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Facultat de Ciències

Memòria del Treball Final de Grau

DESIGN AND OPTIMIZATION OF PRIMERS FOR GENE TRANSCRIPT AND METHYLATION ANALYSIS ON BOVINE EMBRYOS

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ABSTRACT

The cattle industry is constantly evolving due to an increasing demand for livestock products. The need to breed more cattle and the increase of the temperatures due to climate change have motivated different studies with the aim to link heat stress and bovine reproduction problems. This is the case of the ongoing project in Ghent University entitled "*Effects of sperm stressors on sperm methylation and subsequent embryo development in cattle*". My role in this project was: (I) to test and optimize primers for embryo transcript expression analysis and (II) to design, test and optimize primers for bisulfite treated embryos.

In order to achieve the first objective, RNA extracted from frozen tissue was reverse transcribed, and the resulting cDNA was used as a template in RT-qPCRs to assess the specificity and the efficiency of the studied primers. The second objective was achieved by first, testing and optimizing the oxidative bisulfite sequencing protocol on frozen tissue and then on embryos. Afterwards, specific primers were designed and optimized in frozen tissue and then tested on *in vitro* produced embryos.

The specificity of the primer pairs was tested by performing melt curves during the RT-qPCR program and then, characterizing the resulting amplicons. All the studied primers showed a single distinct peak, indicating high specificity. These peaks were characterized by assessing the uniqueness and length of the amplicons via agarose gel and then, sequencing. PCR efficiency was analyzed and all the assays obtained valid efficiency values backed up by optimal R^2 values. On the other hand, the bisulfite conversion protocol showed trustworthy results both on testicle tissue and on embryos. However, the oxidative step results were not satisfactory. The designed primers for the original and the bisulfite treated DNMT1 gene showed solid results after analyzing them via PCR amplification, agarose gel electrophoresis and Sanger sequencing, therefore, the bisulfite sequencing protocol was verified.

In conclusion, different sets of primers were successfully optimized for gene transcript analysis. The bisulfite conversion protocol was optimized, and the DNTM1 based primers for original and bisulfite treated DNA showed satisfactory results.

RESUM

La indústria ramadera està en constant evolució a causa de la creixent demanda de productes pecuaris. La necessitat de criar més bestiar i l'augment de les temperatures a causa del canvi climàtic han motivat diferents estudis amb l'objectiu de vincular l'estrès calòric i els problemes de reproducció bovina. Aquest és el cas del projecte en marxa a la Universitat de Gant titulat "*Effects of sperm stressors on sperm methylation and subseqüent embryo development in cattle*". El meu paper en aquest projecte va ser: (I) provar i optimitzar encebadors per a l'anàlisi d'expressió de transcrits en embrions i (II) dissenyar, provar i optimitzar encebadors per embrions tractats amb bisulfit.

Per aconseguir el primer objectiu, l'ARN extret de teixit congelat es va transcriure de forma inversa, i l'ADNc resultant es va usar com a plantilla en les RT-qPCR per avaluar l'especificitat i l'eficàcia dels encebadors estudiats. El segon objectiu es va aconseguir primer; provant i optimitzant el protocol de seqüenciació de bisulfit oxidatiu en teixit congelat i en embrions. Posteriorment, es van dissenyar i optimitzar encebadors específics en teixit congelat i després es van provar en embrions produïts *in vitro*.

La especificitat dels encebadors estudiats es va provar realitzant corbes de fusió durant el programa de RT-qPCR, i caracteritzant els amplicons resultants. Tots els primers van mostrar un únic i diferenciat pic, indicant alta especificitat. Aquests pics es van caracteritzar per l'avaluació de la singularitat i la longitud dels amplicons a través de gel d'agarosa i posteriorment, seqüenciant el producte. L'eficiència de la PCR també es va analitzar i tots els parells d'encebadors van obtenir valors d'eficàcia vàlids, recolzats per valors òptims de R^2. Per altra banda, el protocol de conversió de bisulfit va mostrar resultats fiables tant en teixit testicular com en embrions. No obstant això, els resultats de l'oxidació no van ser satisfactoris. Finalment, els primers dissenyats per al gen DNMT1, original i tractat amb bisulfit, van mostrar resultats sòlids després d'analitzar-los mitjançant amplificació per PCR, electroforesi en gel d'agarosa i seqüenciació de Sanger, per tant, es va verificar el protocol de conversió per bisulfit.

En conclusió, diferents conjunts d'encebadors es van optimitzar amb èxit per a l'anàlisi de transcripció de gens. El protocol de conversió de bisulfit es va optimitzar i els encebadors basats en DNTM1 per ADN original i tractat amb bisulfit van mostrar resultats satisfactoris.

RESUMEN

La industria ganadera está en constante evolución debido a la creciente demanda de productos pecuarios. La necesidad de criar más ganado y el aumento de las temperaturas debido al cambio climático han motivado diferentes estudios con el objetivo de vincular el estrés calórico y los problemas de reproducción bovina. Este es el caso del proyecto en marcha en la Universidad de Gante titulado "Effects of sperm stressors on sperm methylation and subsequent embryo development in cattle". Mi rol en este proyecto fue: (I) probar y optimizar cebadores para el análisis de expresión de transcritos en embriones y (II) diseñar, probar y optimizar cebadores para embriones tratados con bisulfito.

Para lograr el primer objetivo, el ARN extraído del tejido congelado se transcribió de forma inversa, y el ADNc resultante se usó como plantilla en las RT-qPCR para evaluar la especificidad y la eficacia de los cebadores estudiados. El segundo objetivo se logró primero; probando y optimizando el protocolo de secuenciación de bisulfito oxidativo en tejido congelado y luego en embriones. Posteriormente, se diseñaron cebadores específicos y se optimizaron en tejido congelado y luego se probaron en embriones producidos *in vitro*.

La especificidad de los pares de cebadores se probó realizando curvas de fusión durante el programa RT-qPCR y caracterizando los amplicones resultantes. Todos los cebadores mostraron un único pico distinto, lo que indica una alta especificidad. Estos picos se caracterizaron por la evaluación de la singularidad y la longitud de los amplicones a través de gel de agarosa y luego, por secuenciación de Sanger. La eficiencia de la PCR también se analizó y todos los pares de cebadores obtuvieron valores de eficacia válidos, respaldados por valores óptimos de R^2. Por otro lado, el protocolo de conversión de bisulfito mostró resultados fiables tanto en tejido testicular como en embriones. Sin embargo, los resultados de la oxidación no fueron satisfactorios. Finalmente, los cebadores diseñados para el gen DNMT1 original y tratado con bisulfito mostraron resultados sólidos después de analizarlos mediante amplificación por PCR, electroforesis en gel de agarosa y secuenciación de Sanger, por lo tanto, el protocolo de conversión por bisulfito fue verificado.

En conclusión, diferentes conjuntos de cebadores se optimizaron con éxito para el análisis de transcripción de genes. El protocolo de conversión de bisulfito se optimizó y los cebadores basados en DNTM1 para ADN original y para ADN tratado con bisulfito mostraron resultados satisfactorios.

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Moltes gràcies.

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1. INTRODUCTION

1.1. Background

The livestock sector has been growing over the past few decades, caused by an increased demand for food and other products derived from animals. This sector comprises a wide range of animals, one of the most relevant ones is cattle, represented in around 3.6 million farms across Europe (Ihle et al., 2018). According to the Food and Agriculture Organization of the United Nations (FAO), countries situated in the tropics have the largest cattle inventory, such as Brazil and India. However, several studies evidence the adverse effects of heat on bovine reproduction (Drost et al., 1987; Roth, 2017), which could be exacerbated due to climate change and thus increasing heat. The average yearly temperature in many countries is greater than 23 ºC, as shown in *Figure 1*, leading to more frequent heat stress symptoms (DairyNZ, n.d.). According to a recent study, heat stress reduces the probability of a successful pregnancy in cattle, as the ability to detect estrus is decreased and causes a large reduction in fertility, damaging both, the oocyte and the early embryo (Hansen, 2015). Moreover, heat stress may have adverse effects on bovine semen quality which may ultimately reduce the field fertility (Rahman et al., 2018).

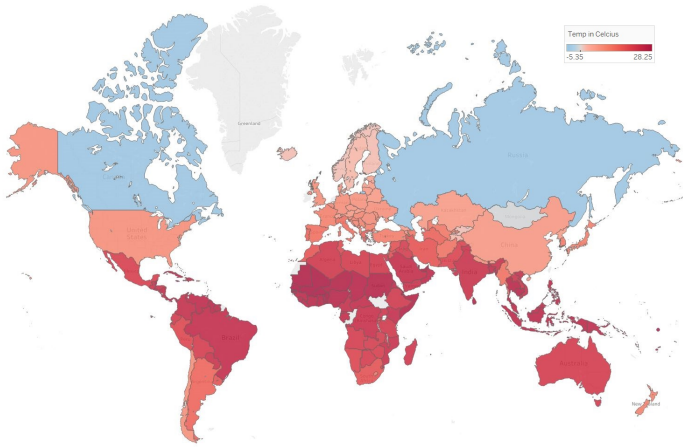


***Figure 1**. Map of countries by average yearly temperature* (Roehl, 2016)

Several studies aim to improve our knowledge on the effect of heat stress on bovine fertility (Dash et al., 2016; Sakatani, 2017). For instance, the ongoing project in Ghent University entitled "*Effects of sperm stressors on sperm methylation and subsequent embryo development in cattle*", hypothesizes that heat stress alters the epigenetic pattern of sperm cells and consequently of the future embryo. Therefore, the objective of this project is to compare different semen samples from the same bulls at different weather conditions.

1.2. Transcript expression analysis

1.2.1. PCR

The polymerase chain reaction (PCR) method was developed in the 1980s by Nobel laureate Kary Mullis. It has ever since been one of the major tools in genetic analysis and has continued to generate new areas of usage (ThermoFisher Scientific, 2003). The basic principle of PCR is summarized in *Figure 2*.

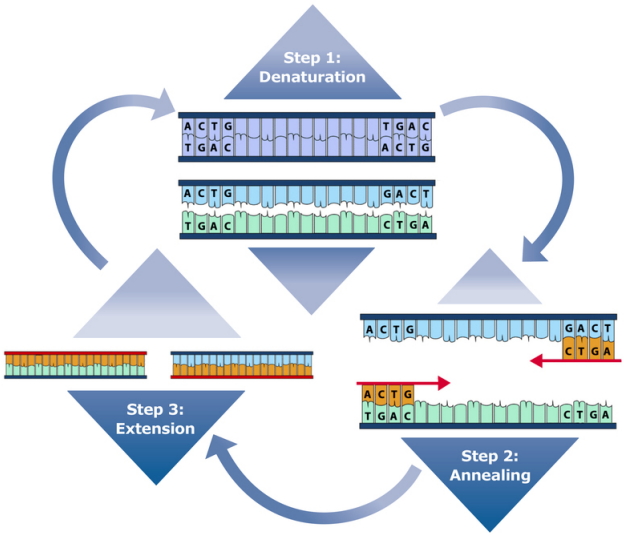


***Figure 2**. The 3 basic steps of PCR* (Waikato, 2015)

The first step is called the denaturation step, in which separation of the double-stranded DNA occurs. In the second step, the annealing step, primers anchor themselves to the recently formed single-stranded DNA-molecules. The temperature in this step is the most variable of all steps. It is desired to have a temperature as close as possible to the melting temperature of the primers to get the most highly specific primer hybridization. Finally, the primer extension step, in which the polymerase completes de complementary strand (Singh et al., 2014). Hot start polymerase can be used to avoid a non-specific amplification of DNA by inactivating the enzyme while at room temperature. When hot start PCR is performed, an initial activation step at 95 ºC is required for activation of the polymerase.

1.2.2. Quantitative Real-Time PCR

Quantitative Real-Time polymerase chain reaction (qPCR) has become a complete technique for detection and quantification of nucleic acids. In conventional PCR, the amplified DNA product is detected in an end-point analysis. In contrast, in qPCR, the accumulation of amplification product is measured as the reaction progresses, in real time, with product quantification after each cycle (Bustin, 2012).

Amplification products are measured as they are synthesized using a fluorescent label. As amplification occurs, a fluorescent dye binds, either directly or indirectly via a labelled hybridizing probe to the accumulating DNA molecules, and fluorescence values are recorded at each cycle of amplification. The fluorescence signal is directly proportional to the DNA concentration, and the linear correlation between the qPCR product and the fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. In other words, if a sample contains more targets, the fluorescence will be detected in earlier cycles. The cycle at which fluorescence can be detected is termed quantification cycle (Cq; formerly known as threshold cycle or Ct), and is the basic result of qPCR: lower Cq values mean higher initial copy numbers of the target.

