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Sex Determination of Bovine Embryos by Polymerase Chain Reaction

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PCR 방법을 이용한 소 수정란의 성판별

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요 약

포유동물 초기 수정란의 성판별에 있어서 biopsied 수정란의 생존성과 성판별의 정확도는 동시에 중요하다. 본 연구자들은 이미 생쥐 수정란에 있어서 biopsy와 PCR을 이용한 성판별의 효율적인 방법을 개발한 바 있다. 본 연구에서는 생쥐 수정란에서 개발된 biopsy방법 (압착방법)을 이용하여 과배란 시킨 상실배기 소 수정란으로부터 몇 개의 할구세포를 분리할 수 있었다. 할구세포가 분리된 13개의 수정란은 biopsy 후 모두 생존하였으며, 이 중 10개의 수정란은 24시간 체외배양에서 정상적인 배반포기로 발달하여 77%의 체외발달율을 나타내었다. 이어서 이렇게 분리된 할구세포에서 PCR 방법으로 수정란의 성을 판별하였는데, 분석된 13개의 수정란 중 수컷은 7개, 암컷은 6개인 것으로 판명되었다. 결론적으로 본 연구에서는 압착방법을 이용하여 상실배기 소 수정란으로부터 적은 수의 할구세포를 쉽게 분리할 수 있었으며, 또한 분리된 할구세포로부터 PCR 방법을 이용하여 소 수정란의 성을 판별할 수 있었다.

Ⅰ. INTRODUCTION

Sex determination of preimplantation embros has important significance in domestic animals for livestock management and breeding. Several approaches have been developed to identify the sex of preimplantation mammalian embryos. These approaches include cytological analysis of sex chromosomes (King, 1984), quantitative comparison of X-linked enzyme activity between male and femals embryos (Monk & Handyside, 1988), detection of mals specific H-Y antigen (Wachtel, 1984), differences in morphological development between male and female embryos (Avery, 1989 ; Xu et al., 1992), and sexing by hybridization with male-specific chromosomal DNA probes (Bondioli et al., 1989). Amplification of male specific DNA sequences by polymerase chain reaction (PCR) has also been

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employed for sexing of embryos (Handyside et al., 1990 ; Bradbury et al., 1990 ; Peura et al., 1991 ; Strom et al., 1991 ; Grifo et al., 1992 ; Kunieda et al., 1992 ; Han et al., 1993). The application of the PCR method, which enables one to detect a specific DNA sequence in small number of cells (Li et al., 1989), should allow accurate and rapid sexing of embryos.

Also important in embryo sexing is the technique for embryo biopsy because it is directly related to embryo viability. Several approaches have been developed to separate a small number of cells from an embryo. These methods include zona drilling (Gordon & Gang, 1990), splitting of embryo (Picared et al., 1985) and aspiration of blastomeres (Wilton & Trounson, 1989). Recently, we have developed an efficient method for isolating single blastomeres from preimplantation mouse embryos which could then be used for sexing by PCR amplification of Y chromosome specific DNA sequence (Han et al., 1993). The biopsy method was designated as a squeeze method. Our results showed that removal of single blastomeres from mouse 4-cell embryos and morulae by this method did not affect the subsequent embryonic development *in vitro* and *in vivo* (Han et al., 1994).

In this study it was investigated whether the squeeze method for embryo biopsy is applicable to bovine embryos. In addition, we determined the sex of bovine embryos from a few blastomeres by PCR amplifying a Y-specific DNA sequence.

Ⅱ. MATERIALS AND METHODS

1. Collection of bovine morulae

Holstein heifers were superovulated using the supplier's recommended dose of follicle-stimulating hormone (FSH: F.S.H.－P.™, Schering-Plough Animal Health Co., USA) beginning on Day 8～12 of estrous cycle. The FSH was given every 12 h for 4 days and 750 μg cloprostenol (Estrumate, USA) was given together with the seventh FSH injection. Cattle displaying estrus behavior 36～48 h after the prostaglandin administration were inseminated with frozen semen every 12 h from the onset of observed standing estrous until 24 h after the end of estrous ; typically, insemination frequency ranged between 2～4. Morular embryos were obtained by flushing of uteri with 500 ml of phosphate buffered saline (PBS) supplemented with 10% fetal bovine serum (FBS) on Day 6 after the onset of standing estrus.

