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Predetermination of Sex in Bovine Preimplantation Embryos Produced *In vitro* using Micromanipulative Biopsy and PCR*

S. W. Seo, H. J. Lee, S. C. Choi, K. D. Kim and S. H. Lee

Gradute School of Biotechnology, Korea University, Seoul 136-701

미세조작 Biopsy와 PCR에 의한 착상전 소 초기배의 성 판정

서승운 · 이홍준 · 최승철 · 김기동 · 이상호

고려대학교 생명공학원

요 약

수정란이식의 주변기술인 초기배의 미세조작 및 성 판정은 가축의 경우, 경제 형질의 유전적 개량에 크게 기여하였다. 본 연구는 미세조작 biopsy와 PCR에 의한 체외생산 소 초기배의 급속·정확한 성 판정 기법을 확립하기 위해 실시하였다. 체외성숙 및 체외수정에 의해 생산된 소 수정란은 소 난관상피세포와 공배양을 통해 8-세포부터 배반포시기까지 체외발생시킨 후 미세조작 biopsy에 이용하였다. 미세조작 biopsy 과정중에 약간의 형태적인 손상이 관찰되었지만 대부분의 demi-embryo는 정상적인 배반포와 나화배반포로 발생하였다. 8~16 세포, 상실배, 초기배반포 시기에서 미세조작 biopsy 후 확장 또는 나화배반포시기까지의 발달율은 각각 62.8(27/43), 83.3(30/36) 및 80.9%(55/68)로 정상적인 초기배와 큰 차이가 없는 것으로 나타났다. 총 136개의 소 초기배로 부터 2~10개의 할구세포를 미세조작 biopsy에 의해 분리하여 소 특이와 Y-특이 염기서열을 가진 두 쌍의 염기서열을 이용하여 PCR을 수행하였다. 이들 중 112(82.4%)개를 성공적으로 성 판정하였으며, 암/수 비율은 각각 34.8%(39/112)/65.2%(73/112)로 나타났다. 본 실험에서 얻은 결과는 확립된 소 초기배의 미세조작기술과 성 판정 방법을 통해 계획적인 암·수 송아지의 선별 생산뿐만 아니라 소 수정란 이식을 통한 유전적 개량을 촉진시키는데 효과적으로 이용될 수 있는 기술이 확립된 것을 보여주었다.

(Key words : bovine embryo, biopsy, PCR, sexing)

INTRODUCTION

Embryo micromanipulation and sexing in embryo transfer programme have made a significant contribution to genetic improvement for economically useful characteristics in domestic animals. The recent development of the polymerase chain reaction(PCR) was significant in this field because this technique allows amplification of Y chromosome-specific repetitive sequences and thus determination of the sex of the embryo within 2 hours and with high reliability(Herr *et al.*, 1990). Embryo biopsy has been utilized in both animals(Wilton and Trounson, 1989; Cui *et al.*, 1993) and recently humans (Handyside *et al.*, 1989; Grifo *et al.*, 1992). Embryo biopsy has become increasingly popular in

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bovine embryo transfer for sexing and production-trait marker selection, and in human infertility clinics for diagnosis of genetic abnormalties. Here we describe a consistent and practical simple procedure for dissection of 8~16-cell to blastocyst bovine embryos using a fine microsurgical blade. In this experiment we tested the viability of the biopsied embryos and investigated whether sex can be determined by the 2~10-cells from cell mass by using IVF embryos of predetermination of sex in embryos can be achieved with combining all the other embryo-related techniques and the sexed embryos may be used for subsequent embryo transfer.

MATERIALS AND METHODS

1. Chemicals and enzymes

Taq DNA polymerase and deoxynucleotides (dNTPs) for PCR were purchased from Promega Co.(Madison, WI, USA). The chemicals for PCR buffer such as tris(hydroxymethyl) aminomethane, bovine serum albumin(BSA), $MgCl_2$, KCl and Triton X-100 were purchased from Sigma Chemical Co.(St. Louis, MO, USA). Tissue culture medium 199(TCM 199) and fetal bovine serum(FBS) for embryo culture were from Gibco Life Science Technologies Inc.(Grand Island, NY, USA) and other chemicals for culture from BDH Chemicals Co.(Poole, UK) or Sigma, respectively.

