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Sequence Characterization of Bovine Antisense to Insulin-Like Growth Factor Type 2 Receptor Non-Coding RNA (*AIRN*)

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Abstract

Bovine insulin-like growth factor type 2 receptor (*IGF2R*) is an imprinted gene whose aberrant expression has been implicated in development of abnormal offspring syndrome. Bovine *AIRN* (*AIRN*) is expressed in post-implantation fetal tissues coinciding with imprinted expression of *IGF2R*. Although expression patterns of bovine *AIRN* have been reported based on PCR analysis, characteristics of this transcript are unknown. Therefore, the objective of this work was to sequence characterize the *AIRN* ncRNA transcript. PCR primer sets (n = 19) were designed based on genomic sequence to "walk" down the predicted *AIRN* ncRNA sequence. Total RNA extracted from gestational Day 150 bovine fetal liver was used as source material for analysis. Extracted RNA was DNase-treated prior to cDNA synthesis, PCR amplified, and sequenced. A putative b*AIRN* promoter was located 623 base-pairs upstream of differentially methylated region 2 (DMR2) within intron 2 of *IGF2R*. A polyadenylation signal was found 117 kb downstream of the promoter. Primer sets designed upstream of the promoter as well as downstream of the polyadenylation signal yielded no PCR amplicons, suggesting that the length of *AIRN* is approximately 117 kb. In conclusion, bovine *AIRN* appears to be an antisense transcript of approximately 117 kb in length with a promoter region located 623 bp upstream of DMR2 within intron 2 of *IGF2R*.

Keywords

AIRN, Antisense RNA, Bovine, *IGF2R*, Non-coding RNA, Sequence

Introduction

Transfer of *in vitro* produced (IVP) or somatic cell nuclear transfer (SCNT) manipulated bovine embryos results in a proportion of conceptuses, fetuses, and offspring that exhibit developmental abnormalities collectively referred to as Abnormal Offspring Syndrome (AOS) [1]. Live offspring are generated from 45% of transferred IVP embryos with 5% to 20% of those exhibiting abnormalities depending on the culture system used [1]. Some of the abnormalities are hypothesized to be derived from the inadequacy of the *in vitro* culture environment and disruption of epigenetic patterns regulating parent-specific expression of imprinted genes [2].

The insulin-like growth factor type 2 receptor (*IGF2R*) is an imprinted gene whose aberrant expression has been directly related to the overgrowth phenotype following the transfer of IVP embryos in sheep and implicated in overgrowth of bovine IVP fetuses [3-6]. However, little is known about how imprinted expression of *IGF2R* is regulated in cattle. The maternal bovine *IGF2R* allele is preferentially expressed and the paternal *IGF2R* allele is repressed in post-implantation tissues [7]. Silencing of paternal *IGF2R* coincides with *AIRN* expression and acquisition of DNA methylation at the paternal *IGF2R* promoter. Recently, it has been demonstrated that the mechanisms governing imprinted expression of *IGF2R* may be tissue specific since the degree to which paternal *IGF2R* is repressed differs between endodermal, mesodermal and ectodermal origins [8]. Because the sequence characteristics of bovine *AIRN* are not known, the objective of the present study was to characterize the bovine *AIRN* non-coding RNA sequence.

Material and Methods

Production of Day 150 Fetuses

All procedures and protocols involving the use of animals in this study were approved by the Institutional Animal Care and Use Committee at North Carolina State University. *In vivo* embryos were produced using superovulated Holstein cows as embryo donors. Cows received two intramuscular (i.m.) injections of 25 mg Prostaglandin $F_2\alpha$ (PGF; Lutalyse; Pfizer Animal Health, USA) administered 14 days apart to synchronize estrus. Between Days 10 and 13 of the estrous cycle (Day 0 = estrus), cows received i.m. injections of 20 to 32 mg follicle stimulating hormone (FSH; FSH-P; Schering-Plough, Piscataway, NJ) in decreasing doses over a 4-day period. Estrus was induced by i.m. injection of 25 mg of $PGF_2\alpha$ on the morning and evening of the third day of FSH treatment. Cows were artificially inseminated 12 to 24 hours after the first observed standing estrus with frozen thawed semen from a proven Holstein bull. Embryos were collected non-surgically on Day 7 (Day 0 = first detected estrus) by uterine lavage and evaluated for stage of development and grade [9].

