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SEX DIAGNOSIS OF PREIMPLANTATION PORCINE EMBRYOS THROUGH PCR AMPLIFICATION OF THE SRY GENE

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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SEX DIAGNOSIS OF PREIMPLANTATION PORCINE EMBRYOS THROUGH PCR AMPLIFICATION OF THE SRY GENE

ABSTRACT

Heather L. Watt M.Sc. Animal Science

Multiplex polymerase chain reaction (PCR) assays were designed that incorporated primer pairs for the sex determining region on the Y chromosome, *Sry*, and one or two control sequences. A triplex and a duplex assay were created involving *Sry* and *Dax*, a single copy X chromosome gene which is involved in the female sex determination pathway. The third sequence in the triplex assay was a repetitive Y chromosome sequence, *YR*. The *Sry* sequences were used for actual sex determination of each embryo. The *Dax* and *YR* sequences represented controls for the presence of DNA in the samples. A nested PCR assay was required since both *Sry* and *Dax* are single copy genes and only a small amount of template DNA would be available for sexing purposes. A minimum of $2.5x10^{-3}$ to $2.5x10^{-4}$ µg/µL of male DNA and 2.5×10^{-5} µg/µL of female DNA was required if a single multiplex PCR was performed. Amplification of both *Dax* and *Sry* DNA sequences in a nested PCR is possible with as little as $2.5x10^{-7}$ µg/µL of DNA in the initial reaction. To demonstrate that sex determination of preimplantation porcine embryos is possible, morulae were collected 5 d post insemination from pregnant mare serum gonadotrophin (PMSG)/ human chorionic

gonadotrophin (hCG)-treated 60-70 kg prepubertal gilts. To recover DNA, embryos were biopsied using a micromanipulator and cells were placed into individual microcentrifuge tubes for further analysis. Embryos were then cultured in Whitten's medium at 39°C in 5% CO_2 to allow development to the blastocyst stage. A total of 315 embryos were sexed via the PCR assay with a resultant female/male (%) ratio of 73/27. When 94 embryos from heavier gilts (90-100 kg) were sexed in a similar assay, the resultant female/male ratio was 60/40. Attempts were made to correlate these results with karyotypes. Five transfers of sexed embryos into synchronized recipients were attempted. None of these resulted in pregnancies; although return to estrus was delayed by two to eight days, in four out of the five recipients. Our findings suggest that PCR amplification of the *Sry* gene can be a reliable method for sexing porcine embryos, particularly those of higher quality as recovered from the heavier donors. It does appear that embryo quality is critical for both the PCR assay and subsequent successful culture.

LE DIAGNOSTIC DU SEXE DES JEUNES EMBRYONS PORCINS PAR L'AMPLIFICATION DU GÈNE SRY PAR LA RÉACTION DE POLYMÉRISATION EN CHAINE (PCR)

RÉSUMÉ

Heather L. Watt M.Sc. Sciences Animales

Des paires d'amorces ont été utilisées simultanément afin d'amplifier par la réaction de polymérisation en chaîne (PCR) le gène sur le chromosome Y déterminant le sexe chez les embryons porcins (*Sry*) et une ou deux séquences de contrôle afin de s'assurer de la présence d'ADN dans les échantillons. Alors que le gène *Dax*, présent sur le chromosome X et impliqué dans la détermination du sexe femelle, a été utilisé comme séquence contrôle dans des amplifications par PCR en duplex, une séquence répetitive sur le chromosome Y (*YR*) a été utilisée comme deuxième séquence de contrôle en plus du gène *Dax* dans des amplifications par PCR triplex. Deux séries d'amplifications étaient nécessaires étant donné que les séquences *Sry* et *Dax* sont représentés en simple copies par génome et que l'amplification à partir d'embryons se base sur des petites quantitées d'ADN. Un minimum d'ADN de $2.5x10^{-3}$ à $2.5x10^{-4}$ µg/µL était nécessaire pour analyser les mâles alors qu'un minimum de $2.5x10^{-5}$ µg/µL était nécessaire pour analyser les femelles. Cependant, en faisant une deuxième série d'amplification, on pouvait obtenir des amplifications à partir de seulement $2.5x10^{-7}$ µg/µL. Dans l'optique de

démontrer que la détermination du sexe des jeunes embryons est possible, des embryons au stade de morula ont été échantillonnés 5 jours après l'insémination des femelles gestantes de 60 à 70 kg ayant été stimulées avec du PMSG/hCG (pregnant mare serum gonadotrophin/human chorionic gonadotrophin). Des échantillons ont été prélevées à partir des embryons afin d'en extraire l'ADN pour les analyses de PCR. Les embryons ont ensuite été placés dans un médium Whitten à 39°C dans 5% de CO_2 pour leur permettre de se développer jusqu'au stade de blastocyste. Le sexe de 315 embryons a été déterminé et l'analyse a démontré que la proportion de femelles/mâles était de 73/27. Aussi, le sexe de 94 embryons de femelles plus lourdes (90-100 kg) a également été déterminé et l'analyse a démontré que la proportion de femelles/mâles était de 60/40. On a aussi tenté de démontrer la présence d'une corrélation entre les résultats de caryotypes et ceux du PCR. Cinq transferts d'embryons ont été tentés chez les femelles ayant été synchronisées mais tous ont échoués, bien qu'il y ait eu un retardement des chaleurs de deux à huit jours chez quatres femelles. Nos résultats suggèrent que l'amplification par PCR du gène *Sry* est une méthode efficace pour déterminer le sexe d'embryons porcins surtout chez les embryons de meilleure qualité comme ceux obtenus à partir des femelles plus lourdes. Par conséquent, il semble que la qualité des embryons soit crucial pour l'analyse par PCR et pour un bon développement embryonnaire *in vitro*.

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GENERAL INTRODUCTION

The processes of sex determination and differentiation involve multiple interacting pathways which lead to the development of male or female characteristics. These pathways must include a switch mechanism responsible for the decision to become male or female. Throughout evolution, several mechanisms have evolved for this purpose. In lower organisms, such as yeast, the switch is a particular allele at an active mating type locus. Mammals have a conceptually similar mechanism in that the presence of the Y chromosome acts as a dominant male determinant (Lovell-Badge, 1992). In several other species, all of the genes responsible for sexual dimorphism are present in both males and females. In reptiles, the chromosomes are identical in both sexes, and the switch is environmental. In Drosophila, the X : autosome ratio is critical for the activation of one or the other pathway and, although a Y chromosome is present, it is only required for fertility.

Muller (1914) hypothesized that the heteromorphic sex chromosomes of *Drosophila* have evolved from a homologous pair of chromosomes. Through repressed recombination and gene function loss, one chromosome evolved into what is now called the Y chromosome. This theory has also been postulated for the evolution of mammalian sex chromosomes.

Classical studies have defined two rules of sex determination (Goodfellow and Lovell-Badge, 1993). The first rule is derived from Jost's lab (Jost, 1947). Gonadal ridges were surgically removed from rabbit fetuses, in utero, which were then allowed to develop to term. If the ridges were removed just prior to gonadal development, all embryos, regardless of chromosomal sex, developed as females indicating that testes must be present for the development of male characteristics. Jost proceeded to demonstrate that the main substance involved was testosterone and predicted the presence of another factor: Mullerian Inhibiting Substance (MIS). The first rule can, therefore, be summarized as follows: "the specialization of the developing gonad into testis or ovary determines the subsequent sexual differentiation of the embryos."[1]

In mammals, the primary event in sex determination is the differentiation of the undifferentiated gonads (or genital ridges) into testes rather than ovaries. In the absence of the Y chromosome, the male differentiation pathway is either not initiated or blocked, leading to the development of a female (Hawkins *et al.*, 1992). Female development was, subsequently, considered to be the default or normal pathway while the Y chromosome diverted development into the testicular pathway (Lovell-Badge and Hacker, 1995).

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1. Goodfellow, P.N. and Lovell-Badge, R. 1993. SRY and sex determination in mammals. Annu Rev Genet. 27:71-92.

Experimental data suggest that, as a rule, the gonads of the homogametic sex are easily altered into those of the heterogametic sex but the reverse is not true (Boczkowski, 1971). In amphibians and birds, where the male is the homogametic sex, the testes can be converted into ovaries with steroid treatment. In mammals, the male is the heterogametic sex and it is much more difficult to transform a testis into an ovary.

The second rule, therefore, relates to the chromosomal basis of gonadal development. In mammals, individuals have an XX or an XY chromosomal constitution which generally leads to the development of females or males, respectively. The same results were seen in *Drosophila melanogaster;* however, the ratio of X chromosomes to autosomes was the crucial factor. When this was discovered, it was assumed that X chromosome dosage was the controlling mechanism for mammals as well. This was, however, proven wrong when a few females with an XO chromosomal constitution and some males with a single Y and multiple X chromosomes were discovered.

It doesn't matter how many X chromosomes are present; as long as there is a normal Y chromosome, the embryo will develop as a male. The fact that the Y chromosome carries a gene or genes that determine maleness implies that genetic sex is established at fertilization through the inheritance of either a Y or X chromosome from the father. This lead to the definition of the second rule: "the Y chromosome carries

genetic information required for male sex determination."[2]

In 1976, Cooke isolated the first sequence specific to the human Y chromosome. Thirteen years later Handyside *et al.* (1989) used a derivative of this sequence in a PCR-based assay to determine the sex of human preimplantation embryos. The following year, Perret *et al.* (1990) hybridized a mouse minisatellite probe to male and female bovine DNA and cloned a repetitive sequence, bt *DYZ-1*. Through multiple other screenings, other male specific sequences have been and continue to be uncovered.

In the pig, two such male-specific DNA sequences have been isolated: 1) a 3.8 kb repeated sequence cloned by McGraw *et al.* (1988) and, 2) another 3.8 kb repeated sequence with an approximate copy number of 200 was isolated by Mileham *et al.* (1988). These two sequences show an eighty percent similarity to each other suggesting that they belong to the same family.

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2. Goodfellow, P.N. and Lovell-Badge, R. 1993. SRY and sex determination in mammals. Annu Rev Genet. 27:71-92.

LITERATURE REVIEW

Defining the testis determining factor

The X and Y chromosomes share a region, the pseudoautosomal region (PAR), on the terminal sections of their short arms which allows for proper pairing during male meiosis. Through crossover of these PARs, during male meiosis, Y-specific sequences and X-specific sequences can be transferred to the X and Y chromosomes, respectively. This can potentially produce XX males (Gubbay *et al.*, 1990) and XY females. Deletion maps have been constructed using the transferred fragments from these individuals to identify Y chromosome specific genes that are involved in testis differentiation.

The master switch that initiates a series of events leading to testicular development and male development has been labelled the testis determining factor, TDF, in humans and testis determining Y gene, Tdy, in mice (Haqq *et al.*, 1993). The genetic program requires, for male development, both positive and negative regulation in view of initiating the development of male characteristics and causing the inhibition or regression of their female counterparts (Berta *et al.*, 1990; Gubbay *et al.*, 1990). Some of the more important downstream genes have been shown to be autosomal, including Müllerian Inhibiting Substance (MIS) and P450 aromatase. MIS, a member of the TGFß (transforming growth

factor) family, causes the regression of the female reproductive tract and its expression is, therefore, up-regulated in males. On the other hand, the expression of P450 aromatase, the catalyst for the conversion of testosterone to estradiol, in females, is down-regulated in male embryos.

Page *et al.* (1987) defined the region in which this TDF has to lie as being 140-280 kilobases (kb) from the PAR boundary. Cloning and screening by hybridization for conserved sequences suggested *ZFY* (zinc finger on Y) as the likely candidate. This gene encodes a putative protein containing several zinc fingers and an "active domain". It is also highly conserved amongst eutherian mammals.

Proteins containing zinc fingers are known to be transcription factors; however, this hypothesis was weakened by several discoveries. Firstly, a closely related gene, ZFX is present on the X chromosome and escapes X-inactivation (Goodfellow and Lovell-Badge, 1993). Sinclair *et al.* (1988) proceeded to show that genes homologous to *ZFY* are autosomal in marsupials. Koopman *et al.* (1989) also noted that the patterns of expression of Zfy-1 and Zfy-2 are inconsistent with a role in sex determination since their presence is germ cell dependant. As a final blow, Palmer *et al.* (1989) found four XX individuals with testicular development who had inherited Y-specific sequences but who lacked *ZFY* in their genomes.

Further tests, by Palmer *et al.* (1989), of these four individuals showed the inheritance of a 35 kb long fragment adjacent to the PAR. Gubbay *et al.* (1990) and Sinclair *et al.* (1990), through cloning and sequencing similar fragments in mice and humans, respectively, isolated a Y-specific sequence, the *Sry/SRY* (sex determining region on Y) gene. In support of its putative role, Yang *et al.* (1993) determined that this gene exhibits both sequential and positional conservation amongst other mammals.

SRY maps to the 35 kb fragment, in humans, which is known to induce testicular development (Sinclair *et al.*, 1990). The phenotype of XX males that lack the *ZFY* sequence but have the one encoding the SRY protein ranges from normal males with undescended testes to true hermaphrodites. This may indicate the involvement of another Y-located gene or it could simply be due to positional effects. In effect, proximity to the breakpoint or X-inactivation of the mutated X chromosome may affect, to differing degrees, the final phenotype of these individuals.

Comparisons of the DNA sequence from human, rabbit and mouse *SRY* genes indicated the presence of a conserved open reading frame (ORF) containing a 79 amino acid domain similar to one found in several known DNA-binding proteins. These other proteins have been classified into three groups: (1) non-histone proteins, eg. High mobility group 1 protein (HMG-1), which have little or no sequence specificity in their

interactions with DNA. This DNA-binding domain is referred to as the HMG box since this is the first group of proteins in which it was recognized (Jantzen *et al.*, 1990). (2) hUBF (human upstream binding factor), a co-factor of RNA polymerase I, contains four such sequences. It shows some sequence specificity in its interactions with ribosomal RNA. (3) transcription factors, including gene products such as the T-cell proteins, TCF-1 and LEF-1 (Travis *et al.*, 1991; van de Wetering *et al.*, 1991). They show higher affinity and greater sequence specificity for DNA targets than the other two groups. The HMG box in *SRY* most resembles those of this last group, with a similarity of 40-50% which suggests that it is a sequence-specific DNA-binding protein and probably a transcription factor.

With a few exceptions, highly conserved regions generally indicate functional domains, whereas, less conserved regions are not so functionally constrained (Tucker and Lundrigan, 1995). Sinclair *et al.* (1990) demonstrated that there is no significant homology of the *SRY* sequences, between different species, outside the HMG box, emphasizing the importance of this DNA-binding domain.

SRY expression

The genital ridge arises as a thickening along the length of the mesonephros and becomes more prominent from 10.5 to 11.5 days post coitum (dpc) in mice. As of day 12.5 post coitum, differences between males and females can be

detected. Sertoli cells proliferate and organize themselves into testis cords, giving the male gonad its characteristic striped appearance.

In contrast, the first visible sign of female development is the entry of germ cells into meiosis (Gubbay *et al.*, 1990): male germ cells remain in mitotic arrest in the testis. This is followed by the differentiation of follicle cells and their aggregation around oocytes. In other words, without germ cells, ovaries do not form.

In order to have a function in sex determination, a gene must be expressed prior to the overt differentiation of the genital ridge into testes. In mice, this event occurs around 12-12.5 dpc. Sry expression in mouse embryos, determined by reverse transcriptase (RT) PCR, begins around 10.5 dpc. By 12.5 dpc, it is still detectable, however, at lower levels. Porcine SRY is expressed concurrently in the genital ridge on days 21 and 23 post coitum and weakly at 31 dpc. This corresponds with fetal pig development: the gonadal ridge only appears around 21 dpc. The tunica albuginea, the first noticeable indicator of testis formation, can only be histologically identified by 24-27 dpc. Data, therefore, support the hypothesized temporal expression profile of the TDF/Tdy: SRY/Sry is expressed just prior to the differentiation of the genital ridge but not later in development (Daneau *et al.*; 1996, Gubbay *et al.*, 1990; Koopman *et al.*, 1990). The timing of its expression also

suggests that it acts to initiate testis determination but is not required to maintain the activity of any testis specific gene (Lovell-Badge and Hacker, 1995).

The developing gonad is composed of four cell lineages: germ cells, supporting cells, steroidigenic cells and connective tissue cells (Lovell-Badge, 1992). Experiments by Burgoyne *et al.* (1988) and by Palmer & Burgoyne (1991) support the theory that the testis determining gene acts cell autonomously within a population of cells called the supporting cell precursors. Studies using chimaeras made with XX and XY embryos suggest that SRY acts solely within the supporting cell line, directing them towards Sertoli cells rather than follicles (Koopman *et al.*, 1990). Sertoli cells would then signal the surrounding cell types causing the steroidigenic cells to develop into Leydig cells, rather than Theca cells, and to generate myoid cells, all in an effort to organize the gonad into the characteristic testis pattern.

Harley and Goodfellow (1994) showed that germ cells are not required for testis determination. Mice whose germ cells have been destroyed or those lacking them (mice homozygous for the W^e, white spotting, mutation) still show somatically normal testis formation. Koopman *et al.* (1990) demonstrated that genital ridges from 11.5 dpc W^e/W^e mouse embryos express comparable levels of Sry with those from normal genital ridges.

The existence of a pathway of genes controlling sex determination can be deduced from several of these facts. For instance, Sry induction around 10.5 dpc suggests the existence of upstream regulatory genes (Koopman *et al.*, 1990). The testicular growth in XX males lacking Y-derived sequences, in turn, supports the presence of downstream genes which, in this instance exhibit gain-of-function mutations (Palmer *et al.*, 1990). It has been recognized that sex-reversing mutations can also occur in other genes along the sex determination pathway.

Transgenic studies

Studies with chimaeric mice have demonstrated that their sex may depend on the number of Sertoli cells present. Koopman *et al.* (1991) injected a 14 kb genomic DNA fragment consisting of approximately 8 kb of sequence upstream and 5 kb downstream of the putative DNA binding domain of *Sry* into fertilized mouse eggs in order to create mice transgenic for *Sry*. The size of the fragment injected was larger than necessary to allow for the possibility of some undiscovered exons and to provide the necessary regulatory sequences for correct tissue and temporal expression (attempts to make sex-reversed transgenic animals using a 3.5 kb fragment containing the *SRY* ORF have been unsuccessful).

At 14 dpc, a group of embryos was sexed by gonadal morphology while chromosomal sex was determined by PCR and Southern analysis. Twenty five percent of the transgenic XX

embryos showed testicular development demonstrating that Sry is sufficient, on its own, to shift female development towards the male pathway. Failure in the other transgenic embryos could be due to positional effects, mosaicism or X-inactivation, since four out of seven transgenic XX females had fewer than one copy of *Sry* per cell.

Within the group of embryos allowed to go to term, one XX male was found. Histological examination of the individual showed normal testes, although they were small compared to normal XY males, with the exception of no spermatogenesis. This phenotype is identical to that of XXSxr (XX sex reversal) male mice where the lack of spermatogenesis is due to the absence of other Y chromosome gene(s) involved in this process. After DNA sequencing of the 14 kb fragment revealed no other genes, it was concluded that *Sry* was the only gene required to initiate testis development (Koopman *et al.*, 1991). One of the other XX transgenic mice produced in this group developed as a fertile female; however, some of her offspring developed into XX males. This suggests that there might be a threshold level for the biochemical activity of Sry (Harley and Goodfellow, 1994).

