minutes. The solution was removed and 4 ml of 60% acetic acid, 20% methanol, and 20% distilled water was added for 20-30 seconds. The solution was removed and 5 ml of 3:1 fixative was added for 10 seconds. The solution was removed and the dish rinsed twice with 3:1 fixative. The culture dish was air dried.

Culture dishes were graded based on the mitotic index and the quality of the metaphase spreads. Only high quality dishes from each sample were used for restriction endonuclease banding. A high quality metaphase spread had long black chromosomes (length of chromosome 1 is over 7  $\mu\text{m}$ ), minimal chromosomal overlap, little debris, and no cytoplasm. Each dish was allowed to dry for 24 hours at 20° C. before being restriction endonuclease banded.

### **C. Blood Lymphocyte Cultures**

#### **1. Preparation and Culturing**

If cultures were not set up immediately, the blood was refrigerated at 4° Celsius. Cultures were not attempted if sample was more than 5 days old. In a laminar flow hood, 0.3 ml of heparinized blood was added to a 15 ml centrifuge tube containing medium (13 ml Dulbecco Modified Eagles Medium (Gibco) supplemented with 1.5 ml FBS, 0.1 ml PSF, 0.1 ml L-glutamine, and 0.1 ml sodium heparin, 0.1 ml Phytohemagglutinin M form (Gibco). The centrifuge tube was sealed and incubated at 37° C. for either 68-72 hours or 92-96 hours.

#### **2. Harvesting**

After the incubation period, 0.1 ml of Colcemid was added to the blood mixture and reincubated for a further 25 minutes. The culture tube was centrifuged at 1000x gravity for 10 minutes. The supernatant was removed and the pellet carefully

resuspended. 10 ml of 37° C. 0.075 M potassium chloride, KCl (BDH) was added, the mixture was pipetted slowly to mix the suspension and solution together. The suspension was divided into two 15 ml centrifuge tubes and made up to 10 ml with 0.075 M KCL and incubated at 37° C. for 15 minutes. The cell suspension was centrifuged at 1000x gravity for 10 minutes. The supernatant was removed until 0.2 ml remained over the pellet. The pellet was gently resuspended and simultaneously, 10 ml of 3:1 fixative was added drop by drop while mixing. The suspension was centrifuged and washed with 3:1 fixative a total of three times. To make slides the pellet was resuspended in 0.4 ml of fixative. 100-200  $\mu$ l of cell suspension was placed onto a precleaned slide and then air dried. By varying the slide making technique (see appendix for details), chromosomes suitable for restriction endonuclease digestion could be obtained. The slides were aged for 24 hours at 20° C. before being restriction endonuclease banded.

#### **D. Restriction Endonuclease Banding**

The restriction enzymes AluI, DdeI, MboI, and RsaI (BRL, BM) were diluted in the manufacture supplied buffer to a concentration of 200 units/ml. 150  $\mu$ l of enzyme was placed on each slide and was covered with a 22 mm x 22 mm and a 22 mm x 60 mm coverglass. 200  $\mu$ l of enzyme was placed on each fibroblast culture dish and was covered with two 22 mm x 22 mm and one 22 mm x 60 mm cover glass. Negative control slides were setup similarly however, buffer solution without restriction enzymes was used in place of the restriction endonuclease solution. The slide/dish was placed into a sealed humidified chamber composed of a wetted filter paper in a 150 mm diameter petri dish. The edges of the petri dish were sealed and incubated at 37° C. for 6 hours. The portion of the petri dish that was exposed to the restriction endonuclease solution was first outlined and the dish/slide was then washed in several changes of distilled water and air dried. Digested slides/dishes were aged for 24 hours at 20° C. before staining with 4% Giemsa stain in Gurr's buffer for 2 1/2 minutes or alternatively stained by using the



trypsin/Giemsa banding technique. The walls of the petri dishes were removed and the remaining base was then mounted on a 75 mm x 50 mm glass slide.

The dishes/slides were initially screened using a compound light microscope. Dishes/slides used for detailed study were selected if differential staining had been attained between the centromeric region and the surrounding arms of the chromosome.

#### **E. Photomicroscopy and Photography**

Representative metaphases were photographed using a Zeiss photomicroscope II and Kodak Technical Pan Film 5152. Metaphases were selected based on the following criteria:

- 1) high contrast between the chromatid arms and the polymorphic areas.
- 2) chromosomes with few overlaps.
- 3) straight chromosomes.
- 4) metaphases with little cytoplasmic debris and or extracellular debris.
- 5) condensation state of the chromosomes. The longitudinal length of chromosome 1 in the range of 7-15  $\mu\text{m}$ .

The photographic film was processed using Kodak HC110 film developer and Kodak Rapid Fixative. Negatives were printed on Kodabrome II F3 paper using an Omega enlarger, Kodak Dektol developer, and Kodak Rapidfix for prints.

Photographs (1850x magnification) were made in duplicates, one for karyotyping and the other as a representative photograph of the metaphase. For each photographic print identical magnification were used and the exposure and development times of the prints were modified so that a consistent level of grey background was achieved.

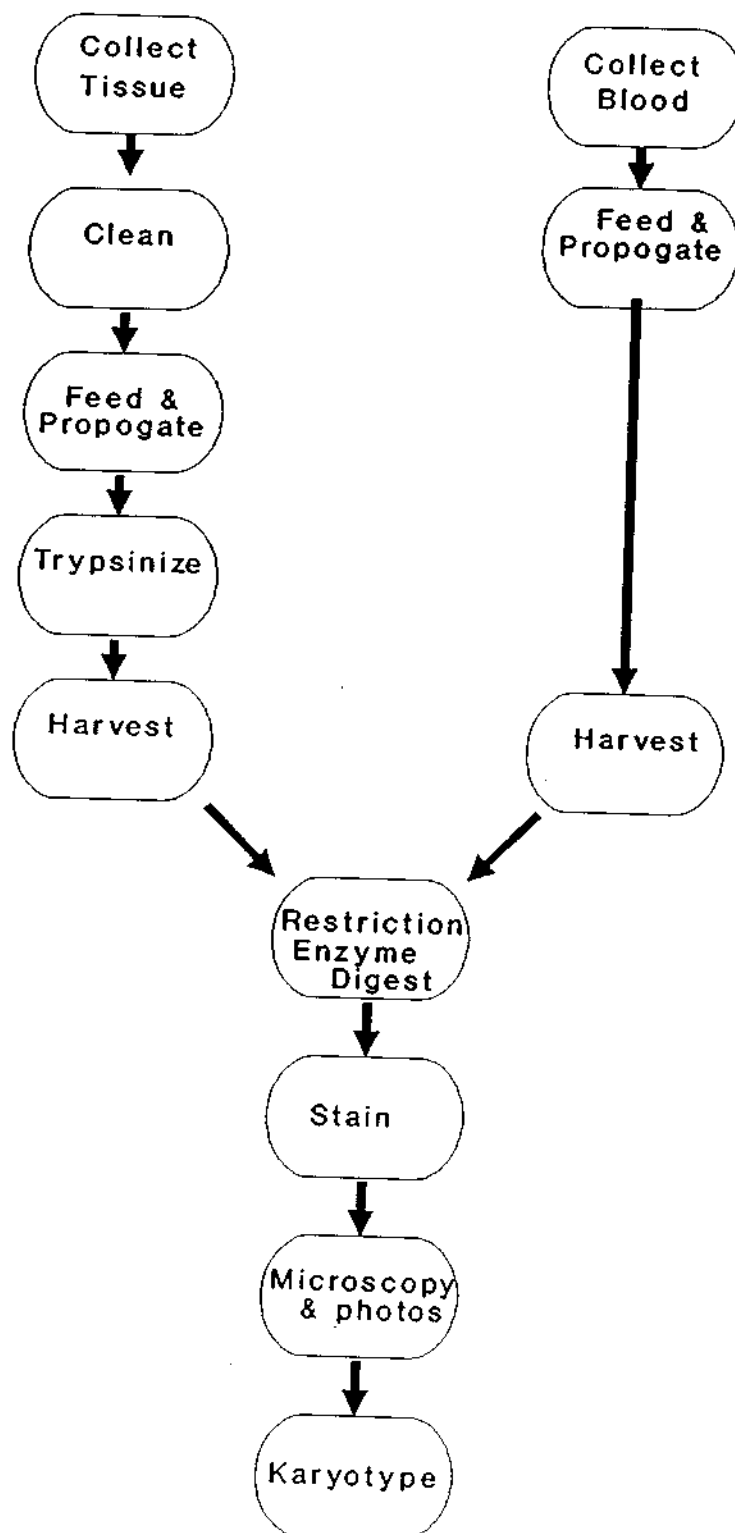


Figure 7. Methodology flowchart

## **F. Selection of Restriction Enzyme for Intensive Study**

One of the four restriction endonucleases AluI, RsaI, DdeI, and MboI was to be chosen for further study. Selection of this enzyme was based on the following criteria.

- 1) the enzyme digestion must be consistent and reproducible.
- 2) the enzyme must be able to reveal polymorphisms on most if not all of the 24 human chromosomes.
- 3) the enzyme must reveal many different alleles at the polymorphic region.

A minimum of five samples (a sample could consist of a combination of fibroblast preparations from diploid or triploid conceptuses and blood lymphocyte preparations from normal adults) were used in the analysis. 12 slides/dishes were prepared from each sample and 3 slides/dishes were used for each of the four enzymes to be evaluated. The slides/dishes were treated with the restriction endonuclease solution and then stained (procedure is as stated in the previous sections). The slide/dish was recorded as a successful experiment if differential staining between the centromeric region and the surrounding chromosome arms were revealed on greater than half of the 24 human chromosomes. Slide/dish that were successful were photographed using a compound light microscope (procedure and criteria is as stated in the previous section). Photographs were karyotyped and the chromosomes analyzed to determine the type of banding each restriction enzyme reveals.

The enzyme to be selected for further analysis based on the above criteria was then used in evaluating methods for quantifying the polymorphic regions.

## **G. Quantification of Polymorphic Region**

The search for uniparental disomy using a cytogenetic technique such as C-like banding revealed by restriction endonuclease treatment, involves the tracing of visible polymorphisms (alleles). Quantification of the centromeric region can be performed using different techniques. Four different techniques were evaluated to quantify the polymorphic regions induced by AluI restriction endonuclease banding: linear

measurements, surface area measurements, polymorphism sizing, and chromosome structures as standards.

### 1. Linear Measurements

Twenty-nine metaphase photographs from one normal adult peripheral human blood specimen treated with AluI were used in the evaluation of the linear measurement technique as a means of quantification of the polymorphic region. The polymorphic regions of both homologues of chromosome 1 and chromosome 9 were measured using a millimeter graded ruler along the long axis of each chromosome and their respective means were calculated. The entire length of both homologues of chromosome 1 were also measured and their mean was calculated (See figure 8). Two scatter plots were produced by plotting the mean of the length of the centromeric region against the mean of the total length of chromosome 1. The numerical analysis of correlation and regression were performed.

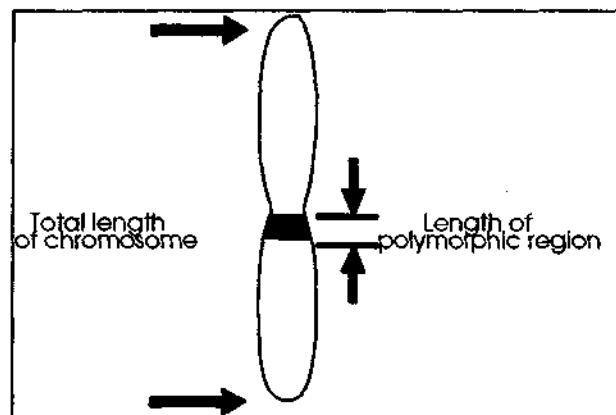


Figure 8. Diagram showing how linear measurements are performed.