2. Primers for PCR

Two sets of primers were used in this experiment. Primers for bovine-specific amplification were 5'-TGGAAGCAAAGAACCCCGCT-3' and 5'-TCGTGAGAAACCGCACACTG-3' of bovine 1.715 satellite DNA(Pluciennicza*k et al.*, 1982). These primers were used as a positive control for cattle genome. Primers for Y chromosome-specific amplification were 5'-GGATCCGAGACACAGAACAGG-3' and 5'-GCTAATCCATCCATCCTATAG-3' of BRY.1(Reed *et al.*, 1988) or 5'-CTCAGCAAAGCACACCAG AC-3' and 5'-GAACTTTCAAGCAGCTGAGGC-3' of BRY4a (Reed *et al.*, 1989). The sizes of bovine- and Y chromosome-specific amplification products were 216 bp and 301 bp, respectively.

3. Optimization of PCR conditions

Genomic DNAs were used to test the precision, sensitivity, and stability of PCR amplification with the designed primers and also to define PCR conditions. PCR conditions, such as pH, concentration of components used for amplification buffer, annealing temperature and the number of cycle, were defined by the procedures of several references(Sambrook *et al.*, 1989; Peura *et al.*, 1991).

4. *In vitro* maturation(IVM)

Bovine ovaries were collected from a local slaughter house and immediately brought to the laboratory. Immature oocytes were recovered by aspiration of antral follicles(2~6mm in diameter) with a 10ml disposable sterile syringe attached with a 18 gauge needle. Oocytes-cumulus complexes(OCCs) were washed three times in Wash medium containing 3mg/ml BSA. For IVM, the OCCs were cultured in 2ml TCM 199 medium supplemented with 20%(v/v) FBS, 10 IU/ml PMSG and 10 IU/ml hCG for 22 for 24 h at 39℃ in an atmosphere of 5% CO_2 in humidified air.

5. *In vitro* fertilization and *in vitro* culture

Bovine embryos were produced by the method of Ng *et al.*(1992) with minor modifications. One 0.5ml straw of frozen Hanwoo(Korean native cattle) semen was thawed at 37℃ in a waterbath for 1 min. The motile spermatozoa were separated using 45 and 90%(v/v) Percoll gradient dil-

uted with fertilization medium without BSA by centrifugation at 2,000 rpm for 30 min. The sperm were subsequently washed in fertilization medium supplemented with 6mg/ml BSA, heparin, hypotaurin, epinephirine and penicillamin by centrifugation at 800 rpm for 10 min. Piror to insemination, the cumulus cells were removed from oocytes by treament of 300 IU/ml hyaluronidase and 3%(w/v) sodium citrate. Cumulus-free oocytes were then placed in 600$\mu\ell$ drops of fertilization medium overlayed with paraffin oil and followed by insemination with sperm at a concentration of 1.5×10^6 spermatozoa/ml. The oocytes and spermatozoa were then incubated for 18 h 39℃ under 5% CO_2 in air. Washed oocytes were then co-cultured with bovine oviductal epithelial cell(BOEC) monolayer in USU-6 medium supplemented with 15% FBS and developed to blastocyst.

6. Micromanipulative biopsy of bovine embryos

Eight-cell to blastocyst stage embryos were biopsied in microdrops of pH equilibrated Wash medium supplemented with 4mg/ml BSA under an inverted microscope equipped with an ultraprecise hydraupneumatic remote-controlled 3-dimensional micromanipulator. Each embryo was fixed with a flame-polished holding pipette and embryonic cells were removed by a single movement of the microblade through the zona pellucida. The zona pellucida was not sliced off, but was partly cut open. The bopsied cell mass(approximately 10%) of embryos was protruded through the slight opening of zona pellucida by further manipulation, and saved for PCR analysis. After the demi-embryos were transferred individually in a drop of USU-6+FBS containing BOEC, they were cultured *in vitro* under same culture conditions as unmanipulated embryos and development to hatched blastocyst stage was recorded.

7. PCR amplification and gel electrophoresis

The resulting demi-embryos were washed 3 times with PBS+BSA. Each demi-emryo was then individually transferred to a 0.5ml sterile Eppendorf tubes containing double distilled water using mouth-controlled micropipette. After repeated freezings(−196℃) and thawings(37℃), the genomic DNA of embryos was prepared by adding 200μg/ml proteinase K and incubating at 50℃ for 1 h, followed by inactivation at 95℃ for 10 min. The extracted DNA sample of each was transferred into different PCR tube for PCR amplifications. The PCR reactions were performed in a total volume of 40$\mu\ell$ containing 1×PCR buffer(10 mM Tris-HCl, pH 8.9, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1%, v/v, Triton X-100 and 0.01%, w/v, BSA), each 0.2 mM of dATP, dCTP, dGTP, dTTP, 2 U of *Taq* DNA polymerase, 20 pmol bovine specific primers and 80 pmol Y chromosome-specific primers. Each cycle consisted of denaturation at 94℃ for 50 sec, annealing at 59℃ for 50 sec followed by extension at 72℃ for 50 sec. This was repeated 40 times. Finally, samples were held at 72℃ for 10 min and cooled to 4℃. Amplification products were separated on a 2.5%(w/v) agarose gel, stained with ethidium bromide and visualized on a UV transilluminator.

