

# Use of a Combined Duplex PCR/Dot Blot Assay for more sensitive genetic characterization

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**Abstract:** A reliable and sensitive method of genetic analysis is necessary to detect multiple specific nucleic acid sequences from samples containing limited template. The most widely utilized method of specific gene detection, polymerase chain reaction (PCR), imparts inconsistent results when assessing samples with restricted template, especially in a multiplex reaction when copies of target genes are unequal. This study aimed to compare two methods of PCR product analysis, fluorescent detection following agarose gel electrophoresis or dot blot hybridization with chemiluminescent evaluation, in the detection of a single copy gene (SRY) and a multicopy gene ( $\beta$ -actin). Bovine embryo sex determination was employed to exploit the limited DNA template available and the target genes of unequal copies. Primers were used either independently or together in a duplex reaction with purified bovine genomic DNA or DNA isolated from embryos. When used independently, SRY and  $\beta$ -actin products were detected on a gel at the equivalent of 4-cell or 1-cell of DNA, respectively; however, the duplex reaction produced visible SRY bands at the 256 cell DNA equivalent and  $\beta$ -actin products at the 64 cell DNA equivalent. Upon blotting and hybridization of the duplex PCR reaction, product was visible at the 1–4 cell DNA equivalent. Duplex PCR was also conducted on 186 bovine embryos and product was subjected to gel electrophoresis or dot-blot hybridization in duplicate. Using PCR alone, sex determination was not possible for 22.6% of the samples. Using PCR combined with dot blot hybridization, 100.0% of the samples exhibited either both the male specific and  $\beta$ -actin products or the  $\beta$ -actin signal alone, indicating that the reaction worked in all samples. This study demonstrated that PCR amplification followed by dot blot hybridization provided more conclusive results in the evaluation of samples with low DNA concentrations and target genes of unequal copies.

**Keywords:** PCR, Southern blot, sexing, embryo, PGD

## Introduction

Polymerase chain reaction (PCR) is the most commonly used technique to amplify a specific gene or gene segment and was first used for genetic diagnosis of a single copy gene from bovine blastocysts in 1988 [1]. Upon the identification of SRY as the gene responsible for sex determination in cattle [2], PCR became a widely used method of sexing bovine embryos [3–7]. Although PCR followed by product detection on gel is frequently used, PCR assays for embryo sexing have been optimized to amplify samples with small amounts of DNA and they are also sensitive enough to amplify contamination by a single DNA molecule, consequently providing inconsistent or inaccurate results.

Almodin et al. [8] reported successful sex determination in only 82.0% of embryos using a single SRY primer set. It is possible that a failed PCR reaction resulted in the misdiagnosis of the embryo as a female. Others have used a duplex PCR protocol in which a male-specific primer set was used to determine sex, while a gene present in both male and female genomes was used as an internal reaction control. A retrospective study conducted by Shea [9] examined the use of duplex PCR for sexing 4,183 embryos in a commercial bovine embryo transfer program. Embryos were recovered at blastocyst stage and up to 20.0% of their mass removed and biopsied. The failure to assign sex ranged from 6.0 to 18.0% over the six year period and 7.0% of the male calves had been misdiagnosed as females. Failures at sexing were attributed to malfunctions of specific PCR reagents, contamination, and insufficient DNA template.

A simple procedure to increase efficiency of multiplex PCR is the use of dot blotting followed by nucleic acid hybridization. Studies have been conducted comparing PCR with gel analysis to Southern

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blot [10–12] and PCR consistently has been found to be more sensitive when each method was used independently. In 1999, Boerner et al. [13] combined the two methods by blotting PCR product to identify bovine Herpesvirus 4 (BHV-4) from experimentally-infected calves. Using PCR and gel analysis, a lower limit of 10 fg of BHV-4 could be detected, whereas by blotting PCR product, as little as 1 fg could be detected: a ten-fold increase in sensitivity. Results of this study indicated that blotting PCR product followed by hybridization to nucleic acid probes allows for the detection of lower quantities of products of interest than gel analysis.

An accurate and, more importantly, reliable method of genetic analysis is necessary to detect specific DNA sequences with samples of limited template and target genes of unequal copies. Although previous studies have compared the sensitivity of gel analysis of PCR product versus blotting crude DNA, and even blotting of PCR product [13], no reports compare the two methods of analysis using a multiplex PCR reaction. This study aimed to compare duplex PCR with product visualization on agarose gel stained with ethidium bromide versus PCR followed by dot blot hybridization in the detection of genes with unequal copies in the bovine genome: the bovine SRY gene, which exists in a single copy on the Y chromosome, and  $\beta$ -actin, in which multiple copies are present [14].

