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Development of Fluorescent In Situ Hybridization Techniques for Physical Mapping in *Ovis aries*

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DEVELOPMENT OF FLUORESCENT *IN SITU* HYBRIDIZATION TECHNIQUES FOR PHYSICAL MAPPING IN *OVIS ARIES*

by

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ABSTRACT

Development of Fluorescent *In Situ* Hybridization Techniques

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by

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Genome maps provide information used to identify economic trait loci and loci proximal to genes affecting economically important traits. This research contributes to the development of the ovine genome map by establishing ovine tissue culture techniques and fluorescent *in situ* hybridization techniques in this laboratory. In addition, a karyotype for sheep affected with spider lamb syndrome was developed. Ovine tissue culture techniques were established using fibroblasts grown from primary explants of sheep and modified cytogenetic procedures. Metaphase chromosomes were obtained from the fibroblast cultures and used for fluorescent *in situ* hybridization and for banding procedures to develop the spider lamb karyotype. Fluorescent *in situ* hybridization procedures were established by hybridizing a fluorescent-labeled probe to fixed ovine chromosome spreads. The probe was a bacterial

artificial chromosome clone containing the bovine 3-ß-hydroxy-5-ene steroid dehydrogenase gene. In this study, the probe hybridized to ovine chromosome 1. This chromosomal location was supported by the previous hybridization of the probe to bovine chromosome 3, which has a high level of homology with ovine chromosome 1. The final objective of this study was to determine whether a chromosomal abnormality is the cause of spider lamb syndrome, a fatal autosomal recessive genetic disease in North American black-faced breeds of sheep. A fibroblast cell line was produced from a four-week-old male homozygous spider lamb. Chromosomes were banded with trypsin using standard structural banding techniques. The lamb had a normal diploid chromosome number of 54 and a karyotype of 3 pairs of large biarmed chromosomes, 23 pairs of autosomal acrocentrics, and a large acrocentric X and a minute biarmed Y chromosome. G-band analysis of all chromosomes within the karyotype did not reveal any abnormality that was attributable to spider lamb syndrome.

(113 pages)

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The development of a genome map involves the differentiation and characterization of chromosomes within an organism and the localization of genes or specific DNA fragments on chromosomes. Concerted efforts to develop species-specific genome maps began in 1988 with the Human Genome Project (HUGO). A primary goal of HUGO is to provide genetic markers 2 centimorgans (cM) apart across the entire human genome (Collins and Galas, 1993). Mammalian geneticists have also embarked on developing genome maps for agricultural animal species. A common goal among mammalian geneticists is to provide genetic markers located 10 cM apart on domestic animal genomes. Despite recent efforts to develop genome maps for livestock species, animal genome map development lags behind the human genome project. For example, the human genome is currently characterized by 16,000 mapped genes and genetic markers (Collins and Galas, 1993). In contrast, the current bovine, porcine, and ovine genome maps have 650, 800, and 430 genes and genetic markers mapped to them, respectively (cattle: Barendse et al., 1994; Bishop et al., 1994; Georges et al., 1995; swine: Rohrer et al., 1994; sheep: Pearce and Broad, 1994). Therefore, much work remains in completing animal genome maps.

Physical mapping involves somatic cell hybridization or *in situ* hybridization techniques. Somatic cell hybridization develops cell lines

containing specific chromosomes or parts of chromosomes. Although somatic cell hybridization has been used extensively in physical mapping efforts, problems arise in identifying individual chromosomes within the cell lines (Stranzinger and Vercoe, 1988). In addition, cell lines carrying species-specific chromosomes, such as sheep, are not readily available. In contrast, *in situ* hybridization is an effective physical mapping technique. This technique involves identifying chromosomes based on a standardized karyotype, hybridizing a labeled DNA probe to its complementary sequence on a fixed metaphase spread, and visualizing the hybridization signal. Radioisotopes were originally used as labels for *in situ* hybridization, but more recent techniques use nonradioactive substances such as fluorescent-labeled nucleotides. Fluorescent probes eliminate the problems associated with radioactive probes such as safety precautions, limited shelf life, time required for autoradiography, and limited spatial resolution. Fluorescence *in situ* hybridization (FISH) reduces processing time by greater than half, improves sensitivity, and provides favorable spatial resolution (Ransom et al., 1992).

The advantages of FISH make it an attractive resource for the development of an ovine genome map. Therefore, one of the objectives of this study was to establish the procedures required for FISH, including preliminary requirements such as ovine tissue culture and chromosome preparation. The second objective of the study was to identify a positive

control for the hybridization of fluorescent-labeled probes to the ovine genome. A bacterial artificial chromosome (BAC) clone, containing the bovine 3-ß-hydroxy-5-ene steroid dehydrogenase (HSD) gene, which had previously been hybridized to bovine chromosome 3, was selected as a potential positive control (Gallagher et al., 1995).

The final objective of this study was to develop a karyotype for sheep affected with spider lamb syndrome (SLS). Spider lamb syndrome is a semi-lethal genetic disorder in North American breeds of black-faced sheep. It has surfaced in the Suffolk and Hampshire sheep breeds within the last 2 decades (Berg et al., 1987) and is believed to be caused by an autosomal recessive gene (Thomas and Cobb, 1986). The presence of the SLS gene results in severe skeletal deformities apparent at birth or that develop by 3 mo of age. These deformities include bent, abnormally long limbs, spinal curvature, and facial deformities (Rook et al., 1988). The concern of introducing the SLS gene into a producer's flock is turning sheep breeders away from the Suffolk and Hampshire breeds. Therefore, it is imperative to reduce the frequency of this gene. At present the only way to decrease the frequency of SLS is by progeny testing potential breeding animals or dramatic culling of all suspected carriers, both of which are expensive and very time consuming. Unfortunately, the identity of the SLS gene and its location within the genome is not known at this time. It is possible that the SLS defect is caused by a major mutation such as a deletion, insertion, or

translocation of a large chromosomal segment. If so, these chromosomal aberrations could be detected by karyotype analysis, providing an accurate method of detecting carrier animals. By comparing a karyotype developed from a sheep known to have the SLS mutation with the Reading standard ovine karyotype (Ford et al., 1980), gross chromosomal abnormalities would be detected. Thus, the third objective of this study was to determine whether chromosomal aberrations were associated with SLS by developing the respective karyotype.

LITERATURE REVIEW

Ovine Karyotype

Ovine cytogenetics began over 6 decades ago in an effort to determine the number of domestic sheep chromosomes. The first study to establish the ovine diploid chromosome number of 54 was by Shiwago (1931). In addition to establishing the chromosome number, Shiwago also identified three pairs of large submetacentric chromosomes within the ovine karyotype. These six chromosomes were also recognized in later studies performed by Berry (1938, 1941) and Ahmed (1940). In 1973, Evans et al. identified the ovine X and Y chromosomes as the longest acrocentric and smallest metacentric chromosomes, respectively.

As cytogenetic technology progressed, chromosome staining techniques preceded the development of banded karyotypes. During the 1970's, banded karyotypes became increasingly important for differentiating the normal from the abnormal karyotype (Hsu, 1979). To avoid discrepancies when describing individual chromosomes, it was deemed necessary to create an internationally standardized ovine karyotype. The result was an international conference held in Reading, England to standardize banded karyotypes of domestic animals. The proceedings of this conference were published by Ford et al. (1980) and accessed by researchers internationally. The principal objective of the Reading Conference was to characterize G-banded karyotypes by providing

descriptions of the main G-bands of each chromosome pair. The proceedings of the Paris Conference (1971) describing human-banded chromosomes served as a model for domestic livestock cytogenetics.

Certain descriptive terms have been used to describe chromosomes. Submetacentric chromosomes use the letters p and q to signify the short (petite) from the long (q) chromatid arms. Centromeres are reference points from which chromosomal classifications are derived. The terms proximal, central, and distal are used subjectively to describe characteristic banding patterns located near the centromere, at the center of the chromatid arm, and at the chromatid arm terminus, respectively. Chromosome bands are described as either light or dark, combined with at least one of the qualifying adjectives: narrow, faint, broad, distinct, prominent, and adjacent to.

The Reading Conference standard arranged ovine chromosomes in order of decreasing size, numbering the autosomes sequentially from 1 to 26. The chromosomes were conventionally stained following G-banding procedures. G-bands were obtained by pretreating chromosomes on slides with proteolytic enzymes. The first three autosomal pairs were large submetacentric chromosomes and the remaining 23 pairs were smaller acrocentric chromosomes. The sex chromosomes were readily differentiated from the autosomes. Chromosome X was the largest acrocentric chromosome within the ovine karyotype, and Y was a minute metacentric chromosome.

Even with the development of this standardized ovine karyotype, efforts have continued to describe and clarify differences between acrocentric chromosomes of similar size. Long (1985) presented additional nomenclature for the ovine G-band karyotype. In her study, she retained the chromosome numbering system implemented at the Reading Conference, and incorporated the system derived from human cytogenetic nomenclature for numbering chromosome regions and bands within regions. With this addition, each band was identified by its corresponding chromosome number, arm symbol, chromosome region number, and band number within that region. An example is 2p21, interpreted as a band on the short arm of chromosome two, the first band within region two. Long also suggested that banding patterns may vary in accordance with the degree of chromosome contraction or elongation. Her findings suggested an error in the previous standardized ovine karyotype. According to her study, the Reading Conference proceedings reversed the descriptions corresponding to the p and q arms of ovine chromosome 3. Long's descriptions of chromosomes 6 and 21 also differed from those presented at the Reading Conference.

The International System for Cytogenetic Nomenclature of Domestic Animals (ISCNDA) meeting in 1989 (DiBerardino et al., 1990) further supplemented the characterization of the ovine karyotype. The purpose of this second standardization conference was to update previous domestic

animal standard karyotypes by correlating G-bands and R-bands (reverse banding), subsequently establishing standard R-band karyotypes (DiBerardino et al., 1990). Because G-bands and R-bands differ in nucleotide content, both can potentially contribute to cytogenetic analyses. Positive G-bands contain high levels of adenine and thymine (Hsu, 1979). Conversely, positive R-bands are guanine and cytosine-rich (Ponce de Leon et al., 1992). By providing standard R-band karyotypes for cattle, goats, and sheep, ISCNDA hoped to universalize cytogenetic nomenclature for classifying chromosomal abnormalities and gene mapping (DiBerardino et al., 1990). ISCNDA also corrected some errors in the ovine karyotype established from the Reading Conference. Specifically, the descriptions that corresponded with chromosomes 8 and 9, and 19 and 20, had been interchanged (DiBerardino et al., 1990; Ansari et al., 1994a). To avoid further confusion, ISCNDA used only the karyotype presented by Long (1985) to establish an updated standard ovine karyotype.

ISCNDA's findings of banding pattern similarities suggested a high level of genetic interspecies homology between cattle and sheep (DiBerardino et al., 1990). The strong correlations between the two species' G-band patterns and R-band patterns created a basis on which the standard R-band ovine karyotype was inferentially derived from the cattle R-band karyotype (Ansari et al., 1994a). The R-band karyotype created by ISCNDA serves as the most recently published standard ovine karyotype, despite a

few modifications proposed by Ansari et al. (1993b, 1994a). These modifications included interchanging chromosomes 4 and 6 from the proposed R-band karyotype in ISCNDA, and resuming the Reading standard order for chromosomes 8 and 9, chromosomes 19, 20, and 21, and chromosomes 23, 24, 25, and 26. To compare banding patterns, Ansari et al. (1993b) used Robertsonian fusions and nucleolus organizer regions (NORs) as references. They also used chromosomes twice the length of the Reading standard, thus producing high resolution karyotypes with many more bands. The progress made in standardizing the ovine karyotype has been imperative for expanding the efficiency of *in situ* hybridization as a means to develop an ovine genomic map.

Gene Mapping

In addition to chromosome differentiation and characterization, a genome map comprises information on the location of genes and genetic markers with respect to each other (Hetzel, 1989). Thus, determination of gene order and the genetic/physical distance between genes and genetic markers is the objective of gene mapping. Genomic maps can be developed using linkage mapping, comparative mapping, and/or physical mapping methods.

Linkage Mapping

Linkage mapping estimates the genetic distance between loci in terms

of meiotic recombination units (Hetzel, 1989). At meiosis, crossing over or recombination takes place between homologous chromosomes, thereby altering allelic combinations. The recombination rate between two genes is closely related to the physical distance between them (Hetzel, 1989). As the distance between genes and genetic markers increases, the likelihood of the loci separated by recombination also increases (Watkins, 1988). The distance between genetic markers is measured in centimorgans (cM) and is equivalent to the physical distance of approximately one million base pairs. Two markers that are 1 cM apart segregate by recombination approximately 1% of the time.

Recombination frequency and therefore gene order can be computed from observations of the genotypes of parents and offspring. The most efficient linkage analyses are performed using large sibship families in which one parent is heterozygous for the gene of interest and the other parent is homozygous (Stranzinger and Vercoe, 1988). This type of pedigree provides reliable information on allele inheritance patterns (White et al., 1985).

The statistical significance of the recombination rate is determined using the logarithm of odds (LOD) score (Morton, 1955). A LOD score of three or greater suggests significant evidence of linkage between two loci (White et al., 1985). A LOD score of three is used as a rule-of-thumb to separate linkage from nonlinkage because the probability of linkage between two loci with a LOD score of three or greater is 1,000 times the probability

that the result is due to chance. Conversely, a LOD score of less than three rejects the probability of linkage (Ruddle and Fries, 1985). In addition, gene order can be predicted using LOD scores to evaluate the probabilities of different gene orders when at least three loci are studied (White et al., 1985).

Of more than 477 loci currently assigned to the ovine genome, approximately 68% have been mapped using family linkage studies (Pearce and Broad, 1994). The genetic markers used to map these loci are polymorphic in DNA sequences surrounding the region. One method of detecting the polymorphic sequences is the restriction fragment length polymorphism (RFLP) assay; RFLPs are highly heritable, providing reliable markers for linkage studies (Botstein et al., 1980).

Comparative Mapping

Comparative gene mapping utilizes the evolutionary conservation of syntenic groups between species (Frezal, 1987; Hetzel, 1989; Ruddle and Fries, 1985). This mapping method is contingent upon the production of high resolution chromosome bands to identify conserved interspecies chromosomal segments. The location of genes and genetic markers in one species can then be extrapolated to another species. Although comparative mapping is not conclusive, it may be used to expedite gene mapping among highly conserved species such as cattle, sheep, and goats (DiBerardino et al., 1990).

Physical Mapping

Physical mapping facilitates the identification of genes and genetic markers in relation to specific chromosome locations. By identifying genes to chromosomes and to regions on chromosomes, a physical map can provide landmarks to estimate genetic distances and define syntenic group locations. The methods used to develop physical maps are somatic cell hybridization and *in situ* hybridization.

Somatic Cell Hybridization. Somatic cell hybridization is a physical mapping method in which cell lines containing specific chromsomes or parts of chromosomes are developed (Watkins, 1988). Cell lines for ovine studies are created by hybridizing nuclei from sheep somatic cells with rodent tumor cells (Saidi-Mehtar et al., 1981; Burkin et al., 1993). To stimulate cell membrane fusion between the two cell types, cells are treated with inactivated Sendai virus or polyethylene glycol (Darnell et al., 1990). The cells are then cultured in a specific enzyme deficient medium that is selective for hybrid cell growth. As the hybrid cells grow and divide, chromosomes from the ovine genome are progressively lost until only single chromosomes or parts of chromosomes remain. This preferential chromosome retention is determined by the location of the gene, which complements the enzyme-deficient medium (Burkin et al., 1993). The rodent chromosomes within the hybrid cells remain stable. Sheep and rodent chromosomes are differentiated by species-specific staining

characteristics and sheep chromosomes are banded and characterized according to the Reading standard ovine karyotype (Burkin et al., 1993). Cell lines containing specific ovine chromosomes are then maintained. Viable sheep somatic cell hybrids were first produced in 1981 by Saidi-Mehtar et al. (1981).

