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(54) Title: METHODS OF DETERMINING THE GENDER OF POULTRY

(57) Abstract

The present invention relates in general to a method of determining the gender of poultry, and more particularly ratites, via the hybridization of DNA In the hybridization technique, the blood cells in a sample withdrawn from a bird are lysed, and the DNA from the lysed cells are affixed to a filter membrane. A labeled probe that is an oligonucleotide which hybridizes with a series of unique target gender specific oligonucleotides is then added to the filter membrane. If the female specific target oligonucleotides are present in the sample, the probe will bind to the complementary oligonucleotide sequences in the sample DNA affixed to the filter membrane. The label on the probe will then allow the filter membrane to be read. The present invention further relates to a general labeling technique using latex particles bound to a probe. This invention also relates to a method of determining the gender of poultry via the monitoring of reproductive hormone levels. This method comprises withdrawing blood from a bird, injecting the bird with an FSH composition, again withdrawing blood from the bird, and comparing the plasma concentrations of testosterone before and after the injection.

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# METHODS OF DETERMINING THE GENDER OF POULTRY

## Cross-Reference to Related Applications

This is a continuation-in-part of U.S. Patent Application Serial No. 08/202,909, filed February 28, 1994, which is incorporated herein by reference.

## Field of the Invention

The present invention relates in general to a method of determining the gender of poultry, and more particularly ratites. The present invention more particularly relates to determining the gender of ratites by DNA hybridization techniques using, for example, latex particles as a label. This invention also relates to a method of determining the gender of poultry via the monitoring of reproductive hormone levels.

## Background of the Invention

Ratites are flightless, generally large, running birds, comprising several orders including the species Ostrich, Emu, Rhea, Cassowaries, and Kiwis. An emu (*Dromiceius novaehollandiae*) is an Australian ratite bird. An average adult emu is approximately 6 feet tall and weighs approximately 150 pounds. A standard adult ostrich (*Struthio camelius*) is approximately 8 feet tall and weighs approximately 325 to 375 pounds, making them the largest living avian species.

The ostrich and emu have long had commercial value in their natural environments of South Africa and Australia, respectively. Ostrich products have been in demand for more than 100 years, and a substantial worldwide market exists for their hides, meat, and feathers. For example, ostrich leather is used in boots, handbags, jackets, attache cases, wallets, and many other articles. Ostrich feathers are used in fashion, costuming, and in feather dusters.

In contrast, the emu is a relative newcomer to the market place. It is valued for the same products with the addition of an essential oil that is used in the cosmetic industry. Emu oil, rendered from a thick layer of subcutaneous fat has deep penetrating properties which make the oil useful in cosmetic creams, such as, wrinkle retardant emollients. Also, possible medicinal uses for emu oil, such as the treatment of arthritis, are currently being investigated. A typical full-grown emu can achieve a height of 1.6 to 1.9 m or more, and weight 30 to 45 kg or more. Emus mature at about one year, and pre- and post-pubescent emus never show gender specific phenotypic differences. Similar to the ostrich, the emu population in the United States has also experienced explosive growth within the last several years. As of 1994, there were approximately 150,000 total emu, including 15,000 breeding pairs, in the United States. It is predicted that the numbers of emus in 1995 will further increase to between 500,000 and 750,000 birds, of which 45,000 are expected to be breeding pairs.

There is a growing demand for ratite products in several countries, including Australia, Belgium, Canada, the Netherlands, Namibia, Israel, South Africa, and Zimbabwe. Accordingly, over the past several years there has been explosive growth in the domestic market for ostrich and emu, and to a lesser extent rhea. In the last five years, the number of breeding ostrich pairs and total bird numbers in the United States has increased 7.5- and 20-fold, respectively. It is estimated that in 1995, 200,000 ostrich, including 20,000 breeding pairs, will exist in the United States. The tremendous interest in breeding these animals is due to the

significant value of adults, as well as immature animals, and especially for proven breeding pairs of ostrich. A majority of the animals are purchased between three and six months of age.

Further, there is interest in ratites as an alternative to more traditional forms of animal agriculture. Several factors relating to ratites make them a superior alternative to the more traditional forms of animal agriculture (i.e., cattle, swine and sheep farming). These factors include: superior feed conversion ratios, a greater ability to be intensively farmed, large animal size, enhanced reproductive capacity, and exceptional nutritional value of their meat. For example, ostrich meat, which is a red meat resembling beef, contains significantly less fat, calories, and cholesterol than chicken or turkey meat. More particularly, a typical 85 gram portion of Ostrich meat contains 2 grams of fat, 58 mg of cholesterol, and 97 calories. In contrast, an 85 gram portion of turkey meat contains 3 grams of fat, 59 mg of cholesterol, and 135 calories. An 86 gram portion of chicken meat contains 3 grams of fat, 73 mg of cholesterol, and 140 calories. An 85 gram portion of beef (steak) contains 15 grams of fat, 77 mg of cholesterol, and 240 calories. And finally, an 85 gram portion of pork contains 19 grams of fat, 84 mg of cholesterol, and 275 calories. (Values for ostrich meat were derived from AMSI Quality Laboratory Report # 0800100. Values for the other meats were derived from U.S.D.A. Handbook No. 8, "Nutritive Value of Foods".) Similar to the ostrich, emu meat is a low fat red meat. More particularly, a 100 gram portion of emu meat contains 1.7 g fat, 57.5 mg cholesterol, and 109 calories. (Values for emu meat were derived from Silliker Laboratories of Texas, Inc.)

Ratites such as ostriches provide approximately 100 pounds of meat at the age of 12 months and therefore produce a substantial amount of meat in a relatively short period of time. An illustration of how ratites are a superior alternative to the more traditional forms of animal agriculture is the following comparison of an ostrich and a cow. First, an ostrich has a gestation and incubation period of 42 days, wherein a cow

requires 280 days. Second, an average female ostrich produces more than 20 offspring per year, whereas a cow produces one offspring per year. Third, the feed conversion ratio of an ostrich is less than 2:1 whereas the feed conversion ratio of a cow is 5:1. Fourth, the days from conception to slaughter is approximately 407 days for an ostrich in contrast to 645 for a cow. Finally, ostriches produce feathers in addition to their meat and leather, whereas cows do not produce products other than meat and leather.

Considering these attributes, and the increasing demands of the world population for meat which is nutritious yet low in fat and cholesterol, and which may be efficiently produced with a minimum negative impact on the environment, the ratite industry has a high potential for future growth.

Currently, the demand for ratites far outweighs the supply. However, ratite producers are hampered by an inability to accurately determine the gender of chicks intended for breeding stock. No sexual dimorphism occurs in ratites as immature animals. For example, ostriches show plumage differences only upon sexual maturity which is at approximately 2 years-of-age. In some ratites, even the mature birds do not show sexual dimorphism. For example, emus never display sexual dimorphism.

Vent sexing or palpation of the sexual organs is one of the most widely used techniques that is currently used to determine the gender of a ratite. However, vent sexing is no more than 80% accurate, it may possibly injure the bird while one conducts the examination, and it increases the risk of spreading disease among ratites. This level of inaccuracy is unacceptable in an industry where it is critical to know the gender of the ratite at the time of its sale as their cost is high in anticipation of the ratite being used in breeding operations.

Another technique that may be used is surgical gender determination. The morphological characteristics of the internal reproductive organs is observed to determine the gender of the poultry. However, surgical determination is invasive, and there is

the risk of injury or death to the bird being studied. Further, this method requires that the bird be anesthetized, that the surgery be performed in sterile conditions, and that a veterinarian perform the surgery. Therefore, although this method is accurate, it is expensive, time consuming, and risky. Generally, this method is undertaken only when other surgery is required to be performed.

In an effort to develop a more accurate and less expensive method for determining the gender of poultry, the structure and function of the gender determining chromosomes in a variety of species have been studied. In most species studied to date the single pair of gender determining chromosomes show morphological, chemical structure, or cytochemical staining differences from the autosomal chromosomes. The morphological difference is typically in the form of reduced size and condensed appearance. The chemical analysis of the gender determining chromosomes also indicates a large portion of non-coding DNA, termed heterochromatin. Therefore, most gender specific chromosomes are termed condensed heterochromatin. Avian species gender chromosomes are designated Z and W, the male having the ZZ karyotype, and the female having the WZ karyotype. Therefore, in avian species the female gamete determines the gender of the embryo.

A variety of methods for determining the gender of animals is known in the art. For example, U.S. Patent No. 5,055,393 teaches a method for determining the gender of mammalian embryos and fetuses employing male-specific nucleic acid probe hybridization methods to assay the genomic DNA of embryonic or fetal cells for the presence of male-specific sequences. However, this method is complex and time consuming. Under optimized conditions, the assay which comprises Polymerase Chain Reaction ("PCR") amplification and non-radioactive detection techniques, requires 5 to 6 hours to complete.

U.S. Patent No. 4,960,690 also describes a method for determining the gender of mammalian embryos and fetuses employing male-specific nucleic acid probe hybridization methods

to assay the genomic DNA of embryonic or fetal cells for the presence of male-specific sequences. This method is even more complex and time consuming than the '393 method described above. The method comprises carrying out hybridizations with labeled probe prepared from male DNA, with increasing dilutions of total male and total female DNAs and determining the smallest quantity of DNA at which hybridization with the male DNA is distinguishable from that with the female DNA when hybridization and detection are conducted over a period of time. The '690 patent discloses that this method of determining the gender of a mammal may be completed in less than a few days.

Several studies have been completed on the gender determining chromosomes of poultry. For example, one study discloses that in the domestic chicken the W chromosome contains a multiple repeat segment of 17 base pairs (repeated up to 10,000 times) that is absent in the Z chromosome. (Kodoma, H., et al, Chromosoma *(Berl),* 96:18-25, 1987). The repetitive 17 base pair sequence was established through the use of restriction analysis. Several laboratories have confirmed this sequence or very similar sequences in a number of avian species. Specific repetitive sequences on W chromosomes in similar avian species, such as turkeys, ducks, quail, and larks, have been determined. (Griffiths et al., *Chromosoma,* 99:243-250, 1990; Saitoh et al., *Chromosoma (Berl),* 98:250-258, 1989; Saitoh et al, *Chromosoma*, 101:32-40, 1991).

These W chromosome-specific sequences have been used to determine gender in the domestic chicken through a relatively simple dot blot procedure as described in Uryu N., et al., *Poultry Science,* 68:850-853, 1989. Briefly, the procedure involves using a biotinylated detection probe specific to the female W chromosome repeat sequences, with the specimen genomic DNA bound to a membrane. A hybridization reaction is performed using the biotin labeled detection probe, and the presence of female or W specific sequences is detected using strepavidin conjugated with alkaline phosphatase. Visualization is by

radiographic film placed over the blots in the presence of a chemiluminescent substrate for alkaline phosphatase.

One currently available DNA based gender diagnostic for poultry is produced by Zoogen. This method requires a blood sample of the poultry to be sent to their facility where a Restriction Fragment Length Polymorphism ("RFLP") analysis of its DNA is conducted to determine the gender of the bird. This method of determining gender also requires a veterinarian for obtaining the blood sample, and requires highly trained personnel for analyzing the sample. The results are received by return mail, and the entire process takes a minimum of approximately two weeks. Accordingly, this method of determining gender is expensive due to the shipping costs, the equipment needed to perform the RFLP analysis, the requirement for highly trained personnel to analyze the DNA, and the requirement for a veterinarian to collect the sample. Therefore, this diagnostic is quite expensive, time consuming, and is not amenable for use in the field.

. As discussed above, methods for determining the gender of ratites are known in the art, yet these methods are complex, expensive, time-consuming, and sometimes inaccurate. Currently, at the age of sale of the majority of ratites, usually after 3 months-of-age, there is no simple, reliable, cost-effective means of positively determining the gender of ratites. This is a very serious problem in the industry, and it costs breeders, brokers and investors millions of dollars annually. Therefore, there is a continuing need for a simple, reliable, cost-effective assay for the determination of the gender of ratites such as ostriches and emus. There is also a need for such an assay which is rapid and can be used in the field by untrained personnel.

## Summary of the Invention

The present invention addresses the need for a simple, reliable, cost-effective assay for the determination of the gender of poultry, and more particularly ratites such as ostrich and emu. The present invention also addresses the need for an assay for the

determination of the gender of poultry such as ratites which is rapid and can be used in the field by untrained personnel.

The present invention relates, in general, to a method of determining the gender of poultry, and more particularly ratites, via hybridization of DNA. Briefly described, one embodiment of the present invention comprises obtaining a sample of blood cells from a bird, lysing the blood cells, and affixing the DNA from the lysed cells to a solid phase. A labeled probe (detection probe or probe) that is an oligonucleotide which hybridizes with unique target female specific oligonucleotides is then added to the filter membrane. If the female specific target oligonucleotides are present in the sample, the probe will bind to the complementary oligonucleotide sequences in the sample DNA affixed to the filter membrane. The label on the probe will then allow the filter membrane to be read. The label may be any detectable label such as horseradish peroxidase, biotin, alkaline phosphatase, and ß-galactodsidase. A preferred label is horseradish peroxidase. Another preferred label is biotin. A preferred means of reading the presence or absence of a biotin labeled probe is the addition of avidin conjugated with horseradish peroxidase. Another preferred means of reading the presence or absence of a biotin labeled probe is the addition of strep-avidin conjugated with horseradish peroxidase.

A preferred probe comprises the purified and isolated nucleotide sequence comprising SEQ ID NO:3. Another preferred probe comprises a fragment of SEQ ID NO:3 capable of hybridizing with a unique target female specific oligonucleotide sequence in genomic ratite DNA. Yet another preferred probe comprises the purified and isolated nucleotide sequence comprising SEQ ID NO:4. The present invention is also directed to host cells containing recombinant DNA molecules comprising SEQ ID NO:3, SEQ ID NO:4, or fragments thereof.

The DNA from the lysed blood cells of the sample may be affixed directly to the solid phase, or in the alternative, the DNA may be affixed to an oligonucleotide sequence (capture probe) that hybridizes with unique target female specific oligonucleotides

where the capture probe is affixed to the solid phase. Where the term "capture probe" is used in the method, it is to be understood that the capture probe is an oligonucleotide sequence that hybridizes with different unique target female specific oligonucleotides of the sample DNA than the detection probe hybridizes to. It is also to be understood that the terms "probe" and "detection probe" are used interchangeably throughout the specification to denote an oligonucleotide sequence capable of hybridizing with female specific target oligonucleotide sequences and which is used for detecting the same. In contrast, the term "capture probe" denotes an oligonucleotide sequence that is used to capture unique target female specific oligonucleotides, by hybridizing with the same, such that a detection probe can later be used for detection purposes.

In another embodiment of the method of the present invention, the method comprises the step of adding to the sample a restriction endonuclease enzyme which cleaves a site in the male specific oligonucleotide sequences but does not cleave a site in the female specific oligonucleotide sequences. After the restriction endonuclease enzyme digestion treatment, a detection probe is added to the sample. The addition of such a restriction endonuclease enzyme allows for the use of a detection probe in the method that ordinarily hybridizes with both male and female specific oligonucleotide sequences because the enzyme cleaves a site in only the male specific oligonucleotide sequences such that the probe can no longer hybridize with the male specific oligonucleotide sequences.

The present invention further relates to a general labeling (assay/detection) technique using particles bound to a nucleic acid probe, and to a method of detecting the presence of a target substance in a medium using a probe which is bound to a particle. As used herein, the term “particle” means a bead or particle that can be optionally generally spherical. However, it is to be understood that the particle can be shapes other than spherical. The particle may be colored or labeled by means well known to those of ordinary skill in the art. The label includes, but is not

limited to, radioactive compounds or elements, enzymes, luminescent or chemiluminescent labels, and fluorescent labels. The method of detecting the presence of a target substance in a medium using a probe bound to a particle comprises the step of contacting the medium with the probe, allowing the probe to selectively interact with the target substance, removing the uninteracted probe, and detecting the presence of interacted probe by the color of the particle or by the label on the particle. Preferably, the particle is colored, and is preferably a latex particle.

The use of particles in analyses results in more accurate, more rapid, and more simple analyses. The use of particles for the visualization of the results eliminates the need for the use of other protein based visualization systems such as biotin-avidin-enzyme-substrate, or antigen and enzyme conjugated antibody reactions. The reduction of the amount of protein involved in the analysis reduces background problems which arise from the affinity of protein to various media such as plastic and cellulose. The reduction of background problems therefore increases the sensitivity of the analysis.

This invention also relates to a method of determining the gender of a bird via the monitoring of its reproductive hormone levels. This method comprises withdrawing blood from a bird and measuring testoterone concentrations in the blood, administering a composition comprising follicle stimulating hormone ("FSH") to the bird, again withdrawing blood from the bird, and comparing the plasma concentrations of testosterone before and after the injection. If the testosterone level of the bird remains relatively constant after the administration of the FSH composition, the bird is female. If the testosterone level of the bird after the administration of the FSH composition increases, then the bird is male. The FSH composition is comprised of FSH and leutinizing hormone ("LH"). Preferably, the FSH composition further comprises pregnant mare serum gonadotropin ("PMSG"). More preferably, the FSH composition

is comprised of mammalian FSH, mammalian LH, and mammalian PMSG.

Accordingly, it is an object of the present invention to provide a simple, reliable, cost-effective assay for the determination of the gender of poultry, and more particularly ratites.

It is another object of the present invention to provide an assay for the determination of the gender of poultry, and more particularly ratites, which is rapid and can be used in the field by untrained personnel.

It is yet another object of the present invention to provide an assay for the determination of the gender of poultry, and more particularly ratites, via the hybridization of DNA.

It is a further object of the present invention to provide an assay for the determination of the gender of poultry, and more particularly ratites, via monitoring hormone levels.

It is another object to provide an assay for the determination of the gender of poultry, and more particularly ratites, which may be utilized on all ages of poultry at any point in their development or at any time with respect to their breeding season.

It is yet another object of the invention to provide a field usable DNA based diagnostic for determining the gender of poultry in approximately one hour.

It is a further object of the present invention to provide a diagnostic assay having a minimum amount of protein present.

It is another object of the present invention to provide a diagnostic assay which is rapid, and has reduced background levels.

These and other objects, features and advantages of the present invention will become apparent after a review of the following detailed description of the disclosed embodiments and the appended claims.

## Brief Description of the Drawings

Figure 1 is a representation of the filter membrane in the gender determining diagnostic kit of the present invention, and the possible outcomes of the assay.

Figure 2 illustrates the quantitative results of radio-immunoassays for testosterone on plasma obtained from six different emus on ten consecutive days, wherein each emu was challenged with exogenous FSH composition on days 4-6.

Figure 3 illustrates the quantitative results of radio-immunoassays for androstenedione on plasma obtained from six different emus on ten consecutive days, wherein each emu was challenged with exogenous FSH composition on days 4-6.

Figure 4 illustrates the quantitative results of radio-immunoassays for testosterone and androstenedione on plasma obtained from a male emu, emu A, on ten consecutive days, wherein emu A was challenged with exogenous FSH composition on days 4-6.

Figure 5 illustrates the quantitative results of radio-immunoassays for testosterone and androstenedione on plasma obtained from a male emu, emu B, on ten consecutive days, wherein emu B was challenged with exogenous FSH composition on days 4-6.

Figure 6 illustrates the quantitative results of radio-immunoassays for testosterone and androstenedione on plasma obtained from a male emu, emu C, on ten consecutive days, wherein emu C was challenged with exogenous FSH composition on days 4-6.

Figure 7 illustrates the quantitative results of immunoassays for testosterone and androstenedione on plasma obtained from a male emu, emu D, on ten consecutive days, wherein emu D was challenged with exogenous FSH composition on days 4-6.

Figure 8 illustrates the quantitative results of radio-immunoassays for testosterone and androstenedione on plasma obtained from a female emu, emu E, on ten consecutive days, wherein emu E was challenged with exogenous FSH composition on days 4-6.

Figure 9 illustrates the quantitative results of radio-immunoassays for testosterone and androstenedione on plasma obtained from a male emu, emu F, on ten consecutive days, wherein emu F was challenged with exogenous FSH composition on days 4-6.

Figure 10 illustrates the quantitative results of radio-immunoassays for testosterone on plasma obtained from six female quail and six male quail in response to exogenous FSH composition challenge on days 1, 2, and 3. Each bar in figure 10 illustrates the average testosterone level of all of six male quail or all six female quail.

Figure 11 is a photograph of low astringency PCR amplification from the genomic DNA of two female and two male ostriches. As can be seen from the female genomic DNA, the PCR yielded a single band that is not found in the male genomic DNA.

Figure 12 illustrates the genomic southern blot of SEQ ID NO:3 hybridized with DNA from individual female ostriches (lanes. 1-6) and individual male ostriches (lanes 7-12), wherein the DNA was first digested with Pst I. The two center wells are pre-biotinylated molecular weight markers, a mixture of Hind III digested lambda DNA and Hae III digested øx174 DNA.

Figure 13 illustrates a restriction map of SEQ ID NO:3, the 1708 base pair gender probe from the gel in Figure 12 inserted into a pUC 19 vector, wherein the fragment Sma1 to Sma1 represents the 1708 base pair gender probe, wherein the Kpn1-BamH1 fragment is the large or "L" fragment, and wherein the Xba1-Xba1 fragment is the small or "S" fragment was used for testing the sex of ostriches and emus. The fragment Xba1 to Sma1 represents SEQ ID NO:4, the 924 base pair gender probe.

Figure 14 illustrates the results of simple dot hybridization of male or female ostrich DNA, from whole blood, hybridized with SEQ ID NO:3 of the L fragment.

Figure 15 illustrates the results of simple dot hybridization of ostrich or emu DNA, from either male or female whole blood, the top four rows representing samples from either a male or

female bird hybridized with SEQ ID NO:4 of the S fragment of the gender probe, and wherein the bottom four rows represent a simple dot hybridization of samples from a male or female bird with SEQ ID NO:3 of the L fragment of the gender probe.

Figure 16 illustrates the results of simple dot hybridization of ostrich or emu DNA from either male or female whole blood, the top four rows representing samples from either a male or female bird hybridized with SEQ ID NO:4 of the S fragment of the gender probe, and wherein the bottom four rows represent a simple dot hybridization of samples from a male or female bird with SEQ ID NO:4 of the L fragment of the gender probe.