RESULTS

1. *In vitro* development of demi-embryos

It is crucial for all techniques that micromanipulative biopsy must not adversely affect developmental potential of the embryos. Therefore, *in vitro* development of demi-embryos after micromanipulation was examined. The embryo biopsy procedures were undertaken at the eight-cell to blastocyst stages and presented in Fig. 1. Despite physical damages of some cells

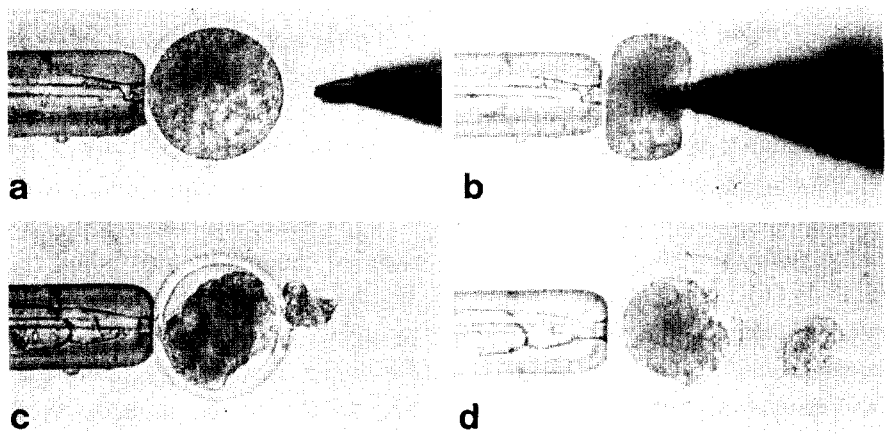

Fig. 1. A sequential procedure for the micromanipulative biopsy used in this study. An early blastocyst was fixed with a holding pipette(a) and a microblade was pushed through the zona pellucida to make a partial cut(b). The biopsied embryonic cell mass was consequently protruded from blastocyst embryo by further micromanipulation(c). After micromanipulation, a demi-embryo and biopsied embryonic cells were shown(d). Magnificatios is 300×.

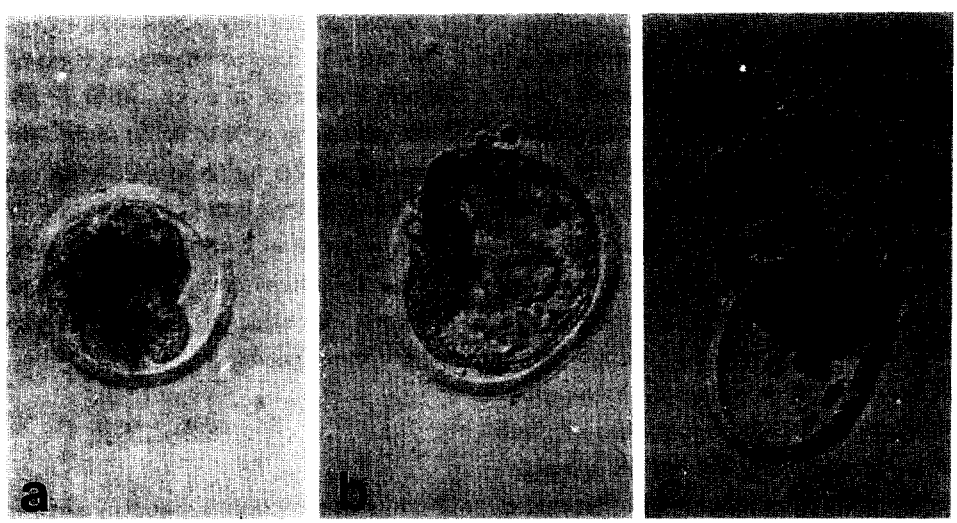

Fig. 2. A representative cavitation and hatching of a micromanipulated embryo during subsequent culture. The resulting demi-embryo biopsied at the morula stage(a) developed into the blastocyst after 24 h culture(b) and the hatched blastocyst after 48 h culture(c). Note that the partial cut on the zona pellucida can be traced in the developing embryo in(b) and(c) Magnification is 300×.