Koopman *et al.* (1991) also created mice transgenic for the human *SRY* gene; however, no sex reversed animals were produced even though, in some cases, the human *SRY* gene was expressed at significant levels in their genital ridges. The *Sry* gene is flanked by a very long inverted repeat in mice,

but not in humans (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990), producing rather different proteins, outside the HMG box motif. Since, *in vitro*, mouse and human Sry/SRY proteins interact with the same DNA sequences, this lack of interaction *in vivo* suggests that the presence of other factors may be required.

Studies using XX↔XY chimaeras have shown that those with less than twenty five percent XY cells may show ovarian development. This suggests that Sry is not forcing the supporting cell precursors to differentiate into Sertoli cells. Since the XY cells that did not differentiate as Sertoli cells remained viable, Sry could potentially be involved in competence rather than commitment. If Sry allows the expression of another gene, there may be a threshold concentration below which it has no effect on the surrounding cells (Lovell-Badge and Hacker, 1995).

SRY mutations in XY females produce females that can be classified into two groups: (1) those with pure or complete gonadal dysgenesis, with dysgenic or "streak" ovaries, identical to those seen in individuals with Turner's syndrome (XO females). The ovaries degenerate relatively early in life and appear only as streaks of connective tissue in adults (Hawkins *et al.*, 1992). (2) those displaying mixed or partial gonadal dysgenesis, where some testicular tissue is present in the gonads.

DNA sequencing of the *SRY* gene from these individuals (Berta *et al.*, 1990; Harley *et al.*, 1992; Hawkins *et al*,, 1992; McElreavey *et al.*, 1992) revealed that, in ten to fifteen percent of XY female cases, point mutations, sometimes inactivating the SRY protein, occurred within the HMG-box. These individuals support the hypothesis placing the *SRY* gene as the TDF. The other 85-90% of sex-reversed individuals where mutations in the HMG box have not been detected may have mutations elsewhere in the *SRY* gene, SRY promoter or in other genes required for the testis-determining pathway. In other words, the underlying causes may be Y-linked, X-linked or autosomal. If an individual has a mutation in the testis-differentiation pathway, one should be able to find some testicular cells within the streak gonads: this can be revealed by full histological examination.

Harley and Goodfellow (1994) showed that most of the point mutations, within the *SRY* gene, found in human XY females fall into codons coding for amino acids that are known to be conserved in SRY proteins between the species. This led to investigations in DNA-binding activity of these mutant SRY proteins. Initially, all cases showed reduced or no DNA-binding activity (Harley *et al.*, 1992), providing strong evidence that these mutations were responsible for the sex reversals.

Jager *et al.* (1992) investigated one family where there was one XY female and four normal males all carrying the same mutation. Normal DNA-binding activity, in vitro, was observed for this protein; however, neither DNA-bending properties, nor interactions with other factors were investigated as possible reasons for the XY female. Other factors which may affect testis determination include both gain of function and loss of function mutations and gene dosage.

Properties of the SRY protein

The HMG-box region is the only portion of the *SRY* gene that shows DNA-binding activity. Point mutations within this region have been shown to cause sex reversals (Harley *et al.*, 1992). When tested, these mutations have been shown to reduce or abolish DNA-binding activity of the protein. The majority of affected amino acid residues are those which are conserved amongst different species.

There are two other biochemical properties associated with this domain. The binding of SRY and LEF-1 to the minor groove of target DNA induces substantial bends (Goodfellow and Lovell-Badge, 1993). This may affect the interaction of other components within the transcriptional apparatus and, thereby, control transcription of these molecules.

Possible roles for SRY could be to act as a structural element which enables multiprotein complex formation, or to de-repress downstream genes, by bending the DNA. SRY may also compete with another factor for the same binding site or may

directly contact other proteins or adjacent DNA sequences, to affect transcription (Harley and Goodfellow, 1994).

The last property distinguishing SRY is that, although HMG-1 is abundant in all vertebrate nuclei (Bianchi *et al.*, 1989; Bianchi *et al.*, 1992), the full length HMG-1 molecule, with its two HMG boxes, is essentially unable to bind linear DNA fragments. Cruciform structures, resembling Holliday junctions produced during recombination, can be produced by linear DNA sequences containing inverted repeats or may occur during DNA wrapping and looping associated with transcription. Ferrari *et al.* (1992) and Goodfellow and Lovell-Badge (1993) showed that the HMG box, in SRY, is indifferent to DNA sequences present in four-way junctions.

There is competition for linear versus junction DNA since both HMG-1 and SRY can bind both types. However, HMG-1 prefers binding with cruciform DNA over linear DNA even if the linear fragment shows same sequence and/or is in 1000 fold excess. SRY binds both cruciform and linear DNA. Linear DNA can compete more effectively with cruciform DNA for the SRY HMG box than is seen with HMG-1 under similar circumstances. This is consistent with a fairly high non-specific binding affinity of SRY-HMG box for linear DNA as seen by its tolerance of certain mutations within the consensus sequence.

Ferrari *et al.* (1992) concluded that the selectivity of SRY for a particular structure (four-way junction) versus a

specific sequence varies between a factor of four and one hundred. It is, therefore, dependent upon the actual sequence present in the DNA which, in turn, gives it a much lower selectivity than that of HMG-1. Since HMG-1 is present in much higher concentration than SRY, it can potentially saturate the sites with kinked DNA. This would force SRY to recognize and bind the appropriate linear DNA sequences, thereby altering the pathway of sex differentiation.

The correct spatial configuration of the complexes formed by SRY is important for gene expression and subsequent cellular differentiation (Pontiggia *et al.*, 1994). In other words, DNA strand bending and affinity towards a particular DNA segment are not correlated. The DNA bending function may, however, be central to the biological activity of SRY. It assumes that the induced bend brings two factors, one on either side of the HMG binding site, together. This is based on two assumptions. (i) SRY does not promote transcription itself. The two neighbouring transcription factors will be activated only when brought into close enough proximity to interact with one another. This is partially supported by the fact that no known transcription activation domain has been found in *SRY* genes, other than in mice and, even here, it may not be functional *in vivo*. In addition, the sequences outside the HMG-box diverge to a great extent. (ii) The two transcription factors only interact in the presence of SRY. This would require specific bending of nucleoprotein

complexes which would not otherwise be seen in nature or with other proteins. Pontiggia *et al.* (1994) have shown that the geometry of the DNA-SRY complex depends on the sequence of the binding site. Mutations within the HMG-box, causing sex reversal in the individuals, exhibit different abilities to bind and bend DNA when compared with wild-type SRY HMG-boxes.

There are currently three known mechanisms for protein-DNA recognition (King and Weiss, 1993). Direct readout of bases involves specific chemical interactions between different proteins and specific nucleic-acid bases. Indirect readout refers to the ability to deform the DNA backbone. A third mechanism involves the partial intercalation of a nonpolar protein side chain through the DNA minor groove (King and Weiss, 1993).

The HMG box is required for DNA binding under many circumstances and is characterized by relatively high concentrations of aromatic, basic and prolyl residues. Point mutations within the HMG box can, depending on the residue affected and the resulting residue, drastically affect the binding ability of the protein. Soulier *et al.* (1994) showed the importance of conserving the three aromatic residues in positions 10, 41 and 52. Others that appear to be slightly less important, and therefore less conserved, are in positions 4, 5, 6, 9, 26, 38 and 70.

HMG box characteristics

SRY's HMG box shows affinity towards the following motif A/TAACAAT/AG (Harley *et al.*, 1992; Harley *et al.*, 1994). The base A (adenosine) is, however, preferred in the -1 position and T (thymine) is preferred in the last position. Van der Wetering and Clevers (1992) demonstrated that the HMG box contacts DNA through the minor groove using three approaches. (1) Methylation interference footprinting resulted in the prevention of SRY binding upon N3 methylation of adenosine within these motifs; however, N7 methylation of guanosines within or surrounding the sequence had no effect. (2) Diethylpyrocarbonate (DEPC) carbothoxylation interference footprinting, which modifies purines located in the major groove, showed that the HMG box may protrude into the major groove at the first four positions in the motif. (3) Replacement of A and T by I (inosine) and C (cytosine), mimicking A→G and T→C substitutions, alters the way the motif is perceived if located in the major groove, but does not affect it within the minor groove.

Both kinked and linear DNA compete with each other for binding to the human SRY HMG-box; however, the SRY protein only has a single binding surface (Rimini *et al.*, 1995). Hydroxyl radical cleavage shows that 7 base pairs are protected and, therefore, are bound by the SRY protein: GAACAAA. Mutated proteins show reduced binding affinity and/or altered angles of induced bend. Mutations that don't

show any significant changes from the wild-type SRY and that don't always have an effect on the final sex of individuals are potentially conditional mutations, dependant upon the specific genetic background and environment.

King and Weiss (1993) demonstrated that the isoleucine side chain, in the SRY HMG-box, enters the minor groove to partially penetrate between two consecutive AT pairs. Following this, it is assumed that the α-helix, surrounding the isoleucine residue, will also enter the minor groove. In B DNA, this groove would be too narrow for this to occur. It, therefore, suggests that the groove is widened at the site of the partial intercalation. According to the "Wedge intercalation" model (King and Weiss, 1993), the flanking base pairs are separated by the insertion and probably rotated. This would cause a partial unwinding of the DNA chain in order to accommodate the side chain. The HMG protein would probably also undergo conformational changes upon forming these complexes with DNA (Ferrari *et al.*, 1992; Harley *et al.*, 1996).

The correct geometrical configuration of the nucleoprotein complex depends not only on the protein but also on the sequence of the target site and its intrinsic ability to bend. In effect, the bending of DNA requires a widening of the minor groove, a compression of the major groove and a distortion of the plane of bases away from being perpendicular to the axis. Four-way junction DNA already has

a widened minor groove; therefore, SRY can bind regardless of sequence. SRY can only widen the minor groove of linear sequences if it can establish specific contacts with a particular sequence of bases.

It is thought that the specificity for the TT sequence is not attributable to direct readout, but instead to the exclusion of other sequences. In other words, it will be affected the least by the partial penetration of an isoleucine. This suggests that SRY should be able to bind to different DNA sequences as long as they can accommodate the intercalation without additional penalties. In other words, pyrimidine to pyrimidine mismatches should be relatively well tolerated while interchanges between purines and pyrimidines would not be tolerated because of altered patterns of twisting (King and Weiss, 1993). Therefore, possible causes of sex reversal, directly involving SRY, could include mutations of isoleucine in the SRY HMG-box and transversions of the sequence within the minor groove, thereby preventing the partial intercalation from occurring.

SRY transcripts

Northern blot analysis has provided most of the evidence supporting SRY/Sry as the TDF/Tdy. Sry transcripts of 1.3 kb are detected only in testicular tissues of adult mice (Gubbay *et al.*, 1990). Koopman *et al.* (1990) demonstrated Sry's germ cell dependency using adult XXSxr and XXSxr' mice which lack germ cells and therefore fail to produce Sry in the testes.

This, however, has not helped determine Sry's function in the adult testes.

Sry transcripts from mouse testes are present as circular RNA molecules which appear to be very stable. Despite the fact that these molecules are neither capped nor polyadenylated, they can still be found within the cytoplasm. They are, however, not associated with polysomes which suggests that they are probably not translated. This circularization may be due to normal splicing processes since *Sry* is embedded within a large inverted repeat (Gubbay *et al.*, 1992). These transcripts have not been detected in differentiating genital ridges or embryonic testes from mice suggesting that they aren't an active form.

Human SRY transcription units are co-linear with the genome. Using cDNA clones isolated from adult testes, Sinclair *et al.* (1990) defined the end of the transcript as position 747 (with respect to the presumed start codon). After further investigation, Clépet *et al.* (1993) found multiple transcription start sites in the human *SRY* gene. The major transcript is about 900 bases, while longer transcripts are also present but at much lower levels within the tissues. In humans, the major transcript seen is polyadenylated, suggesting a linear transcript, rather than a circular one as noticed in adult mouse testes. However, even though they appear to be linear, Clépet *et al.* (1993) don't think that these transcripts are biologically active in adults.

Nevertheless, they don't dismiss the possibility that one of these longer transcripts may be the relevant one in human fetuses.

In adult humans, SRY transcription is not limited to the testis: it has also been detected in the heart, liver and kidneys, but not in the lungs. Fetal human tissues showing expression include the genital ridge, adrenal gland, brain, liver, pancreas, small intestine, spleen, thymus, and heart. The presence of SRY in these tissues, even at very low levels, might suggest a role for SRY outside of the genital ridge and the testis.

The mouse *Sry* transcript is located within inverted repeats and is positioned near the centromeres (Lovell-Badge and Hacker, 1995). The genital ridge transcript is substantially larger than that of the human transcript with a total calculated size of 4929 bp (Daneau *et al.*, 1996). This is mainly due to the size of the 3' untranslated region in the linear transcript: 3481 bp.

The protein is longer than the human protein, being 395 amino acids long with a 942 amino acid carboxy terminal region (region to the right of the HMG box), containing a putative transcriptional activator site. *Mus musculus domesticus*, due to a premature termination codon in the sequence which deletes half of the putative activation domain, has only a 447 bp carboxy terminus. Mouse Sry proteins, however, have a virtually non-existent amino-

terminal segment (region to the left of the HMG box) consisting of only two amino acids. The protein also exists in one or two copies, depending on the strain: New World or Old World (Nagamine *et al*, 1994).

The circular transcript seen in adult testes is also seen in the genital ridge but appears to be a very minor component of the Sry transcripts present (Clépet *et al.*, 1993). The circularization of this transcript may be related to the fact that the gene is flanked by a large inverted repeat. It appears to be the splice product of a long transcript, containing at least part of a palindrome, which originates from a promoter within the repeat. The genital ridge transcript, on the other hand, initiates in the non-repeat region (Clépet *et al.*, 1993).

The human *SRY* transcript, on the other hand, is located 5 kb from the boundary to the pseudoautosomal region and is not positioned within inverted repeats. The total calculated size of the genital ridge *SRY* transcript is 826 bp (Clépet *et al.*, 1993) which is partially due to a 137 bp 3' untranslated region in the linear transcript. The protein is 204 amino acids long with a 58 amino acid amino-terminal region and a 69 amino acid carboxy terminus (Goodfellow and Lovell-Badge, 1993).

Studies in cattle have revealed an *SRY* transcript that has an open reading frame of 687 bp (Daneau *et al.*, 1995). The protein itself has a 52 amino acid amino-terminal region

and a 100 amino acid carboxy terminus (Goodfellow and Lovell-Badge, 1993).

The porcine *SRY* locus contains an open reading frame of 208 amino acids which includes a central 237 bp HMG domain, a 177 nucleotide (nt) 5' translated flanking sequence and a 210 nt translated 3' flanking region (Daneau *et al.*, 1996). It also has a 5' promoter region of 615 bp and a 532 bp long 3' untranslated region. The protein, itself, has a 59 amino acid amino-terminal region and a 71 amino acid carboxy terminus which includes a single polyadenylation site. According to the preceding descriptions of the SRY/Sry transcripts and proteins, pigs may prove to be a more suitable model for human sex determination studies than mice.

Comparison of the above structures of SRY suggests that there are at least two types of SRY molecules (Daneau *et al.*, 1996). The first group would include species where the protein contains the DNA-binding domain of the HMG box but does not have an activation domain. Species that fall within this group would include humans, cattle, pigs, sheep, goats and buffalo. In the second group, Sry proteins, as seen in mice, contain both the HMG box and a transcriptional activation domain.

In theory, SRY could bind any bent damaged or looped DNA; however, HMG-1 is present in much higher quantities and would compete for these sites. Sry, on the other hand, demonstrates a sequence-specific DNA-binding activity *in*

vitro which HMG-1 doesn't. It is probably this activity that is required for male sex determination, but the mode of action of type I SRY proteins *in vivo* is unclear. Several models have been proposed based upon the following two observations: (i) HMG box proteins can both stimulate and repress transcription, and (ii) SRY has no obvious activation domains (e.g. Pro-rich, Gln-rich, Ser-rich, acidic).

Models concerning the mechanism of SRY's involvement in testis determination suggest that: 1) its DNA-bending action may bring ubiquitous factors into proximity to produce an SRY-mediated transcription complex (Soulier *et al.*, 1994), 2) such bending may de-repress downstream genes, 3) SRY competes with a second factor that shares the same binding site (Soulier *et al.*, 1994), 4) it enters into direct contact with other proteins and 5) it may be involved in direct transcriptional activation. Type II SRY proteins which contain both a DNA-binding domain and a transcriptional activation site which is active *in vitro* would, therefore, be likely candidates for this last point. *In vivo* activity of the transactivation domain must, however, be shown for complete support. Mouse Sry could also be included in some of the other models if the putative activation domain is non-functional *in vivo*.

Four proteins have been proposed as candidate players involved in the sex determination pathway: 1) SF-1, 2) Sox-9, 3) WT1 and 4) DAX-1. DAX-1, an X-linked gene, is related to

the nuclear hormone receptor superfamily and may be involved in the development of adrenal glands and the female gonads (Zanaria *et al.*, 1994; Swain *et al.*, 1998). WT-1, Wilms' tumor 1 gene, on the other hand, was originally isolated from kidney cancer in children (Call *et al.*, 1990). It is a zinc finger containing protein which appears to act upstream of SRY. Knockout studies in mice (Kreidberg *et al.*, 1993) suggest it is involved in the commitment and maintenance of the bipotential gonad along with kidney organogenesis. Sox-9, a SRY-related protein, contains both an HMG-box domain and a transcription activating domain at its C-terminus. Expression studies suggest that this protein is part of the molecular cascade involved in the differentiation of Sertoli cells in the developing gonad (Kent *et al.*, 1996). The fourth protein, SF-1, is a transactivating binding factor involved in steroidogenesis. It also plays a role in gonad and adrenal gland formation as demonstrated by knock out experiments. It is expressed in the primitive gonads of both sexes prior to sex differentiation after which it is only expressed in males. This suggests that its presence may be required for gonadal development, up to a specific stage, and for testis determination. It appears to be involved in male sex differentiation: perhaps by activating genes, such as *StAR* (steroidogenic acute regulatory protein) or *MIS* (Mullerian inhibiting substance).

In order for MIS to be a target molecule for SRY, it needs to be: 1) expressed in the same cell lineage as SRY, 2) expressed differentially in males and females at the time when SRY protein is present, 3) a good binding site for SRY, and 4) important for testis differentiation. Murine Sry expression begins around 10.5 days post coitum (dpc), peaks at 11.5 dpc and is not detectable by 12.5 dpc. Mis expression begins around 11.5 dpc, peaks at 13 dpc and remains at that level throughout fetal life (Lovell-Badge and Hacker, 1995). This leaves a 22 hour gap between initial Sry expression and initiation of the expression of Mis, leaving room for an intermediate gene. There are critical regulatory regions close to the *Mis* gene but they don't show any Sry DNA binding consensus sequences. Another point negating its role as a target molecule is that *Mis* is not critical for testis development: mice lacking the Mis gene still show normal testis development.