Since PCR is a geometric amplification, ideally doubling the number of amplicons after every cycle, a linear plot of the data should show a classic exponential amplification as shown in *Figure 3* standard plot.

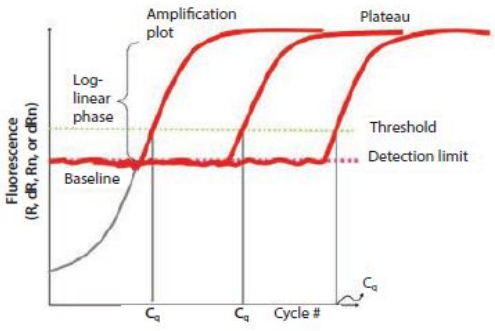


***Figure 3**. Detection of DNA in Real-Time qPCR* (Bio-Rad, n.d.)

The amplification plots can be divided in two phases: an exponential phase and a non-exponential plateau phase. At the first phase, the amount of PCR product approximately doubles in each cycle. As the reaction proceeds, some necessary components are consumed, and ultimately one or more of the components becomes limiting. At this point, the reaction slows and enters the plateau phase.

When a dilution series is amplified, the Cq values can be plotted against the logarithm of the dilution factor to generate a standard curve, which is the regression line through the obtained points.

PCR efficiency can be affected by not properly designed primers as well as suboptimal PCR conditions. Ideally the efficiency of a PCR should be 100 %, meaning that for each cycle the amount of product doubles. Efficiency is calculated from the slope of the standard curve according to the following formula (Stephenson, 2016):

$$E = 10^{\left(-\frac{1}{slope}\right)} - 1;\ \log E = \left(-\frac{1}{slope}\right)\log 10 - \log 1; \log 2 = \left(-\frac{1}{slope}\right); Slope = -\frac{1}{log2}$$

$$Slope = -3.32$$

From this formula, it can be concluded that an efficiency of 100 %, equals a -3,32 slope. A good reaction should have an efficiency between 90 % and 115 %, which corresponds to a slope between -3,59 and -3,00 (Applied Biosystems, n.d.).

An important parameter for evaluating qPCR efficiency is the coefficient of correlation (R^2), which is a statistical term that indicates how good the experimental data fits the regression line. An R^2 value over 0,99 provides good confidence in correlating two values (Taylor et al., 2010).

When using SYBR as an intercalating dye, non-specific PCR products and primer dimers will also generate a fluorescent signal. These signals will be added to the signal of the specific amplicon, leading to an overestimation of the DNA level. To avoid them and check for them, a melt curve can be performed at the end of the qPCR run. The thermocycler starts at a preset temperature and measures the amount of fluorescence. Then, the temperature of the sample is increased incrementally while the instrument continues to measure fluorescence. As the temperature increases, dsDNA denatures becoming single-stranded and the dye dissociates, resulting in decreasing fluorescence (Mecham, 2018). The change in slope of this curve is then plotted as a function of temperature to visualize the melt peaks, as shown in *Figure 4*. This curve allows to derive the melting temperature of the produced amplicon(s), but not always the number of amplicons. For this reason, during the optimization phase, the products length and uniqueness

should also be characterized via agarose gel electrophoresis and the amplified sequence should be characterized by Sanger sequencing.

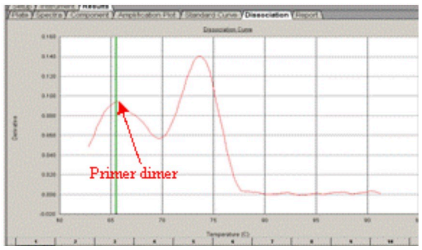


***Figure 4**. Primer dimers peak presence is revealed following melt curve analysis* (Applied Biosystems, 2008)

Over the past 10 years, the popularity of the qPCR method has grown exponentially, as evidenced by the publication of over 20,000 research articles. However, several technical deficiencies can affect qPCR primer performance, including, for instance, improper experimental design, inadequate controls and replicate or poor quality of the RNA sample. To assist the scientific community in producing consistent, high-quality data from qPCR experiments, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were published by Bustin et al. (2009). These guidelines provide a checklist of experimental parameters to ensure high-quality results that will meet the requirements of any journal.

1.2.3. Gene transcript analysis using RT-qPCR

The most commonly-used qPCR application is gene transcript analysis via reverse transcription quantitative real-time PCR (ThermoFisher Scientific, 2010). Transcript expression is the process by which information from a gene is used in the synthesis of a functional gene product. Researchers reverse transcribe mRNA, then use the cDNA produced as template in qPCR reactions to detect and quantitate mRNA products. When studying gene transcripts with RT-qPCR, scientists usually investigate changes in the expression of a particular gene or set of genes by measuring the abundance of the gene-specific transcript. Transcript expression studies can also involve looking at profiles or patterns of expression of several genes.

1.3. DNA methylation

Epigenetics is the study of changes in transcript expression that do not involve changes in the DNA sequence. One of the most known and studied epigenetic mechanisms is DNA methylation. It involves a covalent transfer of a methyl group to the C-5 position of the cytosine (C) ring of the DNA by DNA methyltransferases (DNMTs) (Robertson, 2005) as shown in *Figure 5*.

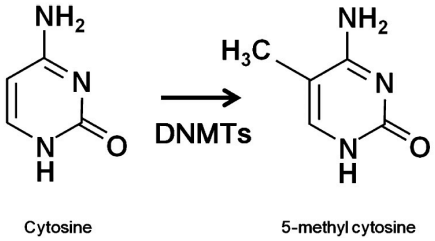


***Figure 5**. DNA methylation catalyzed by DNA methyltransferases* (Nasu et al., 2011)

Most DNA methylation is essential for normal development, and it plays a very important role in a number of key processes, including genomic imprinting, and when dysregulated, it contributes to diseases like cancer (Jin et al., 2011). DNA methylation has also been closely associated with male infertility (Gunes et al., 2016; Xu et al., 2016).

A method that has been used to differentiate and detect unmethylated versus methylated C is bisulfite (BS) sequencing. Treatment of DNA with BS converts C residues to uracil (U) but leaves 5-methylcytosine (5mC) residues and other C-modifications, such as 5-hydroxymethylcytosine (5hmC), unaffected (Booth et al., 2013), because methylated C are resistant to BS conversion. After the BS treatment, a PCR must be performed to selectively amplify the bisulfite-converted region of interest. The U residues are then replaced by thymine (T). On the other hand, methylated C is read as C at the sequencing step, which enables a quantitative differentiation between unmodified C and 5mC (Van Poucke et al., 2017).

Bisulfite treatment doesn't discriminate between 5mC and 5hmC, therefore a new method has been described where an oxidation step of 5hmC to 5-formylcytosine (5fC) is introduced (Booth et al., 2013). Bisulfite treatment will cause 5fC to be deformylated and deaminated to form U, which will be read as T at the sequencing stage (Booth et al., 2012). This added step enables researchers to discriminate between 5mC and 5hmC, as seen in *Figure 6*.

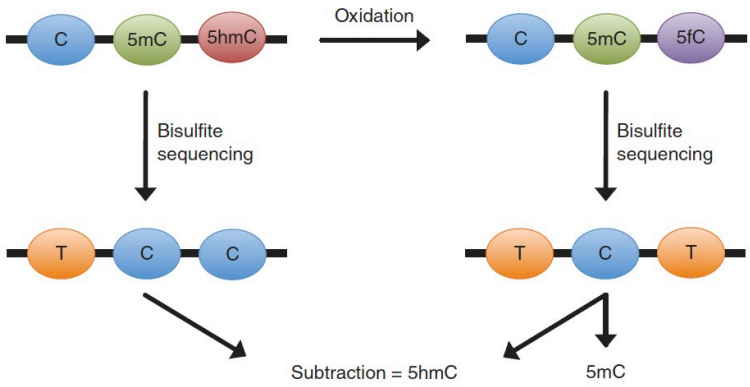


Figure 6. *After bisulfite treatment of DNA, C reads as T, whereas 5mC and 5hmC read as C. With the oxidation of 5hmC to 5fc followed by the bisulfite treatment, C and 5hmC read as T, whereas only 5mC reads as C* (Booth et al., 2013)

2. AIMS OF THE STUDY

This work involves collaborating in the project entitled "*Effects of sperm stressors on sperm methylation and subsequent embryo development in cattle*". My role will be to optimize assays for a battery of genes so that these can be used in this study to find differential expression by RT-qPCR and therefore, only analyze the methylation status of those genes showing differential expression.

Specifically, the objectives are:

- To test and optimize primers for transcript expression: perform RNA extraction from frozen tissues, check RNA quality, synthesize cDNA, perform RT-qPCR and characterize the products via agarose gel electrophoresis and Sanger sequencing.
- To produce bovine embryos: collect ovaries and cumulus-oocyte complexes (COCs), perform *in vitro* maturation (IVM) of COCs, *in* vitro fertilization (IVF) of matured oocytes, *in vitro* culture (IVC) of presumed zygotes and evaluation of cleavage and embryo development rates.
- To freeze day 8 blastocysts for methylation analysis
- To test and optimize the oxidative bisulfite sequencing protocol on frozen tissue: extract DNA from frozen tissues, perform oxidation protocol, bisulfite treatment, amplify the product performing a PCR and analyzing the products via gel electrophoresis.
- To design, test and optimize primers for methylated and original DNA: design and order primers, perform gradient PCR, characterize the products via agarose gel electrophoresis and Sanger sequencing.
- To test the bisulfite sequencing protocol and the designed primers on embryo DNA: perform bisulfite treatment and PCR amplification using the design primers and characterize the products via agarose gel electrophoresis and Sanger sequencing.

3. MATERIALS AND METHODS

3.1. Optimization of primers for transcript analysis by RT-qPCR

3.1.1. RNA Isolation from frozen tissue and quality check

One hundred mg of frozen bovine testicle tissue were transferred to a microcentrifuge tube containing 1 ml of QIAzol (Qiagen, Germany). The tissue was disrupted using a TissueRuptor (Qiagen, Germany), followed by 5 min of incubation at room temperature while rotating to allow the complete dissociation. RNA was extracted by adding 0.2 ml of chloroform, shaking vigorously for 15 s, incubation for 5 min at room temperature while rotating, and centrifuging at 12,000 x g for 15 min at 4 ºC. The mixture was separated into three phases: an upper colorless aqueous phase, a white interphase, and a lower red organic phase. The aqueous phase was transferred to a new tube, as RNA can be found exclusively in this phase, containing, the same amount of 70 % (v/v) ethanol. To proceed with the RNA extraction, the Aurum Total RNA Fatty and Fibrous Tissue Kit (Bio-Rad, USA) was used. An RNA binding column was inserted into a 2 ml capless tube and 700 µl of the lysate were transferred. The sample was centrifuged for 30 s, 700 µl of low stringency wash were added to the column and then, 80 µl of 1:15 diluted DNase were added to avoid DNA contamination. The mixture was incubated for 15 min and at the end, was centrifuged for 30 s. The next step was to add 700 of high-stringency wash, centrifuged it for 30 s and then, 700 µl of low-stringency were added, followed by first, 1 min of centrifuging and then 2 additional min to make sure that no remaining wash solution was present in the column. To conclude the RNA isolation, 30 µl of 70 ºC elution solution were added onto the center of the membrane stack, incubated for 1 min and centrifuged for 2 min to elute the sample. The elution was added once again to the column to recover remaining sample, and it was centrifuged for 2 additional min.

A Nanodrop 1000 spectrophotometer (ThermoFisher Scientific, USA) was used to determine RNA concentration and purity. The concentration was assessed by adding 2 µl of eluted RNA and measuring A260 absorbance. The purity was determined by calculating the A260/A280 and the A260/A230 ratio. Nucleic acids absorb at 260 nm and proteins at 280 nm. A ratio of ~1.8 is generally accepted as "pure" for DNA and a ratio of ~2.0 is generally accepted as "pure" for RNA (Matlock, 2012). On the other hand, expected values of the A260/A230 ratio are commonly in the range of 2.0-2.2 (Geuther, 2007), if the ratio is appreciably lower than expected, it may indicate the presence of contaminants, for instance carbohydates or phenol, which absorb at 230 nm. The integrity of the samples was estimated by analyzing 1 µg of RNA on a 2 % agarose gel. The gel was not treated to be non-denaturing or to have RNase denaturation conditions, as the used conditions have been tested and are accepted in our laboratory. The presence of DNA contamination was evaluated by means of a minus RT-PCR using ubiquitin C gene (UBC) primers. The UBC gene is a highly conserved gene that is used to develop a single, cheap and multi-use PCR-based assay to estimate the presence, integrity and amplificability of DNA and cDNA (Van Poucke et al., 2017). RNA was added as template in the PCR reaction and because DNA polymerase can only amplify DNA and not RNA, if there is amplification, it means that contamination is present and in that case, the length of the bands (137, 365, 593 and 821 bp) will determine the degradation level of the DNA The UBC primers sequences and design are shown in *Figure 7*, and the reaction setup and the thermocycling conditions used for UBC assays are listed in *Table 1*.