2. Micromanipulation

Zona cutting, pushing and holding pipets for micromanipulation were pulled from glass capillary tubing (O.D. 1 mm, I.D. 0.5 mm, length 130 mm ; Garner glass Co., USA) using a vertical pipette puller model 720 (David Korf, USA). Cutting and polishing of pipet tips were carried out using the microforge (Alcatel, France). Micromanipulations were perfomed on an inverted microscope with DIC system (Nikon, Japan) using a Leitz micromanipulator (Leitz, Germany).

3. Embryo biopsy

Bovine morulae were biopsied using a micromanipulator. For micromanipulation, three to five bovine embryos were perpendicularly plasced into 5 μl droplet of PBS under silicone oil (Sigma Chemical Co., USA) on a glass well slide. An embryo was then grasped by a holding pipette and the small part of zona pellucida was dissected with a sharp glass microneedle. The embryo with partially dissected zona pellucida was gently squeezed with holding and blunt glass micropipettes, and blastomeres were consequently protruded from the embryo. The

protruded blastomeres were separated from other blastomeres by gently suction into the blunt glass micropipette using the suction and injection syringe (Alcatel, France) and then expelled from the blunt glass micropipette.

Biopsied bovine embryos were cultured for 24 h in TCM199 medium supplemented with 10% FBS at 39℃, 5% CO_2 in air.

4. Polymerase chain reaction

For the purpose of sexing bovine embryos the DNA sequence of a male-specific gene, BRY.1 (Reed et al., 1988), was amplified using PCR. The primers used for PCR wer 5′-GGATCC-GAGACACAGAACAG-3′ and 5′-CAAGCTAA-TCCATGCATCCT-3′, and the expected size of the specific amplified fragments was 304 nucleotides.

Blastomeres isolated from a bovine embryo were transferred into a tube containing 10 μl of distilled water. Tube with balstomeres was boiled for 10 min, cooled immediately on ice and then briefly centrifuged. The reaction mixture (90 μl) which contains 1 X supplied incubation buffer (Boehringer Mannheim Biochemica, Germany), 200 μM of each of the four deoxynucleotides, 0.1 μM of each primer, and 2.0 units of Taq DNA polymerase (Boehringer Mannheim Biochemica, Germany) was added to each tube. Samples were overlaid with 50 μl of light mineral oil. PCR reactions were carried out in DNA Thermal Cycler 480 (Perkin Elmer Cetus, USA) with the following condition : at 94℃ (1 min), at 61℃ (1 min), and at 72℃ (1 min). After 45 cycles, samples were incubated for 7 min at 72℃ and then cooled on the thermal cycler. Amplified DNA fragments were resolved by electrophoresis on an 8% polyacrylamide gel and visualized under ultraviolet light (UV) after ethidium bromide (EtBr) staining.

5. Isolation of bovine genomic DNAs

Bovine genomic DNAs were isolated from blood as described by Sambrook et al. (1989). Approximately 20 ml of fresh blood was collected in a conical tube containing 3.5 ml of acid citrate dextrose solution B, an anticoagulant. The solution B consists of 0.48 g of citric acid, 1.32 g of sodium citrate and 1.47 g of glucose in 100 ml of H_2O. After centrifugation at 1,300 × g for 15 min, the buffy coat was carefully transferred to a fresh tube and then suspended in 15 ml of extraction buffer which was consisted of 10 mM TrisCl (pH 8.0), 0.1 M EDTA (pH 8.0), 0.5% SDS and 20 μg/ml pacreatic RNAase. The solution containing the buffy coat was incubated for 1 h at 37℃, and then proteinase K was added to a final concentration of 100 μg/ml. After gently mixing the enzyme into the viscous solution, the suspension of lysed tissue was placed in a water bath at 50℃ for 3 h. The equal volume of phenol equilibrated with 0.5 M TrisCl (pH 8.0) was added to the solution. The two phase were gently mixed for 10 min and then separated by centrifugation at 5,000 × g for 15 min. The viscous aqueous phase was transferred to a clean centrifuge tube and the extraction with phenol was repeated twice. After the thired extraction with phenol, the supernatant was transferred to a fresh centrifuge tube and 0.2 volume of 10 M ammonium acetate was added. Two volumes of absolute ethanol was added and the tube was swirled untill the solution is throughly mixed. The DNA precipitate was recovered by centrifugation at 5,000 × g for 5 min and washed twice with 70% ethanol. The pellet dried at room temperature was completely dissolved in TE (pH 8.0) and the concentration of DNA was calculated by electrophoresis through a 0.5% agaros gel. To prevent degradation of genomic DNAs by microorganisms

during long-term storage, three drops of chloroform were added to DNA solution and the DNA solution was stored at 4℃.