during the biopsy procedure, demi-embryos were able to develop to morphologically normal hatched blastocysts in subsequent culture for 24~72 h. As shown in Fig. 2, the demi-embryos showed a blastocoel, eventually hatched, and formed an outgrowth similar to that of normal embryos. These results indicate that biopsy used in this study did not alter the full developmental

potential of embryos *in vitro*. *In vitro* development of biopsied demi-embryos after micromanipulation was summarized in Table 1. Further developmental rates of embryos biopsied at 8~16-cell morula and blastocyst stages were 65.0 (13/20), 83.3(10/22) and 86.4%(19/22), respectively. Thus, the overall survival rate of biopsied embryos was 77.8%(42/52), indicating that it was not significantly different from that of unmanipulated embryos. The developmental rate of biopsied 8~16-cell stage embryos(65%) was lower in comparison with morula and blastocyst stage embryos(85%). The reduction in embryonic survival of 8~16-cell stage demi-embryos was mainly observed when relatively greater number of embryonic cells was removed during biopsy procedures. This may be due to the insufficient embryonic mass to achieve normal development and the damage of zona pellucida required to maintain the integrity of the cleaving embryos. To maintain acceptable viability of demi-embryos in relation with biopsy, it would be essential to remove as few cells as possible. Although washing procedures which minimize contamination might occasionally result in loss of biopsied cells, the requisite increase in the number of biopsied embryonic cells could be achieved, apparently without detrimental effect on the embryo, by performing biopsy at the blastocyst stage. This would allow sampling of greater number of cells in separate tubes, thus reducing potential errors. Improved results are achievable from biopsy of blastocyst stage embryos. Additional benefit is that although the zona pellucida is certainly useful during precompaction development *in vitro*, zona pellucida may not be an essential structural component of an embryo at the(late) blastocyst stage for the normal development following transfer. Therefore, it is a reliable practice to biopsy from embryos beyond morula stage whenever possible.

2. Sex predetermination of bovine demi-embryo after micromanipulation

The sex determination of preimplantation embryos should be accomplished with a minimal amount of embryonic DNA, such as that obtained in a biopsy of 2~10-cells, to ensure maximal embryo viability in applications requiring subsequent embryo transfer. It is very important to isolate a few number of embryonic cells to obtain biopsied embryos. As shown in Fig. 1, it was possible to obtain a small number of cells using a simple micromanipulative biopsy for sex determination. For the PCR-based sexing, biopsied embryonic DNA samples were prepared as for whole embryos. A few number of biopsied embryonic cells obtained were subjected to PCR. The results were summarized in Table 2. When 136 biopsied embryonic cells(containing 2~10 cells per embryo) were subjected to PCR, the sex of 112 biopsied embryonic cells(82.4%) was determined successfully. Amplification was

Table 1. Further development of demi-embryos following micromanipulation[1]

Embryonic stages	No. of the embryos used	No. of embryos at following stages(%)		
		Blastocyst	Degenerated embryo	Development rate(%)
8~16 cell	43	27	16	27(62.8)
Morula	36	30	6	30(83.3)
Blastocyst	68	55	22	55(80.9)
Total	147	112	44	112(76.2)

[1], Embryos were evaluated at 168 hours post-insemination(hpi)

Table 2. Predetermination of sex in biopsied embryonic cells by PCR after micromanipulation

Embryonic stages	No. of the embryos used	No. of embryos showing the following sexes(%)		
		Male	Female	Unable to determine
8~16 cell	42	8	23	11
Morula	36	11	17	8
Blastocyst	58	20	33	5
Total	136	39(28.7)	73(53.7)	24(23.5)

Fig. 3. Amplification of bovine- and Y chromosome-specific sequences in biopsied embryonic cells(2-10 cells). Sample showing only one amplified product(216 bp) were identified as female(lanes 5, and 8 to 10), while those showing two amplified products(216 and 301 bp) as male(lanes 2, 4, 6 and 7). Amplification was failed in lane 2, probably due to the loss of biopsied embryonic cells. Lanes 1 and 2 to 10: negative control without DNA and biopsied embryonic cells, respectively, and P: primers. Lanes M: DNA size marker.