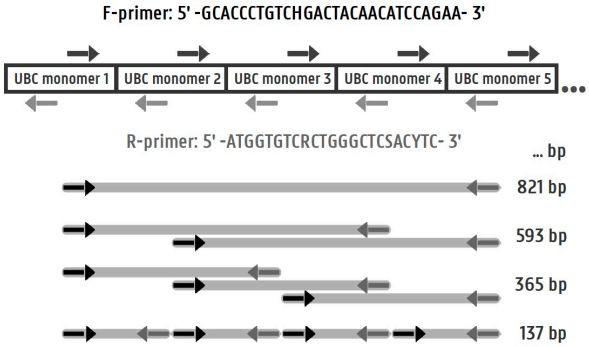


Figure 7. *Design and sequences of the UBC primers* (Van Poucke & Peelman, 2017)

The PCR products were analyzed via 2 % agarose gel electrophoresis. The gels were prepared following the protocol for the i-MyRun electrophoresis system (Cosmo Bio, USA) which consists in 120 ml of 1x TBE, 2.4 g of agarose and 24 µl of 10 µg/µl Ethidium bromide.

Table 1. *Reaction components and thermocycling conditions for UBC assays PCRs*

Component	**10 µl**	**Step**	**Temp.**	**Time**
10X TEMPase Key Buffer	1 µl	**Initial Denaturation**	95 ºC	15 min
40 mM dNTPs	0,2 µl	**40 cycles**	95 ºC	20 s
Forw. and Rev. Primers (5 µM each)	1 µl		68 ºC	20 s
Template DNA	1 µl		72 ºC	40 s
5 U/µl TEMPase Enzyme	0,1 µl	**Final Extension**	72 ºC	2 min
Nuclease Free Water	6,7 µl	**Hold**	15 ºC	

3.1.2. cDNA synthesis and quality check

Reverse transcription was performed using the ImProm-II Reverse Transcriptase kit (Promega, USA). One µg of high quality DNA-free RNA and 0.5 µg of a random and oligo(dT) primer mix were pooled in a final volume of 5 µl. The mixture was incubated for 5 min at 70 ºC and then cooled down at 4 ºC for 5 min. Next, 4 µl of ImProm-II 5X Reaction Buffer, 2.4 µl of 25 mM $MgCl_2$, 1 µl of 40 nM dNTP mix, 1 µl ImProm-II Reverse Transcriptase and 6,6 µl of nuclease-free water were added to the sample until the final volume of 20 µl. After this step, reverse transcription was performed in a Mastercycler (Eppendorf, Germany), with the following settings: primer annealing at 25 ºC for 5 min, reverse transcription at 42 ºC for 60 min and denaturation of the enzyme at 70 ºC for 15 min to stop the reaction.

The cDNA was amplified by adding a 1/10 dilution of the products of the heat-inactivated reverse transcription reaction to a routine PCR mix. The UBC assay was used to check on the cDNA quality and the PCR products were analyzed by 2 % agarose gel electrophoresis following the protocol for the i-MyRun electrophoresis system (Cosmo Bio, USA) as mentioned before.

3.1.3. RT-qPCR

The RT-qPCRs were performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, USA) in a Bio-Rad ® CFX96™ Real-Time PCR System (Bio-Rad, USA). The primers analyzed are listed in *Table 2*. Many genes have more than one transcript due to alternative

splicing; the splice variants for a particular gene are known as transcript variants. When the aim is to design primers to analyze transcript expression, assays can be designed either to amplify a common region that is shared in all the predicted transcripts or to only amplify one variant by making sure that the designed primers are for a region that is unique to that variant.

Table 2. *List and information of the studied primers in Real-Time PCR*

Gene / Accession Number			Sequence (5' – 3')	Prod. Length	Ta (ºC)
H19		*F*	*TCGTTTGCACTGGTTGGGGC*	*129 bp*	*63.86*
NR_003958.2		*R*	*AAGTGTGGGAAGGGTGCTGGG*		*64.64*
IGF2	*1*	*F*	*GTCCAACGCATAGAGGACGGGG*	*173 bp*	*64.91*
		R	*TAGCAGCACGAGGCGAAGGC*		*65.26*
XM_005227270.3	*2*	*F*	*CCCCGTGGGCAAGTTCTTCCA*	*150 bp*	*64.94*
		R	*AGCGGACGGTGACTCTTGGC*		*64.88*
IGF2R		*F*	*CTGCACGTCTAGGCTCGCCC*	*118 bp*	*65.30*
NM_174352.2		*R*	*CCCCACCACCAACACTCTTCCC*		*65.05*
MEG3		*F*	*ACCTTCCGTTTCCCCTCCAACC*	*115 bp*	*64.47*
NR_037684.1		*R*	*CACCTCCTCTGTCCAGGCGTT*		*64.10*
MEG9		*F*	*GGCCCCTACCTCTTCCTAGCC*	*180 bp*	*63.47*
NR_132275.1		*R*	*GGGCACAGCAGGGAACATGAC*		*63.88*
MEST		*F*	*AGAAGACGCTGGGTGGGAGC*	*170 bp*	*64.33*
NM_001083368.1		*R*	*AGCTGTGGATAGTGGCTAATGTGGT*		*63.86*
PEG10	*1*	*F*	*CCCGTCTTTCCAGCCTTCGCA*	*142 bp*	*65.25*
		R	*GCGCGTAGTAGCTTCACTCCTGT*		*64.64*
NM_001109815.5	*2*	*F*	*CGCGTGGTGAGTATGCGAAACA*	*111 bp*	*63.93*
		R	*GCGCGTAGTAGCTTCACTCCTG*		*63.24*
PHLDA2		*F*	*GTCCCACGGCGAATCATGCC*	*147 bp*	*64.14*
NM_001076521.2		*R*	*CAGCACGGAAAGGTCATTGTGTGT*		*64.05*
PLAGL1		*F*	*GCTCGACCACCTCAAAGCCCA*	*112 bp*	*64.96*
XM_015472892.1		*R*	*CCGCACATCCTTCCGGGTGTA*		*64.48*
SNRPN		*F*	*GCAAGATGGTCGTATATTCATTGGCACC*	*162 bp*	*64.50*
NM_001079797.1		*R*	*TCCCCACGCAACAACACCAGA*		*64.53*
DNMT1		*F*	*CTGCGGACCCTGGACGTGTT*	*130 bp*	*64.84*
NM_182651.2		*R*	*GGTTGTTGAGCCGGAACGCC*		*64.54*
DNMT3a		*F*	*TCGCCAACAACCATGACCAGGA*	*153 bp*	*64.44*
NM_001206502.1		*R*	*AGCGGTCCACCTGAATGCCC*		*64.98*
DNMT3b		*F*	*ACCTGCTGAATTACACTCGCCCC*	*104 bp*	*64.93*
XR_003037672.1		*R*	*AGAGATGTCCCGCTTGTCGCC*		*64.78*
NANOG		*F*	*GTGAAACCACTGTCCCCGTCTG*	*101 bp*	*63.08*
NM_001025344.1		*R*	*AGCTGGGTCTGCGAGAACAC*		*63.81*
SOX2		*F*	*AAGGGAGAGAAGTTTGAGCCCC*	*170 bp*	*61.96*
NM_001105463.2		*R*	*GCGAGGAAAATCAGGCGAAGAA*		*61.51*
POU5F1		*F*	*TTCAGCCAAACGACTATCTGCCGT*	*158 bp*	*64.67*
NM_174580.3		*R*	*TTTCGGGCCTGCACAAGGGT*		*64.82*

The reaction mix used for each sample was composed of 5 µl of SsoAdvanced™ Universal SYBR® Green Supermix, 1µl of forward and reverse primers (5 µM each), 2 µl of different dilutions of the cDNA template and 2 µl of nuclease-free H_2O. A 1/4 dilution series of the template was used to generate a standard curve from which the PCR efficiency could be determined. A no template control (NTC) was also used to serve as a control for extraneous nucleic acid

contamination, since SYBR® Green was used in this reaction, this control will also determine the presence of primer dimers. The RT-qPCR program was configured as follows: 3 min at 95ºC to achieve polymerase activation and DNA denaturation, 40 cycles consisting of 15 s at 95ºC for denaturation and 30 s at 65ºC for primer annealing, extension and at the end, fluorescence measurement. Melt curve analyses were performed at the end of the run by heating from 72 to 95ºC, consisting in repeated steps of 0,5ºC for 5 sec and fluorescent measurements. In some cases, when late Cq values were obtained not allowing a complete amplification of some of the dilutions, the RT-qPCR product obtained was used as template to run the program again. In this case, a 1/10 dilution series was used. From the RT-qPCR products 2 µl was additionally analyzed by 2 % agarose gel electrophoresis to verify the length of the single amplicon and the remaining 8µl was used to verify the identity of the amplicon by Sanger sequencing. Both results would then be linked to the melt curve pattern.

3.1.4. Sequencing RT-qPCR products

To ensure maximum efficiency of the sequencing protocol, a cleanup of the RT-qPCR products with Exonuclease I / Antarctic Phosphatase (Exo-AP) was performed. Exonuclease I was used to digest residual single-stranded primers and any extraneous single-stranded DNA produced in the PCR. Antarctic Phosphatase was used to remove the remaining dNTPs from the PCR mixture. PCR Cleanup was performed by adding 0.6 µl of the Exo-AP solution (the mix consisted on 40 µl of 20 U/µL Exonuclease I and 80 µl of 5 U/µl Antarctic Phosphatase) (BioLabs, USA). The following program was applied using a thermal cycler: 30 min at 37ªC to perform the reaction followed by 15 min at 80 ºC to inactivate the enzymes.

The sequencing reaction was performed by preparing the following sequencing mix: 0.5 µl RR-mix (BigDye^{TM} Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems), 2 µl 5x SEQ-Buffer (equals 200 mM Tris-HCl, pH8 and 5 mM MgCl2), 2 µl of 20 ng/µl Exo-AP treated PCR product, 1.5 µl of 2 µM sequence primer, and nuclease-free water to reach 10 µl of total volume. For each RT-qPCR product, two samples were prepared, one containing the forward primer and one with the reverse primer. Afterwards, the following sequencing program was applied: 2 min at 95 ºC, 30 cycles consisting of 20 s at 96 ºC, 10 s at 55 ºC, and 4 min at 60 ºC. The resulting samples were sent to Eurofins Genomics (Germany) for analysis on the ABI 3037XL sequencer (Applied Biosystems, USA).

The obtained sequencing results were aligned and compared to the target sequence using the Nucleotide BLAST tool of the National Center for Biotechnology Information (NCBI) and the results were analyzed using the identities value.

3.2. In vitro production of bovine embryos

3.2.1. *In vitro* maturation of bovine cumulus-oocyte complexes

Ovaries were collected at the slaughterhouse of Zele (Baaikensstraat 33; 9240 Zele) and brought to the Reproduction labs. The mesovaria and the oviduct were removed and the remaining ovarian tissues were washed with physiological saline solution with 1 g / 200 ml kanamycin (Sigma

Aldrich, UK) at 37 ºC. The ovaries were shortly pressed in a towel with ethanol 96 % to remove the excess of blood and washed another 2 times. Follicles with a diameter of 2-8 mm were aspirated with a 10 ml syringe and a sterile needle (Novolab, Belgium) and placed into conical tubes (Greiner Bio-One, Germany) at 38.5 ºC containing tyrode's albumin lactate pyruvate (HEPES-TALP) . After a minimum of 30 min to give the oocytes the time to sag down, the supernatant was removed with a syringe and needle, and the pellet was resuspended in HEPES-TALP and poured into a previously squared 90 mm petridish (Greiner Bio-One, Germany). Oocytes with a complete compact cumulus mass were selected and placed into a 33 mm petridish (Greiner Bio-One, Germany) containing warm HEPES-TALP. The dish was kept covered to prevent evaporation and a resulting increase in osmolality. Good quality COCs were washed by transferring them to fresh HEPES-TALP and separated in 2 groups of 60 COCs were made. A second wash was performed in 500 µl of equilibrated *in vitro* maturation medium in a NUNC 4-well plate (Novolab, Belgium). Lastly, the COCs were transferred to clean maturation medium wells and the plate was placed in the media CO_2 incubator for 18 to 26 h at 39 ºC and 5 % of CO_2 in the air.