### 2. Surface Area Measurements

A normal adult control sample treated with AluI with an obvious heterozygosity of the polymorphic region of chromosome 1 was used in the evaluation of the surface area measurement technique as a means of quantification of the polymorphic region. Eleven

metaphase photographs, a Bioquant II Image Analysis System (R&M Biometrics) in conjunction with a digitizing tablet and a Apple II computer were utilized. The homologue of chromosome 1 with the larger polymorphic region was used in the analysis to reduce the error of the surface area of the polymorphic region due to user input error. The polymorphic region and the entire chromosome was traced using the image analyzer system (See figure 9). The system calculates the surface area corresponding to the traced region on the photograph. Each tracing was repeated 10 times/metaphase and the mean was calculated and recorded. A scatter plot was produced by plotting the surface area of the centromeric region against the surface area of the entire chromosome. The numerical analysis of correlation and regression were performed.

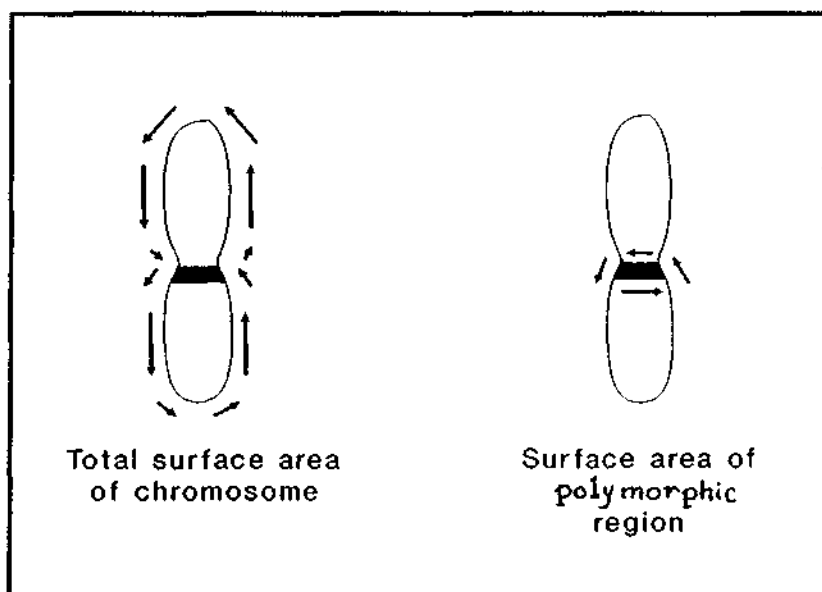


Figure 9. Diagram showing how surface area measurements are performed.

### 3. Polymorphism Sizing

Three normal adult samples (6 karyotypes/sample) were used in evaluating the method of polymorphism sizing as a means of quantification of the polymorphic region. This method involved categorizing the polymorphic region into the 4 categories of large bands, medium bands, small bands, and gaps (See figure 10). Size related reference

structures were not used in the analysis. Repeatability and accuracy of this technique was tested by analyzing the chromosomes from each karyotype a total of 3 times.

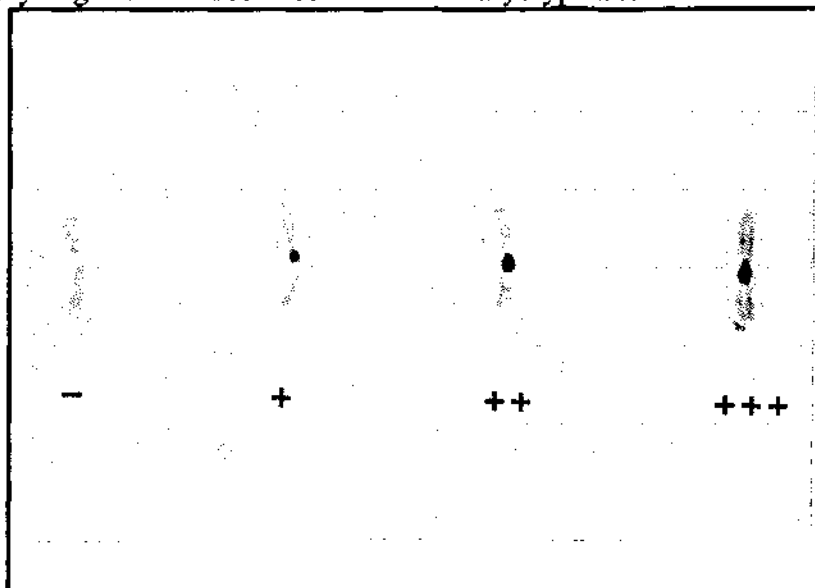


Figure 10. Diagram of large bands, medium bands, small bands, and gaps in the polymorphism sizing technique.

#### 4. Chromosome Structures as Standards

Five normal adult control samples treated with AluI were used in the evaluation of the chromosome structures as standards technique as a means of quantification of the polymorphic region. The centromeric staining of chromosome 20 and the short arm (p-arm) of chromosome 16 were used as size references in order to quantify the polymorphic regions on the other chromosomes. The classification used in this thesis were as follows.

- a) less than centromeric staining of chromosome #20
- b) greater than or equal to centromeric staining of chromosome #20 and less than 1/2 of 16p
- c) size greater than 1/2 of 16p and less than or equal to 16p
- d) size greater than 16p and less than or equal to 3/2 16p
- e) size greater than 3/2 16p and less than or equal to 4/2 16p
- f) size greater than 4/2 16p
- g) not analyzable
- h) complete inversion
- i) inversion involving less than half of the polymorphism
- j) inversion involving more than half of the staining region

Polymorphic regions could have more than one classification. For example, a polymorphic region could have a size greater than  $4/2$  16p and also have an complete inversion. 6-10 karyotypes were analyzed per sample. Chromosomes were classified into the categories if more than half of the analyzable chromosomes but not less than 3 chromosome pairs could be classified similarly.

#### **H. Tracing the Parental Origins of Chromosomes**

The centromeric staining of two sets of diploid conceptuses and parents were first quantified using chromosome structures as standards method. The data collected for each chromosome was used in chromosome tracing analysis. Unambiguous tracing of chromosomes from parent to conceptus was recorded.

The polymorphic region from 5 sets of conceptuses (3 triploid and 2 diploid) and parents (a conceptus and parents are considered as being one set) were analyzed visually. The conceptus and parental samples consisted of a minimum of 6 karyotypes each . The visual method of tracing analysis does not rely on quantification results. Instead the methodology employed here involves comparing the conceptus and parental chromosomes side-by-side to determine the parental origin of the conceptuses chromosomes. A successful tracing was determined by the similarity of polymorphisms in the conceptus and one parent unambiguously in half of the analyzable chromosomes but not fewer than 4 chromosomes.

Chromosome tracing analysis were not attempted on data collected using quantification techniques based on linear measurements, surface area measurements, and polymorphism sizing.

#### **I. Incidence of Heterozygosity of Polymorphic Sites on Homologous Chromosomes**

Homologous chromosomes from 40 normal adult samples were compared both visually and by the chromosome structures as standards method to identify polymorphic regions which were stained dissimilarly, that is were heterozygous, in regards to length, location, and stain uptake. The visual assessment of identifying heterozygosity is

performed by placing homologous chromosomes side by side. The visual assessment also consisted of recording heterozygosity in the satellite stalk length and size and intensity of satellites of the acrocentric chromosomes. This procedure was performed to establish the frequency of heteromorphic staining between homologues on AluI digested chromosomes in a normal population. A total of 6-10 karyotypes/sample were analyzed using both visual assessment and chromosome structures as standards method. Homologous chromosomes were classified as being heterozygous if more than half of the analyzable chromosomes but not less than 3 chromosome pairs could be classified similarly.



### **III. Results**

#### **A. Restriction Endonuclease Digestion**

Chromosomes digested with the restriction endonucleases AluI, RsaI, DdeI, and MboI are characterized by differential staining at the polymorphic region similar to the appearance of C-banded chromosomes; however, not all chromosomes are stained at the centromeric region. These C-like banded staining regions are stained homogeneously (continuously), that is they were not found to appear in separated segments.

Chromosomes were also shown to exhibit staining similar to G-banding. Samples digested using different restriction endonucleases (AluI, RsaI, DdeI, or MboI) revealed centromeric staining on different chromosomes.

#### **B. Selecting a Restriction Endonuclease for Tracing Analysis**

Selection of one of the four restriction endonuclease AluI, RsaI, MboI, and DdeI for further studies was performed based on the the following criteria. This criteria consisted of the consistency and reproducibility of the enzymatic digestion, the presence of centromeric staining ideally on all chromosomes, and polymorphic variability at the centromeric region.

##### **1. Reproducibility of Restriction Endonuclease Digestion**

A restriction endonuclease digestion experiment was considered successful if polymorphic sites were revealed as differential stained structures between the centromeric region and the surrounding chromosome arms on greater than half of the 24 human chromosomes (see Fig 11,12). The success rates using the restriction endonuclease AluI, RsaI, MboI, and DdeI on chromosomes isolated from fibroblast and blood lymphocyte preparations is shown in table 3. AluI was found to be most consistent in producing differential staining between the centromeric region and the surrounding chromosome arms in fibroblast and blood lymphocyte preparations.

	Fibroblast (dish) Preparation		Blood Lymphocyte (slide) Preparation	
Restriction Enzyme	% Success	# of Experiments	% Success	# of Experiments
AluI	100	70	70	50
RsaI	50	13	10	31
MboI	33	3	55	9
DdeI	100	5	0	13

Table 3 Reproducibility of experiments in lymphocyte and fibroblast cultures using different restriction endonucleases. An experiment refers to an enzyme digestion of a single dish/slide. An experiment is considered as being successful when centromeric staining is revealed on more than half of the chromosomes in a metaphase spread.



Figure 11. Photograph of a successfully digested metaphase spread.

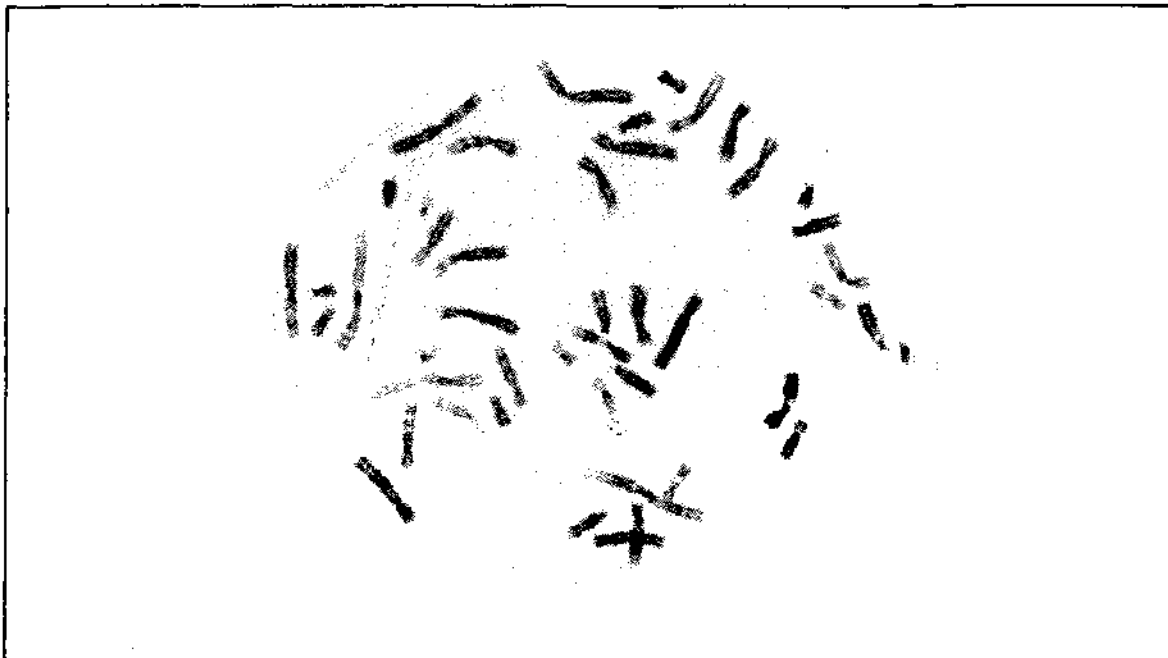


Figure 12. Photograph of an unsuccessfully digested metaphase spread.

