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Development and optimization of a fluorescent differential display PCR system for studying bovine embryo development *in vitro*

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ABSTRACT. Differential display is a widely used methodology to identify genes that are differentially expressed in biological samples. We developed a new protocol for the amplification and recovery of differentially expressed genes from extremely small initial amounts of RNA (10 to 25 pg mRNA) from preimplantation bovine embryos. The cDNAs generated with an anchor primer, associated with a universal sequence, were amplified with an arbitrary primer and a single fluorescently labeled primer. Amplification products were easily visualized with a fluorescence scanner, without the need for radioisotopes. Nineteen isolated fragments were cloned and sequenced, confirming the expected primer sequences and allowing the recognition and identification of gene transcripts involved in bovine embryonic physiology.

Key words: Differential display PCR, Fluorescence, Universal primer, Bovine embryo

INTRODUCTION

Several techniques have been developed for identifying genes differentially expressed among biological samples, including suppressive subtractive hybridization (SSH, Robert et al., 2001), differential display polymerase chain reaction (ddPCR, Liang and Pardee, 1992), serial analysis of gene expression (SAGE, Velculescu et al., 1995), and microrrays (Schena et al., 1995). Such techniques require relatively large initial amounts of RNA, except for ddPCR, which is widely used for identification of new or rare transcripts when RNA samples are limited, such as in preimplantation embryos (Natale et al., 2001; Kanka et al., 2003; Li et al., 2003; Tesfaye et al., 2003).

The original ddPCR method consists of cDNA production from subsets of mRNA that have been reversely transcribed with different primers anchored to the polyadenylated tail, followed by amplification of cDNAs with arbitrary primers, with incorporation of a radioactive label and electrophoresis in polyacrylamide sequencing gels. Several modifications of the original ddPCR protocols have been reported, in attempts to minimize false-positive signals (Luce and Burrows, 1998; Miele et al., 1998; Nagel et al., 1999), increase the efficiency with different primer designs (Verca et al., 1998; Wang et al., 1998; Zhao et al., 1998) and eliminate the need for radioactive markers (Ito et al., 1994; Luehrsen et al., 1997; Cho et al., 2001; Stein and Liang, 2002).

Fluorescence-based ddPCR methods, which use labeled primers or direct incorporation of labeled dNTPs, have been widely used and have replaced radioactive detection in many procedures (Ito et al., 1994; Doss, 1996; Reinhardt et al., 1999; Cho et al., 2001). Smith et al. (1997) described a method that uses a universal fluorescently labeled primer that shares a region of the cDNA sequence incorporated during the reverse transcription-PCR reaction. A DNA sequencer was used to analyze the amplified products, but recovery of fragments required repeating the entire process with radioisotopes.

We developed an alternative protocol for ddPCR that allows the amplification and recovery of differentially expressed cDNA fragments from extremely small initial amounts of RNA (10 to 25 pg mRNA) from groups of early cleavage stage bovine embryos, using a single fluorescently labeled universal primer, without radioactivity steps.

MATERIAL AND METHODS

In vitro production of bovine embryos

Bovine embryos were produced by *in vitro* oocyte maturation, fertilization and culture, according to previously published protocols (Bousquet et al., 1999). Three different groups of embryos were used: 4-cell (S4) and 8-cell stages (F8) at 48 h post-insemination (hpi), and the 8-cell stage at 90 hpi. Embryos were recovered from culture, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction.

RNA extraction and reverse transcription

Total RNA was extracted from groups of 50 embryos of each category (F8, S4 and S8) with the Rneasy® Protect Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's

instructions and eluted in 30 µL RNase-free water. Nine microliters of total RNA from each embryo group was reversely transcribed in 20-µL reactions containing 1 µL *SuperScript*III™ (200 U/µL; Invitrogen™, Carlsbad, CA, USA), 1 µL of an anchor primer (5 µM) 5´- AATACGA CTCACTATAGT$(T)_{12}$NN- 3´ (NN = GC, GA or GG; Table 1), 2 µL dNTPs (2.5 mM each), 2 µL DTT (0.1 M), 1 µL RNaseOUT® Recombinant Ribonuclease Inhibitor (40 U/µL; Invitrogen™), and 4 µL 5X first strand buffer. Reactions were carried out at 42°C for 15 min, 50°C for 50 min and 70°C for 15 min, for enzyme inactivation.

Table 1. Primers used in the modified ddPCR method.