6. Direct hybridization of genomic DNAs in agarose gels

Bovine genomic DNAs (20 μg) were digested with restriction endonuclease EcoRI (Kosco, Korea) at 37℃. Completely digested chromosomal DNAs were electrophoresed on a 0.8% agarose and then photographed under UV after staining with EtBr. Direct hybridizaiton of genomic DNA in agarose gels was carried out as described by Tsao et al. (1983). After electrophoresis the agarose gel was dried at 60℃ for 2 h on a gel dryer under the vacuum. The DNAs of the dried gel were denatured in the solution of 0.5 N NaOH and 0.15 M NaCl for 30 min and neutralized in the solution of 0.5 M TrisCl (pH 7.4) and 0.15 M NaCl with gently shaking for 45 min at room temperature. After briefly washing with 2 × SSC, the gel was prehybridized at 42℃ for 2~4 h in hybridization solution containing 50% formamide, 5 × Denhardt's solution, 5 × SSPE, 0.1% SDS and 100 μg/ml of denatured salmon sperm DNA. Hybridization was performed at 42℃ for 36~40 h in the hybridization solution containing the probe. After washing with 2 × SSC, 0.1% SDS solution for 5 min the gel was stringently rinsed twice in 0.1 × SSC, 0.1% SDS solution. The gel was exposed to a X-ray film at −70℃ and the hybridized DNAs were visualized by autoradiography.

7. Synthesis of double-stranded DNA probe

DNA fragment (304 nucleotides long) used as probe was amplified by PCR. PCR products were eluted from polyacrylamide gels using the "Crush and Soak" technique originally described by Maxam and Gilbert (1977). PCR product was radiolabelled internally with [α-^{32}P] dCTP by nick translation (Rigby, 1977). Nick translation was performed in a 20 μl volume using nick translation kit (Boehringer Mannheim Biochemica, Germany). Reaction mixture consists of 100 ng of template DNA (1 μl), each 1 μl of dATP, dGTP, dTTP, 2 μl of 10 × buffer, 4 μl of [α-^{32}P] dCTP (3,000 Ci/mmole), 2μl of enzyme mixture and 8 μl of distilled water. After incubation at 15℃ for 35 min, 80 μl of 2 × SSC was added to the reaction mixture and the reaction was terminated by addition of 100 μl of phenol/chloroform (1:1). After briefly vortexing the tube was centrifuged at 12,000 rpm for 3 min and the supernatant was transferred to a new tube. Spun-column chromatography (Sambrook et al., 1989) was used to separate the radiolabelled DNA from unincorporated, radioactive mononucleotides that were retained on the sephadex G-50 column. The concentration of radioactive DNA probes used for hybridization was more than 1 × 10^6 cpm/ml.

Ⅲ. RESULTS

1. Biopsy of bovine embryos

The squeeze method developed in mouse embryos was applied to separation of blastomeres from bovine embryos. Compact morulae bovine embryos were obtained by flushing uteri with 500 ml of PBS supplemented with 10% FBS on Day 6 after the onset of standing estrus. The embryos were not decompact even though they had been incubated at 37℃ for at least 1 h in embryo biopsy medium (Gordon & Gang, 1990) free of Ca^{++} and Mg^{++} ions. However, a small number of blastomeres could be separated from the compact bovine embryos by the squeeze method (Fig. 1). Although lysis of some cells occurred during biopsy procedure, the biopsied embryos normally developed to blastocyst stage