## 2. Polymorphisms of Chromosomes Digested Using *RsaI*, *MboI*, *DdeI*, and *AluI*

Not all chromosomes exhibit differential staining at or near the centromeric region (C-like bands) when exposed to a restriction endonuclease. Different restriction endonucleases can give rise to different staining properties at the centromeric region. For example some chromosomes show staining at the centromeric region using one enzyme but no staining at the centromeric region using another enzyme. Those chromosomes that do stain at the centromeric region may exhibit polymorphisms (length variability) in the centromeric region.

### a. *RsaI* Digested Chromosomes

Metaphases digested with *RsaI* reveal 12 out of the 24 human chromosomes exhibit centromeric region staining (see Fig 13). The following is a list of chromosomes ordered in decreasing centromeric region size: 1, 9, 16, 5, 19, 10, 15, 14, 7, 3, 18, and 22.

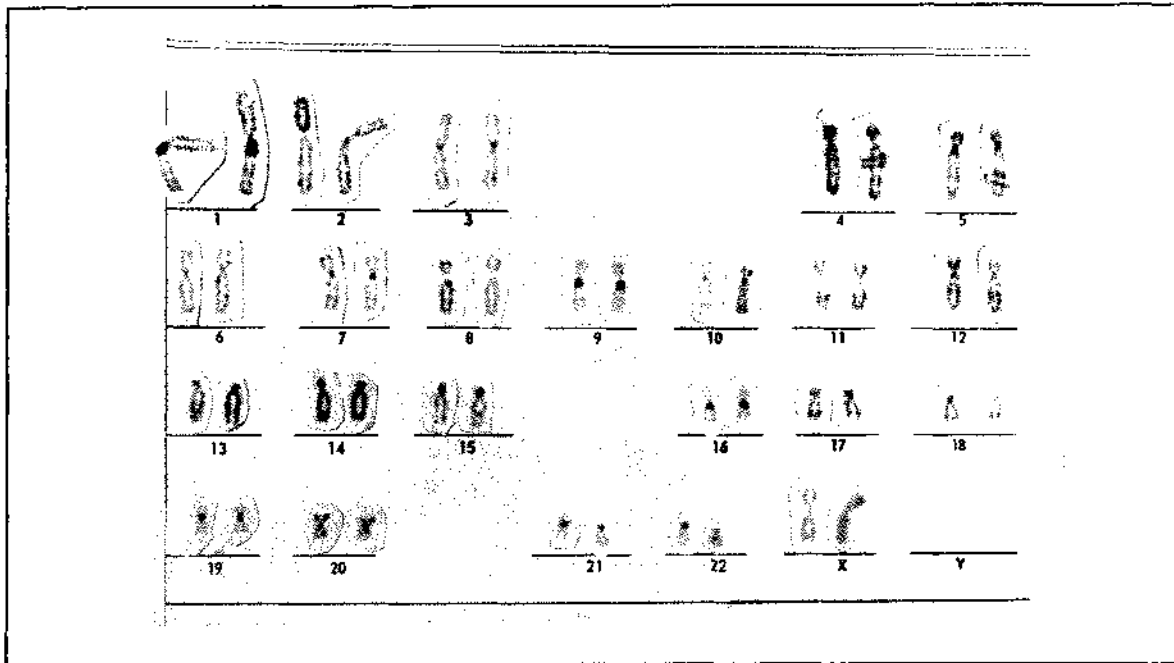


Figure 13. Karyotype of RsaI treated metaphase chromosomes.

#### **b. MboI Digested Chromosomes**

Metaphases digested with MboI reveal 15 out of the 24 human chromosomes exhibit centromeric region staining (see Fig 14). The following is a list of chromosomes ordered in decreasing centromeric region size: 1, 16, 11, 19, 5, X, 7, 12, 17, 20, 10, 9, 22, 15, and 14.

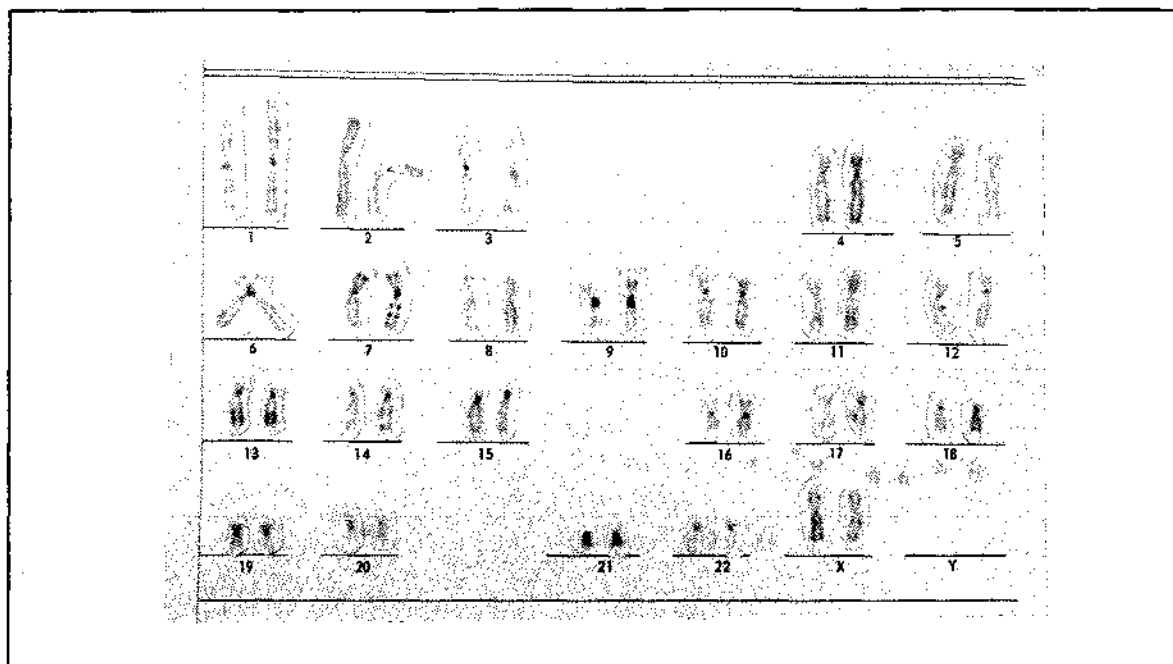


Figure 14. Karyotype of MboI treated metaphase chromosomes.

### c. DdeI Digested Chromosomes

Metaphases digested with DdeI reveal 6 out of the 24 human chromosomes exhibit centromeric region staining (see Fig 15). The following is a list of chromosomes ordered in decreasing centromeric region size: 1, 9, 16, 12, 3, and 22.

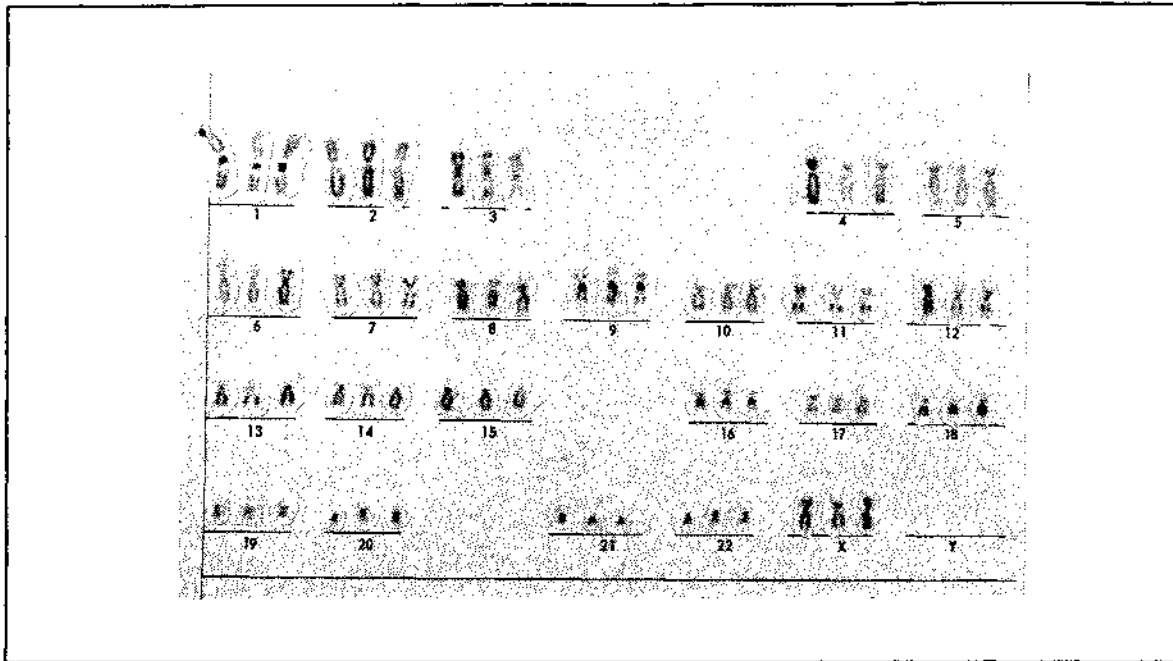


Figure 15. Karyotype of DdeI treated metaphase chromosomes.

#### d. AluI Digested Chromosomes

Metaphases digested with AluI reveal 20 out of the 24 human chromosomes exhibit centromeric region staining (see Fig 16). The following is a list of chromosomes ordered in decreasing centromeric region size: 1, 9, 16, Y, 19, 5, 10, 3, 7, 15, 22, 18, 20, 21, 12, 13, 17, 14, 6, and 4.

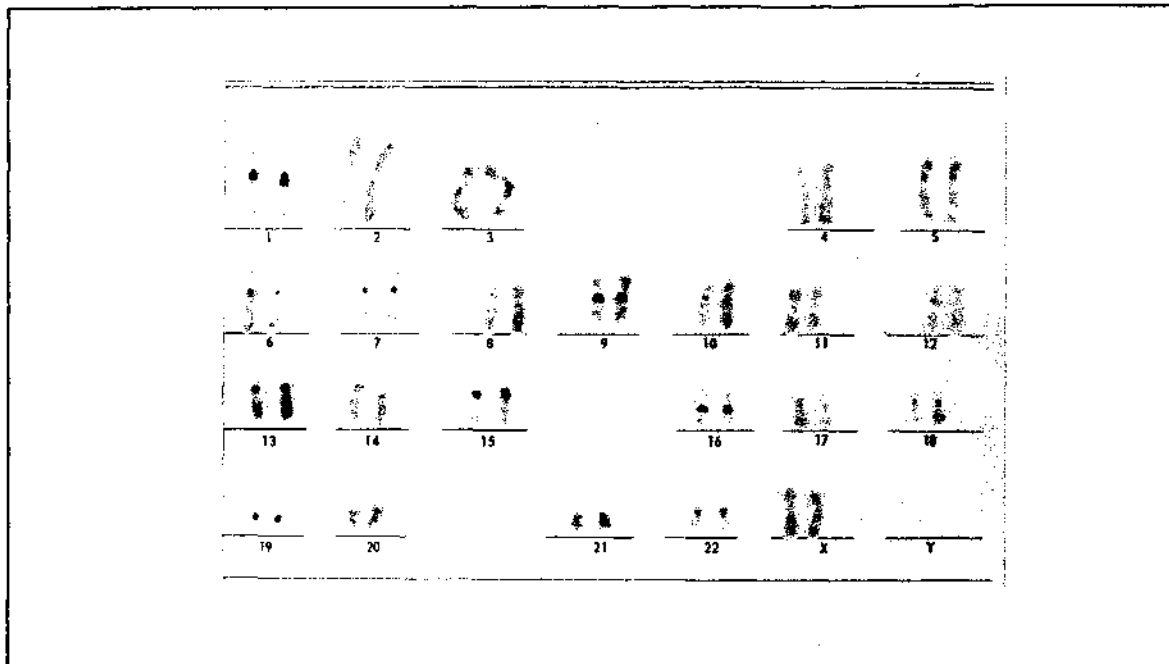


Figure 16. Karyotype of AluI treated metaphase chromosomes.