By hybridizing DNA probes to panels of ovine somatic cell hybrids, chromosome localization of the probe is determined. Although somatic cell hybridization is used extensively in certain species, problems arise in using this technique for ovine genome development because sheep cell hybrids are not readily available. In addition, individual ovine chromosomes are difficult to identify when segregated from other ovine chromosomes of similar size (Stranzinger and Vercoe, 1988). Somatic cell hybrids have been used to assign 13% of the genes and genetic markers to the sheep genome map (Pearce and Broad, 1994).

In Situ Hybridization. *In situ* hybridization is the localization of labeled nucleotide sequences (called probes) to complementary regions on fixed metaphase chromosomes. This technique was introduced by Pardue and Gall in 1969. Using *Xenopus* (toad) oocytes, they successfully hybridized tritium-labeled DNA to fixed cytological preparations. DNA binding sites were detected autoradiographically using photographic emulsifying and developing procedures. Also at this time, John et al. (1969) hybridized ^{3}H-labeled RNA to fixed HeLa cell nuclei. Autoradiography was used to

visualize silver grains in specific locations within the nuclei. The grains represented RNA-DNA hybrid formation and suggested base complementarity between labeled probe nucleotide sequences and DNA (John et al., 1969). In 1974, Evans et al. verified specific binding by observing specific chromosome regions with heavily distributed silver grains. Chromosome regions with low grain yields were proposed as regions of nonspecific probe binding. Later molecular hybridization studies revealed that by increasing the autoradiographic exposure time, specific and nonspecific binding increased (Harper et al., 1981). However, because there were a limited number of specific hybridization sites, the clarity of the labeled probe site decreased with longer exposure times (Harper et al., 1981). This occurrence is described as a decrease in the signal-to-noise ratio. In developing this concept, Gerhard et al. (1981) labeled DNA sequences with the radioisotope ^{125}I using nick translation and hybridized different concentrations of the labeled probe to fixed chromosome spreads. Their work concluded that the optimum probe concentration varied with the size of the probe. In addition, the number of silver grains visualized at the specific hybridization site was directly proportional to probe size (Gerhard et al., 1981). To explain the occurrence of background signals, Gerhard and associates postulated that the probes contained repetitive nucleotide sequences yielding nonspecific binding sites.

High signal-to-noise ratios limit the potential of using *in situ*

hybridization for gene mapping (Lawrence et al., 1988). Another disadvantage of using radiolabeled probes includes the time needed to autoradiographically expose the hybridized chromosome spreads. These times vary from several days to several weeks (Gerhard et al., 1981). In addition to these limitations, probes labeled with radioisotopes provide low resolution when referenced to specific chromosome locations. This limited resolution is a consequence of the radioactive disintegrations which scatter from the specific hybridization site (Lawrence et al., 1988). Statistical analysis of 50-100 chromosome spreads (Lawrence et al., 1988, 1990) is also required when using radioisotopes in chromosomal localization studies. These limitations prompted efforts to develop alternative probe labeling methods and visualization techniques.

One of the first methods to replace radioisotope labeling of nucleotide probes used antibodies tagged with a fluorescent molecule (Rudkin and Stollar, 1977). This technique was developed by preparing antisera in rabbits against sequences rich in A and T nucleotides. Cytological preparations were exposed to the rabbit antiserum and then to a specific anti-rabbit antibody that had been prepared in goats. The antibody was tagged with rhodamine and visualized using fluorescent microscopy. Rudkin and Stollar (1977) demonstrated the specificity of the immunological reagents by identifying the chromosomal localization of the fluorescent signal.

Due to the limited availability of specific anti-RNA-DNA antibodies, researchers developed additional labeling methods. Chemically modified DNA probes were introduced in 1975 when biotin was covalently coupled with an RNA probe via polyamine bridges (Manning et al., 1975). Avidin, a large glycoprotein with a high binding specificity for biotin, was labeled with ferritin or polymethacrylate spheres and sequentially incubated with chromosomes hybridized with the biotin-labeled probe. The large biotin-avidin complex was visualized with scanning electron microscopy. In 1981, Langer et al. simplified the biotin labeling procedure by using nick translation to incorporate biotinylated dUTP directly into a DNA probe. The biotin-labeled polynucleotides were chemically stable and hybridized specifically to complementary nucleotide sequences (Langer et al., 1981). Another indirect labeling method replaced avidin with an antibiotin antibody conjugated to FITC (fluorescein isothiocyanate) or horseradish peroxidase (Langer-Safer et al., 1982). The signals were visualized with fluorescent and light microscopy, respectively. This study reported significantly less nonspecific background than was evident when using autoradiographic detection.

Simultaneous to indirect immunofluorescent studies, endeavors were made to directly label RNA and DNA sequences with fluorescence. Bauman et al., 1980, 1981) initiated this work by covalently binding the fluorescent molecule TRITC (tetramethyl rhodamine isothiocyanate) to the probe sequence. The fluorochrome-labeled probes were then hybridized to

denatured DNA and the red fluorescent signal was visualized microscopically using excitation and emission filters specific for rhodamine. Significant advantages in spatial resolution of the signal and time-conservative procedures made fluorescent *in situ* hybridization attractive for cytogenetic studies.

With the advances in labeling techniques, fluorochrome-labeled probes containing repetitive sequences could be hybridized to complementary DNA sequences and visualized microscopically. However, to visualize most single-copy genes following *in situ* hybridization, autoradiographic detection was required. The rare exception to this limitation were very large unique sequences (25-50 kb) (Lawrence, 1990). Landegent et al. (1985) reported detection of a large single gene after hybridizing a labeled probe containing the unique genomic sequence to human chromosomes. The probe was labeled with AAF (2-acetylaminofluorene) and detected using anti-AAF antibodies in conjunction with reflection-contrast microscopy. Although this method accurately validated the chromosomal location of the gene, a significant limitation was observed because several loci sites showed no hybridization signal (Landegent et al., 1985). Consequently, this single-copy gene mapping procedure was not pursued.

The challenge posed by single-copy gene detection on the efficiency of fluorescence *in situ* hybridization is gradually being overcome. By using

high concentrations of labeled probe DNA, the intensity of the fluorescent signal is increased (Lawrence et al., 1988). Increasing probe concentrations allows the probe to effectively compete with the reannealing chromosomal DNA. In addition, a number of detector molecules have been tested for optimal fluorescent intensity. Among these are rhodamine-conjugated avidin, fluorescein-conjugated avidin, Texas red-conjugated streptavidin, and immunofluorescent detection of antibodies conjugated to biotin. Based on a study performed by Lawrence et al. (1988), fluorescein-conjugated avidin consistently produced the most intense signal with the least background. Since 1988, the fluorescein-conjugated avidin detection system has continued to be refined. Currently, this technique provides a single step detection system that can be used for biotin-labeled probes and digoxigenin-labeled probes (Lawrence et al., 1990). Also, recent advances in detection techniques have enabled visualization of smaller probes and weak signals (Lawrence, 1990). Improved detection techniques include computer-generated image visualization and enhancement.

In developing species-specific gene maps, increasing numbers of probes are being hybridized and detected by fluorescent techniques. Fluorescence *in situ* hybridization, in comparison with autoradiographic techniques, provides some distinct advantages. These advantages include significantly decreased detection time, disposal of radioactive products, lower background noise, increased hybridization efficiency, and increased

probe resolution on specific chromosomes. To date, *in situ* hybridization has facilitated the assignment of 19% of the genes and genetic markers on the sheep genome map (Pearce and Broad, 1994). Table 1 provides specific loci that have been mapped to the ovine genome using *in situ* hybridization. In total, 69 loci have been mapped to specific ovine chromosomes using *in situ* hybridization. Thus, the *in situ* hybridization technique is a powerful method for enhancing the ovine genome map.

Table 1. Loci mapped to the sheep genome by *in situ* hybridization

Gene or marker name	Chromosome	First author of reference
Acetylcholine receptor, muscle, beta 1 subunit	11q14-q22	Pearce et al. (1993)
Acetylcholine receptor, muscle, delta subunit	2q42-qter	Ansari et al. (1994b)
Alpha 2 macroglobulin	3q26-q35	Graphodatsky et al. (1993)
Amelogenin, X-linked	X	Shaw et al. (1993)
Amelogenin, Y-linked	Y	Shaw et al. (1993)
Casein, alpha S2	6q32	Hayes et al. (1993a)
Casein, beta	6q22-q31	Hayes et al. (1992)
CD3 antigen, zeta polypeptide	1p14-p11	Broad et al. (1994a)
cGMP rod phosphodiesterase, alpha	5q31-q33	Pearce et al. (1994)
cGMP rod phosphodiesterase, beta	6q33-qter	Broad et al. (1994b)

(table continues)

Coagulation factor VIII	Xq24-q33	Backfisch et al. (1994)
Coagulation factor 10	10q33-qter	Pearce et al. (1994)
Collagen alpha 1, type III	2q12-q14	Ansari et al. (1994b)
Corticotropin releasing hormone	9q23-q28	Broad et al. (1995)
Cytochrome P450, Subfamily XVII	22q21-q23	Broad et al. (1992)
Cytokeratin, type I, acidic (KRTA)	11q25-q29	Hediger et al. (1991a)
Cytokeratin, type II, basic (KRTB)	3q14-q22	Hediger et al. (1991a)
DNA segment BRY.1	Y	Matthews and Reed (1991)
DNA segment cos1	11qter	Monteagudo et al. (1992)
DNA segment CSRD1613	1q36-qter	Drinkwater et al. (1993)
DNA segment GMBT6	9q13-q17	Gunawardana (1991)
DNA segment GMBT11	17q23-qter	Gunawardana (1991)
DNA segment GMBT11	22q11-q21	Gunawardana (1991)
DNA segment GMBT16	18q24	Georges et al. (1991)
DNA segment GMBT19	7q15-q23	Georges et al. (1991)
DNA segment GMBT22	11q17-qter	Georges et al. (1991)
DNA segment JMP8	6q34-q35	Pearce et al. (1994)
DNA segment OY1.1	Y	Jorgensen et al. (1992)
DNA segment OY1.11	Y	Jorgensen et al. (1992)
DNA segment RP11	10q24-q32	Crawford et al. (1994)
Elastin	24q16-qter	Broad et al. (1992)

(table continues)

Esterase D	3p34-p22	Graphodatsky et al. (1993)
Estrogen receptor	8q25-q27	Pearce et al. (1994)
Fibrinogen, gamma	17q12-q13	Johnson et al. (1993)
Fibroblast growth factor 2	17q23-q25	Pearce et al. (1994)
Fibronectin 1	2q41-q44	Ansari et al. (1994b)
Follicle-stimulating hormone, beta	15q24-qter	Hediger et al. (1991b)
Gelsolin	2pter-p24	Ansari et al. (1993a)
Glucose phosphate isomerase	14q22-q24	Chowdhary et al. (1991)
Growth hormone I	11q25-qter	Hediger et al. (1990)
Hexosaminidase A, alpha polypeptide	7q13-q22	Pearce et al. (1994)
Immunoglobulin M-like	3p23	Hayes and Petit (1993)
Inhibin, beta A	4q26-q31	Ansari et al. (1993a)
Insulin-like growth factor 2	21q21-qter	Ansari et al. (1993a)
Interferon, omega	2p15	Iannuzzi et al. (1993)
Interferon, trophoblast	2p15	Iannuzzi et al. (1993)
Interleukin receptor A	13q12-q15	Ansari et al. (1993a)
Lactalbumin, alpha	3q21	Hayes et al. (1993b)
Lactoglobulin, beta	3p28	Hayes and Petit (1993)
Lactoperoxidase	11q13	Hayes et al. (1993b)
Laminin, gamma I	12q22-q24	Pearce et al. (1994)
Myelin basic protein	23q11-q12.3	Broad et al. (1994a)
Nerve growth factor, beta	1p24-p21	Broad et al. (1994a)
Neurotensin	3q12-q14	Wood et al. (1993)
Nucleoside phosphorylase	7	Burkin et al. (1993)

(table continues)

Ovine lymphocyte antigen	20q15-q23	Hediger et al. (1991a)
Protein kinase C, beta 1 polypeptide	24q13-q18	Ansari et al. (1993a)
Retinoblastoma 1	10q13	Hayes et al. (1993b)
Rhodopsin	19q23-qter	Crawford et al. (1994)
Ribosomal DNA (RNR1)	1pter	Moreno-Millan and Rodero-Franganillo (1990)
Ribosomal DNA (RNR3)	3qter	Broad et al. (1993)
Ribosomal DNA (RNR5)	25qter	Ansari et al. (1992)
Ribosomal protein 4, X-linked	X	Shaw et al. (1993)
Steroid sulphatase	X	Shaw et al. (1993)
Tachykinin 2 receptor	25q14-q22	Broad et al. (1994a)
Transferrin	1q42-q45	Burkin et al. (1993)
Ubiquitin activating enzyme	X	Shaw et al. (1993)

MATERIALS AND METHODS

Tissue Culture

Initially, a one half thickness of epidermal tissue was excised from the axillary region of a sheep. The tissue biopsy was performed using a sterile technique with a scalpel blade and forceps. Immediately following excision, the tissue was placed in RPMI 1640 medium (Gibco BRL) supplemented with 16% fetal bovine serum (Hyclone Laboratories) and 25 μL (one drop) of antibiotic-antimycotic solution (Gibco BRL) was added. The tissue was left in the culture medium at room temperature for 2 h to check for contamination. The tissue was then incubated for an additional 3 h in fresh tissue culture medium and homogenized with a tissue homogenizer. The resulting cell suspension was poured into a 25-cm^2 tissue culture flask and additional medium was added to completely cover the bottom of the flask. RPMI medium 1640 supplemented with 16% fetal bovine serum, 1% penicillin streptomycin (Gibco BRL), and 1% L-glutamine (Gibco BRL) was used to culture the cells. The flask was gassed with 9.98% CO_2 for approximately 20 s and then incubated at 38°C. The flask was left undisturbed for 3 d and then viewed microscopically with a 4X objective to detect cell growth. Medium was changed every 5 to 7 d to stimulate cell proliferation. A monolayer of fibroblasts developed within 2 or 3 wk. At cell confluency, medium was removed from the flask and replaced with 0.25% trypsin (Hyclone Laboratories). The cells were incubated at 38°C for

an additional 5 to 10 min until most cells had "rounded up" on the bottom of the flask. The cells were detached by tapping on a corner of the flask. The trypsin was then inactivated by adding approximately 7 mL of fresh medium to the flask. Equal amounts of the flask contents were divided between the original flask and a new 25-cm^2 flask. The flasks were gassed and left at 38°C. Culture medium was changed regularly until the cells grew to confluency, at which time one of the flasks was passed into four additional flasks. Following a 24-h incubation period, cell attachment was confirmed and culture medium was replaced with 5 mL of fresh medium and fluorouracil (Sigma; 260 ng/mL). Incubation continued for another 17 to 18 h. The synchronization block was then released by rinsing the cells thoroughly with Hank's Balanced Salt Solution (HBSS) (Hyclone Laboratories). Fresh medium supplemented with 5-bromo-2'-deoxyuridine (BrdU) (Sigma; 92.5 μg/mL), a nucleotide analogue to the nucleotide thymine, was added to the culture and incubated for 5 to 6 h. Following the BrdU incubation period, colcemid (Gibco BRL; 40 ng/mL) and ethidium bromide (Sigma; 20 μg/mL) were added to the culture medium for an additional 30 min. Cells were then harvested and chromosome spreads prepared using standard cytogenetic techniques as described by Verma and Babu (1995).