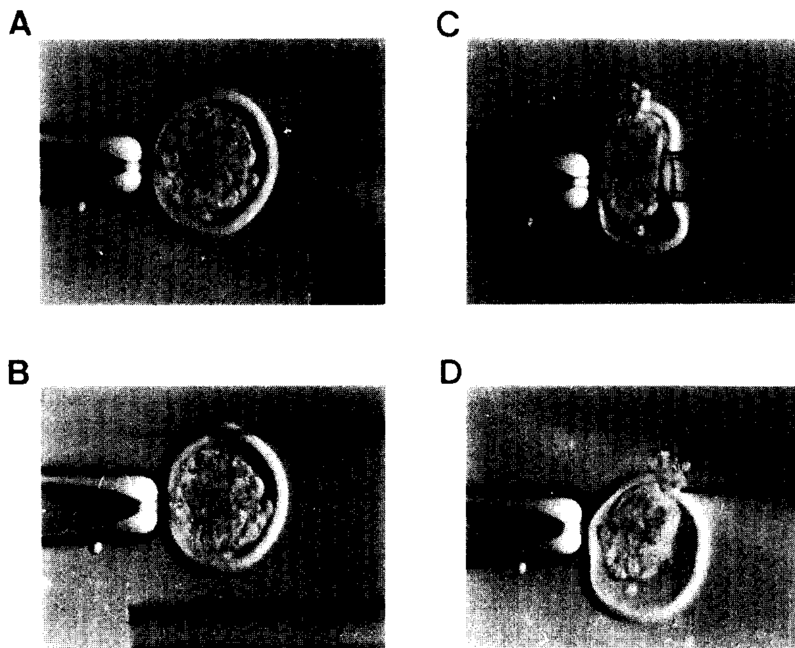

Fig. 1. Biopsy procedures of compact morular embryos. Compact morular embryos were flushed from the uteri of cows on Day 6 after artificial insemination. The embryos were not decompact despite incubation of embryo biopsy medium for 1 h. A, the embryo is immobilized at the end of a holding pipette. B, zona pellucida is partially dissected using a sharp glass microneedle. C, a few cells are protruded from the embryo by squeezing method. D, a few cells are separated by gentle suction through a blunt micropipette.

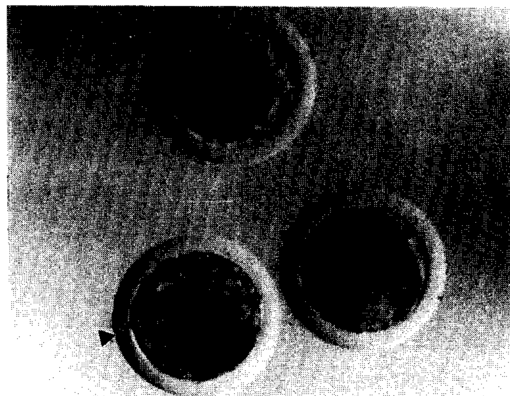

Fig. 2. *In vitro* development of biopsied bovine embryos. Biopsy of bovine embryos is illustrated in Fig. 1. Biopsied embryos were cultured for 24 h in TCM199 medium supplemented with 10% FBS at 39℃, 5% CO_2 in air. After 24 h of culture the embryos formed normal blastocysts in appearance. An arrowhead indicates the hole of zona pellucida created during biopsy procedure.

agter *in vitro* culture at 39℃, 5% CO_2 in air for 24 h. Among 13 bovine embryos biopsied at morular stage, 10 embryos developed to morphologically normal blastocysts (Fig. 2).

2. Sexing of bovine embryos

Prior to sexing of bovine embryos, we checked the specificity of the assay system by carrying out PCR in bovine genomic DNAs using Y-specific primers. Specific DNA fragments (304 nucleotides long) were seen in bovine male genomic DNAs (Fig. 3, lanes 1 &

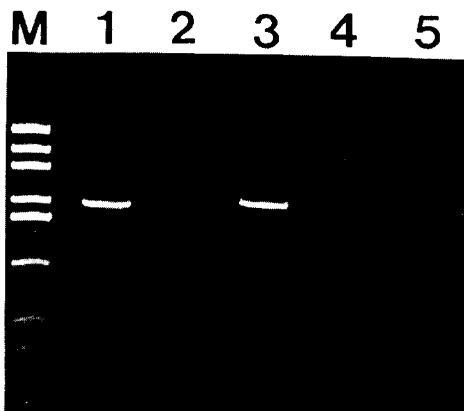

Fig. 3. Amplification of bovine Y specific DNA sequences in bovine genomic DNAs. PCR products were analyzed by 8% polyacrylamide gel electrophoresis and stained with EtBr. DNA size markers (lane M), HaeIII digested pUC18 DNA fragments (0.25 μg) ; male bovine genomic DNA in Holstein or Korea Native cattle (lanes 1 and 3, respectirely) ; female bovine genomic DNA in Holstein or Korea Native cattle (lanes 2 and 4, respectively) ; distilled water as a negative control (lane 5).