failed in 24 cases(17.6%). Males and females were 34.8(39/112) and 65.2%(73/112) cases each. It is suggested that rapid sexing of biopsied embryonic cells using PCR could be useful for practical application to the transfer of sexed embryos. PCR co-amplification with two sets of bovine- and Y-chromosome-specific primers was employed to determine the sex of embryonic sample. Thus, the incidence of misclassification became minimal, especially in the case of amplification failure due to inavailability of embryonic DNA. Bovine-specific sequences were amplified in both male and female, whereas only Y chromosome-specific sequences were amplified in male. As expected, amplified products of bovine- and Y-specific sequences were 216 bp and 301 bp, respectively. As shown in Fig. 3, two amplified products were obtained in male samples(lanes 2, 4, 5, 8 and 9), whereas only one products in female(lanes 3, 6, 7 and 10). The presence of a 216 bp bovine-specific products in all lanes and the absence in the negative control(lane 1) indicated the success of the procedures; thus, confirming specificity of the Y-specific primers to male embryos.

DISCUSSION

There are various methods for removal of cells from preimplantation embryo, including zona drilling using acidified solutions(Handyside *et al.*, 1989; Gordon and Gang, 1990), zona thining (Muggleton-Harris and Findlay, 1991), aspiration using a fine micropipette(Wilton and Tro-

unson, 1989; Wilton *et al.*, 1989) and blastomere displacement(Roudebush *et al.*, 1990). Relatively greater number of cells biopsied from the embryo are used for sex determination in cows. Bondioli *et al.*(1989) separated 10~20% of the cell mass from uterine-stage embryos collected from superovulated cows and determined the sex of the embryos by DNA hybridization using the biopsied sample. Herr and Reed(1991) and Thibier and Nibart(1992) determined the sex of bovine embryos by PCR after cutting a few cells from morulae or blastocysts using microblade; others used bisected blastocysts for sexing by the PCR(Peura *et al.*, 1991). The applicatios of embryo transfer and related techniques such as *in vitro* fertilization, micromanipulative biopsy and sexing in domestic animals can be divided into two general directions : accelerated sex predetermination and improved exploitation of animals based on their natural characteristics, and genetic modification to produce animals with new characteristics. Recent progress in the micromanipulation of domestic animal embryos has been remarkable and is a key component of current procedures in embryo sexing, cloning and gene transfer. Embryo biopsy has been utilized in animals(Gardner and Edwards, 1968; Wilton and Trounson, 1989; Grifo *et al.*, 1990; Cui *et al.*, 1993) and recently in humans (Handyside *et al.*, 1989; Grifo *et al.*, 1992). Embryo biopsy has become increasingly popular in bovine embryo transfer for sexing and production-trait marker selection, and in human infertility clinics for diagnosis of genetic abnormalities.

The usefulness of embryo biopsy is realized when the number of embryos suitable for transfer is limited or when a simple system for production of genetically identical animals is needed. The demi-embryos produced from 8-cell to blastocyst stage embryos developed to expanded and hatched blastocysts at high survival rates (76.2%). Thus, demi-embryos can be obtained using simple biopsy techniques in this study. The PCR-based sexing used in this study was sensitive since most of biopsied embryonic cells could be sexed despite a smaller number of biopsy size(2~10-cells), indicating that biopsy followed by use of Y-specific probes and PCR for embryo sexing can be used routinely. These results also suggest that demi-embryos can be sexed with reliable efficiency and with embryo viability comparable to that of the survival of embryos which have not been biospied.

In conclusion, it is possible to predetermine sex of bovine preimplantation embryos using micromanipulative biopsy and PCR. It was also shown that method employed in this study is an efficient and accurate system. Results imply that genetic improvement for economically useful characteristics in domestic animals can be achieved by the further application to embryos transfer programme of *in vitro* fertilization, embryo manipulation and PCR-based sexing techniques used in this study.

SUMMARY

We exmined the *in vitro* development of demi-embryos following micromanipulative biopsy for sex determination. The biopsy procedures were undertaken at the eight-cell to blastocyst stages. The biopsied embryonic cells were subjected to the PCR-based sexing while demi-embryos were cultured further. Despite physical damages of some cells during the biopsy, the demi-embryos were able to develop to morphologically normal expanded or hatched blastocysts *in vitro*. The further developmental rates of embryos biopsied at 8~16 cell, morula and blastocyst stages were 62.8(27/43) 83.3(30/36) and 90.9%(55/68), respectively. The overall survival rate(77.8%) of demi-embryos was that of un-

manipulated embryos. For the sex predetermination, bovine and Y chromosome-specific sequences were amplified simultaneously. When 136 biopsied embryonic samples(2~10-cells) were subjected to PCR, The sex of 112 biopsied cells (82.4%) was determined successfully; males and females were 34.8(39/112), 65.2%(73/112) cases each. Amplification was failed in 24 cases. It is suggested that the PCR-based sexing method can be applied efficiently when coupled with embyo biopsy procedure; thus, facilitating genetic improvement in the bovine ET programme.