Embryo sexing

The main idea behind cheaply and quickly sexing embryos prior to transplantation is not to increase the number of female embryos available, but to reduce the number of recipients used and, in that way, decrease the cost of the piglets. There is a limit as to the number of embryos that can be collected, biopsied and sexed within a given time period. A fixed number of embryos must be transferred into a synchronized recipient in order for the pregnancy to be

maintained. By removing the male embryos from the group to be transferred, fewer embryos are then available, resulting in the use of fewer recipients. This could prove useful if looking at conditions expressed only in females or in cases where attempting to produce transgenic animals that will produce specific compounds in their milk (Robl and First, 1985). This has been done in ruminants; however, litter sizes are limited. Pigs could provide a rapid means of producing large numbers of these transgenic animals within a short period of time. Another advantage is the shorter gestation seen in pigs, 114 days, compared with 145-151 days, 149 days and 278-293 days seen in sheep, goats and cattle, respectively (Bearden and Fuquay, 1992).

There are two potential ways of altering sex ratios of offspring in mammals: separating X- and Y-bearing spermatozoa or sexing the embryos. Sexing of spermatozoa to alter the sex ratio of fertilized embryos has not proven very efficient. The procedures tried to date take too long, are too inefficient or reduce the viability of the spermatozoa. However, live calves have been produced using flow cytometry to sex spermatozoa followed by *in vitro* fertilization of cultured oocytes (Cartwright *et al.*, 1993).

Previously employed methods for sexing porcine embryos prior to sexual differentiation include: (i) counting chromatin bodies, inactivated X chromosomes (seen in females), in amniotic cells to identify females and males in

thirty- to ninety five-day old fetuses. This method is not considered to be very reliable (Pelliniemi & Salonius, 1976). (ii) Quinacrine mustard staining can also be used to identify males by the presence of fluorescent Y bodies in interphase cells of amnion or liver spreads. This has not yet proven suitable for use in pigs using embryonic interphase spreads (Pelliniemi and Salonius, 1976). (iii) Karyotyping. This requires the spreading of mitotic metaphase chromosomes. The latter method requires actively dividing cells into which a colchicine solution is added to block the chromosomes at the metaphase stage. After expanding the cells in a hypotonic solution, they can be spread and stained using a variety of stains (Gustavsson, 1988; McFeely, 1966).

Sex identification of even younger, preimplantation, embryos is also possible. This would be important for controlling sex ratios of domestic animals and in investigations of sexual differences in the developmental process. Several methods have been developed for this purpose, including karyotyping blastomeres, detecting male specific antigens, such as the HY antigen, monitoring X chromosome-linked enzyme activity, hybridizing Y chromosome-specific probes and, most recently, amplifying Y chromosome-specific repetitive and single copy sequences by Polymerase Chain Reaction (Kunieda *et al.*, 1992). Antigen detection and quantification of X-linked enzymes are non-invasive but tend to be less accurate. Alternatively, cytogenetic analysis and

the use of Y-specific probes are very accurate; however, they are invasive. This means that to produce viable embryos, the size of the biopsy must be limited. Another problem arises when the probes aren't entirely Y-specific.

Y chromosome-specific DNA fragments can be used to predict embryonic sex, in numerous species, using four different methodologies (Miller, 1991): (1) in situ hybridization, (2) Southern/dot blotting, (3) Polymerase Chain Reaction (PCR) based assays, and (4) cytogenetic analysis. While Southern and dot blotting can be used with high accuracy on relatively small numbers of biopsied cells, it takes eight days for the procedure. This, therefore, requires that the embryos be frozen during this time period (Akamatsu *et al.*, 1989; Bondioli *et al.*, 1989; Perret *et al.*, 1990). Cytological methods of sexing embryos require culturing cells in the presence of a mitotic arresting agent to get enough metaphase chromosomes for analysis. This extra time also implies *in vitro* culturing of the embryo which reduces its viability. Another drawback is that the pig has three pairs of metacentric chromosomes in addition to the Y chromosome. Therefore, high quality chromosome spreads are required in order to make an accurate analysis (Polge, 1985).

Analysis of Y chromosome specific sequences can be accomplished through in situ hybridization or PCR. The former method requires repetitive DNA sequences that are entirely male-specific. According to Bondioli *et al.* (1989) a single

copy sequence can be detected in 1 µg samples of DNA; however, the limitations imposed by the preimplantation embryo would provide less than 100 pg samples if 10 cells were removed for analysis. To overcome this, sequences repeated on the Y chromosome 1000 times or more should be used. However, if these sequences are present elsewhere in the genome, the accuracy of the assay will be decreased, detracting from its usefulness. Human (Jones *et al.*, 1987; West *et al.* 1987) and mouse (Jones *et al.*, 1987) preimplantation embryos have been sexed by this method using embryonic cells fixed to microscope slides. This, unfortunately, requires eight days, six of which involve exposure of the film. Leonard *et al.* (1987) modified the technique to sex bovine embryos using non-radioactively labeled DNA probes.

The PCR based assay appears to be the most feasible means of increasing the speed without requiring the use of a gene with a greater number of copies in the genome.

Deoxyribonucleic acid (DNA) sequences specific to the porcine Y-chromosome have been isolated and cloned (McGraw *et al.*, 1988; Mileham *et al.*, 1988). From these sequences, primer pairs can be produced for PCR amplification of these sequences in order to sex embryos.

The Polymerase Chain Reaction allows the specific *in vitro* amplification of individual DNA sequences using a set of flanking oligonucleotides. The three main advantages to

employing this technique over the others are that it is: i) fast, ii) very accurate, with a reproducibility of greater than 90%, and iii) technically straightforward to perform. Amplification of a repetitive sequence can take as little as six hours from the time the embryos are flushed until visualization of the bands under ultraviolet lighting. In addition, accuracy can be further increased by using control autosomal primer pairs in conjunction with the pairs of interest. This would enable one to differentiate between females and samples with too little or no DNA.

PCR amplification of these repetitive sequences is preferred under some circumstances due the increased sensitivity over similar amplification of single copy sequences (Akane *et al.*, 1992). A sequence present in multiple copies requires fewer amplification cycles to detect the product. A single copy sequence requires 10 additional cycles of amplification than a sequence present 1000 times in the genome (Miller, 1991). This, therefore, implies that lower amounts of template DNA can be used, such as in forensic investigations, and that there is a reduced overall time for analysis (Handyside *et al.*, 1989). There is also a reduced risk of amplification failure since one is starting with a larger number of copies of the sequence.

Despite these advantages, certain situations favor the amplification of single copy sequences. Assays containing primers for single copy sequences can potentially reduce the

generation of nonspecific products (Kunieda et al., 1992; Cui et al., 1994) since sequences are specified by four oligonucleotide primers. PCR amplification of such sequences generally requires nested reactions when the amount of template available for analysis is limited, such as in the sexing of preimplantation embryos. Often controls for the success of the amplification necessitate the incorporation of one or more additional sequences into the reaction. If one is multiplexing single copy sequences, they will be of equal (single) copy number, unlike in the analysis of X- and Y-specific repetitive sequences, which will help in the interpretation of the results (Sullivan *et al.*, 1993).

Sex determination in the pig has been done using a variety of methods, including flow cytometry to separate male and female spermatozoa (Cartwright *et al.*, 1993), and immunofluorescence to detect the presence of the HY antigen on male embryos (White *et al.*, 1987). To sex pig embryos by probing for the HY antigen, the zona pellucida must be removed prior to exposing the embryo to antibodies. This is mainly because the large numbers of sperm, present in the zona pellucida, interfere with the sex diagnosis. This test shows an 81% accuracy. Unfortunately, due to compromised survivability of these sperm or embryos, the above methods have not proven to be effective enough for use in the field.

The bovine industry has succeeded in developing a more efficient technique which involves the amplification of

specific Y chromosome sequences by PCR. This permits efficient sex determination while using small quantities of embryonic material which would be insufficient for karyotyping. In pigs, sex determination by PCR was first established using a repetitive Y chromosome sequence, *DYZ1* (Perret *et al.*, 1990). PCR primers for the HMG-box sequence within the porcine *SRY* gene have also been developed (Yang *et al.*, 1993; Sathasivam *et al.*, 1995; Daneau *et al.*, 1996).

Bradbury *et al.* (1990) used PCR-based sexing of eight-cell mouse embryos. By removing a single blastomere from each embryo, they were able to amplify a repetitive sequence, a 1.5 kb *ECO* R1 fragment, and sex a number of embryos. The sexed embryos were reimplanted into surrogate mothers and recovered 14 days later to confirm the PCR results. Out of the ten embryos collected, eight of them had been correctly sexed, while the other two had not had their sex predetermined. Certain problems were encountered by this group such as co-amplification of the sequence in the females and not being able to sex embryos when there was the smallest change in its sequence.

Single copy sequences for PCR

Various single copy sequences have been used for sex determination of embryos including the *ZFY*, amelogenin and *SRY* genes.

The zinc finger protein, ZFY, has been used to determine the sex of individuals and embryos in several species

including humans (Chong et al., 1993), mice (Kunieda et al., 1992) and cattle (Kirkpatrick et al., 1993). The main advantage to amplifying this sequence is that one can synthesize primer pairs that contain some degeneracy which enables them to accommodate the differences in the Zinc Finger Y, *ZFY*, and Zinc finger X, *ZFX*, sequences. These products can then be digested with *Hae* III to give two bands for XX samples and four bands for XY samples. The amplification of X-Y homologous genes offers the advantage of containing internal positive controls since both X- and Y-specific sequences can be amplified in a single reaction. In addition, the presence of a *Hae* III site common to both *ZFX* and *ZFY* provides a control for successful digestion.

Investigations into sex determination of humans (Akane *et al.*, 1992; Sullivan *et al.*, 1993) and cattle (Ennis and Gallagher, 1994) using the amelogenin gene have also been attempted. Amplification of the amelogenin gene is similar to that of the zinc finger proteins in that there is co-amplification of distinguishable X and Y fragments. The X fragment, therefore, acts as an internal control for the amplification. Dual amplification assays have been developed to amplify a homologous region of the X (class I) and Y (class II) amelogenin genes. Since the Y fragment is deleted for a section of one intron, it is possible to distinguish the two products on the basis of size. Analysis of the products shows that there are two bands in male samples, one

stemming from the class I (X chromosome) gene and one from the class II (from the Y chromosome) gene (Ennis and Gallagher, 1994). Females, on the other hand, only produce one band, corresponding to the class I gene. There are several advantages to amplifying the amelogenin gene for sex determination purposes. (1) It is a single copy sequence present on both sex chromosomes which eliminates the problems encountered when amplifying sequences of different copy numbers. 2) The segment amplified on the Y-homologue is shorter than that of the X-homologue. This ensures amplification of the male sequence provided that it is present in the sample. (3) Since the same primer pair is used to amplify both homologues, the PCR conditions are optimized for the amplification of both genes. In addition, the presence of the class I product acts as an internal control for the amplification of the Y-homologue.

The third sequence used in humans (Cui *et al.*, 1994), mice (Kunieda *et al.*, 1992) and pigs (Sathasivam *et al.*, 1995) for such assays involves the HMG box contained within the *SRY* gene. This is a single copy Y chromosome gene with no known homologue in the genome. There is, therefore, no need for restriction enzyme digestion of the PCR products to differentiate between males and females such as with the zinc finger proteins, *ZFX* and *ZFY*. However, a second sequence should be co-amplified in the reaction as a control for the amplification and the presence of DNA in the tube.

Embryonic development

In many species, male embryos tend to develop faster than their female counterparts due to higher circulating levels of androgens, synthesized by the testes. Kaminski *et al.*, (1996) have recently shown that these differences begin prior to testicular differentiation in certain species including, rats, mice and cattle.

In pigs, genetics play a major role in embryonic and fetal development, as seen by the wide variance in birth weights among different breeds. Fetal androgen levels show increased concentrations at the beginning of the second trimester of pregnancy and at the time of birth. It has been hypothesized that the rate of embryonic development can affect embryo survival in pigs, particularly, once the more advanced embryos change the uterine environment, e.g., by producing estrogen. This change may prevent or hinder further growth by the less mature embryos. According to Kaminski *et al.* (1996), no effect of sex has been seen in pig embryonic and foetal growth. It is only in extremely large sample sizes that one can find a statistically significant 2-5% difference in birth weight.

Menino *et al.* (1989) have demonstrated that fewer embryos from first estrus are able to develop to the blastocyst stage compared to embryos collected from the same gilts at their third estrus. This appears to be due to increased numbers of morphologically abnormal embryos from

first estrus collections. The most prominent abnormality was the failure of blastomeres to incorporate into the morula or blastocyst. This implies that there are fewer cells involved in embryonic development. There may, therefore, be some factor present in first estrus gilts, and probably prepuberal gilts, which influences compaction of cells and cavitation.

The culture requirements of pig embryos changes depending upon the stage of development (Petters and Wells, 1993; van der Hoeven *et al.*, 1985). There is a 'block' *in vitro* at the four cell stage in pigs. Another stage at which medium composition must be changed is at the blastocyst stage. In order for continued development, including hatching and trophoblast expansion, ten to twenty percent serum must be added (Rosenkrans *et al.*, 1989).

During embryo manipulation, embryonic cells are removed for sexing. Other cells may be excluded from the developing embryo if damaged during the procedure, further reducing the number of cells available for embryo development. Heyman (1985) showed, through transferring demi embryos, that there is a minimal number of embryonic cells required to ensure a strong enough signal in the uterus for pregnancy to be initiated.

The survival of pre-morulae stage embryos *in vivo* may depend upon the presence of an intact zona pellucida (Martin *et al.*, 1991). Niemann *et al.* (1983) have shown that, following a 48 h culture period, after microsurgical

manipulation, fewer nuclei can be detected in the manipulated blastocysts when compared with untouched blastocysts. One explanation for this would be that the microsurgical treatment of the zona pellucida and the death of microsurgically-damaged blastomeres inhibit and/or delay development *in vitro* (Niemann *et al.*, 1983). It was also proposed that the incision in the zona pellucida was having a detrimental effect on *in vivo* growth. The incision may also result in the trophoblast exiting the zona early.

Embryo transfer

Superovulation and embryo transfer can potentially be used to increase the rate of growth of new lines or increase selection pressure within already established lines. Embryo transfer has also been used to establish disease-free herds and could potentially be used to introduce new genetic material into herds while minimizing the risk of transmitting diseases.

Porcine embryos are able to develop quite well *in vitro* given appropriate culture conditions. However, transfer of *in vitro* cultured embryos versus freshly collected embryos results in substantially reduced litter sizes (Pope and Day, 1977; Davis and Day, 1978). Hyttel and Niemann (1990) observed structural differences between embryos that developed to the blastocyst stage *in vitro* versus *in vivo*. Nucleolus function appeared to be altered in a number of the *in vitro* grown embryos. Smooth endoplasmic reticula also

showed altered distributions in some *in vitro* cultured embryos which may indicate a reduced steroid synthesizing capability.

Immediate transfer of embryos collected three to seven days after the onset of oestrus into synchronized recipients can result in an 82-85% pregnancy rate and an overall embryo survival rate of 40-70% (Polge, 1985; Davis, 1985; Niemann and Reichelt, 1993). This is similar to what is observed after artificial insemination or natural service. Extending the culture periods up to twenty seven hours can reduce it to 20-26%. This embryonic loss has also been seen in mice and cows. One explanation is that resorption frequency appears elevated in embryos that had been cultured *in vitro* at the blastocyst stage (Davis, 1985).

Potential factors involved in conception failure include season, mode of insemination, age of the sow and the number of times the female is inseminated (Drickamer *et al.*, 1997). According to Drickamer's study, seasonal effects were not significant factors; however, age played a role. Females younger than 1.5 years were more likely to have unsuccessful matings, whereas the older sows were more likely to conceive. Natural matings showed greater success rates than AI. Mating two or more times per estrus period also helped.

Another factor appears to be the sex ratio of the litter into which the sow had been born. Females born into litters with low proportions of males show improved reproductive

performance compared to females born into litters with a high proportion of males. Females developing between two males may be more influenced by the androgens produced by their neighbours than females developing between two females. Drickamer *et al.* (1997) also showed that females from litters with high proportions of males reach puberty at a later date than those from low male litters.

In this study, our initial goal was to design a multiplex PCR protocol that would include primer pairs for a single copy Y chromosome sequence, the *SRY* gene, a control sequence, *DAX-1* or *GAPDH*, and a repetitive Y chromosome sequence, *YR*. Genomic DNA and white blood cells from both male and female pigs were used to test whether or not the different combinations worked. Once this was achieved, sexing of embryos by removing small biopsies with the use of a micromanipulator and running the biopsies through the PCR assay was performed. Karyotyping of the surviving embryos was attempted to test the reliability of the multiplex PCR assay. The final step in this project was to sex day five embryos and transfer them into synchronized recipient gilts.

EXPERIMENTAL DESIGNS

1.1 Experiment 1

The purpose of this series of experiments was to design a multiplex PCR assay capable of determining the sex of preimplantation porcine embryos.

The steps involved in preparing pools of genomic DNA and WBC from both male and female pigs are shown in Table 1a. The pools of WBC were promptly stored at -20°C until required, while the genomic DNA samples underwent further processing such that the WBC membranes, cellular and nuclear, were lysed, followed by overnight exposure to proteinase K. DNA could then be precipitated out of solution and dissolved in Tris buffer. The DNA concentrations of these samples were calculated prior to their being stored at -20°C.

These pools of DNA were used to determine the conditions required for a multiplex PCR reaction that could be employed for sexing embryos. Serial dilutions were done to ensure that the reaction would work at low DNA concentrations and to show the increased sensitivity of a nested PCR reaction over a one-step reaction when amplifying single copy genes. Serial dilutions of the WBC were also done to demonstrate that the amount of DNA contained within one cell was sufficient for amplification of the genes of interest and that sexing embryos using 1-3 cell biopsies would be possible.

The procedure involved in purifying DNA from the various tissue samples of both male and female pigs is described in

Table 1b. The DNA concentrations were also determined for each sample prior to storage at -20°C. These samples were then used to show that sexing of individuals can be done regardless of the source of the DNA.

1.2 Experiment 2

The objective of this experiment was to sex day 5 porcine embryos using the above multiplex assay and to demonstrate its accuracy through cytogenetic analysis of the embryos.

Sixty to seventy kilogram prepuberal gilts were hormonally induced to ovulate. Five days after insemination, the embryos were surgically removed, biopsied and placed into culture medium to allow further development (see Table 2 for a more detailed time line) overnight. Meanwhile, the biopsies were stored at -20°C until run in a PCR reaction, for sex determination. The development of these embryos was monitored to determine survival rates of biopsied embryos and to karyotype the viable ones.

1.3 Experiment 3

The aim of this experiment was to confirm the accuracy and feasibility of the multiplex assay through the transfer of sexed embryos into recipients and the production of litters of one sex.

This last set of experiments required cycling pigs (see Table 3). Once natural cycling was demonstrated, these females were placed onto the same restricted feeding regimen as the breeding sows: 3 kg of feed/pig/day. This allowed top

feeding of altrenogest, a synthetic progestin, with the aim of synchronizing their heats with those of their corresponding donors.