3). To learn whether the specific band was derived from bovine male DNA, a variety of genomic DNAs were digested with restriction endonuclease EcoRI and electrophoresed on a 0.8% agarose gel (Fig. 4A). It was shown by Southern hybridization analysis that the PCR products were specific for male bovine genomes because it hybridized only with genomic DNAs from male Holstein or Korea Native cattle (Fig. 4B, lanes 1 & 3), whereas female bovine (lanes 2 & 4), human (lanes 5 & 6) and the mouse (lanes 7 & 8) genomic DNAs did not give specific hybridization with the probe. This result demonstrates that the PCR products were indeed specifically amplified from bovine Y chromosome. Subsequently, PCR was performed

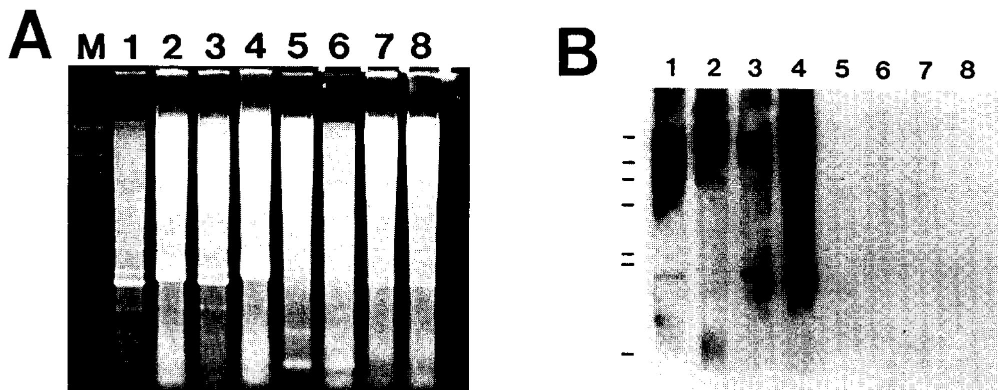

Fig. 4. Southern blot analysis of bovine genomic DNAs. (A) Genomic DNAs of the bovine (lanes 1 to 4), the human (lanes 5 and 6) and the mouse (lanes 7 and 8) were digested with restriction endonuclease EcoRI. Odd and even numbers represent males and females, respectively. The digested chromosomal DNAs were electrophoresed on 0.8% agarose gel, and visualized by EtBr staining and UV illumination. (B) Hybridization of bovine genomic DNAs was directly carried out on a dried agarose gel. Probes were made from bovine Y-specific PCR product by nick translation. The specific bands were shown in male genomic DNAs of Holstein and Korean Native cattle (lanes 1 and 3). The result shows that DNA sequences used for primers might be composed of repeated unit or multicopies.

in a few blastomeres to identify the sex of preimplantation bovine embryos. Among 13 embryos analyzed, 7 embryos were determined as males and 6 embryos as females. Thus, sex of bovine embryos could be successfully determined from a few blastomeres by PCR.