Studies evaluating the embryonic genome have centered on the ability to determine sex; however, as more of the bovine genome is mapped, genetic screening of embryos will expand to include genes impacting production traits. Therefore, multiplex PCR utilizing limited DNA template that is both accurate and repeatable needs to be developed. Multiplexing is challenging in that non-specific priming can yield false products, the primer sets must function under similar conditions, and the possibility of reduced sensitivity for gene detection must be considered.

## Materials and Methods

### DNA isolation

Genomic bovine DNA was isolated from bovine testis and ovary by using the Wizard<sup>®</sup> Genomic DNA Purification Kit (Promega, Madison, WI, USA) and quantified using spectrophotometry.

Day 7 bovine embryos ( $n = 186$ ) were warmed to room temperature and 3  $\mu$ l Proteinase K (1  $\mu$ g/ $\mu$ l; Sigma-Aldrich, St. Louis, Missouri, USA) was added. Tubes were placed in a thermocycler (Mastercycler<sup>®</sup> gradient; Eppendorf, Ontario, Canada) and incubated at 55 °C for 12 min to allow digestion of the zona pellucida and to lyse cell membranes. Embryos were heated to 98 °C for 10 min to inactivate the Proteinase K, and then held at 4 °C for immediate use in the PCR assay.

### PCR

Primer sets for all experiments were generated to the genomic bovine  $\beta$ -actin gene and bovine SRY [15]. The  $\beta$ -actin product was 385 bp in size (forward: 5'-CCGAGGACTTGATTGTACATGG-3'; reverse: 5'-ACTGGTCTCAAGTCAGTGTACAGG-3'). A 532 bp segment of the male-specific SRY gene was amplified (forward: 5'TCTTCC TTG TGCACA GAC AG-3'; reverse: 5'TTATTG TGG CCC AGG CTT GT-3'). Primers were diluted to 250 pmoles/ $\mu$ l in TE stock and then diluted 1:10 in DepC water for a working stock. PCR mixture: 10  $\mu$ l Go-taq (Promega), 1  $\mu$ l of each  $\beta$ -actin primer and 2  $\mu$ l of each SRY primer. Reaction conditions were optimized for each primer set by performing PCR in an Eppendorph gradient thermocycler. Optimal reaction conditions were 95 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30s, primer annealing at 60 °C for 30s, extension at 72 °C for 30s, a hold at 72 °C for 3 min, and then a final hold at 4 °C.

Male bovine genomic DNA was diluted to a concentration representative of 256, 64, 16, 4, and 1 cell, assuming 7 pg per cell [16], and subjected to PCR with either each primer separately or together in a duplex reaction. Reactions were subjected to gel electrophoresis on a 1.6% agarose gel containing 6  $\mu$ l ethidium bromide 1% (Fisher Scientific, Fair Lawn, NJ, USA) in 5X Tris/Borate/EDTA buffer at 85 V. Product was visualized by exposure to ultraviolet light. Sequences were confirmed by subcloning into pDrive Cloning Vectors (Qiagen Inc., Valencia, CA, USA) and sequenced at Clemson University Genomics Institute (Clemson, SC, USA).

### Dot blot

PCR product was denatured with 0.1 vol 1N NaOH, incubated at 37 °C for 5 min, then neutralized by

addition of 20X SSPE (3M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.02 M EDTA) to a final concentration of 6X. Using a dot blot manifold, samples were added to 6X SSPE pre-soaked nylon membrane (Biobond™-Plus Nylon Membrane; Sigma, St. Louis, MO, USA) over two pieces of Whatman paper. Embryo samples were run in duplicate, along with positive controls ranging from 2.0 ng to 0.08 ng of DNA purified from PCR product (QIAquick® PCR purification kit; Qiagen, Valencia, CA, USA). All membranes were cross-linked twice (Spectrolinker; Spectronics Corporation, Westbury, NY, USA) under UV light. Membrane hybridization overnight at 55 °C was conducted using Amersham Gene Images AlkPhos Direct Labelling and Detection System™ (GE Healthcare, Buckinghamshire, UK). Blots were washed and product detection conducted by the addition of CDP-Star™ chemiluminescent detection reagent (GE Healthcare). The blots were exposed to autoradiographic film (Kodak BioMax Light Film; Cedex, France) for 20 min. Membranes were first probed for the SRY gene segment and then stripped in boiling 6X SSPE/ 0.5% SDS for 15 min, twice. Membranes were then probed for the β-actin segment.