The restriction endonuclease AluI was selected for a more intensive study since digestions using this enzyme were more successful and more chromosomes were found to show centromeric region staining when compared to the other restriction endonucleases.

### **C. Evaluation of Techniques for Quantifying the Polymorphic Region Revealed by AluI Restriction Endonuclease Digestion**

#### **1. Linear Measurements of Polymorphic Regions**

The length of the polymorphic region of chromosome 1 and 9 and the entire length of chromosome 1 in 29 metaphases from one sample were measured. Although the differential staining between the centromeric region and the surrounding chromosome appear obvious at a quick glance, the demarcation between the two areas was not definite. This boundary is characterized by an increasingly stained continuous gradient. Polymorphic region measurements were taken at the middle of the boundary. A scatter

plot of polymorphism length versus the length of chromosome 1 is shown in figure 17 and figure 18.

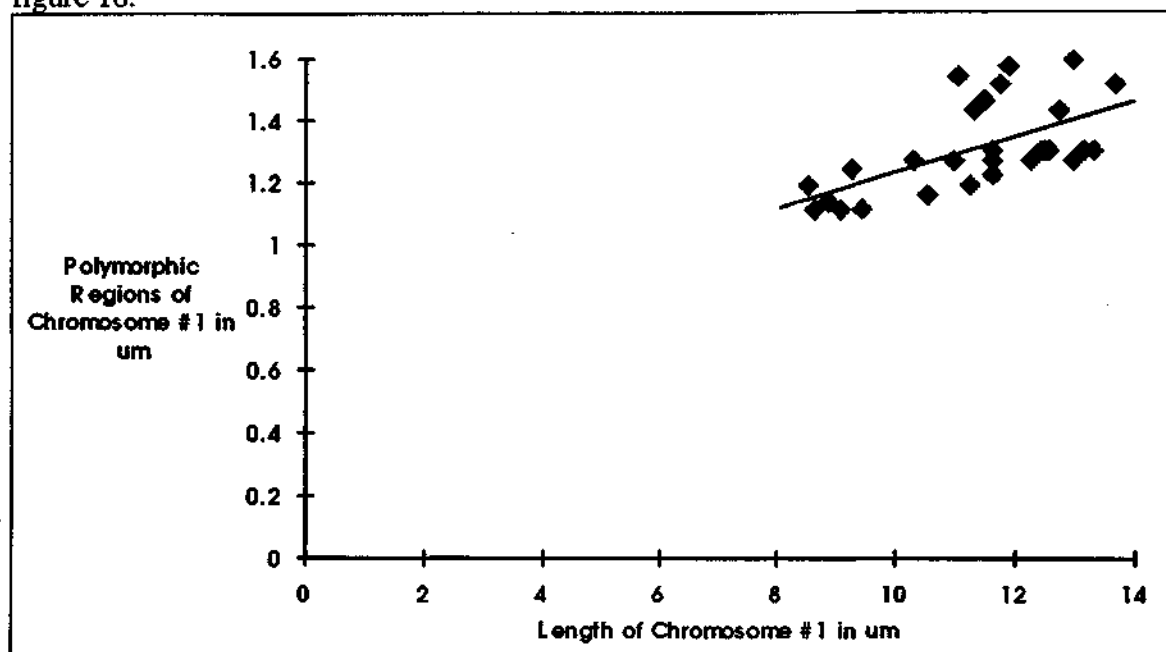


Figure 17 Linear measurement of the polymorphisms of chromosome #1

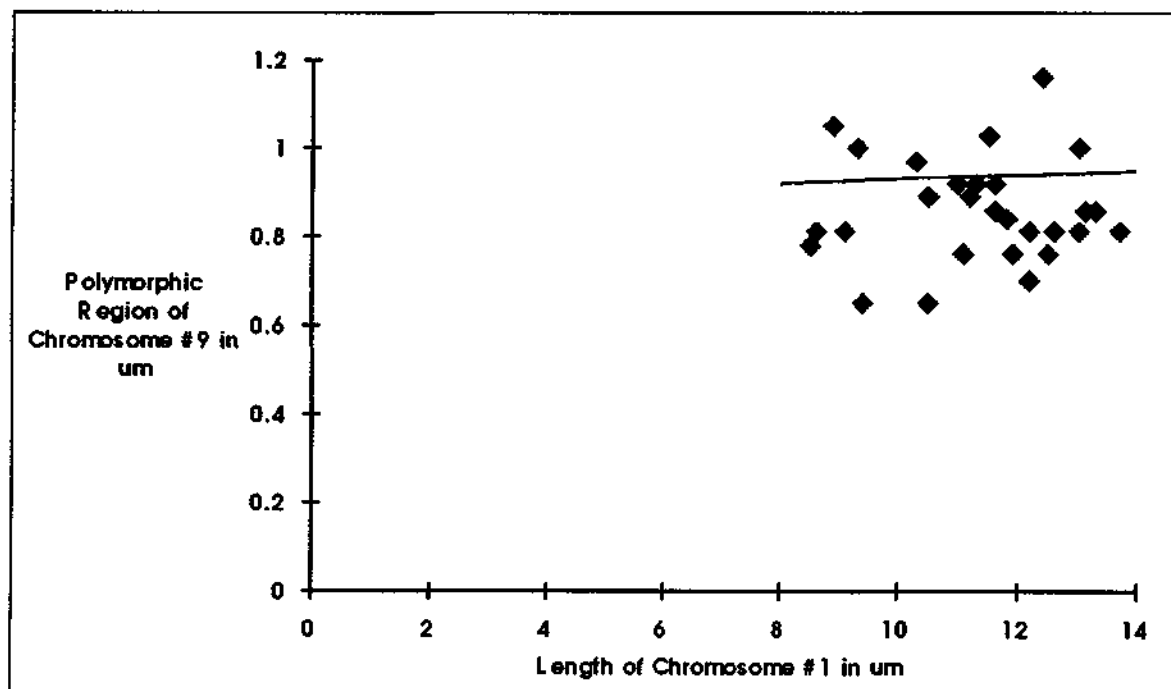


Figure 18 Linear measurement of the polymorphisms of chromosome #9



Linear regression analysis of the polymorphic region of chromosome 1 gives a linear regression model with  $\beta_1$  (slope) = 0.06,  $\beta_0$  (y-intercept) = 0.64. F-test of  $\beta_1 = 0$  gives a F value of 15.721. Since  $F(0.05;1,27) = 4.21 < 15.721$ ,  $\beta_1 \neq 0$ . Correlation is found to be 0.639.

Linear regression analysis of the polymorphism of chromosome 9 gives a linear regression model with  $\beta_1 = 0.0016$ ,  $\beta_0 = 0.84$ . F-test of  $\beta_1 = 0$  gives a F value of 0.0106. Since  $F(0.05;1,27) = 4.21 > 0.0106$ ,  $\beta_1 = 0$ . Correlation is found to be 0.0372.

Based on the 95% confidence interval limits of the data collected the technique cannot resolve structures that are less than 0.2  $\mu\text{m}$  different in length.

## **2. Surface Area Measurements**

The surface area of the polymorphic region of chromosome 1 and the entire surface area of chromosome 1 from 11 metaphases from one sample were measured directly from photographs. Figure 19 is a scatter plot of the surface area of the polymorphic region of chromosome 1 versus the total surface area of chromosome 1. Linear regression analysis gives a linear regression model with  $\beta_1 = 0.087$ ,  $\beta_0 = 1.476$ . F-test of  $\beta_1 = 0$  gives a F value of 8.314. Since  $F(0.05;1,9) = 5.12 < 8.314$ ,  $\beta_1 \neq 0$ . Correlation is found to be 0.709. However, 95% confidence interval limits indicate that this method lacks the resolution for quantifying polymorphic regions revealed using AluI restriction endonuclease banding.

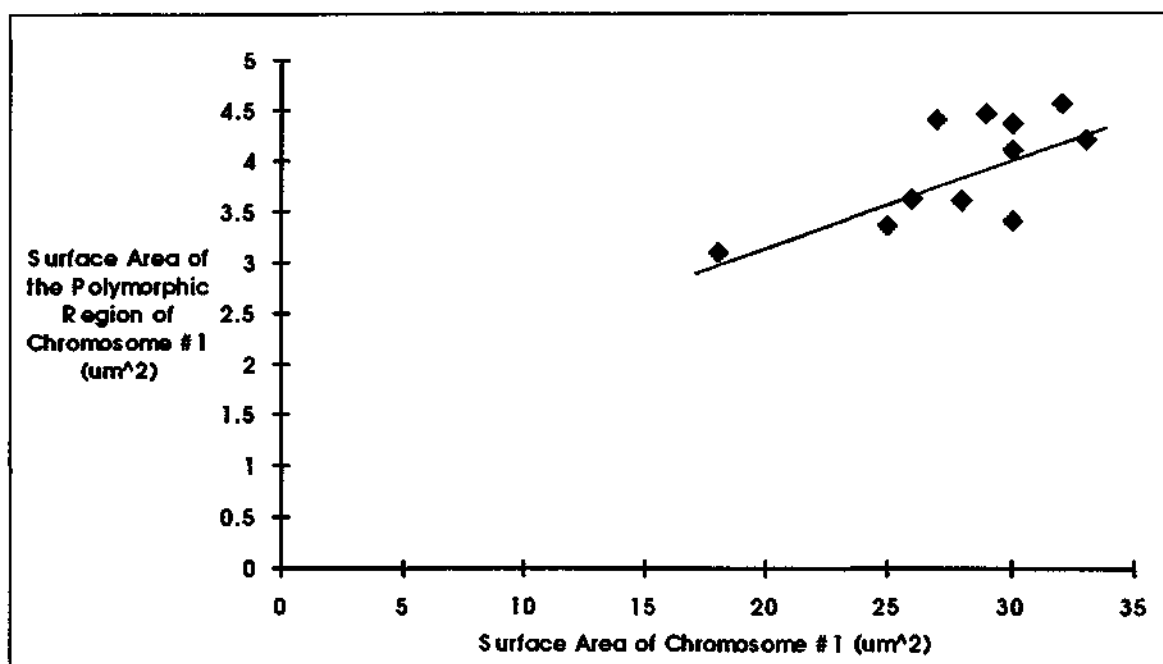


Figure 19 Surface area measurements of the polymorphisms of chromosome #1

### 3. Polymorphism Sizing

The centromeric staining revealed by the restriction endonuclease AluI from 3 normal adult blood samples (6 karyotypes/specimen) were analyzed. Variations in the length of the centromeric region were classified into one of the four groups: large bands, medium bands, small bands, and gaps. Repeat measurements of chromosomes revealed that the size of the centromeric regions were easily misclassified (see table 4).

Chromosome	Specimen 1	Specimen 2	Specimen 3
1	++ / +++ ++ / +++ +++ / ++	++ / +++ ++ / +++ ++ / +++	+++ / +++ ++ / +++ +++ / +++
3	- / + - / + - / +	- / + - / + - / +	+ / ++ - / ++ - / +
7	+ / + - / + + / +	+ / + + / + + / +	+ / + + / ++ + / +
9	+ / +++ + / ++ ++ / -	+ / ++ ++ / ++ ++ / +++	+ / ++ + / ++ + / ++
16	+ / ++ + / ++ + / ++	+ / + ++ / ++ + / +	+ / ++ + / ++ ++ / ++

Table 4. Sample results of 3 specimens (one karyotype per specimen) and each karyotype analyzed 3 times using the method of polymorphism sizing. Legend: '+++' large bands, '++' medium bands, '+' small bands, '-' gaps.

#### 4. Chromosome Structures as Standards

Quantification of the polymorphic staining region using chromosome structures as standards was evaluated. The technique involved comparing the staining at the centromeric region of chromosomes to the size of the centromeric staining of chromosome 20 or the p-arm (short arm) of chromosome 16.