Cross-bred Angus heifers were given two i.m. injections of 25 mg PGF 10 to 12 days apart to synchronize estrus and were used as embryo recipients. On Day 7 of the estrous cycle (Day 0 = estrus), a single Grade 1 *in vivo* produced blastocyst was transferred non-surgically into the uterus and at Day 150 of gestation, heifers were slaughtered and fetuses recovered. Fetal liver samples were collected and immediately snap frozen in liquid nitrogen and stored at -80˚C for extraction of whole cell RNA (wcRNA).

RNA extraction of Day 150 bovine fetal liver

Whole cell (wcRNA) from a gestational Day 150 bovine fetal liver was extracted as previously described [10]. Briefly, frozen tissue (25 to

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Table 1: Primers used for RT-PCR to detect the non-coding RNA, *AIRN*.

Primer Set		Primer	Annealing Temp. (°C)	Length (bp)	Accession No.
1	Forward	5' TGT TGG GGT CTA AAC AGT GC 3'	55	3064	AC_000166.1
	Reverse	5' CTG AAT CCC CAC TTA CCA TT 3'			
2	Forward	5' GAG CCC AGC AGT TAT GAG GC 3'	55	1283	AC_000166.1
	Reverse	5' TGA AGG AGA GAC AGC CCA GA 3'			
3	Forward	5' TTG CCA CAG TTC TAA ATC AG 3'	55	1341	AC_000166.1
	Reverse	5' CTG TTG TAT CGT GTC TTT CG 3'			
4	Forward	5' GTG GAC TAG GAG AGG TTG GT 3'	55	1242	AC_000166.1
	Reverse	5' TTC CCA CAA GGG TTA GAG AC 3'			
5	Forward	5' CGG ATG TGA AAG CAG GAG GT 3'	55	1594	AC_000166.1
	Reverse	5' GCA AGA AGC CAA GCA AGT CC 3'			
6	Forward	5' TCC ACG GTG ATT GGA AAG GT 3'	55	1300	AC_000166.1
	Reverse	5' GCC ACA CCA TCA TCA CCA GT 3'			
7	Forward	5' GGT TAT GGA AGT CTT AAG CTT GAA AGT GGC 3'	60	225	AC_000166.1
	Reverse	5' GTC TTC AAG TCA TGC ATA CAG ACA GCA CT 3'			
8	Forward	5' CTC TTG GGG AGA CTG CTT GT 3'	55	1312	AC_000166.1
	Reverse	5' TGG TAT CCA CTG CTT CCC AT 3'			
9	Forward	5' ATC TCC AGG CAG TTG TGA TG 3'	55	127	AC_000166.1
	Reverse	5' GTT CCT ACC TTC CCG ATA CTG 3'			
10	Forward	5' AGG AAA TGC GTG TGT GGG TG 3'	55	3887	AC_000166.1
	Reverse	5' CTT GGT TGC TGG CAG ATT GG 3'			
11	Forward	5' CCC TGG TGG TCG TGT CTA AG 3'	55	431	AC_000166.1
	Reverse	5' ACA AAC CTG TGG CAA TGT GA 3'			
12	Forward	5' GAT GGA TAT TTG CGA CCC CG 3'	55	3222	AC_000166.1
	Reverse	5' CTG GAA TTG CCT TCG GGT TG 3'			
13	Forward	5' TCC TTC TGT GGT TGG TCC ATT G 3'	57	3672	AC_000166.1
	Reverse	5' TCT GTA ACT GTT GGG CTC CGA A 3'			
14	Forward	5' TCC CAA GTA CAA ACT GCG GCT C 3'	57	824	AC_000166.1
	Reverse	5' TCC ATC GCG GAC ATC TCG TT 3'			
15	Forward	5' TTT CAA GGA CTC TTT GAA CCG C 3'	57	1302	AC_000166.1
	Reverse	5' TCG AAT TCT AAC GCG GTT AAC C 3'			
16	Forward	5' TAGT TCA CAG ACT GCC GCC TTT 3'	57	3482	AC_000166.1
	Reverse	5' TGT CTC TGG AAT CCG TTC GTT C 3'			
17	Forward	5' GTG TAT TCT TGC CTC CTC TT 3'	55	3368	AC_000166.1
	Reverse	5' CCC ACA GCA TCT CCA CTT 3'			
18	Forward	5' TAT GTG CCA ACG CTG CGA A 3'	57	1942	AC_000166.1
	Reverse	5' GGC TCG ATA AGG ACA GAA ACG G 3'			
19	Forward	5' AGA TGT CTG CTC TGT GTT GCC AAA AAC 3'	60	1642	AC_000166.1
	Reverse	5' GGT TCT TCG CAG CGT TGG CAC ATA 3'			