### Assay sensitivity

In the PCR reaction, sensitivity curves were performed in order to determine the lowest detectable

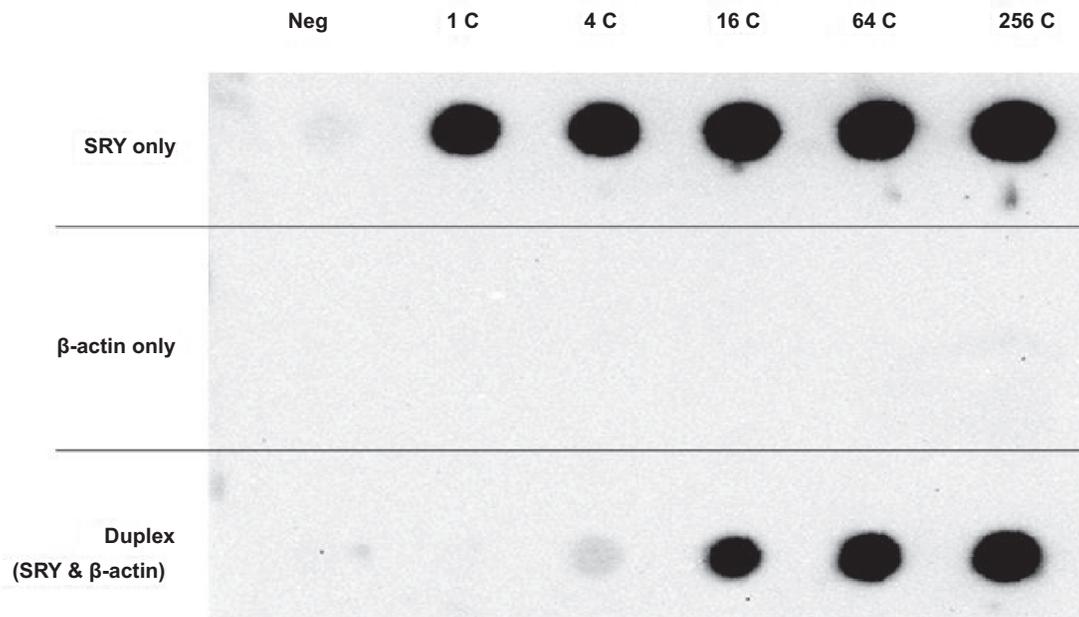
amount of PCR product using primer sets both individually and together in a duplex reaction. Male bovine genomic DNA ranging from the equivalent of 1 cell (zygote; 7.0 pg) to 256 cells (expanded blastocyst; 1.8 ng) was used as starting template. The products were visualized on a gel or dot blot membrane as described. In addition, PCR products from duplex reactions with starting template ranging from the DNA equivalent of 1 cell to 256 cells were spotted on a nylon membrane, crosslinked, and hybridized as described (Figure 1).

### Data analysis

Embryos were divided into two groups: ‘transferable’ included viable embryos while ‘degenerate’ encompassed those embryos 8 cells or less or those that appeared to have deteriorated. SAS Chi-square analysis was used to evaluate differences in proportions of embryos to which a sex was assigned.

### Results

Comparison of gel analysis of PCR products to dot blotting of PCR products in both single primer pair and duplex primer reactions showed that blotting could detect lower quantities of product than gel analysis (Table 1). Whole embryos subjected to duplex PCR amplification showed that



**Figure 1. Results of Dot Blot hybridization following male-specific probe on single primer and duplex reactions.** Figure 1 is a photograph showing the results of a dot blot hybridization using the SRY probe in single primer pair and duplex primer PCR reactions using a range of the 1 cell DNA equivalent to the 256 cell DNA equivalent.

**Table 1.** Starting template (cell equivalent) required to visualize product in both single and duplex primer PCR reactions using two detection methods.

	Primers used independently		Duplex reaction	
	SRY	$\beta$ -actin	SRY	$\beta$ -actin
Gel analysis	4 c	1 c	256 c	64 c
Dot blot analysis	1 c	1 c	4 c	1 c