**Detailed Description**

The present invention relates, in part, to a method and test kit for determining the gender of poultry via the hybridization of DNA. More particularly, the present invention relates to a method for determining the gender of a ratite. Briefly stated, the method comprises the steps of obtaining a sample of the ratite's blood, lysing the blood cells in the sample, contacting the sample with a labeled oligonucleotide sequence (probe) which hybridizes to target female specific oligonucleotide sequences, allowing the labeled probe to hybridize with female ratite DNA if it is present in the sample, separating any labeled probe which did not hybridize with the sample from any labeled probe that did hybridize with the sample, and determining the presence or absence of labeled probe hybridized to the sample.

In one embodiment of the present invention, the blood cells in a sample withdrawn from a bird are lysed, and the DNA from the lysed cells are affixed to a solid phase. A probe that is an oligonucleotide which hybridizes with unique target female specific oligonucleotides (hereinafter "the probe") is labeled, and is then added to the solid phase. If the female specific target oligonucleotides are present in the sample, the probe will bind to the sample DNA which is bound to the solid phase. The label on the probe will then allow the visualization of the presence of the gender specific DNA thereby indicating that the bird is female.

The method of the present invention is facilitated in avian species because the red blood cells of this group are nucleated, providing sufficient quantities of DNA in a very small and easy to collect sample. Blood may be obtained from a ratite by any method known in the art. For example, the blood may be obtained by pulling a feather from the bird and collecting the blood in the quill of the feather, or the blood may be obtained by lancet. Also, the blood may be obtained during a surgical or other procedure performed on the bird. For example, identification microchips are often surgically implanted in the pipping muscle of an ostrich or emu while it is hatching or one day after it has hatched. Blood may be simultaneously obtained for the determination of the bird's gender while the microchip is being implanted in the bird. The method of the present invention requires between approximately 10 μL and 500 μL of blood. Preferably, the procedure requires approximately 100 μL of blood. Therefore, approximately 100 μL or more of blood is to be obtained from the bird whose gender is to be determined.

The blood cells in the sample can be lysed by any method known in the art including, but not limited to, chemical lysis and mechanical lysis. Chemical lysis of cells can be achieved using any of a number of types of solutions. Chemical lysis of cells is preferred to minimize equipment needed to perform the method and to minimize the cost of the method. For example, a 0.5 N NaOH solution is preferable as it lyses the red blood cells in the sample of blood, denatures the DNA and other proteins in the sample, and clears the sample by oxidizing the heme iron so that the solution is no longer red.

Visualization techniques in hybridization assays are well known in the art. The labels used to visualize the probe of the present invention include, but are not limited to, bioluminescent labels, chemiluminescent labels, fluorescent labels, and radioactive labels. The determination of whether the target gender specific oligonucleotide sequences are present in a sample may be conducted by the following corresponding means:

luminescent means, enzymatic means, fluorometric means, and radiometric means.

The preferred visualization means of the present invention is a means which is inexpensive, rapid, and does not require specialized laboratory equipment or trained personnel. Accordingly, enzymatic visualization means are a preferred method of determining the presence of target oligonucleotide sequences. Although any of the enzyme/substrate systems known in the art may be used in the present invention, preferred enzymes include horseradish peroxidase ("HRP"), alkaline phosphatase, and ß-galactosidase. Another preferred visualization system is avidin conjugated to an enzyme, wherein the oligonucleotide sequence (probe) is labeled with biotin. Enzymes that can be used in the biotin:avidin visualization system are well known to those of ordinary skill in the art.

A desired label is horseradish peroxidase ("HRP") and the preferred substrate for HRP is Nitro Blue Tetrazolium ("NBT"). The label HRP is chemically coupled directly to the probe. After a probe is labeled with HRP, it is allowed to hybridize with the target oligonucleotide sequences. The remaining unhybridized probe is removed from the hybridized probe, and an enzyme substrate is added to the hybridized probe for visualization. Another desirable label is biotin, wherein the biotin labeled probe is detected by adding a strepavidin conjugated enzyme, washing, and then adding a substrate for visualization of the presence of the enzyme. Any enzyme/substrate pair known in the art may be used with the biotin/avidin system. A desirable enzyme/substrate used with the biotin/strepavidin detection system is HRP/NBT.

A unique and unexpected method of determining the presence of target oligonucleotide sequences is by labeling the probe with a latex particle. This method of visualization will be more fully discussed later in the specification.

The probe of the present invention is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA. A preferred probe of the present invention is SEQ ID NO:3. Another preferred probe of

the present invention is any fragment of SEQ ID NO:3 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA. Such fragments are hereinafter referred to as "functional fragments". Yet another preferred probe of the present invention is SEQ ID NO:4. Still another preferred probe is any fragment of SEQ ID NO:4 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA.

Any method known in the art for synthesizing, manufacturing or isolating SEQ ID NO:3 and SEQ ID NO:4 may be used in the present invention. A preferred method of obtaining SEQ ID NO:3 and SEQ ID NO:4 is discussed more fully below. Also, any method known in the art for synthesizing, manufacturing, or isolating fragments of SEQ ID NO:3 capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA, the "functional fragments", may be used in the present invention. A preferred method of obtaining such functional fragments are described in Examples 9 and 10.

Hybridization techniques are well known in the art. See Nucleic Acid Hybridization, A Practical Approach, edited by B.D. Hames & S.J. Higgins, Practical Application Series, edited by D. Rickwood & B.D. Hames, IRC Press (1985). Therefore, the gender specific target oligonucleotide sequences from the lysed blood cells of a sample may be detected by hybridization with the probe of the present invention by any hybridization techniques known in the art. For example, the target oligonucleotide sequences may be immobilized by covalent bonds to a solid substrate and then hybridized to a labeled probe, or a portion of the target oligonucleotide sequences can be hybridized with an immobilized capture probe and then another portion of the immobilized target oligonucleotide sequences can be hybridized with a second probe (a detection probe) which is labeled, and which interacts with a different portion of the gender specific DNA than the capture probe.

More particularly, in one embodiment of the present invention, the presence of gender specific oligonucleotides in the sample is determined by direct assay. This method comprises affixing the DNA from the lysed cells in the sample to a solid phase, adding a probe comprising DNA complimentary to loci specific to the gender specific DNA, incubating to allow the sample to hybridize with the probe, washing unreacted probe from the solid phase, and reading the filter membrane to determine the gender of the bird tested.

Any solid phase currently used in hybridization assays may be used in the present method. The solid phase can include, but is not limited to, cellulose, nitrocellulose, or glass filters. In a preferable embodiment, the solid phase is a filter membrane or a dip stick. More preferably, the filter membrane is a dipstick which is pre-blocked with a nonreactive protein such as bovine serum albumin (BSA), and pre-spotted. Most preferably, the dipstick has three areas, two of which have been pre-spotted with gender specific genomic DNA or gender specific oligonucleotide sequences. The first area has male specific oligonucleotide sequences bound to that area as the male control. The second area is left unbound. The third area has target female specific oligonucleotide sequences bound to that area as the female control, the target oligonucleotide sequences being complementary to the labeled probe.

It is to be understood that the "probe" of the present invention includes, but is not limited to, an oligonucleotide sequence which hybridizes to target female specific oligonucleotide sequences in female ratite DNA. The probe of the present invention includes, but is not limited to, SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, functional fragments of SEQ ID NO:3, functional fragments of SEQ ID NO:4, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:1, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:3, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:4, and oligonucleotide sequences containing substantially the

same sequence as the functional fragments thereof. The probe of the present invention also includes detection probes and capture probes as described herein.

In another embodiment, gender specific genomic DNA is used to pre-spot the dipstick instead of gender specific oligonucleotide sequences. It is preferred that genomic gender specific DNA be used for pre-spotting the dipstick. Genomic DNA, as used herein, is the totality of genetic information in an organism, and refers to DNA that is obtained by isolating DNA from animal cells. In contrast, oligonucleotide sequences may be derived by many different methods including, but not limited to, recombinant DNA synthesis, chemical synthesis, restriction enzyme ligation, and isolation from animal cells.

The solid phase is pre-spotted and pre-blocked according to methods that are well known in the art. Preferably, the solid phase is pre-spotted by hybridizing the gender specific genomic DNA to the solid phase as follows. First, approximately 1.0µl of approximately 360µg/µl gender specific DNA (male or female) in a solution of Tris-EDTA (5mM Tris - 0.1M EDTA) is spotted on the membrane. The membrane is then heated at 80°C for one hour. The membrane is then prehybridized at 42°C for two hours, preferably in a prehybridization solution having the following concentrations: 5x "SSC"; 5x "Denhardts"; 25 mM NaP04 pH 6.5; 50% Formamide; 0.5mg/ml Salmon Sperm DNA (Sigma) in an aqueous solution. Wherein 20x "SSC" = 3M NaCl, and 0.3M Na Citrate having a pH of 7.0 in an aqueous solution. And wherein 100x "Denhardts" = 2% Ficol; 2% polyvinylpyrrolidone; and 2% BSA (Bovine Serum Albumin) in an aqueous solution. The components of the above compositions are described by their concentration, and all are in aqueous solutions. More particularly, 20x SSC is the stock solution, and 5x SSC is a 4 to 1 dilution of the stock solution with water. Accordingly, 100 ml of the prehybridization solution, by volume or weight, is approximately 25 ml of 20x SSC stock + 5 ml of 100x Denhardts stock + 50 ml Formamide + 50 mg Salmon Sperm DNA +340 mg $NaH_2PO_4$ + 20 ml water.

As shown in Figure 1, where the bird tested is male, only the female control area of the dipstick binds with the labeled probe of the present invention. In contrast, where the bird tested is female, both the specimen area and the female control area bind with labeled probe of the present invention. If all areas of the dipstick bind with the labeled probe, or if none of the areas bind with the labeled probe, then the test is a false test which must be repeated.

Preferably, the rate the sample DNA is affixed to the solid phase is accelerated by heat. Any heat source may be used that is capable of heating the solid substrate and the sample DNA to between approximately 50°C to 90°C. The length of time the solid phase is heated varies according to the solid phase used. Generally, the solid substrate and the sample DNA should be heated until the DNA is affixed to the solid phase. More particularly, the solid phase is heated to a temperature of between approximately 50°C to 90°C, and preferably between 60°C to 80°C for approximately 3-5 minutes with a hairdryer.

After the sample DNA has been affixed to the solid phase, the labeled probe of the present invention is contacted with the sample DNA, and incubated under hybridizing conditions. If the sample DNA is from a female bird, the probe will hybridize with the sample DNA. As stated above, methods of hybridization are well known in the art. Although any hybridization method and conditions known in the art may be used, the following is a preferred hybridization method. The affixed sample DNA is incubated for approximately 15 minutes at 25-42°C in approximately 1.5 to 2.0 ml of an aqueous solution having the following concentrations: 5x SSC; 1x Denhardts; 25 mM $NaPO_4$ pH 6.5; 45% Formamide; 0.2 mg/ml Salmon Sperm DNA; and 400 ng/ml of the probe.

In another embodiment of the present invention, one probe can be used as a capture probe, and another probe can be labeled and used as a detection probe. In this hybridization technique, the capture probe and the detection probe are complementary to different portions of the gender specific oligonucleotide

sequences. The capture probe is affixed to a solid phase portion of a device such as a filter membrane or a dipstick. A sample containing lysed blood cells is brought into contact with the capture probe, and if the target oligonucleotide is present in the sample, it will bind with the capture probe. After the first binding step, a second probe ("detection probe") which is a labeled oligonucleotide with a sequence known to hybridize with different target oligonucleotide sequences than the capture probe, is added to the reaction mixture. If the gender specific target oligonucleotides are present in the sample, the detection probe will selectively bind to the gender specific target oligonucleotides in the sample. The label on the probe will then allow the visualization of the presence of the gender specific DNA thereby indicating that the bird is female.

The DNA-based assay of the present invention may be utilized on all ages of ratites at any point in their development or at any time with respect to their breeding season. Although a ratite's gender may be determined at any time by the method of the present invention, one of ordinary skill in the art would understand that ratites are often tested after hatching, and also at each sale.

Another aspect of the present invention is the probe itself, and the method of its synthesis. As stated above, the probe is comprised of a series of nucleotides which hybridize with one or more unique female specific sequences in a female bird's DNA. It is to be understood that the term "detection probe", as used herein, is a diagnostic probe, or probe, that has been labeled. A diagnostic probe, or probe, as used herein, is an oligonucleotide sequence that is specific for a target oligonucleotide sequence.

*Attempts at obtaining a gender specific probe for ratites.*

Numerous attempts were made to identify probes capable of determining the gender of ratites. More particularly, several literature reported sex specific synthetic oligonucletotides and cloned DNA sequences from reptile (Epplen et al., 1982, *Base sequences of a cloned snake W-chromosome DNA fragment and*

*identification of a male-specific putative mRNA in the mouse*, PNAS, USA 79:3798-3802), avian (Kodama et al., 1987, *Nucleotide sequences and unusual electrophoretic behavior of the W chromosome-specific repeating DNA units of the domestic fowl, Gallus gallus domesticus*, Chromosoma 96:18-25; Saitoh et al, 1989, *Occupancy of the majority of DNA in the chicken W chromosome by bent-repetitive sequences*, Chromosoma 101:32-40; Griffiths and Holland, 1990, *A novel avian W chromosome DNA repeat sequence in the lesser black-backed gull (Larus fuscus)*, Chromosoma 99:243-250), and mammalian (Jeffreys et al., 1985; *Hypervariable 'minisatellite' regions in human DNA*, Nature 314:67-73; Rabenold et al., 1991, *Polymorphic minisatellite amplified on avian W chromosome*, Genome 34:489-493) species were tested for their ability to predict gender in ratites. These sequences either did not hybridize with male or female genomic DNA of ratites, or they hybridized without gender specificity.

For example, bases 1300-2000 of the 2.5 kb sex-specific satellite DNA from the *Colubrid* snake species (Epplen et al., 1982) was screened by a computer program (MacVector™, IBI/KODAK, version 4.5) and a set of primers for Polymerase Chain Reaction ("PCR") were selected. These primers amplified bases 1480-1906 of the snake sex-specific satellite DNA. Primers, 5' CCACTGGCAAAAGTGCATATTAC 3' (SEQ ID NO: 5), and 5' CTACACTGGGGCTCCATTAAGATC 3' (SEQ ID NO:6), were synthesized on a Cruachem PS 250 Automated DNA Synthesizer. PCR amplifications were done in 100 uL reaction mixtures containing 100 ng of genomic DNA, each dNTP at 200 uM, 100 ug/mL BSA, 100 nM of each primer, and 2.5 units of Taq DNA polymerase in standard buffer. Amplification was done using three different $MgCl_2$ concentrations (1.5, 2.5 and 3.5 mM). The samples were overlaid with mineral oil, incubated at 95 C for 5 min and then subjected to 40 cycles (1 min at 95 C, 1 min at 56 C and 2 min at 72 C) in a programmable DNA Thermal Cycler (Eppendorf, Westbury, NY). A final incubation was performed at 72 C for 10 Min.

Amplified DNA was subjected to agarose gel electrophoresis. DNA bands that appeared to be sex-specific were excised and purified using the Prep A Gene DNA Purification Matrix (Bio-Rad).

DNA obtained from each purified band was subjected to the Random Primer Biotinylation Reaction (Amersham Life Science, Arlington Heights, Illinois) and was tested as a probe against ostrich genomic DNA. Ostrich male or female genomic DNA was digested by Hae III enzyme and subjected to agarose gel electrophoresis, alkaline blotting to a positively charged Nytran membrane, and used for Southern hybridization according to the protocol for long DNA biotinylated probes (Tropix, Inc., Bedford, MA). (The protocol is in a manual that comes with the Southern hybridization kit supplied by Tropix.) Several of the probes hybridized equally well with female and male ostrich genomic DNA. Some probes did not show any sequence homology with ostrich genomic DNA, male or female.

With respect to Saitoh et al., hybridizations performed using the Kodama probe on genomic DNA from male or female chickens, ostrich and emu, digested with either Pst I enzyme or Taq I enzyme, showed the usefulness of the Kodoma probe in determining the gender of chickens, but not in determining the gender of ostrich or emu. Also, the same enzyme cuts of turkey (Pst I) and pheasant (Taq I) genomic DNA (Saitoh et al., 1989) resulted in gender specific hybridizations with the Kodoma probe. These results using Kodama's probe with turkey, pheasant, and chicken genomic DNA suggest that sequences present in genomic DNA of turkeys, pheasants, and chickens are absent in ostrich and emu genomic DNA.

With respect to Griffiths and Holland (1990), part of the female specific repetitive DNA sequence (298 bp) from *larus fuscus* was screened on computer software, MacVector™, and an oligonucleotide was selected. (The oligonucleotide selected is a 25-mer illustrated on page 246 of Griffiths and Holland, Fig. 2, base pairs 255 to 279 of P2000-17.) This probe was synthesized and biotinylated using a Cruachem PS 250 automated DNA

synthesizer, and was used as a probe for Southern hybridizations performed on three male and three female ostrich genomic DNA samples cut with BamHI and AluI. Southern hybridizations were done according to procedures described by Tropix, Inc. for biotinylated oligonucleotide probes.

Hybridizations conducted using the above 25-mer oligonucleotide, derived from Griffiths and Holland's black-backed gull probe, with ostrich genomic DNA digested with Pst I did not show any significant sequence homology. Hybridizations performed on ostrich DNA digested with BamHI and AluI showed individual bird sequence recognition, but no sex specificity.

As for Jeffreys et al (1985), the first 25 bp of the 33 bp repeat sequence capable of detecting human mini-satellites was synthesized and biotinylated using a Cruachem PS 250 Automated DNA Synthesizer. (This 25-mer is the first 25 base pairs of the sequence listed in Fig. 1 of Jeffreys et al.) This oligonucleotide was used as a probe for Southern hybridizations with male and female ostrich genomic DNA cut with BamHI, BamHI and EcoRI, BamHI and Hind III, and BamHI and SalI, according to procedures described by Tropix Inc., for biotinylated oligonucleotide probes.

Ostrich genomic DNA hybridized with the oligonucleotide derived from Jeffreys' probe, but this oligonucleotide did not exhibit gender specificity. Therefore, the hyper-variable mini-satellite sequence used here (Jeffreys' 33.15) does not appear to be sex linked in ostriches.

Note, however, that although the Jeffrey's probe did not demonstrate sex specificity in ostriches, in Rabenold et al., 1991, it was reported that the Jeffreys' probe demonstrated sex specificity (female) in the striped-back wren.

*Method of obtaining the probe, and its characterization.*

As described below, low stringency PCR of random 10-mer primers has been successfully employed to identify DNA sequences that are female specific in Struthioniforms. These

DNA sequences hybridize with SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:4, functional fragments of SEQ ID NO:3, functional fragments of SEQ ID NO:4, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:1, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:3, oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:4, and oligonucleotide sequences containing substantially the same sequence as the functional fragments thereof.

To obtain the sex-specific sequence for ostriches, low-stringency PCR was used to screen randomly selected primers for their ability to amplify sex-specific loci. See Richard Griffiths and Bela Tiwari, *The Isolation of Molecular Genetic Markers For the Identification of Sex*, PNAS, USA, Vol. 90, pp. 8324-8326, September 1983. More particularly, several 10-mers were randomly generated, and PCR was conducted using each of the 10-mers separately. Southern Blots with male or female ostrich DNA were conducted with each of the 10-mers. Of the randomly selected and screened primers, the primer SEQ ID NO:2 best amplified sex-specific loci.

To isolate DNA, whole blood was used which was collected into heparinased Vacutainer tubes. The blood was then frozen. After washing twice with phosphate buffered saline ("PBS"), the processed cells were placed in a solution of proteinase K and sodium dodecyl sulfate and incubated until most of the cellular proteins were degraded. The digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extraction, recovered by ethanol precipitation, dried, and resuspended in buffer. See Current Protocols in Molecular Biology, (Laboratory Manuals), Unit 2.2; Unit 2.9.7, 1994, John Wiley & Sons.

PCR amplifications were carried out in 100 µl volumes, containing 50-500ng of genomic DNA, each dNTP at 200µM, 25pkM of primer (SEQ ID NO:2), 2.5 Units of Taq DNA polymerase (Promega, of Madison, Wisconsin), and 1.5mM $MgCL_2$ in standard buffer from Promega. PCR conditions were as follows:

| | | |
|---|---|---|
| 95°C | 5 minutes | |
| 94°C | 1 minute | \ |
| 34°C | 1 minute | 36 cycles |
| 72°C | 3 minutes | / |
| 72°C | 10 minutes | |

For PCR, two DNA samples were used. PCR products were visualized after electrophoresis through a 1.5% agarose gel. Figure 11 is a photograph of the visualization after electrophoresis after low astringency PCR amplification from the genomic DNA of two female and two male ostriches. The first six columns are from 500µg of DNA, and the last six columns from 50µg of DNA. The first two columns in Figure 11 are from 500µg of DNA from each individual female ostrich separately (F1; F2). The next two columns in Figure 11 are from 500µg of DNA from each individual male ostrich separately (M1; M2). The fifth column is from 500µg of DNA from both female ostriches together (F1 plus F2), and the sixth column is from 500µg of DNA from both male ostriches together (M1 plus M2). The center two columns (columns 7 and 8) are the following standards: λHind III; and pBR322-Msp1. Columns 9 and 10 in Figure 11 are from 50µg of DNA from each individual female ostrich separately (F1; F2). The next two columns in Figure 11 are from 50µg of DNA from each individual male ostrich separately (M1; M2). The fifth column is from 50µg of DNA from both female ostriches together (F1 plus F2), and the sixth column is from 50µg of DNA from both male ostriches together (M1 plus M2).

As can be seen from the female genomic DNA in Figure 11, the PCR with SEQ ID NO:2 yielded a single band that is not found in the male genomic DNA.

Female-specific amplified sequence was extracted from the gel shown in Figure 11 by using Prep-A-Gene® DNA Purification Systems (Bio-Rad, Hercules, CA, Catalog #732-6010), and used as a probe for hybridization. The NEBlot™ Phototop™ Kit (New England Biolabs, Beverly, MA.

Catalog #7550) was used for the random primer biotin labeling of DNA. The female specific sequence extracted from the gel illustrated in Figure 11 contains SEQ ID NO:3.

