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Comparative study of PCR-sexing procedures using bovine embryos fertilized with sex-sorted spermatozoa

B. Trigal[1,*], E. Gómez[1], C. Díez[1], J. N. Caamaño[1], M. Muñoz[1], J. F. Moreno[2], S. Carrocera[1], D. Martín[1], F. Goyache[1] and I. Álvarez[1]

[1] *Área de Genética y Reproducción Animal. Centro de Biotecnología Animal-SERIDA. Camino de Rioseco 1225, La Olla-Deva. 33394 Gijón (Asturias). Spain*
[2] *Sexing Technologies, Navasota, TX, USA*

Abstract

Sex determination in bovine embryos is a useful tool in reproductive biotechnology. This work compares two techniques of embryo sexing by polymerase chain reaction (PCR). Embryos were produced *in vitro* with sex-sorted sperm and two techniques of DNA lysis were tested (proteinase K *versus* heat shock). Subsequently, halves of each lysed sample was amplified both by amelogenin and BRY4a/SAT1 primers. Male embryos treated by both digestion methods and amplified by BRY4a/SAT1 gave higher rates of false negatives. Amelogenin amplification failed with embryos previously digested by proteinase K. In contrast, the lysis method allowed obtaining the best accuracy in terms of sex verification when using amelogenin amplification.

Additional key words: amelogenin; blastocyst; BRY4a/SAT1; sex technique.

Resumen

Estudio comparativo de dos métodos de sexaje por PCR en embriones bovinos producidos con semen sexado

La determinación del sexo en embriones bovinos es una herramienta útil en las biotecnologías reproductivas. Este trabajo compara dos técnicas de determinación del sexo en embriones mediante la reacción en cadena de la polimerasa (PCR). Los embriones fueron producidos *in vitro* con semen sexado y se analizaron dos técnicas de lisis de ADN (proteinasa K y choque térmico). Posteriormente, la mitad de cada muestra lisada fue amplificada por la amelogenina y por los cebadores BRY4a/SAT1. Los embriones machos tratados por ambos métodos de digestión y amplificados por BRY4a/SAT1 dieron mayores tasas de falsos negativos. La amplificación de la amelogenina no dio resultados en embriones previamente digeridos por proteinasa K; sin embargo, el método de lisis por choque térmico junto con la amplificación por amelogenina permitió obtener la mayor precisión en términos de verificación de sexo.

Palabras clave adicionales: amelogenina; blastocistos; BRY4a/SAT1; técnica de sexaje.

Introduction

Although different laboratories may have tried several sexing protocols, there is a general lack of studies comparing different methods. Primers design within bovine embryo sexing by polymerase chain reaction (PCR) is often based on male specific sequences that can be used alone (Bredbacka *et al*., 1995; Lu *et al*., 2007) or combined with somatic sequences (male-female). Combinations include independent primer, which consist of either two pairs of gene primer sequences (Pomp *et al*., 1995; McClive & Sinclair, 2001; Kageyama *et al*., 2004; Nedambale *et al*., 2004; Almodin *et al*., 2005; Bermejo-Alvarez *et al*., 2008, 2011; Diez *et al*., 2009;

*Corresponding author: beatriztr@serida.org
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Abbreviations used: BSA (bovine serum albumin); CCO (cumulus-oocyte complexes); ET (embryo transfer); FCS (fetal calf serum); FERT-TALP (fertilization medium-tyrode albumin lactate piruvate); FSH (follicle stimulating hormone); IVF (*in vitro* fertilization); LH (luteinizing hormone); PBS (phosphate buffer saline); PCR (polymerase chain reaction); PVA (polyvinyl alcohol); SOF (synthetic oviduct fluid); TCM199 (tissue culture medium 199).

Alves *et al*., 2010; Martinhago *et al*., 2010; Rattanasuk *et al*., 2011), or multiple primer sequences that lead to a loop-mediated isothermal amplification (Agung *et al*., 2006; Hirayama *et al*., 2004; Zoheir & Allam, 2009). Also, the so called built-in control techniques consist of a single pair of primers that amplify a Y-chromosome specific sequence and a second byproduct. In bovine embryo sexing, only two techniques with a single pair of primers have been reported performing double amplification: amplification of a male-specific 178 base pairs (bp) fragment and an unidentified 145 bp by product common to male and female, which acts as an internal control (Hirayama *et al*., 2004; Kageyama *et al*., 2004), and amelogenin amplification (Levinson *et al*., 1992; Ennis & Gallagher, 1994). Amelogenin, as any other built-in control techniques, is theoretically advantageous over multiple primers and sequences amplification, as the use of the same primers to male and female sequence eliminates misdiagnosis errors associated to specific primer amplification efficiency (Levinson *et al*., 1992; Ennis & Gallagher, 1994; Pajares *et al*., 2007; Colley *et al*., 2008).