The chromosome preparation from 5 normal blood lymphocyte cultures were digested using AluI. All chromosomes from this normal population were analyzed (except chromosome 20 which was found not to be polymorphic) in the analysis to determine the polymorphism frequency of the centromeric region of chromosomes in a normal population (see Fig 20).

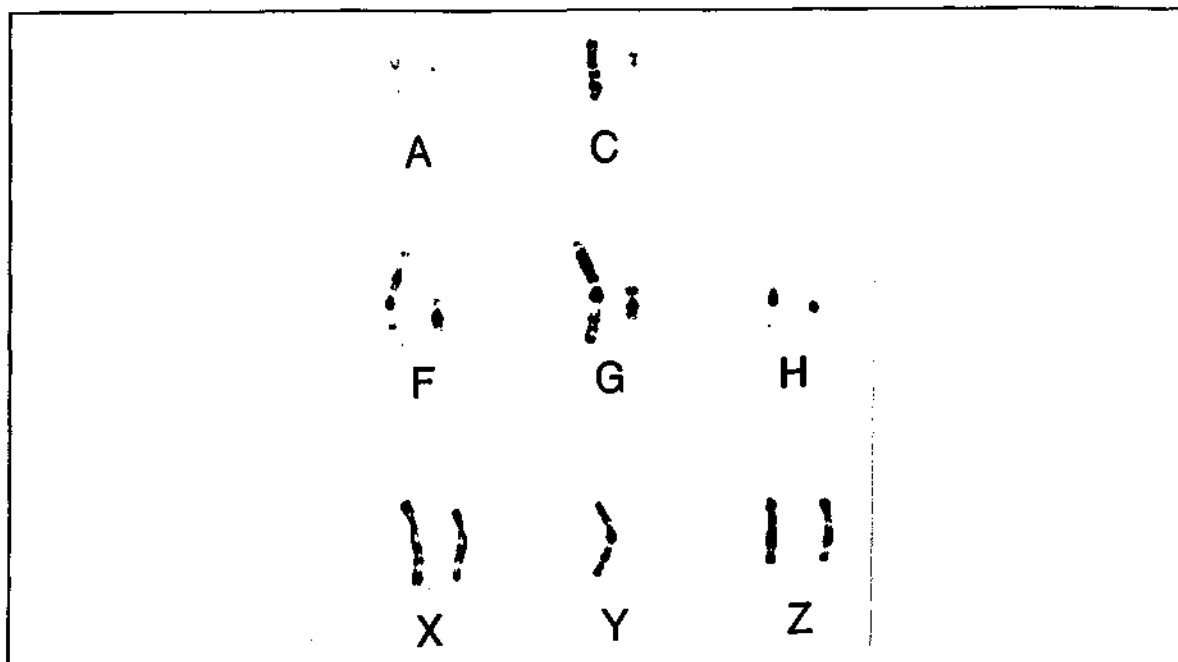


Figure 20. Example of chromosomes and their classification using chromosome structures as standards. For classifications A, C, F, G, and H, chromosomes on the right are the standards and chromosomes on the left are the examples. For classifications, X, Y, and Z, chromosome on the right are examples of different inversion classes and chromosomes on the left are examples of chromosomes that are most commonly found.

A-less than polymorphisms of chromosome #20

C-greater than or equal to centromeric staining of chromosome #20 and less than 1/2 of 16p

F-size greater than 1/2 of 16p and greater or equal to 16p

G-size greater than 16p and greater or equal to 3/2 16p

H-size greater than 3/2 16p and greater to or equal to 4/2 16p

I-size greater than 4/2 16p

X-complete inversion

Y-Inversion involving less than half of the polymorphism

Z-Inversion involving more than half of the staining region

Preliminary analysis of the 5 normal samples gave indications that this was the method of choice for quantifying the polymorphic regions. Therefore, an additional 35 samples were analyzed and the results are shown in table 5.

Polymorphic variability, that is a measure of variability of the polymorphism at the centromeric region was also calculated based on the 40 normal samples and are listed in table 5. The index ranges between 0 and 1 where 0 represents no variability and 1

represents infinite variability. The variability index was calculated using the following formula:

$$\text{variability} = \sum_{i=1}^n \sum_{j=i+1}^n ij, \text{ with } i \leq j (j \text{ is the number of different alleles/chromosome})$$

The summary of results listed in table 5 suggest that in the normal samples, chromosome 1,16, 6,and Y have the most variable polymorphic regions when digested using AluI.

Chromosome	Class												Variability
	A	C	F	G	H	XC	XG	YC	YF	YG	YH	ZC	
1		0.03	0.46	0.30			0.01		0.03	0.13	0.01		0.310
2	0.98	0.02											0.020
3	0.20	0.76	0.02										0.171
4	0.71	0.28											0.198
5		0.96										0.04	0.038
6	0.60	0.36	0.02										0.235
7		0.92	0.06			0.01							0.065
8	1.00												0
9		0.05	0.82	0.09	0.02								0.139
10	0.02	0.92	0.03									0.01	0.056
11	1.00												0
12	0.74	0.24						0.02					0.195
13	0.05	0.95											0.048
14	0.20	0.80											0.160
15		0.97	0.02										0.019
16		0.30	0.62	0.07									0.250
17	0.92	0.07											0.060
18	0.16	0.83											0.133
19		0.92	0.07										0.064
21	0.73	0.26											0.190
22	0.01	0.98											0.010
X	0.95	0.04											0.038
Y			0.73	0.20	0.06								0.202

A-less than polymorphisms of chromosome #20

C-greater than or equal to centromeric staining of chromosome #20 and less than 1/2 of 16p

F-size greater than 1/2 of 16p and greater or equal to 16p

G-size greater than 16p and greater or equal to 3/2 16p

H-size greater than 3/2 16p and greater to or equal to 4/2 16p

I-size greater than 4/2 16p

X-complete inversion

Y-Inversion involving less than half of the polymorphism

Z-Inversion involving more than half of the staining region

Table 5. Frequency of the classes of polymorphisms found in a normal population (n=40)

### **E. Tracing the Origin of Chromosomes**

Tracing the parental origin of chromosomes in aborted specimens was attempted using chromosome structures as standards. Two diploid conceptus and parental sets were analyzed. None of the 92 chromosomes from the conceptuses could be traced back to the individual parents unambiguously.

Tracing the parental origin of chromosomes in aborted specimens was attempted for 5 parental and conceptus sets visually. Two of the conceptuses were cytogenetically diploid and 3 were triploid. The parents were 46,XX and 46,XY. 17 out of 299 chromosomes (one chromosome 1, two chromosome 3, three chromosome 13, five chromosome 14, one chromosome 21, and five chromosome 22) from the conceptuses could be traced back to an individual parent.

### **F. Heterozygosity of Polymorphic Sites on Homologous Chromosomes**

Heterozygosity (heteromorphic) of polymorphic sites on homologous chromosomes are useful in ruling out uniparental isodisomy (see Fig 21). Two methods were used for this analysis: chromosome structures as standards and visual assessment.

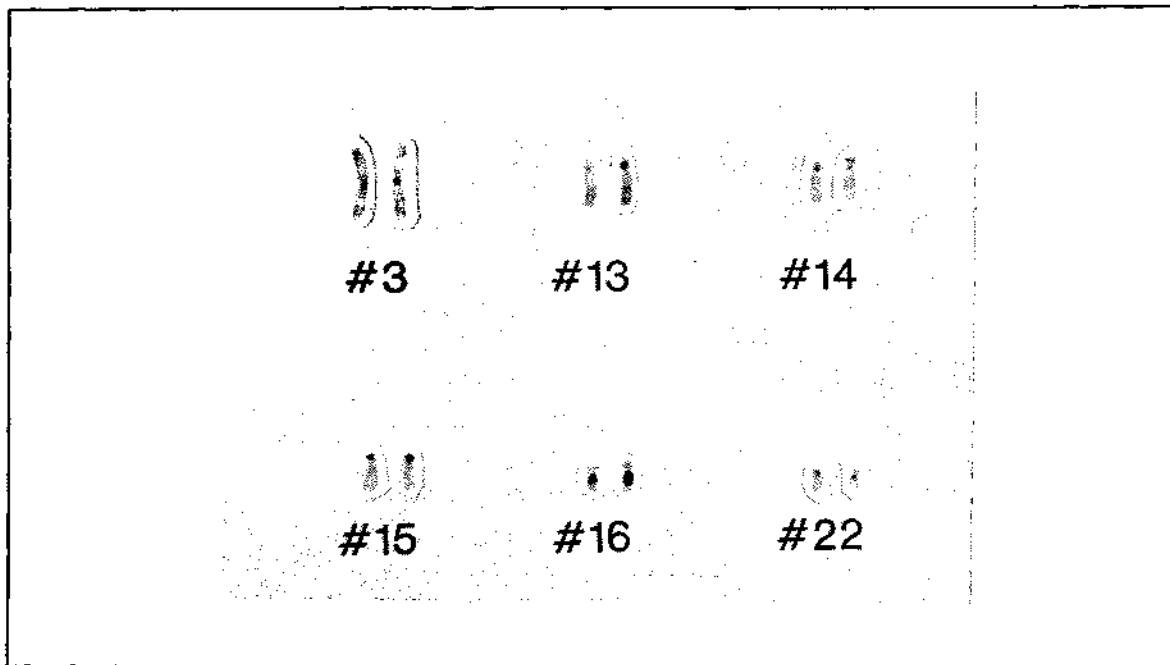


Figure 21. Examples of heterozygous polymorphic regions on homologous chromosomes..

### 1. Chromosome Structures as Standards

Figure 22 identifies the number of homologous chromosomes having allelic differences at the centromeric region on homologous chromosomes in a normal population ( $n=40$ ) using the chromosome structures as standards method. Chromosome 1, 3, 16, and 4 are most likely to show heterozygosity at the centromeric region with probabilities of 0.55, 0.53, 0.20, and 0.20 respectively.

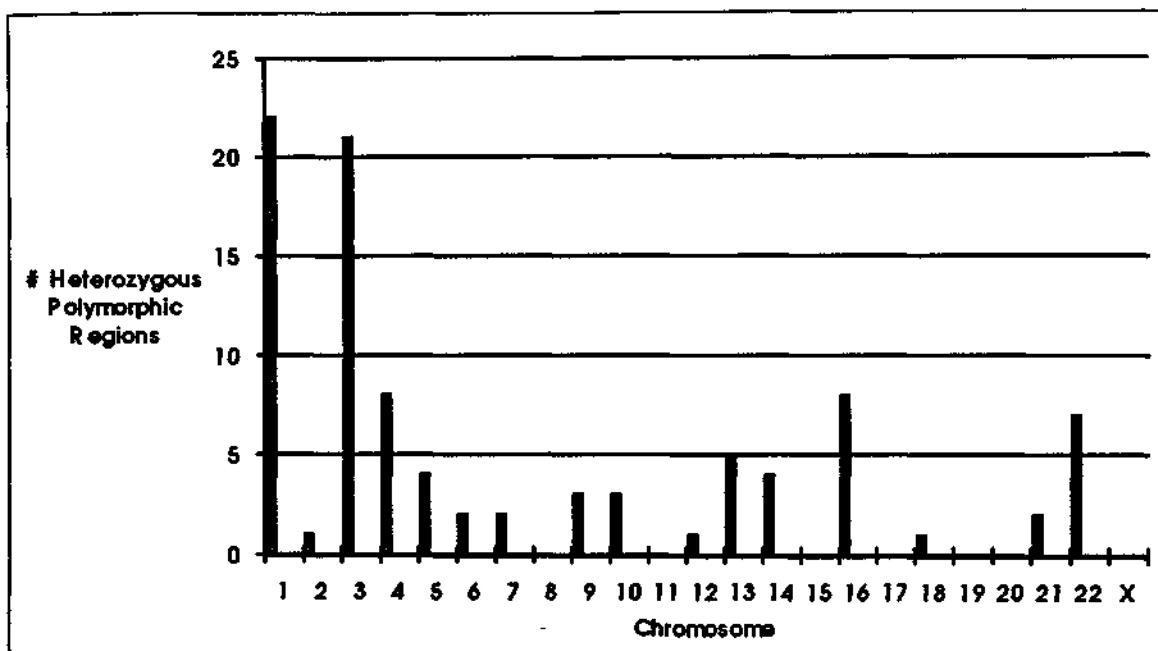


Figure 22 Heterozygosity of the polymorphic region on homologous chromosomes using chromosome structures as standards (n=40, for X-chromosome n=25)

## 2. Visual Assessment

Figure 23 identifies the number of homologous chromosomes found to have allelic differences on homologous chromosomes in a normal population (n=40) using visual assessment method. The visual assessment method involves the visual analysis of polymorphisms located at the centromeric region as well as the satellites and satellite stalk region on the acrocentric chromosomes. Chromosome 15, 21, 13, 3, 14, 1, and 22 are most likely to have heterozygous polymorphisms with probabilities of 0.78, 0.73, 0.73, 0.70, 0.68, 0.65, and 0.65 respectively.