3.2.2. *In vitro* fertilization of bovine in vitro matured oocytes

The sperm separation procedure performed in this experiment was a Percoll gradient. The gradient was prepared in a 15 ml conical tube by slowly and carefully adding 2 ml of Percoll 45 % (v/v) on top of the same volume of Percoll 90 % (v/v). To prepare the 45 % (v/v) Percoll solution, a Percoll 90 % solution was mixed 1:1 with sodium pyruvate and gentamicin (SP-TL) (-) BSA. The sperm straws were thawed in a warm water bath at 38 ºC, dried and emptied by cutting both sides. To bring the sperm suspension on the Percoll gradient a 10 ml syringe and a needle were used to carefully release the sperm suspension on top of the Percoll 45 % (v/v) layer. The gradient column with the sperm sample were centrifuged at 700 g for 30 min at 22 ºC using the Jouan C3i centrifuge (ThermoFisher Scientific, USA). Once the centrifuge stops, the supernatant above the sperm pellet is immediately aspirated using the needle and syringe. To wash the sperm pellet, 5 ml SP-TL (+) BSA were added to the tube and the second centrifuging step was performed at 100 g for 10 min at 22 ºC. After the second centrifuging step, the supernatant was removed and the pellet was kept ina volume of approximately 100 µl.

To define the sperm concentration, a Bürker counting chamber (Novolab, Belgium) was used. The cover glass was placed over the chambers and 10 µl of a sperm dilution 1:10 was brought into the Bürker chambers. The sperm cells in each chamber were analyzed by counting only the intact sperm cells in 40 small squares. Sperm cells lying on the border of a square were only counted when laying in two of the four designated sides. The sperm concentration was obtained by calculating the average of both counting chambers multiplied per one million cells. The dilution to obtain a sperm suspension of 2 million sperm cells/ml was calculated for fertilization.

The correct sperm concentration was obtained diluting the sperm solution in IVF medium. 250 µl of the dilution were brought into the IVF wells where matured oocytes had been previously transferred. The presence of sperm in the wells and their mobility was checked under the stereo microscope and the IVF dishes were placed back into the CO2 incubator.

3.2.3. *In vitro* culture of bovine presumed zygotes

All the zygotes were aspirated and placed into a conical 15 ml tube containing 2.5 ml HEPES-TALP. The tube was shaken in a vortex for 3 min thoroughly. The suspension was poured into a 33 mm petridish standing on the heating stage and the presumed zygotes were brought into the fresh HEPES-TALP in the lid, while counting. The presumed zygotes were divided into two groups and transferred into a petri dish with SOF culture medium and mineral oil (Sigma Aldrich, UK). Afterwards, each group of zygotes was aspirated and transferred in a 4-well dish (Novolab, Belgium) with 50 µl SOF droplets covered with mineral oil. The zygotes were cultured in the Trigas (6 % O_2, 6 % CO_2, 88 % N_2) incubator (Thermo Fisher Scientific, USA). The cleavage rate was evaluated 24 h after incubating the zygotes, and the blastocyst rate was evaluated at day 7 and day 8 post-fertilization. At day 8 post-fertilization, the cultured embryos were washed 3 times with PBS and placed in a tube with 13 µl of M-Digestion Buffer from the EZ DNA Methylation-Direct kit (Zymo Research, USA) and frozen at -80 ºC until needed to optimize the oxidative bisulfite conversion protocol.

3.3. Design, test and optimization of primers for methylation

3.3.1. DNA isolation from frozen tissue and quality check

Approximately 100 mg of frozen tissue was sliced and transferred to a microcentrifuge tube containing 500 µl of lysis buffer (composed of 1ml 1M Tris-HCl pH 8, 2.5 ml 10 % SDS, 2.5 ml 0,5 M EDTA, 1 ml 5 M NaCl and 43 ml H_2O) and 20 µl of 10 µg/µl proteinase K (Roche, Germany). The mixture was shaken thoroughly using a vortex and then incubated overnight at 56 ºC while rotating to allow the enzymatic digestion of proteins and non-nucleic acid cellular components. Afterwards, the sample was centrifuged at 16.168 x g for 2 min and the cell debris free supernatant was transferred to a new tube. The next steps were done to promote the partitioning of lipids and proteins into the organic phase and interphase, leaving the isolated DNA in the aqueous phase. To start, 500 µl of phenol/chloroform were added to the mixture, the sample was mixed with a vortex and centrifuged at 16.168 x g for 5 min. The top phase was transferred to a new tube and 500 µl of chloroform were added, the sample was mixed with a vortex and centrifuged at 16.168 x g for 5 min. The supernatant was brought to a new tube and 50 µl of 5 M NaCl (VWR International Leuven, Belgium) and 500 µl of isopropanol were added. The sample was mixed using a vortex and incubated on the bench for 10 min. Thereafter, the sample was centrifuged at 16.168 x g for 5 min and the supernatant was removed. To wash the pellet, 500 µl of 70 % ethanol were added. The sample was shaken thoroughly using a vortex and then centrifuged at 16.168 x g for 5 min. The supernatant was removed, the tube was centrifuged briefly and left open for one min to dry. Then, the mixture was resuspended by adding 100 µl of nuclease-free water and mixing with a vortex. To analyze the DNA isolation results, a Nanodrop 1000 spectrophotometer was used to determine DNA concentration and purity. The concentration was determined by adding 2 µl of the eluted DNA and measuring A260 absorbance for each sample. The purity was assessed by calculating the A260/A280 and the A260/A230 ratio.

In this study, the oxidation protocol was tested to be able to discriminate between 5mC and 5hmC. To do it successfully, the extraction could not contain traces of the reagents used for the DNA

extraction. For this reason and to concentrate the sample, the use of an Amicon Ultra 100 k centrifugal filter for DNA purification (Merck KGaA, Germany) was tested. The overall operation of the column is represented in *Figure 8*. In first place, 100 µl of the sample and 100 µl of nuclease-free water were added to the column and it was centrifuged for 10 min at 16.000 x g. Afterwards, the flowthrough was removed, 180 µl of nuclease-free water were added and the column was centrifuged for 10 min at 16.000 x g. The last step was repeated and then the product was obtained by placing the filter in a new tube upside down and centrifuging the sample for 3 min at 1.000 x g. After this step, the DNA concentration and purity were assessed using the Nanodrop 1000 spectrophotometer.

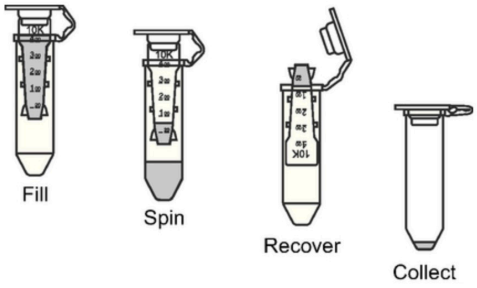


Figure 8. *The Amicon Ultra was used to purify and concentrate the sample* (Merck Millipore Corporation, 2015)

3.3.2. DNA oxidation and quality check

To test the DNA oxidation protocol, part of the DNA isolated from frozen tissue was oxidized and the other part was left without oxidizing. The DNA oxidation was carried out following the protocol described by Booth et al., (2013), with the differences that a Mini Quick Spin Oligo Column (Roche, Germany) was used instead of a Micro Bio-Spin P-6 SS column (Bio-Rad Laboratories, California), and the AMPure XP beads (Beckman Coulter, USA) were not used. The Mini quick spin oligo column was washed 4 times with the aim to remove up to 99.99 % of the salts present in the matrix by adding 300 µl of water, and it was centrifuged at 1.000 x g for 60 s. Afterwards, 20 µl of the sample were added to the column and it was centrifuged at 1.000 x g for 240 s. The obtained product was analyzed with Nanodrop.

To denature the DNA, 1 µg of the sample was mixed with 1.25 µl of 1M NaOH and Nuclease-free water to reach 23 µl. The sample was incubated at 37 ºC for 30 min in a shaking incubator (Sheldon Manufacturing, USA). Once finished, it was immediately cooled by placing the tube in an ice-water bath for 5 min. To proceed with the oxidation, 2 µl of a freshly prepared $KRuO_4$ (15 mM in 0.05 M NaOH) solution were added to the tube with denatured DNA. The oxidation reaction was placed in the ice-water bath for 60 min. The tube was briefly vortexed four times: at the start, after 20 min, after 40 min and at the end. The followed protocol said that the solution had to remain orange and that any other color of the solution, green or brown/black, would imply that the oxidant was reacting with traces of contaminants. If these colors were observed, the oxidation had to be re-started with a fresh sample of genomic DNA.

After the oxidation reaction, the sample was purified with a Mini Quick Spin Oligo Column, previously washed using the same protocol as described earlier. The sample was added to the column and centrifuged at 1.000 x g for 480 s. The obtained product was analyzed with Nanodrop.

3.3.3. DNA bisulfite conversion

The DNA bisulfite conversion was performed using the EZ DNA Methylation- Lighting kit (Zymo Research, USA). Two different samples were treated in parallel, the oxidized DNA and the native DNA. First, the CT-conversion reagent was prepared by dissolving it with 790 µl of M-Solubilization Buffer and 300 µl of M-Dilution Buffer. The mixture was vortexed for 10 min and 160 µl of M-Reaction Buffer were added to the sample. This reactive is light sensitive, therefore, the exposure to light was minimized. To perform the bisulfite conversion, 130 µl of the CT-conversion reagent were added to 20 µl of the samples and the mixtures were placed in a thermal cycler that performed the following steps: 98 ºC for 8 min and 64 ºC for 3.5 h.

To progress with the BS treatment, the Zymo-Spin IC Column was prepared by adding 600 µl of M-Binding Buffer. Afterwards, the sample was loaded into the column, inverted vigorously, centrifuged at 16.000 x g for 30 s and the flow-through was discarded. The sample was washed with 100 µl of the M-wash buffer and it was centrifuged at 16.000 x g for 30 s. To desulfonate the DNA, the sample was incubated for 20 min at room temperature after adding 200 µl of M-Desulphonation buffer. After the incubation, the column was centrifuged at 16.000 x g for 30 s. The BS converted DNA was washed twice by adding 200 µl of M-wash buffer, centrifuging it at 16.000 x g for 30 s and discarding the flow-through. Lastly, the column was placed into a 1.5 ml microcentrifuge tube, 10 µl of M-Elution Buffer were added directly to the column matrix and the sample was centrifuged for 30 s at 16.000 x g to elute the DNA. The BS converted DNA samples were analyzed with the Nanodrop to determine the concentration. Integrity of the treated DNA was evaluated by performing the UBC bisulfite assay (Van Poucke et al., 2017). The sequences of the UBC bisulfite primers are shown in *Table 3* and the thermocycling conditions used are listed in *Table 4*.

Table 3. *Sequences of the forward and reverse Bisulfite UBC primers and the expected amplicon lengths*

Name		Sequence	Amplicons length
UBC Bisulfite	Forward	GAARGAGTTTATTTTGTATTT	789, 561, 333 and 105 bp
	Reverse	TCACTAAACTCMACYTCC	

Table 4. *Thermocycling conditions used for UBC bisulfite assay*

Step	Temperature	Time
Initial Denaturation	95 ºC	14.5 minutes
40 cycles	95 ºC	30 seconds
	54 ºC	30 seconds
	72 ºC	2 minutes
Final Extension	72 ºC	5 minutes
Hold	15 ºC	

3.3.4. Primer design for methylation analysis and optimization on frozen tissue

At this point, the expression of the selected genes in embryos had not yet been analyzed via RT-qPCR, hence the choice of genes to analyze the BS conversion was done theoretically. The gene of interest was DNA (cytosine-5)-methyltransferase 1 (DNMT1). This maintenance gene belongs to the DNMTs family, which is a group of greatly conserved proteins, highly involved in the DNA methylation process. The FASTA sequence of this gene was retrieved from the NCBI database and the CpG islands were located *in silico* using the UCSC software. CpG islands are defined as stretches of DNA 500-1500 bp long with a CG:GC ratio higher than 0.6. Cytosines in CpG dinucleotides can be methylated to form 5-methylcytosine; in mammals, 70 % to 80 % of cytosines located in CpG islands are methylated (Jabbari & Bernardi, 2004). To design primers for the BS converted DNA, the CG an CNG sites were selected, given that cytosines in this position are susceptible to methylation. The other C were converted to T residues due to bisulfite conversion. The folding of this new sequence was checked using the m-fold software (RNA Institute at the University of Albany) and using the obtained information, the primers were designed using the NCBI Primer-Blast software and are shown in *Table 5*. The CG and CNG regions should be avoided in the primers because these positions could be methylated or not. If included, when designing it you have to include both options in order to have primers that can bind all options. To solve this, degenerated bases are used; Y equals C or T residues and R equals A or G residues. The same primers were also designed for the non-BS converted DNA to serve as a control, however, poor results were achieved at first instance. For this reason, these primers were redesigned to amplify a different amplicon but in this case, instead of amplifying the same exact fragment, only a partial matching sequence was set to be amplified. The primer designs were sent to Integrated DNA Technologies (IDT, Belgium) to be manufactured. Then, a gradient PCR was performed to determine the optimal annealing temperature. The conditions followed are showed in *Table 6*.