Fluorescent In Situ Hybridization

Nick translation was used to introduce a fluorescently-labeled

nucleotide (biotin-21-dUTP) into the DNA probe according to manufacturer's directions (Biotin-21-dUTP Nick Translation Kit, Clontech Laboratories, Palo Alto, CA). Immediately prior to hybridization, chromosomes on prewarmed microscope slides were denatured by immersing in 70% formamide/2X SSC at 74°C for 2 min, dehydrated in a 70%-, 80%-, and 100%-ice cold ethanol series and allowed to air dry at room temperature. The labeled probe was also denatured in a 74°C water bath for 5 min and spotted onto the denatured chromosomes. Hybridization was performed overnight in a 37°C humidified chamber. Slides were washed in three changes of 50% formamide/2X SSC at 45°C for 5 min each, followed by three washes in 2X SSC at 45°C for 5 min each. To detect the probe, slides were first incubated at 37°C with blocking buffer for 30 min and then incubated at 37°C with fluorescein-labeled avidin (Oncor) for 20 min. Slides were washed three times in 4X SSC/0.1% Tween 20 at 45°C for 5 min each. The detection steps were repeated three times as described, with the following two alterations. Slides were incubated with blocking buffer for 5 min at room temperature during the second and third detection rounds and fluorescein-labeled avidin was replaced with biotinylated antiavidin in the second detection round. Chromosomes were counterstained with propidium iodide (Sigma; 200 ng/mL) and overlaid with *p*-phenylenediamine antifade (pH 11) (Sigma) (Lemieux et al., 1992; Ponce de Leon, personal communication). By using a fluorescent microscope with a propidium iodide

filter, suitable chromosome spreads were first located with a 10X objective. The hybridized probe was visualized with a 100X objective and a Hi-Q FITC longwave pass band filter. Photomicrographs of selected chromosome spreads in which probe hybridized to a single chromosome pair were taken using a camera attached directly to the fluorescent microscope. The camera ASA was set at 800 and Fuji slide film (ASA 400) was used. The camera control panel was set on manual exposure and film was exposed to the image for varying exposure times, i.e., 0.5 s, 1 s, 2 s, 4 s, and 8 s.

Karyotyping

To develop a spider lamb karyotype, tissue was collected from a 4-wk-old male homozygous spider lamb and fibroblasts were cultured *in vitro* following the previously described procedures. Because the chromosomes were to be banded using structural banding techniques, the addition of fluorouracil and BrdU to the cell culture was omitted. Colcemid (40 ng/mL) and ethidium bromide (20 μg/mL) were added to the culture, cells were harvested, and chromosome spreads prepared using standard cytogenetic techniques as described by Verma and Babu (1995). Microscope slides containing several metaphase spreads were incubated at 38.5°C for 5 d. Slides were immersed in 2% Enzar trypsin solution (pH 7.0) (Intergen Co.) for 10-50 s. The trypsin exposure time varied between cell preparations and was determined empirically. The enzymatic activity of trypsin was blocked by briefly rinsing the slides in 70% ethanol. Banded chromosomes were

stained with 2% Giemsa (Sigma) and visualized through a light microscope at 1000X. Selected chromosome spreads were photographed with black and white Kodak 100 speed film. The camera speed was set at 400 ASA and the film was exposed using the automatic exposure settings '-1' and '0'. To make the karyotype, photographs were enlarged using standard procedures. The karyotype was compared to the Reading standard G-band ovine karyotype (Ford et al., 1980).

RESULTS AND DISCUSSION

Tissue Culture

Blood cultures are frequently used in cytogenetic studies because they are easy to obtain and require little attention following collection (Verma and Babu, 1995). However, blood cultures do not provide a continuous cell source without multiple collections. Conversely, fibroblast cultures require some additional effort to collect and establish *in vitro* but provide a continual source of cells for cytogenetic studies. In this study, fibroblasts were successfully cultured and provided adequate chromosome spreads for fluorescent *in situ* hybridization (FISH) procedures and karyotype development.

The steps and techniques provided in the materials and methods section have been adapted from standard cytogenetic techniques (Verma and Babu, 1989, 1995). Several adaptations were made to original cytogenetic procedures for improvement of cell culture efficiency and to provide quality chromosome spreads for FISH and banding studies. One of these modifications was implemented by using a tissue homogenizer to separate individual cells from the epidermal tissue sample. In the original procedure, the sample was collected from the axillary region on a sheep and incubated for 2 to 3 h in tissue culture medium supplemented with 16% fetal bovine serum and an antibiotic antimycotic solution to rinse possible contaminants from the tissue. The culture medium was then replaced with

medium supplemented only with fetal bovine serum and incubation continued for 4 to 5 d. The tissue sample was minced into a paste by simultaneously cutting across the sample in opposite directions with two sterile scalpel blades. The paste was then spread across the bottom of a tissue culture flask and medium was carefully layered on top. The culture was continued by gassing the contents of the culture flask with 9.98% CO_2 and incubating at 38.5°C. The more recent method used in this study followed original procedures to collect the tissue sample. However, tissue incubation time in medium supplemented with serum and an antibiotic antimycotic solution was shortened from 2 to 3 h to 1 h. The 4 to 5 d incubation in antibiotic antimycotic-free medium was shortened to 2 to 3 h, followed by pouring the tissue sample and culture medium into a tissue homogenizer bag. The tissue was homogenized for 60 to 90 s. After homogenization, the medium appeared cloudy, which was attributed to the fibroblasts separating from the tissue sample and being dispersed in the medium. The tissue was also smaller and looked white instead of pink because of connective tissue which did not homogenize. The cloudy medium was then poured into a 25-cm^2 tissue culture flask and standard cytogenetic procedures were resumed by gassing and incubating the flask contents. Compared with the original method, the tissue homogenizing method is faster and less tedious. Cell cultures that had been prepared using a tissue homogenizer also became established more quickly than the

other cell cultures.

As cells attached to the plastic surface of the culture flask and began to proliferate, tissue culture medium was replaced every 7 to 10 d. When the flasks reached cell confluency, cells were detached from the flask surface with 0.25% trypsin, diluted with 20 to 25 mL of fresh medium, and passed into several new culture flasks. Typically, confluent cell cultures were passed into five 25-cm^2 culture flasks or a ratio of 1:5. However, cell lines that grew exceptionally fast were passed 1:6 and cell lines with a slow cell proliferation rate were passed 1:4. Thus, the proportion of cells passed into new flasks was dependent on the rate of cell proliferation and was determined empirically for individual cell cultures.

Besides inherent differences in proliferation rates of individual cell lines, other factors were found to influence cell proliferation rates. One critical factor was the concentration of serum used to supplement the RPMI medium. Tissue culture medium was prepared using two different serum concentrations, 11.26% and 16%. Each concentration of serum supplemented medium was added to different culture flasks containing cells from the same cell line. Cells incubated in medium supplemented with 16% fetal bovine serum consistently proliferated faster than cells incubated in 11.26% serum supplemented medium. Another factor that affected cell proliferation rate was the type of serum used to supplement the tissue culture medium. In this study, two types of serum were used, fetal bovine

serum and Fetal Clone III (Hyclone Laboratories, Logan, UT). Fetal Clone III is a serum that was recently developed to replace the cost prohibitive fetal bovine serum. Separate preparations of medium supplemented with 16% fetal bovine serum and medium supplemented with 16% Fetal Clone III were added to different flasks containing cells from the same ovine cell line. Cells were grown to confluency and passed into new flasks with the respective serum-supplemented medium. Cell proliferation rates between the two cultures were compared through four generations of cell growth. Cells incubated in fetal bovine serum-supplemented medium proliferated faster than cells in the Fetal Clone III-supplemented medium. This study concluded that medium supplemented with 16% fetal bovine serum provided faster cell proliferation rates than did medium supplemented with 11.26% fetal bovine serum and medium supplemented with 16% Fetal Clone III.

Cell proliferation rates were maintained by replacing the tissue culture medium 24 h after passing the cells. Immediately prior to passing, cells were exposed to 0.25% trypsin. Trypsin is a proteolytic enzyme that causes fibroblasts to detach from the plastic surface of the flask. Exposing cells to trypsin can be deleterious and cause irreversible cell damage to some cells. By replacing the culture medium 24 h after trypsin treatment, damaged cells were removed and the growth rate of healthy cells increased. When culture medium was not replaced until 5 to 7 d after passing, cells exhibited a slower proliferation rate. Cell proliferation rates were also

optimized by adjusting the pH of the medium to approximately 7.0. This was done by gassing the contents of the flask with 9.98% CO_2 gas for 15 to 20 sec. Within 10 to 15 min following gassing, the culture medium turned from dark pink to a pale orange-pink color, indicating a pH change. If the cells were not gassed when the medium was dark pink, cell growth was drastically inhibited. The cell proliferation rate seemed best when an orange-pink medium color was maintained.

In addition to cell-culturing modifications, some modifications were incorporated in cell-harvesting procedures. To synchronize mitotically active cells at the DNA synthesis phase, traditional cytogenetic methods add methotrexate to the tissue culture medium for 17 to 18 h. Initially in this study, concentrations of methotrexate between 0.45 ng/mL and 500 ng/mL were used to synchronize ovine cells for chromosome banding. However, an inadequate number of chromosome spreads were obtained when methotrexate was added to the cell culture. In later studies, methotrexate was replaced with 260-ng/mL fluorouracil as described by Verma and Babu (1995). Cells were effectively synchronized and adequate numbers of chromosome spreads were observed. Some dynamic R-banded chromosomes that were obtained from cells synchronized with fluorouracil and banded by BrdU incorporation and exposure to UV light are shown in Figure 1. Thus, incubation of cells in fluorouracil replaced methotrexate for obtaining dynamic banded chromosomes.

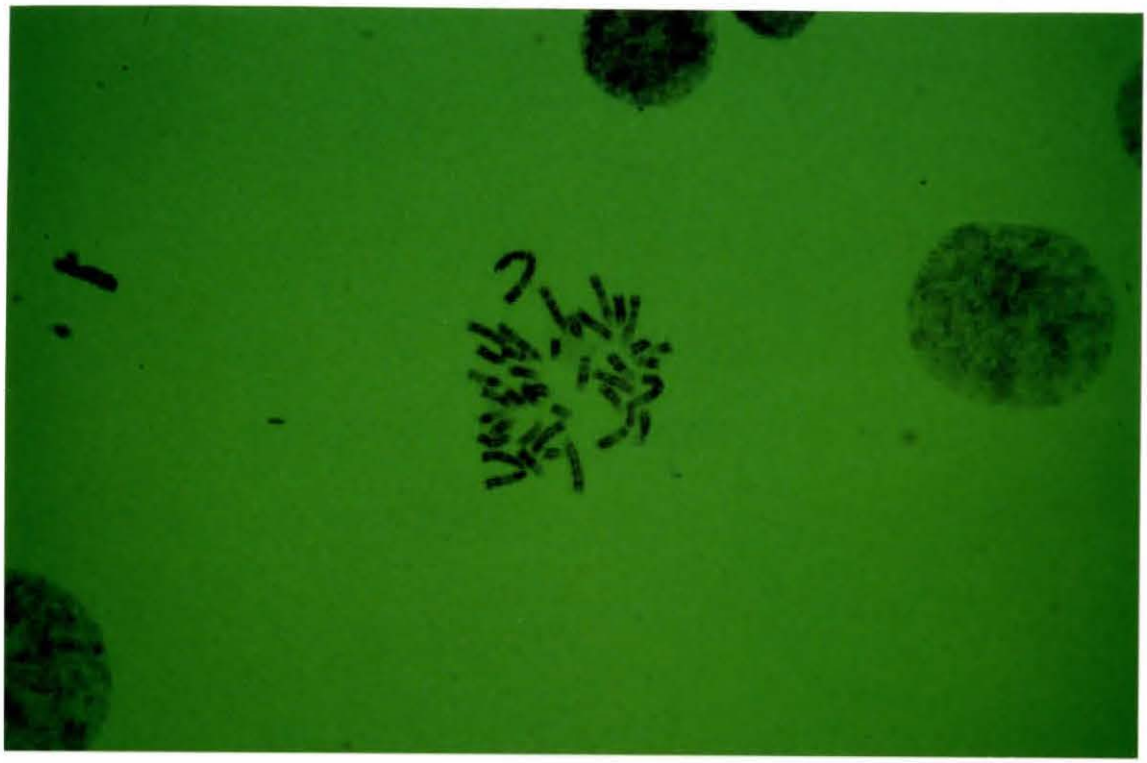

Figure 1. A photomicrograph of dynamic R-banded ovine chromosomes. (1000X)

When chromosomes are identified using banding patterns, it is essential that chromosome length is sufficient to contain an informative number of bands. To obtain chromosome elongation, ethidium bromide was added to the cell culture 30 min prior to harvesting. The optimum concentration of ethidium bromide was 0.02 mg/mL, determined empirically (0.01 mg/mL to 0.03 mg/mL). Lower concentrations of ethidium bromide (0.01 mg/mL) resulted in short chromosomes with few bands, and higher ethidium bromide concentrations (0.03 mg/mL) created extremely long, overlapping chromosomes. Colcemid was added (at a standard

concentration of 40 ng/mL) to cell cultures simultaneous with ethidium bromide to increase the mitotic index.

For cytogenetic studies, it is important that when cells are splashed onto microscope slides, the chromosomes from a single cell do not overlap. Chromosomes that are inadequately spread create difficulty in identifying and characterizing individual chromosomes and chromosome bands. To decrease chromosome overlap, the length of time and temperature at which cells are exposed to hypotonic solution can be modified. Hypotonic solution causes the cells to swell. Consequently, the chromosomes within the cells spread so when the cells are dropped onto a microscope slide and the cell membranes rupture, the chromosomes will adequately spread. Typically, cytogenetic procedures recommend using hypotonic solution at room temperature for 20 min (Verma and Babu, 1995). If cells are exposed to hypotonic solution too long and(or) at warm temperatures, the cells will prematurely burst in suspension and few chromosome spreads will be observed on the microscope slide. In this study, some ovine cell suspensions were treated with hypotonic solution at room temperature for times of 20 to 50 min. Others were treated with hypotonic solution at 37°C for times of 20 to 50 min. After the cell suspensions had been washed in fixative and splashed onto cold microscope slides, each slide was examined for the ratio of chromosome spreads to the number of cells. The slides with the highest ratio of chromosome spreads were from cell

suspensions that had been treated with hypotonic solution at 37°C for 40 min. Also, compared with other hypotonic treatments, fewer chromosomes surrounded by cytoplasm were observed from cell suspensions treated with hypotonic solution at 37°C for 40 min. Cytoplasm that surrounds the chromosomes (cytoplasmic residues) can interfere with trypsin activity in obtaining highly resolved chromosome bands and with hybridization efficiency of a labeled probe to the chromosomes (Verma and Babu, 1989).

Following hypotonic treatment, cell suspensions were washed twice in fresh fixative and a test slide was prepared from the cell suspension. The necessity of additional washes was determined by the amount of cellular debris on the test slide. If debris interfered with the visualization of several chromosome spreads, one to two additional washes were performed. Slides were prepared following standard cytogenetic techniques (Verma and Babu, 1989, 1995) or stored at -20°C to preserve chromosomes for FISH studies. When tissue culture and cell harvesting methods were followed as described, an average of three or four chromosome spreads was visible in a low power microscope field.