However, there could be a need to compare two PCR systems for accuracy purposes. All digestion methods published in the amplification of the Y chromosome and somatic sequence were performed by proteinase K (McClive & Sinclair, 2001; Bermejo-Alvarez *et al*., 2008; Diez *et al*., 2009). In contrast, embryos subjected to amelogenin amplification were lysed by thermal shock (Ennis & Gallagher, 1994; Kageyama *et al*., 2004).

The objective of the present work was to compare the efficiency of two PCR procedures. First, the best lysis treatment (proteinase K *vs*. heat shock) for DNA of bovine embryos produced with sex-sorted sperm was determined. Thereafter the accuracy of amplification with amelogenin or two sets of PCR primers (Y-chromosome-specific primers [BRY4a], and bovine-specific satellite sequence primers [SAT1]) was also analyzed. Sex-sorted sperm (male or female) usually allows getting embryos of the desired sex at rates of 90% or higher (Garner & Seidel, 2008). Such embryos might be used as a simple and rapid method of phenotypic validation and comparison of efficiency between PCR methods.

Material and methods

All reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise stated.

In vitro embryo production

Embryos were produced *in vitro* from oocytes recovered from slaughterhouse ovaries and previously reported (Gomez *et al*., 2008). Briefly, cumulus-oocyte complexes (COCs) were matured in TCM199, $NaHCO_3$ (2.2 g L^{-1}), FCS (10% v/v), porcine FSH-LH (Stimufol, Rhone-Mérieux, France) and 17β-estradiol (1 μg mL^{-1}). *In vitro* fertilization (IVF) was performed with frozen/thawed male and female sex-sorted sperm from a single bull (donated by Sexing Technologies, Navasota, TX, USA). Spermatozoa were separated using Percoll gradients and fertilized in droplets of 20 μL with FERT-TALP medium under mineral oil (Trigal *et al*., 2010). Presumptive zygotes were cultured in SOF droplets with 6 g L^{-1} BSA at 38.7ºC, 5% CO_2, 5% O_2 and 90% N_2. Embryos were cultured up to Day-7 or Day-8 post IVF. The source of embryos for sexing was blastocysts.

Embryo sexing by PCR

After embryo incubation with 5 mg mL^{-1} pronase in PBS for 2 min to remove zona pellucida and any attached spermatozoa, embryos were individually washed 3-4 times in PBS + 0.1 mg mL^{-1} PVA. Washed blastocysts were collected in 0.3 μL of medium into 0.2 mL PCR tube strips (BIO-RAD) and individually snap-frozen in liquid nitrogen in Eppendorf tubes and stored at –20°C until analysis. Samples were thawed at room temperature and centrifuged at 8,000 g for 1 min prior to being mixed with PCR reagents.

According to the experimental design, embryonic DNA was lysed by two methods: (1) overnight digestion with 8 μL of a 100 μg mL^{-1} proteinase K solution at 55ºC and subsequently heated at 95ºC for 10 min for enzyme inactivation (Diez *et al*., 2009); (2) a procedure modified from Hirayama *et al*. (2004), consisting of a heat shock 95ºC for 10 min in 6 μL deionized water replacing the original 10 mM Tris HCl.

Embryonic sex was analyzed by PCR either with amelogenin primers or two sets of primers: Y- chromosome specific primer (BRY4a) and bovine specific satellite sequence primer (SAT1) (Diez *et al*., 2009). Bovine amelogenin *c*DNA sequences were obtained by GenBank accession no: NM_001014984 (AMELX) and NM_174240 (AMELY). The PCR reactions were carried out with a total volume of 20 μL containing 8 μL of proteinase K digested sample or 6 μL of heated water. The reaction mixture were formed by 2x QIAGEN Multiplex