The donor pigs were prepared in a similar fashion as in experiment two. The embryos were collected five days after insemination, biopsied and cultured in medium overnight. Meanwhile, the biopsies were run through the PCR assay overnight so that the following morning, viable embryos of known sex could be transferred into the synchronized recipients.

Table 1: Overview of the steps involved in the isolation of a: DNA or WBC from large samples of blood, and b: DNA from small samples of various tissues including blood, muscle, adipose tissue and hair.

a)

collect blood samples	isolate WBC layer	remove RBC (*Stop here for WBC isolation*)	lyse WBC	lyse WBC nuclei	incubate with proteinase K overnight	extract DNA (EtOH)	OD_{260} -> [DNA]

b)

collect tissue samples	Lysis buffer 1X (clean blood & muscle samples)	PCR buffer with non-ionic detergents	incubate with proteinase K overnight	heat inacti-vation	OD_{260} -> [DNA]

Table 2: Schedule for embryo collections and karyotyping.

hours:	0	72	96	108	112				
days:	-4	-1	0	1	1	5	5-6	6	6-7
	750IU PMSG	500IU hCG	AI	AI	ovulation	embryo collection & biopsies	colcemid® (six hour incubation)	spread embryos	stain slides

Table 3: Experimental design for embryo transfers. Time table for synchronizing the recipients with the donor pigs.

Donor pigs (3-4)	days	Recipient pigs (1-2)
		daily heat checks
	0 (M/Th)	
		restricted feeding starts
	1	
		11 mg Regu-Mate®/pig/day
	18	
		last day of Regu-Mate®
	19	
750 IU PMSG		750 IU PMSG
	22 (Sa/Th)	
500 IU hCG		500 IU hCG
	23 (day 1	of recipient oestrus
		cycle)
heat -> A.I.		heat
	28 (M/Th)	
surgery (collection)		
& overnight sexing	29 (Tu/F)	
		surgery (embryo transfer)
	38	
		heat checks (pregnancy
		checks)

MATERIALS AND METHODS

2.1 Genomic DNA and WBC Isolation

Forty-five to fifty millilitre blood samples were collected from finishing gilts and barrows at the Macdonald Campus Farm swine facility using heparinized vacutainers. The whole blood samples were brought back to the lab and pooled, by pig, into 50 mL centrifuge tubes. After addition of 2.5 mg of sodium citrate/ mL of blood, the samples were centrifuged at 1100Xg for 15 min at 4°C. The white blood cell (WBC) layer along with a portion of the red blood cell (RBC) layer were transferred to another tube and the RBC were subsequently lysed over multiple washes with 0.83% ammonium chloride. WBC were lysed with a solution consisting of one part buffer C1 (320 mM saccharose, 5 mM $MgCl_2$, 10 mM Tris/HCl, 1% Triton X-100, pH 7.5; QIAGEN Genomic DNA Handbook) to three parts water. Nuclear membranes were removed with 20 mL of buffer G2 (800 mM GuHCl, 30 mM EDTA, 30 mM Tris/HCl, 5% Tween 20, 0.5% Triton X-100, pH 8.0; QIAGEN Genomic DNA Handbook). After overnight digestion with 5 mg proteinase K at 55°C, 0.5 volumes of NaCl were added to deactivate it. DNA was then precipitated out of solution with two volumes of 100% ethanol and resuspended, at room temperature, in a solution of Tris (10 mM, pH 8.0). Concentrations of DNA within these samples were calculated with the use of a spectrophotometer (λ=260nm).

Generation of WBC pools followed the above procedure until the step utilizing buffer C1.

To test the precision, sensitivity and stability of the PCR amplification using the designed primers, both genomic DNA and leucocytes were used. Serial dilutions of the genomic DNA were set up such that the initial template amounts (concentrations) present in the PCR tubes decreased by factors of two or ten between 0.1 µg (5.0×10^{-3} µg/µL) and 5.0×10^{-6} µg (2.5×10^{-7} µg/µL). WBC were also used for these tests to compare cell numbers with concentration of DNA. In order to mimic the amount of DNA potentially present in the tubes containing biopsies, 1-4 WBC were placed into individual PCR tubes. A micromanipulator (Jena instrumentation) was used to pick out individual WBC. Freezing the tubes at -20°C followed by preheating them to 95°C did not damage the membranes sufficiently for the PCR reactants to gain access to the DNA. Therefore, the tubes were placed into liquid nitrogen and thawed three times to ensure exposure of the DNA.

To demonstrate the increase in sensitivity of a nested PCR over a single run when initial levels of template DNA are quite low, samples from both the inner and outer reactions were run on gels.

2.2 Purification of DNA from a Variety of Tissues

Samples of blood, muscle, adipose tissue, skin and hair were taken from freshly slaughtered pigs at the Bouché Primo

abattoir (St. Louis de Gonzague). They were stored on ice until DNA was isolated following the succeeding protocols. For fresh blood, 500 µL (≈ 10^4 cells) was placed in 500 µL of 1X Lysis buffer (0.32 M sucrose, 10 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1% Triton X-100), whereas, 0.079 to 0.094 g of muscle was placed in 800 µL of 1X Lysis buffer. After brief centrifugation, 20-30 seconds at 9300 X g, the supernatant was removed and the pellets resuspended in additional buffer and recentrifuged. The muscle was subjected to this procedure an additional two times before being suspended in 200 µL of the PCR buffer with non-ionic detergents (10 mM Tris/HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.45% NP-40, 0.45% Tween 20) while the pellets from the blood were suspended in 500 µL of this buffer without further washes. Six to eight hairs, cut near the roots, and 0.05 to 0.75 g of adipose were each placed immediately into 50 and 200 µL of the PCR buffer with non-ionic detergents without prior exposure to the 1X Lysis buffer. Each tube received 0.04 mg of proteinase K prior to overnight incubation at 56°C in a shaking water bath. Following incubation, the samples were boiled at 95°C for 20 minutes to inactivate the proteinase K. After a one minute centrifugation, the supernatant was transferred to a clean tube and stored at -20°C until required for PCR amplification (Balnaves *et al.*, 1991).

The final concentration of DNA, in each tube, was estimated using a spectrophotometer. A 1 mL solution, containing 20 µL of the sample DNA in 980 µL of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), was placed in a quartz cuvette and its optical density (OD) at a wavelength of 260 nm was determined. This value was then multiplied by the dilution factor of the solution, 50, and 40/1000. The final equation was, therefore, as follows:

$$[DNA] = OD_{260} \times 40/1000 \times 50$$

PCR reactions using the above samples were run with 2.5 x 10^{-3} µg/µL (0.05 µg total DNA) aliquots as template.

2.3 Oligonucleotide Primers, Polymerase Chain Reaction and Agarose Gel Electrophoresis

2.3.1 Sequences amplified

SRY gene:

SRY, the sex determining region on the Y chromosome, is a single copy Y chromosome gene. A nested PCR was set up since we were anticipating running samples containing very low template numbers: embryo biopsies would only be providing 1-3 copies of the sequence as a starting point for DNA amplification. Three outer primers were tested along with one inner pair. The HMG Exp1/A_{red}, HMG 1/A_{red}, and HMG 2/B primer pairs yielded fragments of 526 bp, 346 bp and 286 bp, respectively (see Table 4 for the primer sequences and Table 5 for the approximate lengths of the amplified sequences).

Final protocols used pHMG 1/A_{red} and pHMG 2/B as the outer and inner primer pairs.

DAX-1 gene:

The "dosage sensitive sex reversal-adrenal hypoplasia congenita locus critical region on the X gene 1" (*DAX-1*) is a single copy X chromosome gene potentially involved in the female sex differentiation pathway. It was used in these experiments as a control for the presence of DNA in the tubes. A nested reaction was performed using pDAX i/12 and pDAX j/13 as the outer and inner primer pairs. They yielded 300 and 200 bp fragments, respectively (see Table 4 for the primer sequences) in both males and females.

YR sequence:

YR is a repetitive Y chromosome sequence that, therefore, only requires a simple, one-step, PCR amplification for visualization of the final product. There are some homologous sequences elsewhere in the genome resulting in amplification in females. However, the sequence is present 200 times more frequently on the Y chromosome than in the rest of the genome (Mileham *et al.*, 1988) which allows for much greater amplification in males. The amplified fragment was 192 bp long (see Table 4 for the primer sequences).

GAPDH sequence:

A one-sided nested PCR reaction was set up in an attempt to amplify the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. The expected band lengths for the outer (pGAPDH 1/A) and inner (GAPDH 1/B) primer pairs were approximately 750 bp and 500 bp, respectively (see Table 4 for the primer sequences).

2.3.2 PCR protocols

The PCR was performed under the following conditions: 20 µL reaction mixes were set up containing the sample, 2 µL of 10X PCR buffer (100 mM Tris/HCl, pH 8.3 (at 25°C); 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatin), water and primers for the sequence(s) of interest. A drop of mineral oil was placed on top of the mix in each tube to minimize evaporative losses. Sample tubes were placed in a thermal cycler (Perkin Elmer) and denatured at 95°C for 7 min, prior to addition of the AmpliTaq® DNA polymerase (5 U/µL)/dNTP mix. This was followed by 40 cycles at the following temperatures: denaturation at 95°C for 30 sec, annealing at 68°C for 30 sec and elongation at 72°C for 1 min. The cycle was completed with a 7 minute extension period at 72°C and cooling to 4°C.

The PCR products were analyzed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide and viewed under ultraviolet lighting.

All primer pairs were initially tested in uniplex PCR reactions to ensure that amplification was specific towards the sequence in question even at low template concentrations. These reactions required 0.2 µM of each primer, 0.1 µM dNTP mix and 0.5 U AmpliTaq® DNA polymerase. The inner set of reactions for *HMG*, *DAX* and *GAPDH* required a 1 µL sample from the initial amplification as a template to be able to visualize the final product.

Multiplex PCR reactions required alterations of the conditions for both products to be seen in the same sample. Attempts to set up reactions using GAPDH primers in combination with YR and/or HMG primer pairs were unsuccessful so alternate combinations were tested.

The other multiplex combinations investigated involved DAX, HMG and YR primer pairs. HMG and YR multiplex reactions used 0.2 µM each of pHMG Exp1/A_{red} and pYR 1/2, 0.4 µM dNTP mix and 1 U AmpliTaq® DNA polymerase. Templates of 2 µL were required for the inner reaction. The HMG, YR and DAX multiplex PCR mix required 0.3 µM each of pHMG 1/A_{red} and DAX i/12, 0.2 µM each of pYR 1/2, 0.6 µM dNTP mix and 1.5 U AmpliTaq® DNA polymerase. The nested reaction used 0.3 µM each of pHMG 2/B and DAX j/13, 0.4 µM dNTP, 1.0 U AmpliTaq® DNA polymerase and a 2 µL sample from the initial amplification. These two multiplex combinations did not show consistent amplification of the *HMG* fragment; therefore, work

with the *YR* sequence was discontinued. The final combination consisted of 0.1 µM each of pHMG $1/A_{red}$ and pDAX i/12, 0.2 µM dNTP mix and 0.5 U AmpliTaq® DNA polymerase in the initial run. The inner reaction was composed of a 2.0-2.5 µL sample from the first amplification series as template, 0.25 µM each of pHMG 2/B, 0.1 µM each of pDAX j/13, 0.2 µM dNTP mix and 0.75 U AmpliTaq® DNA polymerase. This combination gave the most reliable results; however, at low concentrations, amplification of the HMG sequence was still sometimes compromised.

2.4 Animals

Prepuberal Landrace X Yorkshire F2 gilts from the Macdonald Campus Farm swine facility (see Appendix Table 1), weighing 60-70 kg along with one batch of twelve pigs weighing 88-105 kg, received intramuscular (IM) injections of 750 IU pregnant mare serum gonadotrophin (PMSG/Equinex®, Ayerst Laboratories) followed 72 hours later with 500 IU human chorionic gonadotrophin (hCG/APL®, Ayerst Laboratories). Gilts were inseminated with 80-100 mL of semen from CIPQ (Centre d'insemination porcine de Québec) or collected from boars at the Macdonald Campus Farm swine facility, 20-24 and 36-42 hours after hCG.

Gilts were transported to the Large Animal Research Unit (LARU), Macdonald Campus, one to three days after

insemination for surgery. They were returned to the swine facility three to five days post-surgery.

2.5 Embryo Collection

Anaesthesia was induced with approximately 0.2 mL/kg of a KRT cocktail (Ketamine 50 mg/mL, xylazine 10 mg/mL, butorphanol 1 mg/mL) and 4.4 mg atropine and maintained on an inhalation closed circuit system of 2-5% isofluorane and 1-1.5 L/mL oxygen. Once anaesthesia was induced, the gilt was placed on the surgical table and its abdomen was washed, shaved and cleaned with alcohol and an iodine solution.

Embryos were collected from the uterine horns using a standard mid-ventral laparotomy method. The reproductive tract on each animal was exposed and the number of corpora lutea (CL), follicles and cysts were counted on each ovary. A small incision was made, using mosquito forceps, in the uterine horn about 7-9 inches distal to the uterotubal junction. A one way Foley catheter was introduced into this incision and threaded anteriorly until the opening of the catheter was 5-7 inches below the uterotubal junction. The bulb, on the catheter, was then inflated using 5-8 mL of saline to help maintain its position within the horn and to prevent passage of the embryos beyond this point. Using a syringe attached to a mouse feeding tube inserted 2-3 cm past the ostium into the oviduct, 30 mL of flushing medium (Dulbecco's phosphate buffered saline (GIBCO), 0.4% BSA and 0.050 mg gentamicin) was passed through the oviduct into the

uterine horn. The medium was collected into a 50 mL centrifuge tube and stored in an incubator at 37°C until the search for embryos was carried out. This procedure was done on both horns, then the tract was placed back into the body cavity and the incision was sutured closed.

The embryos were found using a stereomicroscope, retrieved, and placed into small droplets of flushing medium in a small petri dish. They were then kept at 37-39°C until biopsied.

2.6 Embryo Manipulation

2.6.1 Embryo biopsies and culture

Biopsies of the embryos were taken using a micromanipulator. This required two glass micropipettes; one for holding the embryo (40 µm inner diameter) and the other for taking the biopsies (15 µm inner diameter). Each was attached to a micromanipulator and manipulation was done under 400X magnification. The micropipettes were connected by rigid-walled plastic tubing to syringes for control of fluid movement within the micropipettes.

The embryos were placed into individual 6-7 mL droplets of flushing medium, on a petri dish, covered with mineral oil. One to three cells were taken at biopsy and placed into the same droplet as the embryo. They were immediately removed along with 3 µL of the fluid, using a P20 pipetteman with a C10 tip, and placed into labelled 0.5 mL eppendorf tubes. Once all biopsies had been taken, embryos were placed into

individual 175 µL droplets of Whitten's medium (68.49 mM NaCl, 4.78 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25.00 mM $NaHCO_3$, 0.33 mM Na pyruvate, 1.71 mM Ca lactate·$5H_20$, 24.58 mM Na lactate, 1% Phenol Red, 5.56 mM glucose) supplemented with 15 mg/mL BSA and 0.050 mg gentamicin/mL. Two drops of mineral oil were placed on top of each well to prevent evaporation prior to being placed in an incubator for overnight culturing at 39°C in a 5% CO_2 atmosphere.

2.6.2 Cytogenetic Analysis

Embryos which developed to the blastocyst stage were subsequently exposed to 12.5 mL of 1:3 deacetylmethyl colchicine (Colcemid®)/Whitten's medium solution for six hours. Higher concentrations or longer exposure times tended to cause excessive contraction of the chromosomes.

These embryos were fixed and stained according to the methods of Tarkowski (1966) and King *et al.* (1987). They were placed into 5-10 µL droplets of hypotonic solution (0.9% sodium citrate) for four minutes to partially dissolve the zona pellucida. They were then transferred to labelled precleaned slides (soaked in methanol) with as small a drop of hypotonic solution as possible. One to two drops of freshly prepared mounting solution (one part methanol: one part acetic acid) were dropped onto the embryo from a height of 12-15 cm. Blowing on the slide helped break the zona pellucida and spread the cells. The slides were then placed

into freshly prepared Carnoy's fixative solution (one part glacial acetic acid: three parts methanol) for six hours at room temperature or overnight at 4°C. They were then dried for at least one hour, stained with freshly prepared 4% Giemsa for four to five minutes, rinsed and allowed to dry.

Slides were subsequently viewed under 20X magnification. All slides with potential spreads were sent to Dr. A. King's laboratory, University of Guelph, for reading of the karyotypes. This involved scanning the spread in from the Leica microscope into an IP Lab Spectrum and then importing the image into Adobe Photoshop where chormosomes could be cut and positioned in a standard karyotype arrangement.

2.7 Embryo Transfer

2.7.1 Donor Pigs

Donor pigs were prepared as previously described. Donors were used in groups of three initially; however, they yielded insufficient numbers of good quality embryos. This meant that insufficient numbers of embryos developed to the blastocyst stage; therefore, transfers were not warranted. Groups were subsequently increased to four gilts per collection.

2.7.2 Recipient Pigs

Potential recipient gilts, weighing between 88 and 112 kg, were removed from the main group of finishing pigs, at the Macdonald Campus Farm swine facility, and housed in a naturally ventilated room alongside a boar. Daily heat checks, performed in the morning, were done until the gilt

showed signs of two estruses (see Table 6 for time line). Many first heats went unnoticed so a good heat one to two months after starting the heat checks was accepted as a sign that the animal was cycling on its own. Once cycling was determined, the pigs, in groups of two, were moved over to LARU and placed into individual stalls with a feeder and watering spout at the front. They were kept on a restricted feeding regimen (three kg/pig/day) to minimize fat deposition and to allow top-feeding of Regu-Mate® (17α-allyl-17ß-hydroxyestra-4,9,11-trien-3-one, a synthetic progestin: altrenogest). To synchronize the heats of the recipients with those of the donors, the cycling sows were placed on an 11 mg altrenogest/day treatment for 18 consecutive days. Twenty-four hours after the recipients received the last dose of Regu-Mate®, recipients and donors received IM injections of 750 IU of PMSG followed 72 hours later by 500 IU hCG.

2.7.3 Embryo Collection, Biopsying, Sexing

Embryo collections and biopsying were performed as previously described. Sexing of the embryos was done overnight while they continued to develop in Whitten's medium. This was required in order to transfer embryos of known sex, into the recipients, the following morning.

2.7.4 Embryo Transfer

Preparation and surgical procedures were as stated for embryo collections, up until exposure of the reproductive tract. In order to minimize handling of the tracts, they were

not completely exposed. Ovaries were not exteriorized; however, they were checked to see if ovulation had occurred. A blunt 18 gauge needle was used to penetrate the uterine horn wall approximately 5 cm posterior to the uterotubal junction. A tom cat catheter attached to a length of rigid-walled plastic tubing and a 1 mL syringe was inserted into the lumen through the blunt ended needle or directly through the incision. The embryos along with 0.1 to 0.5 mL of culture media were then expelled into the lumen 1-2 cm from the uterotubal junction. The uterine horn was then gently placed back into the abdominal cavity and the incisions were sutured closed. The body wall was closed using simple interrupted stitches approximately 5 mm apart using #1 polydioxanone (PDS) absorbable suture material. Non-absorbable #2 polyamid suture material was then used to loosely stitch the fat layer together, more to minimize fluid accumulation than for support. Following this, the skin was sutured closed using a continuous horizontal mattress with the length of each stitch not exceeding 1.5 cm.