Southern blots were prepared from genomic DNA digested with Pst I, electrophoresed, and transferred to a positively charged nylon membrane (Nytran Plus, Schleicher@Schuell, Keene, New Hampshire), by using alkaline transfer buffer. Hybridization and chemiluminiscent detection were carried out using the parameters described for a long DNA probe in the Southern-Light™, DNA Detection Kit (Tropix, Inc., Bedford, MA, Catalog #SL100C, Version N3, Feb. 1993). The results of the Genomic Southern blot are shown in Figure 12. Figure 12 illustrates the genomic southern blot of the probe containing SEQ ID No:3 hybridized with DNA from individual female ostriches (lanes 1-6) and individual male ostriches (lanes 7-12), wherein the DNA from the ostriches was first digested with Pst I. The two center wells were pre-biotinylated molecular weight markers, a mixture of Hind III digested lambda DNA and Hae III digested øx174 DNA. As shown in Figure 12, the probe containing SEQ ID NO:3 hybridizes with female ostrich DNA, not male ostrich DNA.

As shown in Figure 12, all six genomic southern blots of female ostriches have a specific band that is not present in any of the genomic southern blots of the male ostriches. The sequence of this specific band, SEQ ID NO:3, was cloned into the Smal-site of the plasmid vector pUC19 using methods well known in the art. SEQ ID NO:3 is represented in Figure 13 as the portion of the vector from Sma1 to Sma1. SEQ ID NO:4 is represented in Figure 13 as the portion of the vector from xba1 to Sma1. The portion of the vector from kpn1 to BamH1 is the large or "L" fragment. The portion of the vector from xba1 to xba1 is the small or "S" fragment.

The sequences of the fragments of ostrich DNA, SEQ ID NO:3 and SEQ ID NO:4, were determined by using Sequenase®7-DEAZA-dGTP DNA Sequencing Kit with

Sequenase Version 2.0 T7 DNA Polymerase (United States Biochemical Corporation, Cleveland, OH, Catalog #70990).

The above method is a preferred method of obtaining the probe of the present invention. However, any other method known to one skilled in the art that is capable of producing the probe of the present invention, or probes having substantially the same sequence, may be used. See for example, Sambrook, Fritsch, and Maniatis, *Molecular Cloning, A Laboratory Manual, 2nd Edition*, Vols. I, II, III, Cold Spring Harbor Laboratory Press, 1989.

Although not wanting to be limited by the following hypothesis, it is theorized that the right third, and more particularly the right end (the 3' end), of SEQ ID NO:3 is more specific for the female specific target oligonucleotides in ratites. It is also theorized that the portion of the female ratite genome following after where the PCR primer SEQ ID NO:2 hybridizes with the female ratite DNA is also female specific. In other words, it is theorized that the 1708 base pair sequence, SEQ ID NO:3, may only hybridize with a part of a larger female specific oligonucleotide sequence in the ratite genome, and that upon further investigation it may be discovered that SEQ ID NO:3 is only the first portion of a larger female specific probe capable of hybridizing with a larger portion of the female specific sequence.

The cloned female-specific ostrich sequence was used as a probe for the determination of the sex of an ostrich and emu by using whole blood, without any purification, by simple Dot hybridization. More particularly, the L fragment and the S fragment were used as probes for the determination of the gender of ostriches and emus as described in Examples 20, 21, and 22 below, and as shown in Figures 14 through 16. As can be seen in Figures 14 through 16, the S fragment probe is preferable over the L fragment probe for determining the gender of emus. Also, as seen in Figures 14 through 16, both the S fragment probe and the L fragment probe readily determine the gender of ostriches.

As stated above, the L fragment, the S fragment, SEQ ID NO: 3, SEQ ID NO: 4, probes containing substantially the same

sequence thereof, and fragments of all of these probes that are capable of hybridizing with female specific target oligonucleotide sequences of ratites may be used in the method of the present invention.

In another embodiment of the invention, the probe is obtained by using Representational Difference Amplification (RDA) on genomic DNA. (See Lisitsyn et al., 1993, *Cloning the differences between two complex genomes*, Science 259:946-951 which is incorporated herein by reference).This system clones the differences between two complex genomes. Subtractive and kinetic enrichment is used to purify restriction endonuclease fragments that are present in one population of DNA fragments but not in another. This method is used to isolate unique DNA sequences such as those found within gender chromosomes. In principle, this method can be used to isolate unique DNA sequences such as those found within sex chromosomes.

Briefly, RDA is based on PCR amplification of genomic sequences previously digested with restriction endonucleases that cut DNA relatively infrequently. Digested DNA is ligated to oligonucleotide adapters and amplified by PCR. This step is performed for both tester and driver genomes, with tester being the genome that is targeted for isolation of specific differences. Here, the tester genome would be the W genome (female), and the driver genome would be the Z genome (male). In a second step, both PCR reactions from tester and driver samples are melted and reannealed together in the presence of excess driver amplicon. Driver DNA acts as a competitive inhibitor for the self-annealing of those tester DNA fragments common to the driver DNA. Only self-reannealed tester molecules have 5' adapters at each end of the duplex DNA, and thus are filled in at both 3' ends. Therefore, only self-reannealed tester fragments are subsequently amplified in PCR using the primers represented in the ligated adapters (Lisitsyn, et al., *Cloning the Differences Between Two Complex Genomes*, SCIENCE, 259: 946-951, 1993.)

This process of PCR, reannealing-subtraction and re-PCR is repeated a number of times, and the final PCR products are

cloned. Each cloned DNA fragment is then subsequently used as a probe in Southern dot blots to test whether it is specific for male or female DNA. To ensure that different regions of the genomes are represented, a number of different restriction enzymes are used in the initial digestion of the genomic DNA and the ligation of different adapters.

Another embodiment of the present invention is a method of obtaining a probe by the technique called Random Amplified Polymorphic DNA ("RAPD"). RAPD utilizes amplification of genomic DNA with single short (10-nucleotide) primers of arbitrary sequence (Levin et al., 1993, *Genetic map of the chicken Z chromosome using Random Amplified Polymorphic DNA (RAPD) markers*, Genomics 16:224-230 which is incorporated by reference). DNA fragments produced by RAPD that are different between male and female ratites are cloned and tested independently for their potential as sex specific probes.

Yet another embodiment of the present invention is a method for obtaining a probe of the present invention by isolating gender specific chromosomes and using that highly enriched DNA in an RDA protocol. Morphological differences have been reported in the gender chromosomes in emu and rhea. Capitalizing on these differences, a technique called chromosome sorting using dual beam laser separators and flow microflouremetry is used.( See Hashimoto, K., *Flow Karyotyping and Chromosome Sorting*, Japanese Journal of Clinical Medicine, Oct. 50(10), 2484-2488, 1992; and Albright, K. et al, *Molecular Mapping of Mouse Chromosomes 4 and 6: use of a Flow-sorted Robertsonian Chromosome*, Genomics, Jul. 13(3), 761-769, 1992.) This process essentially results in 90%+ pure W and Z chromosomes. These pure chromosomes are then used in an RDA to isolate a number of gender specific sequences. This approach is preferred over the RDA on genomic DNA as described above because although it successfully yields unique gender specific DNA sequences, a field diagnostic which utilizes that sequence as a probe would have a lower sensitivity and therefore would not produce an accurate field test. Although the

chromosome isolation followed by RDA does not yield repeated sequences, it does yield sufficient numbers of gender specific sequences that in total act as a repeated sequence. Preferably, the probe is obtained by using the above described protocol wherein isolated chromosomes are used in an RDA protocol.

The probes of the present invention, as described above, are significant in that gender specific repetitive sequences do not appear to be available in female ratite DNA. None of the restriction enzymes such as Psa I, HindIII, SaeIIIa, XhoI, as well as several others, show a repetitive fragment that is gender specific in ostrich or emu DNA. Further, as described above, the probes derived from other animal's DNA which are used to determine the gender of that animal do not work on ostriches or emus. For example, the domestic chicken probe reported in Kodoma H., et al., *Nucleotide sequences and unusual electrophoretic behavior of the W chromosome-specific repeating DNA units of the domestic fowl, Gallus gallus domesticus*, Chromosoma (Berl), 96:18-25, 1987, which contains a repetitive 17 base pair sequence, does not show significant binding to either male or female ostrich DNA. Also, the human probes reported in Miyaki, et al., do not show significant binding to either male or female ostrich DNA. Further, snake (*E. radiata*) W chromosome primers comprising the following oligonucleotide sequences show considerable binding to both male and female ostrich DNA: CTTTGAGTAACCAAGCAGCAGTGTC (SEQ ID NO:7); ACTGATGTTCTTCCAAGGATGTGGG (SEQ ID NO:8); and GGACTTAATGATCTTAATGGAGCCCC (SEQ ID NO:9).

Accordingly, the existing probes can not be used to determine the gender of ratites, and prior to the present invention, there was no probe, method, or test kit available for determining the gender of a ratite by DNA hybridization techniques.

*RDA Separation Method with Latex Particles*

In another aspect of the present invention, it has been discovered that the above described RDA protocols are improved

by the use of latex particles in the step where male and female specific DNA is separated from each other. This method of separation is preferred during the preparation of the probe as it significantly reduces the separation time.

In the RDA method known in the art, it takes approximately 4 to 5 hours to separate the male DNA from the female DNA using conventional gel separation techniques. In the improved method, the driver (male) DNA is labeled with biotin prior to the amplification. After the amplification, the male and female DNA is separated as follows: latex particles with strepavidin bound to its surfaces are added to the mixture; the particles then bind to the male DNA via the biotin label; the mixture is centrifuged for approximately 10 minutes wherein the DNA bound to the latex particle forms the pellet at the bottom of the centrifuge tube; the supernatant contains the female DNA. By this separation method, the female and male DNA is separated in approximately 10 minutes instead of 4 to 5 hours. Preferably, the latex particle is less than 0.5μ in diameter, and most preferably it is less than 0.2μ in diameter.

It is to be understood that a probe comprising oligonucleotide sequences which hybridize with target male specific oligonucleotide sequences may be utilized in the above method and test kit for the determination of the gender of a ratite. In this embodiment of the invention, the probe would detect the presence of male specific DNA in the sample. Such a probe would be synthesized according to the above method wherein the driver genome would be the W genome (female), and the tester genome would be the Z genome (male).

*Test Kit*

Another aspect of the present invention is a test kit for determining the gender of an animal. The test kit of the present invention for the determination of the gender of an animal such as a ratite, and more specifically an ostrich or emu, comprises the following: a means for transferring the blood collected from a bird such as a capillary pipette, a solution to lyse the collected

blood cells, a solid phase such as a nitrocellulose filter membrane, a means for transferring the lysed cells onto the solid phase, a solution containing a female specific oligonucleotide sequence probe conjugated to a label, washing solution, solution containing a substrate for the label if required by the label, and a stop solution if required by the label, and any other solution for visualization of the label as required by the label system used. It should be understood that one or more of the elements in the test kit may be combined depending on the configuration of the kit.

The solid phase can be any solid phase but is preferably in a dip stick configuration. The nitrocellulose solid phase is preferably pre-blocked and pre-spotted. The solid phase is pre-blocked with a nonreactive protein such as bovine serum albumin (BSA). Preferably, the solid phase is pre-spotted in different areas with female specific and male specific genomic DNA to provide both positive and negative controls for the assay. In another embodiment, the solid phase is pre-spotted with gender specific oligonucleotide sequences instead of gender specific genomic DNA. Preferably, the solid phase is pre-spotted with gender specific genomic DNA. It is to be understood that the solid phase in the test kit of the present invention does not require that controls be prespotted on the solid phase.

More preferably, the solid phase is a dip stick having three areas. The first area has male specific genomic DNA bound to that area as the male control. The second area is left unbound, or in the alternative the second area may have a portion of female specific oligonucleotide sequences bound to it as a capture probe. The third area has female specific genomic DNA bound to that area as the female control. The dipstick is pre-spotted by hybridizing the DNA to the filter membrane by any means known in the art, but preferably as follows. First, 1.0µl of 360µg/µl gender specific DNA (male or female) in a solution of Tris-EDTA (5mM Tris - 0.1M EDTA) is spotted on the membrane. The membrane is then heated at 80°C for one hour. The membrane is then prehybridized at 42°C for two hours, preferably in a prehybridization solution having the following

concentrations: 5x "SSC"; 5x "Denhardts"; 25 mM NaP04 pH 6.5; 50% Formamide; 0.5mg/ml Salmon Sperm DNA (Sigma) in an aqueous solution. Wherein 20x "SSC" = 3M NaC1 and 0.3M Na Citrate having a pH of 7.0 in an aqueous solution. Wherein 100x "Denhardts" = 2% Ficol; 2% polyvinylpyrrolidone; and 2% BSA (Bovine Serum Albumin) in an aqueous solution.

Where the second area of the solid phase is left unbound, a heat source is preferably included in the above test kit to increase the rate of covalently binding the sample DNA to the filter membrane. Any heat source capable of heating the solid media to a temperature of between approximately 50°C to 90°C may be used in the present invention. A hair dryer can be the source of heat in the present invention. More particularly, it is preferable that the solid phase is heated to between approximately 60°C and 80°C for approximately 3 to 5 minutes. In an embodiment where the second area of the solid phase is prespotted with a portion of female specific genomic DNA or female specific oligonucleotide sequences (a capture probe), no heat source is needed.

As discussed above, the label conjugated to the probe, and the visualization means may be any label or visualization means known in the art. A preferred label is horseradish peroxidase ("HRP"), and its preferred substrate is Nitro Blue Tetrazolium ("NBT"). Another preferred label is biotin-strepavidin conjugated to an enzyme such as HRP, its preferred substrate being NBT. Yet another preferred label is a colored latex particle as described more fully below.

The diagnostic kit of the present invention is an easy to use, accurate test conducted in the field, with results available in approximately one hour or less depending on the visualization means used. The total time for determining the gender of a ratite by a preferred test kit, after collecting the sample, is between approximately 1.0 and 1.5 hours where the label is an enzyme, and is approximately 45 minutes where the label is a colored latex particle.

A preferred probe of the test kit is a probe containing substantially the same sequence as SEQ ID NO:3. Another

preferred probe of the test kit is any fragment of SEQ ID NO:3 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA. Yet another preferred probe is substantially the same sequence as SEQ ID NO:3 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA.

Another preferred probe of the test kit is a probe containing substantially the same sequence as SEQ ID NO:4. Another preferred probe of the test kit is any fragment of SEQ ID NO:4 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA. Yet another preferred probe is substantially the same sequence as SEQ ID NO:4 that is capable of hybridizing with the target female specific oligonucleotide sequences present in ratite DNA.

*Restriction Endonuclease Enzyme Embodiment*

In another embodiment of the present invention, the method further comprises the step of adding to the sample a restriction endonuclease enzyme which cleaves a site in the male specific oligonucleotide sequences but does not cleave a site in the female specific oligonucleotide sequences. After the restriction endonuclease enzyme digestion treatment, the probe is added to the sample under hybridizing conditions as described above. The addition of such a restriction endonuclease enzyme allows for the use of a probe in the method that ordinarily hybridizes with both male and female specific oligonucleotide sequences because the enzyme cleaves a site in only the male specific oligonucleotide sequences such that the probe can no longer hybridize with the male specific oligonucleotide sequences.

This embodiment also includes the step of extracting the DNA from the protein in the sample prior to the addition of the restriction endonuclease enzyme to the DNA. Therefore, after the blood has been obtained from the bird, the genomic DNA in the sample is extracted. Methods for extracting the genomic DNA from the sample are well known in the art. Therefore, the genomic DNA may be extracted by any means known in the art.

A preferred means for extracting the genomic DNA is the commercially available genomic DNA isolation kit QuickGeno™, from CloneTech, of Palo Alto, California.

After the DNA has been extracted from the sample, an aqueous solution comprising the restriction endonuclease enzyme is added to the DNA. After digesting the sample DNA with the restriction endonuclease enzyme, a probe is added to the sample under hybridizing conditions as described above.

Any restriction endonuclease enzyme which is capable of cleaving a site in male, but not female, specific oligonucleotide sequences such that a probe can no longer hybridize with the male specific oligonucleotide sequences and can still hybridize with female specific oligonucleotide sequences may be used in the present invention. A preferred restriction endonuclease enzyme is Pst I (New England Biolabs, Beverly, MA). It is critical to this embodiment that a sufficient amount of the restriction endonuclease enzyme be added such that all of the male oligonucleotides present be cleaved. Therefore, it is preferred that an excess of the restriction endonuclease enzyme be added to the sample DNA. A preferred concentration of the Pst I solution is between approximately 3 and 10, and more preferably is approximately 5 units/μl Pst I restriction endonuclease enzyme, wherein 1 unit digests 1μg DNA per hour at 37°C. Preferably, the sample DNA is digested with 100 units of restriction endonuclease enzyme solution between approximately 5 to 40 minutes, and more preferably for 20 minutes.

One embodiment of the method of the present invention is the use of Pst I restriction endonuclease enzyme, and the use of a probe containing substantially the same sequence as SEQ ID NO:1: GCGCTACATCCCCAAGAACCCCTAC. This probe ordinarily hybridizes with both male and female ostrich genomic DNA, and with chicken hen genomic DNA. However, it has been unexpectedly discovered that after digesting the sample genomic DNA in Pst I, the probe no longer hybridizes with the ratite male genomic DNA, nor with the chicken hen DNA. Therefore, this probe may be used to determine the gender of an ostrich after the

ostrich DNA has been digested with the restriction endonuclease enzyme Pst I.

The probe is labeled with any label known in the art, and visualized by any method known in the art. A preferred label is horseradish peroxidase ("HRP"), and its preferred substrate is Nitro Blue Tetrazolium ("NBT"). Another preferred label is biotin-strepavidin conjugated to an enzyme such as HRP, its preferred substrate being NBT. Yet another preferred label is a colored latex particle as described more fully below. The probe can also be labeled with biotin conjugated with strepavidin-alkaline phosphatase, and the substrate is a chemiluminescent substrate such as AMPPD™ (Disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-tricyclo[3,3,1,1$^{3,7}$] decan)-4-yl)phenyl phosphate), or CSPD™ (Disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3,3,1,1$^{3,7}$]decan)-4-yl)phenyl phosphate), from Tropics, Inc. of Bedford, Massachusetts. Preferably, after the chemiluminescent substrate is added, the solid phase is covered with X-ray film in the dark, and hybridization of the probe with female specific oligonucleotide sequences is detected by development of the film.

In another embodiment of the test kit of the present invention, the test kit further comprises a solution comprising a restriction endonuclease enzyme which cleaves a site in the male specific oligonucleotide sequences but does not cleave a site in the female specific oligonucleotide sequences. This embodiment of the test kit also includes a means for extracting the DNA from the protein in the sample prior to the addition of the restriction endonuclease enzyme to the DNA. In the assay, after the blood has been obtained from the bird, the genomic DNA in the sample is extracted. The genomic DNA may be extracted by any means known in the art. A preferred means for extracting the genomic DNA is the commercially available genomic DNA isolation kit QuickGeno™, from CloneTech, of Palo Alto, California.

After the DNA has been extracted from the sample, an aqueous solution comprising the restriction endonuclease enzyme is added to the DNA. After digesting the sample DNA with the

restriction endonuclease enzyme, a probe is added to the sample under hybridizing conditions as described above. A preferred probe in this embodiment contains substantially the same sequence as SEQ ID NO:1. The addition of such a restriction endonuclease enzyme allows for the use of a probe in the method that ordinarily hybridizes with both male and female specific oligonucleotide sequences because the enzyme cleaves a site in only the male specific oligonucleotide sequences such that the probe can no longer hybridize with the male specific oligonucleotide sequences. Therefore, in this embodiment of the present invention, the probe need not be an oligonucleotide sequence that is complementary to only female specific oligonucleotide sequences.

Any restriction endonuclease enzyme which is capable of cleaving a site in male, but not female, specific oligonucleotide sequences such that a probe can no longer hybridize with the male specific oligonucleotide sequences and can still hybridize with female specific oligonucleotide sequences may be used in the present invention. A preferred restriction endonuclease enzyme is Pst I. Further, it is preferred that an excess of the restriction endonuclease enzyme be added to the sample DNA. A preferred amount of the Pst I solution is approximately 20µl of an aqueous solution comprising 5 units/µl Pst I restriction endonuclease enzyme, wherein 1 unit digests 1µg DNA per hour at 37°C. Therefore, a preferred amount of Pst I is 100 units per analysis. Preferably, the sample DNA is digested with the restriction endonuclease enzyme solution for approximately 10 to 20 minutes.

*Latex Particle as Label of Probe*

The present invention further relates to a general diagnostic method using colored latex particles bound to a probe or a protein as the means for visualization of results in diagnostic analyses. In this method, the surface of a colored latex particle may either be chemically bound directly to the probe, or its surface may be chemically bound to a spacer which is bound to

the probe. A spacer is used between a probe and a colored latex particle to avoid any stearic problems which may hinder the reaction of the probe with its target Whether or not a spacer is used varies according to the application involved, and one of ordinary skill in the art could readily ascertain whether a spacer is needed by routine experimentation. Many means for binding the surface of a latex particle to a probe or a spacer are known in the art. Any such means for binding the surface of a colored latex particle to a probe or to a spacer may be used in the present invention.

The colored latex particle may be any color desired for visualization purposes. Preferably, the latex particle is yellow, red, blue, or green. The colored latex particle may be hollow or solid. Preferably, the latex particle is hollow to minimize its weight. The size of the latex particle varies according to its intended use and according to the size of the probe. Preferably, the particle used is as small as possible to eliminate stearic problems between the probe and its intended nucleotide target, while retaining the ability to easily visualize the results of the analysis. More preferably, the latex particle is between approximately 0.1 and 0.5μ in diameter, and most preferably it is between 0.1 and 0.2μ in diameter.

For example, a latex particle could be used as the label for the above described oligonucleotide sequence (probe) which hybridizes to target female specific oligonucleotide sequences in the above described method for determining the gender of ratites. In this method, a biotin-labeled nucleotide with a sequence known to hybridize with the target oligonucleotide is added to the reaction mixture and allowed to interact with the sample. Preferably, a higher molecular weight probe is used if it is to be labeled with a latex particle to minimize background problems. Generally, the probe may be less than a 30-mer, whereas with a latex particle it is preferable to use a probe having several hundred base pairs. A strepavidin labeled latex particle is next added to the reaction mixture. The particle binds to the biotin-labeled oligonucleotide to form a biotin/avidin bond. The latex

particles can be colored or an enzyme can be attached to the particle. If the target oligonucleotide is present in the sample, the labeled latex particle will be immobilized and can be visualized.