Fluorescent In Situ Hybridization

Fluorescent *in situ* hybridization (FISH) utilizes cell culture techniques to produce quality chromosomes on which unique DNA sequences, or probes, are hybridized. FISH techniques were established using procedures received from Clontech and Oncor Laboratories and personal communication

with Dr. Dan Gallagher and Dr. Abel Ponce de Leon.

Prior to hybridization, chromosome spreads were prepared on microscope slides and aged to maintain chromosome morphology throughout the FISH procedure. Aging techniques included storing the slides at room temperature for 2 wk, storing them at 38.5°C for 2 to 3 d, incubating the slides in 2X SSC at 37°C for 1 h, incubating the slides at 65°C for 1 h, or combinations of these techniques. The optimum aging technique was determined empirically by varying the aging techniques among several slides containing chromosome spreads. Following FISH, all slides were observed microscopically for the quality of chromosome morphology. Chromosomes that were not adequately aged appeared fuzzy and were paler than chromosomes that had been adequately aged. Based on the sharpness and high resolution of chromosomes that had been aged at room temperature for 2 wk and further aged by incubation in 2X SSC at 37°C for 1 h, this aging technique was selected to prepare ovine chromosomes for FISH.

After chromosome spreads were aged, they were viewed under a phase contrast microscope. Microscope slides were selected that contained several chromosome spreads in which the chromosomes appeared grey-black. This characteristic increased the likelihood that chromosome morphology would be maintained throughout the FISH procedure. Chromosomes that appeared glassy or pale grey were not used for FISH due to a potential loss of morphology during the FISH procedure. This loss of

chromosome morphology would also adversely affect the hybridization efficiency of the labeled probe. Chromosomes were then dehydrated by immersing the selected slides in increasing concentrations of ice cold ethanol (70%, 80%, and 100%). Dehydration with ethanol hardens the chromosomes, making them more resistant against loss of morphology during the denaturing technique. Chromosomal DNA is denatured, or separated into single strands, to allow the denatured probe to hybridize with its complementary sequence on the chromosomes. The chromosomes were denatured by immersing the microscope slides in a preheated solution of 70% formamide. The temperature was maintained at 74°C and the chromosomes were left in the denaturation solution for 2 min. To ensure that chromosome morphology was preserved during denaturation, chromosome spreads were again viewed under a phase contrast microscope. Maintaining chromosome morphology is essential to obtain adequate results from FISH.

The probe used to develop FISH techniques in this laboratory was 3-ß-hydroxy-5-ene steroid dehydrogenase (HSD), a bovine BAC clone that had been previously hybridized to bovine chromosome 3 (BTA 3). The probe was donated by Dan Gallagher at Clontech Laboratories, where it had been labeled using Clontech's Biotin-21-dUTP Nick Translation Kit. Salmon sperm DNA was added to the labeled probe solution. Salmon sperm DNA is used as carrier DNA to protect the labeled probe DNA from being lost during the

probe preparation procedures. Prior to adding the salmon sperm DNA to the labeled probe solution, the salmon sperm DNA was denatured by dissolving it in NaOH and boiling it for 45 min. The salmon sperm DNA was then mechanically sheared into DNA fragments between 100 and 500 bp long by forcing it through decreasing sizes of hypodermic needles.

The efficacy of using bovine competitor DNA (COT-1 DNA) to suppress hybridization of HSD with repetitive sequences on ovine chromosomes was tested by adding bovine COT-1 DNA to specific probe solutions. Figure 2 depicts an ovine chromosome spread that was hybridized with HSD in the presence of bovine COT-1 DNA. The competitor DNA effectively prevented nonspecific hybridization of the fluorescently labeled HSD probe. Specific hybridization of the HSD probe is evident by the two pairs of fluorescent yellow signals being emitted from both chromatids of a single large submetacentric chromosome pair. When compared with Figure 3, in which the labeled probe solution was not supplemented with competitor DNA, it is apparent that bovine COT-1 DNA is essential to suppress nonspecific hybridization of the labeled probe. Therefore, to provide quality FISH results, the probe solution contained biotin-labeled HSD probe DNA (75 ng), sheared salmon sperm DNA (50 μg), and competitor (COT-1) DNA (2 μg).

The DNA probe solution was denatured for 5 min at 74°C and spotted onto prepared microscope slides containing denatured

Figure 2. A photomicrogragh of ovine chromosomes hybridized to bovine HSD: with competitor DNA. (1000X)

chromosomes. During incubation at 37°C for 16 h, the labeled HSD probe hybridized to its complementary sequence on the chromosomal DNA and the COT-1 DNA hybridized to repetitive chromosomal DNA sequences. Following hybridization, the microscope slides were washed in 2X SSC at 45°C to remove excess probe solution and prepare the hybridized chromosomes for the detection procedure. To detect the chromosomal location of the hybridized HSD probe, slides were spotted with blocking buffer and incubated at 37°C for 30 min. Blocking buffer minimizes nonspecific binding of the detection reagents. Nonspecific binding inhibits

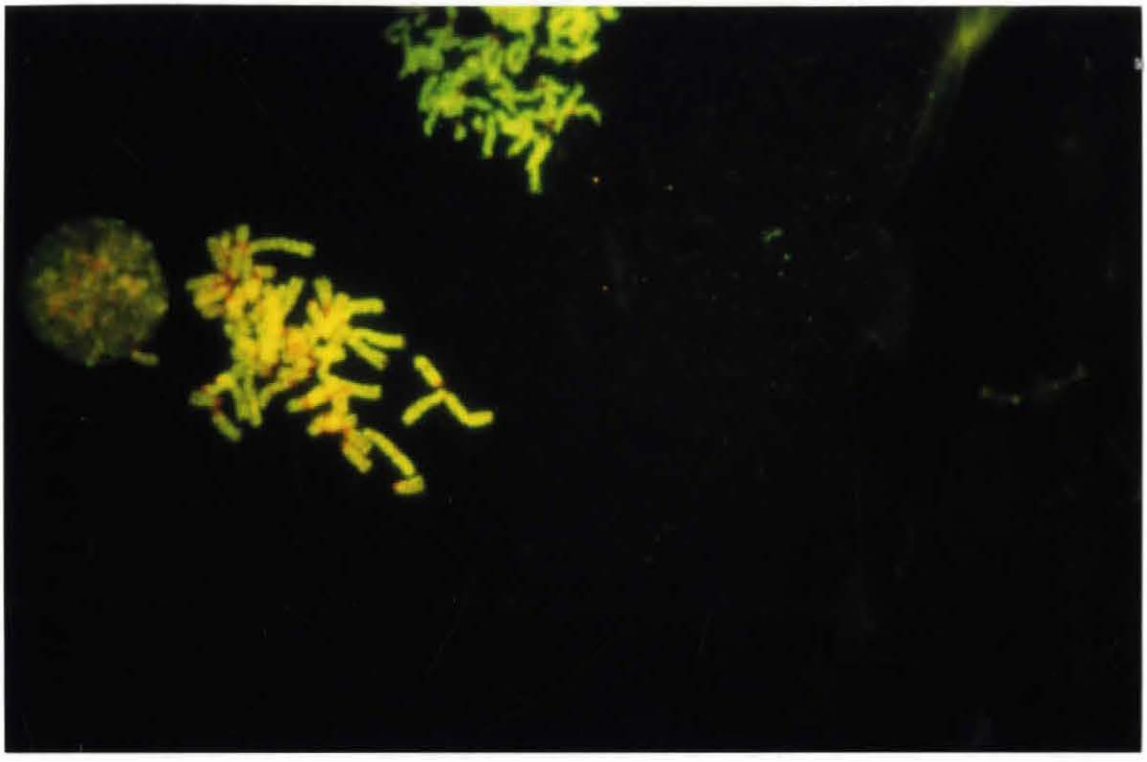

Figure 3. A photomicrograph of ovine chromosomes hybridized to bovine HSD: without competitor DNA. (1000X)

the visualization of the fluorescent signal emitted from the labeled probe. The detection reagent used in this procedure was fluorescein-labeled avidin. Avidin has a strong affinity for biotin. Therefore, by spotting the slide with fluorescein-labeled avidin and incubating at 37°C for 20 min, the avidin binds to the hybridized biotin-labeled probe. After chromosome spreads are stained, fluorescein-labeled avidin emits a fluorescent signal, indicating the chromosomal location of the hybridized probe.

If the fluorescent signal emitted from the fluorescein-labeled avidin molecule is pale or too weak to visualize, the signal must be amplified. When amplification of the fluorescent signal is necessary, it is performed following the detection procedure. The signal amplification procedure used in this study included spotting the microscope slide with blocking buffer to minimize nonspecific binding of the amplification reagents, followed by spotting an anti-avidin antibody solution onto the slide and incubating the slide at 37°C for 20 min. Anti-avidin antibody has a strong affinity for avidin and will bind to the localized fluorescein-labeled avidin molecule on the microscope slide. Chromosomes were exposed to the blocking buffer a second time after excess anti-avidin antibody had been washed from the slide with three washes in 4X SSC at 45°C. Next, fluorescein-labeled avidin was spotted onto the slide and incubated at 37°C for 20 min. Having an affinity for the anti-avidin antibody, this application of fluorescein-labeled avidin will bind to the antibody. Thus, a chain of molecules is established to identify the chromosomal location of an unique DNA sequence: biotin-labeled probe DNA (HSD) hybridized with a complementary sequence on the chromosomal DNA, fluorescein-labeled avidin bound to the biotin-labeled probe, anti-avidin antibody bound to the fluorescent avidin molecule, and a second round of fluorescein-labeled avidin bound to the anti-avidin antibody. In this way, the intensity of the DNA probe signal was increased. The efficacy of amplification for the HSD probe was determined empirically by

microscopically viewing nonamplified and amplified ovine chromosome spreads hybridized with the HSD probe. Figure 4 displays a chromosome spread hybridized with the HSD probe in which amplification was not performed. Although the fluorescent probe signal is light compared with the probe signal emitted from chromosomes in Figure 5, it is visible on a specific ovine chromosome pair. The chromosome spread in Figure 5 was hybridized with the HSD probe and amplification was performed. The fluorescent signal in Figure 5 is more intense than the signal observed on chromosomes in Figure 4, but fluorescent background signals are also more intense. Background signals result from nonspecific binding of detection and amplification reagents and can potentially mask chromosomal locations of specific hybridization. Therefore, this study determined that signal amplification procedures are not essential to visualize the location of the HSD probe to the ovine genome.

Chromosome spreads in Figures 2, 3, 4, and 5 were photographed using a Hi-Q FITC longwave pass band filter following hybridization, detection, and staining procedures. Chromosomes were stained with a fluorescent red stain, propidium iodide (PI), at 200 ng/mL. Fluorescein, the molecule used to visualize the HSD probe, fades rapidly when exposed to light. Therefore, *p*-phenylenediamine antifade (pH 11) is overlaid on the stained microscope slide. Hybridized chromosomes were observed using fluorescent microscopy and appropriate microscope filters.

The Hi-Q FITC filter was essential to detect the fluorescent yellow probe signal. The chromosome spread in Figure 6 is the same chromosome spread as seen in Figure 2. However, Figure 6 was photographed using a propidium iodide (PI) filter that highlights the fluorescent red-stained chromosomes.

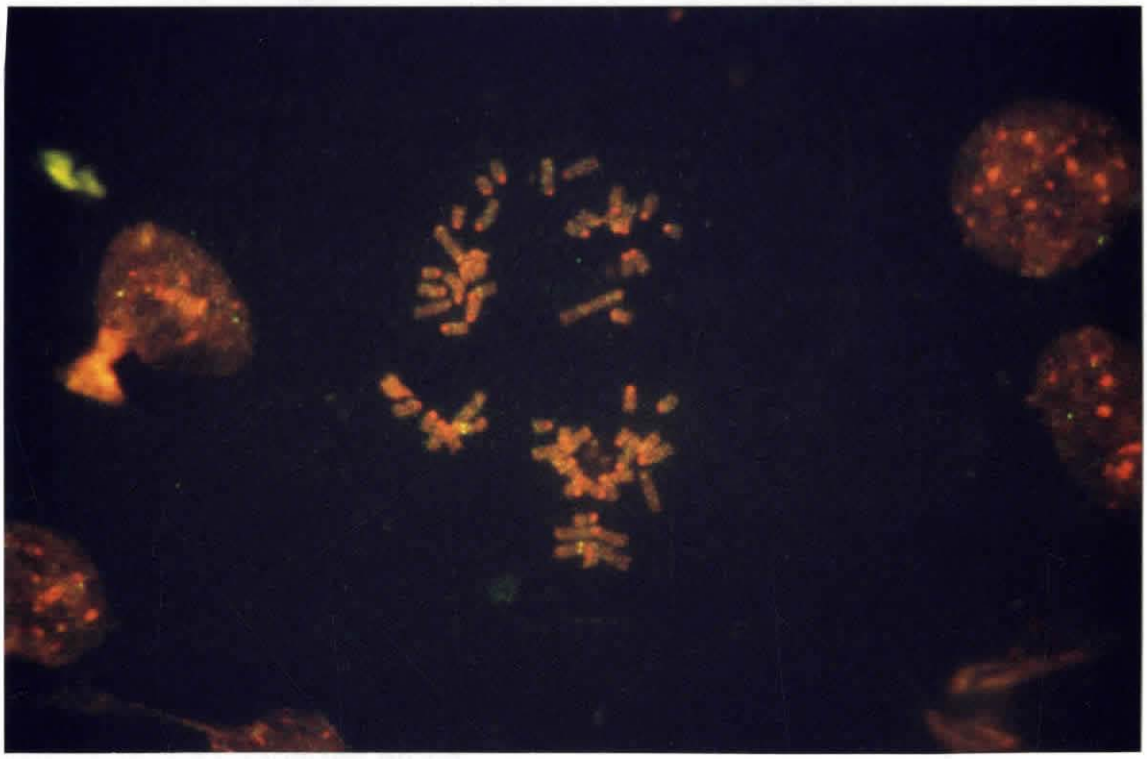

Figure 4. A photomicrograph of ovine chromosomes hybridized to bovine HSD: with amplification. (1000X)

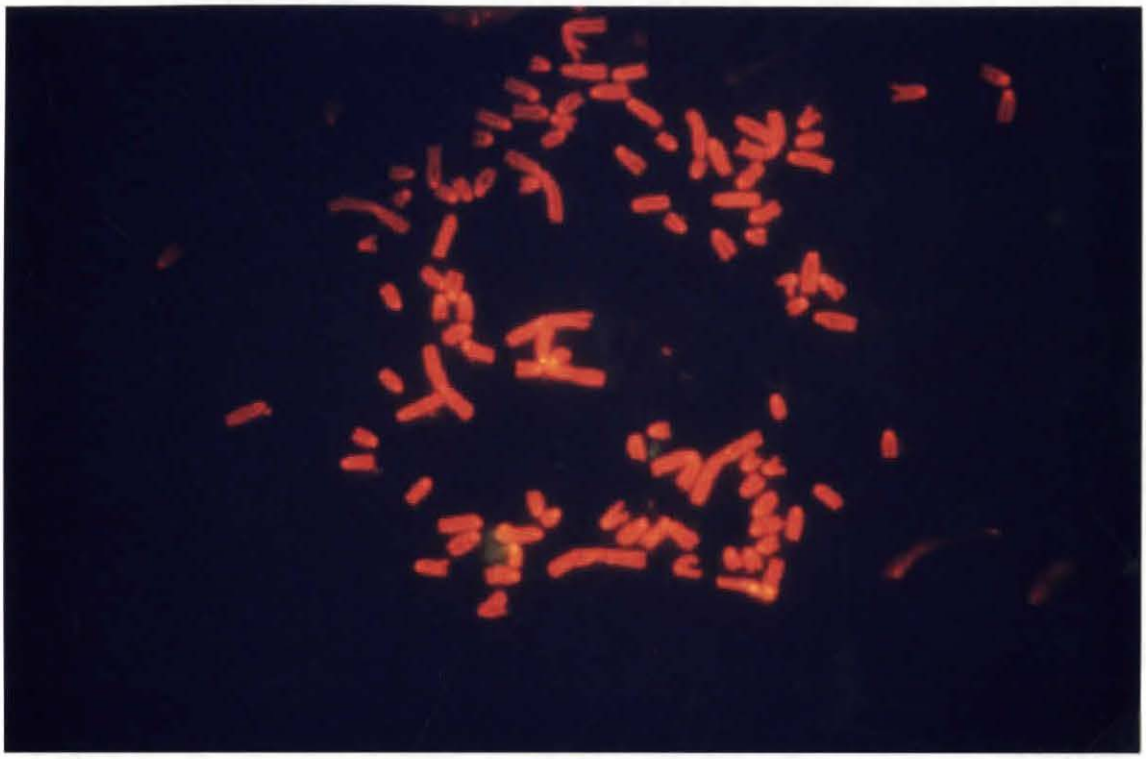

Figure 5. A photomicrograph of ovine chromosomes hybridized to bovine HSD: without amplification. (1000X)

The probe signal is not visible with the PI filter, indicating the importance of the specific excitation and emission wavelengths of the Hi-Q FITC filter in visualizing the hybridized probe.