master mix (containing HotStar Taq DNA Polymerase, Multiplex PCR Buffer, dNTP mix), Q-solution 5x, 0.65 µM BRY4a forward (5'-CTCAGCAAAGCACACCAGAC) reverse (5'-GAACTTTCAAGCAGCTGAGGC) (Manna *et al*., 2003); 0.15 µM SAT1 forward (5'-TGGAAGCAAAGAACCCCGCT) reverse (5'-TCGTGAGAAACCGCACACTG); or 3.0 µM amelogenin forward (5'-CAGCCAAACCTCCCTCTGC) reverse (5'-CCCGCTTGGTCTTGTCTGTTGC). Both PCR rounds with each set of primers were performed in a Bio-Rad thermocycler with one cycle of 95 Cº for 15 min, 35 cycles of 94ºC for 20 sec, annealing temperatures of 60ºC (aMEL) and 67.5ºC (BRY4a/SAT1) for 40 sec, and 72ºC for 20 sec, and a final extension step of 72ºC for 10 min. Products were visualized on a SYBR® Safe stained 2% agarose gel. The gel was visualized under ultraviolet illumination for the positive 300 bp band of BRY4a and 216 bp of the satellite sequence. Samples which exhibited two bands were assigned as male, while samples exhibiting only a satellite sequence band were assigned as female. For amelogenin, single fragments of 270 bp were assigned as female, while two fragments of 270 bp and 214 bp were considered as male. Every PCR reaction was carried out with three controls: male (unsorted sperm) and female (ovarian tissue) genomic DNA, and a negative control with deionized water. Control DNA was extracted with proteinase K and the phenol/chloroform method (Strauss, 2001). DNA concentration was measured in genomic DNA and embryo samples with a spectrophotometer (NanoDrop ND1000-Technologies-Inc, Wilmington, DE, USA).

Experimental design

— Experiment 1: Embryos were lysed either with proteinase K solution or heated at 95ºC in deionized water for 5 min to lyse DNA samples. Subsequently embryos were processed for sexing with amelogenin primers or BRY4a-SAT1 primers in a 2×2 factorial design.

— Experiment 2: According to the results of Experiment 1, DNA lysis was performed in embryo samples by heating at 95ºC in deionized water for 5 min. Subsequently, each treated sample was vortexed and split in halves of 3 µL each, which received an equal volume of PCR mixture including the corresponding primers. This way, comparisons could be limited to the subjected amplification and gel-identification steps, while omitting manipulation of embryos, which could have introduced undesired variability. Thereafter, embryos were processed for sexing with amelogenin primers or BRY4a-SAT1 primers. Sex -male or female- diagnosis by PCR was estimated to be correct when: (1) it was in agreement with the reference sex (*i.e.,* the sex-sorted sperm origin of the embryo); and (2) it was different from the reference sex, but both amelogenin and BRY4a-SAT1 resulted in the same sex. Other situations were neither considered nor observed (see Results section and Tables 1 and 2). A flow chart of experiments 1 and 2 is shown in Figure 1.

Statistical analysis

Performance of sexing methodologies was statistically assessed via a χ^2-test using the Proc Freq of SAS/STAT® v8.2 (SAS Institute Inc., 1999, Cary NC). Sexing success was considered "1" when the expected and the actual sex of the embryo were consistent. Otherwise, sexing success was coded as "0". In Experiment 1, the χ^2-test used the DNA lysis protocol and the primers as sources of variation. In Experiment 2, sources of variation were the primers used and embryo type (male-female). Efficiency of embryo sexing data was

Table 1. Efficiency of proteinase K and water-heating as treatments for DNA lysis of bovine embryos produced with sex-sorted sperm, subjected to PCR using amelogenin or BRY4a/SAT1 amplification

Amplification	DNA-lysis	N[1]	Sexed	Male	Female
Amelogenin	Proteinase K	16	0% (0)	0% (0)	0% (0)
Amelogenin	Water-heating	16	100% (16)	43.7% (7)	56.2% (9)
BRY4a/SAT1	Proteinase K	16	62.5% (10)	25% (4)	37.5% (6)
BRY4a/SAT1	Water-heating	16	93.7% (15)	43.7 % (7)	50% (8)

χ^2-value = 30.6145; $p < 0.0001$. [1]N: Processed samples. Embryos were taken from three IVF and PCR replicates.