After giving the recipients a few days to recuperate from the surgery, they were brought back to the barn. Pregnancy was monitored by daily heat checks beginning on day 15 of their estrus cycle. Return to estrus would indicate failure of the transfer to take.

Table 4: Description of the primers used to amplify the different sequences†.

Seq[a]	Primer ID	Length	Primer Sequence (5' TO 3')	GC[b]
SRY[1]	pEXP.A (outer)	22 mer	ATGGTGCAGTCATATGCTTCTG	10
	pHMG.A_{red} (outer)	31 mer	GAATTCGACAATCATAGCTCAAACGATGGAC	13
	pHMG.1	24 mer	ACTTGGCTGCATAGTACTGCCGCC	14
	pHMG.B (inner)	25 mer	ACTAGAGGAAGTGGTAGAGAGAGTG	12
	pHMG.2 (inner)	25 mer	GGAAGCAAATTCTGTGCCCTCTCTC	13
DAX[1]	pDAX.12 (outer)	23 mer	CTGCTCACAGCTCCTGTACTTGG	13
	pDAX.i (outer)	23 mer	CCAGGGCTTCAGTGCGTGAAGTA	13
	pDAX.13 (inner)	31 mer	CTCGAGTCATAACTTTGCACAGAGCATTTCC	13
	pDAX.j (inner)	24 mer	ACTTCAGTGGGGAACTCAGCAGAT	12
YR[2]	pYR.1	24 mer	GCCCTATCTTACCATGGTCAGCCC	14
	pYR.2	23 mer	CCTCACCAGCAACCTCACAGACC	14
GAPDH[1]	pGAPDH.A (outer)	23 mer	TCCTGCACCACCAACTGCTTAGC	13
	pGAPDH.B (inner)	24 mer	CAAGGTCATCCATGACAACTTTGG	11
	pGAPDH.1	23 mer	AGGTCCACCACCCTGTTGCTGTA	13

†Primers were generously provided by: (1) Dr. D. Silversides and (2) Pig Improvement Company (PIC).
[a]Seq= sequence from which the primer originates.
[b]GC= GC content of the primer

Table 5: Comparison of the length of the PCR amplification products from the different primer pairs.

Reaction[1]	Primer Pair	Size[2]	Lengths of products[3]
outer	pHMG.A_{red}·pHMG.1	346	
inner	pHMG.B·pHMG.2	286	
outer	pDAX.i·pDAX.12	256	
inner	pDAX.j·pDAX.13	213	
single	pYR.1·pYR.2	192	

[1]Outer reaction refers to the initial series of amplifications in a nested PCR. Inner reaction refers to the nested, or second set of PCR amplifications. Single refers to a sequence that is amplified once within the PCR assay.
[2]Size refers to the length of the PCR amplification products for a given primer pair.
[3]The lines represent the PCR amplification products for the different primer pairs drawn to scale.

Table 6: Schedule for recipient pigs:

Days	
	daily heat checks
- 0	(M/Th)
	restricted feeding starts
- 1	
	11 mg Regu-Mate®/pig/day
- 18	
	last day of Regu-Mate®
- 19	
	750 IU PMSG (2 recipients & 3-4 donors)
- 22	(Sa/Th)
	500 IU hCG (2 recipients & 3-4 donors)
- 23	
	heat (AI: donors only)
- 29	(Tu/F)
	surgery (embryo transfer)
- 38	
	heat checks (pregnancy checks)

RESULTS

3.1 Sensitivity Tests

3.1.1: Genomic DNA

Initially, primer pairs were tested individually to ensure that they amplified the expected sequences with reasonable accuracy and sensitivity. The *GAPDH* sequence, a potential control sequence for the multiplex reaction, was amplified in a one-sided nested PCR reaction. The outer primer pairs, GAPDH 1/A, showed good amplification in both male and female samples, with DNA concentrations as low as 10^{-3} µg/µL. Weak bands could, however, still be visualized at 10^{-4} µg/µL. The inner primer pairs were not tested at this point, in a dilution series, since samples containing DNA isolated from the different tissues showed good amplification when the GAPDH 1/B primer pair was used on its own.

Primers for the repetitive Y chromosome sequence, YR 1/2, exhibited differential amplification between the male and female samples with the bands being stronger in the males and visible when template DNA was as low as 10^{-5} µg/µL. Conversely, amplification could only be seen with female DNA concentrations greater than 10^{-4} µg/µL.

The third sequence proposed as a control sequence in the multiplex PCR was the *DAX* gene. This sequence showed

amplification in all samples from both sexes with slightly better amplification seen in females.

The sequence intended to be used to sex the embryos incorporated the *SRY* HMG box. *SRY* is a single copy gene on the Y chromosome, thereby requiring a nested PCR reaction. Two outer primer pairs were tested: HMG $1/A_{red}$ and HMG $Exp1/A_{red}$. No amplification could be seen in females with either pair, while bands could be seen in males with DNA concentrations as low as 10^{-3} µg/µL. HMG $Exp1/A_{red}$ was, however, eliminated once contamination was seen in female samples. Amplification of the inner sequence could be seen when the initial template levels were as low as 10^{-5} µg/µL.

The next step involved testing different combinations of primers in multiplex reactions to determine the best combination. Attempts were made to use the GAPDH primers; however, perhaps due to the large size of its product, amplification of this sequence could not be seen.

Two of the combinations tested showed promise: (i) a triplex reaction involving the amplification of the *HMG*, *DAX* and *YR* sequences and (ii) a duplex reaction amplifying the two single copy sequences, *HMG* and *DAX*. The triplex reaction was run as a nested reaction incorporating primer pairs HMG $1/A_{red}$, DAX i/12 and YR 1/2 into the outer reaction (see Figure 1a) and HMG 2/B and DAX j/13 as part of the inner

reaction. The HMG $1/A_{red}$ sequence was not seen in female samples. In male samples, good amplification was seen with DNA concentrations ranging from $5.0x10^{-3}$ µg/µL to $3.1x10^{-4}$ µg/µL. Weak bands could still be visualized with concentrations between $1.6x10^{-4}$ µg/µL to $3.9x10^{-5}$ µg/µL. On the other hand, DAX i/12 products could be seen in all samples. However, noticeable decreases in band widths, implying decreasing amounts of product, were seen as template levels decreased. The YR 1/2 product could be visualized in all male samples, while it was only detected in female samples as long as the template DNA levels were above $3.1x10^{-4}$ µg/µL. The inner reaction resulted in one set of bands corresponding to the DAX j/13 product in all samples and another for the HMG 2/B sequence, in male samples only, where the initial DNA concentrations ranged from $5.0x10^{-3}$ µg/µL to $2.0x10^{-5}$ µg/µL.

The duplex reaction (see Figure 1b and c) showed increased sensitivity with respect to *HMG* sequence amplification. The outer reaction showed no amplification of the HMG $1/A_{red}$ in females; however, in males, bands were visualized with DNA concentrations as low as $2.5x10^{-3}$ µg/µL, while very faint bands could still be seen at $2.5x10^{-4}$ µg/µL. DAX i/12 amplification resulted in products in female samples when template levels were as low as $2.5x10^{-5}$ µg/µL, although

weakly once they were below $2.5x10^{-3}$ µg/µL. Males showed similar amplification patterns, for the DAX sequence; however, no products were seen below $2.5x10^{-4}$ µg/µL. The inner run, as expected, showed increased sensitivity (see figure 1c). HMG 2/B bands could be visualized in male samples where the initial template levels were between $2.5x10^{-3}$ µg/µL and $2.5x10^{-7}$ µg/µL. DAX j/13 could be seen in all samples within this range.

3.1.2: White blood cells

Duplex PCR amplifications using WBC as the template resulted in visible bands corresponding to the outer *DAX* sequence with as few as two cells, in females, and four cells, in males, for template. One of the tubes with three male WBC also showed weak amplification; however, no amplification of the *HMG* sequence was seen, implying that more than four copies of the sequence would be required if attempting to sex embryos in a single run.

The inner reaction resulted in the amplification of the *DAX* sequence in all samples, corresponding to one to four initial copies, in the male samples, and two to eight initial copies, in females. The *HMG* amplification product could also been seen in all male samples where the initial template contained two or more WBC. One out of eight samples containing only one copy of the *SRY* gene, as template, did not show *HMG* amplification, whereas the other seven did.

3.2 DNA isolated from various tissues

The triplex PCR reaction was run with $2.5x10^{-3}$ µg/µL samples of DNA isolated from muscle, adipose, skin or hair. Amplification products of all three sequences - HMG $1/A_{red}$, DAX i/12 and YR 1/2 - could be seen in all male samples, after the initial set of amplifications. Bands corresponding to the *YR* and *DAX* sequences could also be seen in all female samples. Similarly, the duplex reaction, also using $2.5x10^{-3}$ µg/µL of the DNA samples used in the triplex one, showed amplification of the DAX j/13 sequence in all samples, while the HMG 2/B bands were only seen in male samples (see figure 2).

3.3 Embryo biopsies

3.3.1 Biopsies

Embryos were collected five days after insemination which corresponded with the embryos being between the sixteen cell and morula stages. The collections, however, yielded a substantially more diversified group of embryos (see Table 7). This suggests that the embryos that were less advanced in development were showing signs of delayed growth or developmental arrest: an indication of low viability. According to Table 9, morula were the least susceptible to damage from the biopsy technique as seen by their survival rate. The eight to sixteen cell stage embryos also showed

relatively high survival rates while younger embryos and those already at the blastocyst stage showed marked decreases in viability after manipulation (see Appendix Table 4).

The medium used in these experiments did not allow embryos to pass through the four-cell stage block; therefore, none of the younger embryos survived. See Tables 7, 8 and Appendix Table 3 for details concerning the success of the collections. The results are grouped according to the treatment/use to which the biopsies and/or embryos were submitted. The first group of biopsies was sexed using the triplex reaction. Due to the low success rate, the multiplex reaction was modified into the duplex reaction (see Table 10). This was then employed for all subsequent collections (see Figure 3). The percentage of embryos not sexed decreased from 57.7% to 28.6-34.6% upon removal of the YR primers from the assay (see Table 10). The percentage of embryos diagnosed as males subsequently appeared to increase from 8.5% to 24.7%; however, this percentage decreased to 13.8% when embryos were collected for transfers into recipients, presumably due to smaller biopsies being removed in an attempt to increase survival rate of the embryos. Larger biopsies were, therefore, removed from the final group of embryos resulting in a 13.8% PCR failure rate, and an increase in percentage of embryos diagnosed as male.

The second group of embryos was collected to generate karyotypes which could be compared to the sexes determined by

PCR. This was expected to show the accuracy of the duplex reaction. Unfortunately, no karyotypes were obtained partially because the embryos were left overnight to develop to the blastocyst stage. By the time the colchicine solution was added to the medium, the embryos appeared to have already started degenerating since the medium was not able to support further growth. The embryo transfer collections made this apparent when it was noted that fewer embryos were alive just before the transfer than a few hours earlier. The final group of pigs was, therefore, used to obtain some karyotypes. These pigs were heavier than the other donors due to the lack of availability of pigs of the appropriate size at that time. These gilts, however, produced better quality embryos. The development of these embryos was monitored every five to six hours so that those that had reached the blastocyst stage could receive colchicine while they were still able to develop. This, therefore, produced more metaphase spreads implying a better chance of obtaining readable karyotypes.

3.3.2 Cytogenetic analysis

Twenty-five chromosome spreads obtained from embryos originally destined for transfer into recipients were sent to Dr. King's laboratory to be analyzed (see Appendix Table 5). To date, karyotype analysis of nine samples has indicated that five are males and the other four are females (see Table 11). Of these, two males and two females were in accordance with the results from the PCR assay. The other three male

embryos determined by the karyotypic analysis only showed amplification of the DAX sequence, therefore, placing them as female. The other two putative female embryos determined by karyotypic analysis showed no amplification of either sequence in the PCR assay, hence were not sexed.

3.4 Embryo Transfer

3.4.1 Embryo Donors

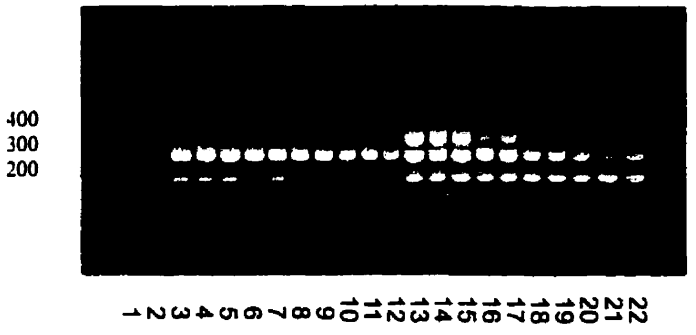
A total of 326 embryos were collected from 39 PMSG/hCG-primed donor gilts (see Table 7). Within this group, five were blastocysts, 249 embryos were between the sixteen cell and morulla stage, and 73 were at the eight cell stage or earlier. Of the 279 embryos that were biopsied, 108 developed to the blastocyst stage while culturing overnight in Whitten's medium and 185 were sexed by PCR (see Table 8 and Table 10).

3.4.2 Embryo Transfer Recipients

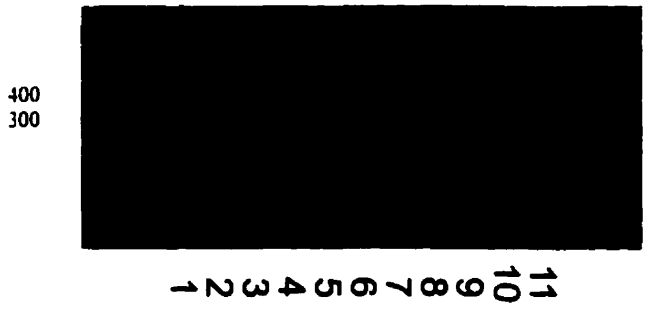
Twenty one cycling pigs were placed on Regu-Mate® treatment (see Appendix Table 6). One of these was dropped from the program when she developed an ear infection and stopped eating. Three others were placed back on the treatment after the embryo collections did not provide sufficient numbers of embryos for transfers. Five were used for embryo transfer. They received between seven and eleven embryos each (see Table 12). None of the transfers were successful; however, their return to estrus was delayed in four out of five recipients by two to eight days.

Figure 1. PCR amplification products from serial dilutions of both female and male porcine genomic DNA. a: outer triplex PCR reaction amplifying the HMG $1/A_{red}$ (400 bp), DAX i/12 (300 bp) and YR 1/2 (200 bp) DNA sequences. Lane 1: molecular weight markers. Lane 2: control (water). Lanes 3-12: female DNA. Lanes 13-22: male DNA. The DNA decreased by factors of two from $5.0x10^{-3}$ (lanes 3 and 13) to $9.8x10^{-6}$ µg/µL (lanes 12 and 22). b: outer duplex PCR amplification of the DAX i/12 and HMG $1/A_{red}$ DNA sequences. Lane 1: molecular weight markers. Lanes 2-6: female DNA. Lanes 7-11: male DNA. The DNA decreased by factors of ten from $2.5x10^{-3}$ (Lanes 2 and 7) to $2.5x10^{-7}$ µg/µL (lanes 6 and 11). c: inner duplex PCR showing amplification of the DAX j/13 (300 bp) and HMG 2/B (200 bp) sequences. Two microlitre samples from the outer reaction (products shown in b) were used as template. PCR products were analyzed by gel electrophoresis on a 1% agarose gel.

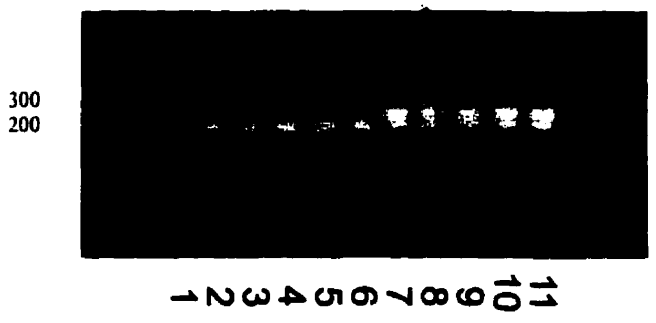
a)


400
300
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

b)


400
300
1 2 3 4 5 6 7 8 9 10 11

c)


300
200
1 2 3 4 5 6 7 8 9 10 11


300
200
1 2 3 4 5 6 7 8

Figure 2: PCR amplification of the HMG 2/B (300 bp) and DAX j/13 (200 bp) DNA sequences in female and male porcine DNA samples isolated from various tissues. Lanes 1-4: female DNA. Lanes 5-8: male DNA. DNA was isolated from blood (lanes 1 and 5), skin (lanes 2 and 6), hair (lanes 3 and 7) and muscle (lanes 4 and 8). The PCR products were analyzed by gel electrophoresis on a 1% agarose gel.

Table 7: Distribution of embryonic cell stages collected from prepuberal gilts 5 days post-insemination†.

	Pig (n)	Dead	<4¢‡	4-8¢	8-16¢	M	Bl	Total
HYD[1]	30	12 (6.2)	2 (1.0)	12 (6.2)	44 (22.8)	122 (63.2)	2 (1.0)	193
HD[2]	30	51 (19.0)	8 (3.0)	31 (11.5)	53 (19.7)	126 (46.8)	0 (0.0)	269
Trnsfr[3]	38	30 (9.2)	13 (4.0)	30 (9.2)	28 (8.6)	221 (67.8)	5 (1.5)	326
Final[4]	10	9 (7.9)	6 (5.3)	1 (0.9)	6 (5.3)	89 (78.8)	2 (1.8)	113
Total	108	102 (11.3)	29 (3.2)	74 (8.2)	131 (14.5)	558 (61.9)	9 (1.0)	901

[1] HYD refers to the group of embryos sexed using the multiplex PCR reaction which amplified the HMG, YR and DAX sequences.
[2] HD refers to those sexed using the HMG and DAX sequences.
[3] Trnsfr refers to embryos collected in view of transferring them into synchronized recipients.
[4] Final group refers to the last batch of donor pigs which weighed between 88 and 105 kg.
‡ ¢=cells
† Values reported are the numbers (%) of embryos collected at a specific stage.

Table 8: Results from the embryo collections†.

