QUANTIFICATION OF EXPRESSION PATTERN OF SOME IMPORTANT HOUSEKEEPING GENES DURING *IN VITRO* MATURATION OF BUFFALO OOCYTES

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

MASTER OF VETERINARY SCIENCE
IN
DAIRYING
(ANIMAL BIOTECHNOLOGY)

BY
AJAY PAL SINGH ASWAL

ANIMAL BIOTECHNOLOGY CENTRE
NATIONAL DAIRY RESEARCH INSTITUTE
(I.C.A.R.)
KARNAL-132001 (HARYANA), INDIA
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This is to certify that the thesis entitled, **"QUANTIFICATION OF EXPRESSION PATTERN OF SOME IMPORTANT HOUSEKEEPING GENES DURING *IN VITRO* MATURATION OF BUFFALO OOCYTES"** submitted by **Dr. Ajay Pal Singh Aswal** towards the partial fulfilment of the requirements for the award of the degree of **MASTER OF VETERINARY SCIENCE IN DAIRYING (ANIMAL BIOTECHNOLOGY)** of the **NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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Ajaypal Singh Aswal

(Ajay Pal Singh Aswal)

Abstract

In the present dissertation, work was taken up with two principal objectives of standardizing a method of RNA extraction from buffalo oocytes to study the expression pattern variation of house keeping genes (HKG) and also to establish the stability of expression pattern of these genes in course of their in vitro maturation. Buffalo oocytes were collected from the ovaries of slaughtered animals and RNA was extracted to generate cDNA following RT. Temporal expression stability of two house keeping genes viz. 18s rRNA and G3PDH were assessed using q-RTPCR and competitive RTPCR approach over the period of 24 hours during in vitro maturation. Using standardized parameters for q-RTPCR both 18s rRNA and G3PDH genes were found to be expressed in a stable manner but 18s rRNA was interpreted as a better candidate house keeping gene than G3PDH for its use as internal standard in relative q-RTPCR method. A composite primer strategy was developed to generate homologous cDNA competitor for 18s rRNA and used in a competitive RTPCR protocol to quantitate expression pattern variability of 18s rRNA. The competitive RTPCR approach followed was found to be valid for absolute quantification of 18s rRNA with high level of confidence. The competitive quantitative RTPCR method also confirmed the stability of the expression pattern of 18s rRNA in the course of in vitro maturation of oocytes in buffalo.

सारांश

यह शोधकार्य दो मुख्य उद्देश्यों के साथ किया गया है आर.एन.ए. को भैंस के अंडाणु निकालने की विधि स्थापित करना और हाउस कीपिंग जीन की अभिव्यक्ति में बदलाव और उन ान की अभिव्यक्ति में स्थिरता को परखनली परिपक्वन के दौरान स्थापित करना। भैंसों की अंडाशय अंडाणु को निकालकर आर.टी. के द्वारा सी.डी.एन.ए के निर्माण के लिए आर.एन.ए. को निकाला या तथा परखनली परिपक्वन के चौबीस घंटे के दौरान दो हाउस कीपिंग जीन के समय के साथ भिव्यक्ति की स्थिरता में बदलाव का अध्ययन आर.टी.पी.सी.आर और सी.आर.टी.पी.सी.आर कनीक के द्वारा किया गया। क्यु.आर.टी.पी.सी.आर के लिए स्थापित मापदंड के अनुसार 18 एस. ार.आर.एन में और जी 3 पी.डी.एच जीन की अभिव्यक्ति में स्थिरता पाई गई। परन्तु क्यू.आर. .पी.आर. से सम्बन्धित आंतरिक स्टैंडर्ड के प्रयोग के लिए जी 3 पी.डी.एच. की अपेक्षा 18 एस. ार.आर.एन.ए. जीन को ज्यादा उपयुक्त पाया गया। 18 एस.आर.आर.एन.ए. के सामानार्थी सी. .एन.ए. बनाने के लिए सम्मलित प्राईमर की रचना की गई और उनका उपयोग ु.आर.टी.पी.सी.आर. विधि में 18 एस.आर.आर.एन.ए. जीन की अभिव्यक्ति में बदलाव को त्रित करने के लिए किया गया। इस कार्य में यह भी पाया गया कि क्यु.सी.आर.टी.पी.आर. विधि 18 एस.आर.आर.एन.ए. की शुद्ध मात्रा को निर्धारित करने के लिए ज्यादा विश्वसनीय है। भैंस अंडाणु का परखनली परिपक्वन के दौरान 18 एस.आर.आर.एन.ए. के अभिव्यक्ति की स्थिरता ा क्यु.सी.आर.टी.पी.आर. विधि द्वारा प्रमाणित किया गया।