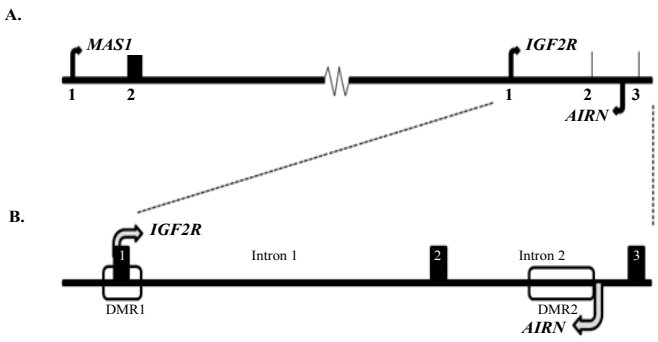

Figure 1: Illustration of the bovine gene region on Chromosome 9 specific to *IGF2R* and *AIRN*.

A. Region illustrating the orientation of the sense genes *MAS1* and *IGF2R* to the antisense gene for the non-coding RNA, *AIRN*. Numbers indicate exons 1 and 2 or exons 1, 2, and 3 of the *MAS1* and *IGF2R* gene sequences, respectively; B. Illustration of the overlap between *IGF2R* and *AIRN*. The numbered blocks represent exons 1, 2, and 3 of the *IGF2R* gene sequence. The open rectangles indicate the location of Differentially Methylated Regions 1 and 2 (DMR1 and DMR2) with the *IGF2R* gene sequence.

40 mg) was removed from -80°C storage, weighed, placed in a mortar, covered with liquid nitrogen, and subsequently crushed to a fine powder. The powder was homogenized (Brinkmann Homogenizer PT 10/35; Westbury, NY) and dissociated in lysis solution with mercapto-ethanol. RNA was extracted using a total RNA extraction kit (Sigma, GenElute Total Mammalian RNA Extraction Kit) following the manufacturer's protocols. The quality and integrity of the wcRNA was assessed by nanodrop using ratio of absorbances at 260 nm and 280 nm, which yielded an A260/A280 ratio of 2.11 for gestational Day 150 bovine fetal liver.

cDNA synthesis

Prior to cDNA synthesis, 2 µg of wcRNA from a previously extracted fetal liver sample was DNase treated by incubation of the wcRNA with 1.5 µl of DNase and 2 µl of DNase buffer at 37°C for 20 minutes. Following the manufacturer's instructions, 2 µg of DNase-treated wcRNA was incubated with 1 µg of random primers (Promega; Madison, WI), 1 µl of 10 mM dNTP mix (PCR Nucleotide Mix, Roche; Mannheim, Germany) and distilled water

at 65°C for 5 minutes. After placement on ice for 1 minute, samples were incubated with 4 µl of 5X First Strand Buffer, 1 µl of 0.1 M DTT, and 1 µl of reverse transcriptase (200 U/µl); (Superscript III, Invitrogen; Carlsbad, CA) at 25°C for 5 minutes. This was followed by incubation at 50°C for 60 minutes and inactivation by heating to 70°C for 15 minutes. The synthesized cDNA was subjected to purification using the QIAquick Purification Kit (Qiagen; Qiagen Sciences, MD) according to the manufacturer's instructions.