The PCR products were analyzed with 2 % agarose gel electrophoresis and were sequenced following the protocol shown in *Paragraph 3.3.6*. The obtained sequencing results were aligned and compared to the target sequences to assess the specificity of the designed primers. The BS converted and the original DNA amplified sequences were compared to verify the bisulfite treatment protocol.

Table 5. *Sequences of the designed primers for the original and bisulfite treated DNA for the DNMT1 gene. The original DNA primers had to be redesigned after obtaining poor results*

Type		Sequence	Product Length
Original DNA	Forward	CTGACCTCAAAGTAGAGCAAGC	273
	Reverse	GCGGCATCTGTCTCTGGA	
BS Treated DNA	Forward	GGYYGTTTGGYGTTAAAATG	280
	Reverse	AATACCCTCRRAAACCARACC	

Table 6. *Thermocycling conditions used for the gradient PCR for designed primers for the DNMT1 Gene*

Step	Temperature	Time
Initial Denaturation	95 ºC	15 minutes
40 cycles	95 ºC	30 seconds
	53 – 60 ºC	30 seconds
	72 ºC	1 minute
Final Extension	72 ºC	5 minutes
Hold	15 ºC	

3.3.5. Bisulfite conversion and primer test on bovine embryos

The DNA bisulfite conversion on embryos was performed out using the EZ DNA Methylation - Direct kit (Zymo Research, USA). The previously produced embryos were thawed and transferred into a tube. One µl of 10 µg/µl Proteinase K (Zymo Research, USA) and 7 µl of water were added into the tube to perform the digestion of the sample. The mixture was incubated for 3 h at 50 ºC. Then, BS conversion was executed following the protocol shown in *Paragraph 3.3.3*.

The designed primers were tested on the BS treated embryos following the optimized thermocycling conditions shown in *Table 8*. The PCR products were analyzed with 2 % agarose gel electrophoresis and were sequenced following the protocol shown in *Paragraph 3.3.6*. The obtained sequences were analyzed to assess the specificity of the primers and the BS treatment protocol in embryos.

3.4. Ethics and sustainability

In this study, ethics and sustainability guidelines were followed at all times: for the obtainment and manipulation of samples, and for the proper elimination of the material used, as well as the residues generated.

The obtainment of the ovaries and testicle was done at a regulated slaughterhouse from the Flanders Meat Group (FMG). Besides the controls imposed by the Belgian government (AFSCA), the FMG does further checks and analysis to guarantee the quality of the product and the animal welfare.

All the residues generated during the manipulation of the samples and the material used, were eliminated by disposing them in their specific container in order to avoid any harm to the laboratory facilities, environment or health.

4. RESULTS

4.1. Optimization of primers for transcript analysis by RT-qPCR

4.1.1. RNA isolation and cDNA synthesis from frozen tissue and quality check

Following RNA extraction, concentration and purity of the sample were measured using Nanodrop. The resulting concentration was 850 ng/µl, the A260/A280 value was 2.08 and the A260/A230 was 1.96, which is generally accepted as "pure" RNA. The presence of DNA contamination was evaluated by means of a routine PCR using UBC primers which can generate four amplicons of 821, 593, 365 and 137 bp long. The 137 bp amplicon was amplified, meaning that a slight DNA contamination was detected (*Figure 9*). Since the aim of this protocol was to generate a standard curve to assess the efficiency of the designed primers and not to quantify samples, the study was performed despite the genomic DNA contamination present in the sample. RNA integrity in the samples was assessed by analyzing 1 µg of RNA on a 2 % agarose gel. Partial RNA degradation was present in the sample, as shown in *Figure 9*.

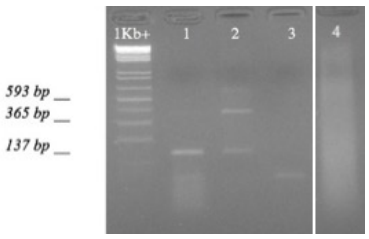


Figure 9. *Agarose gel showing the PCR product generated to verify the presence of DNA contamination in the RNA sample (1). Moreover, RNA degradation was also analyzed in the gel (4). The 1Kb+ DNA ladder (ThermoFisher Scientific, USA) was used.*

After cDNA synthesis, the product was amplified with UBC primers to verify the cDNA extraction. As shown in *Figure 10*, the 137, 365, 593 and the 821 bp amplicons were amplified and both, positive and negative controls were correct, meaning that the RNA sample was successfully converted into cDNA and that the quality of the RNA was good enough to produce cDNA amplicons of at least 800 bp.

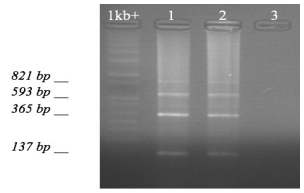


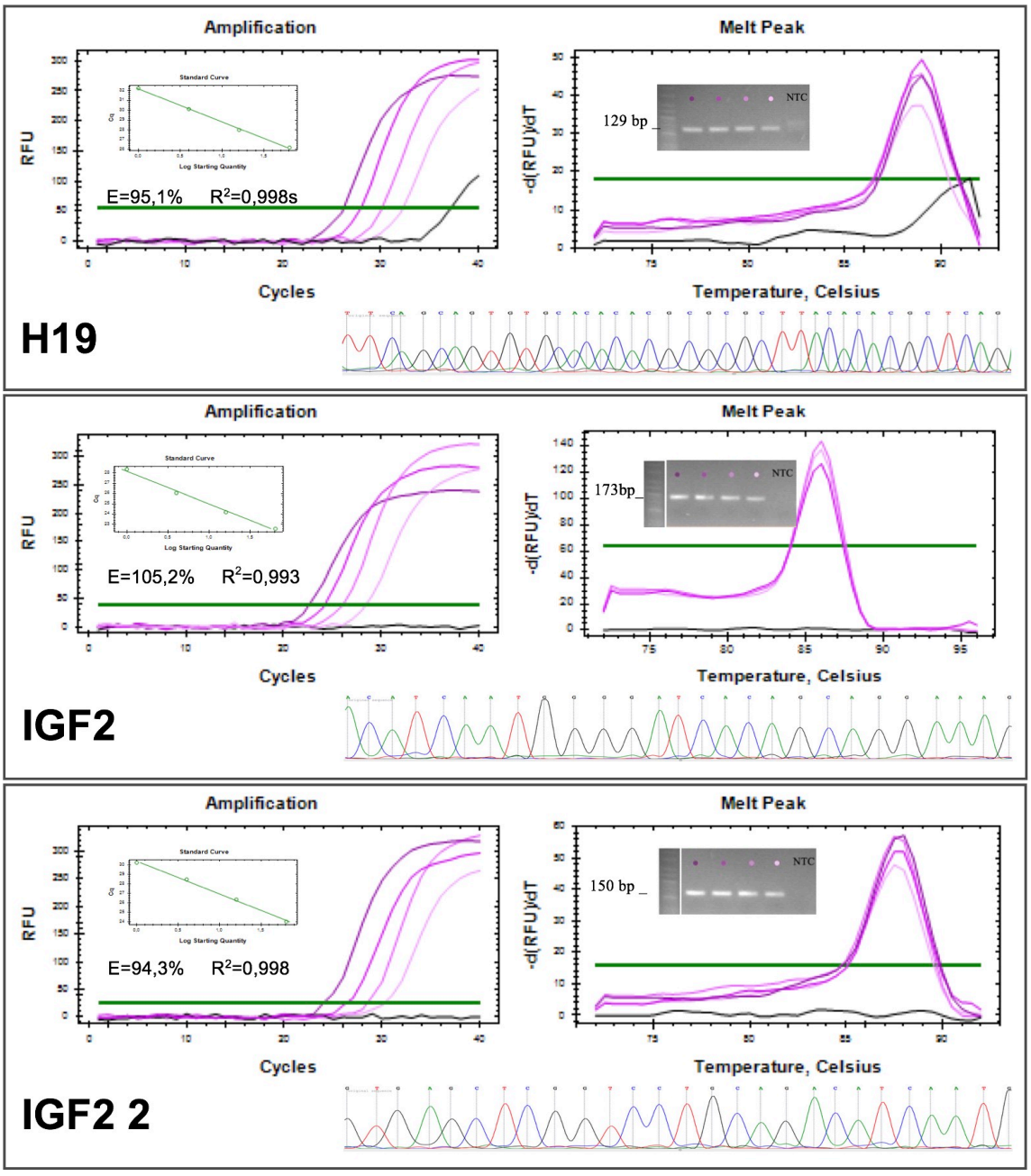
Figure 10. *The amplified cDNA sample (1), the positive (2) and the negative (3) control are shown in this agarose gel*

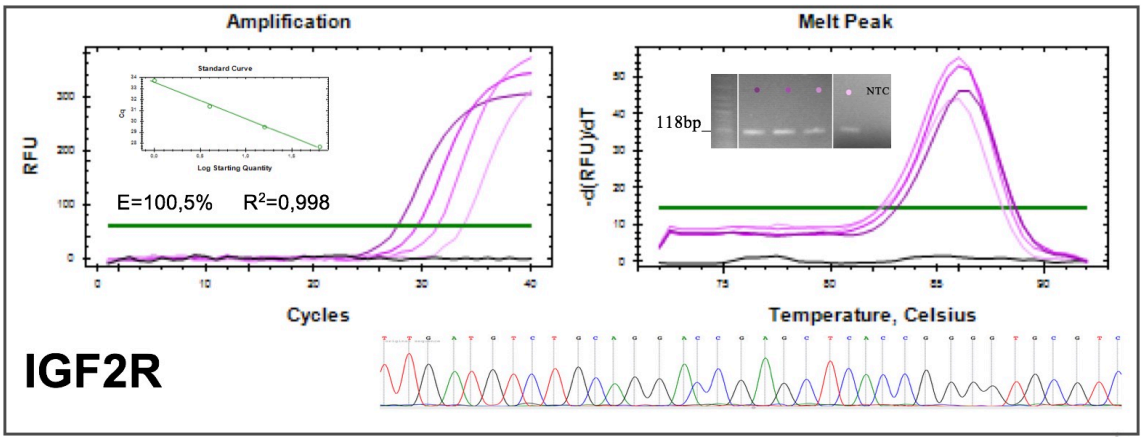
4.1.2. RT-qPCR

This study was based on a research that compares DNA methylation in different embryo samples. The genes to be compared had to be chosen ahead of time by gene transcript analysis via RT-qPCR in embryos. Before this, the primers to be used were tested and optimized as part of this project in bovine testicle tissue to avoid the misuse of *in vitro* produced bovine embryos, given its difficulty and high price of production.

The primer sets, shown in *Table 2*, were tested via RT-qPCRs and the products were analyzed by gel electrophoresis to verify the uniqueness and correct length of the resulting amplicons, as shown

in *Figure 11*. All the studied primers showed only one amplicon of the expected fragment length. Moreover, the obtained amplicons were sequenced and aligned to the target sequence using the Nucleotide BLAST tool, and all the sequences showed high identities values, meaning that the specificity of the studied primers was verified. In *Figure 11*, the obtained sequencing chromatograms are shown. The analysis of the amplicons on gel and the sequencing results were used to characterize the melt peaks obtained after each RT-qPCR run. To evaluate the performance of each primer set, a serial dilution of the target was analyzed, which generated a standard curve for each set. These standard curves are shown in *Figure 11* and were verified using the R^2 value, which was higher than 0,99 in all the cases. PCR efficiency was determined from the slope of the standard curves. The PCR standard curve efficiency was approximately 100 % for most of the primer pairs, as shown in *Figure 11*, meaning that in each cycle the amount of product doubled.