Table 2. Efficiency of the BRY4a-SAT1 and amelogenin primers for sexing bovine embryos *in vitro* produced with male- or female-sorted spermatozoa

Sex	N_1	N_2	PCR technique	Diagnosed sex (PCR)	
				Expected	Dissenting
Male IVF	88	86			
			BRY4a-SAT1	86% (74) males	14% (12) females [(1)]
			Amelogenin	97.6% (84) males	2.4% (2) females [(1)]
Female IVF	91	90			
			BRY4a-SAT1	96.6% (87) females	3.3% (3) males [(2)]
			Amelogenin	96.6% (87) females	3.3% (3) males [(2)]

χ^2-value = 33.5851; $p < 0.0001$. N_1: Blastocysts processed for PCR. N_2: Blastocyst successfully sexed. [(1)]: Dissenting females diagnosed by amelogenin (n = 2) were confirmed among those detected by BRY4a-SAT1 (n=12). [(2)]: Dissenting males diagnosed by BRY4a-SAT1 and amelogenin were the same embryos (n = 3). Embryos were taken from 12 replicates and sexed in 10 PCR replicates.

transformed to frequency percentages. Preliminary analysis included the assessment of the effect of both blastocyst age (*i.e.,* Day-7 and Day-8) and replicate. However, no statistical significance was assessed for these effects and therefore, they were excluded from subsequent analysis.

Results

Experiment 1: A total of 64 embryos were used from three IVF replicates, out of which n = 32 embryos were processed for DNA extraction by proteinase K and n = 32 embryos were subjected to heat shock in a cross amplification with amelogenin and BRY4a/SAT1 primers in three PCR replicates. As seen in Table 1, amelogenin amplification did not work with embryos lysed by proteinase K, but all samples diluted in water and heated were successfully amplified. In contrast, BRY4a/SAT1 amplification led to different proportions of amplified samples, and no differences were observed for PCR sexing when DNA was extracted using the heated water method.

Experiment 2: A total of 179 embryos from 12 IVF replicates were processed for sexing, out of which 176 could be correctly sexed in a total of 10 replicates (Table 2). Accuracy of sexing with blastocysts produced by sorted sperm was 96.6% in females and 97.6% in males. Dissenting diagnosis was recorded in males, where BRY4a/SAT1 failed to identify 10 male embryos out of 87 male embryos sexed, leading to the appearance of a proportion of false positive female embryos. However, amelogenin amplification was 100% effective within all sexed samples.

A χ^2-test confirmed statistically the better performance of the amelogenin-based sexing protocol ($p < 0.0001$; d.f. = 3). This better performance was due to the high false negative rates (Type II error), which took place when embryos expected to be males were sexed using BRY4a/SAT1. Figure 2 shows a representative image of gel electrophoresis containing PCR products.

Discussion

Embryonic lysis with proteinase K preceding amplification led to failure with amelogenin primers, while BRY4a/SAT1 primers were in part effective. However, in the absence of proteinase K (*i.e.* heat shock), all amelogenin samples (n=16) and 15 out of 16 BRY4a/SAT1 treated samples could be amplified. Therefore, optimization of DNA digestion was carried out by heat shock in Experiment 2. Several studies have reported induced lysis by heat shock with amelogenin, although to our knowledge none of them has compared heat shock against other DNA extraction procedures (Ennis & Gallagher, 1994; Kageyama *et al*., 2004).

The amelogenin gene amplification is advantageous for sex-testing in adult cells as the reaction contains a built-in reaction control which detects PCR failure (Eastoe, 1965). On the other hand, amelogenin amplifies a single copy gene, and therefore the amounts of available DNA represent a theoretical limitation. Multicopy sequence methods have been used to sex very low amounts of DNA, such as 2-cell embryos (Dominko & First, 1997; Bermejo-Alvarez *et al*.,