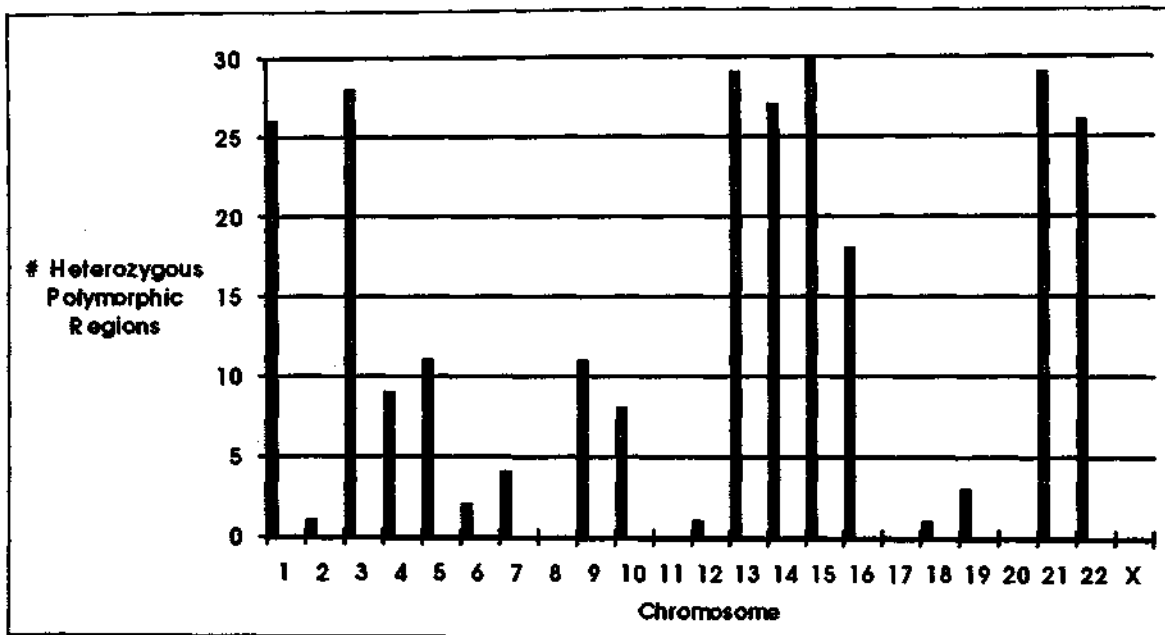


Figure 23 Heterozygosity of the polymorphic region on homologous chromosomes using visual assessment (n=40, for X-chromosome n=25)

Identification of homologous chromosomes with heterozygous polymorphisms were better achieved by using visual assessment.

### 3. Incidence of Polymorphism Heterozygosity on Homologous Chromosomes

Using visual assessment, figure 24 summarizes the number of homologous chromosomes found to have heterozygous polymorphisms in each individual in a normal population. In the control population of 40 normal adults, the mean is found to be 6.3 (standard deviation=1.96) homologous chromosomes exhibiting heterozygous polymorphisms per individual.

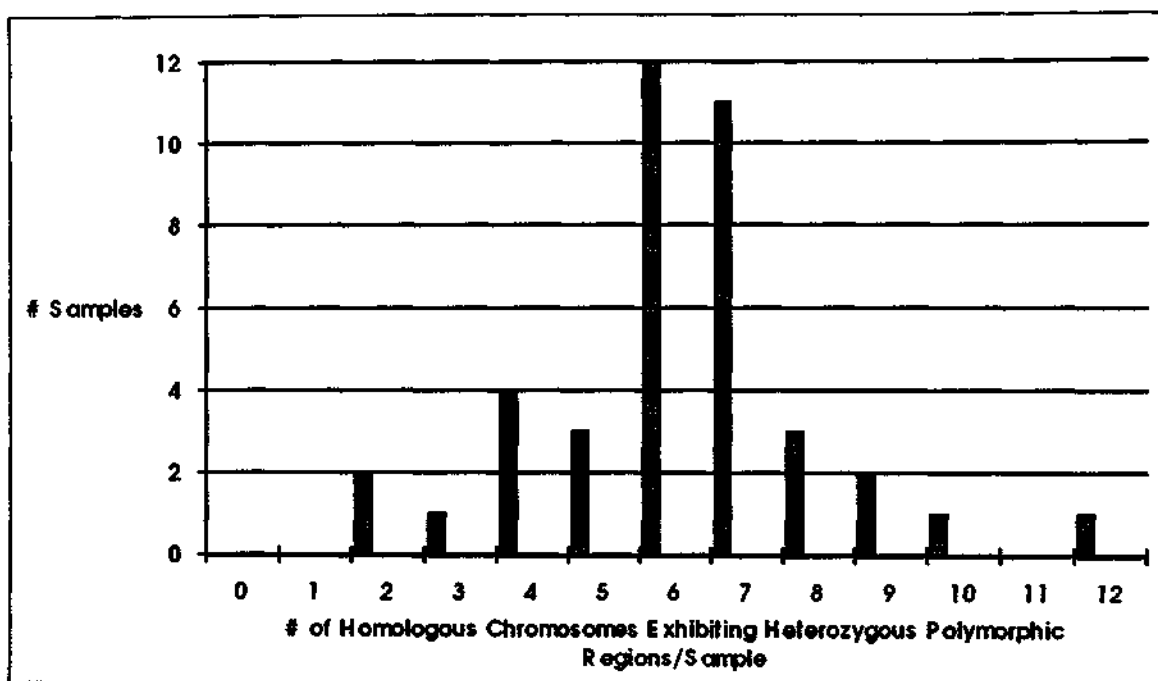


Figure 24 Distribution of the number of homologous chromosomes exhibiting heterozygosity at the polymorphic region. The mean is 6.3 homologous chromosomes exhibiting heterozygosity at the polymorphic region/sample (n=40 samples)

#### **IV. Discussion**

The objective of this study was to determine if uniparental disomy in diploid early spontaneous abortuses could be detected using a cytogenetic technique. The method of approach in searching for uniparental disomy was to first select a cytogenetic technique best suited for chromosome tracing analysis. This led to the selection of the restriction endonuclease banding, since reports in the literature suggested that the polymorphic variability of the centromeric regions revealed by this technique is greater than that shown by conventional cytogenetic techniques. (Babu and Verma, 1986b; Babu and Verma, 1986c; Babu and Verma, 1988). Since there are many restriction endonucleases available that can reveal polymorphism at the centromeric region, one enzyme had to be selected based on its ability to reveal polymorphisms reproducibly on most chromosomes. The cytogenetic method of detecting uniparental disomy relies on chromosome tracing analysis involving the parents and the conceptus. Successful tracing analysis using chromosomes treated with a restriction endonuclease is based on the detection of polymorphism of centromeric regions which are variable in terms of size, location, and stain uptake, between individuals. This variability of the centromeric region must also be accurately quantified so that the task of chromosome tracing analysis can be performed. For this reason, four methods of quantifying the polymorphic region were evaluated: linear measurements, surface area measurements, polymorphism sizing, and chromosome structures as standards. Quantification of polymorphism also allows the observation of the polymorphic variability in the normal population and consequently has biological as well as clinical significance. Several aspects of this study will be discussed, these include the morphology of the digested chromosomes, banding reproducibility, chromosomal polymorphism quantification, polymorphic frequencies, tracing analysis, detection of uniparental disomy, and future directions and technical improvements.

### **A. Morphology of Restriction Endonuclease Digested Chromosomes**

Numerous studies describing the morphology of human chromosomes after restriction endonuclease digestion have been reported (Mezzanotte et al., 1983a; Bianchi et al., 1985; Babu and Verma, 1986; Babu et al., 1988). Different restriction endonucleases were found to reveal chromosomes which were G-like banded, C-like banded, and gap banded.

Chromosomes treated with the restriction endonucleases *RsaI*, *DdeI*, *MboI*, and *AluI* were reported to produce differential staining between the centromeric region (C-like banded) and the surrounding chromosome arms in metaphase chromosomes (Mezzanotte et al., 1983a; Bianchi et al., 1985; Babu et al., 1988). Furthermore, these chromosomes were also G-like banded which were useful in chromosome identification (Mezzanotte et al., 1983a; Bianchi et al., 1985; Babu et al., 1988). Each restriction endonuclease capable of revealing C-like banding had a characteristic staining pattern that was unique to each enzyme (Babu and Verma, 1986; Babu et al., 1988). Suggestions had been made that the differences in the banding at the centromeric region and the satellited regions between the different enzymes was due to the prevalence and/or accessibility of restriction enzyme recognition sites where the enzyme was able to act (Mezzanotte et al., 1983; Bianchi et al., 1985; Babu and Verma, 1990). Variations of the chromosomal polymorphic regions in terms of size, location, and stain uptake, are generally consistently observed from cell to cell (Babu and Verma, 1986; Babu and Verma, 1990). Babu and Verma (1986) reported that polymorphic regions exposed using restriction enzymes were predominantly homogeneously stained. When uneven staining characterized by interstitial bands in the polymorphic region was observed, these structures were likely due to chromosome breaks (Babu and Verma, 1986).

In my experiments, I have found that the centromeric regions and the satellited regions of chromosomes were stained darker than the surrounding chromosome arms.

Both the centromeric region and the satellited region were found to be polymorphic on some chromosomes. All chromosomes in addition to C-like banding were also G-like banded. Interstitial banding in the centromeric region were observed in some of the metaphases, however, these were rare events and therefore they were likely due to chromosome breaks. These findings were similar to the findings reported in the literature.

Even though restriction endonuclease banding using *RsaI*, *DdeI*, *MboI*, and *AluI* revealed chromosomes which appear C-like banded, the mechanism involved in C-banding and restriction endonuclease banding are not similar. The banding pattern seen after restriction endonuclease banding depends on the method of preparation of the chromosomes (methanol fixed versus methanol/acetic acid fixed) and the cleaving of DNA by the restriction endonuclease (Peretti et al., 1990). The banding pattern of C-banded chromosomes depends on the extraction of DNA preferentially on the chromosome arms leaving DNA in the centromeric region intact (Babu and Verma, 1986; Verma et al., 1978). Therefore, results comparing the polymorphic nature of the centromeric region obtained from studies involving C-banding and restriction endonuclease banding are not directly comparable.

#### **B. Banding Reproducibility of the Restriction Endonuclease Banding Technique**

Babu et al. (1987) and Babu and Verma (1988) had reported that digestions using restriction endonuclease to reveal C-like banding have not always been successful. That is, metaphases treated with restriction endonucleases did not consistently reveal differential staining between the centromeric region and the surrounding chromosomal arms. Similar comments were also made by Hedemann et al. (1988). They reported that the restriction endonucleases *AluI* and *DdeI* were more consistent in producing the desired C-like bands when compared to the restriction endonucleases *PvuII*, *RsaI*, *MboI*, and *HaeIII*.

Hedemann et al. (1988) suggested that contamination on the slides (e.g. nuclear proteins) may inactivate restriction endonucleases and that it is this inactivation of the enzyme which can lead to the varying success of experiments. The enzyme dependent

success rate may also be related to the half life of the cleaving ability of the enzymes.