In another embodiment, a colored latex particle is bound directly to the probe prior to the hybridization of the probe with the target female specific oligonucleotide sequences. This method is a one-solution addition step method: the latex particle-labeled probe is added to the sample under hybridization conditions; after incubation (hybridization), unhybridized probe is removed; and the hybridized probe is visualized by the colored particle.

The colored latex particles may be used with all types of probes and proteins in all types of analyses providing that the probe or protein remains adhered to the particle and does not stearically hinder the reaction between the probe or protein and its target.

The use of colored latex particles in analyses results in more accurate, more rapid, and more simple analyses. The use of latex particles for the visualization of the results eliminates the need for the use of other protein based visualization systems such as biotin-avidin-enzyme-substrate, or antigen and enzyme conjugated antibody reactions. The reduction of the amount of protein involved in the analysis reduces background problems which arise from the affinity of protein to various media such as plastic and cellulose. The reduction of background problems therefore increases the sensitivity of the analysis. Further, the use of colored particles also eliminates one or more steps from an analysis as no enzyme substrate solution or stop solution need be added which also eliminates the need for the required intermediate washing steps between the addition of such solutions. The reduction of the number of solutions that need to be added in an analysis not only results in a more rapid and simple analysis, it also increases the accuracy of the analysis as fewer contaminants or operator errors would be introduced into the analysis.

*FSH challenged Testosterone Monitoring*

Monitoring the absolute amounts as well as the circulating levels of reproductive hormones in ostriches and emus in an effort to develop a reliable method to determine gender has been found to be difficult. The monitoring of the reproductive hormones was done using commercially available radioimmunoassay diagnostics for testosterone, androstenedione, and estradiol-17-ß. At several different ages both ostrich and emu show significant differences in absolute values as well as testosterone: androstenedione ("T/A") ratios as a function of gender. As the hormone levels of ratites constantly change during growth, development, and breeding seasons, determining the gender of ratites by monitoring their reproductive hormones would therefore require a more diverse product line, and as different tests would be required at different ages and during different seasons, this method of determining gender would be quite complex. Further, the results based on monitoring reproductive hormones of ratites have sometimes been inconclusive.

However, we have discovered that by challenging a bird, such as a ratite, with a composition comprising follicle stimulating hormone ("FSH") and leutinizing hormone ("LH"), the hormonal response of a female bird is significantly and consistently different than the hormonal response of a male bird. This surprising and unexpected difference in the response to the FSH composition is a highly accurate method of determining the gender of a bird. Therefore, this invention also relates to a method of determining the gender of birds which comprises administering an exogenous FSH composition to a bird, and monitoring the bird's plasma concentrations of testosterone before, and after the administration of the FSH composition.

Briefly described, the method comprises withdrawing blood from the bird, inoculating the bird with FSH composition, again withdrawing blood from the bird, and then performing an RIA or ELISA on the blood samples to compare the quantity of testosterone in the blood before and after challenge by the FSH

composition. If the testosterone level of the bird remains relatively constant after the FSH composition challenge, the bird is female. If the testosterone level of the bird after the FSH composition challenge increases, then the bird is male.

The FSH composition is comprised of FSH and LH. Preferably, the FSH composition is comprised of mammalian FSH and LH. A preferred ratio of FSH to LH is between approximately 7.5 : 1 to 16 : 1. More preferably, the FSH composition is further comprised of pregnant mare serum gonadotropin ("PMSG"). Most preferably, the FSH composition is comprised of mammalian FSH, mammalian LH, and mammalian PMSG. A preferred amount of hormones in the FHS composition is as follows: between approximately 30 and 80% FSH; approximately 30 and 80% LH; and 30 and 80% PMSG. Another preferred amount of hormones in the FHS composition is as follows: between approximately 75-80% FSH; approximately 5-10% LH; and approximately 10-15% PMSG.

The FSH composition used in the present invention may be obtained from SeroImmuno Diagnostics (Tucker, Georgia) as the product Ratitrophin™. The FSH composition of the present invention may also be obtained by separately purchasing FSH, LH, and optionally PMSG, from any commercial source and then mixing these hormones together in the appropriate ratios. Commercial sources of such hormones includes Sigma Chemical Company (St. Louis, Missouri), among others.

The amount of FSH composition administered to a bird varies according to the weight, and species of the bird, and the number of times the bird will be challenged with the FSH composition. In the embodiment where the composition will be administered more than once, between approximately 5 and 25 IU of the FSH composition per kilogram of weight of the bird are administered. Preferably, for multiple challenges, between approximately 10 and 20 IU/kg are administered to the bird. Most preferably, for multiple challenges, between approximately 13.5 and 16 IU/kg is administered to the bird, the most preferred amount being approximately 14.7 IU/kg. For example, with a 68

kg emu, approximately 1000 IU of the FSH composition of the present invention is administered per dose. For a 0.25 - 0.4 kg quail, approximately 100 IU of the FSH composition is administered per dose. For a 136 kg ostrich, approximately 2000 IU of the FSH composition of the present invention is administered per dose. The FSH composition may be administered one or more times on consecutive days depending on the size, species and age of the bird. Preferably, the FSH composition is administered on three consecutive days.

In another embodiment, the FSH composition is administered in a single dose, and the blood sample is obtained approximately two to three days later. In the single-dose embodiment, between approximately 30 to 60 IU/kg is administered to a ratite, and preferably between 40 and 50 IU/kg is administered. More preferably, approximately 45 IU/kg is administered to a ratite in the single-dose embodiment. For example, approximately 3000 IU of the FSH composition of the present invention would be administered in a single-dose method to a 68 kg emu.

The composition may be administered to a bird by any means known in the art. For example, the composition may be administered subcutaneously, intraperitonealy, or intramuscularly. Preferably, the composition is injected intramuscularly.

The blood samples are obtained from the bird by any method known in the art. Plasma levels of testosterone and or androstenedione in the blood samples may be determined using any radioimmunoassay kit such as Coat-A-Count® Radioimmunoassay Kit, a commercially available RIA kit for testosterone from Diagnostic Products Corporation of Los Angeles, California. As stated above, if the testosterone level after challenge with the FSH composition increases, then the bird is male. If the testosterone level remains substantially the same, then the bird is female.

As demonstrated in Figures 2 and 3, blood was collected from six 3.5 to 6.5 year old emus daily for 10 consecutive days.

The six emus were housed as three breeding pairs as determined by veterinary genital examination as juveniles (< 3 months of age) and again approximately 6 months prior to the experiment. Baseline plasma levels of androstenedione and testosterone were obtained from the samples collected on days 1-3. Each emu was injected with 1000 IU of an exogenous FSH composition, such as Ratitrophin™, daily for three consecutive days (days 4-6). Each emu's response to the exogenous FSH composition was determined from the blood samples collected on days 7-10. Figures 4 through 9 illustrate the hormone levels of each of the six emus, emus A through F, individually.

Plasma levels of androstenedione and testosterone in the blood samples were determined using radioimmunoassay kits from Diagnostic Products Corporation, of Los Angeles, California. The baseline hormone levels are the average of the preinjection levels of the hormones in the blood samples collected on days 1-3. Baseline levels for these emus were about 1.8 ng/ml testosterone. Male emu hormone levels after injection with exogenous FSH composition were elevated by approximately 5 to 15 fold for testosterone. Female emu testosterone levels after injection with the exogenous FSH composition did not differ substantially from baseline levels. These results are illustrated in Figures 2-9, wherein emu E is female, and emus A, B, C, D, and F are male. The gender of these birds was confirmed by DNA testing by Sex Made Easy® by Zoogen, Inc., of Davis, California.

As shown above, the three breeding pairs of emus, as determined by veterinary genital examination (vent-sexing), were not three females and three males, but instead were actually one female and five males. The above experiment demonstrates that the determination of gender of a bird by traditional methods is far less accurate than determining the gender of a bird by challenging the bird with a FSH composition and monitoring its hormone response.

Similarly, the gender of six pairs of twelve week old Japanese quail (*Coturnix coturnix japonica*) was determined using the above method. The gender of the quail was determined at six

weeks of age by plumage coloration. The above method was modified in that only 100 IU of the FSH composition was administered to each quail. The above method was also modified in that blood was obtained from each quail on days 1, 2, and 4, and the FSH composition was administered on days 1, 2, and 3 after the blood samples had been obtained for the day. As shown in Figure 4, the plasma responses in male in comparison to female quail mimicked the androgen hormone responses in the emus. The plasma testosterone levels of the male quail were substantially elevated after challenge with the FSH composition, while the change in plasma testosterone levels of the female quail were negligible.

Accordingly, the gender of a bird is determined by observing the bird's hormonal response to the administration of exogenous FSH composition. If the testosterone level of the bird remains relatively constant after the FSH composition challenge, the bird is female. By relatively constant it is to be understood that the testosterone level of the bird remains within approximately 3 times the pre-FSH administration level. If the testosterone level of the bird after the FSH composition challenge increases significantly, then the bird is male. By a significant increase it is to be understood that the testosterone level of the bird after the FSH composition challenge increases by more than three times the pre-FSH level. Preferably, the testosteron level increases by at least approximately five fold. More preferably, the testosterone level of the bird increases by approximately 5 to 15 fold.

This invention is further illustrated by the following examples, which are not to be construed in any way as imposing limitations upon the scope thereof. On the contrary, it is to be clearly understood that resort may be had to various other embodiments, modifications, and equivalents thereof which, after reading the description herein, may suggest themselves to those skilled in the art without departing from the spirit of the present invention and/or the scope of the appended claims.

## EXAMPLE 1

*A test kit containing an enzyme labeled probe for the determination of the gender of a ratite.*

A test kit for the determination of the gender of a ratite comprises the following: a capillary pipette to transfer the blood collected from a bird, a solution to lyse the collected blood cells, a second capillary pipette to transfer the lysed cells onto a filter membrane, a hair dryer (or some other directed heat source, such as a light bulb) to increase the rate of covalent binding of the sample DNA to the filter membrane, a solution containing biotin labeled SEQ ID NO:3 (probe) which hybridizes to target female specific oligonucleotide sequences, a washing solution, a solution containing strepavidin conjugated to HRP, a solution containing Nitro Blue Tetrazolium ("NBT"), and a stop solution.

The filter membrane is a dipstick which has been pre-blocked and pre-spotted. The dipstick has been pre-blocked with a nonreactive protein such as bovine serum albumin (BSA), and has three areas, two of which have been pre-spotted with sex specific genomic ratite DNA. The first area has male specific genomic DNA bound to that area as the male control. The second area is left unbound. The third area has female specific genomic DNA complementary to the labeled probe bound to that area as the female control. The dipstick is pre-spotted by hybridizing the sex specific genomic DNA to the filter membrane as follows. First, 1.0µl of 360µg/µl gender specific DNA (male or female) in a solution of Tris-EDTA (5mM Tris - 0.1M EDTA) is spotted on the membrane. The membrane is then heated at 80°C for one hour. The membrane is then prehybridized at 42°C for two hours, preferably in a prehybridization solution having the following concentrations: 5x "SSC"; 5x "Denhardts"; 25 mM NaP04 pH 6.5; 50% Formamide; and 0.5mg/ml Salmon Sperm DNA (Sigma) in an aqueous solution. Wherein 20x "SSC" = 3M NaC1 and 0.3M Na Citrate having a pH of 7.0 in an aqueous solution. Wherein 100x "Denhardts" = 2% Ficol; 2% polyvinylpyrrolidone; and 2% BSA (Bovine Serum Albumin) in an aqueous solution.

EXAMPLE 2

*A test kit containing a capture probe and a latex particle probe for the determination of the gender of a ratite.*

A test kit containing a probe conjugated to a latex particle for the determination of the gender of a ratite comprises the following: a capillary pipette to transfer the blood collected from a bird, a solution to lyse the collected blood cells, a second capillary pipette to transfer the lysed cells onto a filter membrane, a solution containing SEQ ID NO:3 (probe) conjugated to a colored latex particle, and washing solution.

The filter membrane is a dipstick which has been pre-blocked and pre-spotted. The dipstick has been pre-blocked with a nonreactive protein such as bovine serum albumin (BSA), and has two areas which have been pre-spotted with sex specific genomic DNA, and one area which has been pre-spotted with female specific oligonucleotide sequences. The first area has male specific genomic DNA bound to that area as the male control. The second area has a portion of female specific oligonucleotide sequences complementary to the labeled probe bound to it as a capture probe. The third area has female genomic DNA complementary to the labeled probe bound to that area as the female control. The dipstick is pre-spotted by hybridizing the sex specific genomic DNA and oligonucleotide sequences to the filter membrane under the conditions as described in Example 1.

EXAMPLE 3

*The determination of the gender of an ostrich by DNA hybridization by the test kit of Example 1.*

The following method describes the use of the test kit described in Example 1 for the determination of the gender of an ostrich:

1. Collect approximately 100-200µl of blood from the ostrich.
2. Dispense approximately 75µl of blood by capillary pipet into a 0.5 N NaOH solution. The solution lyses the red blood cells in the sample of blood, denatures the DNA and other

proteins in the sample, and clears the sample by oxidizing the heme iron so that the solution is no longer red.

3. After the sample has been treated with the solution of step 2 for approximately five minutes, transfer between approximately 75 to 100µl of the mixture by capillary pipet onto the pre-blocked and pre-spotted filter membrane.

4. Heat the filter membrane to a temperature of approximately 60°C to 80°C for approximately 3-5 minutes with a hairdryer.

5. Add between approximately 400 to 1000 ng of SEQ ID NO:3, labeled with biotin, and incubate for approximately 15 minutes. The affixed sample DNA is incubated for approximately 15 minutes at 25-42°C in approximately 1.5 to 2.0 ml of an aqueous solution having the following concentrations: 5x SSC; 1x Denhardts; 25 mM $NaPO_4$ pH 6.5; 45% Formamide; 0.2 mg/ml Salmon Sperm DNA; and 400 ng/ml of the probe.

6. Wash for ten minutes with an aqueous solution comprising 2x SSC and 0.1% SDS (sodium dodecyl sulfate). Repeat until the filter membrane has been washed at least three times, 3 minutes each time.

7. Step 7 followed by Step 8 is a combination of conjugate binding and blocking to minimize any non-specific binding signal.

Step 7 comprises blocking the filter for 10 minutes at room temperature in an aqueous Buffer containing 3% BSA; 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

8. Incubate filter for 25 minutes in an aqueous solution comprising strepavidin-HRP conjugate in 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

9. Wash three times in an aqueous solution comprising 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

10. Wash one time in an aqueous solution comprising 0.1M NaCl; 0.1M Tris-HCl, pH 9.5; and 50 mM $MgCl_2$.

11. Add an excess of enzyme substrate (NBT) and incubate for approximately 15 minutes in 1.0 to 2.0 ml of the following aqueous solution: 32µl of Nitro Blue Tetrazolium ("NBT") at 50 mg/ml in 70% methyl alcohol; 16µl of 5-bromo-4-chloro-3-

indolyl-phosphate ("BCIP") 50 mg/ml in dimethylformamide; in 5 ml of an aqueous solution comprising 0.1M NaCl; 0.1M Tris-HCl, pH 9.5; and 50 mM $MgCl_2$.

12. Add an aqueous stop solution comprising 1mM EDTA, and wait for approximately two minutes.

13. Read the filter membrane to determine the gender of the ostrich tested. As shown in Figure 1, where the bird tested is male, only the female control area of the dipstick turns blue. In contrast, where the bird tested is female, both the specimen and the female control area turn blue. If all areas of the dipstick are blue, or if none of the areas turn blue, then the test is a false test which must be repeated.

Therefore, the total time for determining the gender of the ostrich by this method, after collecting the sample, is approximately 1.5 hours.

## EXAMPLE 4

*The determination of the gender of an ostrich by DNA hybridization by the test kit of Example 2.*

The following method describes the use of the test kit described in Example 2 for the determination of the gender of an ostrich:

1. Collect approximately 100-200µl of blood from the ostrich.

2. Dispense approximately 75µl of blood by capillary pipet into a 0.5 N NaOH solution. The solution lyses the red blood cells in the sample of blood, denatures the DNA and other proteins in the sample, and clears the sample by oxidizing the heme iron so that the solution is no longer red.

3. After the sample has been treated with the solution of step 2 for approximately five minutes, transfer between approximately 75 to 100µl of the mixture by capillary pipet onto the pre-blocked and pre-spotted filter membrane having the capture probe prespotted in the second area of the dipstick.

4. The sample is incubated with the dipstick having the capture probe on it for approximately 15 minutes, and is then

washed with an aqueous solution comprising 2x SSC and 0.1% SDS for approximately 10 to 15 minutes.

5. Add approximately 5µg of SEQ ID NO:3 conjugated with blue colored latex particles, and incubate for approximately 15 minutes. Hybridization (incubation with detection probe) conditions are as described in Example 3.

6. Wash for ten minutes with phosphate buffered saline at pH 6.8. Repeat until the filter membrane has been washed at least three times, 3 minutes each time.

7. Read the filter membrane to determine the gender of the ostrich tested. As shown in Figure 1, where the bird tested is male, only the female control area of the dipstick turns blue. In contrast, where the bird tested is female, both the specimen and the female control area turn blue. If all areas of the dipstick are blue, or if none of the areas turn blue, then the test is a false test which must be repeated.

Accordingly, the total time for determining the gender of the ostrich by this method, after collecting the sample, is approximately 45 minutes.

## EXAMPLE 5

*A test kit containing an enzyme labeled probe and a restriction endonuclease enzyme for the determination of the gender of a ratite.*

A test kit for the determination of the gender of a ratite comprises the following: a capillary pipette to transfer the blood collected from a bird, a means for isolating the DNA in the sample from the protein in the sample which is removed, a solution containing Pst I restriction endonuclease enzyme, a solution to lyse the collected blood cells, a second capillary pipette to transfer the lysed cells onto a filter membrane, a hair dryer to increase the rate of covalent binding of the sample DNA to the filter membrane, a solution containing a biotin labeled oligonucleotide sequence (probe) which hybridizes to target female and male specific oligonucleotide sequences and contains substantially the same sequence as SEQ ID NO:1:

GCGCTACATCCCCAAGAACCCCTAC, a washing solution, a solution containing strepavidin conjugated to HRP, a solution containing Nitro Blue Tetrazolium ("NBT"), and a stop solution. The means for isolating the DNA is preferably a commercially available kit such as QuickGene™ from CloneTech, of Palo Alto, California.

## EXAMPLE 6

*The determination of the gender of an ostrich by DNA hybridization by the test kit of Example 5.*

The following method describes the use of the test kit described in Example 5 for the determination of the gender of an ostrich:

1. Collect approximately 100-200µl of blood from the ostrich.
2. Extract protein from the blood sample using the genomic isolation kit QuickGeno™, from CloneTech of Palo Alto California. This step takes approximately 20 to 30 minutes.
3. Add approximately 20 µl of an aqueous solution comprising 5 units/µl Pst I restriction endonuclease enzyme, wherein 1 unit digests 1µg DNA per hour at 37°C.
4. Digest the sample DNA with the restriction endonuclease enzyme solution for approximately 30 minutes.
5. Dispense approximately 75µl of the above reaction mixture by capillary pipet into a 0.5 N NaOH solution.
6. After the sample has been treated with the solution of step 5 for approximately five minutes, transfer between approximately 75 to 100µl of the mixture by capillary pipet onto the pre-blocked and pre-spotted filter membrane.
7. Heat the filter membrane to a temperature of approximately 60°C to 80°C for approximately 3-5 minutes with a hairdryer.
8. Add between approximately 400 to 1000 ng of a probe comprising oligonucleotide sequences complimentary to target female and male specific oligonucleotide sequences containing substantially the same sequence as SEQ ID NO:1: GCGCTACATCCCCAAGAACCCCTAC, labeled with biotin, and

incubate for approximately 15 minutes. The affixed sample DNA is incubated for approximately 15 minutes at 25-42°C in approximately 1.5 to 2.0 ml of an aqueous solution having the following concentrations: 5x SSC; 1x Denhardts; 25 mM $NaPO_4$ pH 6.5; 45% Formamide; 0.2 mg/ml Salmon Sperm DNA; and 400 ng/ml of the probe.

9. Wash for ten minutes with an aqueous solution comprising 2x SSC and 0.1% SDS (sodium dodecyl sulfate). Repeat until the filter membrane has been washed at least three times, 10 minutes each time.

10. Step 10 followed by Step 11 is a combination of conjugate binding and blocking to minimize any non-specific binding signal. Step 10 comprises blocking the filter for 10 minutes at room temperature in an aqueous Buffer containing 3% BSA; 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

11. Incubate filter for 25 minutes in an aqueous solution comprising strepavidin-HRP conjugate in 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

12. . Wash three times in an aqueous solution comprising 0.2M NaCl; 0.1M Tris-HCl, pH 7.5; and 0.05% Triton X-100.

13. Wash one time in an aqueous solution comprising 0.1M NaCl; 0.1M Tris-HCl, pH 9.5; and 50 mM $MgCl_2$.

14. Add an excess of enzyme substrate (NBT) and incubate for approximately 15 minutes in 1.0 to 2.0 ml of the following aqueous solution: 32µl of Nitro Blue Tetrazolium ("NBT") at 50 mg/ml in 70% methyl alcohol; 16µl of 5-bromo-4-chloro-3-indolyl-phosphate ("BCIP") 50 mg/ml in dimethylformamide; in 5 ml of an aqueous solution comprising 0.1M NaCl; 0.1M Tris-HCl, pH 9.5; and 50 mM $MgCl_2$.

15. Add an aqueous stop solution comprising 1mM EDTA, and wait for approximately two minutes.

16. Read the filter membrane to determine the gender of the ostrich tested.

As shown in Figure 1, where the bird tested is male, only the female control area of the dipstick turns blue. In contrast, where the bird tested is female, both the specimen and the female

control area turn blue. If all areas of the dipstick are blue, or if none of the areas turn blue, then the test is a false test which must be repeated.