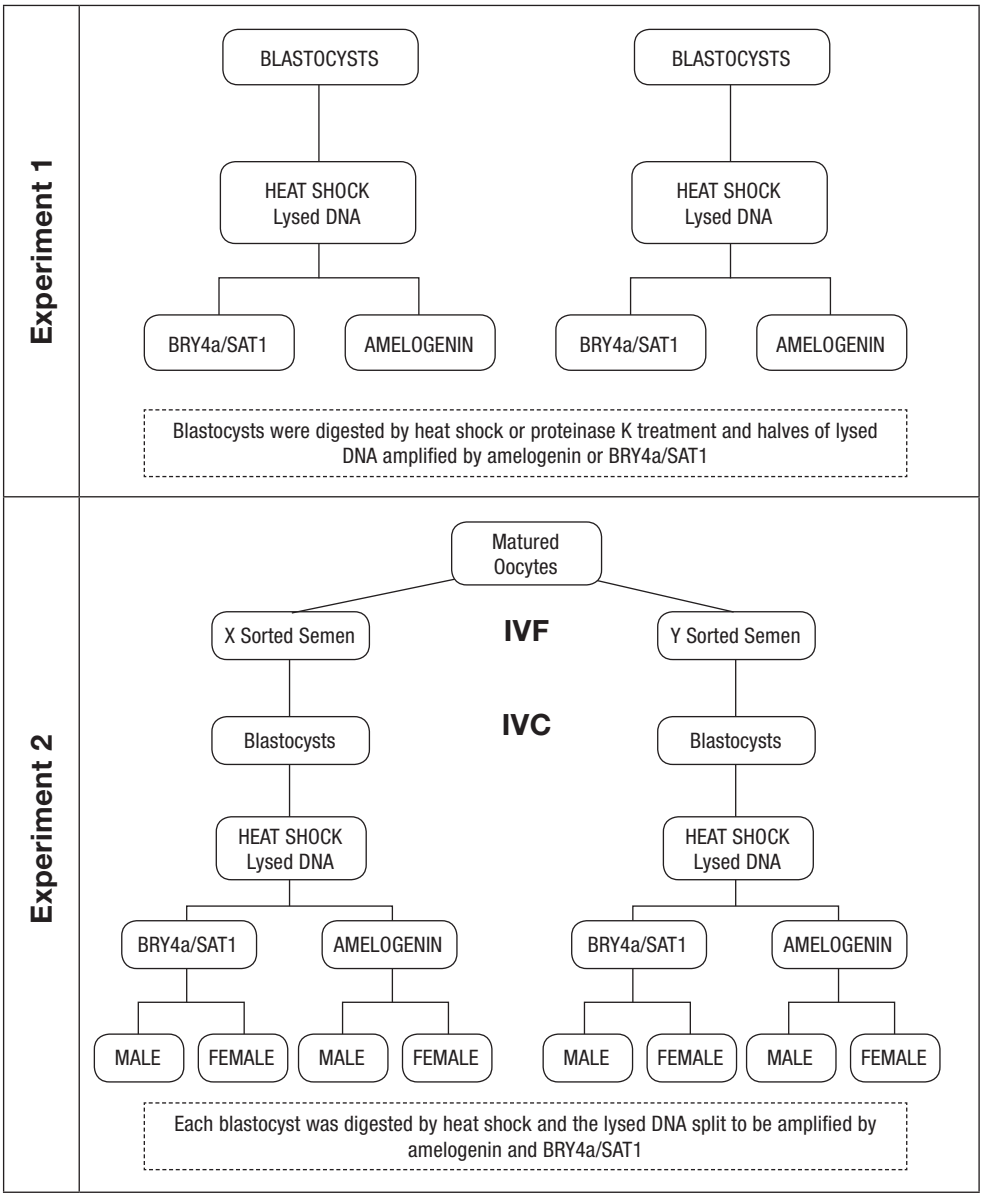

Figure 1. A schematic diagram showing the flow of the experiments.

2008), or very small embryo biopsies (Kageyama *et al*., 2004). In contrast, it has been reported that the amelogenin method requires DNA preamplification or more than 4 cell stage embryos (Ennis & Gallagher, 1994) or at least 40 pg DNA from somatic cells (Weikard *et al*., 2006). Our controls with digested ovarian tissue (female) and unsorted sperm (male) contained 25 ng DNA, a concentration found to be lower in our blastocysts (*i.e.:* 2 ng/blastocyst). Prior to testing embryos, sensitivity tests were performed by dilution of genomic DNA from our controls; the dilutions used contained 20, 2 and 0.2 ng respectively. Such low DNA amounts did not preclude to obtain constant and successful sex validation by PCR in controls when heat shock followed by amelogenin amplification was used. Amelogenin amplification does not require an external control gene, but its actual efficiency needs to be verified both *vs.* conventional PCR sexing procedures into which each pair of primers amplifies a single, specific sequence and, ultimately, contrasted with an appropriate phenotype validation. An answer to this question requests a phenotype, which when dealing with early embryos is normally not available unless biopsy and embryo transfer (ET) are made. Biopsy is time consum-