In this study, HSD (3-ß-hydroxy-5-ene steroid dehydrogenase), a bovine BAC clone, consistently hybridized near the centromere of a single large submetacentric ovine chromosome, either OAR 1, 2, or 3. The successful hybridization of HSD to the ovine genome indicates that FISH techniques were performed correctly. The HSD probe will serve as a

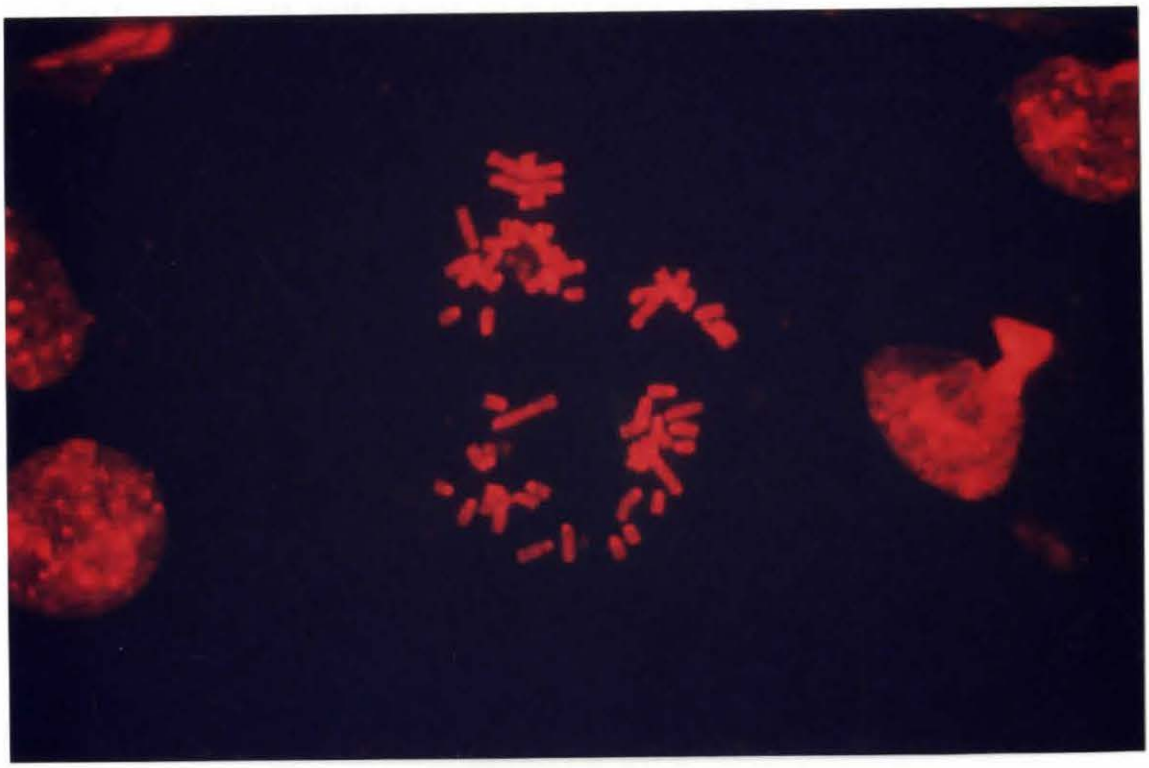

Figure 6. A photomicrograph of ovine chromosomes hybridized to bovine HSD: visualized with a propidium iodide (PI) filter. (1000X)

positive control for FISH techniques performed in this laboratory in the future. The hybridization of HSD to a single ovine chromosome further demonstrates the high level of genetic homology between livestock species. Genetic homology is the evolutionary conservation of genes, chromosome segments, and chromosomes between species and has been supported by The International System for Cytogenetic Nomenclature of Domestic Animals (ISCNDA) (DiBerardino et al., 1989). ISCNDA correlates cattle, sheep, and goat karyotypes by identifying homologous chromosomes among the three species. ISCNDA's results suggest that bovine chromosome 3 (BTA 3) is

highly homologous with ovine chromosome 1 (OAR 1). Based on ISCNDA's karyotypes and this FISH study, in which HSD was hybridized with a single large submetacentric ovine chromosome pair (OAR 1, 2, or 3), the probable location of the HSD probe is on ovine chromosome 1 (OAR 1). To verify this location, additional studies utilizing banding for chromosome differentiation are necessary.

Karyotyping

To develop a karyotype representing the chromosome morphology of a sheep affected with SLS, epidermal tissue from a 4-wk-old male homozygous spider lamb was collected and cultured. Structural banding was performed on aged chromosome spreads using Enzar trypsin (Hopwood and Pathak, 1994). As with FISH, the age of the chromosomes was a crucial factor. If chromosomes were not adequately aged, they exhibited complete loss of morphology following trypsin treatment. Aging was empirically tested using a variety of techniques. These techniques included placing microscope slides containing chromosome spreads at room temperature for 1 to 2 wk, incubating the slides at 38.5°C for 2 to 5 d, incubating the slides at 65°C for 24 to 48 h, and immersing freshly prepared microscope slides in 15% H_2O_2 for 5 min prior to trypsin treatment. The aging optimum for this cell line was determined by comparing the resolution of chromosome bands following different aging techniques and exposure to trypsin. Chromosomes that had been aged at 38.5°C for 5 d produced

chromosome bands with the sharpest resolution. Trypsin treatment was also determined empirically. Slides containing adequately aged chromosome spreads were immersed in a 2% buffered Enzar trypsin solution for times ranging from 10 to 90 s. Enzar trypsin treatment alters chromosome morphology by digesting protein at chromosomal sites rich in the nucleotides guanine and cytosine (Hsu, 1979). When chromosomes were stained following trypsin treatment, the highest quality positive G-bands were observed on chromosomes that had been treated with trypsin for 40 to 45 s. To block the enzymatic activity of the trypsin, slides were immediately rinsed in 70% ethanol. G-banded chromosomes were prepared for visualization by staining the microscope slides in a 4% Geimsa solution. G-banded chromosome spreads were selected based on a complete number of chromosomes ($2n = 54$) and on banding quality. Selected chromosome spreads were microscopically photographed and photomicrographs were enlarged using standard photography techniques. To assemble the karyotype, chromosomes were cut from the enlarged photograph and chromosome pairs were identified according to size and banding pattern. The paired chromosomes were arranged in order of decreasing size (Figure 7). The SLS karyotype was compared to the Reading standard ovine karyotype (Ford et al., 1980). No differences between the Reading standard karyotype and the SLS karyotype were detected. The spider lamb had a diploid chromosome number of 54 and a karyotype of 3 pairs of large

submetacentric chromosomes, 23 pairs of autosomal acrocentrics, and a large acrocentric X and a minute biarmed Y chromosome. G-band analysis of all chromosomes within the karyotype did not reveal any abnormality that was attributable to SLS. The results suggest that the SLS defect is not associated with gross chromosomal abnormalities. Therefore, animals that are carriers for the SLS gene (heterozygous) cannot be detected using karyotype analysis.

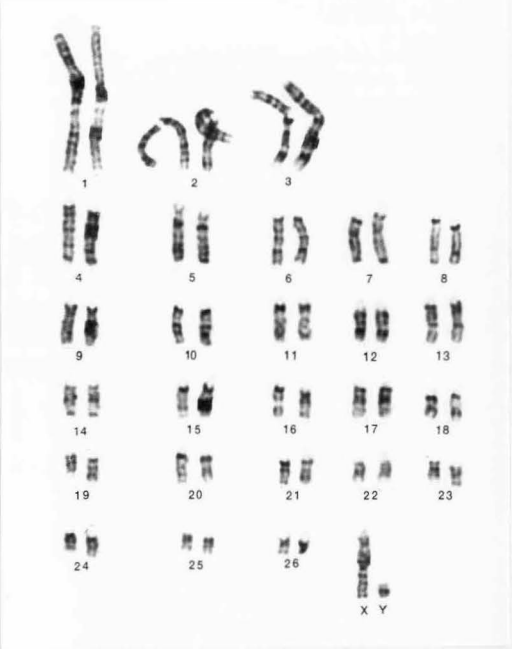

Figure 7. Spider lamb syndrome (SLS) karyotype.

IMPLICATIONS

In situ hybridization is the physical mapping technique of choice for many researchers because it provides high resolution location of genes and important loci on chromosomes. Genetic markers mapped by *in situ* hybridization will provide anchors for markers and genes mapped by linkage analysis, thereby aiding in the identification of economic trait loci. Also, because some species maintain evolutionarily conserved chromosomes and chromosome segments, mapping probes developed in other species to ovine chromosomes will enhance comparative mapping efforts between the ovine genome and other species. The techniques developed in this project will allow researchers at Utah State University to map additional loci to specific ovine chromosomes, thereby contributing to the development of genome maps for domestic animal species.

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APPENDICES

APPENDIX A

Karyotype Development

References:

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Equipment:

Laminar flow hood such as SterilGARD Hood, model SG-600, The Baker Co., Sanford, ME 04073.

Tissue homogenizer such as Stomacher Lab-Blender 80, model #STOM 80, from Tekmar Co., Cincinnati, OH 45222.

Gas regulator such as Veriflo, Richmond, CA 94801.

Incubator such as CO_2 Incubator, model 2500, VWR Scientific, Philadelphia, PA 19101-9711.

Low power microscope such as Olympus Tokyo, L.A. Scientific Instrument Co.

Timer such as 3-Channel Alarm Timer, cat. C6510-1, Baxter Scientific Products, McGaw Park, IL 60085-6787.

Centrifuge with fixed angle rotar such as IEC Clinical Centrifuge, International Equipment Co.

Water bath such as Circulating Water Bath, model 260, Precision Scientific, Chicago, IL 60647.

pH meter such as Corning pH meter, model 240, Baxter, McGaw Park, IL 60085-6787.

Hair dryer with cool air setting such as Revlon, model RV406.

High power microscope such as Zeiss Axioskop, Carl Zeiss Inc., San Leandro, CA 94577.

Microscope camera such as MC 80 microscope camera, Carl Zeiss Inc., San Leandro, CA 94577.

Pipet such as V3-Series, cat. V200TE, Ulster Scientific, New Paltz, NY 12561-0819.

Pipet filler such as pipet filler, cat. P5310, Baxter Healthcare Corp., McGaw Park, IL 60085-6787.

Ultraviolet (UV) light such as Spectroline UV Light, model ENF-260C, Spectronics Corporation, Westbury, NY 11568.

Negative enlarger such as Beseler 23 CII-XL-Enlarger, Charles Beseler Company, Linden NJ 07036.

Safelight such as Kodak Adjustable Safelight Lamp, Model B, Eastman Kodak Company, Rochester, NY 14650.

Focus finder such as Micro Focus Finder, product PTP643, Paterson Products, Borehamwood Herts, England.

Supplies:

Permanent marking pen

Surgical blade (sterile)

Forceps (sterile)

Cotton swabs

Iodine

Alcohol swabs

RPMI medium 1640
-from Gibco BRL, Grand Island, NY 14072
-cat. #11875-069

Defined fetal bovine serum
-from Hyclone Laboratories, Logan, UT 84321
-cat. #A-1111-L
Aliquot in 20 mL. Store at -80°C.

Penicillin streptomycin
-from Gibco BRL, Grand Island, NY 14072
-cat. #15075-013

Rehydrate in 20 mL RPMI medium 1640. Aliquot in 1.5 mL. Store at -20°C.

L-glutamine-200 mM
-from Gibco BRL, Grand Island, NY 14072
-cat. #15039-019

Rehydrate in 20 mL RPMI medium 1640. Protect from light. Aliquot in 1.5 mL. Store at -80°C.

Antibiotic-Antimycotic (100X)
-from Gibco BRL, Grand Island, NY 14072
-cat. #15245-012

Rehydrate in 20 mL sterile, ddH_2O. Aliquot in 50 μL. Store at -80°C.

Trypsin (0.25%)
-from Hyclone Laboratories, Logan, UT 84321
-cat. #B-3004

Colcemid (10 μg/mL)
-from Gibco BRL, Grand Island, NY 14072.
-cat. #15212-012

Ethidium bromide
-from Sigma, St. Louis, MO 63178
-cat. #E-8751

Hank's balanced salt solution (HBSS)
-from Hyclone Laboratories, Logan, UT 84321
-cat. #B-4006-AA

Potassium chloride (KCl)
-from Mallinckrodt, Paris, KY 40361
-cat. #6858

Methyl alcohol (anhydrous)
-from Mallinckrodt, Paris, KY 40361
-cat. #3016

Acetic acid (glacial)
-from Mallinckrodt, Paris, KY 40361
-cat. #8817

Sodium phosphate (monobasic) (NaH_2PO_4 H_20)
-from Mallinckrodt, Paris, KY 40361
-cat. #7892

Sodium phosphate (dibasic heptahydrate) (Na_2HPO_4 $7H_2O$)
-from Mallinckrodt, Paris, KY 40361
-cat. #7914-(05)

Hydrochloric acid (HCl) (12N)
-from Mallinckrodt, Paris, KY 40361
-cat. #2612

Giemsa stain, modified
-from Sigma, St. Louis, MO 63178
-cat. #GS-500

Tissue homogenizer bags
-such as Seward Medical Stomacher '80' Bags

Tissue culture flasks (25 cm^2)
-from Corning, Corning, NY 14831
-cat. #25100-25

Conical polypropylene centrifuge tubes (15 mL)
-from Corning, Corning, NY 14831
-cat. #25319-15

Gas -9.98% CO_2
-19.98% O_2
-70.04% N

9 inch pasteur pipets
-from Kimble, Toledo, OH 43666
-cat. #72050

Microscope slides
-from Corning, Corning NY 14830
-cat. #2948

Coplin staining jars
-from Fisher Scientific, Pittsburgh, PA 15219-4785
-cat. #08-817

Ice

Pipets (1 mL)
-from Corning, Corning, NY 14831
-cat. #7075-1

Pipets (10 mL)
-from Corning, Corning, NY 14831
-cat. #27003-10

Pipets (25 mL)
-from Corning, Corning, NY 14831
-cat. #7075-25

Pipet tips (1-200 μL)
-from VWR Scientific, West Chester, PA 19380
-cat. #53508-810

Photography film
-from Kodak
-#TMX 135-24

Enzar-T (40X trypsin solution)
-from Intergen Co., Purchase, NY 10577
-cat. #7000-65

Hank's balanced salt solution (calcium and magnesium free)
-from Gibco BRL, Grand Island, NY 14072
-cat. #14170-039

Sodium bicarbonate (7.5%)
-from Hyclone Laboratories, Logan, UT 84321
-cat. #B-3002-D

Ethyl alcohol
-from Mallinckrodt, Paris, KY 40361
-cat. #7018

5-Fluorouracil
-from Sigma, St. Louis, MO 63178
-cat #F-6627

5-bromo-2'-deoxyuridine (BrdU)
-from Sigma, St. Louis, MO 63178
-cat #B-2398

Hoechst (stain) No. 33258
-from Sigma, St. Louis, MO 63178
-cat #B-1782

Film developing canister

Photography developing trays

Wooden tongs

Clothes pins or photo clips

Negative squee-gee

Photography easel

Paper cutter

Scraper with rubber blade

Multigrade Contrast Filters
-from ILFORD Limited, Moberly Cheshire, England, 13996141 J89
-cat #624 232

Polycontrast III RC glossy print paper
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #128 4645

Kodak D76 Developer
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4817

Kodak Indicator Stop Bath
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4247

Kodak Rapid Fixer
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4106

Kodak Dektol Developer
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4726

Kodak Hypo Clearing Agent
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4254

Kodak Photo-Flo 200 Solution
-from Eastman Kodak Company, Rochester, NY 14650
-cat. #146 4502

TISSUE COLLECTION AND CULTURE

Solutions/Reagents:

Tissue culture medium

RPMI medium 1640	100 mL
Defined fetal bovine serum	20 mL
Penicillin streptomycin	1.3 mL
L-glutamine	1.3 mL

Protect from light. Store at 4°C.