Therefore, the total time for determining the gender of the ostrich by this method, after collecting the sample, is approximately 2 to 3 hours.

## EXAMPLE 7

*The determination of the gender of an emu by monitoring reproductive hormones.*

The following method determines the gender of an emu by monitoring the reproductive hormone levels of the emu before and after it has been injected with an exogenous FSH composition. Whole blood is collected from the emu daily for 10 consecutive days. The blood is collected directly into heparinized vacutainers via jugular vein harvest. Baseline plasma levels of androstenedione ("A") and testosterone ("T") are obtained from the samples collected days 1-3. The emu is injected with 1000 IU exogenous FSH composition, Ratitrophin™, daily for three consecutive days (days 4-6). The emu is injected intramuscularly, in the psoas major muscle. The emu's response to the exogenous FSH composition differs according to the gender of the emu, and its response is determined from the blood samples collected on days 7-10.

Plasma levels of androstenedione and testosterone in the blood samples are determined by Coat-A-Count® Radioimmunoassay kits (Diagnostic Products Corporation, Los Angeles, California). The baseline hormone levels are the average of the preinjection levels of the hormones in the blood samples collected on days 1-3. Baseline levels for an emu is about 1.8 ng/ml testosterone. Male emu hormone levels after injection with exogenous FSH are elevated by approximately 5 to 15 fold for testosterone. Female emu testosterone levels after injection with the exogenous FSH do not differ substantially from baseline levels. These results are illustrated in Figures 2-9, wherein Emu

E is female, and Emus A, B, C, D, and F are male. All six emus were from 3.5 to 6.5 years of age.

EXAMPLE 8

*The determination of the gender of quail by monitoring reproductive hormones.*

The method described in Example 7 was used to determine the gender of quail by monitoring the reproductive hormone levels of the quail before and after it had been injected with an exogenous FSH composition. The above method was modified in that only 100 IU of the FSH composition was administered to each quail intramuscularly, in the gastrocnemius muscle. The above method was also modified in that blood was obtained via cardiac puncture on each quail on days 1, 2, and 4, and the FSH composition was administered on days 1, 2, and 3 after the blood samples had been obtained for the day. The whole blood was collected into heparanized containers using tuberculin syringes fitted with 25 gauge needles.

The plasma responses to exogenous FSH challenge by twelve Japanese quail (ten weeks old) are shown in Figure 10. The FSH challenge caused significant elevations, of at least three fold, in plasma testosterone levels in male quail, while the plasma testosterone response in female quail was negligible. The plasma testosterone responses in male quail, in comparison to female quail, mimicked the testosterone plasma responses found in the emus as shown in Figures 2-9.

EXAMPLE 9

*Generating the restriction map of SEQ ID NO:3 .*

The following restriction map of SEQ ID NO:3 was generated using the following computer software: MacVector™ Version 4.5.

## Table 1
## The Restriction Map of SEQ ID No:3
## With Respect to the Following Enzymes

| Enzyme | #Cuts | Positions | | | | | |
|---|---|---|---|---|---|---|---|
| Aci1 | 12 | 308 | 317 | 363 | 414 | 443 | 591 |
| | | 606 | 632 | 921 | 1212 | 1298 | 1565 |
| Acy1 | 1 | 1117 | | | | | |
| Afl1 | 4 | 138 | 417 | 911 | 1552 | | |
| Afl3 | 1 | 600 | | | | | |
| Aha1 | 4 | 456 | 851 | 972 | 1704 | | |
| Aha2 | 1 | 1117 | | | | | |
| Alu1 | 8 | 165 | 578 | 796 | 934 | 1303 | 1309 |
| | | 1349 | 1549 | | | | |
| Alw1 | 3 | 625 | 728 | 1682 | | | |
| AlwN1 | 1 | 754 | | | | | |
| Aoc1 | 1 | 1257 | | | | | |
| Aoc2 | 3 | 38 | 930 | 1043 | | | |
| Apa1 | 1 | 930 | | | | | |
| Apo1 | 1 | 170 | | | | | |
| Apy1 | 4 | 1161 | 1191 | 1377 | 1556 | | |
| AspA1 | 1 | 1379 | | | | | |
| AspH1 | 1 | 1043 | | | | | |
| Asu1 | 10 | 138 | 417 | 762 | 911 | 926 | 927 |
| | | 1157 | 1283 | 1436 | 1552 | | |
| Ava2 | 4 | 138 | 417 | 911 | 1552 | | |
| Ban1 | 1 | 1116 | | | | | |
| Ban2 | 2 | 38 | 930 | | | | |
| Bbe1 | 1 | 1120 | | | | | |
| Bbv1 | 9 | 174 | 235 | 326 | 707 | 921 | 1058 |
| | | 1312 | 1380 | 1558 | | | |
| Bfa1 | 3 | 543 | 785 | 1570 | | | |
| Bgl1 | 1 | 1068 | | | | | |
| Bmy1 | 3 | 38 | 930 | 1043 | | | |
| Bpm1 | 2 | 351 | 528 | | | | |
| Bsa1 | 1 | 1582 | | | | | |
| BsaH1 | 1 | 1117 | | | | | |
| BsaJ1 | 4 | 175 | 1190 | 1235 | 1703 | | |
| Bsg1 | 2 | 591 | 1199 | | | | |
| BsiY1 | 13 | 84 | 378 | 461 | 759 | 760 | 881 |
| | | 920 | 978 | 1022 | 1341 | 1403 | 1499 |
| | | 1616 | | | | | |
| Bsl1 | 13 | 84 | 378 | 461 | 759 | 760 | 881 |
| | | 920 | 978 | 1022 | 1341 | 1403 | 1499 |
| | | 1616 | | | | | |
| BsmA1 | 3 | 221 | 1123 | 1582 | | | |
| Bsp1286I | 3 | 38 | 930 | 1043 | | | |
| BspH1 | 1 | 703 | | | | | |
| BspM1 | 1 | 332 | | | | | |
| BspW1 | 11 | 42 | 162 | 314 | 440 | 527 | 629 |
| | | 638 | 934 | 1068 | 1209 | 1306 | |
| Bsr1 | 4 | 89 | 437 | 738 | 1493 | | |
| BstE2 | 1 | 1379 | | | | | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| BstN1 | 4 | 1161 | 1191 | 1377 | 1556 | | |
| Bsu36I | 1 | 1257 | | | | | |
| Cfo1 | 3 | 436 | 504 | 1119 | | | |
| Cfr1 | 1 | 621 | | | | | |
| Cfr13I | 10 | 138 | 417 | 762 | 911 | 926 | 927 |
| | | 1157 | 1283 | 1436 | 1552 | | |
| Cvn1 | 1 | 1257 | | | | | |
| Dde1 | 7 | 148 | 941 | 1058 | 1142 | 1257 | 1476 |
| | | 1514 | | | | | |
| Dpn1 | 4 | 244 | 619 | 722 | 1689 | | |
| Dpn2 | 4 | 242 | 617 | 720 | 1687 | | |
| Dra2 | 3 | 417 | 926 | 1436 | | | |
| Dra3 | 2 | 378 | 1403 | | | | |
| Dsa1 | 1 | 1235 | | | | | |
| Dsa5 | 8 | 454 | 849 | 970 | 1159 | 1189 | 1375 |
| | | 1554 | 1702 | | | | |
| Eae1 | 1 | 621 | | | | | |
| Eco47I | 4 | 138 | 417 | 911 | 1552 | | |
| Eco81I | 1 | 1257 | | | | | |
| EcoO109I | 3 | 417 | 926 | 1436 | | | |
| EcoR1 | 1 | 170 | | | | | |
| EcoR1* | 9 | 21 | 170 | 186 | 204 | 516 | 654 |
| | | 699 | 772 | 1288 | | | |
| EcoR2 | 4 | 1159 | 1189 | 1375 | 1554 | | |
| Esp3I | 1 | 1123 | | | | | |
| Fnu4H1 | 13 | 163 | 249 | 315 | 318 | 444 | 591 |
| | | 696 | 935 | 1072 | 1298 | 1301 | 1394 |
| | | 1547 | | | | | |
| Fok1 | 4 | 438 | 858 | 1214 | 1686 | | |
| Gsu1 | 2 | 351 | 528 | | | | |
| Hae2 | 3 | 437 | 505 | 1120 | | | |
| Hae3 | 8 | 590 | 623 | 764 | 928 | 1159 | 1285 |
| | | 1297 | 1437 | | | | |
| Hap2 | 5 | 141 | 455 | 851 | 972 | 1703 | |
| HgiA1 | 1 | 1043 | | | | | |
| Hha1 | 3 | 436 | 504 | 1119 | | | |
| Hinf1 | 1 | 492 | | | | | |
| Hinl1 | 1 | 1117 | | | | | |
| HinP1 | 3 | 434 | 502 | 1117 | | | |
| Hpa2 | 5 | 141 | 455 | 851 | 972 | 1703 | |
| Hph1 | 3 | 191 | 865 | 902 | | | |
| Kas1 | 1 | 1116 | | | | | |
| Mae1 | 3 | 543 | 785 | 1570 | | | |
| Mae2 | 4 | 3 | 844 | 1132 | 1626 | | |
| Mae3 | 7 | 218 | 553 | 835 | 845 | 853 | 890 |
| | | 1379 | | | | | |
| Mbo1 | 4 | 242 | 617 | 720 | 1687 | | |
| Mbo2 | 4 | 998 | 1419 | 1567 | 1597 | | |
| Mnl1 | 27 | 23 | 216 | 397 | 403 | 451 | 454 |
| | | 502 | 607 | 691 | 751 | 792 | 879 |
| | | 974 | 1058 | 1058 | 1087 | 1106 | 1130 |
| | | 1143 | 1266 | 1310 | 1345 | 1412 | 1426 |
| | | 1485 | 1523 | 1670 | | | |
| Mse1 | 6 | 27 | 48 | 304 | 977 | 1291 | 1313 |
| Msp1 | 5 | 141 | 455 | 851 | 972 | 1703 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Mst2 | 1 | 1257 | | | | | |
| Mun1 | 1 | 654 | | | | | |
| Mva1 | 4 | 1161 | 1191 | 1377 | 1556 | | |
| Nar1 | 1 | 1117 | | | | | |
| Nci1 | 4 | 456 | 851 | 972 | 1704 | | |
| Nde2 | 4 | 242 | 617 | 720 | 1687 | | |
| Nla3 | 12 | 604 | 707 | 730 | 889 | 918 | 999 |
| | | 1012 | 1047 | 1226 | 1391 | 1417 | 1596 |
| Nla4 | 9 | 37 | 418 | 419 | 763 | 912 | 927 |
| | | 928 | 1118 | 1560 | | | |
| Nsp(7524)1 | 4 | 604 | 1012 | 1047 | 1226 | | |
| Nsp(7524)2 | 3 | 38 | 930 | 1043 | | | |
| Nsp1 | 4 | 604 | 1012 | 1047 | 1226 | | |
| NspB2 | 3 | 317 | 632 | 1549 | | | |
| NspH1 | 4 | 604 | 1012 | 1047 | 1226 | | |
| Pal1 | 8 | 590 | 623 | 764 | 928 | 1159 | 1285 |
| | | 1297 | 1437 | | | | |
| Pfim1 | 3 | 378 | 1403 | 1499 | | | |
| PflM1 | 3 | 378 | 1403 | 1499 | | | |
| PpuM1 | 1 | 417 | | | | | |
| Pss1 | 3 | 420 | 929 | 1439 | | | |
| Pst I | 1 | 253 | | | | | |
| Pvu2 | 1 | 1549 | | | | | |
| Rma1 | 3 | 543 | 785 | 1570 | | | |
| Rsa1 | 6 | 483 | 636 | 710 | 1002 | 1360 | 1481 |
| Sau1 | 1 | 1257 | | | | | |
| Sau3A1 | 4 | 242 | 617 | 720 | 1687 | | |
| Sau96I | 10 | 138 | 417 | 762 | 911 | 926 | 927 |
| | | 1157 | 1283 | 1436 | 1552 | | |
| ScrF1 | 8 | 456 | 851 | 972 | 1161 | 1191 | 1377 |
| | | 1556 | 1704 | | | | |
| Sdu1 | 3 | 38 | 930 | 1043 | | | |
| Sec1 | 4 | 175 | 1190 | 1235 | 1703 | | |
| SfaN1 | 3 | 1314 | 1641 | 1669 | | | |
| Sfc1 | 3 | 249 | 1217 | 1304 | | | |
| Sty1 | 1 | 175 | | | | | |
| Taq1 | 2 | 723 | 1127 | | | | |
| Tfi1 | 1 | 492 | | | | | |
| Xba1 | 1 | 784 | | | | | |
| Xcm1 | 1 | 375 | | | | | |

## EXAMPLE 10

*The method of generating fragments of SEQ ID NO:3 capable of hybridizing with female specific oligonucleotide sequences of ostrich DNA*

Table 1 in Example 9 defines the library of restriction sites of SEQ ID NO:3. Accordingly, one of ordinary skill in the art can use Table 1 to choose enzymes to produce fragments of SEQ ID NO:3. Then, one can determine which of these fragments of SEQ ID NO:3 are capable of hybridizing with female specific

oligonucleotides of ostrich DNA by routine experimentation. More specifically, the fragment under study would be labeled, and then placed in contact with male and female ostrich genomic DNA under conditions that would allow hybridization of the fragment with the genomic DNA. After unhybridized labeled fragments are removed, visualization techniques appropriate for the label on the fragment would then be utilized to determine if the fragment hybridized with both male and female ostrich genomic DNA, with neither, or with only female ostrich genomic DNA. Accordingly, one of ordinary skill in the art, in view of the sequence of SEQ ID NO:3 and the restriction sites listed in Table 1, using routine experimentation, can determine the fragments of SEQ ID NO:3 that are capable of hybridizing with female specific oligonucleotides of ostrich DNA.

## EXAMPLE 11

*The method of generating fragments of SEQ ID NO:3 capable of hybridizing with female specific oligonucleotide sequences of emu DNA*

Table 1 in Example 9 defines the library of restriction sites of SEQ ID NO:3. Accordingly, one of ordinary skill in the art can use Table 1 to choose enzymes to produce fragments of SEQ ID NO:3. Then, one can determine which of these fragments of SEQ ID NO:3 are capable of hybridizing with female specific oligonucleotides of emu DNA by routine experimentation. More specifically, the fragment under study would be labeled, and then placed in contact with male and female emu genomic DNA under conditions that would allow hybridization of the fragment with the genomic DNA. After unhybridized labeled fragments are removed, visualization techniques appropriate for the label on the fragment would then be utilized to determine if the fragment hybridized with both male and female emu genomic DNA, with neither, or with only female emu genomic DNA. Accordingly, one of ordinary skill in the art, in view of the sequence of SEQ ID NO:3 and the restriction sites listed in Table 1, using routine experimentation, can determine the fragments of SEQ ID NO:3

that are capable of hybridizing with female specific oligonucleotides of emu DNA.

EXAMPLE 12

*A test kit containing an enzyme labeled probe for the determination of the gender of a ratite.*

The test kit for the determination of the gender of a ratite of the present example is as described in Example 1, wherein instead of the kit containing biotin labeled SEQ ID NO:3, the kit contains biotin labeled SEQ ID NO:4.

EXAMPLE 13

*A test kit containing a capture probe and a latex particle probe for the determination of the gender of a ratite.*

The test kit for the determination of the gender of a ratite of the present example is as described in Example 2, wherein instead of the kit containing biotin labeled SEQ ID NO:3, the kit contains biotin labeled SEQ ID NO:4.

EXAMPLE 14

*The determination of the gender of an ostrich by DNA hybridization by the test kit of Example 12.*

The method of determining the gender of an ostrich is described in Example 3, wherein instead of using the test kit of Example 1, the test kit of Example 12 is used.

EXAMPLE 15

*The determination of the gender of an ostrich by DNA hybridization by the test kit of Example 13.*

The method of determining the gender of an ostrich is described in Example 4, wherein instead of using the test kit of Example 2, the test kit of Example 13 is used.

EXAMPLE 16

*The determination of the gender of an emu by DNA hybridization by the test kit of Example 12.*

The method of determining the gender of an emu is described in Example 3, wherein instead of using the test kit of Example 1, the test kit of Example 12 is used. Clearly, the blood to be tested with the test kit of Example 12 will be collected from an emu, not an ostrich.

EXAMPLE 17

*The determination of the gender of an emu by DNA hybridization by the test kit of Example 13.*

The method of determining the gender of an emu is described in Example 4, wherein instead of using the test kit of Example 2, the test kit of Example 13 is used. Clearly, the blood to be tested with the kit of Example 13 will be collected from an emu, not an ostrich.

EXAMPLE 18

*The determination of the gender of an emu by DNA hybridization by the test kit of Example 1.*

The method of determining the gender of an emu is described in Example 3, using the test kit of Example 1. Clearly, the blood to be tested with the test kit of Example 1 will be collected from an emu, not an ostrich.

EXAMPLE 19

*The determination of the gender of an emu by DNA hybridization by the test kit of Example 2.*

The method of determining the gender of an emu is described in Example 4, using the test kit of Example 2. Clearly, the blood to be tested with the kit of Example 2 will be collected from an emu, not an ostrich.

EXAMPLE 20

*The determination of the gender of ostriches by simple Dot DNA hybridization.*

The cloned female-specific ostrich sequence containing SEQ ID NO:3 was used as a probe for the determination of the sex of ostriches by using whole blood, without any purification, by simple Dot hybridization. More particularly, the L fragment was used as a probe for the determination of the gender of ostriches.

The Dot hybridization method was performed as follows. First, the ostrich DNA was collected and prepared for use in a hybridization procedure. Accordingly, 50 µl of whole blood was collected from an ostrich. The 50 µl of whole blood was mixed with 1.2 mL phosphate buffered saline ("PBS") (1X) plus 300µL of 0.5 M EDTA by repeatedly pipetting. Next, 1 mL of 1M sodium hydroxide (NaOH) was added to the above mixture, and the resultant mixture was repeatedly pipetted to thoroughly mix the components of the mixture. The volume of the resultant mixture was adjusted to 15 mL with 0.4M NaOH. The resultant volume adjusted mixture is hereinafter referred to as the "DNA solution". The ostrich DNA was then ready for use in hybridization procedure with a nylon membrane as described below.

Next, approximately 100µl of the above DNA solution was blotted onto the top four rows of a positively charged nylon membrane (via a vacuum). Approximately 20µl of the above DNA solution was blotted onto the bottom four rows (rows five through eight) of the nylon membrane (via a vacuum). The blood of several male and female ostriches was spotted onto the nylon membrane as described above, and as illustrated in Figure 14. (M represents a male ostrich, and F represents a female ostrich.) More particularly, the top row of designations (F or M) are in reference to the first, second, fifth, and sixth rows. The bottom row of designations (F or M) are in reference to the third, fourth, seventh, and eighth rows.

Following transfer of the ostrich DNA onto the nylon membrane the membrane was allowed to dry. Once dried, the nylon membrane with the blotted DNA was hybridized with biotin labeled L fragment as described in the manual by Tropix. More particularly, the probe solution contained 200ng/5ml biotin labeled L fragment probe (which contains SEQ ID NO:3). Once the hybridization procedure has been completed, the nylon membrane is allowed to dry and the presence of the hybridized L fragment on the nylon membrane is visualized by autoradiography.

Figure 14 illustrates the results of the above simple dot hybridization of male or female ostrich DNA, from whole blood, hybridized with SEQ ID No:3 in the L fragment. As can be seen in Figure 14, the L fragment probe hybridized with the DNA in the blood from the female ostriches, and not the DNA in the blood from the male ostriches. Accordingly, the L fragment probe containing SEQ ID NO:3 determines the gender of ostriches in DNA hybridization methods.

## EXAMPLE 21

*The determination of the gender of a ostriches and emus by simple dot blot DNA hybridization.*

The L fragment containing SEQ ID NO:3 and the S fragment containing SEQ ID NO:4 were used as probes for the determination of the gender of ostriches and emus by using whole blood, without any purification, by simple dot hybridization, as shown in Figure 15.

Figure 15 illustrates the results of simple dot hybridization of ostrich or emu DNA, from either male or female whole blood, the top four rows representing samples from either a male or female bird hybridized with SEQ ID NO:4 of the S fragment of the gender probe, and wherein the rows five through eight represent a simple dot hybridization of samples from a male or female bird with SEQ ID NO:3 of the L fragment of the gender probe.

The Dot hybridization method was performed as described in Example 20, with the exception of the concentrations and amount of blood blotted onto the nylon membrane, and of the use of emu blood, and the S fragment probe. More particularly, the first, second, fifth, and sixth rows of Figure 15 had approximately 100µl of the DNA solution blotted onto the nylon membrane. The third, fourth, seventh, and eighth rows had approximately 50µl of the DNA solution blotted onto the nylon membrane.

As can be seen in Figure 15, the S fragment probe appears to be preferable (having more specificity) over the L fragment probe for determining the gender of emus. As can be seen in Figure 15, the L fragment and the S fragment probes hybridized with the DNA in the blood from the female ostriches, and not the DNA in the blood from the male ostriches. Accordingly, the L fragment probe containing SEQ ID NO:3 and the S fragment probe containing SEQ ID NO:4 determines the gender of ostriches in DNA hybridization methods.

## EXAMPLE 22

*The determination of the gender of ostriches and emus by simple dot blot DNA hybridization.*

The L fragment containing SEQ ID NO:3 and the S fragment containing SEQ ID NO:4 were used as probes for the determination of the gender of ostriches and emus by using whole blood, without any purification, by simple dot hybridization, as shown in Figure 16.

Figure 16 illustrates the results of simple dot hybridization of ostrich or emu DNA, from either male or female whole blood, the top four rows representing samples from either a male or female bird hybridized with SEQ ID NO:4 of the S fragment of the gender probe, and wherein the rows five through eight represent a simple dot hybridization of samples from a male or female bird with SEQ ID NO:3 of the L fragment of the gender probe.

The Dot hybridization method was performed as described in Example 20, with the exception of the concentrations and amount of blood blotted onto the nylon membrane, and of the use of emu blood, and the S fragment probe. More particularly, the first, second, fifth, and sixth rows of Figure 16 had approximately 100µl of the DNA solution blotted onto the nylon membrane. The third, fourth, seventh, and eighth rows had approximately 50µl of the DNA solution blotted onto the nylon membrane.