Primer name	Sequence 5' → 3'	Primer name	Sequence 5' → 3'
Universal T7	AATACGACTCACTATAGT	ARP9	TAAGACTAGC
AP1	AATACGACTCACTATAGT$_{(12)}$GA	ARP10	GATCTCAGAC
AP2	AATACGACTCACTATAGT$_{(12)}$GC	ARP11	ACGCTAGTGT
AP3	AATACGACTCACTATAGT$_{(12)}$GG	ARP12	GGTACTAAGG
ARP1	CGACTCCAAG	ARP13	GTTGCACCAT
ARP2	GCTAGCATGG	ARP14	TCCATGACTC
ARP3	GACCATTGCA	ARP15	CTTTCTACCC
ARP4	GCTAGCAGAC	ARP16	TCGGTCATAG
ARP5	ATGGTAGTCT	ARP17	CTGCTAGGTA
ARP6	TACAACGAGG	ARP18	TGATGCTACC
ARP7	TGGATTGGTC	ARP19	TTTTGGCTCC
ARP8	TGGTAAAGGG	ARP20	TCGATACAGG

AP, anchor primer; ARP, arbitrary primer.

Fluorescent differential display

The cDNA samples from each embryo group, generated with one of the three different anchor primers, were used in ddPCR amplifications with one of the 20 arbitrary (decamers) and a TAMRA-labeled T7 primer (Table 1). PCR amplification conditions were optimized by testing several reaction parameters. The quantity of cDNA to be used was evaluated using 1, 1.5 and 2 embryos in each reaction (Figure 1A), which is equivalent to 10, 15 and 20 pg of starting mRNA, respectively, according to previously published estimates (Pikó and Clegg, 1982; Zimmermann and Schultz, 1994). Three different $MgCl_2$ concentrations (2, 3, and 4 mM; Figure 1B) and variations in $MgCl_2$ concentration within cycling protocol were also tested (Figure 1C). Optimal results were achieved with the following procedures: a first amplification step was carried out in 10-µL reactions containing 1.33 µL of the RT reaction products (equivalent to ~15 pg of starting mRNA, 1 µL 10X *Taq* polymerase buffer, 2 µL dNTPs (2.5 mM each), 1.33 µL TAMRA-labeled T7 (5 µM), 1.33 µL random primer (5 µM), 0.1 µL *Taq* DNA polymerase (5 U/µL; Invitrogen™), and 0.2 µL $MgCl_2$ (50 mM). PCR cycling conditions were: 95ºC for 2 min, followed by 10 cycles at 92°C for 15 s, 42°C for 30 s and 72°C for 2 min, with a final step of 72°C for 10 min. Subsequently, 10 µL of a second PCR mix containing 1.8 µL $MgCl_2$ (50 mM), and the same concentrations of the other components, were added to each tube, followed by another round of amplification of 25 cycles at 92°C for 15 s, 42°C for 30 s and 72°C for 2 min, with a final step of 72°C for 10 min.