Amplification
Melt Peak
Standard Curve
Log Starting Quantity
RFU
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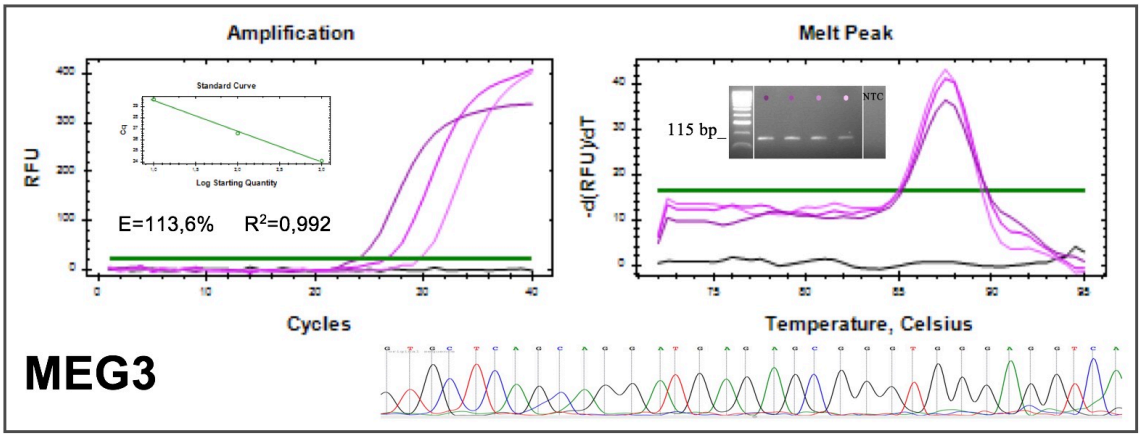
R²=0,998
Cycles
-d(RFU)/dT
118bp_
NTC
Temperature, Celsius
IGF2R


Amplification
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Standard Curve
Log Starting Quantity
RFU
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R²=0,992
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-d(RFU)/dT
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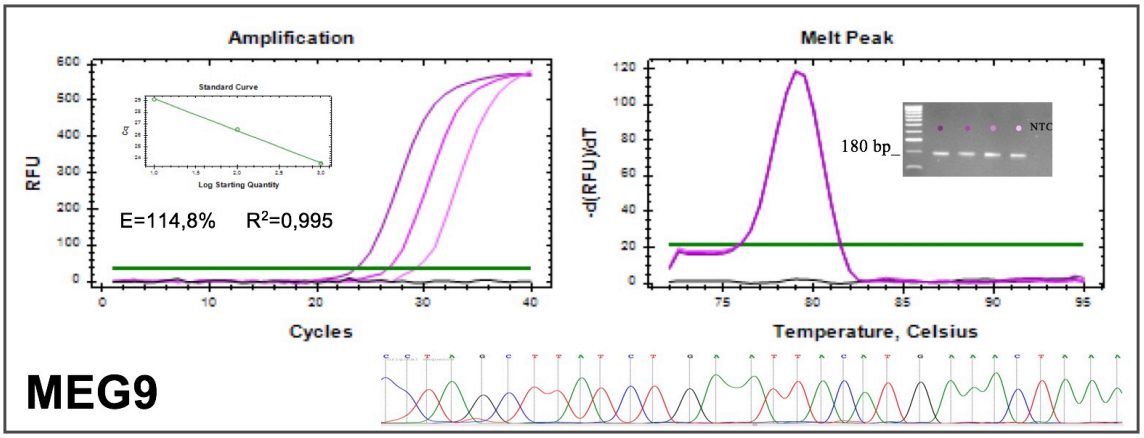
NTC
Temperature, Celsius
MEG3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Standard Curve
Log Starting Quantity
RFU
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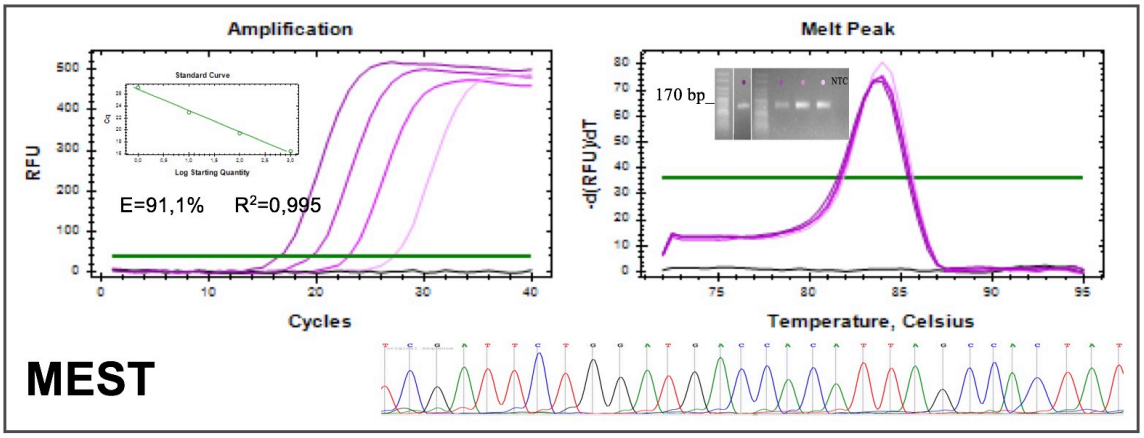
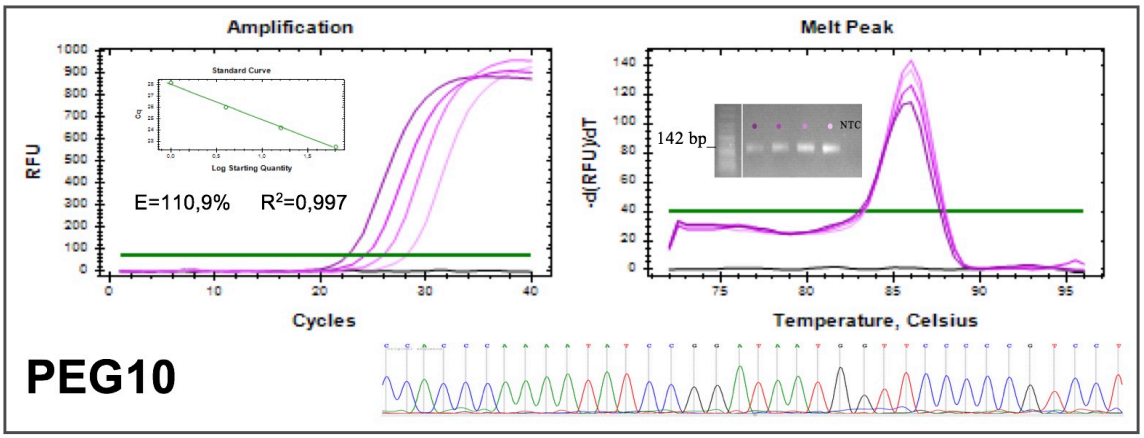
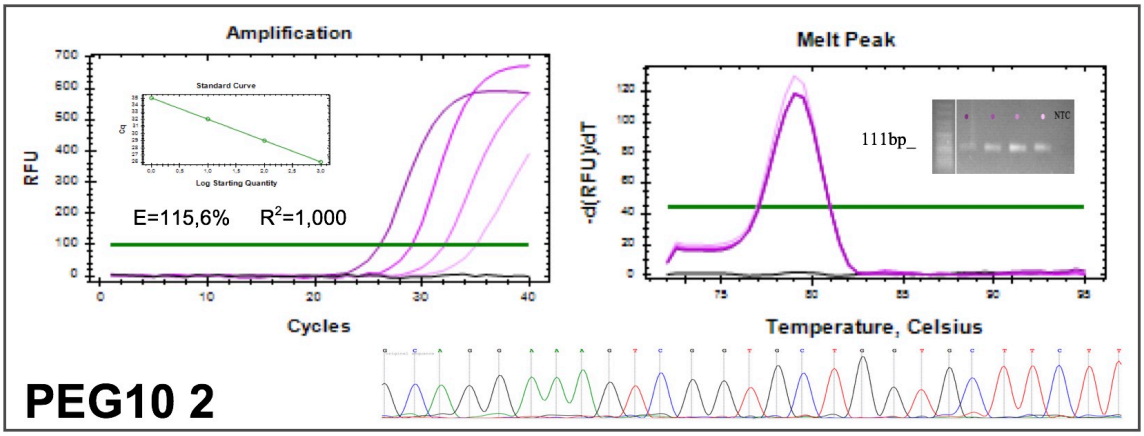
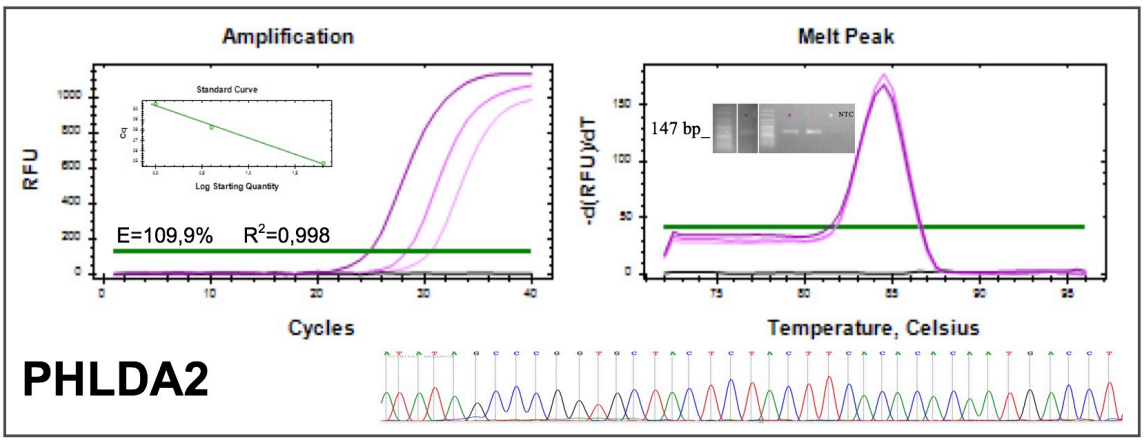
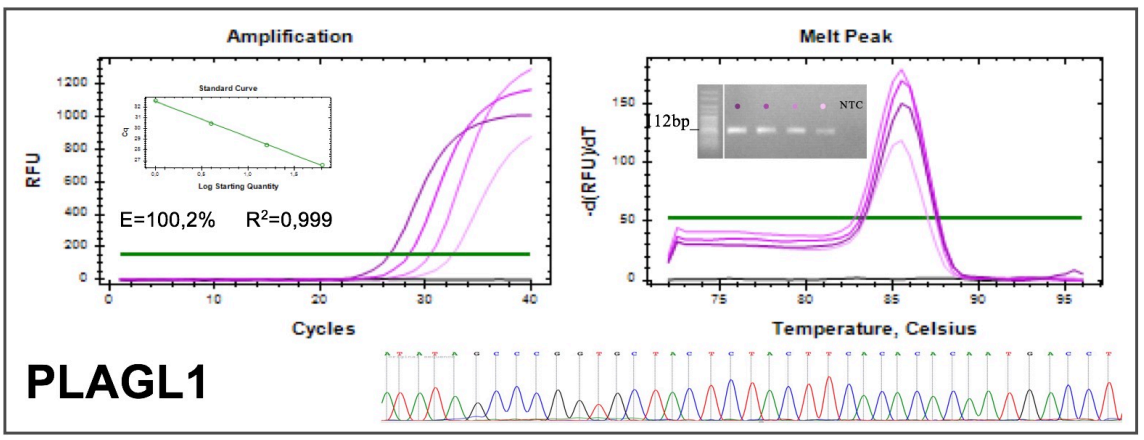
R²=0,995
Cycles
-d(RFU)/dT
180 bp_
NTC
Temperature, Celsius
MEG9

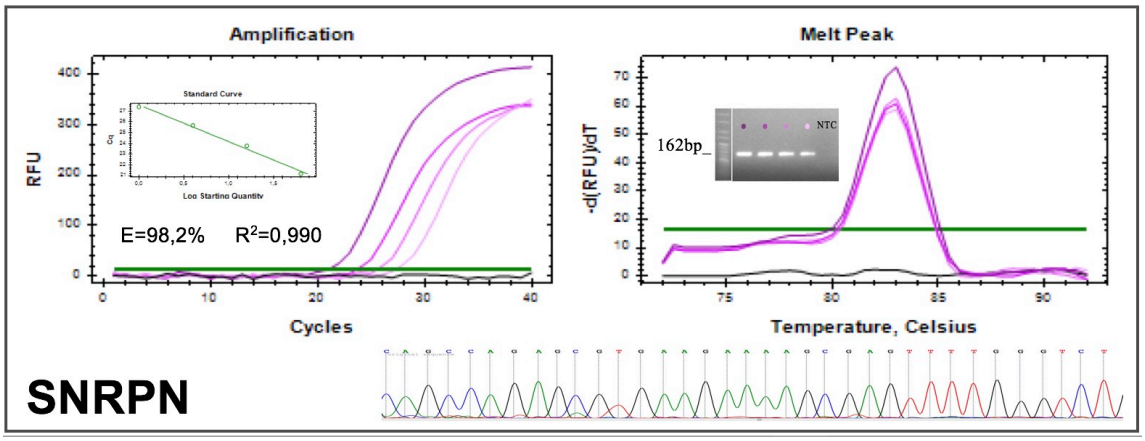

Amplification
Melt Peak
Standard Curve
Log Starting Quantity
RFU
E=91,1%
R²=0,995
Cycles
-d(RFU)/dT
170 bp_
NTC
Temperature, Celsius
MEST