As can be seen in Figure 16, the S fragment probe appears to be preferable over the L fragment probe for determining the gender of emus. As can be seen in Figure 16, the L fragment and the S fragment probes hybridized with the DNA in the blood from the female ostriches, and not the DNA in the blood from the male ostriches. Accordingly, the L fragment probe containing SEQ ID NO:3 and the S fragment probe containing SEQ ID NO:4 determines the gender of ostriches in DNA hybridization methods.

It should be understood, of course, that the foregoing relates only to preferred embodiments of the present invention and that numerous modifications or alterations may be made therein without departing from the spirit and the scope of the invention as set forth in the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(ii) TITLE OF INVENTION: Methods of Determining the Gender of Poultry

(iii)NUMBER OF SEQUENCES: 9

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.50
(B) COMPUTER: MacIntosh
(C) OPERATING SYSTEM: 7.0
(D) SOFTWARE: Microsoft Word

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/202,909
(B) FILING DATE: February 28, 1994

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGCTACATC CCCAAGAACC CCTAC 25

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCGGATGGT 10

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1708 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TAACGTGGCA GATGAAAACC CAATTATTAA GAGGGGCTCC AAGTGCCTTA AAGCCATTAC   60
TACTTGGCAC AGTAATGCCT GCAACTGGGT CAGTAGGGGC TTTGGTAAAC AATATAAGGG  120
ATTTGGCACT TGTTGGTGGA CCGGATACTA AGCAAGCGAG AGCAGCTTGG AATTCCAAGG  180
TGAAAAAATT GGCAGGGACT ACTAAATTGA GTGGAAAGGT AACGAGGAGA CAGATGTGGA  240
CTGATCTGCT GCAGGCAGGG GGATTATGTG ATGAGATAAA CGGTCTTTCT ACTGCTGACT  300
TGTTTAAGCG GTGGCAGCGG CTACCTGCCA ATCAGCAATA TCGGACACAG CCTACCGTCC  360
ACCCGCTCCA GCCACAGCGT GGAAACTGCC AGCCAAAAGA CAATGAGGGG GAGGCGGGTC  420
CCCCACAATA CGAGCGCCAG TTGCGGCTTA CCATCCGGGA GGAGGGGACT AACGCCCTTA  480
TGTACCCCTG TGAATCTGCT GGCGCTCTGG AGGAGAAATT GCTGTGCTTG CTTTAGTGGA  540
TACTAGGGCA GAAGTTACTG TATTACATAG TGCAGTAGCT CTGGGGAAGG CCGCTACCCA  600
CATGTGCGGG TTGGGAGGAT CGGCCACCCC AGCGGTACAG GCTGTAATGT TAGCAATTGG  660
CAAAACCCCC CCATTTTCCA CCTCTGTTTT ATTAGCAGCA ATTCATGAGT ACATCTTGGG  720
GATCGACATG ATACTGGGCA GAGATATTCA GACCCTGCGA GGGGCCACAG CAAATTATAC  780
ACCTCTAGAA AAACAGCTTT TGGCTGTGGG GTGGGCTTTG CAGGATACCG AAGGAGTTAC  840
AGGACGTGAC CCGGTGACTT TACATACCCC TCATCCCCAT TCAGGCATGG GTGACTGATG  900
ATACAGTCAG GGTCCATGTG GCGGGGGGCC CAAGCTGCAA CTCAGTGGAA ATGGAAACAC  960
TATTTACAAG CCCGGTTTAA GGAGGGAAGA AAGGACATGA GTACCCTACA TGCAACCTTG 1020
TTAGGGGAAA TATATTTTGA GCACATGCCT CTGTTGTCTG AGCCAGAGGG GCTGCCCCTC 1080
CCTGCTCCCC CAATAGAGCC ATCGCCTGTC CAAGAGGCGC CTCCTTTCGA GACGTTATCC 1140
CCTGAGCAAC GAGGGTGGGC CTGGTTTCCT GATGGCAGTG CAGTGTATTC CTGGGCAGGA 1200
GAGCGAGCGT GGCGGTCTGT AGCATGTCAT CCTGCCACGG ATACTATCTG TTTTGCCTCA 1260
GGAACAGGAT ACTCAAGTCA GTGGGCCGAA TTAAAGGCCG CAGCTATAGC TCTTAATGAG 1320
GGAGATGCAC GCTACCTCTA TACGGATAGC TGGGTAGTGT ACAAGGGTCT GCGAACCTGG 1380
TTACCAACAT GGGCTGCCAC ACAGTGGAAG ATACATGATG AGGAACTCTG GGGGAGGCCC 1440
TTGTGGGAAA GGATTTGGGA CATTGTGCAA ACACCCTCAG TACACTCTTT AGCCAGTGTA 1500
TGGATACTAA CTCCTCAGAA GGAAAAATGG TCTGGCACTG TTTTAGCAGC TGGTCCAGGA 1560
GCCACCGCTC TAGTTCTTCC TGATGGCGAG ACCATGCCTT TCTATCTTCC CATCAACAGG 1620
TTATCACGTC GGCATCTGTA AGTTGCTGTT GGAATGTTGC CTCTTGCTGA CTATGACAGA 1680
TGCCATTGAT CCACAAGACC ATCCGGGG                                     1708
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(5) INFORMATION FOR SEQ ID NO:4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 924 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
CTAGAAAAAC AGCTTTTGGC TGTGGGGTGG GCTTTGCAGG ATACCGAAGG AGTTACAGGA    60
CGTGACCCGG TGACTTTACA TACCCCTCAT CCCCATTCAG GCATGGGTGA CTGATGATAC   120
AGTCAGGGTC CATGTGGCGG GGGGCCCAAG CTGCAACTCA GTGGAAATGG AAACACTATT   180
TACAAGCCCG GTTTAAGGAG GGAAGAAAGG ACATGAGTAC CCTACATGCA ACCTTGTTAG   240
GGGAAATATA TTTTGAGCAC ATGCCTCTGT TGTCTGAGCC AGAGGGGCTG CCCCTCCCTG   300
CTCCCCCAAT AGAGCCATCG CCTGTCCAAG AGGCGCCTCC TTTCGAGACG TTATCCCCTG   360
AGCAACGAGG GTGGGCCTGG TTTCCTGATG GCAGTGCAGT GTATTCCTGG GCAGGAGAGC   420
GAGCGTGGCG GTCTGTAGCA TGTCATCCTG CCACGGATAC TATCTGTTTT GCCTCAGGAA   480
CAGGATACTC AAGTCAGTGG GCCGAATTAA AGGCCGCAGC TATAGCTCTT AATGAGGGAG   540
ATGCACGCTA CCTCTATACG GATAGCTGGG TAGTGTACAA GGGTCTGCGA ACCTGGTTAC   600
CAACATGGGC TGCCACACAG TGGAAGATAC ATGATGAGGA ACTCTGGGGG AGGCCCTTGT   660
GGGAAAGGAT TTGGGACATT GTGCAAACAC CCTCAGTACA CTCTTTAGCC AGTGTATGGA   720
TACTAACTCC TCAGAAGGAA AAATGGTCTG GCACTGTTTT AGCAGCTGGT CCAGGAGCCA   780
CCGCTCTAGT TCTTCCTGAT GGCGAGACCA TGCCTTTCTA TCTTCCCATC AACAGGTTAT   840
CACGTCGGCA TCTGTAAGTT GCTGTTGGAA TGTTGCCTCT TGCTGACTAT GACAGATGCC   900
ATTGATCCAC AAGACCATCC GGGG                                          924
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(6) INFORMATION FOR SEQ ID NO:5:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCACTGGCAA AAGTGCATAT TAC 23

(7) INFORMATION FOR SEQ ID NO:6:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTACACTGGG GCTCCATTAA GATC 24

(8) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTTTGAGTAA CCAAGCAGCA GTGTC 25

(9) INFORMATION FOR SEQ ID NO:8:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACTGATGTTC TTCCAAGGAT GTGGG 25

(10) INFORMATION FOR SEQ ID NO:9:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGACTTAATG ATCTTAATGG AGCCCC 26

## Claims

We claim:

1. A method for determining the gender of ratites, comprising the steps of:

a) contacting under hybridizing conditions a labeled probe with a sample containing DNA from the ratite whose gender is to be determined;

b) removing any unhybridized labeled probe; and

c) detecting the presence of hybridized labeled probe by visualizing the label,

wherein the labeled probe is an oligonucleotide sequence capable of hybridizing with a unique target female specific oligonucleotide sequence in genomic ratite DNA.

2. The method of Claim 1, wherein the labeled probe is a probe having or containing substantially the same sequence as SEQ ID NO:3.

3. The method of Claim 1, wherein the labeled probe is a fragment of a sequence containing substantially the same sequence as SEQ ID NO:3, wherein the sequence is capable of hybridizing with a unique target female specific oligonucleotide sequence in genomic ratite DNA.

4. The method of Claim 1, wherein the labeled probe is a probe having or containing substantially the same sequence as SEQ ID NO:4.

5. The DNA hybridization method of Claim 1, wherein the sample is a sample of whole blood.

6. The DNA hybridization method of Claim 1, wherein the label is selected from the group consisting of horseradish peroxidase, biotin, alkaline phosphatase, and ß-galactosidase.

7. The DNA hybridization method of Claim 6, wherein the label is horseradish peroxidase.

8. The DNA hybridization method of Claim 6, wherein the label is biotin

9. The DNA hybridization method of Claim 8, wherein the label is visualized by the addition of avidin conjugated with horseradish peroxidase.

10. The DNA hybridization method of Claim 8, wherein the label is visualized by the addition of strep-avidin conjugated with horseradish peroxidase.

11. The DNA hybridization method of Claim 5, further comprising the step of obtaining the blood by removing a quill from the ratite whose gender is to be determined.

12. A purified and isolated nucleotide sequence comprising SEQ ID NO:3.

13. The sequence of Claim 12, further comprising a recombinant DNA molecule comprising SEQ ID NO:3.

14. The composition of Claim 13, further comprising a recombinant host cell.

15. A fragment of the nucleotide sequence of Claim 12, wherein the fragment is capable of hybridizing with a unique target female specific oligonucleotide sequence in genomic ratite DNA.

16. The sequence of Claim 15, further comprising a recombinant DNA molecule comprising the fragment of Claim 15.

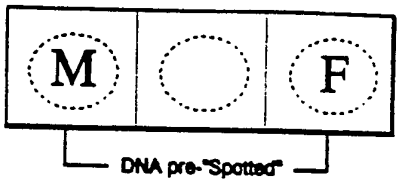
17. The composition of Claim 16, further comprising a recombinant host cell.

18. A purified and isolated nucleotide sequence comprising SEQ ID NO:4.

19. The sequence of Claim 18, further comprising a recombinant DNA molecule comprising SEQ ID NO:4.

20. The composition of Claim 19, further comprising a recombinant host cell.

FIGURE 1.


M
F
DNA pre-"Spotted"

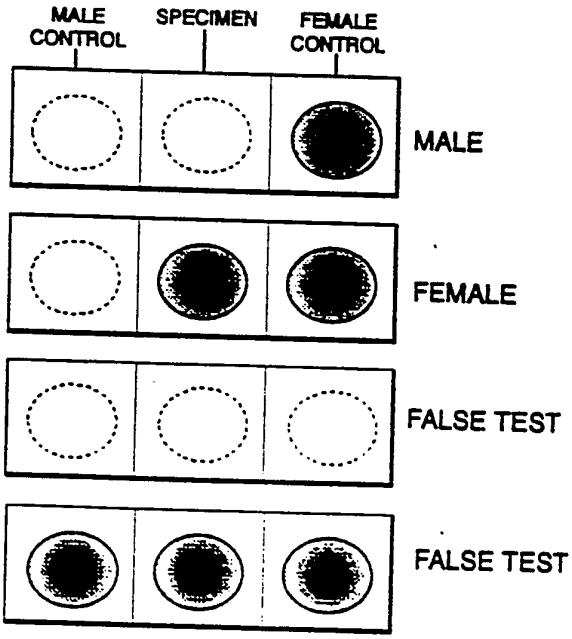

MALE CONTROL
SPECIMEN
FEMALE CONTROL
MALE
FEMALE
FALSE TEST
FALSE TEST

FIGURE 2

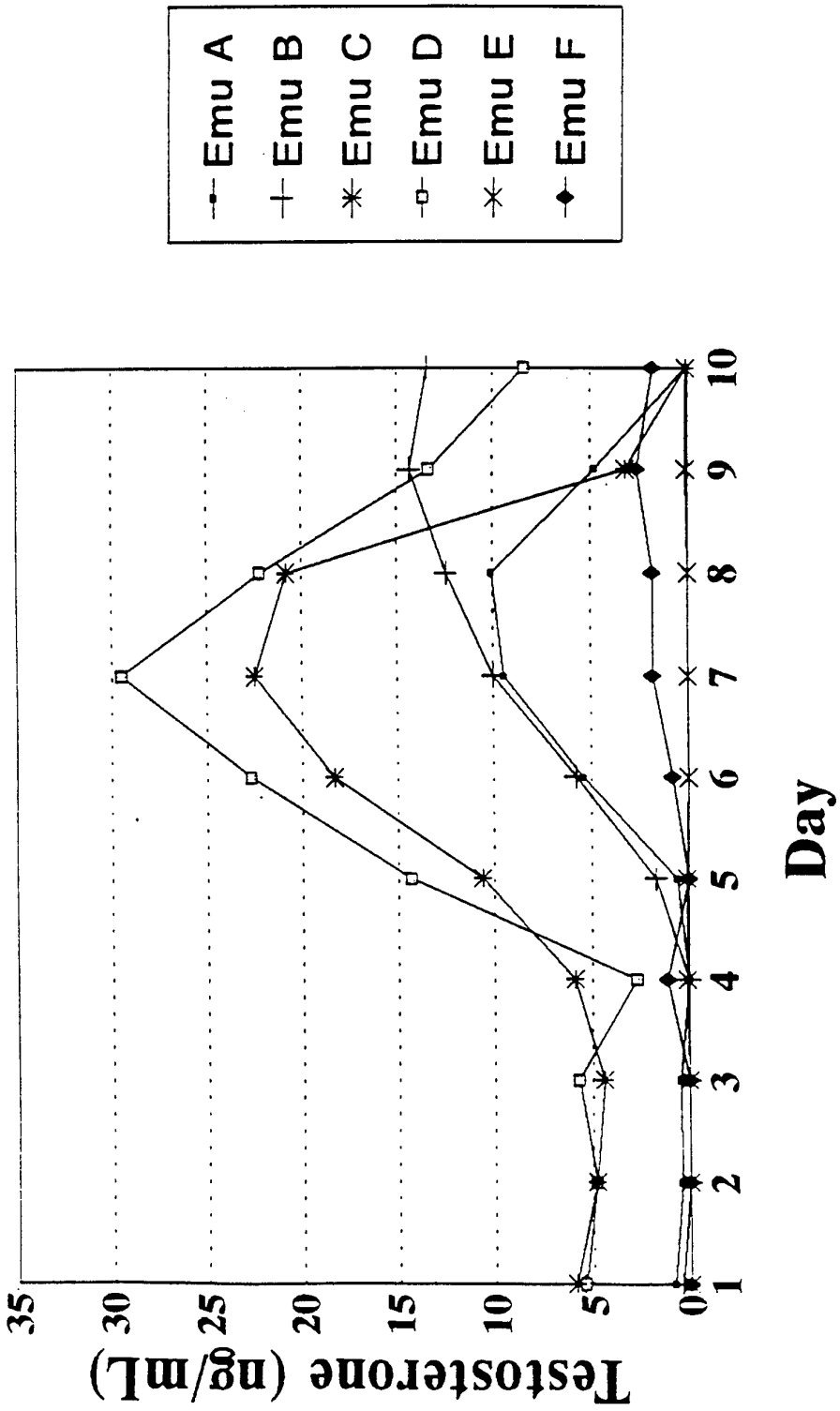

Emu A
Emu B
Emu C
Emu D
Emu E
Emu F
Testosterone (ng/mL)
0
5
10
15
20
25
30
35
Day
1
2
3
4
5
6
7
8
9
10

FIGURE 3

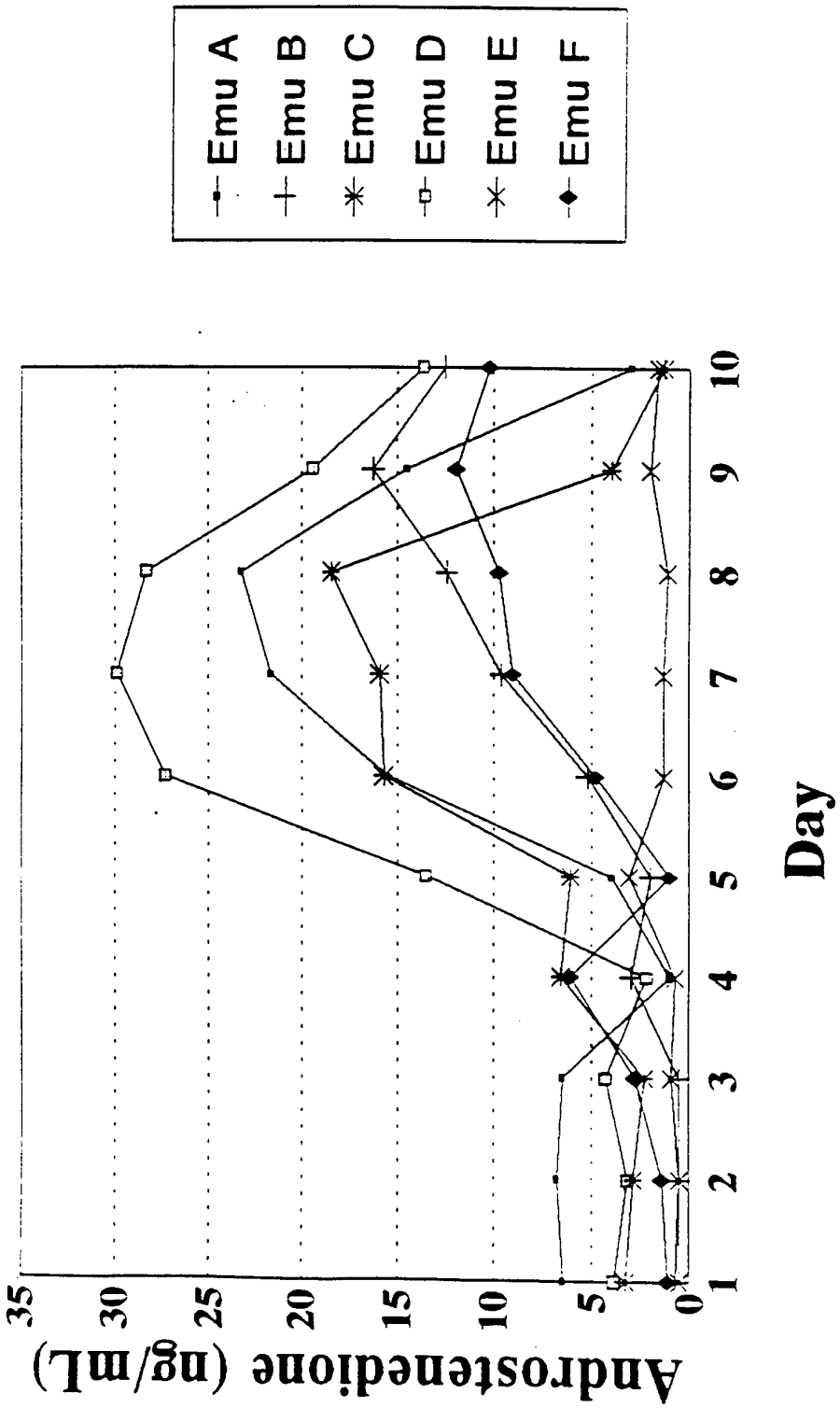
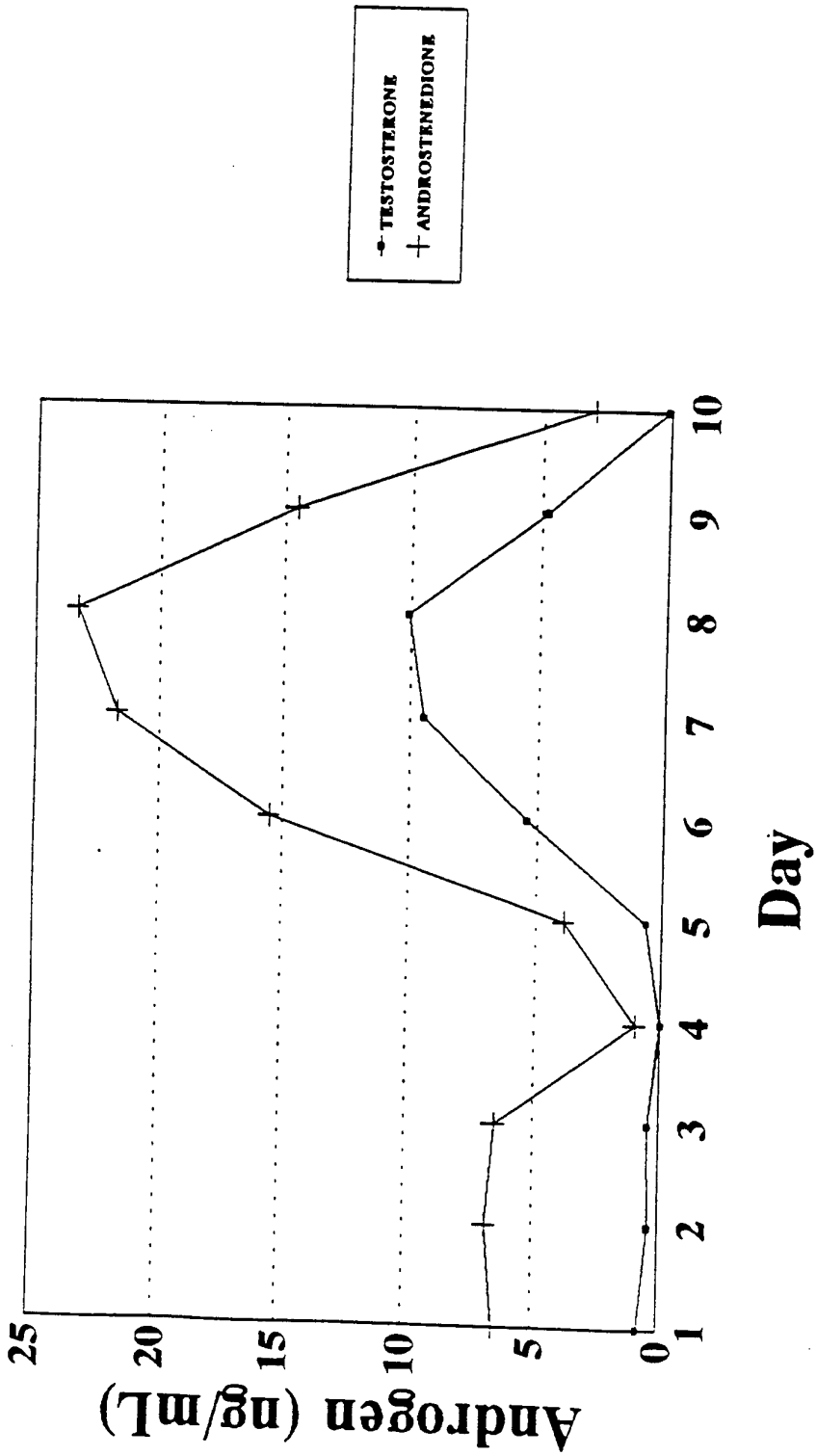