	HYD[1]	HD[2]	Final[3]	Transfer[4]	Total[5]
Age(days)	132.0 (1.9)	137.3 (2.1)	170.4 (4.8)	135.2 (1.9)	138.3 (1.6)
Weight (kg)	64.9 (0.7)	68.4 (1.0)	86.7 (3.8)	64.6 (0.6)	67.8 (0.9)
Follicles	0.8 (0.5)	0.5 (0.3)	0.2 (0.1)	1.7 (0.9)	1.0 (0.3)
C.L.	11.6 (1.3)	16.0 (2.1)	13.0 (1.5)	11.1 (1.0)	12.6 (0.7)
Embryos recovered	6.5 (0.9)	9.2 (1.4)	11.3 (1.7)	8.5 (1.1)	8.3 (0.6)
Embryos biopsied	5.5 (0.7)	7.0 (1.3)	9.2 (1.6)	7.3 (0.9)	7.0 (0.5)
Embryos survival	1.8 (0.3)	2.5 (0.5)	5.9 (1.3)	2.8 (0.6)	2.8 (0.3)
Embryos sexed	2.3 (0.5)	5.0 (1.0)	7.8 (1.6)	4.9 (0.7)	4.5 (0.4)
# Pigs	31	26	12	38	114

1: HYD refers to the group of embryos sexed using the multiplex PCR reaction which amplified the HMG, YR and DAX sequences. The total number of observations is 31 except for weight (27 observations)
2: HD refers to those sexed using the HMG and DAX sequences. There are 26 observations for all but age (21) and weight (16).
3: Transfer refers to embryos collected in view of transferring them into synchronized recipients.
4: Final group refers to the last batch of donor pigs which weighed between 88 and 105 kg. 5: There are 114 observation for all but age (109), weight (100), cls (113), embryo survival (113) and embryos sexed (107).
†. Values reported are the average numbers (±se).

Table 9: Survival of embryos based on morphology at collection and destination of biopsy and embryo†.

	2-4 cell	4-8 cell	8-16 cell	Morula	Blastocyst
HYD[1]	0.50	0.08	0.35	0.29	0.00
HD[2]	0.12	0.20	0.31	0.44	0.00
Transfer[3]	0.00	0.04	0.19	0.46	0.33
Final[4]	0.00	0.00	0.57	0.70	0.00
Total	0.05	0.10	0.31	0.46	0.33

†Values are reported as percent of biopsied embryos that developed to the blastocyst stage
[1]HYD: biopsies sexed using the triplex PCR assay
[2]HD: biopsies sexed using the duplex PCR assay
[3]Transfer: biopsies collected for transfer into synchronized recipients
[4]Final: biopsies collected from the heavier donors

Table 10: PCR Results from the embryo biopsies†.

	HYD[1]	HD[2]	Final[3]	Transfer[4]	Total
# biopsied	189	182	109	283	763
males	16 (8.5)	45 (24.7)	38 (34.9)	39 (13.8)	138 (18.1)
females	55 (29.1)	85 (46.7)	56 (51.4)	146 (51.6)	342 (44.8)
unknown	109 (57.7)	52 (28.6)	15 (13.8)	98 (34.6)	274 (35.9)

1: HYD refers to the group of embryos sexed using the multiplex PCR reaction which amplified the HMG, YR and DAX sequences.
2: HD refers to those sexed using the HMG and DAX sequences.
3: Trnsfr refers to embryos collected in view of transfering them into synchronized recipients.
4: Final group refers to the last batch of donor pigs which weighed between 88 and 105 kg
†Values reported are the numbers (%) of embryos.

Figure 3: PCR results from sexing porcine embryo biopsies. Biopsies were taken using a micromanipulator, from freshly collected day 5 embryos from PMSG/hCG-primed gilts. Results show amplification of both the HMG 2/B (300 bp) and DAX j/13 (200 bp) DNA sequences in males (lanes 1, 4-6, 8, 12,16, 19-20, 23-25). Female embryos (lanes 3, 7, 9-11, 13-15, 17-18, 21-22, 26-27) showed amplification of only the DAX j/13 DNA sequence. Lane 2: molecular weight markers. PCR products were analyzed by gel electrophoresis on a 1% agarose gel.

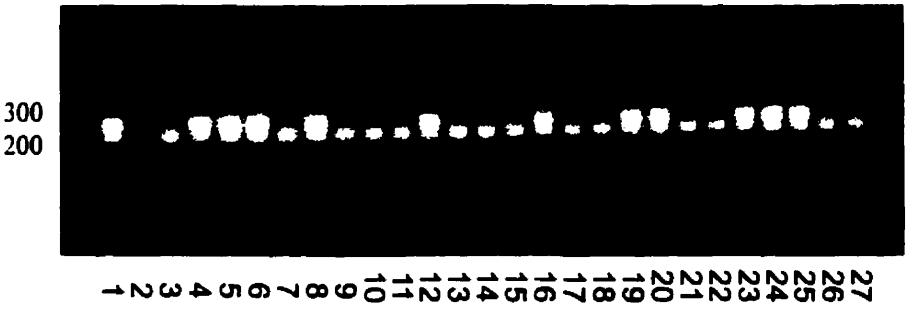

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Table 11: Cytogenetic analysis of biopsied embryos along with their sex as determined through the duplex PCR assay.

biopsy id	karyotype sex[1]	PCR sex
448	male	female
581	female	unknown
661	male	male
677	female	unknown
683	male	female
698	female	female
770	female	female
816	male	female
840	male	male

[1] karyotypes determined by M. Neal at the University of Guelph

Table 12. Results from the embryo transfers.

ID	195	232	132	213	137
Age at heat	188	179	222	208	229
ReguMate® treatment	09/9-26/9	12/9-29/9	23/9-10/10	23/9-10/10	26/9-13/10
Surgery date	7/10	10/10	21/10	21/10	31/10
# embryos transferred	10	8	10	7	11
Sex of embryos	2 females 8 unknown	8 females	6 females 4 males	7 unknown	11 females
Expected heat	19/10	22/10	2/11	2/11	12/11
Actual heat	22/10	28/10	10/11	2/11	14/11
Delay in heat	3	6	8	0	2

DISCUSSION

Multiplex PCR assays are often advantageous when sexing young embryos. The number of cells that can be removed from the embryo while maintaining its viability is rather limited prior to expansion of the trophoblast. This implies that copy number in the sample will be rather low unless amplifying a repetitive sequence. However, the repetitive sequences used often show amplification in females, albeit at lower levels. Single copy sequences specific to the Y chromosome could, therefore, potentially provide a more accurate means of determining the sex of normal young embryos.

The sex determining region on the Y chromosome, *SRY*, the proposed testis determining factor, TDF, is a likely candidate for sexing embryos. It is a single copy gene on the short arm of the Y chromosome and is required for testis determination early in the development of male embryos. With such a low copy number, a two-step PCR assay is recommended. This is especially true if expecting to employ the assay under conditions where the sample will only contain a few copies of the sequence, such as when sexing embryos biopsies. A repetitive sequence, on the other hand, could undergo the less time-consuming one-step assay.

The accuracy of sexing embryos requires the detection of two or more sequences, not all of which need to be Y chromosome specific. In fact, increased accuracy could

probably be accomplished by amplifying two Y chromosome sequences along with an X-chromosome or autosomal sequence. This latter sequence is necessary as an internal control to check for the presence of an embryo, or embryonic cells, in the sample. Without it, a sample could be falsely concluded to be female when the PCR was actually unsuccessful.

Such a triplex PCR assay would allow for non-amplification of one Y chromosome sequence within any given male sample: the presence of the product for one or the other locus would indicate a male embryo. This is one of the reasons why multiplex assays with the *YR* sequence were attempted. However, unless the amplification of both Y chromosome sequences are Y-specific, problems can arise. In our case, the *YR* sequence also showed amplification in the female samples. This created problems when the *YR* and *DAX* sequences amplified but the *HMG* sequence did not. According to the assays with the serial dilutions, females show weaker amplification of the *YR* sequence. However, in several samples, strong amplification of the *YR* sequence occurred alongside moderate amplification of the *DAX* sequence. Without the product for the *SRY* sequence, these embryos could potentially be considered females; however, the degree of amplification of the *YR* sequence would suggest that they are males.

Multiplex PCR assays are quite sensitive to the conditions imposed on it. When amplifying two or more

sequences, the different primer pairs will, in effect, compete with one another for the nucleotides and other components within the reaction mix. One sequence will, therefore, tend to amplify more efficiently under a given set of conditions. The problem encountered is that optimization of the PCR conditions for one set of primers will often result in suboptimal conditions for the other pair(s) within the reaction.

In order to set up an efficient multiplex assay, unequal amplification of the different sequences could potentially be eliminated if the following aspects were considered when deciding which sequences and primers to use. First of all, the sequences should have similar copy numbers within the genome. If one sequence is present in greater numbers, it will show much greater amplification within fewer steps in the assay. Generally, one round of amplification will double the number of copies and, therefore, templates within the reaction mix. If, on the one hand, a single copy gene is being amplified in an assay that requires forty cycles of amplification and three cells (i.e., three copies of the gene) are present at the start, then by the end of the reaction, there could potentially be 1.2×10^{19} ($[3 \times 1]^{40}$) copies. When, on the other hand, one is amplifying a sequence that is repeated twice, such as the *DAX* gene, or two hundred times, such as the *YR* sequence, throughout the genome, the final copy number could be 1.3×10^{31} ($[3 \times 2]^{40}$) or substantially

greater than 10^{99} ($[3x200]^{40}$). When amplifying a repetitive sequence, the final copy number may not be so high once the solution becomes saturated: the components of the reaction mix may become rate limiting. If running a PCR assay with just the one set of primers, the number of amplification steps can be reduced until a decrease in the amount of final product is seen.

The amplified products should also be of similar lengths since shorter segments are amplified more efficiently than longer ones. This was seen when duplex reactions with the GAPDH primers were attempted. There was probably too great a difference in the size of the amplified fragments: 200 bp for the *YR* sequence or 400 bp for the *SRY* sequence compared to over 700 bp for the *GAPDH* sequence in the outer reaction. In cases like this, it might be worthwhile to decide which sequence within the multiplex assay is more important and arrange the other primer pairs such that they amplify larger sequences.

The YR primers were created to amplify a sequence that was present in much greater numbers than the other two in the multiplex assay and, at the same time, it ended up being the shortest one amplified. Therefore, in order to get better amplification of the *SRY* sequence, the basis for this project, the YR primers were removed from the assay.

In the DAX/SRY duplex assay, non-amplification of the *DAX* sequence appears to have occurred less frequently than

for the *SRY* sequence. In females, there are two copies of the sequence present in the genome, one on each X chromosome. The males, however, only carry one copy of this gene, similar to the *SRY* gene. Only five of the embryos sexed as male did not show amplification of the *DAX* sequence, whereas many more did not show amplification of the *SRY* sequence.

Sexing the embryos may have been more successful if the amplified *DAX* sequence was slightly larger, up to one hundred base pairs, than the *SRY* sequence. This would provide the right conditions for optimal amplification of the *SRY* sequence in the male samples. Even if amplification of the other sequence was not 100% in the male samples, this would not affect the end results since the presence or absence of the *SRY* sequence dictates whether or not the embryo is a male. The female samples would show amplification of the second sequence regardless of its size since the other primer pair would have no template to work with. In other words, as long as the male samples show amplification of the *SRY* sequence, sexing embryos should be possible.

The last point for setting up a good multiplex assay is to use primers that require similar PCR conditions. This entails creating primers with similar GC contents or creating slightly longer primers if the GC content is slightly lower in some of them. This is to ensure that the temperature at which denaturation occurs is similar for all primers. Guanosines and cytosines contain three hydrogen bonds, in

comparison with adenosine and thymidine which only contain two such bonds. Therefore, the higher the GC content, the more energy is required to break the bonds and the higher the temperature requirements for denaturation to occur.

Fulfilling the above requirements is not easily accomplished. Therefore, defining right from the start which sequence is more important would be best. With this in mind, the PCR conditions can be arranged to optimize amplification of this product under the different multiplex assays. This would ensure that when running small samples, such as embryo biopsies, non-amplification, at one of the other loci will not affect the final outcome to as large an extent as non-amplification of the primary sequence.

In this project, the triplex and the duplex PCR assays ran into problems when amplification failed at one or the other locus with the former method being more susceptible.

Other labs have seen this along with, in general, weaker amplification from embryo biopsies in comparison with equivalent numbers of white blood cells (Ennis and Gallagher, 1994). Perhaps this is due to differences in the efficiency of the DNA extraction procedures. The leucocytes were placed in liquid nitrogen several times to ensure that the membranes were broken; however, the embryonic cells were aspirated from the embryos using a micropipette. It is possible that some of the cells were removed in a relatively intact state such that the DNA was not exposed to the PCR reaction mix. There were

also several samples where DNA was lost in the transfer of the biopsy from the micropipette into the eppendorf tube. This could have been minimized if the whole drop was placed into the tube. Since all of the biopsies were being taken prior to placing the embryos into the final culture medium, some of the flushing medium had to remain covering the embryo. A second biopsy was not possible, in these cases, if the embryos were to survive.

In the dilution series using leucocytes, all samples showed amplification of the *DAX* sequence. The WBC isolated from males, in general, also showed amplification of the *SRY* sequence. One out of eight samples containing one WBC did not show amplification of the *SRY* sequence; however, when a second sample was taken from the initial tube and the nested PCR assay was repeated, the sequence showed normal amplification of both sequences. This suggests that either the sample did not contain any amplified products from the SRY primers or that this was one of those occasions where amplification sometimes fails at one locus and not at the other. The first situation seems unlikely since copies of the other sequence were transferred and were further amplified. The PCR reaction mix was made, in bulk, for the ten tubes, five each for the female and male nested assay. Since amplification was seen in the other four male samples, it appears unlikely that the composition of the mix was at fault. Another explanation, though unlikely, is that the

problem could have occurred when placing the enzyme/nucleotide mix into the tubes. If nucleotides were the limiting factor, the primers for the DAX sequence may have outcompeted the other primers since they amplify a shorter sequence or perhaps because they have a lower proportion of G and C residues: 0.5 and 0.45 for primers DAX j/13 compared with 0.52 and 0.48 for primers HMG 2/B (see Table 4).

Since one out of eight samples containing a single male white blood cell as template showed non-amplification of the SRY sequence, it can be expected that at least the same proportion of male embryo biopsies containing only one copy of the SRY gene will show similar results. This means that 12.5% of the time this PCR assay may result in a misdiagnosis of a male as a female. According to observations by Ennis and Gallagher (1994) concerning lower amplification in embryos compared with that seen in WBC samples initially containing the same number of copies, one can expect a higher rate of misdiagnosis with one cell biopsies from male embryos. This can perhaps be reduced by: 1) altering the PCR assay such that the primers for the SRY gene are amplifying the shortest segment and the other primer pairs are amplifying slightly longer fragments, and 2) by placing the biopsies in liquid nitrogen prior to being subjected to the assay. This could lead to increased exposure of the DNA to the components of the PCR mixture if the removal of biopsies does not break the cell membranes to a large enough extent. Samples containing

two or more white blood cells showed 100% reliability which suggests that the embryo biopsies containing two or more cells should be accurately sexed, as long as the DNA is successfully transferred.

Contamination of the PCR can sometimes occur when handling large numbers of copies of the sequence(s) of interest. When transferring samples from the first set of amplifications to the next set of tubes, care must be taken not to get any sample DNA on the gloves nor on the equipment: this can contaminate subsequent samples. Contamination of a male sample with DNA from a female sample should not be a problem since the *DAX* sequence is amplified in both samples; however, it may result in preferential amplification of the *DAX* sequence if it results in large excesses of this product over that of the *SRY* products. Transfer of male DNA into female samples would result in the amplification of the *SRY* sequence and in its subsequent false labelling as a male.

In the initial testing of primer pairs, a contamination problem did arise with the outer set of SRY primers. However, after a few changes in the way samples were handled and changing the pairs of primers used, it did not appear to be a problem when sexing embryos. Filtered pipette tips were used when preparing the PCR mixtures and when handling the samples between amplification steps. On the other hand, transfer of the biopsies from the droplet containing the embryo into the

tube did not require filtered tips nor did the removal of samples to be run on the agarose gels. The purity of the samples was determined by the fact that individual runs in the PCR machine did not produce embryos of entirely one sex. Most groups contained both male and female embryos and a number of them also showed no amplification in some of the samples. These latter samples served as controls, for contamination.

The steps at which contamination problems may occur appear to be when handling the primers and the other components of the PCR mix and when handling amplified products if the samples are to be used for further amplification. The micropipettes do not appear to be a source of contamination since the same one was used for biopsying embryos from up to three different collections. This depended on the quality of the biopsy needle and whether or not it became blocked as part of the solution inside of it evaporated.

According to Kunieda *et al.* (1992), the detection of a target sequence by a two-step PCR method can be accomplished in less than ten hours. This time was sufficient for successful transfer of half embryos into synchronized recipients, in mice. In my experiments, it took from two to five hours from the time the embryos were flushed from the tract until they had been biopsied and placed into the culture medium. The two-step PCR reaction required an

additional five and a half hours, followed by one and a half hours before the bands were visualized on the gel. In effect, it took between nine and twelve hours to sex the embryos; however, the samples that showed no amplification were run through the second step of the assay again, using a larger sample from the original reaction. This allowed for a few more embryos to be sexed but required an additional 5 hours.

The objective of the second study was to examine the feasibility of sexing porcine embryos using a two-step PCR assay to amplify multiple DNA sequences.

Ovulation can extend over 6 h (Pope & First, 1985) which explains why the collected embryos were expected to be between the sixteen cell and morula stages. In order to sex embryos, biopsies of one or more cells were required. This should be less detrimental to the embryo's development if it is slightly more mature. When removing biopsies from eight cell or younger embryos, more damage was incurred, whereas, biopsies from healthy morulae were more efficient. Slightly larger biopsies could be taken without risking extensive damage to the embryo.

Table 9 shows that the more mature embryos, between the eight cell and morula stage, survived the manipulations better. There were problems removing cells from the blastocysts which explains their decreased survival rate. The younger embryos also showed higher mortality rates partially because the biopsies involved removing a greater proportion

of the cells. Other problems may have also been due to a lower viability to begin with since they were exhibiting delayed development compared to their litter mates. Those not having reached the four cell stage would not have been able to continue developing since Whitten's medium does not permit development past the four cell block. Another medium could have been used for these embryos for them to continue developing. The four cell stage embryos that did continue developing may have been on the verge of becoming an eight cell embryos at the time of biopsy: in other words, they would have already passed this *in vitro* block.

Menino *et al*. (1989) investigated the ability of embryos collected from gilts mated at first and third estrus to develop *in vitro*. Significantly more of the embryos between the three and eight cell stage developed to the blastocyst stage when collected from the older gilts. There were also significantly more abnormal blastocysts from those collected at the first estrus compared with those collected at the third estrus. Other studies support this finding: Underhill (1987) showed that embryos collected from older gilts were more likely to survive than those from younger gilts. According to Menino *et al*. (1989), the most commonly encountered abnormality was the inability of some blastomeres to incorporate into the morula or blastocyst. They suggested that some factor present in the first estrus gilts was preventing normal compaction of cells and inhibiting normal

cavitation. They were, however, not able to determine whether this factor could be attributed to larger numbers of immature ova being shed, chromosomal aberrations or to the ova or embryos being susceptible to some biochemical 'poison' in their environment. Induction of superovulation with hormonal treatment can also cause an increase in fertilization failure and the presence of dead or degenerate embryos (Underhill, 1987).

Pigs are short day breeders which involves decreased fertility during the summer and fall months. The decreasing daylengths of late summer-autumn alters their diurnal melatonin secretion patterns and lengthens the exposure time of the animal to high melatonin concentrations as the length of the night increases. Wild boars want to farrow in early spring, therefore, rutting season is in the late fall-early winter period. In domestic pigs, this period can be associated with delayed puberty in gilts, prolonged anoestrus, failure of farrowing after mating and reduced numbers of piglets born within the litter. Love *et al.* (1993) have also noticed that this delay in attainment of puberty is more pronounced in the absence of boars. However, genetics also play a large role in this delay.