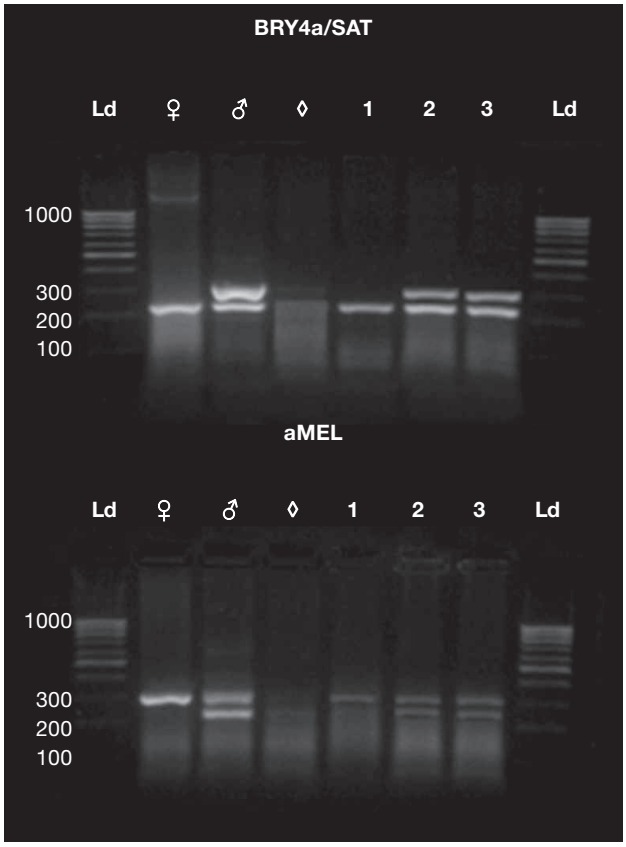

Figure 2. Gel electrophoresis representative of polymerase chain reaction (PCR) sexing reactions. Top samples were processed by BRY4a/SAT1 into which the BRY4a band amplifies to 300 bp, while the satellite sequence (SAT1) amplifies to 216 bp. Bottom samples were processed by amelogenin (aMEL), which shows a 270 bp band for females and two (270 bp and 214 bp) bands for males. From left to right: female control (♀); male control (♂); blank (◊); embryo 1 (female); embryo 2 (male); embryo 3 (male). Ld: 100 bp ladder.

ing, and ET is very expensive as to be used as a contrasting phenotype (Trigal *et al*., 2010). Therefore, we propose the use of embryos made with sexed semen as a probabilistic phenotype, provided that absolute accuracy is not necessary (*i.e.* testing the percent of coincidence between the predicted sex —sperm— and the analyzed sex —PCR—) when a discrepancy between both PCR systems occurs. Such analysis cannot obviously be made with unsorted spermatozoa, as sex rates around 50% would not allow to assign the correct sex to a system in case of discrepancy (Garner & Seidel, 2008).

The observed divergent results between amelogenin and BRY4a/SAT1 were always resolved in favour of amelogenin, as endorsed by the reference embryonic sex (*i.e.* the sorted sperm batch). Sperm sex sorting does not involve full separation of spermatozoa according to their sex, but it is currently the best choice to obtain progeny of the desired sex according to its high rates of accurately sorted spermatozoa, usually larger than 90% (Garner & Seidel, 2008) but it is impossible to regulate the sex ratio of *in vitro* fertilized embryos completely.

In our experimental model, it was not necessary to confirm the proportions of spermatozoa bearing the correct sex in straws, and rates of blastocyst sexed were highly consistent with the reference sperm sex.

Contrary to DNA from adult cells, amplification of DNA from blastocysts by the double pair of primers BRY4a-SAT1 under the conditions described herein, the technique led to errors in the diagnosed sex, despite the use of equal or lower DNA amounts in adult DNA controls than within embryos. Therefore, using built-in control techniques (*i.e.* amelogenin) for embryo sexing is strongly recommended when an accurate embryonic sex diagnosis is necessary. As an example, development of techniques to predict sex or viability in single cultured embryos, based on metabolomics and proteomics analysis of embryo culture medium (Nagy *et al*., 2008), requires an accurate identification of embryonic sex, as cumulative errors (*i.e.* diagnosis of diagnosis) might significantly distort the results.

In conclusion, amelogenin PCR was successful after heating and not proteinase K treatment, while embryos produced with sex-sorted sperm provide embryo phenotyping to discriminate between PCR sexing systems.

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