Bovine AIRN primers

Bovine *AIRN* primers were designed using the known bovine genomic DNA sequence of chromosome 9 UMD 3.1 Primary Assembly AC_000166.1 (97,540,000 bp to 97,710,000 bp). All *AIRN* primer sets (Table 1) were designed using the primer design program, Primer3Plus (Untegrasser and Nijveen 2007, Wageninger University). All amplification products were sequence verified.

AP1
97664989 AGCCAGACAGGC**TCTGACTAA**AGTAAGAGAGAAAAAAG

TATA-box *AIRN*>> INR-site
97664951 GAAATGTTGG**TATA**CAGGATGTGGGCAAATGA**TTACCTC**

DPE
97664912 CAGTGAGGAGAGACAGGAGCATGG**AGAGATA**GTGGTTA

Figure 2: Putative promoter for *AIRN*. Sequence showing transcription initiation site and consensus binding sites of core promoter elements. Nucleotide numbers refer to the location on chromosome 9 Primary Assembly UMD 3.1 (ACC_000166).

Reverse transcription-polymerase chain reaction (RT-PCR)

PCR reactions consisted of a 20 µl reaction volume that contained 100 ng of cDNA, 10 mM dNTP mix, Taq DNA polymerase (1.25 U per 20 µl reaction), sense and anti-sense primers (20 ng of each) and PCR water. PCR reactions were performed in a PTC-100 thermocycler (MJ Research, Inc., Watertown, MA). Annealing temperatures varied (Table 1) and were specific for each primer set evaluated in the primer walk.

Results and Discussion

In cattle, *AIRN* ncRNA is transcribed from the paternal allele in an antisense direction from a transcriptional start site located in exon 2 of *IGF2R* continuing into intron 1 of the neighboring protein-coding gene, MAS1 (Figure 1A). Within intron 2 lies a differentially methylated region, DMR2 (Figure 1B), that is 2620 bps in length and is unmethylated on the paternal allele and methylated on the