Crouse and Amorese (1986) reported measurements of the activity of restriction endonucleases during extended incubation in the digestion of  $\lambda$  or SV40 DNA. DdeI and MboI were found to have full activity until the end of the first hour and partial activity for the remaining 4 hours. RsaI was found to have full activity until the end of the first hour, partial activity until the end of the second hour and no activity in the third hour. AluI was reported to clearly have the most activity as it was found to have full activity up until the end of the fifth hour.

In my experiments, the digestion of the slide/dish was considered successful when half of all chromosomes in a metaphase showed high contrast between the polymorphic region and the surrounding portions of the chromosomes. Of the four enzymes studied, AluI, RsaI, DdeI, and MboI, AluI digestion of fixed metaphase chromosomes of fibroblast or blood lymphocyte preparations were most successful according to the criteria given previously.

### **C. Chromosomal Polymorphism Quantification Methods**

Based on both the high level of experimental reproducibility and the largest number of chromosomal polymorphic regions exposed on different chromosomes, AluI was selected for further study. Four methods involving quantifying the polymorphic regions (linear measurements of polymorphic region, surface area measurements, polymorphism sizing, and chromosome structures as standards) were evaluated in order that one could be selected to study polymorphic variability in the normal population and in performing chromosome tracing.

## **1. Linear Measurements of Polymorphisms**

Quantification of the polymorphic region by measuring the length of the polymorphic region should not be performed without a chromosomal condensation reference, since the length of the centromeric region is proportional to the condensation state of the chromosome (Schmidady and Sperling, 1976; Balicek et al., 1977; Balicek et al., 1978). Sampaio et al. (1989) reported that in chromosomes that are C-banded using traditional methods, they were successful in using the entire length of chromosome 1 as a condensation standard in measuring polymorphism size in C-banded 1, 9, 16, and Y chromosomes. I am not aware of any studies in the literature which attempt to quantify the polymorphic regions revealed by restriction endonuclease banding by using a method of linear measurements. In my experiments, the length of the polymorphisms of chromosome 1 and 9 were plotted against the entire length of chromosome 1 to determine if a linear relationship existed.

A linear relationship between the chromosome condensation of chromosome 1 with the condensation of the centromeric region of chromosome 1 was established. A linear relationship between the chromosome condensation of chromosome 1 with the condensation of the centromeric region of chromosome 9 could not be established. This was due to large variations (up to 50%) of the length of the centromeric region of chromosome 9 for a set length of chromosome 1. Therefore, the results suggest that the length of chromosome 1 is not a good standard for measuring the condensation state of the centromeric region of chromosome 9 revealed by AluI.

Since any form of measuring involves some imprecision, the percentage error, that is the percent difference between the measured size versus the actual size, can be reduced by measuring structures that are longer versus structures that are shorter in length. Since, the centromeric region of chromosomes are thought to vary linearly with the condensation state of the chromosome, the chromosome with the larger polymorphic region would be more accurately measured, that is less percentage error, compared to a chromosome with

a smaller polymorphic region. The polymorphic region of chromosome 1 is larger (1.0 to 1.6  $\mu\text{m}$  when the length of chromosome 1 is between 8  $\mu\text{m}$  and 14  $\mu\text{m}$ ) than the polymorphic region of chromosome 9 (0.6 to 1.2  $\mu\text{m}$  when the length of chromosome 1 is between 8  $\mu\text{m}$  and 14  $\mu\text{m}$ ). It is likely that error in measurement is responsible for the failure in finding a linear relationship between the centromeric region of chromosome 9 and the total length of chromosome 1.

Thus, quantification using linear measurements are only useful for chromosomes with large polymorphic regions. The accuracy and resolution of the linear measurement method for the purpose of quantifying the polymorphic regions of chromosomes has the potential of increasing if the number of chromosomes to be measured are increased. However, increasing the number of chromosomes to measure would involve a direct increase in time to analyze, making this technique an unfavorable option for chromosomal tracing analysis.

## **2. Surface Area Measurements**

The polymorphic region of chromosome 1 in a sample treated with AluI was chosen for analysis using surface area measurements since the chromosome has a large polymorphic region and is also the largest chromosome with respect to its total surface area. These qualities assist in minimizing user input error. The 95% confidence interval of the linear regression line revealed that this technique would not be useful in quantifying polymorphic regions since a linear relationship between the surface area of the polymorphic region and the total surface area of chromosome 1 was not found.

A similar study to the one presented here was performed by Mason et al. (1975). Their study involved quantifying the surface area of one of the C-banded region of chromosome 1 in two specimens. The C-banded region from each specimen was selected for analysis using a computer controlled microscope and scanner. They found that the a linear relationship exists at the 95% confidence interval ( $n=50$ ).



The reason why a linear relationship was not found in my study is due to the small sample size used ( $n=11$  in this study and  $n=50$  in Mason et al.). By increasing the sampling size, this technique has the potential of being a useful technique in quantifying the polymorphic region. However, the time needed to carry out the sampling of 50 chromosomes would make this technique unfavorable.

### **3. Polymorphism Sizing**

The most primitive method of polymorphism evaluation is sizing using the multiple "+" method (Bianchi et al., 1985). In this method, classification of polymorphism depends primarily on the analyzer's individual experience in being able to classify structures based on previous experience. My results indicated that there was a prevalence to misclassify polymorphisms and hence, this method of quantifying the polymorphic region was abandoned. This type of analysis should not be used since data collected in this manner is not transferable from one laboratory to another since chromosome condensation reference structures are not utilized in this technique.

### **4. Chromosome Structures as Standards**

The short arm (p-arm) of chromosome 16 has been used for recording size heteromorphisms in C-banded chromosomes since its length has been determined to vary proportionately with the condensation state of the other chromosomes and the length of the 16 p-arm is found not to be variable within the normal population (Verma et al, 1978; Verma and Dosik, 1980; Lopetegui, 1980). In my study, preliminary observations revealed that both intracellular or intercellular variability of the centromeric region of chromosome 20 did not occur. Therefore, in this study the centromeric region on chromosome 20 was selected as an additional reference structure. Babu and Verma (1986) indicated that no intracellular (referring to the same metaphase) variability of chromosome 20 were found in samples treated with AluI. However, size polymorphisms were recorded intercellularly (referring to between different metaphases) (Babu and

Verma, 1986). The reason for the discrepancy between my observations and that reported by Babu and Verma (1986) is unknown.

The chromosomes preparation from 40 blood lymphocytes cultures from normal patients were digested using AluI. The centromeric regions of these samples were measured with respect to the centromeric staining of chromosome 20 and the p-arm of chromosome 16. Polymorphic regions of chromosomes 1, 9, 16, and Y stained typically greater than or equal to classification F (size greater than 1/2 of 16p and greater or equal to 16p). The polymorphic regions of chromosomes 2, 4, 5, 6, 8, 11, 12, 13, 14, 17, 18, 21, 22, and X never exceeded classification C (greater than or equal to centromeric staining of chromosome #20 and less than 1/2 of 16p). The advantage of using chromosome structures as standards as a method of quantification is that it is simple to perform, variations in chromosome condensation are taken into account by using chromosomal reference structures, and data collected from one lab is easily transported to other labs. The disadvantage of this technique is that since classification of centromeric region is done by sight, that is without instruments than can perform this task more objectively, the technique can not be used to classify the centromeric region into finer classifications. There was also some bias in the method of classification since polymorphic regions are classified only if half or more than half of the polymorphic regions are similarly classified.

Studies regarding the classification of the polymorphic region on AluI restriction endonuclease banded chromosomes have not been reported within the literature, therefore, these results can not be compared with others.

The ability to quantify polymorphisms is a necessary step in evaluating the polymorphic variability in the normal population and can be used in performing chromosome tracing. The four methods, linear measurements of polymorphisms, surface area measurements, polymorphism sizing, and chromosome structure as standards, were evaluated to determine the method best suited for quantifying the polymorphic region.

Chromosome structure as standards was selected since the technique was found to be simple to use and the time necessary for analysis was less than that required by the other techniques. If an increased sample size was used, surface area measurements and linear measurements are potentially superior methods to quantify the polymorphic region. However, as stated previously, increasing the sample size will proportionately increase the time necessary to analyze.

#### **D. Polymorphic Frequencies in Control Population**

The polymorphic frequency data for individual chromosomes is useful since this information can be used in calculating the probability of a particular chromosome to be successfully traced. The classification of the centromeric region revealed by AluI restriction endonuclease in terms of size and location as well as their frequencies in the human population have not been reported in the literature. Babu and Verma (1986) had briefly commented that in AluI restriction endonuclease digested chromosomes, chromosomes with the larger polymorphisms (i.e. chromosome 1, 9, 16, and Y) have the highest variability. In my experiment, AluI restriction endonuclease chromosomes from 40 normal adults were analyzed using the chromosome structure as standards methods and a variability index was calculated for each chromosome (see Table 5). According to the data collected in this study, chromosome 1, 16, 6, and Y should have the highest variability of the 24 human chromosomes. This is similar to the results reported by Babu and Verma (1986), however, they did not report that chromosome 6 is highly variable and in my experiment, chromosome 9 was not found to be highly variable. The reason for this discrepancy is unknown.

#### **E. Tracing Analysis using AluI Restriction Endonuclease Digested Chromosomes.**

Studies involving chromosome tracing rely on a unique differentiating marker enabling the chromosome to be unambiguously traced from parent to progeny. Therefore, by selecting a technique that is capable of revealing highly polymorphic markers, the likelihood of successfully tracing a chromosome's origin is increased.

Tracing analysis of chromosomes treated with restriction endonucleases have not routinely been performed. However, a report by Babu and Verma (1986b) discussed a successful tracing analysis of an extranumerary chromosome 18 treated with the restriction endonuclease AluI in a trisomy 18 patient.

In my experiments, the segregation of chromosomes from parent to conceptus could not be traced using the quantification method of chromosome structure as standards in 2 diploid spontaneously aborted specimens (0%, 0 out of 92 chromosomes). However using visual assessment, the segregation of chromosomes from parent to conceptus could be traced in 6% (17 out of the 299 chromosomes from 2 diploid and 3 triploid spontaneously aborted specimens) of chromosomes using a visual comparison method.

The discrepancy between being able to trace chromosomes using chromosomal structure as standards and visual comparison is due to the fact that the visual comparison method is able to distinguish smaller variations in structures than the chromosome structure as standards method. Furthermore, the visual comparison method uses the structure of the satellite regions of the acrocentric chromosomes to assist in tracing analysis.

Balicek et al. (1978) had reported that tracing analysis of C-banded chromosomes in 10 families, that is 30 individuals, revealed that chromosomes 1, 9, 16, and Y were traced at a 94% success rate (n=67). In my experiment, of the 17 chromosomes that could be traced, one chromosome belongs in the subgroup consisting of chromosome 1, 9, and 16. Therefore, 1 out of 30 chromosomes (3%) in the subgroup consisting of chromosome 1, 9, and 16 could be traced using a visual comparison method. This would indicate that for chromosomes 1, 9, and 16, C-banding exhibits an increased polymorphic variability compared to AluI restriction endonuclease banding.

My experiments have shown that chromosomes 1, 16, 6, and Y had the highest polymorphic frequency when chromosome structures as standards is used as a mean of quantification. These results suggest that chromosomes 1, 16, and 6 are most likely to be

successful in chromosomal tracing analysis. This is important in view of the fact that 30 % of spontaneously aborted trisomic specimens are trisomy 16 (Jacobs and Hassold, 1987). The AluI restriction endonuclease technique could be used in determining the parental origin of the extranumerary chromosome in these specimens.

Using chromosomal polymorphism, uniparental disomy detection is possible when the polymorphisms of both parents and the conceptus are compared. After AluI restriction endonuclease banding, chromosomal tracing analysis revealed that tracing acrocentric chromosomes is more successful than tracing of metacentric and submetacentric chromosomes. In this study, chromosomes tracing analysis was performed on 5 conceptuses of which 2 were diploid. No evidence of uniparental disomy was found in the two diploid conceptuses studied.