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List of Abbreviations used

BSA	Bovine Serum Albumin
cDNA	Complementary Deoxyribonucleic Acid
DNA	Deoxyribonucleic Acid
DPBS	Dulbecco's Phosphate buffer saline
EtBr	Ethidium Bromide
FSH	Follicle Stimulating Hormone
G3PDH	Glyceraldehyde-3-Phosphate dehydrogenase
hn-RNA	Heteronuclear Ribonucleic Acid
ISH	In Situ Hybridization
mRNA	Messenger Ribonucleic Acid
NB	Northern Blot
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
qc-RT-PCR	Quantitative competitive Reverse Transcriptase Polymerase Chain Reaction
Q-RT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
RPA	Ribonuclease Protection Assay
r-RNA	Ribosomal RNA
RT	Reverse Transcription
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
HKG	HouseKeeping Genes
IVM	In vitro maturation
IVF	In vitro fertilization
DEPC	Diethylpyrocarbonate

1. INTRODUCTION

The intensive research efforts on Embryo Transfer Technology per se and Embryo Biotechnologies in general have emerged as a potential tool for faster multiplication of superior germplasm and incorporating genetic modifications in livestock species for bringing in desirable qualities or for using the animal system as a bioreactor for production of useful proteins. In vitro fertilization (IVF) constitutes an important area in 'Embryo Bio-Technology', which has been demonstrated to be feasible in producing live offspring in a variety of livestock species. India is considered as a country of world's best buffalo germplasm. Out of total milk produced in India cow accounts for 39.70% whereas buffalo contributing about 54.47% of the total milk even though the buffalo population is very low (96.62 million) than indigenous and cross bred cattle (187.38 million) population in country (Govt. of India Report, 2003). However, the overall success rate of embryo production following IVF method and more specifically their subsequent development rate have remained low, which presents a principal bottleneck for the successful implementation of high-end embryo biotechnologies. The oocytes obtained from ovarian follicles vary in terms of their maturation competence depending upon their physiological status. The maturation of the mammalian oocytes is about to be redefined by the complexity of the molecular changes that occur during this period. The fine-tuning of gene expression and accumulation of crucial mRNA and proteins are important determinants of the future ability of an egg to become an embryo. Persistent alterations of the normal gene expression patterns under in vitro condition may be responsible for poor development competence of IVF embryos resulting into lower implantation rates. It has been recently found that the basic culture system along with the various kinds of supplementations had pronounced effects on transcript levels of a series of developmentally important genes and *"messenger RNA phenotyping"* and studying expression pattern of genes, essential in early development will provide a useful tool to assess the IVF process and optimizing the in vitro culture conditions for embryo production following IVF. In mammals, the study of gene expression in the oocyte has been difficult because the limitation inherent to the molecular approaches namely paucity of the amount of starting material.

Quantitative measurements of mRNA levels can be achieved using several approaches such as northern blotting, in situ hybridisation, RNAse protection assay, reverse transcription polymerase chain reaction (RT-PCR); however, some of these approaches such as Northern blot analysis and RPA are not suitable when working with oocytes because of the less sensitivity and the large amount of starting material needed to obtain enough RNA to perform