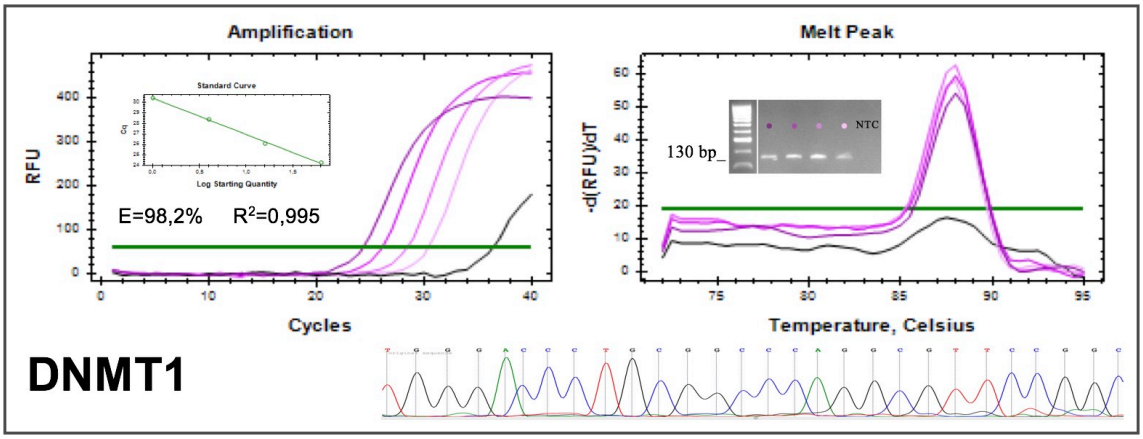

Amplification
Melt Peak
RFU
Standard Curve
Log Starting Quantity
E=110,9% R²=0,997
Cycles
-d(RFU)/dT
142 bp_
NTC
Temperature, Celsius
PEG10

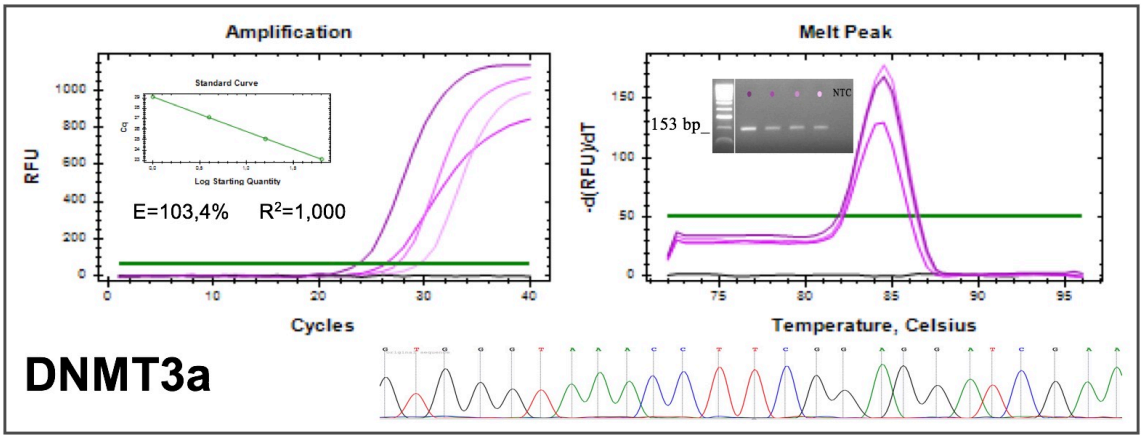

Amplification
Melt Peak
RFU
Standard Curve
Log Starting Quantity
E=115,6% R²=1,000
Cycles
-d(RFU)/dT
111bp_
NTC
Temperature, Celsius
PEG10 2

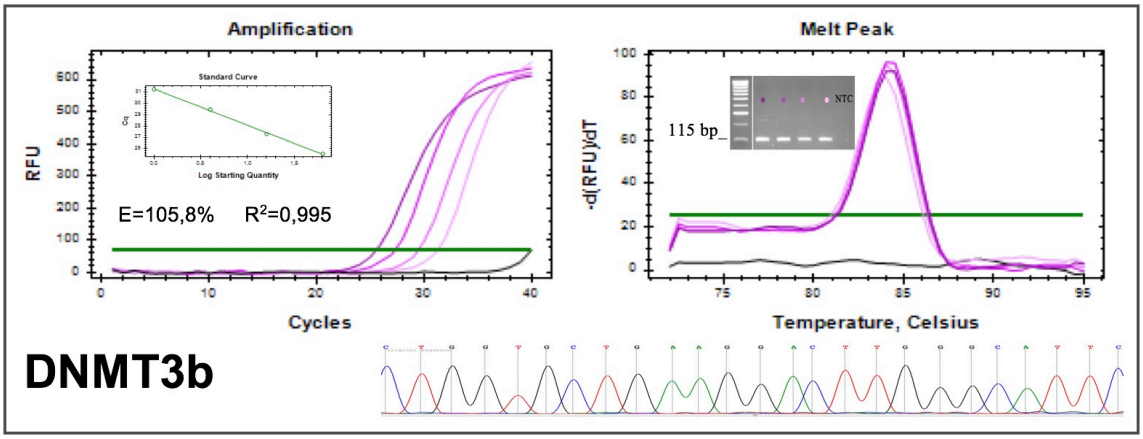

Amplification
Melt Peak
RFU
Standard Curve
Log Starting Quantity
E=109,9% R²=0,998
Cycles
-d(RFU)/dT
147 bp_
NTC
Temperature, Celsius
PHLDA2


Amplification
Melt Peak
RFU
Standard Curve
Log Starting Quantity
E=100,2% R²=0,999
Cycles
-d(RFU)/dT
112bp_
NTC
Temperature, Celsius
PLAGL1


Amplification
Melt Peak
RFU
Cycles
-d(RFU)/dT
Temperature, Celsius
Standard Curve
Log Starting Quantity
E=98,2%
R²=0,990
162bp_
NTC
SNRPN


Amplification
Melt Peak
RFU
Cycles
-d(RFU)/dT
Temperature, Celsius
Standard Curve
Log Starting Quantity
E=98,2%
R²=0,995
130 bp_
NTC
DNMT1


Amplification
Melt Peak
RFU
Cycles
-d(RFU)/dT
Temperature, Celsius
Standard Curve
Log Starting Quantity
E=103,4%
R²=1,000
153 bp_
NTC
DNMT3a


Amplification
Melt Peak
RFU
Cycles
-d(RFU)/dT
Temperature, Celsius
Standard Curve
Log Starting Quantity
E=105,8%
R²=0,995
115 bp_
NTC
DNMT3b

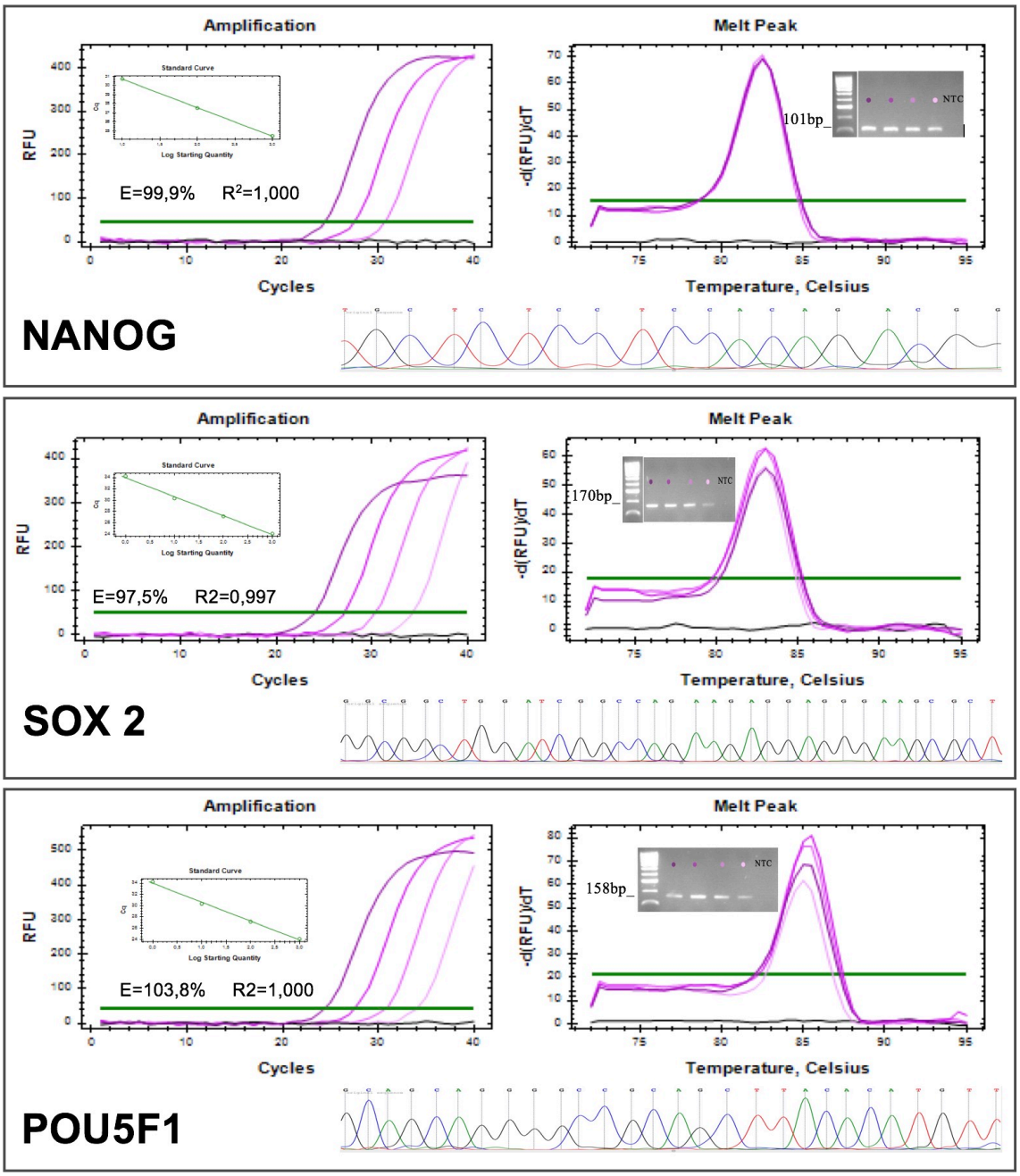


***Figure 11**. Analysis of the primers, consisting on the amplification curves obtained with 1/4 and 1/10 dilution series, the standard curves and the corresponding R^2 and efficiency values and the representation of the obtained melt peak and the agarose gel and sequencing chromatogram used to characterize the peaks.*

4.2. In vitro production of bovine embryos

Two groups of 19 and 17 zygotes were obtained. After incubating them for 24 hours in a Trigas incubator, the respective percentages of cleaved zygotes were 82 and 90 %. At day 7 post fertilization the blastocyst shown in *Figure 12* were analyzed and the blastocyst rate was determined and at day 8 after fertilization, 10 embryos were selected and frozen to optimize the oxidative bisulfite conversion protocol.

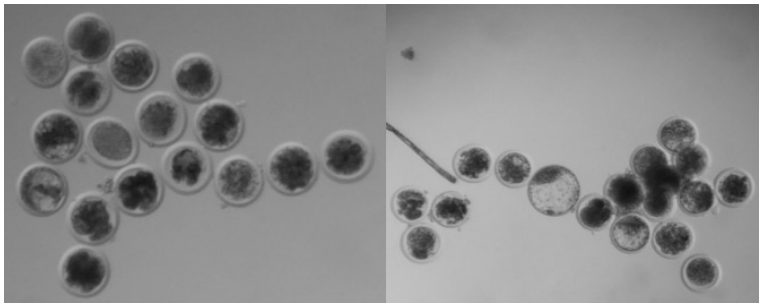


Figure 12. *Cultured bovine embryos on day 5 after insemination*

4.3. Design and optimization of primers for methylation

4.3.1. DNA isolation and oxidation from frozen tissue and quality check

DNA was isolated from frozen bovine testicle tissue using the phenol/chloroform method described in the paragraph 3.3.1. The quantity and purity were measured with Nanodrop and the results are shown in *Table 7*. Part of the DNA sample was used to test the oxidation step, since this process is extremely sensitive to contaminants, an extra purification on an Amicon filter was performed to investigate if it would substantially improve the quality of the DNA. Nanodrop analysis showed that this column concentrated 5 times the sample and increased the A260/A230 value from 1.41 to 2.19. Expected values of this ratio are commonly in the range of 2.0-2.2 (Geuther, 2007), if the ratio is appreciably lower than expected, it may indicate the presence of contaminants, for instance ethanol, which absorb at 230 nm. The extra purification step appeared to be necessary because the color of the purified sample remained orange after oxidation, while the color of the non-purified DNA sample changed from orange to brown/black as shown in *Table 7*. At this point, all the DNA extraction had been purified with the Amicon Column, part of it was oxidized and the other part was left non-oxidized.