Ⅳ. DISCUSSION

1. Biopsy of embryos

Several approaches have been developed to separate a small number of cells from an embryo. These methods include zona drilling (Gordon & Gang, 1990), splitting of embryo (Picard et al., 1985) and aspiration of blastomeres (Wilton & Trounson, 1989). Zona drilling is done by making a hole on the zona pellucida of an embryo using acidified Tyrode's solution (pH 2.3). One or more blastomeres of the embryo are spontaneously protruded through the hole by suction pressure from the holding pipette. Although mouse embryos tolerate zona drilling with acid Tyrode's solution very well (Gordon & Talansky, 1986 ; Conover & Gwatkin, 1988), human embryos are highly sensitive to acid (Garrisi et al., 1990 ; Malter & Cohen, 1989 ; Ng et al., 1989). For this reason, mechanical zona drilling methods have been favored for human embryos (Garrisi et al., 1990 ; Malter & Cohen, 1989). The method of splitting has mainly been used to bisect mammalian embryos for producing monozygotic twin animals or sexing embryos. The demi-embryos produced by this method could suffer some damages because approximately one half embryo is cut out. Aspiration of single blastomeres from 4-cell mouse embryos using a micromanipulator with an aspiration pipette was first developed by Wilton & Trounson (1989). In this method, some of embryos are destroyed during the micromanipulation procedure. Although biopsy of embryos by the aspiration method did not affect their continued development to the blastocyst stage *in vitro*, the implantation rate was significantly reduced after transferring to recipients (Wilton & Trounson, 1989). Recently, we have developed an efficient method, which was designated as the squeeze method, for separating sigle blastomeres from preimplantation mouse embryos (Han et al., 1993). The removal of single blastomeres from mouse 4-cell embryos and morulae by this method did not affect *in vitro* and *in vivo* viabilities (Han et al., 1994). In this study the squeeze method developed in the mouse system was applied to bovine embryos. Although the blastomeres could not be separated as single cells from compact morular bovine embryos in despite that the embryos had been incubated in embryo biopsy medium free of Ca^{++} and Mg^{++} ions for at least 1 h, a few cells of the embryos could be biopsied by this method. It seems that cell to cell contacts of bovine embryos did not decrease in embryo biopsy medium free of Ca^{++} and Mg^{++} ions. Picard et al. (1985) reported that only 42% of bisected bovine embryos were viable after 24 h of culture. In this study, however, embryos biopsied by the squeeze method showed high developmental rate (77%, 10/13 embryos). Thus, we could show that the squeeze method is useful for biopsy of bovine embryos as well.

2. Sexing of bovine embryos

Cytogenetic analysis and DNA hybridization have been the most widely used methods for sexing preimplantation mammalian embryos. In the former procedure, embryo was bisected and the resulting one half of the embryo was used for sexing and the remaining half for transferring (Picard et al., 1985). However, this process has problems such that the chance of obtaining cells with metaphase chromosomes is very low.

The DNA hybridization method has been reported by Bondioli et al. (1989) who successfully determined the sex of bovine preimplantation embryo using Y-chromosome specific DNA probe. In the method, the embryo must be kept frozen during the relatively analytical time-consuming (8 d) process. The embryo could easily be damaged during the freezing step. These problems could be circumvented by PCR technique to sex the embryo. It is possible to amplify a specific DNA sequence by PCR from a few cells of mammalian preimplantation embryos, and thus, identification of a specific gene can be accomplished within several h. The embryo sexing by amplification of male specific DNA sequences using PCR has been performed in livestocks such as sheep (Herr et al., 1990) and cattle (Peura et al., 1991 ; Utsumi et al., 1992 ; Kirkpatrick & Monson, 1993 ; Kudo et al., 1993 ; AppaRao et al., 1994). In this study the sex of preimplantation bovine embryos could be determined in a few blastomeres by PCR.

In conclusion, we showed that procedures developed for embryo biopsy of mouse embryos and PCR amplification of Y-specific DNA sequence from a few isolated blastomeres could be successfully applied to sexing embryos in cattle.

V. SUMMARY

In sexing early mammalian embryos, viability of biopsied embryos and accuracy of sexing are both important. We have been previously developed efficient methods for biopsy of mouse embryos and sex identification from a single blastomere by PCR. In this study, squeeze method used for biopsy of mouse embryos was applied to bovine embryos. Compact bovine morulae were obtained by flushing uteri on Day 6 after the onset of standing estrus. A small number of blastomeres could be isolated from bovine morulae by the biopsy method. All 13 biopsied morulae were survived and 10 embryos developed to normal blastocyst after 24 h of culture. Subsequently, sex of the bovien embryos was identified from a few blastomeres by PCR amplifying a Y-specific bovine DNA sequence. Among 13 embryos analyzed, 7 embryos were determined as males and 6 embryos as females. Thus, bovine embryos at morular stage could be successfully biopsied by the squeeze method and sex of the bovine embryos determined from biopsied material by PCR.

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