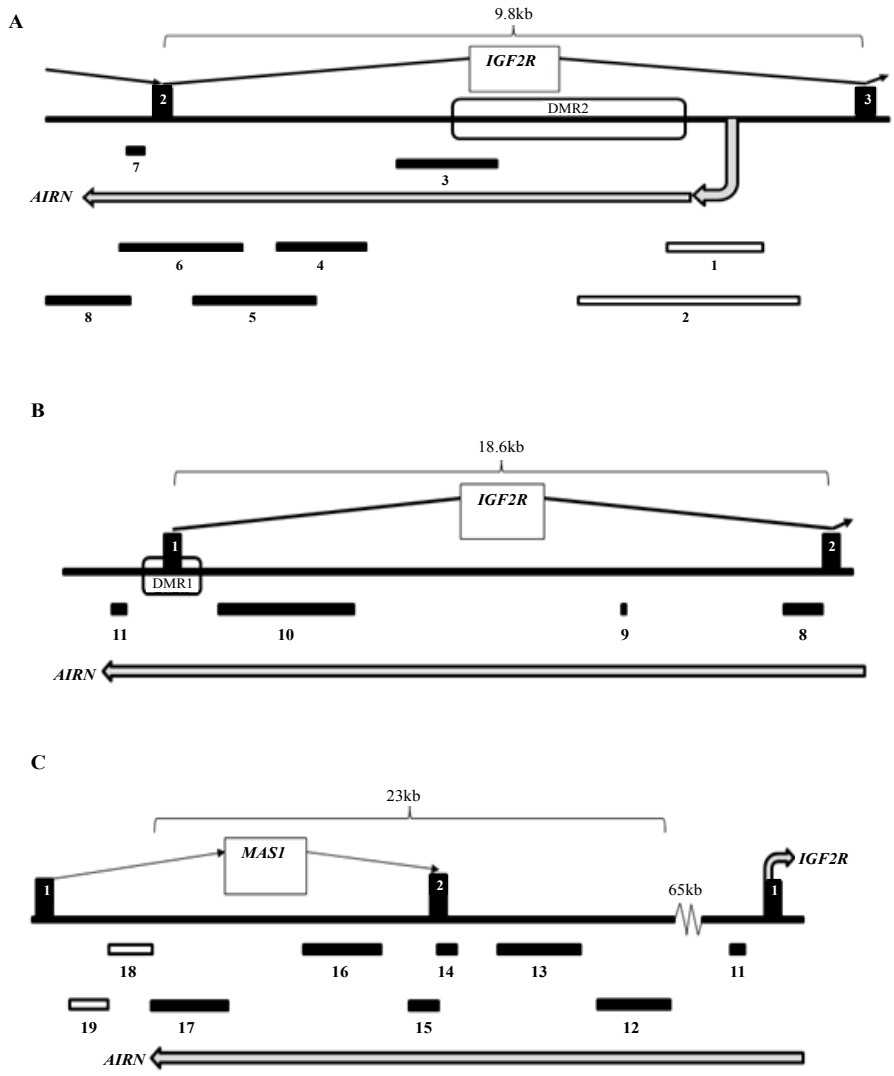

Figure 3: (A) Illustration of the overlap between *IGF2R* and *AIRN* at exons 2 and 3 of *IGF2R*. Vertical Bars depict exons 2 and 3 of the *IGF2R* gene sequence connected by arrows indicating direction of transcription. Horizontal Bars 1 to 8 indicate the location of PCR primer products for *AIRN* through the first 10 kb of transcription; (B) Illustration of the overlap between *IGF2R* and *AIRN* between exons 1 and 2 of *IGF2R*. Vertical bars depict Exons 1 and 2 of the *IGF2R* gene sequence connected by arrows indicating the direction of transcription. Horizontal Bars 8 to 11 indicate the location of PCR primer products for *AIRN* from 10 kb to 29 kb of the putative *AIRN* transcript; (C) Illustration of the overlap between *MAS1* and *AIRN* and depiction of PCR amplicons for AIRN for the terminal portion of transcription. Vertical Bars depict the exons 1 and 2 or exon 1 of the *MAS1* and *IGF2R* gene sequences, respectively. Horizontal Bars 12 to 19 indicate the location of PCR primer products for *AIRN* through the last 23 kb of *AIRN* transcription. For all panels; (A-C) open horizontal bars indicate that amplicons were not generated, closed horizontal bars indicate that PCR amplicons for *AIRN* were produced.

maternal one [5]. Moving in an antisense direction, a putative *AIRN* transcriptional start site was identified 623 bp upstream of the DMR. The promoter region was identified by analyzing the genomic DNA sequence (AC_000166, 97664989 to 97664875) upstream of the DMR for essential promoter elements. A TATA-box was identified 20 bp upstream of the putative transcriptional start site [11,12] (Figure 2). An Activator Protein-1 (AP1) binding site, also known as a 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) response element, was located upstream of the TATA-box [13]. In addition, downstream of the TATA-box we found an initiation response element (INR) and a down-stream promoter element.

Interestingly, as has been observed in the murine *Airn* sequence, there are 4 initiation sites that are clustered together in a 200 bp region in the bovine *AIRN* promoter region [11]. Also intriguing, is the observation that spread out over a 500 bp region are various promoter elements. Within this region are additional TATA boxes, CAAT boxes, AP1 binding sites, INR elements, and DPEs. These observations are consistent with those made of the murine promoter for *Airn* [11].

Primers for *AIRN* (Sets 1 and 2) were designed to amplify regions upstream of the putative promoter and downstream of *IGF2R* exon 3 (Figure 3A). These upstream primers were subjected to varying PCR conditions, including varied concentrations of cDNA template (50 ng, 100 ng, 150 ng), varied annealing temperatures (50 to 65°C), and altered annealing and extension times (10 to 60s). All attempts to generate a PCR amplicon within this region failed. This suggests that the true promoter region for bovine *AIRN* is located approximately 623 bp upstream of *DMR2* within intron 2 of *IGF2R*.