Table 7*. Concentration and purity results, of the non-purified DNA sample and the purified sample using the Amicon filter, obtained by Nanodrop*

	Concentration ng/ µl	**Purity of DNA 260/280**	**Purity 260/230**	**Picture after oxidation**
Non-purified DNA	326,6	1,91	1,41	
Purified DNA	1631,1	1,88	2,19	

4.3.2. Bisulfite conversion

Bisulfite conversion was performed to the oxidized and the non-oxidized samples separately. After completing the treatment, both samples were quantified using Nanodrop. The protocol used aims for a percentage of DNA recovery of 80 %. The percentage of recovery for the non-oxidized sample was 77.4 % and for the oxidized sample it was 64.5 %. This result was recognized as being acceptable for further analyses because some level of degradation was expected after the bisulfite conversion.

Integrity of the DNA after BS conversion, was evaluated by performing the UBC bisulfite integrity assay on the oxidized and the non-oxidized BS-converted DNA. This assay can generate four amplicons of 789, 561, 333 and 105 bp long, depending on the integrity of the sample. The resulting agarose gel is shown in *Figure 13*.

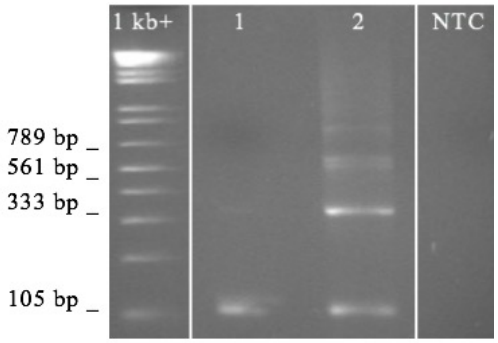


Figure 13. *Agarose gel from the UBC bisulfite integrity assay. The non-oxidized sample (2) does not show degradation. However, degradation is shown in the oxidized sample (1).*

Despite bisulfite conversion being known for damaging the DNA, our results on the non-oxidized sample showed the four expected amplicons, meaning that the sample had not been degraded. However, the oxidized sample showed degradation, as only the 105 bp fragment is clearly defined.

4.3.3. Primer design for methylation analysis and optimization on frozen tissue

Two sets of primers were designed, one for BS treated DNA and one for original DNA. These assays were optimized on testicle tissue by performing a gradient PCR to determine the optimal annealing temperature. The primers were tested on a sample of original DNA, a sample of BS treated DNA and a sample of oxidative BS treated DNA respectively. At 58 ºC the amplification was optimal and the obtained amplification is shown in *Figure 14*.

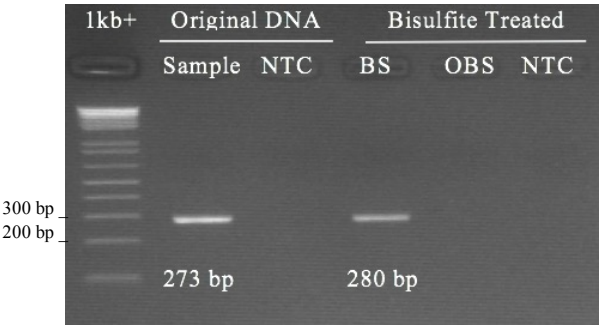


Figure 14. *Agarose gel resulting from the PCR amplification using the BS treated and the original DNA based primers. The BS treated set was tested on oxidized OBS) and non-oxidized (BS) BS treated DNA sample.*

The original DNA based primers successfully amplified the 273 bp desired fragment and the primers designed for the BS treated DNA were also successful in the amplification of the 280 bp desired amplicon, but only on the non-oxidized sample. This confirmed that the oxidization step had clearly degraded the sample and therefore, the oxidative step was not used to analyze the embryo samples.

To assess the specificity of the original and the BS treated DNA primers, the resulting amplicons were sequenced. After aligning the obtained sequences with the expected ones, both sets of

primers were verified. As shown in *Figure 15*, both sequences were compared to prove the efficiency of the BS treatment protocol. The fact that all non-CG/CNG cytosines present in the original DNA sample were read as thymines in the BS treated DNA indicated that the BS treatment worked. The methylation pattern of this bovine testicle tissue was also determined by analyzing the cytosines present in the original DNA sequence; if a cytosine is methylated, after performing the BS sequencing, this base will remain unaffected, because methylated C are resistant to BS conversion. After analyzing the 9 CG fragments and the 12 CNG fragments, no methylations were detected in the bovine testicle DNA.

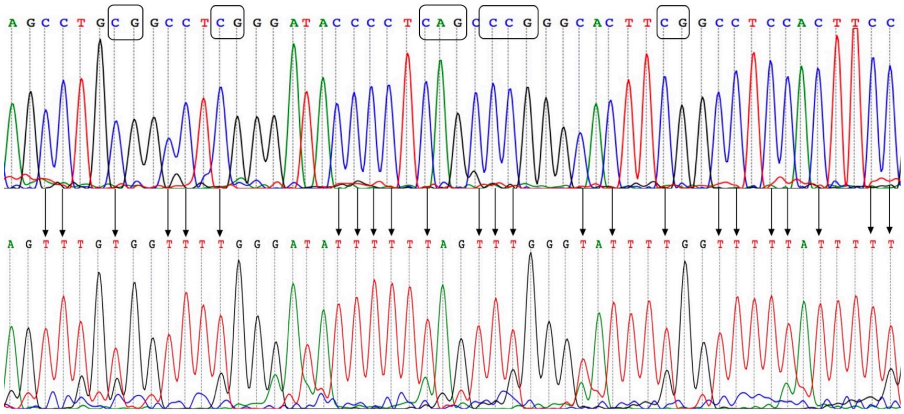


Figure 15. *Analysis of the obtained chromatograms via Sanger sequencing*

4.3.4. Bisulfite conversion and primer test on bovine embryos

The DNA extraction and BS conversion on embryos was carried out and it was tested by amplifying the original and the BS treated DNA with the optimized primers. Both amplifications were successful, since the correct length of the primer was shown in the agarose gel electrophoresis (*Figure 16).*

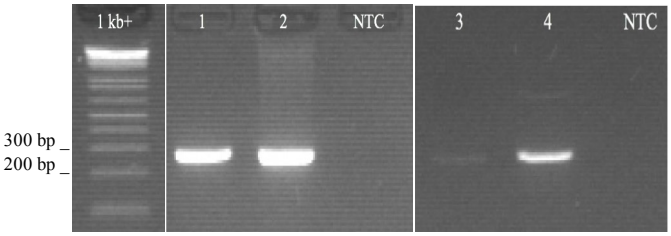


Figure 16. *Agarose gel from the original DNA primers (1) and the BS treated primers (3 amplification on embryos). The already tested original DNA and BS treated DNA from testicle were used as positive controls (2 and 3)*

The amplicons were sequenced and the analysis verified the efficiency of the BS conversion protocol and the specificity of the primers on embryos.

5. DISCUSSION

Primers for a battery of genes, to study the methylation pattern on, were optimized to be used in the project entitled "*Effects of sperm stressors on sperm methylation and subsequent embryo development in cattle*".

This optimization required high-quality cDNA and it was obtained from bovine testicle tissue instead of embryos, because working with these was more difficult and expensive. Extraction of RNA was a critical step towards the transcript expression analysis and to avoid DNA contamination, DNase was used to treat the isolates. Despite the Nanodrop results suggesting that the sample was pure of sugars, proteins and reagents, a slight DNA contamination was detected after PCR evaluation. Since the aim of this study was to verify primers via a standard curve and not to quantify samples, the present genomic DNA contamination did not affect the results. In future studies, to avoid DNA contamination, higher quantities of DNAse, longer incubation time or higher temperatures in this step could be used. In addition, a partial degradation of the RNA was found after analyzing the integrity of the sample, as the 18S and 28S ribosomal RNA bands could not be detected. However, after assessing the reverse transcription results with the UBC assay, it was concluded that high-quality cDNA was obtained. Our working conditions have been tested and are accepted in this laboratory. For this reason, we conclude that this degradation was not caused by not using a non-denaturing gel or RNAse denaturation conditions. Moreover, during the RNA isolation protocol, the sample was always kept on ice, gloves were changed frequently, RNAse-free reagents, tubes and tips were used and all the steps were performed quickly. The degradation could have happened during sampling, in future experiments, it would be important to make sure that the sample is quickly obtained and frozen immediately.

A dilution series was prepared from the obtained cDNA and used as a template to validate each of the transcript expression primer sets used in the present study. The melt peaks obtained after the RT-qPCR run were characterized by gel electrophoresis and sequencing. If in future experiments, the same peak is obtained, we will know the characteristics of the resulting fragment without the need to analyze it again. Based on the standard curve obtained after performing the RT-qPCR, the efficiency was also assessed since it is an indicative that the primers are properly designed and therefore, that can be used to calculate the expression level. The obtained values were between 90 % and 115 %, meaning that the used primers performed as expected; after each cycle the amount of product was approximately doubled. After assessing the specificity and the efficiency of the primers, it was verified that all the studied assays could be used to quantify transcript expression in embryos, but in future studies with the aim to quantify one of these genes, interpolation within the dynamic range will be accurate but extrapolation beyond this range will not be valid. If the Cq values obtained are found outside the studied dynamic range, the dilution series would have to be adapted to avoid extrapolations.

The expression of the studied genes had already been confirmed in cattle in previous studies, but not specifically in testicle tissue. This worked certified the expression of these genes in bovine testicle. In addition, two different sets of primers were designed to amplify different transcript variants of the IGF2 and the PEG10 genes. These transcripts were only considered predictions before the test, but after performing and analyzing RT-qPCR, the presence of these transcript variants was confirmed in bovine testicle.

These assays were optimized with the aim to find differential expression via RT-qPCR in different genes and therefore, only study the methylation of the genes showing differential expression. Since this experiment had not been concluded at time of the development of this thesis, one of the previously studied genes was chosen to optimize the bisulfite sequencing protocol. This method is used to determine the methylation pattern by converting all the C residues to U unless these are methylated or have other modifications. Even though these other modifications are not usual and most of the time do not affect the results, an oxidative step was tested to discriminate between 5mC and 5hmC. DNA quality is an important factor that can affect the efficiency of this step because any trace of a contaminant in the sample could inhibit the oxidation process. Most bisulfite conversion protocols use kits to isolate the DNA with the aim to extract pure and higher quality DNA. In this case, DNA from testis was successfully extracted using the phenol/chloroform extraction protocol, which is more accessible than the kits. In addition, the need of using an Amicon Ultra Column to purify the sample was confirmed by assessing the A260/A230 ratio value and the color of the sample.

The oxidation protocol had to be tested as it had never been performed in our laboratory. Part of the testicle DNA sample was oxidized and the BS conversion protocol was carried out simultaneously in this sample and in non-oxidized DNA. After performing the UBC bisulfite integrity assay on both BS treated samples it was determined that the oxidation step degraded the sample to an extent that it could not be used for further experiments. In the future, a new study could be developed to revert this situation and reduce the DNA degradation after the oxidation step, since in this case, the protocol was followed correctly by maintaining the oxidation sample in iced water, using high quality DNA and assessing the sample color to detect contaminants that could inhibit the oxidation.

The verification of the BS conversion was carried out by amplifying the sample with a pair of specific primers and sequencing the resulting amplicon. Two sets of primers were designed based on the DNMT1 gene, one for BS converted DNA and one for original DNA, to amplify the same fragment. Once the obtained amplicons had been accordingly sequenced, both sequences were compared and the BS conversion protocol was verified because all the every non-CG/CNG C were read as T residues.

Moreover, the analysis of the sequencing results also corroborated the specificity of the designed primer sets based on the DNMT1 gene. The methylation pattern of this gene in bovine testicle DNA was assessed and it was determined that in the analyzed fragment there were no DNA methylations present. This result cannot be extrapolated to the whole gene because the amplified fragment solely consisted of 164 bp and only 9 CG and 12 CNG regions were analyzed.

To conclude the experiments, the BS sequencing protocol was tested and verified on *in vitro* produced embryos by using the optimized primers.

6. CONCLUSIONS

- High-quality bovine testicle cDNA was obtained and it can be used in the future to optimize different assays via standard curve with RT-qPCR.
- The melt curves of all the studied primers were characterized, and in future experiments, gel electrophoresis and sequencing won't be needed.
- The presence of all the studied genes was confirmed in bovine testicle and all the optimized primers were verified and can be used in future studies to quantify transcript expression in embryos.
- Although the oxidation step was not successful, the optimization of the bisulfite conversion was achieved in bovine testicle and embryos.
- The designed and optimized assays for BS treated DNA and original DNA based on the DNMT1 gene can be used in future studies to analyze the methylation pattern in bovine embryos.

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