Prepuberal gilts within the 60-70 kg weight range but younger than 100 d did not show stimulation of follicular growth after injection with PMSG and hCG. This suggests that this new group of pigs at the barn are growing faster than

the group that Underhill's research (1987) was based on. The average age of pigs used in this study, excluding the last group of pigs, was 134.4 ± 10.9 d, while that of the last group was 170.4 ± 16.5d. The older prepuberal gilts tended to produce embryos that looked healthier: the cells were more symmetrical and evenly sized than in those from the younger gilts. In general, the blastomeres appeared to be more compact, giving a rounder, smoother shape to the morula and they seemed to have more sperm in the zona pellucida. These embryos were easier to biopsy without appearing to damage as many adjoining cells which not only led to a higher survival rate but also led to a more accurate sex determination. This group of embryos yielded a higher percentage of embryos sexed. Of those that were sexed, a sex ratio much closer to the anticipated 50:50 male:female ratio was achieved (see Appendix Table 2).

French *et al.* (1993) showed that manipulation of embryos reduces the number of zygotes that are able to develop to the blastocyst stage regardless of the medium. This entails that, even though multiple studies show an increased ability of Whitten's medium to support porcine embryonic growth, it cannot compensate for the deleterious effects of the manipulation. It has also been observed that microsurgical manipulation of the zona pellucida inhibits *in vitro* development. They contain significantly fewer nuclei and produce fewer metaphase spreads compared with control embryos

(Niemann and Reichelt, 1983). Other studies (Martin *et al.*, 1991) have not seen this detrimental effect on *in vitro* development. This is partially due to the fact that, in the latter group's case, they removed any embryos that contained microsurgically damaged blastomeres from their analysis.

Niemann and Reichelt (1983) also showed that microsurgically manipulated embryos did not exhibit cell mass expansion prior to protrusion of the trophoblast through the zona pellucida. They also failed to show signs of thinning of the zona pellucida prior to the hatching of the embryo.

In order to demonstrate the reliability of the multiplex PCR assay, cytogenetic analysis of the embryos that survived the micromanipulation was attempted. It was, however, for the main part unsuccessful.

Initially, embryos had been allowed to culture unmonitored overnight, therefore, the time at which each embryo underwent blastulation was unknown. Since the culture medium was not able to support blastocyst hatching nor trophoblast expansion, the colchicine solution should have been added to the medium soon after attainment of the blastocyst stage. By waiting until morning before adding colcemid®, the embryos had probably been at the blastocyst stage for several hours already. They may have reached the point where further development was blocked and were, therefore, beginning to degenerate. Regardless, they were not

able to continue developing for the six hours required in order to get chromosome spreads.

The rate of development was monitored more closely with the group of embryos collected for transfer into recipients. It was observed that some of the embryos that were blastocysts four to five hours prior to the transfer were degenerating by the time of surgery. Therefore, on collections that did not produce sufficient numbers of embryos for a transfer the next morning, development was observed at different times throughout the night and colchicine was added to the medium as soon as they reached the blastocyst stage. This was still relatively unsuccessful since, for the main part, it was only in the morning that the surgeries for embryo transfer were canceled. However, a few readable karyotypes were obtained from these embryos.

The last group of pigs from which embryos were collected were heavier and older than the preceding groups. This was due to the fact that the gilts of the appropriate size were being used in another study and were, therefore, unavailable. In retrospect, this may have been to our advantage: the embryos collected from these gilts appeared to be healthier in terms of being more compact, more symmetrical and having more sperm in the zona pellucida. The recovery rate also tended to be higher, 86.5% versus 56.6%, 57.2% and 76.9% for the first, second and third groups, respectively (see Appendix Table 3). The development of these embryos was

monitored even more closely such that in as little as four hours after culturing, colcemid® could be added to the medium of some embryos. In general, within twelve hours after biopsying, the embryos that had the capacity, had developed to the blastocyst stage. This would suggest that culturing for twelve hours prior to transfer would allow one to determine which ones are viable without culturing them for so long that they are already in the process of degenerating.

Of the karyotypes analyzed, four, two males and two females, were in agreement with the sex determined by the PCR assay. However, three other embryos determined to be males from their chromosomes were females according to the PCR assay, while two chromosomally female embryos remained unsexed via the PCR method. The latter did not show any amplification after going through the two-step assay. This could have been due to too small a biopsy being taken, insufficient exposure of the DNA to the reaction mix or the biopsy not having been transferred into the tubes. In terms of the males, the PCR assay appeared to amplify the DAX sequence preferentially over the SRY one; therefore, with a small biopsy containing one cell or perhaps a fragment of the genome, only the DAX sequence appeared.

The sexing of embryos by PCR did not result in 50:50 female:male ratios. This may be because there are more female embryos at the earlier stages of development. Studies have shown that male embryos tend to develop faster than their

female counterparts relatively early in development (Valdivia *et al.*, 1993). This suggests that male embryos are stronger and, therefore, more viable. This could result in a larger percentage of female embryos dying throughout gestation resulting in an approximately 50:50 male:female sex ratio at birth. Studies to determine the ratio of males to females in day 5 or earlier porcine embryos may help to demonstrate the reliability and accuracy of the multiplex PCR assays designed in this project.

Karyotyping was expected to help support the results from the assay; however, the quality of the slides and the embryos appear to have hindered this aspect of the project. Karyotyping embryos that have not yet expanded requires substantial skill with the technique, especially with pigs. Since the X chromosome cannot be distinguished from some of the other chromosomes with Q banding, the sex of the embryo is determined almost by default. The Y chromosome is a small metacentric chromosome; therefore, when analyzing the spreads, the first step is to count the number of chromosomes to ensure that there is a full complement. The next step involves finding and counting the small metacentric chromosomes. If there is an odd number of these chromosomes, the embryos are said to be male. If, on the other hand, there is an even number, a female embryo is diagnosed. A larger piece of debris can sometimes hide the Y chromosome resulting in the false determination of a female. However, if the

debris is small, it can sometimes be confused with a small metacentric chromosome, thereby resulting in the false detection of a male. Karyotyping may, therefore, not be 100% accurate under such circumstances. Consequently, one cannot support or reject the accuracy or reliability of the multiplex PCR assays in this project.

Excluding the two embryos where the PCR assay was unsuccessful in the amplification of either sequence, the karyotypes were in agreement (disagreement) with the sex of the embryos, as determined by the PCR assay 57% (43%) of the time. This level of agreement can be increased by better karyotyping techniques and by modifying the methods of PCR analysis to increase its efficiency and sensitivity.

Placing blastocysts into a culture medium that better supports further development once they reach the blastocyst stage will permit expansion of the trophoblast and hatching of the embryo. This would increase the number of cells available for cytogenetic analysis. With potentially more metaphase spreads, the chance of obtaining an accurate analysis for each embryo would increase.

Alternatively, the embryos could be sexed by fluorescent in situ hybridization (FISH) to the nuclei of the embryos using probes specific for the Y and X chromosomes (Munné *et al.*, 1994). This involves fixing embryos to slides followed by exposure to fluorochrome-labeled specific DNA probes for chromosomes Y and X and a final counterstaining. Fluorescence

microscopy is subsequently used to analyze the slides. It may prove more useful than karyotyping since this technique can be applied to embryos that have stopped developing (Munné *et al.*, 1994). This would allow for a larger percentage of embryos to be sexed, enabling one to assess the accuracy of the multiplex PCR assay more easily.

Without evidence supporting or refuting a 50:50 male:female sex ratio in early preimplantation embryos, one cannot draw any conclusions as to the accuracy of the PCR assay with the last group of embryos. According to the sensitivity tests using white blood cells, 12.5% of male embryos where only one cell is removed for analysis will be incorrectly sexed as female. This can be reduced by taking larger biopsies whenever possible, freeze-thawing the biopsies to ensure adequate exposure of the DNA to the components of the PCR mix, designing a new multiplex assay where the SRY primers amplify the shortest fragment and/or designing a more sensitive assay. This last point would involve: 1) running the amplification products through an acrylamide gel, 2) using chromophore-labeled primers, 3) running the assay as a multiplex reaction in the first series of amplifications with subsequent steps involving single reactions, 4) purifying DNA from the initial reaction to increase template numbers in the nested step, and 5) employing primer extension preamplification.

Acrylamide gels are substantially thinner than their agarose counterparts. This increases the sensitivity of the assay by enabling one to detect lower amounts of product. The number of amplification steps can, therefore, potentially be reduced decreasing the time required for analysis. Decreased culture periods could also increase the probability of successfully transferring embryos into recipients.

Using N-labeled primers in a PCR assay would eliminate the need for gels. After the series of amplifications are complete, the excess chromophore-labelled primers could be removed from the reaction mix by running the samples through a filter. The level of fluorescence within this final solution could subsequently be determined with a spectrophotometer. By labeling the different primer pairs with chromophores that fluoresce at different wavelengths, one could distinguish between the various amplification products.

The optimal conditions for PCR amplification is different for each primer pair causing the amplification process to become less efficient when multiple primer pairs are placed in the same reaction mix. With limited numbers of cells available, initially, the first series of amplifications must incorporate the outer primer pairs for all sequences of interest. Since a small aliquot of the initial reaction is used to initiate the nested series, multiple assays containing single primer pairs can be run in

parallel. This would permit single sequence amplification in each reaction at the optimal PCR conditions for each primer pair. However, the main drawback to this option is the lack of internal controls for the presence of DNA or amplification failure when amplifying a Y-specific sequence.

If the DNA is purified after the initial series of amplifications and the entire sample is subsequently used as a template for the nested reaction, fewer amplification cycles will be required initially. While the success of the nested reaction relies more on template numbers available rather than the volume used to transfer the template, too large a volume can be detrimental to the success of the assay. By minimizing the volume transferred, fewer contaminants will be present, reducing the risk of amplification failure. The inner primers pair could also be designed to require a higher annealing temperature than the outer primer pairs (Chong *et al.*, 1993). This will avoid amplification of the outer fragment due to residual primers in the nested reaction.

The amount of template DNA available for multiplex PCR analysis in a one to three cell biopsy is rather limited. To circumvent this, Zhang *et al.* (1992) developed a strategy for whole genome amplification called primer extension preamplification (PEP). This would be followed by locus-specific amplification by PCR. PEP involves the lysing of the cells followed by the addition of reagents and multiple

amplification cycles. The use of random 15-mer primers, rather than shorter primers, reduce the probability that any given primer extension product will be present in substantially greater or lower amounts in the final solution. According to Zhang *et al.* (1992), the probability of amplifying any sequence in the genome to a minimum of thirty copies is 0.78. There is, however, locus dependant variation in amplification efficiency. Therefore, the final solution must be verified for the presence of the loci of interest before incorporating this into the final methodology. In general, shorter sequences appear to amplify more efficiently (Schaaf *et al.*, 1996); however, under some circumstances, such as non-amplification of a specific locus of interest, this procedure may not be viable.

Single cells can only be analyzed once preventing independant confirmation of the genotype. PEP reduces the potential for misdiagnosis when using single cells in a PCR assay by increasing the number of loci available for analysis (Kristjansson *et al.*, 1994). In other words, several loci can be investigated in parallel PCR assays. Simple PCR requires internal controls which frequently involve designing multiplex assays. This can result in decreased amplification efficiency at one or more loci and amplification failure at different loci as seen with the SRY sequence. PEP provides the opportunity to create optimal amplification conditions

for each set of primers since each locus is amplified in a separate reaction.

On average, the initiation of pregnancy requires 14-16 viable embryos. Of these, nine to ten will be born and seven to eight will be weaned (Pope and First, 1985).

Prolonged culturing of embryos has been demonstrated to greatly reduce pregnancy rates in recipient pigs (Pope and Day, 1977). After collecting two cell embryos and culturing them to the four or eight cell stage followed by transfer into recipient gilts, the embryo survival rate could be as high as 63%. However, if the embryos were cultured for 48 h prior to transferring them, the survival rate dropped to 15%. Another study showed a 21.4% survival rate within the first twenty five days of gestation if morula were immediately transferred into synchronized recipients (McDonald, 1982). In this study, non-cycling 60-80 kg gilts were used as recipients. This study also showed that the transfer of blastocysts was less successful than that of the morula.

Studies where manipulated bovine embryos have been transferred also show some success (Baker and Shea, 1985; Rorie *et al.*, 1985). Transfers of embryos that have been surgically split in half show a 78% pregnancy rate if the demi embryos are cultured for two to four hours prior to transfer in order to determine their viabilities. If they are cultured overnight or for longer than twelve hours, about 34% of the demi embryos retain their viability while the success

rate of their transfer decreases to less than 40%.

A late return to estrus may indicate that some embryonic losses have occurred after transfer.

FUTURE STUDIES

Further studies involving *SRY* and/or other single copy sequences and sex determination of preimplantation embryos are still warranted. The first step would involve designing a more sensitive assay, perhaps through the use of different primer pairs for the control sequences such that the *SRY* sequence is amplified more efficiently. Other modifications could include using acrylamide gels such that lower amounts of template can be detected, eliminating the gels and using chromophore-labeled primers or running the assay as a multiplex reaction in the first series of amplifications with subsequent steps involving single reactions. Purification of the DNA from the initial reaction also has the potential to increase the number of templates available for the nested reaction after fewer amplification steps. Whole genome amplification through primer extension preamplification may also increase the efficiency and reliability of the PCR assay.

Once the assay has been developed, cytogenetic analysis or fluorescent in situ hybridization (FISH) can be used to support the reliability of the assay. Cytogenetic analysis can be very accurate when enough metaphase spreads are obtained; however, this would probably entail culturing embryos beyond hatching and trophoblast expansion. FISH can

potentially provide more embryos for comparison since those that have ceased developing can still be analyzed.

GENERAL CONCLUSIONS

Two-step multiplex PCR assays can be designed to efficiently sex embryos through the amplification of single copy DNA sequences. Proper selection of the Y chromosome and control sequence(s) is necessary for reliable sex determination. This should involve the following criteria: (1) The amplified fragments corresponding with the control sequences should be larger than that of the sequence being used to sex the embryo. (2) The sequences being amplified should be equally represented within the genome. In other words, they should all be single copy sequences or, if repetitive, the sequence being used to sex the embryos should have the same number of copies or perhaps a few more than the control sequences. (3) The primers for the different sequences should require the same PCR conditions. If this is not possible, the primers for the control(s) should require slightly higher denaturation temperatures than those required for the main set of primers.

Serial dilutions using genomic DNA and white blood cells can help in the determination of the optimal PCR conditions. However, in general, embryo biopsies tend to show lower amplification than equivalent numbers of leucocytes.

Sexing of pigs using DNA extracted from a variety of tissues is also possible. This could also be used in other

species for forensic purposes when blood is not readily available.

The triplex PCR assay did not show reliable enough results to be used; however, the duplex assay has the potential to be more successful if embryos are collected from more mature gilts. This increased maturity is required not only for the success of the PCR but also for the survival of the embryos after biopsy. The embryos from more mature donors appear to be more compacted and lesss damaged by the removal of a few cells. In order to be able to remove large enough biopsies from the embryos for sex determination through PCR amplification of different sequences, embryos between the eight cell and morula stages should be used. Older embryos, such as blastocysts, are more difficult to biopsy while younger embryos, less than eight cells, do not appear to survive the manipulation as well.

Transfer of embryos into recipients should be done within the first ten to twelve hours to get some pregnancies. Longer culture periods tend to reduce the viability of the embryos, while a minimum of ten to twelve hours is required for both the assay itself and to determine the viability of the individual embryos. Larger numbers of embryos should also be transferred so that pregnancy is recognized by the recipient.

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Appendix Table 1.
Donor gilts listed.
SG= biopsies sexed with triplex PCR (1), sexed with duplex PCR (2), sexed for transfers (3), sexed for karyotypes (4). Grp= grouped by date of surgery. I.D.= donor identifictaion number. Age= age in days at surgery. Wt= weight when PMSG injection was administered. F= number of follicles on both ovaries. Ct= number of cysts on ovaries. CL= number of corpora lutea on ovaries. E.R.= number of embryos recovered in flush. EBi= number of embryos biopsied. ES= number of embryos sexed. ESU= number of embryos not sexed. ESM= number of males embryos. ESF= number of female embryos. EB= number of embryos surviving to the blastocyst stage.