these techniques. The reverse transcriptase polymerase chain reaction (RT-PCR) has provided new tools for exploration of gene expression in oocytes because of its high sensitivity, suitability for quantifying rare transcripts and its ability to analyze a slight change in abundance of developmentally important transcripts. However all aforesaid techniques including real time PCR requires a standard which is essential for normalization. The normalization is the process of correcting the sample for differences in quantity & quality caused by variation in initial sample amount, variation in RNA recovery, possible RNA degradation of sample material, differences in sample and/or nucleic acid quality, tube to tube variation in RT reaction, variation in sample loading / pipetting errors and variation in cDNA synthesis efficiency. The relative quantification using RT-PCR need a standard against which the quantification of target transcript in PCR products are visualised on a gel usually stained with ethidium bromide and the relative band intensity is normalised to that of the standard also called as control. The quantity of the RT-PCR product is determined by comparing the product obtained from the standard with the product of the gene of interest. The expression pattern of the endogenous standard, often referred as a housekeeping gene, for which the mRNA level is considered however, has to be constant to make the quantitation results valid. However, expression pattern of different house keeping genes have been reported to vary in different tissues. So to validate their use as internal standard in oocytes studies, their expression pattern has to be established as stable in oocytes temporally and with respect to different culture conditions during in vitro maturation. The search for such a stable house keeping gene in oocyte context is still on and this area has not been evaluated at all in buffalo IVM/ IVF studies. Since the PCR based RT-PCR methods are error prone so a validation is needed by use of another approach where absolute quantification is possible. With this background information in mind and looking at the importance of buffalo in Indian dairy economy the present study was undertaken with following objectives.

Objectives of the project:

1. To standardize the process of RNA extraction and quantitative RT-PCR for mRNA transcripts of selected house keeping genes in buffalo oocytes.
2. To find out the expression pattern variation of selected house keeping gene's transcripts in oocytes during in vitro maturation.

2. Review of Literature

The oocytes and embryos present one of the most interesting subject to study the events of gene expression because these cells form the very basis of a highly specialized, accurate and orderly cellular development process leading to the formation of fully developed multicellular organism and any alteration during the course of the chronology of these events leads to either termination of the developmental process or more seriously to the development of an malformed organism (Young et al., 1998). Since the time that it was made possible to mimic some of the early developmental process under purely in vitro conditions following in vitro fertilization (IVF) procedure the importance of gene expression studies have gained further momentum because ultimately any given protocol of successful IVF demanded a certification for expression of right genes, in right order, in right magnitude and in right time (Eichenlaub et al., 2002; DeSousa et al., 1998). The in vitro maturation of oocytes (IVM) in the cascade of events leading to IVF presents a challenging step because the events happening during IVM have been demonstrated to affect not only the process of fertilization but the cell divisions during early cleavage, blastocyst formation and implantation are dependent on the genes expressed during this time (Knijn et al., 2002). In context of in vitro production of embryos, oocyte quality is the first parameter that determines the success rate of IVF (Blondin and Sirard, 1995). High developmental competence of oocytes is reported to be dependent on normal follicular and oocyte growth, its maturation and gene expression pattern (Desouza et al, 1998). The essential developmental function of an oocyte is to transfer both paternal and maternal genomes in to a single totipotent functional zygote. The extra large size of oocytes is related to the requirement for an abundant store of biomolecules such as proteins, and mRNA in order to sustain oocytes maturation and early embryonic development after fertilization till embryonic transition of genetic control takes place (Dalbies et al., 2003 a). The present review presents description of work done with respect to gene expression studies and expression studies in mammalian oocytes in particular.

2.1 Different methods available to quantify levels of 'Gene Expression':

The characterization of gene expression pattern in different cells and tissues is a highly informative way of providing insight into cell's development, maintenance, cell-to-cell interaction and behavior of a cell in a given environment. The generation of mRNA molecule is an important consequence of the transcription process and the abundance of RNA transcripts varies in tissues at different developmental stages and also at different states of physiological

and functional status. The level of transcription varies with the induction or repression under influence of different external or internal stimuli. It also varies at different stages of cell cycle as the cell progresses from resting/ quiescent to functional status and in that process ultimately reaches to apoptosis/ senescence. Fundamentally different methods available to detect mRNA expression pattern utilize the principle of specific hybridization of a complementary DNA or RNA strand present in the cell with its counterpart, often called as a probe molecule. Several methods such as Northern Blot (NB), in situ hybridization (ISH), Ribonuclease Protection Assay (RPA) and Reverse Transcription Polymerase Chain Reaction (RT-PCR) have been developed to analyze varying cellular distribution and concentration of mRNA transcripts. Studies carried out on gene expression in different contexts involve the comparison of mRNA population between two samples viz. treated versus untreated, or among different developmental stages.