Procedures:

1. Thoroughly scrub the axillary region on a sheep with iodine. Rinse and wipe with alcohol swabs. Using sterile forceps, gently raise the skin and perform a one half thickness skin biopsy. It is not necessary to cut deep into the tissue.

2. Transfer the excised tissue directly from the sterile forceps into a sterile 15 mL centrifuge tube containing 7 to 10 mL tissue culture medium.

3. Add 1 drop of fungizone (antifungal agent) to medium using a 9 in pasteur pipet.

4. Leave tissue in fungizone-supplemented medium at room temperature for 1 to 2 h.

5. Under laminar flow hood, transfer tissue sample to a new, sterile 15 mL centrifuge tube containing 7 to 10 mL fresh medium (fungizone-free).

6. Leave tissue in medium at room temperature for 2 to 3 h.

   To avoid contamination, all additional steps in which tissue is manipulated or exposed to air need to be performed under the laminar flow hood (unless otherwise stated).

7. Pour medium and tissue into homogenizer bag.

8. Place bag in tissue homogenizer for 1 to 1½ min. After homogenizing, the medium should appear cloudy and the size of the tissue sample should decrease significantly. Tissue will look white instead of pink and contain connective tissue which does not homogenize. If these conditions are not observed, tissue may require additional homogenizing for 10 to 30 s.

9. Label a 25-cm^2 tissue culture flask with date and animal identification number.

10. Pour medium from bag into tissue culture flask. Intact tissue (connective tissue) should be discarded with stomacher bag.

11. Gas contents of flask by attaching a sterile pasteur pipet to gas regulator and holding pipet for 15 to 20 s with tip inside culture

flask, just beyond neck of flask. Immediately replace cap.

12. With cap tight, place culture flask in horizontal position at 38.5°C and leave undisturbed for 3 to 4 d.

13. Check fibroblast growth after 3 to 4 d by microscopically viewing cells with 4X objective.

14. Following 4 to 5 d incubation, pour medium off flask and replace with fresh medium. If fresh medium is dark pink, gas flask for 10 to 20 s. This will lower the pH to provide an optimum growing environment for the fibroblasts. Within 10 to 15 min following gassing, culture medium should be orange-pink. Medium may be changed every 24 to 48 h to enhance the rate of cell proliferation, although fresh medium is required only every 7 to 10 d to maintain the cell culture.

15. When the cell culture is confluent, remove medium and add approximately 1 mL of trypsin (enough trypsin to cover bottom of flask). Tilt the flask from side to side to rinse cells.

16. Pour off trypsin from flask and replace with approximately 1½ mL fresh trypsin. Incubate at 38.5°C for 3 to 5 min. Cells should be checked every 2 min for "rounding up" and detaching from the flask surface. Once cells have begun to round up, a corner of the flask may be tapped against the palm of the hand to expedite the process. This limits the length of time cells are exposed to trypsin. Overexposing cells to trypsin can cause irreversible cell damage.

17. When most of the cells have detached, immediately add 6 to 7 mL fresh medium to the flask. Serum-supplemented medium inhibits trypsin activity and prevents further damage to the cells. Swirl the medium to evenly distribute cells.

18. Pour half of medium into new culture flask.

19. Gas both flasks for 15 to 20 s, immediately replacing caps.

20. Incubate at 38.5°C for 12 to 24 h.

21. Replace medium in both flasks. There may be several dead cells floating in medium. By removing these cells and adding fresh medium, the growth rate of healthy cells should increase.

22. Continue to grow cells *in vitro* by repeating steps 14 to 21.

CELL HARVESTING

Solutions\Reagents:

Tissue culture medium

RPMI medium 1640	100 mL
Defined fetal bovine serum	20 mL
Penicillin streptomycin	1.3 mL
L-glutamine	1.3 mL

Protect from light. Store at 4°C.

Ethidium bromide working solution (1mg/mL)

Ethidium bromide	10 mg
ddH_2O	10 mL

Protect from light. Store at 4°C.

Hypotonic working solution (0.56% or 0.075 M)

Potassium chloride (KCl)	0.56 g
ddH_2O	100 mL

Store at room temperature for 1 to 2 wk.

Fixative (3:1 Methanol:Acetic Acid)

Methyl alcohol (anhydrous)	30 mL
Acetic acid (glacial)	10 mL

Make fresh.

Procedures:

Cell cultures should be established using tissue collection and culture procedures. After cultures have been passed 2 to 3 times, their growth rate is generally adequate to obtain a sufficient number of chromosome spreads for cytogenetic studies.

1. Select a culture flask in which the cells have reached confluency and remove cells from flask surface using the protocol described in Tissue Collection and Culture, steps 15 and 16.

2. Add 20 to 25 mL of medium to flask and swirl medium to evenly distribute cells.

3. Transfer equal amounts of medium from this flask to four new flasks, leaving an equal portion of medium in original flask. (This ratio is given as a starting point. It may be modified depending on the rate of cell proliferation, i.e. cells that grow slower may be passed 1:4 and cells that grow extremely fast passed 1:6.) Cell proliferation rate can vary greatly between cell lines. Therefore, the optimum passing ratio must be determined empirically.

4. Gas all flasks for 15 to 20 s and incubate at 38.5°C for 24 h.

5. Replace medium in all flasks with 5 mL fresh medium and incubate an additional 24 h.

6. View with light microscope (magnification 40X). Cell confluency should be 60 to 70%, with several cells appearing rounded up (10 to 20 per microscope field) among the majority of cells which are flattened against the plastic surface with numerous cytoplasmic processes. To obtain a sufficient number of chromosome spreads, it is critical that cell confluency be around 60 to 70%. The time following passing may be altered if cell proliferation rate is particularly fast. This must be determined empirically for specific cell lines.

7. Select 3 to 4 flasks and add 20 μL colcemid (final concentration 40 ng/mL) and 100 μL ethidium bromide (final concentration 20 μg/mL) solution to each. Remaining flasks will be kept to maintain the culture.

8. Incubate at 38.5°C for 30 min.

9. During the 30 min incubation, prewarm hypotonic solution in 37°C water bath.

10. Pour medium from flasks into separate, sterile, labeled 15 mL centrifuge tubes.

11. Remove cells from flasks following steps 15 to 16 in tissue collection and culture procedures.

12. Pour contents from each flask into respective centrifuge tube.

13. Before discarding flasks, rinse with 2 to 3 mL of Hank's balanced salt solution (HBSS) and add contents to centrifuge tubes.

14. Replace caps on tubes and gently invert.

15. Centrifuge tubes at 800 to 1000 RPM for 8 min using a fixed angle rotor.

    It is not necessary to work with cells in laminar flow hood in the remaining steps.

16. Pour off supernatant without disturbing cell pellet. Approximately ½ mL of supernatant will remain.

17. Resuspend cell pellet by tapping on side of tube or by gently bubbling air through remaining supernatant with pasteur pipet until pellet is dislodged and broken up, forming a slightly cloudy suspension.

18. Slowly add 6 to 8 mL prewarmed hypotonic solution to each tube, replacing caps and inverting gently. Hypotonic solution causes the cells to swell and cell membranes to weaken. Therefore, in all future steps cells must be resuspended and handled gently.

19. Incubate tubes in a 37°C water bath for 40 min.

20. Centrifuge tubes at 800 to 1,000 RPM for 8 min.

21. While centrifuging, prepare fixative solution. (Fixative may be used at room temperature.)

22. Carefully pour off supernatant and resuspend pellet using one of the methods described in step 17.

23. By holding a 9 inch pasteur pipet against inside of centrifuge tube near the opening, slowly layer 5 to 7 mL of fresh fixative onto cell suspension.

24. Replace cap and gently invert tube.

25. Place at room temperature for 5 to 10 min.

26. Centrifuge tubes at 800 to 1,000 RPM for 8 min.

27. Repeat steps 22 to 26 at least two times. The number of fixative washes required varies between cell preparations. A recommended approach to determine the number of washes is to follow the procedure through step 27, then prepare a test slide and view microscopically to identify whether excess cellular debris is present. If the cell preparation appears clean, additional washes are unnecessary. If the cell preparation contains debris that interfere with visualization of chromosome spreads, additional fixative washes are needed.

28. Cell preparations may be splashed onto microscope slides immediately, stored at 4°C overnight, or stored at to 20°C for several weeks.

SLIDE PREPARATION AND CHROMOSOME STAINING

Solutions\Reagents:

Fixative

Methyl alcohol (anhydrous)	30 mL
Acetic acid (glacial)	10 mL

Make fresh.

Hydrochloric acid (HCL) (6N)

Hydrochloric acid (HCl) (12N)	10 mL
ddH_2O	10 mL

Phosphate buffer solution [0.01M] (pH 6.8)

ddH_2O	1 L
Sodium phosphate (monobasic) (NaH_2PO_4 H_2O)	0.469 g
Sodium phosphate (dibasic heptahydrate) (Na_2HPO_4 $7H_2O$)	1.77 g

Adjust pH to 6.8 with 6N HCl.

4% Giemsa staining solution

Phosphate buffer [0.01M] (pH 6.8)	48 mL
Giemsa stain, modified	2 mL

Make fresh every 1 to 2 h.

Procedures:

1. Immediately prior to splashing cell preparations, wash with 5 to 7 mL fresh fixative. (Steps 23 to 26 of cell harvesting procedure.)

2. Carefully pour off supernatant and resuspend the cell pellet by tapping on side of tube.

3. Add fresh fixative, one drop at a time, to the cell suspension until it appears only faintly cloudy. It is likely that cell suspensions will require no additional fixative to dilute the cell concentration. This will vary between tubes and is determined by experience in recognizing the degree of cloudiness in the cell suspension. When initially working with cell cultures, test slides from different cell suspensions may be made to determine cell concentrations.

4. Label microscope slides to correspond with individual cell suspensions.

5. Place new microscope slides in ice for several seconds.

6. Transfer microscope slides to paper towel and place on solid surface.

7. Immediately draw up a small portion of cell suspension into pasteur pipet and release two or three drops 3 to 4 ft directly above cold slide. Aim drops so they fall along length of slide with little overlap.

8. Place slides at 38.5°C for 10 min or until cell suspension is dry.

9. While slides are drying, prepare 4% Giemsa staining solution in Coplin jar.

10. Stain slides in Giemsa solution for 3 to 5 min.

11. Remove slides from staining jar and quickly dip in Coplin jar containing ddH_2O.

12. Immediately dry the slide by holding a hair dryer 4 to 8 in. from slide surface and blowing cool air onto it. This quick-drying technique minimizes visualization problems associated with Giemsa stain residues binding to the glass slide.

13. Visualize chromosome spreads with a light microscope and 100X objective.

14. Adust light sources from high power microscope to provide high contrast level between chromosomes and background. Set automatic exposure panel at '0' or '-1' and photograph using 35mm black and white film.

STRUCTURAL BANDING WITH TRYPSIN

Solutions\Reagents:

Enzar trypsin working solution (pH 7.0)

Enzar-T (40X trypsin solution)	1 mL
Hank's balanced salt solution	49 mL

When these 2 reagents are combined, solution should turn bright yellow. Adjust to pH 7.0 with sodium bicarbonate (7.5%). At pH 7.0, solution color should be pink-orange. Solution may be used for 3 to 4 d. Store at 4°C.

70% Ethanol solution

Ethyl alcohol	35 mL
ddH_2O	15 mL

Hydrochloric acid (HCL) (6N)

Hydrochloric acid (12N)	10 mL
ddH_2O	10 mL

Phosphate buffer [0.01M] (pH 6.8)

ddH_2O	1 L
Sodium phosphate (monobasic) (NaH_2PO_4 H_2O)	0.469 g
Sodium phosphate (dibasic heptahydrate) (Na_2HPO_4 $7H_2O$)	1.77 g

Adjust pH to 6.8 with HCl (6N).

4% Giemsa staining solution

Phosphate buffer [0.01M] (pH 6.8)	48 mL
Giemsa stain, modified	2 mL

Make fresh every 1 to 2 h.

Procedures:

1. Splash several slides following slide preparation procedures, steps 1 to 8.

2. Leave slides at 38.5°C for 5 d.

3. Prepare 2% buffered Enzar trypsin solution in Coplin jar.

4. Treat incubated slides for 10 to 50 s in trypsin solution. Optimum time for exposure to trypsin must be empirically tested by exposing several slides to trypsin for varying times and comparing banding quality. There is a tremendous variation in the time required to band chromosome spreads from different cell preparations and from different cell lines. Chromosome spreads on a single slide also vary in banding quality. Therefore, it is essential to locate several chromosome spreads before conclusively determining trypsin exposure time.

5. Immediately after removing slides from trypsin solution, briefly rinse in 70% ethanol.

6. Transfer slides directly from ethanol rinse into a Coplin jar containing 4% Giemsa stain.

7. Stain and view banded chromosome spreads using steps 10 to 13 in slide preparation and chromosome staining procedures.

8. Adust light sources from high power microscope to provide high contrast level between light and dark bands. Set automatic exposure panel at '0' or '-1' and photograph using 35mm black and white film.

DYNAMIC BANDING

Solutions\Reagents:

Tissue culture medium

RPMI medium 1640	100 mL
Defined fetal bovine serum	20 mL
Penicillin streptomycin	1.3 mL
L-glutamine	1.3 mL

Protect from light. Store at 4°C.

Fluorouracil working solution

Stock solution I:

Fluorouracil	130 mg
Sterile ddH_2O	10 mL

Protect from light. Shake on shaker until dissolves (approximately 6 h).

Stock solution II:

Stock solution I	100 μL
Sterile ddH_2O	9.9 mL

Protect from light.

Working solution:

Stock solution II	1 mL
Sterile ddH20	9 mL

Protect from light. Store in 100 μL aliquots at -80°C.