#### **F. Heterozygous Polymorphism on Homologous Chromosome**

Uniparental isodisomy can be ruled out by the analysis of polymorphisms on homologous chromosomes. It should be noted that these polymorphisms do not necessarily have to be revealed by restriction endonuclease banding as other polymorphism revealing methods, such as Q-banding or G-11 banding, can be used. If the polymorphic region on homologous chromosomes appeared identical the progeny may have uniparental isodisomy. If the polymorphisms on homologous chromosomes are dissimilar, uniparental isodisomy is excluded.

Babu et al. (1988) reported in a study discussing the heteromorphic nature of human chromosomes digested with AluI, DdeI, MboI, and DdeI, that AluI digestion can reveal heteromorphisms at the polymorphic region on chromosome 1, 3, 4, 5, 6, 7, 9, 10, 13, 14, 15, 16, 18, 19, 20, 21, 22, and Y. They also report that chromosome 2 is rarely heteromorphic and that chromosomes 8, 11, 12, 17, and X have never been observed to be heteromorphic. In my experiments, based on a visual assessment, chromosomes 1, 2, 3, 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 18, 19, 21, and 22 (Y not analyzed) were found to

show at least some chromosomal heteromorphism in the 40 normal adults (see figure 23). These results are similar to the finding reported by Babu et al. (1988).

Figure 24 shows that on average 6.3 chromosomes/metaphase (about 25 % of the chromosomes in an individual) are heteromorphic using visual assessment. Chromosome 1, 3, 13,14,15,16, 21, and 22 are the most likely chromosomes to be ruled out as showing uniparental isodisomy since they have been found to be the most heteromorphic pair of the 24 human chromosomes.

### **G. Methods to Improve Polymorphic Variability of Human Chromosomes**

Banding of chromosomes using restriction endonuclease is dependent on the ability of the restriction endonuclease to cleave DNA at specific sites. Since restriction endonucleases have specific recognition and cleaving sites, different restriction endonucleases can induce different banding patterns on chromosomes. Therefore, it is possible that different combination of enzymes may show a banding pattern that is unique compared to the action of a single enzyme when used alone. Hedemann et al. (1988) used this approach and used combination of enzymes to determine if the centromeric region of chromosome 1 and 9 would stain differently compared to single enzyme digestion. They used a variety of enzyme combinations, however, most did not reveal any difference in staining compared to using the enzymes alone. However, one combination AluI + DdeI revealed polymorphic regions that were larger than polymorphic regions revealed by DdeI when used alone.

Further multienzyme digestion studies are warranted since Hedemann et al. (1988) focused on chromosome 1 and 9 and disregarded the other 22 human chromosomes. Other combinations of restriction endonuclease could also be tested.

## **H. Future Direction**

The polymorphic nature of the centromeric region revealed by AluI restriction endonuclease has some uses in chromosomal tracing analysis. Since it has been established that this technique is capable of revealing the polymorphism of the centromeric regions of chromosomes, this property could be potentially useful in confirming paternity or evaluating the status of purged leukemic cells in bone marrow transplantation patients.

A cytogenetic technique is beneficial over a molecular technique in screening for uniparental disomy since uniparental disomy is a chromosomal phenomenon and a cytogenetic method has the potential to screen all chromosomes in one experiment. Multienzyme digestions may aid in revealing more polymorphisms than can be currently revealed using one enzyme. Since linear or surface area measurement techniques have the potential of classifying the polymorphic region into finer classifications, automation of these techniques would be the next logical step.

Molecular techniques such as analyzing the hypervariable DNA regions or by studying restriction fragment length polymorphisms could also be used in the screening for uniparental disomy. The advantage of using this technique is that the likelihood of errors occurring are reduced. The disadvantage is that without automation a molecular based method would require much time to screen even one sample.

## **I. Conclusion**

The initial objective of this investigation was to investigate the etiology of abnormal phenotypes in spontaneously aborted diploid embryos. I have postulated that uniparental disomy in diploid chromosomal complement could be correlated with the abnormal phenotype of the spontaneously aborted diploid embryos. Experiments were designed to evaluate the feasibility of using chromosomal polymorphic markers revealed by restriction endonuclease banding in chromosome tracing analysis. The study has shown the following:

- 1) Compared to RsaI, MboI, and DdeI, the restriction endonuclease AluI was found to most reliably stain the centromeric region of chromosomes prepared from fibroblast and blood lymphocyte cultures.
- 2) AluI revealed centromeric staining in 20 out of 24 human chromosomes.
- 3) Using the quantification technique of chromosome structure as standards, the centromeric regions of AluI digested chromosomes 1, 6, 16, and Y were found to be the most polymorphic.
- 4) Tracing analysis using polymorphic regions as markers in AluI digested chromosomes revealed that 6% of chromosomes can be traced using a visual assessment method.



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## **V. Appendix**

### **A.1 Variations in Slide Making**

Varying the methods used in the air drying technique known as 'slide preparation' can change the appearance of the chromosome visualized using the compound light microscope. Furthermore, variations in the technique affects the quality of banding produced.

#### **Trouble Shooting Guide** (Observations followed by corrective measures)

**Black Chromosomes**-Appearance of black metaphase chromosomes using phase contrast microscopy. These chromosomes are found to stain optimally using trypsin/gymsa banding technique or AluI restriction endonuclease banding.

**Shiny Chromosomes**-Appearance of shiny metaphase chromosomes using phase contrast microscopy.

- Heat the slide above room temperature when drying slide
- If using prewetted slide (distilled water), use a dry slide
- Dry suspension using dry air

**Grey Chromosomes**-Appearance of grey metaphase chromosomes using phase contrast microscopy.

- Use a slide chilled to 4° C.
- Use a slide prewetted in distilled water
- Dry suspension using humid air

#### **Congestion of Chromosomes in Metaphase Spread**

- Drop suspension onto an angled slide
- Dry dropped suspension using blown air

#### **Chromosomes in Metaphase Spread Far Apart**

- Drop suspension onto a horizontal slide carefully
- Let suspension fix for an increased length of time in 3:1 methanol/acetic acid fixative for a greater length of time. Refrigerate at 4° C or -20° C overnight if necessary. Drawback is that cytoplasm of the cell may not eventually disperse in the air drying process causing a stainable material to overlay the chromosome which may affect the subsequent treatment of AluI banding.

### **A.2 Artifacts Created by Microscopy**

Resolution is defined as the ability to differentiate two points separated by a set distance and observing it as two separate points. The compound light microscopes (CLM) resolution is limited by two factors: lens quality and light source. Numerical aperture of the objective lens, immersion techniques, and the wave length of light used in the transmittance contribute to the resolving power of the microscope (Southworth, 1975;

Welford, 1984). The most critical element limiting the resolving power of the microscope is the objective lens.

The numerical aperture of the 100 X objective lens used in this work is 1.3. The wavelength of light used in the transmission is 550 nm and therefore, the theoretical maximum resolution is 0.27  $\mu$ m. The actual resolution of the microscope is also dependent on the accurate positioning of the substage condenser and a non-distorted light path from the light source to the photographic negative.

The diffraction properties of light will also affect the "sharpness" that is the edge between structures having different opacity. The shadow cast by a fully opaque object on the photographic film will not be "sharp". This is analogous of a diffused light source creating a shadow cast by an object against a wall at some distance. The distance of the transition from zero transmittance to full transmittance of the image on the photographic film is finite due to the diffraction property of light. This finite distance cast on the photographic negative is 0.3 mm and 1.4 mm on the photographic paper after the image on the film is transferred. This translates to a distance of 0.76  $\mu$ m at the level of the chromosome (see calculations in A.3). Since the largest polymorphisms (chromosome 1) in a typical sample is 1.5  $\mu$ m long, the property of light can adversely affect any attempts at quantifying chromosomal polymorphisms.

This property of light is responsible for the lack of definition between the polymorphic staining region and the surrounding chromosomal material. Thus, visually the photographic prints would appear "out of focus". If exposure of the photographic negative and photographic prints are carefully controlled (ie. consistent), these optical artifacts will not be as detrimental in quantifying chromosomal polymorphisms.

### **A.3 Light Microscopy Resolving Power**

From Welford (1984) *Optics* Oxford University Press

The maximal resolving power of the light microscope is dependent on the the wavelength of light used for illumination and the numerical aperture of the objective lens. The resolution of the microscope is defined as the ability to differentiate two points as two separate points.

$$\text{resolution} = \delta = \lambda / (\text{numerical aperture} * 2)$$

Example: an illumination source with a wavelength of 550 nanometer and an objective lens with a numerical aperture of 1.3 gives a resolving capability of 0.21 micrometers.

The analysis of the centromeric region involves analysis of a stained object which appears relatively opaque using CLM. This opaque object is similar to a straight edge impeding the passage of light. Light passing any object causes a diffraction pattern to appear in the far-away field according to the formula:

$$z = ((2/\lambda\zeta) * |h|)^{0.5}$$

z as outlined on graph

h = distance relative to z

$\lambda$  = wave length of illumination

$\zeta$  = distance from object

If  $z = 2$ ,  $\lambda = 500\text{nm}$ ,  $\zeta = 0.4826$  meters then  $h = 0.5309$  micrometers

Therefore, the total distance between 0% transmission to 130% transmission (as shown in figure 1A) when light of wavelength 500nm passes a straight edge and the shadow is casted on a screen (photographic film) a distance 0.4826 meters away, is 0.75 micrometer.

Since the image is magnified 400x before being recorded on photographic film, the 0.75 micrometer distance is amplified to 0.3 millimeter.

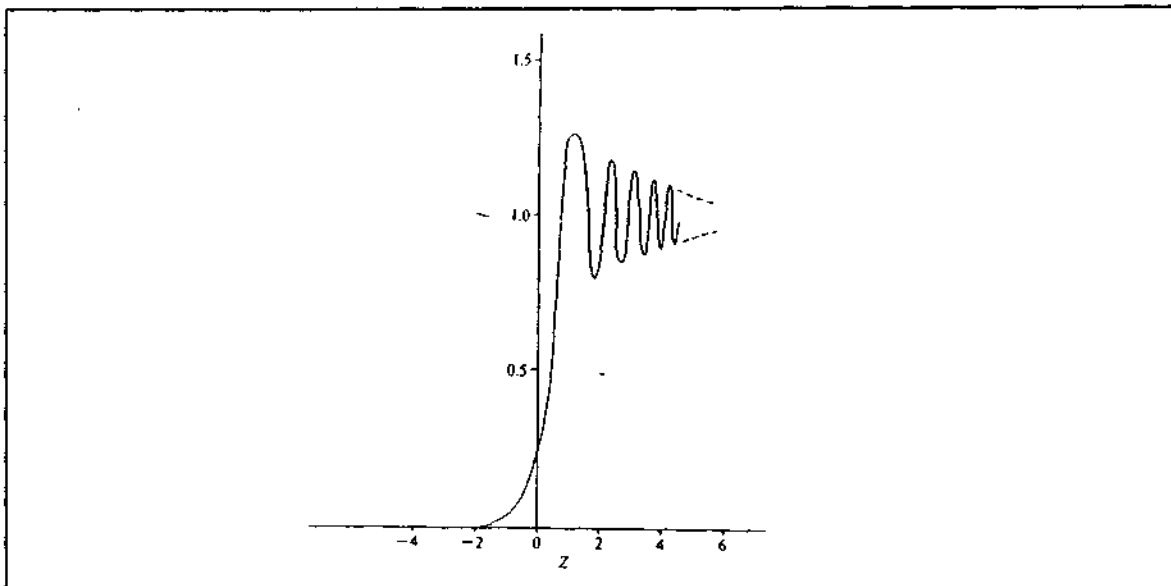


Figure 25. The near-field diffraction pattern from a straight-edge. The ordinate is the light intensity. The abscissa is the argument  $((2/\lambda\zeta) * |h|)^{0.5}$  of the Fresnel integrals. The geometrical optics shadow edge is at  $z = 0$ . Taken from Welford (1984).