2.1.1 Northern Blotting: Northern Blotting is the counterpart of the 'Southern Blotting' technique. It involves the quantification of total RNA or poly $(A)^+$ mRNA isolated from cells or tissue(s) of interest. Following this method the RNA samples are size fractionated using denaturing formaldehyde/agarose gel electrophoresis followed by transferring the RNA product from gel to a membrane usually by capillary transfer. The membrane is then hybridized with a labeled DNA or RNA probe complementary to the RNA of interest (Alwine et al., 1997). Washing is done to remove nonspecific labels. The hybridization signal is generally detected by exposing blots to X-ray film or phosphor storage plates or by staining with ethidium bromide. (Sambrook et al., 2001; Brown and Mackey, 1993). The resulting signals/bands identified by the probe indicate the size of mRNA and the intensity of the band corresponds to its relative abundance. Autoradiographed band intensities may be quantified by densitometry, phospherimaging or scintillation counting of excised bands. The Northern blotting is often used as a key method of mRNA size determination. However, as a quantitative technique the Northern Blot presents several limitations such as it is not suitable for determining the concentration of a relatively rare mRNA transcripts, sensitivity of the method is low, and it involves technical uncertainty in the efficiency of RNA transfer and membrane binding as well as ill defined factors that often affect kinetics of nucleic acid hybridization on a solid support (Reue., 1998).

In the simplified Northern blot modification known as dot or slot blot, the RNA samples or cell extracts are directly applied to the membrane without prior size fractionation on a gel. The samples are deposited onto a filter in a fixed array of dots or slots by use of vacuum filtration. After hybridization and detection by autoradiography, signal may be quantified by densitometry, phospherimaging or scintillation counting of spots (signals). However in bypassing the

fractionation step of a classical Northern blot protocol the accuracy of a dot blot assay is often compromised in terms of false positive results obtained by non-specific hybridization signals (Kafatos et al., 1979).

2.1.2 Ribonuclease Protection Assay (RPA): In RPA unlike northern blotting the hybridization is performed within a solution containing both labeled antisense RNA probe and target mRNA without prior gel fractionation or blotting. After incubation for several hours' unhybridized probes and sample RNA are enzymatically digested by RNase treatment (Azrolan et al., 1990). The probe-target hybrids are then electrophoresed through a denaturing polyacrylamide gel and visualized by autoradiography or phospherimaging. Alternatively the hybrids are precipitated and then bound to filter for direct quantification. Over an improvement to the Northern Blot technique the RPA is a method that provides better sensitivity, rapid and robust quantitative measurements. The two major strength of the RPA are sensitivity and the ability to determine absolute mRNA levels. It offers at least 10 fold higher sensitivity than Northern blot analysis allowing the detection of low abundance mRNAs (Sambrook et al., 2001). Even partially degraded cellular RNA can be analyzed for expression quantification following RPA. On contrary, the feature of absolute size determination of target RNAs is compromised and extremely rare transcripts cannot be quantified by this method. Moreover it requires expert operation, use of radioisotopes and extensive handling risking loss of quantifiable RNA. As with blot techniques, a meaningful quantification of targeted RNA require the concurrent hybridization of an invariant control mRNA to allow normalization between samples. However RPA remained, as one of the most sensitive non-PCR based method for quantification of gene expression (Meisinger and Grothe, 1996; Genovese et al., 1989).

2.1.3 In Situ Hybridization:

The technique of In Situ hybridization (ISH) was originally developed by two independent groups in 1969 (Paradue and Gall, 1969). The principle behind ISH is the specific annealing of a labeled nucleic acid probe to its complementary sequences in fixed tissue or cells, followed by visualization of the hybridization signal with radioactive decay, fluorescent signals or enzyme histochemistry. The critical aspect of the procedure is that the target nucleic acid be presented in situ and be accessible for hybridization to the probe. The RNA in situ technique is used for examining mRNA expression in tissues and to localize specific RNA expression at the individual cell levels. Unlike other technique for RNA analysis such as Northern Blot, RPA and RT-PCR which require the homogenization of the tissue or cell sample the ISH procedure is done on the intact tissue sample itself and thereby gives information about the expression level localization as well. The labeled probes used in ISH are either cDNA or RNA probe (Hudson et

al., 1981). The ISH has been used to analyze gene expression under different experimental or physiological conditions and within different tissues (Gerfen., 1989). Fluorescence in situ hybridization has been applied to RNA in situ hybridization on tissue sections with the introduction of the fluorescent dye, propidium iodide as a counter stain to allow histological orientation (Wuif et al., 1995). RNA in situ hybridization is a powerful tool in examining tissue distribution of gene expression in development, allowing serial reconstruction for the precise structural identification of the rapidly changing complex embryo anatomy with the use of computer image analysis (Wilkinson and Green, 1990).