BrdU working solution (2.5 mg/mL)

BrdU	3 mg
Sterile ddH_2O	1.2 mL

Protect from light. Store in 200 μL aliquots at -80°C.

Ethidium bromide working solution (1 mg/mL)

Ethidium bromide	10 mg
ddH_2O	10 mL

Protect from light. Store at 4°C.

Hoechst stain working solution (150 μg/mL)

Hoechst (stain) No. 33258	1.5 mg
ddH_2O	10 mL

Protect from light. Store in 1 mL aliquots at -80°C.

20X SSC stock solution

	2 L	Molar []
NaCl	350.7 g	3 M
Na Citrate	176.4 g	.3 M

Bring to 2 L volume with ddH_2O. Use 6M HCl to adjust pH to 7.0.

2X SSC

20X SSC	10 mL
ddH_2O	90 mL

4% Giemsa staining solution

Giemsa stain, modified	2 mL
.01 M PO_4 buffer	48 mL

Make fresh every 1 to 2 h.

Procedures:

BrdU incorporation for R-band production

1. When fibroblasts in 25-cm^2 flask are 60 to 75% confluent and numerous mitoses are observed, add 100 μL fluorouracil in 5 mL fresh media (260 ng/mL). Incubate for 17 to 20 h at 38.5°C.
2. Pour off medium. Rinse flask 3X with HBSS. Add 185 μL BrdU in 5 mL fresh media (92.5 μg/mL). Incubate 4½ to 5½ h at 38.5°C.
3. Add 100 μL ethidium bromide (20 μg/mL) and 20 μL colcemid

(40 ng/mL) to flask. Incubate 30 min at 38.5°C.

4. Harvest and splash slides using standard cell harvest procedure, steps 9 to 28 and slide preparation procedures, steps 1 to 8.

5. Perform FPG on slides after 1 d at room temperature or age slides for *in situ* hybridization procedure.

Fluorescence Photolysis Giemsa (FPG)

1. Mount slide incubate in 150 μL [150 μg/mL] Hoechst stain for 15 to 20 min in the dark.

2. Rinse slide with ddH_2O and allow to air dry.

3. Cover slide with 2X SSC and place under UV light (long wavelength) for 30 to 60 min.

4. Rinse slide with ddH_2O. Place in prewarmed 2X SSC at 65°C for 1 h.

5. Rinse slide with ddH_2O and stain in 4% Giemsa staining solution for 3 to 5 min.

6. Rinse slide with ddH_2O. Blow dry. View and photograph under light microscope.

BLACK AND WHITE PHOTOGRAPHY

Solutions\Reagents:

D76 developer working solution

Bring 2 L H_2O to 52°C.
Slowly pour contents of packet into 3.32 L H_2O stirring until dissolved.
Bring volume to 2 L with 52°C H_2O.
Store in dark bottle at RT.
For working solution, dilute 1:1 with H_2O.

Indicator stop bath working solution

Kodak Indicator Stop Bath	16 mL
H_2O	1984 mL

Store in dark bottle at RT.
(Solution may be reused until the color changes.)

Rapid fixer working solution

Bring 3.8 L H_2O to 16 to 27°C.

	Films	Papers
H_2O	1.9 L	1.9 L
Solution A	946 mL	473 mL
Solution B	104 mL	52 mL

Agitate rapidly while adding Solution B. Bring to 3.8 L volume with H_2O. Store in dark bottle at RT.

Dektol developer working solution

Bring 3.8 L H_2O to 32 to 38°C.
Slowly add contents of package to 3.32 L H_2O stirring until dissolved.
Bring to 3.8 L volume with H_2O. Store in dark bottle at RT.

For working solution, dilute 1:1 with H_2O.
(Working solution may be reused for several days.)

Hypo clearing agent working solution

Bring 3.8 L H_2O to 26.5°C.
Slowly add contents of package to 3.8 L H_2O stirring until dissolved.
Store in dark bottle at RT.
For working solution, dilute stock solution 1:4 with H_2O.

Photo-Flo working solution

Add 1 capful Photo-Flo to 1 L H_2O.

Procedures:

Negative Development

1. Take photographs using Kodak 100 speed film in Zeiss MC 80 camera. Rewind when roll is finished.

2. In complete darkness, load film onto reel and place in light-free developing canister.

3. Add 300 mL D76 developer working solution. Develop for 13½ min at 22°C, turning for the first 30 s and then once every 30 s.

4. Pour out developer and add 300 mL indicator stop bath working solution. Turn for 30 s.

5. Pour out stop bath and save. Indicator stop bath may be reused as long as color is yellow. Add 300 mL rapid fixer working solution for films to canister. Fix for 4 min, turning the first 30 s and then once every 30 s.

6. Pour out fixer solution and save for recycling. Add 300 mL H_2O. Turn for 30 s.

7. Pour out H_2O and add 300 mL hypo clearing agent working solution. Leave in hypoclearing agent for 2½ min, turning for first 30 s and then once every 30 s.

8. Pour out hypo clearing agent solution. Remove reel from canister and unwind negatives from reel. Rinse negatives in running H_2O for 20 min.

9. Bathe negatives for 30 s in Photo-Flo working solution.

10. Strip negative roll with water-soaked squee-gee and hang overnight at RT with photo clips or clothes pins on each end.

11. Cut negative roll into lengths short enough to fit into plastic photo sleeves.

Print Development

1. Pour approximately 2 L of Dektol developer working solution, indicator stop bath working solution, and rapid fixer working solution for papers each into a separate developing tray. Place a pair of wooden tongs in each of the three trays.

2. Place negative in enlarger slot so full image can be seen on the white board of the enlarger.

3. With enlarger aperture open to 5.6, focus negative using focus finder until silver grains appear.

4. Shut off all light sources except for a safe light.

5. Close enlarger aperture to 16. Cut one sheet of RC glossy print paper into small strips. Hold contrast filter #3 under enlarger lens. Place test strip on white board. Expose test strip for 20 sec.

6. Using wooden tongs, place strip in tray of Dektol developer working solution. Agitate for 2 min.

7. Immerse strip in tray of indicator stop bath working solution with wooden tongs. Agitate for 30 s.

8. Using wooden tongs, place strip in tray of rapid fixer working solution for prints. Agitate 2 min.

9. Turn on light. Remove strip with wooden tongs. Check strip for contrast and darkness.

10. Make new test strips using steps 5 to 9, exposing the test strips for varying time increments, if the desired results were not achieved. Change contrast by varying the contrast filter. A higher filter number increases contrast. A lower filter number decreases contrast. Change the color intensity by increasing exposure time for a darker print and decreasing exposure time for a lighter print.

11. When a desirable test strip is produced, place one sheet of RC paper on easel and develop using the time and contrast filter used to produce the optimum test strip. Adjust print size using easel, trim prints with paper cutter.

12. Develop using steps 6 to 9. Turn on lights and immerse print in 500 mL hypo clearing agent working solution for 2 min.

13. Wash print in running H_2O for 20 min.

14. Scrape H_2O from print with rubber blade. Hang print for several hours or until dry.

15. When print developing is finished for the day, save rapid fixer from tray for recycling. Save indicator stop bath (if color is yellow) from tray and Dektol developer (if fresher than 3 d) from tray.

APPENDIX B

Fluorescent *In Situ* Hybridization (FISH)

Equipment:

Vortexer such as Vortex-Genie 2, Scientific Industries, Bohemia, NY 11716.

Microcentrifuge such as Microcentrifuge, model 235C, Fisher Scientific, Pittsburgh, PA 15219.

Microwave

Timer such as 3-Channel Alarm Timer, cat. C6510-1, Baxter Scientific Products, McGaw Park, IL 60085-6787.

Water baths such as Circulating Water Bath 260, model 260, Precision Scientific, Chicago, IL 60647.

Miniature gel bed such as Mini Gel Bed Unit, model HE33, Hoeffer Scientific Instruments, San Francisco, CA 94102-12.

Voltage meter such as model FB 154 voltage meter, Fisher Scientific, Pittsburgh, PA 15219.

UV light box with camera such as Foto Prep I, model 3-3500, Fotodyne, Inc., New Berlin, WI 53151.

Cold water bath such as Isotemp Refrigerated Circulator, model 900, Fisher Scientific, Pittsburgh, PA 15219.

Microcentrifuge with variable temperature and RCF such as Avanti 30 Centrifuge with F2402 Rotor, Beckman Instruments, Palo Alto, CA 94303.

Shaker such as model 5S shaker, Reliable Scientific.

Incubator such as CO_2 Incubator, model 2500, VWR Scientific, Philadelphia, PA 19101-9711.

Oven such as drying oven model 1305, VWR Scientific, Philadelphia, PA 19101-9711.

-80°C freezer such as Cryo Fridge, Baxter Scientific Products, McGaw Park, IL 60085-6787.

Phase contrast microscope such as Nikon Diaphot Inverted Microscope, Nikon, Japan.

High power microscope equipped with fluorescence such as Zeiss Axioskop, Carl Zeiss Inc., San Leandro, CA 94577.

Fluorescent filters to detect propidium iodide or FITC such as Propidium Iodide filter, model 31005, and Hi-Q FITC Long Pass filter, model 41012, Chromatechnology, Brattleboro, VT 05301.

Microscope camera such as MC 80 microscope camera, Carl Zeiss Inc., San Leandro, CA 94577.

Supplies:

Microcentrifuge tubes (1.5 mL)
-from Fisher Scientific, Pittsburgh, PA 15219
-cat. #4202

Ice

Biotin-21-dUTP Nick Translation Kit
-from Clontech Laboratories, Inc., Palo Alto, CA 94303
-cat. #K1022-1

Phi X RF DNA/ HAE III Fragments
-from Gibco BRL, Grand Island, NY 14072
-cat. #15611-015

Ethidium bromide
-from Sigma, St. Louis, MO 63178
-cat. #E-8751

CHROMA SPIN+ TE-30 spin columns
-from Clontech Laboratories, Inc., Palo Alto, CA 94303
-cat. #K1321-1

Hybond-N Hybridization transfer membranes
-from Amersham, Arlington Heights, IL 60005
-cat. #RPN.303N

Metal forceps

Plastic containers
-small (12 x 13 x 3 cm)
-medium (16 x 27 x 6 cm)

Sure Blot Blue Detection Reagents DR Kit
-from Oncor, Gaithersburg, MD 20884
-cat. #S4200-BOX

Membrane Blocking Solution (M.B.S.) 2.5X
-from Oncor, Gaithersburg, MD 20884
-cat. #S4200-12

10X Staining Buffer
-from Oncor, Gaithersburg, MD 20884
-cat. #S4200-13

100% Ethanol (ETOH)
-from McCormick Distilling Co Inc., Weslo, MO 64098
-cat. #6505-00-105-0000

Whatman Chromatography Paper (3M Paper)
-from Fisher Scientific, Pittsburgh, PA 15219
-cat. #05-714-4

Bovine COT-1 DNA
-from Clontech Laboratories Inc., Palo Alto, CA 94303
-Dan Gallagher, personal communication

Bovine BAC DNA (HSDB)
-from Clontech Laboratories Inc., Palo Alto, CA 94303
-Dan Gallagher, personal communication

DNA-sodium salt type II, salmon testes (salmon sperm DNA)
-from Sigma, St. Louis, MO 63178
-cat. #D-1626

Ultrapure formamide
-from Gibco BRL, Grand Island, NY 14072
-cat. #5515UB

Amberlite MB-1 ion exchange resin
-from Baxter Scientific Products, McGaw Park, IL 60085-6787
-cat. #IR-120

Humidified chamber
-16 x 10 x 3 cm plastic container with airtight lid
-water-soaked paper towels overlaid with plastic mesh

Glass coverslides (22 x 22 mm)
-from Fisher Scientific, Pittsburgh, PA 15219
-cat. #12-524B

Rubber cement

Permanent marking pen

Microscope slides
-from Corning, Corning, NY 14830
-cat. #2948

Fluorescein Detection of Biotin Labeled Probe kit
-from Oncor, Gaithersburg, MD 20884
-cat. #S1333-BF

Coplin staining jars
-from Fisher Scientific, Pittsburgh, PA 15219-4785
-cat. #08-817

Chromosome *In Situ* System Plastic Coverslips
-from Oncor, Gaithersburg, MD 20884
-cat. #S1331-6

Tween 20
-from Sigma, St. Louis, MO 63178
-cat. #P-7949

Propidium Iodide (PI)
-from Sigma, St. Louis, MO 63178
-cat. #P-4170

Dulbecco's Phosphate Buffered Saline (PBS)
-from Gibco BRL, Grand Island, NY 14072
-cat. #14040-026

Glycerol
-from Baxter Scientific Products, McGaw Park, IL 60085-6787
-cat. #509 1-500

***p*-Phenylenediamine (PPD)**
-from Sigma, St. Louis, MO 63178
-cat. #P-6001

Glass coverslides (50 x 22 mm)
-from Fisher Scientific, Pittsburgh, PA 15219
-cat. #12-531E

Clear fingernail polish

BIOTIN LABELING OF PROBE DNA

Solutions\Reagents:

Biotin-21-dUTP Nick Translation Kit. Solutions include:
10X Reaction buffer
Biotin-21-dUTP
Control lambda DNA
Enzyme mix
Sterile dH_20
Stop solution

10X TAE

	2L	Molar []
Tris	97 g	.4 M
Na Acetate	13.5 g	.05 M
EDTA	7.5 g	.01 M

Bring to 2 L volume with ddH_20. Use acetic acid to adjust pH to 7.8.

Ethidium bromide (10 mg/mL)

Ethidium bromide	100 mg
ddH_2O	10 mL

Protect from light. Store at 4°C.

1% Agarose gel

SeaKem LE agarose	.5 g
10X TAE	5 mL
ddH_2O	45 mL

Combine in 125 mL flask. Cover with plastic wrap. Microwave on medium power until mixture boils. Boil until dissolved. Cool to 60°C in water bath.Add 2.5 μL ethidium bromide. Swirl gently. Pour into mini-gel bed. Cool 10 to 20 min.

1X TAE Buffer

10X TAE	25 mL
ddH_2O	225 mL
Ethidium bromide (10 mg/mL)	12.5 μL

Pour into mini-gel bed chamber.

STOP Buffer

Glycerol	2.5mL
EDTA [.25 M]	.2 mL
ddH_2O	2.3 mL
Bromophenol blue	150 μL

10% SDS

SDS	100 g

Bring to 1 L volume with ddH_2O.

5% SDS

10% SDS	5 mL
ddH_2O	5 mL

TE

	500 mL	Molar []
Tris (1M, pH 7.6)	5 mL	10 mM
EDTA [.25 M]	2 mL	1 mM

Bring to 500 mL volume with ddH_2O. Filter with sterile unit.

TE + .1% SDS

TE	10 mL
10% SDS	100 μL

20X SSC

	2 L	Molar []
Na Cl	350.7 g	3 M
Na Citrate	176.4 g	.3 M

Bring to 2 L volume with ddH_2O. Use 6 M HCl to bring pH to 7.0.

2X SSC

20X SSC	5 mL
ddH_2O	45 mL

.1X SSC

20X SSC	250μL
ddH_2O	44.75 mL

Sure Blot Blue Detection Reagents DR Kit. Solutions include:

Detection Solution **I** (Streptavidin)
Detection Solution **II** (Alkaline Phosphatase)
NBT
BCIP

1X Membrane Blocking Solution (M.B.S.)\Detection Solution I

2.5X M.B.S.	4 mL
ddH_2O	6 mL
Detection Solution I	10 μL

Make fresh.

1X SSC

20X SSC	2.5 mL
ddH_2O	47.5 mL

1X M.B.S.\Detection Solution II

2.5X M.B.S.	4 mL
ddH_2O	6 mL
Detection Solution II	10 μL

Make fresh.