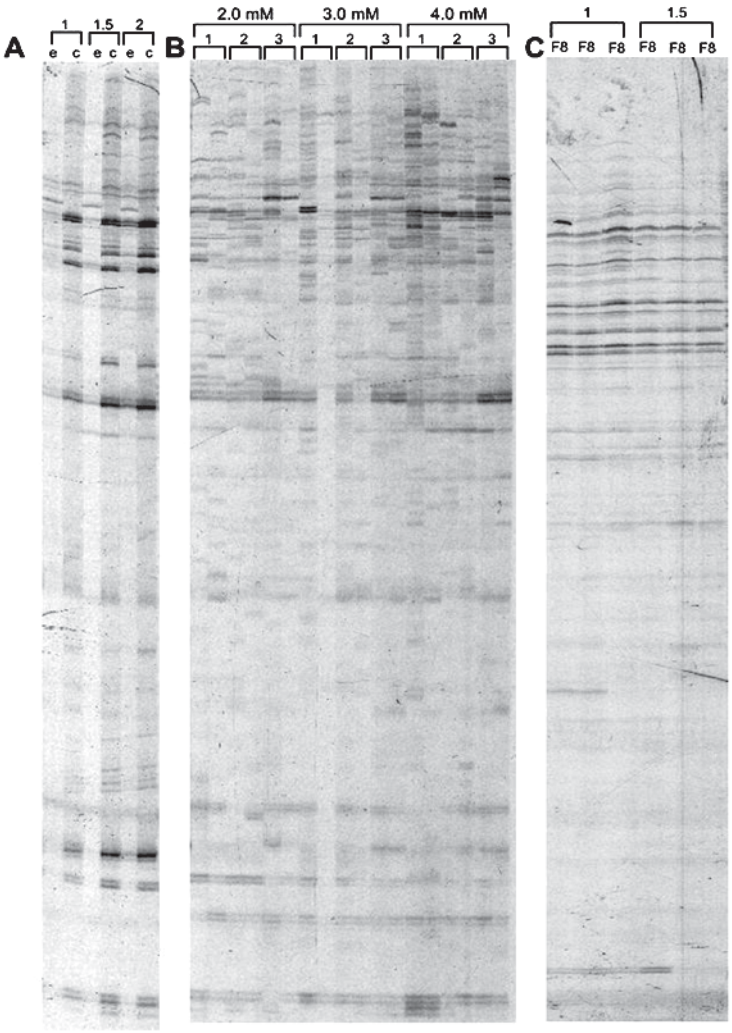

Figure 1. Differential display PCR optimization. **A.** Effect of cDNA quantity in the amplification reactions: e = 1, 1.5 and 2 embryos and c = 200 ng of total RNA from granulosa cells. **B.** Effect of $MgCl_2$ concentration on the amplification reaction: 1, 2, 3 = different groups of F8 embryos amplified in duplicate. **C.** Two rounds of amplification and $MgCl_2$ variation performed in triplicate using 1 and 1.5 embryos per reaction.

A total of 8 µL of each amplification reaction was denatured at 95ºC for 2 min with 1.5 µL of loading buffer (20 mM EDTA, 0.05% xylene cyanol, 95% formamide), chilled on ice; 6 µL was separated in 6.5% denaturing polyacrylamide gels in 1X TBE buffer for 5 h at 1,500 V. After electrophoresis, the gels were scanned with a Fuji FLA3000 fluorescence scanner and fragments were visualized with Image Reader FLA-3000 Series (v.1.11) and Image Gauge (v.3.12) software (Fuji Photo Film Co.). Following the removal of the upper glass plate, the gel was dried at 80ºC and rinsed with double-distilled H_2O repeatedly to completely remove urea residues. Fragments differentially amplified in all triplicates in at least one of the embryo groups were precisely excised from the dried polyacrylamide gel with a scalpel, eluted in 80 µL ultra-pure water at 80ºC for 10 min and stored at -80ºC until analysis.

Cloning and sequencing of different amplified fragments

Each excised fragment was re-amplified with a non-labeled T7 primer and the corresponding arbitrary primer. Re-amplifications were carried out in 25 µL-reaction mixes, containing 0.5 µL of a solution containing the eluted ddPCR fragment, 2.5 µL 10X *Taq* polymerase buffer, 4 µL dNTPs (2.5 mM each), 3.25 µL non-labeled T7 (5 µM), 3.25 µL arbitrary primer (5 µM), 0.2 µL *Taq* DNA polymerase (5 U/µL; Invitrogen™), and 1.5 µL $MgCl_2$ (50 mM). PCR cycling conditions were: 95°C for 2 min, followed by 30 cycles of 92°C for 15 s, 42°C for 30 s, and 72°C for 2 min, with a final step at 72°C for 10 min. Re-amplified fragments were cloned into the TOPO TA Cloning® Vector (Invitrogen™) following the manufacturer's instructions and were sequenced with BigDye dideoxy terminator chemistry (Applied Biosystems, Foster City, CA, USA). The sequences were submitted to BLAST analysis for identification at the GenBank website.

RESULTS AND DISCUSSION

Although several modifications in the original ddPCR protocol have been made since its first publication, our goal was to develop a procedure that could deal with extremely small amounts of starting RNA and use a fluorescently labeled universal primer for detecting and isolating differentially expressed fragments, avoiding the use of radioactivity. ddPCR with a fluorescently labeled universal primer, and detection with an automated sequencer, was previously described by Smith et al. (1997). However, the reported strategy involved a second procedure, using radioactivity, to recover the differentially expressed fragments.

Hundreds of putative fragments differentially expressed in triplicate among the three bovine embryo groups were identified and recovered using the outlined procedures (Figure 2A and B). Re-amplification and direct sequencing were performed with a few fragments but the results indicated the presence of different fragments with similar size that co-migrate with the band of interest, as previously described (Bauer et al., 1993).

Nineteen isolated fragments were cloned and a total of 85 different colonies were sequenced. The results clearly showed the expected sequence structure of ddPCR products (i.e., anchor primer with T7 tail and arbitrary primer; Figure 3). In some instances, we observed the presence of 2 to 4 heterogeneous sequences in the same isolated ddPCR fragment, showing that sequencing of more than one colony is necessary for more precise identification and characterization of differentially expressed mRNAs (Ripamonte P, Mesquita LG, Cortezzi SS, Merighe GK, Caetano AR, Watanabe YF and Meirelles FV, unpublished results). Blast searches with the obtained sequences resulted in hits with similarity to genes related to embryo physiology, as well as bovine genomic and/or unknown sequences, clearly showing that the amplified fragments were not merely PCR artifacts and/or derived from contaminating ribonucleic acids.