A series of primers for *AIRN* (n = 19) were designed to PCR amplify regions throughout the predicted length of b*AIRN* (Figure 3). The PCR amplicons generated covered a total of 27,202 bps of the predicted *AIRN* transcript. Representative gel images of amplicons produced from PCR primer sets are illustrated in Figure 4.

A putative poly-A signal (AATAAA) was identified 270 bps downstream of *AIRN* primer set 17 (Figure 5). Two additional *AIRN* primer sets (18 and 19) were designed to amplify products downstream of the poly-A signal (Figure 3C). Amplicons were not obtained from PCR amplification using these primer sets. Therefore, the putative length of bovine *AIRN* is 117 kb. Amplification of PCR products from primers spread over a 120 kb region from a putative promoter in intron 2 of *IGF2R* to a putative poly-A signal in intron 1 of MAS1 suggest that *AIRN* is intron-less. However, because only 23% of the putative *AIRN* noncoding RNA was sequenced in this effort, the possibility remains that the bovine *AIRN* transcript may be shorter than 117 kb or may contain introns. Additional poly-A signals (n = 3) can be found throughout the region between the second exon of *MAS1* and the first exon of *IGF2R*. These additional poly-A signals may represent additional transcript termination sites.

The murine *Airn* ncRNA sequence has been reported and was found to be 118 kb in length [11,14]. Although the length of bovine *AIRN* ncRNA appears to be similar to that of the mouse, there is a lack of sequence homology between the two species and some of the core promoter elements also differ. Eukaryotic genomes exhibit a vast range of ncRNAs, however, closer examination of these lncRNAs demonstrated that they may not share close sequence homology between mammalian species [15]. Differences in sequence homology may arise from the presence of transposable elements that are common to mature lncRNAs but rarely occur in transcripts from protein-coding genes [15-17]. Therefore, it may not be surprising that bovine *AIRN* and murine *Airn* do not share extensive sequence homology throughout their given lengths.

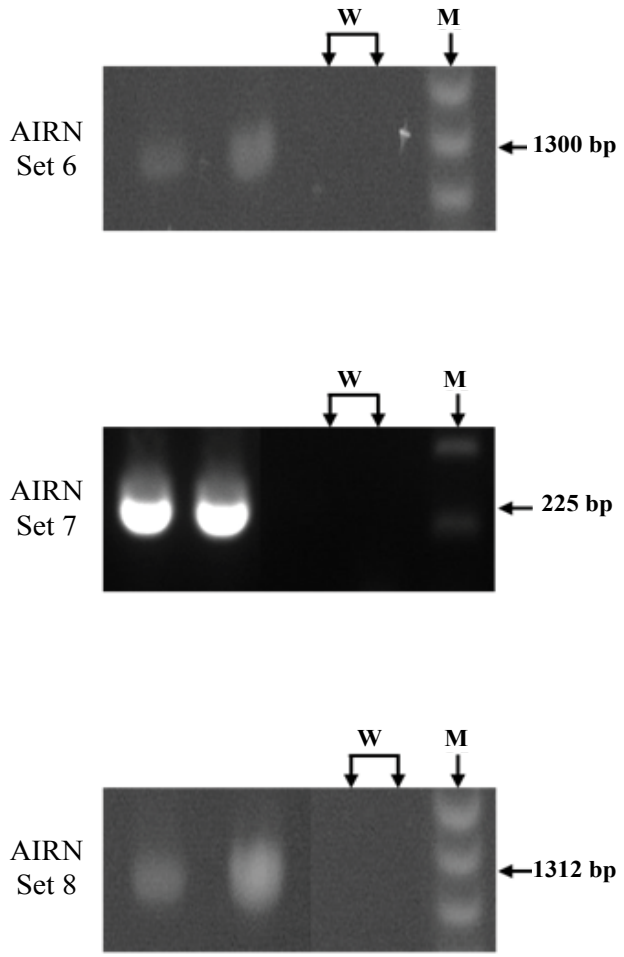

Figure 4: Representative gel images of PCR amplicons for bovine *AIRN* non-coding RNA. Primer sets 6, 7, and 8 (W, Water Blank; M, Ladder Marker).