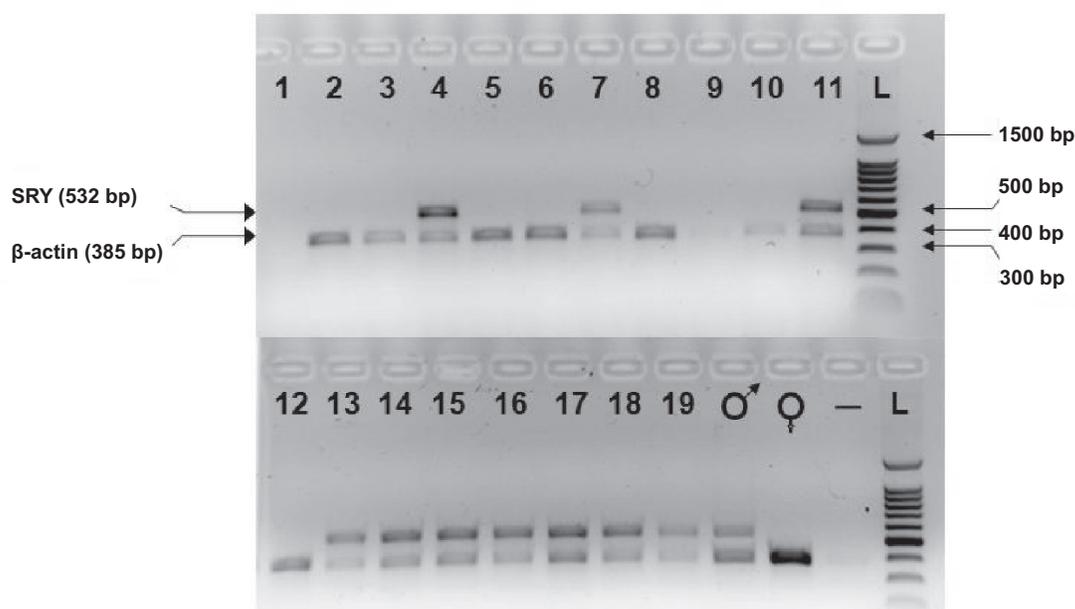
dot blot analysis provided more definitive results ( $p < 0.05$ ) when compared to gel analysis of PCR product. Visualization of PCR product on a gel concluded that 37.6% of embryos were male ( $n = 70$ ), 39.8% were female ( $n = 74$ ), and 22.6% ( $n = 42$ ) showed inconclusive results (Figure 2). No samples showed only the 532 bp (male-specific) band. All samples were then subjected to dot blot analysis and revealed 48.4% male ( $n = 90$ ) and 51.6% female ( $n = 96$ ) embryos, with no inconclusive samples. Of the samples deemed inconclusive by PCR, blotting showed that 38.1% were male ( $n = 16$ ) and 61.9% were female ( $n = 26$ ). Interestingly, dot blot analysis revealed six samples that contradicted the gel analysis, representing 3.2% of the total samples evaluated: one female that the gel demonstrated as male and five males that the gel indicated were females. There was no correlation between embryo

cell grade (transferable versus degenerate) and inconclusive results.

## Discussion

Results of this experiment support the hypothesis that duplex PCR amplification followed by dot blot hybridization allowed for sensitive genetic characterizations and can be applied to more consistently determine the presence of desirable (or undesirable) genes. Previous studies have shown that PCR often conveys considerable failure rates (8.0%–18.0%) in gene amplification from samples with restricted DNA template [8, 9], due to defective reagents, contamination, or insufficient reaction optimization [17].

Following PCR alone, none of the embryo samples showed the male-specific band without the presence of  $\beta$ -actin. This is likely a result of the multiple occurrences of  $\beta$ -actin in the genome, thus



**Figure 2. Agarose Gel following multiplex PCR Reaction on 19 bovine embryos.** Figure 2 is a photograph of 1.6% Agarose gel stained with ethidium bromide. The lanes with one band at 385 bp indicate a female embryo, while lanes with two bands, one at 385 bp and one at 532 bp, denote a male embryo. On this gel, two samples showed inconclusive results (Samples 1 and 9). The male, female, and negative controls are labeled in the bottom right corner. The 100 kb ladder is in the lanes on the right and is labeled 'L'.

providing more starting template relative to the male-specific sequence. Unlike the agarose gel, in which the presence of some bands was debatable, the results of the dot blots were indisputable. In the six dot blot samples that contradicted the gel analysis, it is impossible to determine the 'true' sex of those embryos because the assay described required the consumption of the entire embryo. The 5 males that the gel suggested were females were probably due to the SRY band containing less DNA than the lowest detectable amount by gel analysis. The dot blot designated female that the gel indicated as male may have been due to sample contamination or gel artifact. To determine which method is more accurate, an embryo biopsy could be performed with the blastomere subjected to the PCR/dot blot assay. The embryo could then be transferred to a surrogate and the sex of the fetus or resulting calf determined.

It is possible that embryos containing a smaller number of cells (less DNA template) would be less likely to reveal PCR product than the more developed embryos; however, in this study there was no correlation between embryo cell number or grade (transferable versus degenerate) and inconclusive PCR results. This supported previous findings [9, 18] that PCR frequently imparts inconsistent results, often for seemingly inexplicable reasons.

Future applications of a multiplex PCR/dot blot assay include pre-implantation genetic diagnosis in human assisted reproduction laboratories in which genetic diseases, such as Cystic Fibrosis, Huntingdon's disease, and chromosomal aberrations, could be identified. When combined with blastomere biopsy, an embryo can be screened prior to freezing or transfer. This study demonstrated that duplex PCR amplification followed by dot blotting and nucleic acid hybridization analysis provided accurate, sensitive, and consistent results in the evaluation of samples with low DNA concentrations and targets with unequal gene copies.

## Disclosure

The authors report no conflicts of interest.

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