10X Wash Buffer

	1 L	Molar []
NaCl	81.82 g	1.4 M
Kcl	2.24 g	30 mM
Na_2HPO_4	21.45 g	80 mM
KH_2PO_4	2.04 g	15 mM

Bring to 1 L volume with ddH_2O. Stir 20 min to dissolve.

1X Wash Buffer

10X Wash Buffer	90 mL
ddH_2O	810 mL

1X Staining Buffer

10X Staining Buffer	12.5 mL
ddH_2O	112.5 mL

1X Staining Buffer + NBT + BCIP

1X Staining Buffer	50 mL
NBT	100 μL
BCIP	100 μL

Procedures:

Labeling

1. Add the following in the order listed to a microcentrifuge tube on ice:
   5 μL 10X reaction buffer
   2 μL Biotin-21-dUTP
   17 μL YAC DNA (1 μg)
   Add 21 μL sterile, dH_2O for a final volume of 45 μL.

2. Prepare an identical tube using 10 μL control lambda DNA or 1 μg of test DNA (instead of YAC DNA) and bring the final volume to 45 μL with sterile, distilled H_2O.

3. Add 5 μL of enzyme mix to each tube.

4. Gently vortex and briefly centrifuge the tubes to mix the contents.

5. Incubate at 16°C for 2½ to 3 h.

6. Place tubes on ice immediately.

7. Prepare a 1% agarose gel containing 50 ng/mL ethidium bromide in a miniature gel bed with 225 mL 1X TAE buffer containing 50 ng/mL ethidium bromide.

8. Add 5 μL of each probe to a separate microcentrifuge tube. Add 5 μL STOP buffer to each tube. Vortex.

9. Load each 10 μL sample and 4 μL phi-X marker onto the gel and run the gel at 90 volts for 35 min or until samples are half way down the gel.

10. View the gel under ultraviolet (UV) light and take a picture of the gel.

11. If >80% of the probe DNA is between 150 and 500 bp (as determined by the phi-X marker) then add 5 μL stop solution to each sample on ice. Pull samples off ice and add 1.5 μL 5% SDS to each. Proceed to column purification steps.

12. If the majority of the probe is <150 bp, then begin the procedure again at step 1 and incubate at 16°C for 2 h or less.

13. If the majority of the probe is >500 bp, add 1 μL enzyme mix to tube. Vortex. Incubate for an additional 30 min at 16°C. Repeat steps 6 to 11.

Column purification of probe

1. Tap the column to settle the matrix.

2. Hold the CHROMA SPIN+ TE-30 upright, snap off the break-away end. Place the end of the column into a sterile microcentrifuge tube. Remove and save the cap.

3. Centrifuge for 3 min at 700 X g.

4. Discard the collection microcentrifuge tube. Place column end into a new tube.

5. Load 50 μL TE + .1% SDS carefully into the center of the column.

6. Centrifuge at 700 X g for 5 min.

7. Measure the volume in the microcentrifuge tube. If the volume is approximately 50 μL, then proceed to step 8. If the volume is >50 μL, then repeat steps 5 to 7.

8. Discard the microcentrifuge tube. Place column end into a new tube.

9. Load probe sample carefully into the center of the column.

10. Centrifuge at 700 X g for 5 min.

11. Sample is in the bottom of the microcentrifuge tube. Do not change tubes. Load 20 μL TE + .1% SDS carefully into center of column.

12. Centrifuge at 700 X g for 5 min.

13. Sample volume should be approximately 70 μL. Proceed to dot blot detection or place sample at -20°C.

Dot blot detection

1. Pipette 9 μL ddH_2O into 6 1.5 mL centrifuge tubes for each sample to be detected.

2. Add 1 μL biotin-labeled sample [10 ng/mL] to first tube.

3. Vortex. Add 1 μL from first tube to second tube.

4. Repeat procedure in step 3 for second and third tubes, third and fourth tubes, and fourth and fifth tubes. The sixth tube will remain ddH_2O. The final concentration of probe DNA in each tube will be: first tube = 1 ng/mL, second tube = 100 pg/mL, third tube = 10 pg/mL, fourth tube = 1 pg/mL, fifth tube = .1 pg/mL, sixth tube = 0 pg/mL.

5. Cut nylon membrane to 15 x 10 cm. With pencil, label each sample and draw six circles for each. With forceps, lay nylon membrane on 50 mL H_2O in small plastic container. Submerge membrane until soaked with H_2O.

6. Pour off H_2O. Add 50 mL 2X SSC. Shake at moderate speed for 5 min.

7. Pour off 2X SSC. Add 50 mL .1X SSC. Shake at moderate speed for 5 min.

8. Remove membrane with forceps and lay on stack of 3M paper. For each sample, load 1 μL from first tube on first dot, 1 μL from second tube on second dot, and so forth for all tubes. Allow membrane to air dry.

9. Pour 10 mL 1X M.B.S./Detection Solution I into small plastic container. Immerse membrane with DNA side up. Shake gently at RT for 10 min.

10. Place membrane in 150 mL 2X SSC in medium plastic container. Shake gently at RT for 5 min. Pour off 2X SSC and wash 2X more in 150 mL 2X SSC for 5 min each wash, shaking gently at RT.

11. Pour 10 mL 1X M.B.S./Detection Solution II into small plastic container. Immerse membrane with DNA side up. Shake gently at RT 10 min.

12. Place membrane in 225 mL 1X washing buffer in medium plastic container. Shake gently at RT for 5 min. Pour off washing buffer and wash 3X more in 225 mL washing buffer for 5 min each wash, shaking gently at RT.

13. Place membrane DNA side down in 75 mL 1X staining buffer in small plastic container. Shake gently at RT for 2 min. Pour off

1X staining buffer and add 50 mL 1X staining buffer + NBT + BCIP. DNA side should remain down.

14. Incubate at 37°C 8 to 12 h. Check hourly for staining. When the membrane turns a light purple, staining is complete.

15. Immerse filter in 150 mL 75% EtOH in medium plastic container. Shake gently at RT 5 min.

16. Place membrane on stack of 3M paper and bake in oven at 80°C for 30 min.

17. Sample should be detected to at least the 10 pg/mL level or to the same level as a control with known biotin incorporation. Store membrane in the dark at RT.

IN SITU HYBRIDIZATION

Solutions\Reagents:

Sheared salmon sperm DNA

ssDNA	1 g
NaOH (1M)	40 mL
ddH_2O	60 mL

Gently agitate overnight at RT. Place in boiling H_2O for 45 min. Place on ice for 15 min. Neutralize to pH 4 to 7 using approximately 3 mL glacial acetic acid. Pour supernatant into 2 new tubes. Add 100 mL 100% ETOH to each tube. Place at -20°C overnight. Centrifuge for 10 min at 10,000 RPM. Add 10 mL 80% ETOH to each tube. Centrifuge for 10 min at 10,000 RPM. Dry 10 min. Dissolve in 80 mL ddH_2O. Adjust to [10 μg/mL]. Force 10 mL through a series of 16, 18, 20, 21, 22 gauge needles. Pass through each size 20X.

3 M Na Acetate

Na Acetate	81.6 g

Bring to 200 mL volume with ddH_2O. Use acetic acid to adjust pH to 5.2.

Deionized Formamide

Ultrapure formamide	500 g
Amberlite	25 g

Stir overnight at 4°C. Protect from light. Filter. Store at 4°C.

10% Dextran Sulfate

Dextran Sulfate	10 g
ddH_2O	100 mL

Hybridization Solution

Deionized formamide	5 mL
10% Dextran Sulfate	2 mL
20X SSC	1 mL

Vortex well. Store in 1 mL aliquots at 4°C.

Denaturing Solution

Deionized formamide	70 mL
20X SSC	10 mL
ddH_2O	18 mL

Using 6 M HCl, adjust pH to 7.0. Bring to 100 mL volume with ddH_2O. Make fresh.

Formamide Wash Solution

Deionized formamide	75 mL
20X SSC	15 mL
ddH_2O	58 mL

Using 6 M HCl, adjust pH to 7.0. Bring to 150 mL volume with ddH_2O. Make fresh.

2X SSC

20X SSC	15 mL
ddH_2O	135 mL

Fluorescein Detection of Biotin Labeled Probes Kit. Solutions include:

Fluorescein-Labeled Avidin
Anti-Avidin Antibody (Ab)

4X SSC\.1% Tween 20

20X SSC	100 mL
Tween 20	.5 mL
ddH_2O	400 mL

20% Sodium Azide

Sodium Azide	2 g
ddH_2O	10 mL

Blocking Buffer

20X SSC	20 mL
Sterile ddH_2O	60 mL
Tween 20	100 μL
20% Sodium Azide	400 μL
BSA, fraction V	3 g

Combine liquid reagents in sterile 250 mL beaker. Gently swirl BSA on top until all dissolved (30 min). Adjust to 100 mL volume with sterile ddH_2O. Store in 1 mL aliquots at -20°C.

Propidium Iodide Staining Solution

Stock solution (1 mg/mL):

Propidium iodide	10 mg
ddH_2O	10 mL

Protect from light. Store at 4°C.

Staining solution (200 ng/mL):

Stock solution	10 μL
2X SSC	50 mL

2X SSC\.05% Tween 20

2X SSC	50 mL
Tween 20	25 μL

PPD 11 Antifade

PPD	10 mg
PBS	1 mL
Glycerol	9 mL

Dissolve PPD in PBS. Add glycerol. Using 4 M NaOH, adjust pH to 11. Protect from light. Store at -20°C up to 1 wk.

Procedures:

EtOH precipitation, dissolution and denaturation of probe

1. Add the following to a 1.5 mL microcentrifuge tube:

	+ COT	- COT
For YAC probes:		
Biotin-labeled probe DNA	15.0 µL	15.0 µL
COT DNA	2.0 µL	-
Sheared salmon sperm DNA	5.0 µL	5.0 µL
ddH_2O	78.0 µL	80.0 µL
For BAC test probe:		
Biotin-labeled probe DNA	7.5 µL	7.5 µL
COT DNA	2.0 µL	-
Sheared salmon sperm DNA	5.0 µL	5.0 µL
ddH_2O	85.5 µL	87.5 µL

2. Vortex. Add 10 µL 3 M Na Acetate (pH 5.2) to each tube.

3. Vortex well. Add 220 µL ice cold 100% ethanol.

4. Vortex well. Place in -80°C freezer for one hour.

5. Thaw for 10 min. Centrifuge for 20 min at 15,000 RPM at 0°C. White pellet should appear.

6. Pour ethanol off gently. Add 300 µL ice cold 80% ethanol. Centrifuge for 20 min at 15,000 RPM at 0°C.

7. Pour ethanol off gently. Aspirate remaining ethanol, being careful to not aspirate pellet. Mark pellet size and location on outside of tube. Air dry overnight.

8. Add 10 µL 1X hybridization solution directly onto pellet. Vortex. Spin briefly.

9. Place tube in 37°C waterbath for 6 to 8 h. Vortex periodically.

10. Place tube at 4°C overnight.

11. Prewarm tube in 37°C waterbath for 2 to 3 min. Vortex. Spin briefly.

12. Denature probe in 74°C waterbath for 5 min. Vortex. Spin briefly.

13. If probe contains COT DNA, place in 37°C waterbath for 19 min to preanneal.

14. If probe contains no COT DNA, place on ice immediately for 5 min or longer.

15. Bring probe to RT immediately prior to hybridizing to denatured, dehydrated chromosome preparations.

Prehybridization treatment of slides

1. Examine air dried chromosome preparations under a phase contrast microscope at 200X magnification. Select those that have adequate numbers of chromosome spreads. The chromosomes should be moderate to high contrast (gray-black).

2. Place preparations at room temperature for 2 wk. (If preparations are newer than 2 wk, place at 37 to 38.5°C for 2 d.)

3. Incubate slides for 1 hr in 2X SSC at 37°C.

4. Dehydrate slides in a 70%-, 85%-, and 100%-ice cold ethanol series for 2 min each. Allow slides to air dry.

5. Prewarm slides to 37 to 42°C.

6. Prewarm denaturation solution in 50 mL coplin jars to 74°C. Check temperature of denaturation solution directly. Plunge slides (1 per jar) into denaturation solution and incubate for 2 min.

7. Rapidly transfer slides to ice cold 70% ethanol. Dehydrate slides in the ice cold 70%, 85%,and 100% ethanol series for 2 min each. Allow slides to air dry.

8. Prewarm slides to 37°C.

Probe hybridization and posthybridization washes

1. Spot 10 μL probe in hybridization solution onto denatured, prewarmed slide. Mount with 22 x 22 mm coverslip and seal with rubber cement. Mark the location of the probe on a blank slide with a permanent marker.

2. Place slides in humidified chamber prewarmed to 37°C. Allow hybridization to occur for 16 h at 37°C.

3. Remove rubber cement. Wash slides 3X in formamide wash solution at 45°C for 5 min each wash. Do not shake. Coverslip should float off in first wash.

4. Wash slides 3X in 2X SSC at 45°C for 5 min each wash. Do not shake.

5. Place slides in room temperature 4X SSC/.1% Tween 20 if detection is to be done immediately. Slides may be stored for 1 to 7 d in 4X SSC before detection.

Detection and amplification of probe

Do not allow slides to dry out at any point during the detection and amplification procedures!

1. Remove slides from wash bath one at a time and spot 100 μL blocking buffer on each slide. Mount with a 22 x 60 mm plastic coverslip or parafilm. Incubate for 30 min in humidified chamber at 37°C.

2. Remove coverslip. Drain slide. Add 60 μL fluorescein-labeled avidin. Mount with same coverslip and incubate for 20 min in humidified chamber at 37°C.

3. Remove coverslip and wash slides 3X in 4X SSC/.1% Tween 20 at 45°C for 5 min each wash with shaking.

4. Perform amplification on all YAC probes. BAC probe may be stained at this point.

5. For amplification, remove slides from wash solution one at a time. Spot 100 μL blocking buffer on each slide. Mount with new plastic coverslip or parafilm and incubate at room temperature for 5 min.

6. Remove coverslip. Drain slide. Add 60 μL anti-avidin antibody. Mount with same coverslip and incubate for 20 min in humidified chamber at 37°C.

7. Remove coverslips and wash slides 3X in 4X SSC/.1% Tween 20 at 45°C for 5 min each wash with shaking.

8. Remove slides from wash bath one at a time and spot 100 μL blocking buffer on each slide. Mount with new plastic coverslip or parafilm. Incubate 5 min at room temperature in humidified chamber.

9. Remove coverslip. Drain slide. Add 60 μL fluorescein-labeled avidin . Mount with same coverslip and incubate 20 min in humidified chamber at 37°C.

10. Remove coverslips and wash slides 3X in 4X SSC/.1% Tween 20 at 45°C for 5 min each wash with shaking.

11. Place slides in 4X SSC at room temperature. Perform chromosome staining and microscopy.

Chromosome staining and microscopy

1. Stain slides in propidium iodide staining solution (200 ng/mL) for 10 min.

2. Rinse slides in 2X SSC/.05% Tween 20 for 2 min at RT.

3. Spot one drop of PPD 11 antifade onto each slide and mount with 22 x 50 mm glass coverslip. Seal with clear fingernail polish. Allow polish to air dry.

4. Locate chromosome spreads using fluorescent microscope with PI filter. View probe and bands with FITC filter.

5. Photograph using Fujichrome 400 slide film. On MC 80 camera, bracket manual exposure times from .5 s to 4 s for pictures with PI filter. For pictures with FITC filter, bracket manual exposure times from 1 s to 8 s.