Using the described methodology we were able to identify a transcript with similarity to bovine phosphatidyl inositide 3-kinase (PI3K), which was originally found to be expressed only in S4 embryos, that we later confirmed to be differentially expressed in the bovine oocytes and embryos with different developmental potential (Ripamonte P, Emanuelli IP, Mesquita LG, Cortezzi SS, Merighe GK, Caetano AR, Watanabe YF and Meirelles FV, unpublished results), confirming the effectiveness of the system at identifying differentially expressed transcripts from minute

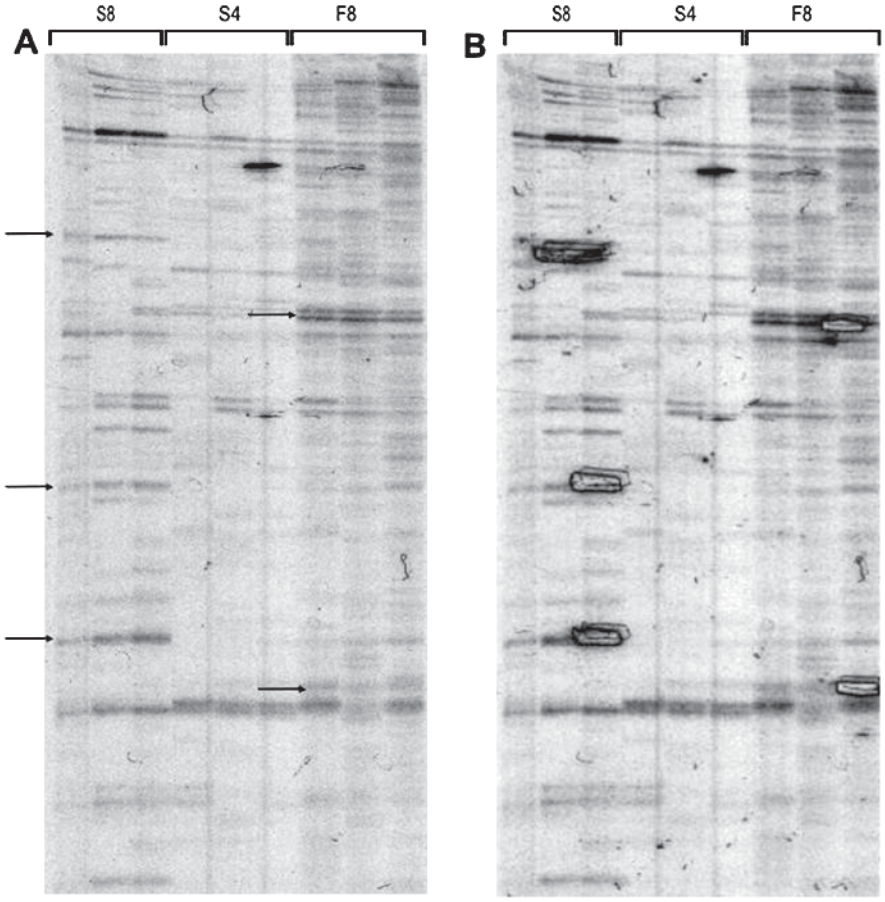

Figure 2. Example of ddPCR experiment showing **A.** differential amplification of bovine embryo groups with high (F8) and low (S4 and S8) development potential in triplicate and **B.** polyacrylamide gel after recovery of selected bands.

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5′GCTAGCAGAC CAGAAGTGCC AGTCAGAGGC CCAGCAGCCT TCTGCCACCA TCTCCCAGCT
  GCCAGTGGGT GCAAGTATGC CTCTGCCAAA CGCAAAGTGG AGGAAATGGA AGTGGACGAC
  TTCTACGATG GGATCAAACG GCTCTATAAC GAAGATAACG CTTCCGAAAG TGTGGGTTCT
  GTGTGTGGCA CTGATTTATC ACGGCAAGAG GGACAGGCTT CTCCTTGTCC ACCTCTGCAG
  CCTGTTTCTG TCACGTAGCT GCAAGAGTTA CCTTCAAGTG CAAACTAAGG TAGACGACTC
  GGGATGGGAG CATGGAAAAC CAGGATAGGC TACGTAAGGT ATATATCTTT TCAGGCTGAT
  TTGAAATGAG CCAGAGGAAA AAACCCAGTT GATTTGTATG ACTAATTATA ATTCAACAAT
  ATTTAAGCAC TTAAAGACCA AAAAAAAAAA ACTATAGTGA GTCGTATT 3′
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Figure 3. Sequence structure of ddPCR products after cloning and sequencing. Arbitrary primer (5') and anchor primer with T7 tail (3') are underlined and in bold.

quantities of starting RNA. The ddPCR methodology we developed, although requires a fluorescence scanner, offers several advantages such as high sensibility and reproducibility, reduced costs and greater lab safety.