Emu A
Emu B
Emu C
Emu D
Emu E
Emu F
Androstenedione (ng/mL)
0
5
10
15
20
25
30
35
Day
1
2
3
4
5
6
7
8
9
10

FIGURE 4


TESTOSTERONE
ANDROSTENEDIONE
Androgen (ng/mL)
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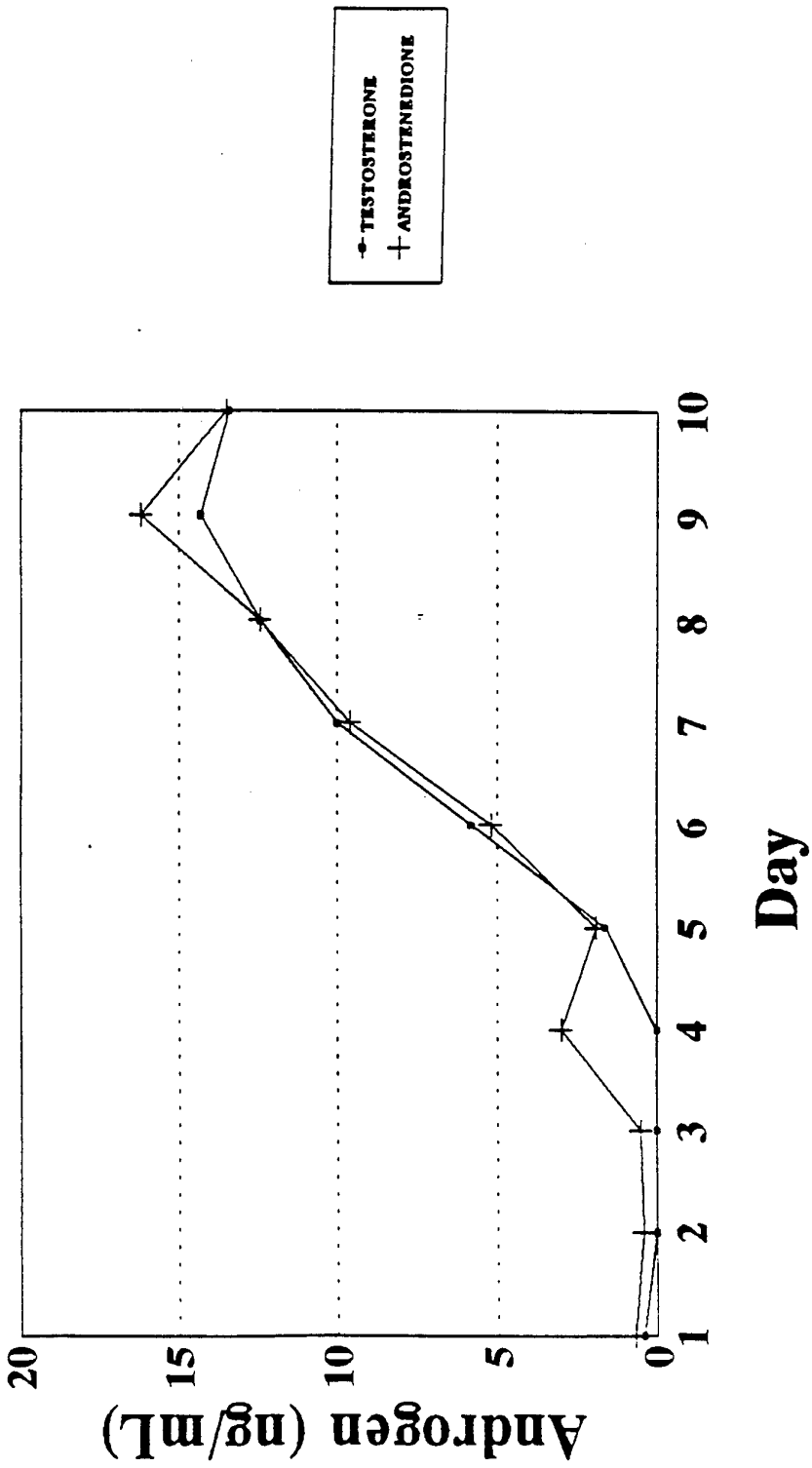
5
10
15
20
25
1
2
3
4
5
6
7
8
9
10
Day

FIGURE 5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ANDROSTENEDIONE
Androgen (ng/mL)
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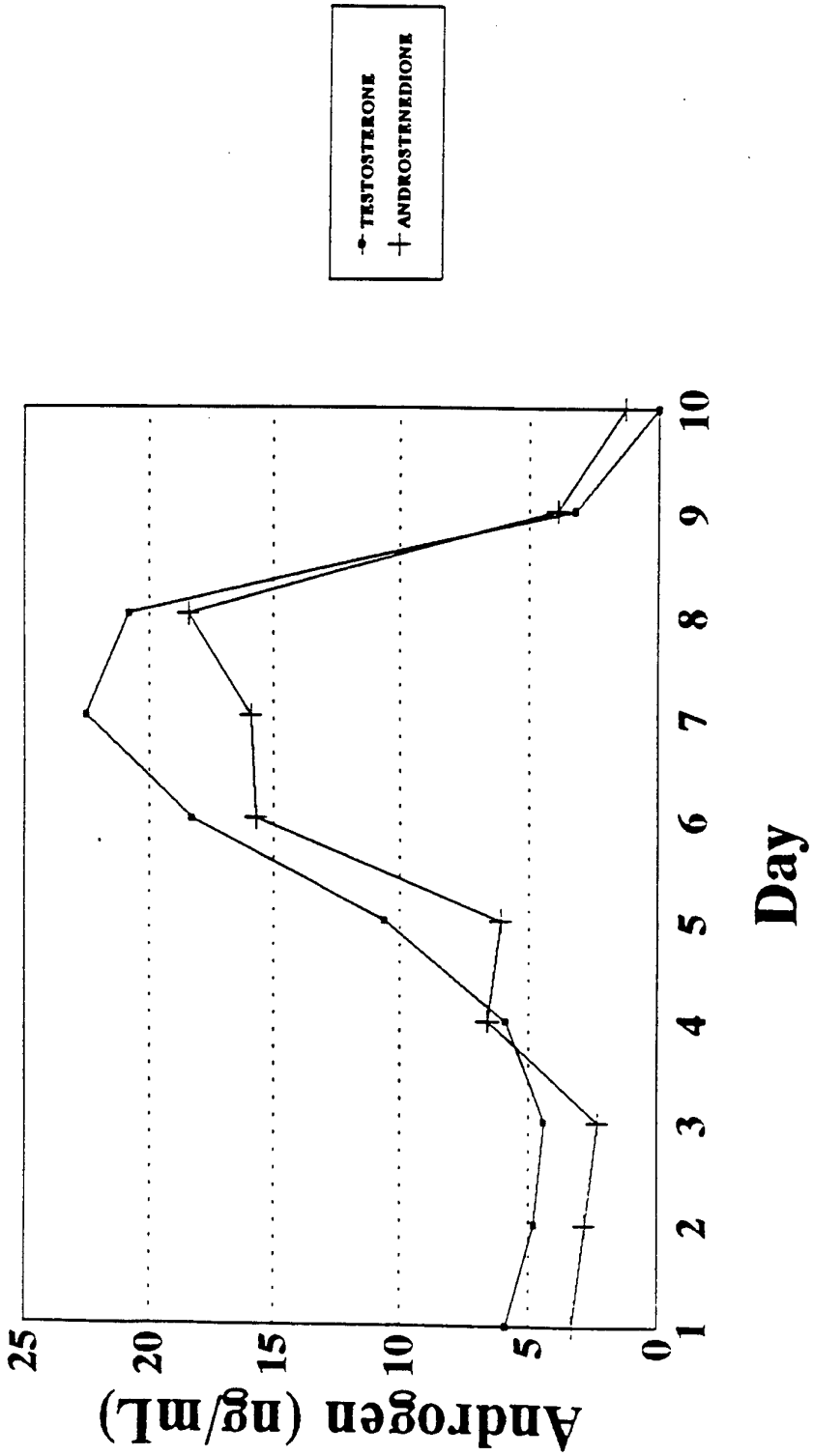
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Day

FIGURE 6


TESTOSTERONE
ANDROSTENEDIONE
Androgen (ng/mL)
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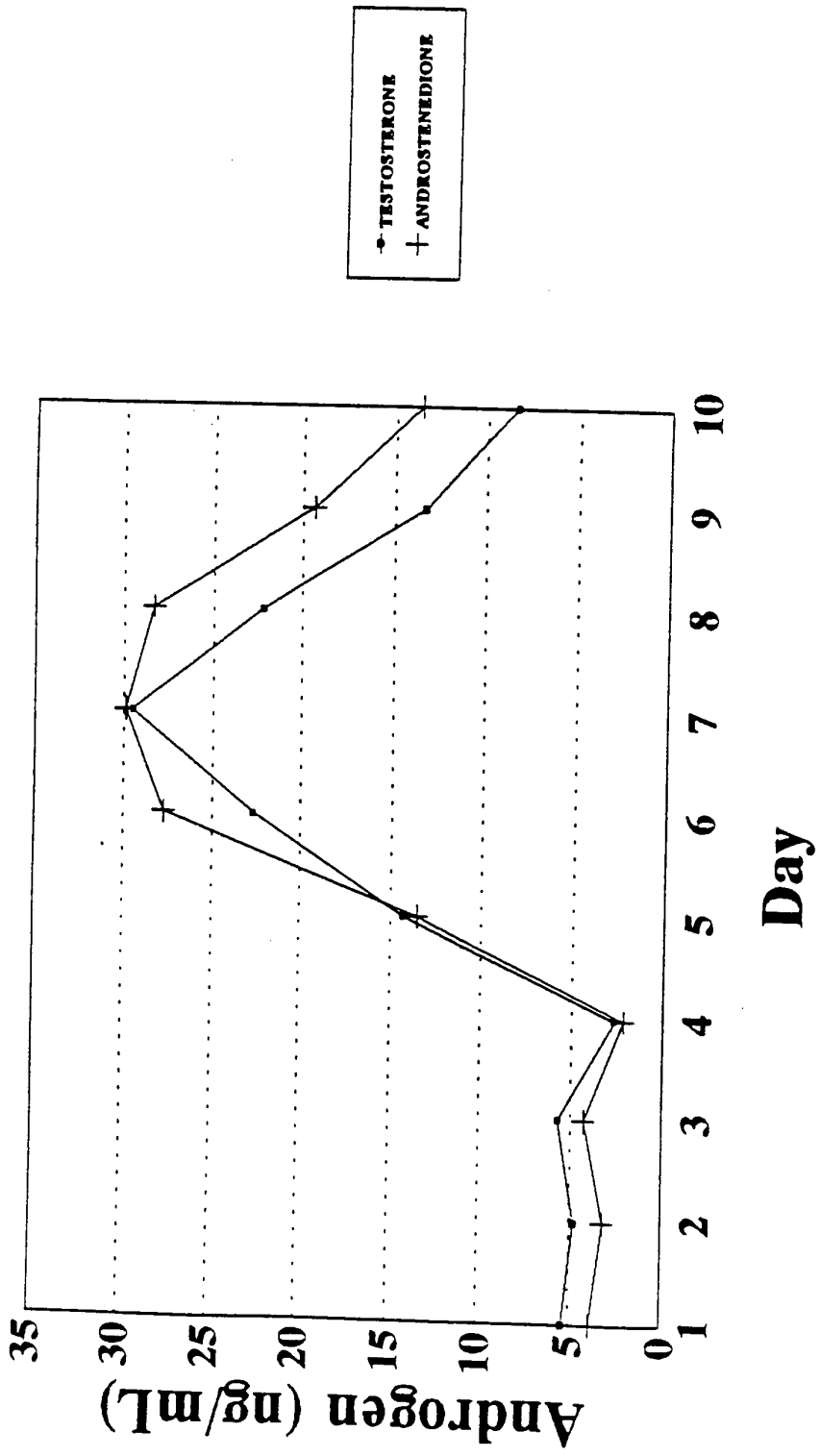
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
Day

FIGURE 7


TESTOSTERONE
ANDROSTENEDIONE
Androgen (ng/mL)
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30
25
20
15
10
5
0
Day
1
2
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5
6
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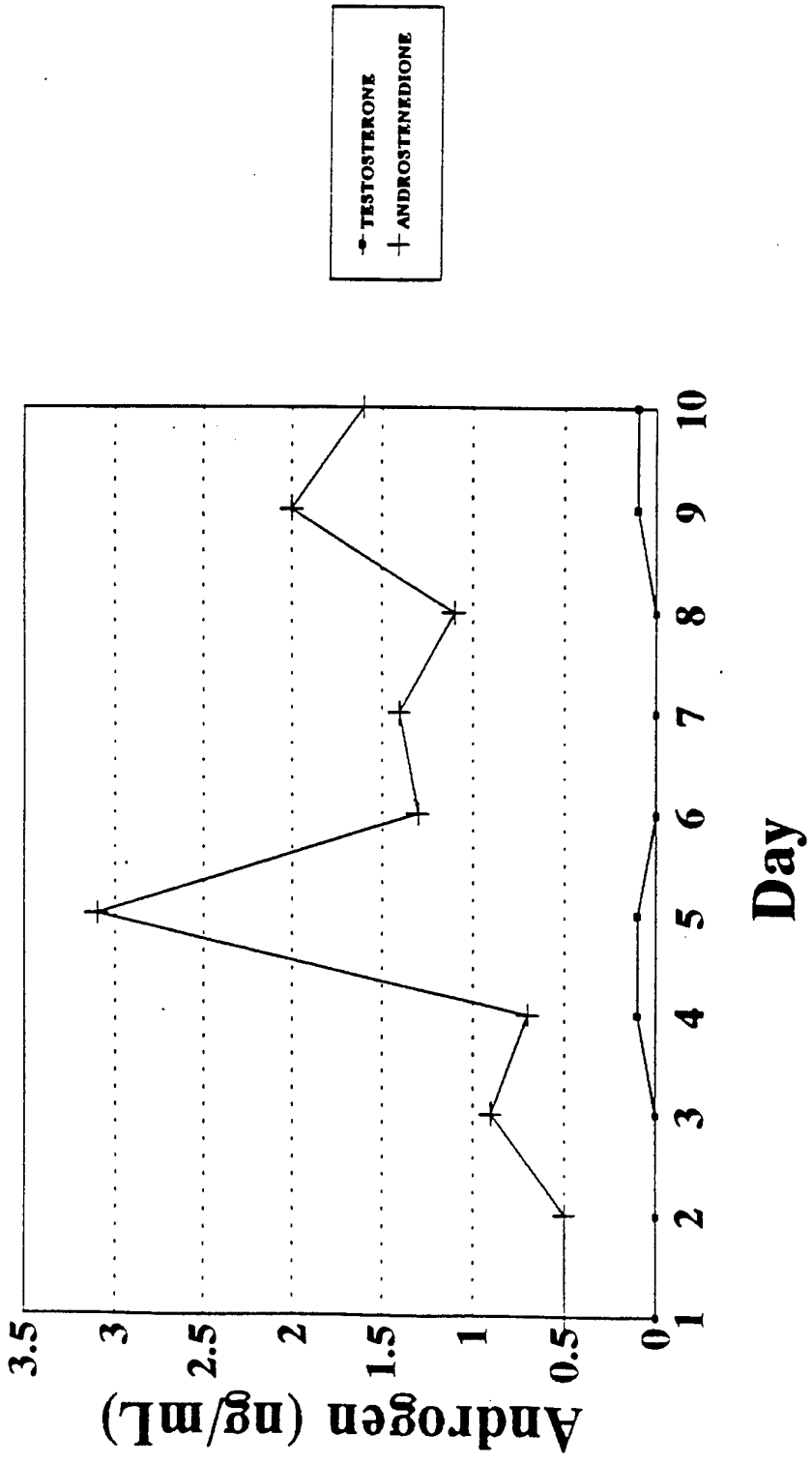
8
9
10

FIGURE 8


TESTOSTERONE
ANDROSTENEDIONE
Androgen (ng/mL)
3.5
3
2.5
2
1.5
1
0.5
0
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2
3
4
5
6
7
8
9
10
Day

FIGURE 9

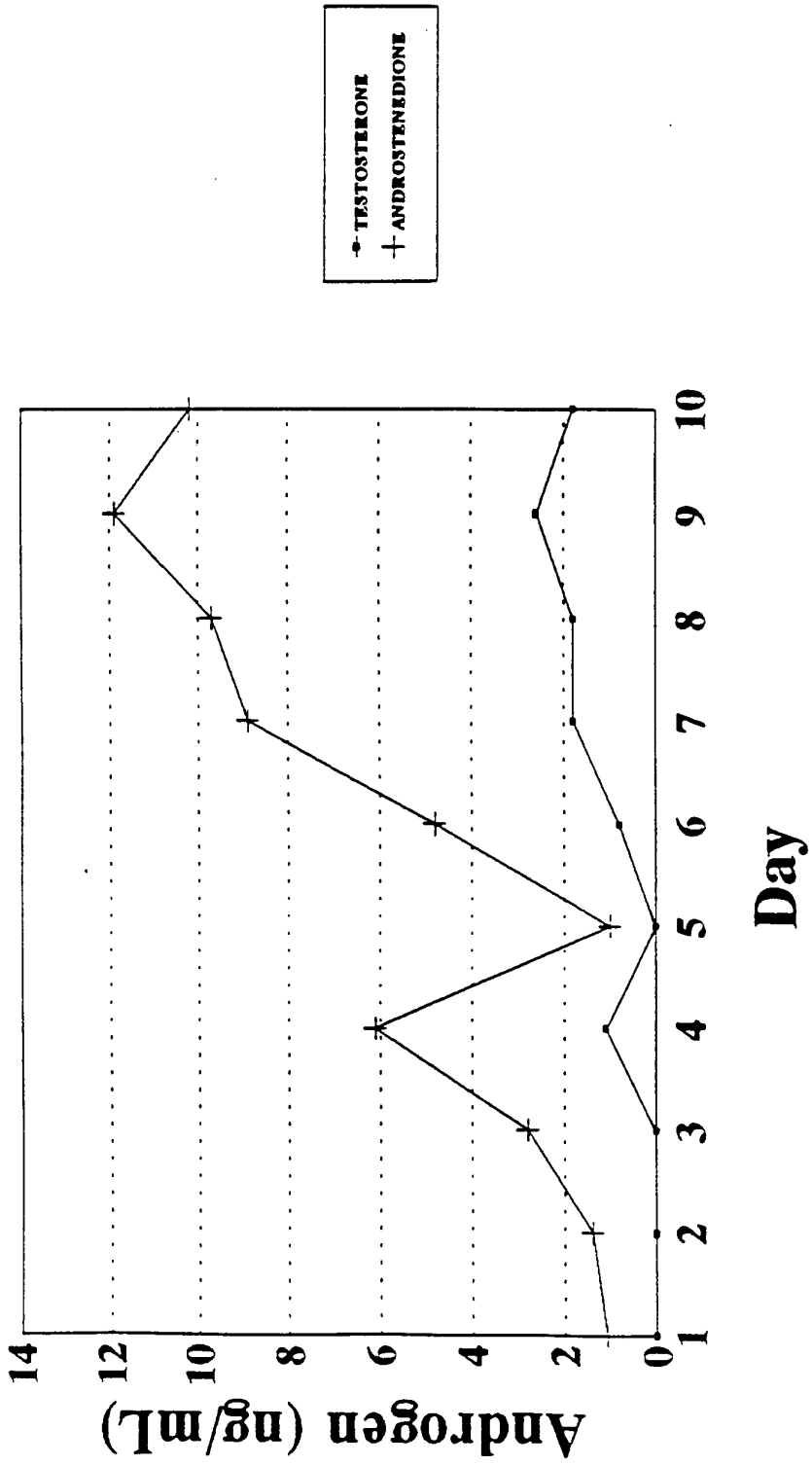
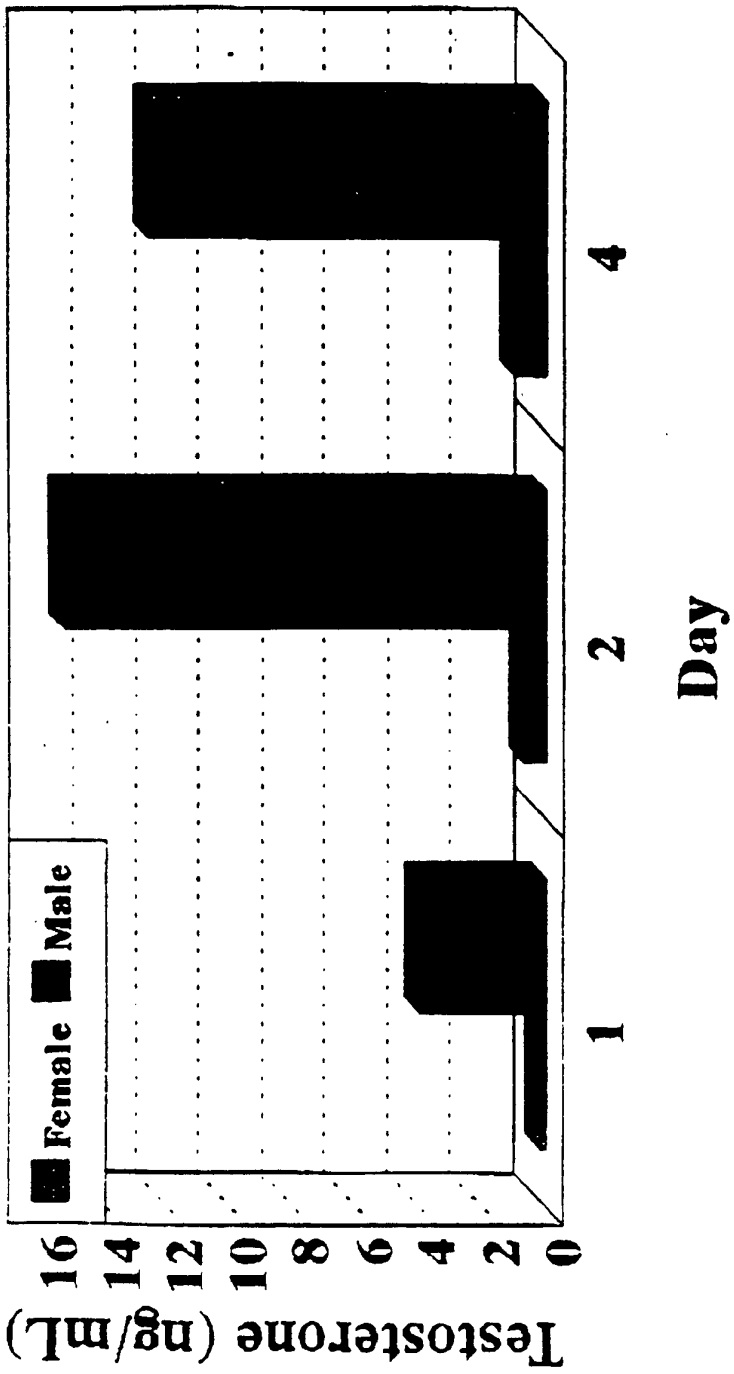