Conclusion

Bovine *AIRN* has been characterized as a long non-coding RNA that is estimated to be approximately 117 kb in length. The transcriptional start site is located outside of the imprint control region located within intron 2 of *IGF2R*. Transcription appears to be continuous from the putative promoter to a poly-A site within intron 1 of *MAS1*. The *AIRN* transcript is repeat rich and collinear with the genomic sequence. Based on the known function of murine *Airn* [18,19], it is presumed that bovine *AIRN* functions to regulate imprinted expression of *IGF2R* [20,21]. Further studies will be needed to confirm the functional role of bovine *AIRN* in regulating imprinted expression of *IGF2R* or other protein-coding genes.

97547801 TGCACTCAAAATGCCAGCAAATTTGGAAAACTCAGTAGTGGCCACAGGAC

Poly-A

97547751 TGGAAAAGGTCTATTTTCATTCCAATCCCA**AATAAA**GGCAATGCCAAAGA

97547701 ATGTTCAAACTACCACACAATTGTACTCATCTCACACGCTAGCAAAGTAA

Figure 5: Putative polyadenylation (Poly-A) signal for bovine *AIRN*. Nucleotide numbers refer to the location on chromosome 9 Primary Assembly UMD 3.1 (ACC_000166).

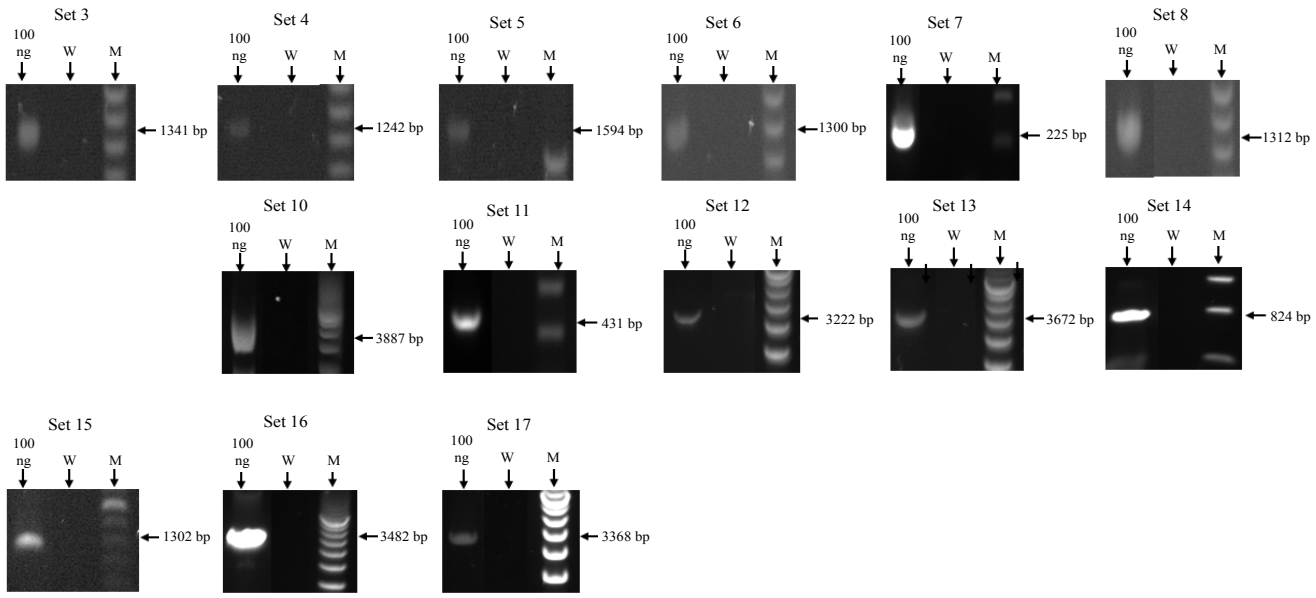

Figure 6: Supplemental Material: Individual gel images of PCR amplicons for bovine *AIRN* non-coding RNA. Set numbers correspond to PCR primer sets listed in table 1 (W, Water Blank; M, Ladder Marker).

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