REFERENCES

Bondioli KR, Ellis SB, Pryor JH, Williams MW and Harpold MM. 1989. The use of male-specific chromosome DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology, 31:95-104.

Cui KH, Putland RA, Seamark RF and Matthews CD. 1993. Precise sex selected births of mice following single cell embryo biopsy and Y-linked testis-sepcific gene analysis. Hum. Reprod., 8:621-626.

Gardner RL and Edwards RG. 1968. Control of the sex rato at full term in the rabbit by transferring sexed blastocysts. Nature, 218: 346-348.

Gordon JW and Gang I. 1990. Use of zona drilling for safe and effective biopsy of murine oocytes and embryos. Biol. Reprod., 42: 869-876.

Grifo JA, Boyle A, Fischer E, Lavy G, DeCherney AH, Ward DC and Sanyal MK. 1990. Preembryo biopsy and analysis of blastomeres by *in situ* hybridization. Am. J. Obstet. Gynecol., 163:2013-2019.

Grifo JA, Boyle A, Tang YX and Ward DC. 1992. Preimplantation genetic diagnosis : *In vitro* hybridization as a tool for analysis, Arch. Pathol. Lab. Med., 116:393-397.

Handyside AH, Pattison JK, Penketh RJA, Delhanty JDA, Winston RML and Tuddenham EGD. 1989. Biopsy of human preimplantation embryos and sexing by DNA amplification. Lncet., 1:347-349.

Handyside AH, Pattison JK, Penketh RJA, Delhanty JDA, Winston, R. M. L. 1990. Pregnancies from biopsied human preimplantation embryos sexed by Y-specific DNA amplification. Nature., 344:768-770.

Herr CM, Holt NA, Matthaei KI and Reed KC. 1990a. Sex of progeny from bovine embryos sexed with a rapid Y-chromosomeo-detection assay. Theriogenology, 33:247(Abstr.).

Herr CM and Reed KC. 1991. Micromanipulation of bovine embryos for sex determination. Theriogenology, 35:45-54.

Muggleton-Harris AL and Findlay I. 1991. *In vitro* studies on 'spare' human preimplantation embryos in culture. Hum. Reprod., 6:85-92.

Ng SC, Bongso A, Chang SI, Sathananthan H and Rotnam S. 1989. Transfer of human sperm into the perivitelline space of human oocytes after zona-drilling of zonapuncture. Fertil. Steril., 52:73-78.

Peura T, Hyttinen JM, Turunen M and Janne J. 1991. A reliable sex determination assay for bovine preimplantation embryos using the polymerase chain reaction. Theriogenology, 35:547-555.

Reed KC, Matthews ME and Jones MA. 1988. Sex determination in ruminates using Y chromosome-specific polynucleotides. Patent Cooperation Treaty, NO. WO 88/01300.

Reed KC, Matthews ME and Jones MA. 1989. Determination of genetic sex in ruminants using Y chromosome-specific polynucleotides. Patent Cooperation Treaty, NO. WO 89/07154.

Roudebush WE, Kim JG, Minhas BS and Dodson MG. 1990. Survival and cell acquistion rates after preimplantation embryo biopsy : use of two mechanicaaaal techniques and two mouse strains. American J. Obstet. Gynec, 162:1084-1090.

Sambrook J, Fritsch EF and Maniatis T. 1989. Molecular cloning : A laboratory manual, 2nd eds. Cold Spring Harbor Laboratory Press. USA.

Thibier M and Nibart M. 1992. Bovine embryo sexing by a DNA probe on the field. Reprod. Domestic Anim., 27:29-33.

Wilton LJ and Trouson AO. 1989. Biopsy of preimplantation mouse embryo : development of micromanipulated embryos and proliferation of single blastomeres *in vitro*. Biol. Reprod., 40:145-152.

Wilton LJ, Shaw JM and Trouson AO. 1989. Successful single-cell biopsy and cryopreservation of preimplantation mouse embryos. Fertil. Steril., 51:513-517.

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