SG	Grp	I.D.	AGE	WT	F	Ct	CL	E.R.	EBi	ES	ESU	PSM	PSF	EB
0	1	325	137	67	2	0	8	8	3	0	3	0	0	5
0	1	333	137	62	0	0	5	5	3	0	3	0	0	3
0	1	333	137	62	0	0	5	5	3	0	3	0	0	3
0	2	344	144	62	0	0	11	5	5	0	5	0	0	5
0	3	488	136	61	0	0	20	11	8	0	18	0	0	7
1	4	482	143	74	5	2	10	8	7	4	3	2	2	6?
1	4	561	106	.	6	0	8	3	3	3	0	1	2	3?
1	5	509	141	.	0	0	26	17	17	15	0	5	10	1
1	5	534	119	.	0	0	6	6	6	5	1	0	5	1
1	5	562	113	.	14	3	3	1	1	1	0	0	1	0
1	6	515	141	63	0	0	8	4	4	2	2	1	1	0
1	6	544	124	64	0	0	11	3	3	2	1	0	2	0
1	6	563	106	60	0	0	8	4	1	1	0	0	1	2
1	7	555	130	64	0	0	29	20	14	3	11	0	3	4
1	7	556	130	65	0	1	27	6	6	1	5	0	1	0
1	8	541	138	68	0	1	6	2	2	1	1	0	1	0
1	8	597	131	63	0	0	9	1	1	0	1	0	0	1
1	9	612	121	59	0	0	4	4	4	3	1	1	2	1
1	9	635	121	61	0	1	14	12	12	5	7	3	2	2
1	10	732	127	61	0	0	2	2	2	2	0	0	2	0
1	10	742	127	62	0	0	8	5	5	5	0	0	5	0
1	11	723	134	69	0	0	13	8	8	5	0	1	4	2
1	11	734	134	69	0	0	9	2	2	1	0	0	1	1
1	11	753	133	69	0	1	8	1	1	0	1	0	0	1
1	12	771	138	68	0	0	9	6	6	5	1	1	4	3
1	12	773	138	66	0	3	5	4	4	4	0	1	3	0
1	12	812	138	65	0	0	9	4	4	2	2	0	2	3
1	13	762	135	65	0	0	7	6	6	0	6	0	0	4
1	13	784	135	64	0	1	23	13	13	0	13	0	0	3
1	13	796	134	71	0	0	14	7	7	0	7	0	0	2
1	14	774	142	64	0	1	6	2	1	0	1	0	0	?
1	14	785	142	63	0	0	16	13	12	0	12	0	0	6
1	14	821	134	60	0	0	13	9	9	0	9	0	0	1
1	15	789	148	65	0	0	24	16	16	1	12	0	1	0

1	15	791	148	65	0	0	12	4	4	0	4	0	0	3
1	15	823	141	65	0	0	12	10	10	0	10	0	0	3
1	16	884	127	60	0	0	8	6	5	.	.	.	.	0
1	16	893	120	65	5	0	2	1	1	.	.	.	.	0
1	16	927	120	68	0	0	20	8	7	.	.	.	.	0
2	17	875	141	.	0	0	35	33	23	21	2	10	11	5
2	17	905	134	.	0	0	29	8	1	1	0	0	1	0
2	18	925	141	72	0	0	11	7	6	4	2	0	4	0
2	18	953	133	62	0	0	5	2	2	2	0	1	1	0
2	19	903	157	72	0	0	43	14	10	6	4	2	4	4
2	19	928	157	72	0	0	15	7	7	7	0	5	2	2
2	19	962	144	66	1	0	18	14	14	14	0	4	10	1
2	20	34	128	72	0	0	29	12	12	11	1	7	4	10
2	20	51	128	68	0	0	8	3	2	2	0	1	1	2
2	20	53	128	66	1	0	9	6	6	6	0	2	4	4
2	21	36	142	70	0	0	20	8	5	4	1	3	1	4
2	21	37	142	65	0	0	11	3	3	3	0	0	3	2
2	21	62	138	73	0	0	3	3	2	2	0	0	2	2
2	22	73	137	74	0	0	35	29	28	16	12	2	14	9
2	22	112	120	67	0	0	16	8	5	2	3	0	2	0
2	23	131	143	63	0	0	17	10	5	3	2	0	3	2
2	23	134	143	67	1	0	12	10	9	4	5	2	2	3
2	23	141	143	70	0	0	12	7	5	3	2	0	3	0
2	24	223	.	75	0	0	9	7	5	3	2	1	2	0
2	24	244	.	70	0	0	4	4	2	1	1	0	1	0
2	25	303	128	67	8	0	28	1	1	0	1	0	0	0
2	25	304	128	67	0	0	6	6	6	3	3	1	2	5
2	25	307	128	61	2	0	9	8	3	1	2	0	1	1
2	26	293	.	62	0	0	8	7	3	1	2	0	1	1
2	26	322	.	71	0	0	8	7	7	5	2	2	3	3
2	26	337	.	68	0	2	16	14	10	5	5	2	3	6
3	27	333	146	68	0	0	7	3	3	2	1	0	2	2
3	27	342	143	69	0	0	8	6	5	3	2	1	2	2
3	28	348	146	65	0	0	8	8	8	5	3	0	5	0
3	28	372	150	64	0	0	7	5	4	4	0	2	2	2
3	28	422	124	61	0	0	7	2	2	1	1	0	1	0
3	29	381	129	69	0	0	14	13	13	3	10	0	3	10
3	29	393	150	69	0	0	9	8	6	2	4	0	2	0
3	29	491	119	68	0	2	11	6	6	1	5	0	1	1
3	30	383	132	67	0	0	7	2	2	2	0	1	1	1
3	30	395	153	68	0	0	9	10	9	7	2	1	6	3
3	30	412	125	67	1	0	22	20	13	7	6	1	6	6
3	30	433	125	65	0	0	5	7	6	6	0	2	4	3
3	31	391	160	65	0	0	4	3	3	2	1	1	1	1
3	31	432	132	60	0	0	8	4	4	1	3	0	1	0
3	31	441	125	65	0	0	4	2	2	2	0	1	1	0
3	31	482	131	61	0	0	9	6	5	1	4	1	0	2
3	32	453	123	67	1	0	12	11	10	4	6	3	1	5
3	32	457	123	69	0	0	11	7	6	4	2	1	3	4
3	32	484	133	68	0	0	12	11	11	6	5	3	3	10
3	33	415	163	61	0	0	9	8	5	2	3	1	1	0

3	33	4510	125	63	25	0	10	7	7	4	3	2	2	0
3	33	474	142	66	0	0	11	12	12	6	6	0	6	4
3	33	504	139	63	0	0	4	2	2	0	2	0	0	0
3	34	501	142	59	4	0	29	29	24	18	6	1	17	2
3	34	505	142	61	0	0	11	9	8	5	3	0	5	0
3	34	506	142	61	0	0	30	28	24	18	6	3	15	19
3	35	455	136	60	0	0	12	8	8	3	5	1	2	6
3	35	462	129	59	0	0	18	9	9	9	0	1	8	8
3	35	516	121	63	0	0	9	7	5	5	0	2	3	0
3	35	517	121	63	0	0	6	6	6	4	2	0	4	1
3	36	531	128	64	5	0	9	8	8	7	1	1	6	7
3	36	535	128	73	5	0	17	11	10	8	2	3	5	4
3	36	536	128	63	0	0	19	8	8	6	2	2	4	1
3	36	571	128	73	25	0	7	5	4	3	1	0	3	0
3	37	483	160	62	0	0	9	4	4	4	0	1	3	1
3	37	552	131	58	0	0	.	4	4	4	0	1	3	0
3	37	553	131	65	0	0	24	22	15	15	0	2	13	3
3	37	561	131	61	0	0	8	2	2	1	1	0	1	0
4	38	591	177	.	0	0	16	17	14	13	1	6	7	5
4	38	602	172	.	0	0	8	5	4	4	0	3	1	4
4	38	603	172	91	1	0	14	14	10	8	2	3	5	5
4	38	621	161	91	0	0	14	12	10	10	0	6	4	10
4	39	615	179	80	0	0	7	4	3	3	0	0	3	2
4	39	623	168	75	0	0	13	9	6	6	0	0	6	4
4	39	656	155	88	1	0	18	20	18	17	1	5	12	14
4	39	695	143	73	0	0	18	15	11	9	2	3	6	9
4	40	582	200	70	0	0	12	7	6	4	2	2	2	4
4	40	593	197	80	0	0	4	3	1	1	0	1	0	0
4	40	632	161	76	0	0	22	19	18	17	1	8	9	13
4	40	655	160	.	0	0	10	10	8	2	6	1	1	1

Appendix Table 2.
Chi-square for 107 donor gilts.
Embryo sexes were determined by PCR.
HMG/YR/DAX= embryos sexed using the triplex PCR. HMG/DAX= embryos sexed using the duplex PCR. Transfer= embryos sexed for potential transfer into synchronized recipients. Final= final group of embryos collected for karyotypes.

Frequency Percent Row Pct Col Pct	HMG/YR/DAX	HMG,DAX	Transfer	Final	TOTAL
Male	17	45	39	40	141
	3.53	9.36	8.11	8.32	
	12.06	31.91	27.66	28.37	
	24.29	34.88	21.08	41.24	
Female	53	84	146	57	340
	11.02	17.46	30.35	11.85	
	15.59	24.71	42.94	16.76	
	75.71	65.12	78.92	58.76	
TOTAL	70	129	185	97	481

TEST STATISTIC	VALUE	DF	PROB
PEARSON CHI-SQUARE	15.492	3	0.001
LIKELIHOOD RATIO CHI-SQUARE	15.451	3	0.001

Appendix Table 3.
The tables detail the relative success rates during embryo collections, biopsying and sexing for the four collection groups.

a) Embryo collections with biopsies being sexed by PCR amplification of HMG, YR and DAX sequences.

	Total	Average/Pig	Percent
Pigs	31	-	-
CLs	359	11.58	-
Embryos recovered	203	6.55	56.55
Embryos biopsied	180	5.81	88.67
Embryos surviving	55	1.77	30.56
Embryos killed	7	0.23	3.89
Embryos dead	118	3.81	65.56
Embryos not sexed	109	3.52	60.56
Embryos sexed	71	2.29	39.44
Male embryos	16	0.52	22.53
Female embryos	55	1.77	77.46

b) Embryo collections with biopsies being sexed by PCR amplification of HMG and DAX sequences.

	Total	Average/Pig	Percent
Pigs	26	-	-
CLs	416	16.00	-
Embryos recovered	238	9.15	57.21
Embryos biopsied	182	7.00	76.47
Embryos surviving	66	2.54	36.26
Embryos killed	10	0.38	5.49
Embryos dead	106	4.08	58.24
Embryos not sexed	52	2.00	28.57
Embryos sexed	130	5.00	71.43
Male embryos	45	1.73	34.62
Female embryos	85	3.27	65.38

c) Embryo collections for transfer into synchronized recipients. Biopsies were sexed by PCR amplification of HMG and DAX sequences.

	Total	Average/Pig	Percent
Pigs	38	-	-
CLs	420	11.05	-
Embryos recovered	323	8.50	76.90
Embryos biopsied	283	7.45	87.62
Embryos surviving	108	2.84	38.16
Embryos killed	33	0.87	11.66
Embryos dead	142	3.74	50.18
Embryos not sexed	98	2.58	34.63
Embryos sexed	185	4.87	65.37

Male embryos	39	1.03	21.08
Female embryos	146	3.84	78.92

d) Final embryo collections for karyotypes. Biopsies were sexed by PCR amplification of HMG and DAX sequences.

	Total	Average/Pig	Percent
Pigs	12	-	-
CLs	156	13.00	-
Embryos recovered	135	11.25	86.54
Embryos biopsied	109	9.08	80.74
Embryos surviving	71	5.92	65.14
Embryos killed	5	0.42	4.59
Embryos dead	33	2.75	30.28
Embryos not sexed	15	1.25	13.76
Embryos sexed	94	7.83	86.24
Male embryos	38	3.17	40.43
Female embryos	56	4.67	59.57

Appendix Table 4.
Correlation between cell stages biopsied and survival.
Biogrp= final destination of the embryo and its biopsy. 1.000= biopsies were sexed with the triplex PCR assay. 2.000= biopsies were sexed with the duplex PCR assay. 3.000= embryos were collected for transfer into synchronized recipients. 4.000= embryos collected from the heavier donors.
Columns represent final morphology: 0.000= unknown. 1.000= dead. 2.000= blastocyst.
Rows represent the morphology of the embryos upon collection. .= unknown morphology. 0.000= dead or degenerating. 1.000= two to four cell stage. 2.000= four to eight cell stage. 3.000= eight to sixteen cell stage. 4.000= morula. 5.000= blastocyst.

Survival of embryos based on morphology at collection and destination of biopsy and embryo†.

	2-4 cell	4-8 cell	8-16 cell	Morula	Blastocyst
HYD[1]	1/1	1/11	11/20	30/70	0/0
HD[2]	1/6	5/15	12/26	47/60	0/0
Transfer[3]	0/8	1/27	5/22	100/117	1/2
Final[4]	0/5	0/2	4/3	67/29	0/0
Total	2/20	7/55	32/71	244/276	1/2

†Values are reported as number surviving/number dead after biopsy
[1]HYD: biopsies sexed using the triplex PCR assay
[2]HD: biopsies sexed using the duplex PCR assay
[3]Transfer: biopsies collected for transfer into synchronized recipients
[4]Final: biopsies collected from the heavier donors

THE FOLLOWING RESULTS ARE FOR:
BIOPGRP = 1.000
TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

FREQUENCIES

	0.000	1.000	2.000	TOTAL
.	3	5	8	16
0.000	0	3	0	3
1.000	0	1	1	2
2.000	0	11	1	12
3.000	0	20	11	31
4.000	5	70	30	100
TOTAL	8	110	51	169

TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

PERCENTS OF TOTAL OF THIS (SUB)TABLE

	0.000	1.000	2.000	TOTAL	N
.	1.78	2.96	4.73	9.47	16.00
0.000	0.00	1.78	0.00	1.78	3.00
1.000	0.00	0.59	0.59	1.18	2.00
2.000	0.00	6.51	0.59	7.10	12.00
3.000	0.00	11.83	6.51	18.34	31.00
4.000	2.96	41.42	17.75	62.13	105.00
TOTAL	4.73	65.09	30.18	100.00	
N	8	110	51	169	

TEST STATISTIC	VALUE	DF	PROB
PEARSON CHI-SQUARE	19.229	10	0.037
LIKELIHOOD RATIO CHI-SQUARE	20.360	10	0.026

THE FOLLOWING RESULTS ARE FOR:
BIOPGRP = 2.000
TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

FREQUENCIES

	0.000	1.000	2.000	TOTAL
1.000	1	6	1	8
2.000	5	15	5	25
3.000	1	26	12	39
4.000	0	60	47	107
TOTAL	7	107	65	179

TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

PERCENTS OF TOTAL OF THIS (SUB)TABLE

	0.000	1.000	2.000	TOTAL	N
1.000	0.56	3.35	0.56	4.47	8.00
2.000	2.79	8.38	2.79	13.97	25.00
3.000	0.56	14.53	6.70	21.79	39.00
4.000	0.00	33.52	26.26	59.78	107.00
TOTAL	3.91	59.78	36.31	100.00	
N	7	107	65	179	

TEST STATISTIC	VALUE	DF	PROB
PEARSON CHI-SQUARE	28.408	6	0.000
LIKELIHOOD RATIO CHI-SQUARE	24.453	6	0.000

THE FOLLOWING RESULTS ARE FOR:
BIOPGRP = 3.000
TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

FREQUENCIES

	1.000	2.000	TOTAL
1.000	8	0	8
2.000	27	1	28
3.000	22	5	27
4.000	117	100	217
5.000	2	1	3
TOTAL	176	107	283

TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

PERCENTS OF TOTAL OF THIS (SUB)TABLE

	1.000	2.000	TOTAL	N
1.000	2.83	0.00	2.83	8.00
2.000	9.54	0.35	9.89	28.00
3.000	7.77	1.77	9.54	27.00
4.000	41.34	35.34	76.68	217.00
5.000	0.71	0.35	1.06	3.00
TOTAL	62.19	37.81	100.00	
N	176	107	283	

TEST STATISTIC	VALUE	DF	PROB
PEARSON CHI-SQUARE	29.438	4	0.000
LIKELIHOOD RATIO CHI-SQUARE	37.512	4	0.000

THE FOLLOWING RESULTS ARE FOR:
BIOPGRP = 4.000
TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

FREQUENCIES

	1.000	2.000	TOTAL
1.000	5	0	5
2.000	2	0	2
3.000	3	4	7
4.000	29	67	96
TOTAL	39	71	110

TABLE OF MORPHINI (ROWS) BY MORPHCUL (COLUMNS)

PERCENTS OF TOTAL OF THIS (SUB)TABLE

	1.000	2.000	TOTAL	N
1.000	4.55	0.00	4.55	5.00
2.000	1.82	0.00	1.82	2.00
3.000	2.73	3.64	6.36	7.00
4.000	26.36	60.91	87.27	96.00
TOTAL	35.45	64.55	100.00	
N	39	71	110	

TEST STATISTIC	VALUE	DF	PROB
PEARSON CHI-SQUARE	14.066	3	0.003
LIKELIHOOD RATIO CHI-SQUARE	15.864	3	0.001

Appendix Table 5.
Results from slides containing embryo spreads sent to Dr. A. King's lab at the University of Guelph for reading.
Source= group from which the embryo originated. #Sli= number of slides sent to be read. #Unr= number of unreadable slides. #Mal= number of male karyotypes. #Fem= number of female karyotypes. #PMa= number of males according to the duplex PCR assay. #PFe= number of females according to the PCR assay. #PUn= number of biopsies where the PCR assay did not amplify the sequence(s). #Sam= number of cases where the readable karyotypes match those from the PCR. #Dif= number of cases where the karyotype does not match those from the PCR.

Source	#Sli	#Unr	#Mal	#Fem	#PMa	#PFe	#PUn	#Sam	#Dif
HMG/DAX	4	4	0	0	1	0	3	-	-
Transfer	25	19	3	3	5	13	7	2	4
Final	61	56	2	1	18	30	1	2	1

Appendix Table 6.

Embryo transfer recipient data.

Gp= group with respect to Regu-Mate® treatment and transfer dates. ID= pig identification number. DOB= date of birth. Wt= weight of the pig when removed from the main herd in order to check heats. Dt= date the pig was weighed. HC= period during which pigs were checked daily for heat. DH= date of estrus demonstrating that the pig was naturally cycling. RMT= Period of altrenogest treatment. DP= date of PMSG injection. Dh= date of hCG injection. TD= Transfer date. Pig #184 was removed from the protocol due to an ear infection. Pigs #94 and 195 did not receive PMSG nor hCG injections on the 27/9 and 30/9. Pig #195 showed heat around 1-2/10 allowing it to be used as a recipient. Pigs #94, 241 and 242 were placed back on Regu-Mate® for potential transfers at a later date.

Gp	ID	DOB	Wt	Dt	HC	DH	RMT	DP	Dh	TD
1	181	6/3	92	15/7	17/7-18/8	18/8	19/8-05/9	6/9	9/9	no transfer
1	199	4/3	92	30/7	31/7-17/8	17/8	19/8-05/9	6/9	9/9	no transfer
2	182	6/3	99	23/7	24/7-19/8	19/8	22/8-08/9	9/9	12/9	no transfer
2	198	4/3	91	30/7	31/7-21/8	21/8	22/8-08/9	9/9	12/9	no transfer
3	142	13/2	90	30/7	31/7-23/8	23/8	26/8-12/9	13/9	16/9	no transfer
3	184	6/3	88	23/7	24/7-24/8	24/8	26/8-27/8	N/A	N/A	N/A
4	94	28/1	88	15/7	17/7-30/8	30/8	09/8-26/9	N/A	N/A	no transfer
4	195	4/3	90	30/7	31/7-07/9	7/9	09/8-26/9	N/A	N/A	7/10
5	113	7/2	88	23/7	24/7-07/9	7/9	12/9-29/9	30/9	3/10	no transfer
5	232	14/3	88	30/7	31/7-08/9	8/9	12/9-29/9	30/9	3/10	10/10
6	241	14/3	99	18/8	19/8-15/9	15/9	19/9-06/10	7/10	10/10	no transfer
6	242	14/3	102	18/8	19/8-13/9	13/9	19/9-06/10	7/10	10/10	no transfer
7	132	13/2	97	18/8	19/8-22/9	22/9	23/9-10/10	11/10	14/10	21/10
7	213	6/3	99	18/8	19/8-22/9	22/9	23/9-10/10	11/10	14/10	21/10
8	145	13/2	91	3/9	04/9-22/9	22/9	26/9-13/10	14/10	17/10	no transfer
8	333	19/9	93	24/9	19/9-26/9	26/9	26/9-13/10	14/10	17/10	no transfer
9	94	28/1	88	15/7	17/7-30/8	30/8	03/10-20/10	21/10	24/10	no transfer
9	137	13/2	95	3/9	04/9-29/9	29/9	03/10-20/10	21/10	24/10	31/10
10	201	5/3	112	24/9	19/9-04/10	4/10	07/10-24/10	25/10	28/10	no transfer
10	231	14/3	103	24/9	19/9-06/10	6/10	07/10-24/10	25/10	28/10	no transfer
11	285	2/4	100	24/9	19/9-08/10	7/10	14/10-31/10	1/11	4/11	no transfer
11	346	17/4	88	24/9	19/9-08/10	7/10	14/10-31/10	1/11	4/11	no transfer
12	241	14/3	99	18/8	19/8-15/9	15/9	17/10-02/11	3/11	7/11	no transfer
12	242	14/3	102	18/8	19/8-13/9	13/9	17/10-02/11	3/11	7/11	no transfer