TESTOSTERONE
ANDROSTENEDIONE
Androgen (ng/mL)
0
2
4
6
8
10
12
14
Day
1
2
3
4
5
6
7
8
9
10

FIGURE 10



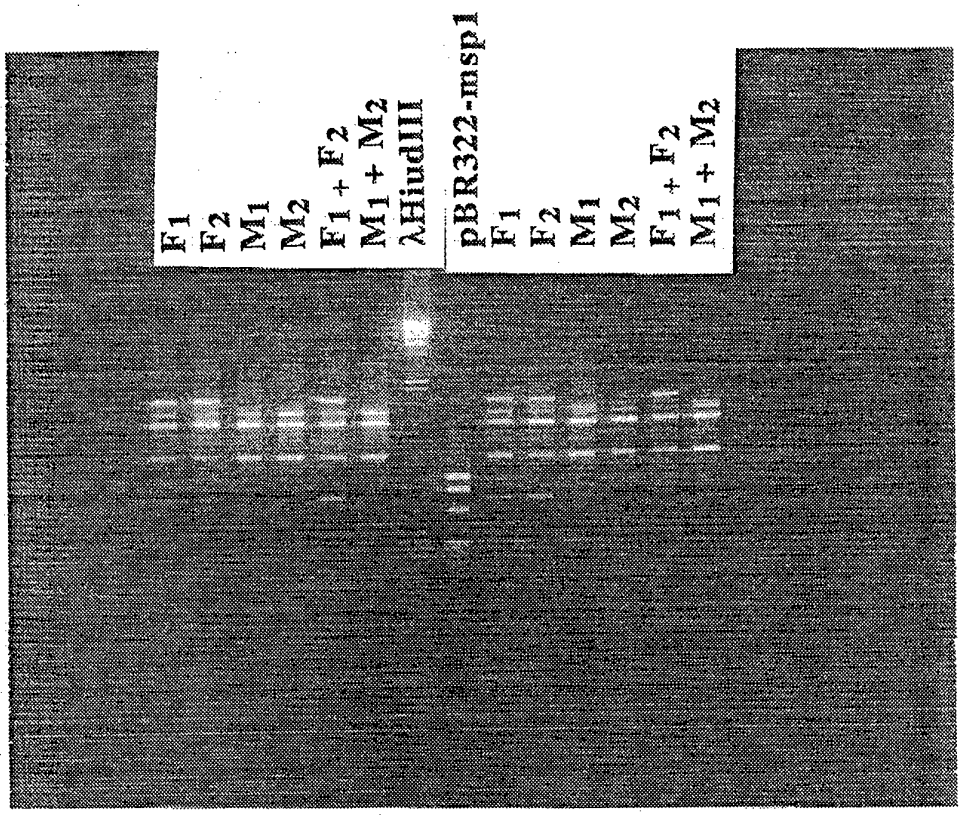

F1
F2
M1
M2
F1 + F2
M1 + M2
λHindIII
pBR322-msp1
F1
F2
M1
M2
F1 + F2
M1 + M2

FIGURE 11

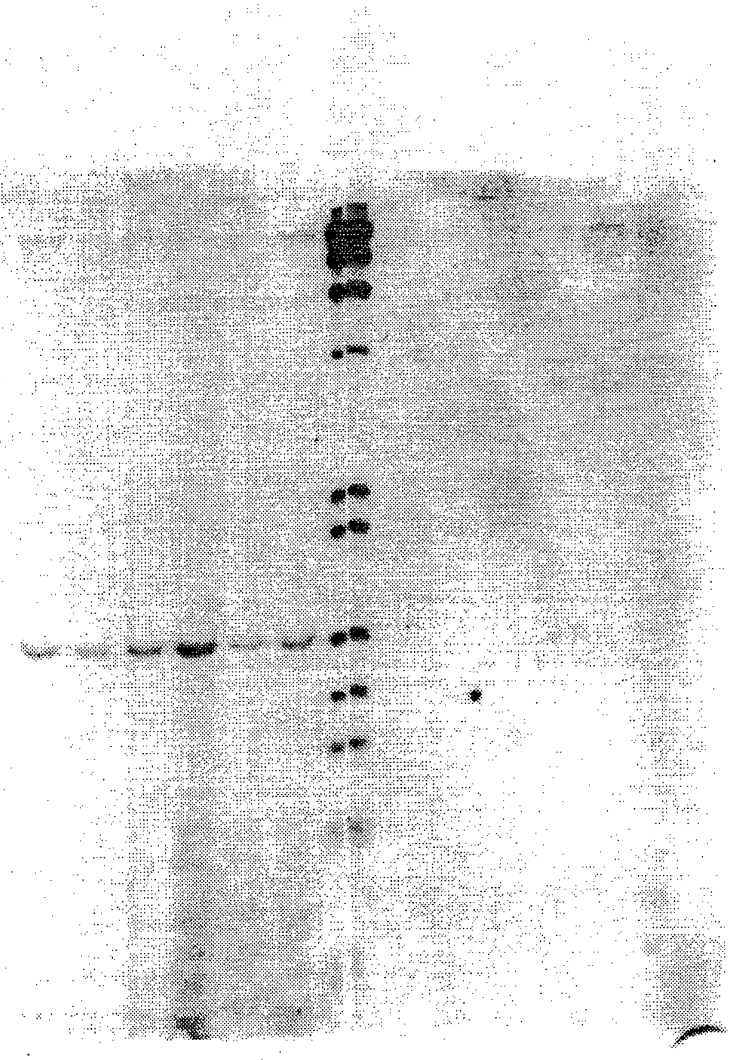
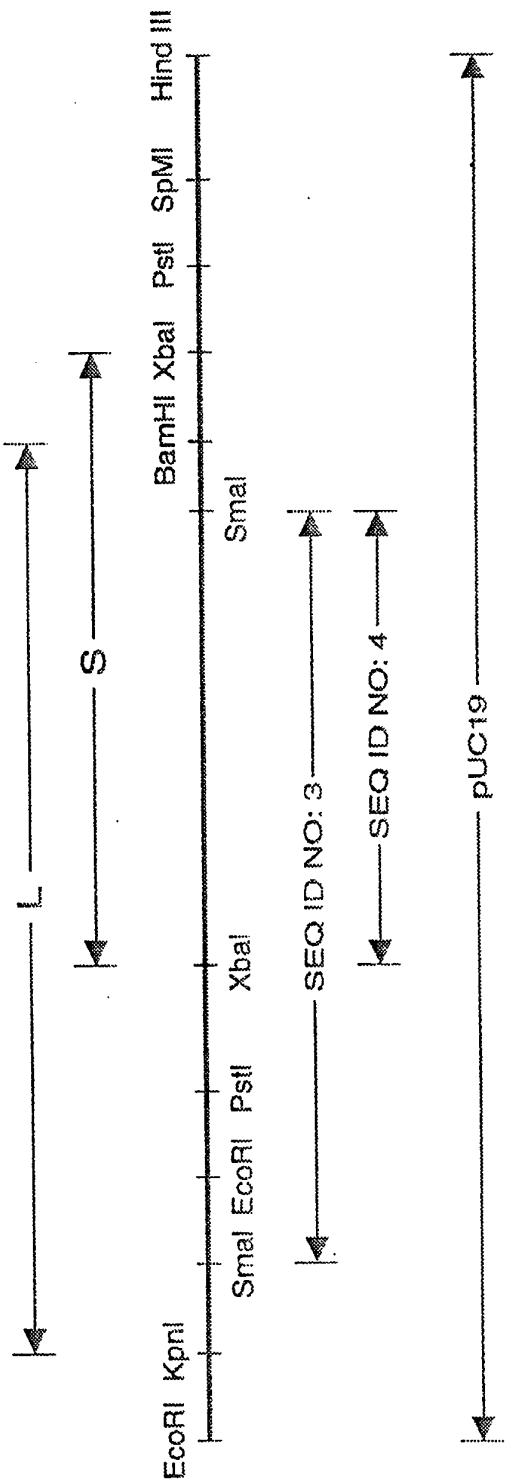


FIGURE 12


Hind III
SpMI
PstI
XbaI
BamHI
SmaI
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PstI
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KpnI
EcoRI
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S
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SEQ ID NO: 4
pUC19

Figure 13

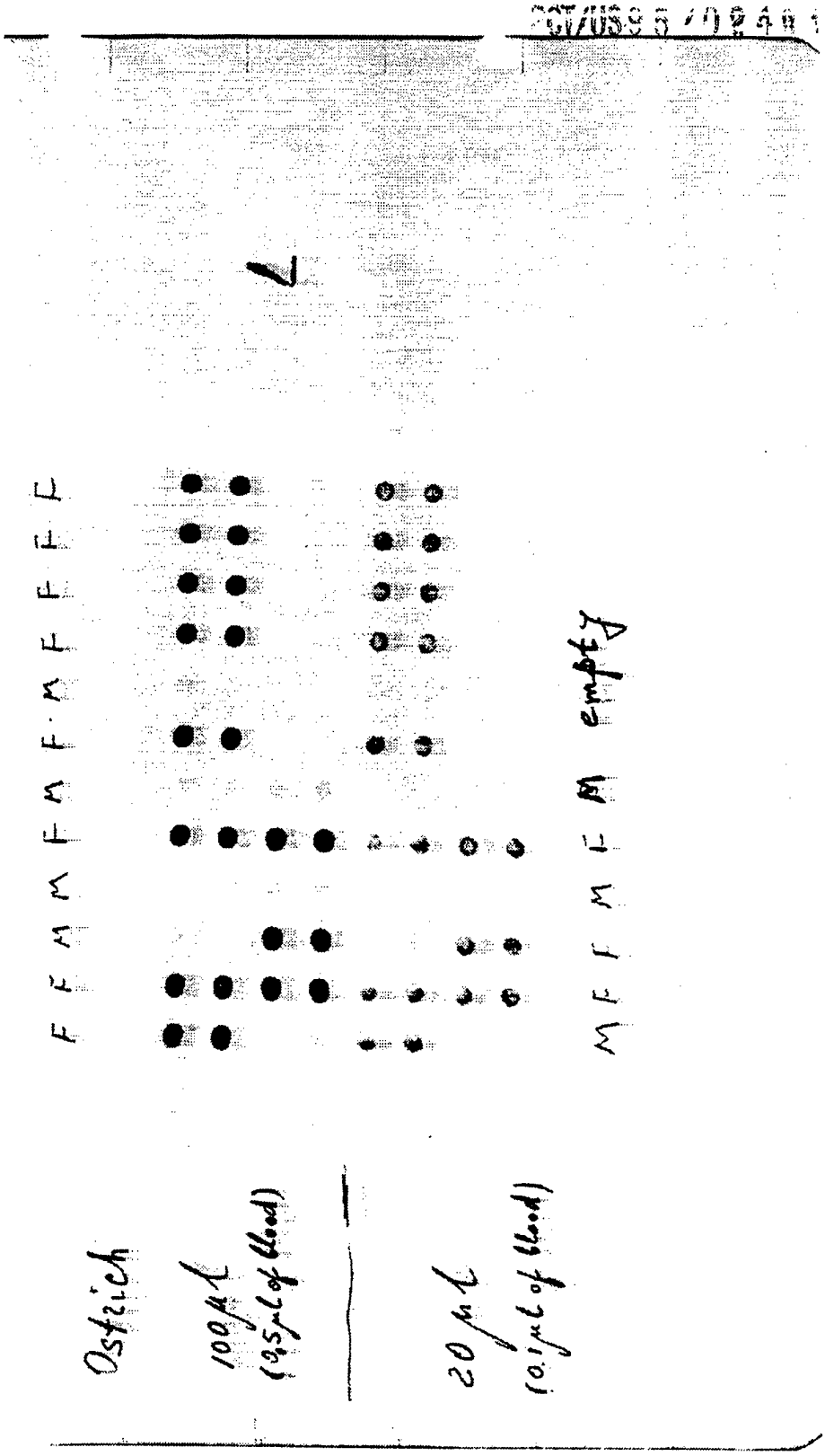


FIGURE 14

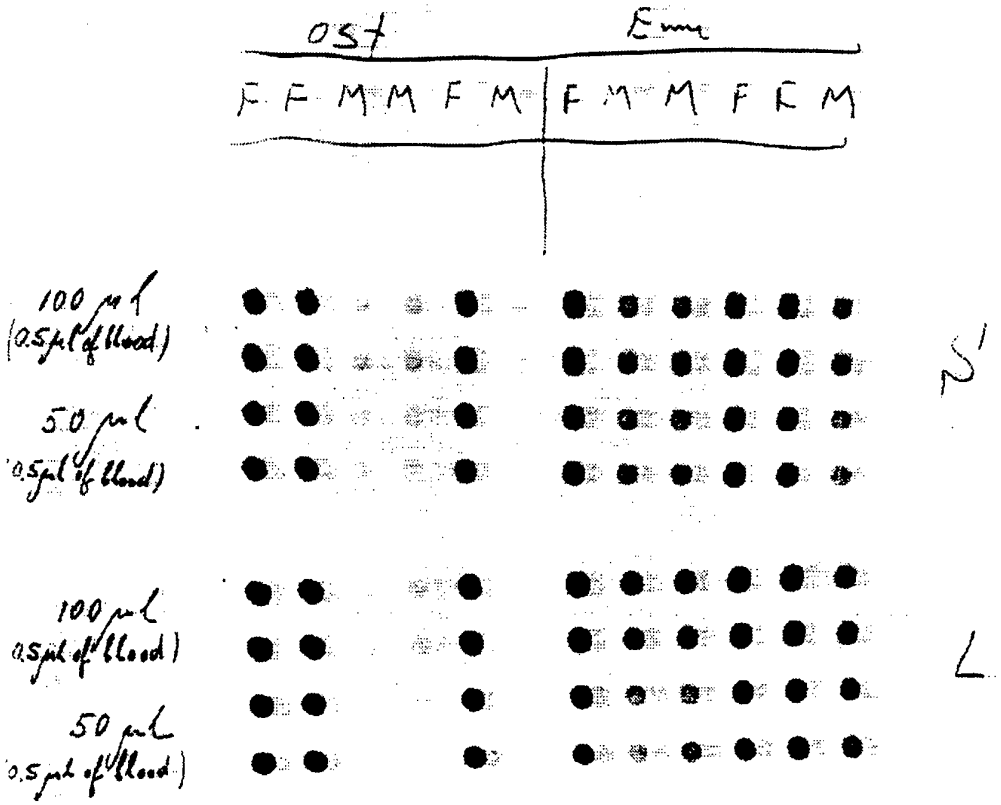


FIGURE 15

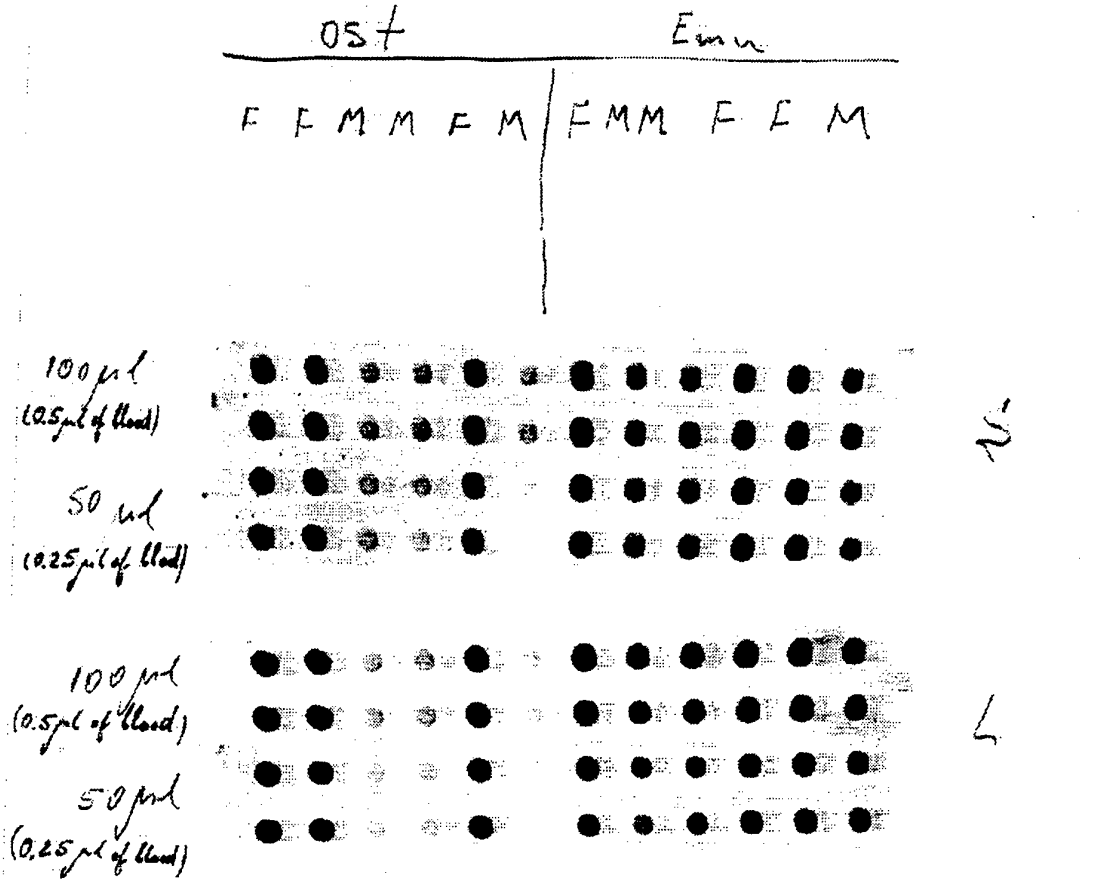


FIGURE 16

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/02481

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C07H 21/02
US CL : 435/6; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, BIOSIS, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | POULTRY SCIENCE, VOLUME 68, ISSUED 1989, URYU ET AL, "RESEARCH NOTE: DETERMINATION OF THE SEX OF CHICKENS BY A BIOTIN-LABELED DEOXYRIBONUCLEIC ACID PROBE", PAGES 850-853, SEE ENTIRE DOCUMENT. | 1-20 |
| Y | CYTOGENETIC CELL GENETICS, VOLUME 47, ISSUED 1988, ANSARI ET AL. "MORPHOLOGICAL DIFFERENTIATION OF SEX CHROMOSOMES IN THREE SPECIES OF RATITE BIRDS", PAGES 185-188. SEE ENTIRE DOCUMENT. | 1-20 |
| Y | GENOME, VOLUME 34, ISSUED 1991, RABENOLD ET AL, "POLYMORPHIC MINISATALLITE AMPLIFIED ON AVIAN W CHROMOSOME", PAGES 489-493, SEE ENTIRE DOCUMENT. | 1-20 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

| | | | |
|---|---|---|---|
| * | Special categories of cited documents: | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention |
| "A" | document defining the general state of the art which is not considered to be of particular relevance | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone |
| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" | document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family |
| "O" | document referring to an oral disclosure, use, exhibition or other means | | |
| "P" | document published prior to the international filing date but later than the priority date claimed | | |

Date of the actual completion of the international search
17 APRIL 1995

Date of mailing of the international search report
03 MAY 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
EGGERTON CAMPBELL
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/US95/02481

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | HALVERSON ET AL, SYMPOSIUM: "CURRENT ADVANCES IN REPRODUCTION", PUBLISHED 1992, PAGES 890-896. SEE ENTIRE DOCUMENT. | 1-20 |
| Y | NATURE, VOLUME 327, ISSUED 14 MAY 1987, "DNA FINGERPRINTING IN BIRDS", PAGES 149-152. SEE ENTIRE DOCUMENT. | 1-20 |
| Y | NUCLEIC ACID RESEARCH, VOLUME 20, NUMBER 19, ISSUED 1992, MIYAKI ET AL, "SEX TYPING OF ARATINGA PARROTS USING THE HUMAN MINISATELLITE PROBE 33.15, PAGES 5235-5236, SEE ENTIRE DOCUMENT. | 1-20 |
| Y | US, A, 5,215,884 (MCGRAW III) 01 JUNE 1993, SEE ENTIRE DOCUMENT. | 1-12 |