2.1.4 Real Time PCR:

The real time fluorescence based reverse transcription (RT-PCR) enables the simultaneous amplification and detection of specific DNA sequence and thereby eliminating the need for post-PCR processing. It monitors the amounts of DNA produced during each PCR cycle and its sensitivity, specificity and wide dynamic range has revolutionized the approach to PCR based quantification of RNA, it is the method of choice for quantifying steady state RNA and mRNA levels (Bustin,2000). The original system involved the addition of an intercalator, ethidium bromide to the PCR reaction (Higuchi et al., 1993) and a thermal cycler with an ultraviolet light source and a computer controlled charge coupled device CCD camera (Higuchi et al., 1992). A plot of increased fluorescence caused by ethidium bromide binding to newly synthesized DNA against cycle number allows the calculation of the quantity of starting material. Currently, the use of fluorescent dye combines the process of amplification and detection of an RNA target to permit the monitoring of PCR reaction in real time during the PCR. The simplest method uses fluorescent dye e.g. SYBR Green that binds specifically to double stranded DNA. The unbound dye exhibit little fluorescence in solution but during elongation increasing amount of dye binds to the nascent double stranded DNA. When monitored in real time this results in an increase in fluorescence signal that can be observed during polymerization step and that falls off when DNA is denatured. Fluorescence measurements are then performed at the end of elongation step of every PCR cycle to monitor the increasing amount of amplified DNA (Morrison et al., 1998). The alternative probes to SYBR green are Taq Man probe, Molecular beacons (Bustin, 2000) and Scorpions (Whitcombe et al., 1999).

2.2 Methods used to study the 'Gene Expression' pattern during IVM/ IVF

2.2.1 Availability and processing of RNA in oocytes:

The cellular activities start in oocytes in the prenatal life itself when they enter in the process of meiosis after an extensive phase of mitotic divisions. In postnatal life and till the time that the female reaches its age of sexual maturity the oocytes have the capacity to remain dormant

(meiotic process suspended at prophase-I/ dictyate stage) for years without compromising its ability to resume meiosis and growth. The ability of an oocyte to develop into a viable embryo depends on the accumulation of specific information and molecules in the form of maternal mRNAs and proteins synthesized during oogenesis (Gandolfi and Gandolfi, 2001). In the primordial follicle, the oocytes appear transcriptionally quiescent and synthesis of both ribosomal and heteronuclear RNA (hnRNA) is first detected in the secondary follicle. Transcription become extremely intense within tertiary follicle until oocytes reaches to a diameter of 110µm and is enclosed in a 2 to 3 mm follicle (Fair et al., 1997). After this stage only a low-level hnRNA synthesis is observed which can be detected up to GV stage while at metaphase-II a sharp decrease is observed (Memili and First, 2000). After germinal vesicle breakdown, gene expression is mainly under posttranscriptional control, which involves differential degradation, stabilization, storage of transcripts and their timely recruitment by the translation machinery. Minor embryonic genomic activation has been detected as early as the 2- cell stage but the major activation i.e maternal to embryo transition occurs at a 8/16 cell stage in bovines (Desouza et al, 1998, Memili et al., 2000).Studies on bovine oocytes suggest that the developmental competence of oocytes based on the morphological criteria differ substantially (both quantitatively and qualitatively) from the point of view of their mRNA content (DeSouza et al., 1998; Blondin and Sirard, 1995). The pool of mRNA present in oocytes sustains a highly orchestrated program of gene expression for its own development, maturation and fertilization as well as for early embryonic growth (Dalbies et al, 2003 b). Oocyte as such is a large cell presenting a diameter of 100µm in most mammals typically 10 fold larger that that of a somatic cell (Dalbies and Mermillod, 2003). They remain transcriptionally hyperactive during maturation and inherit a large quantity of preformed mRNA suitably modified to confer extra stability to these transcripts (Dalbies et al., 2003 b). It has been shown that oocyte's competence to sustain embryonic development depend upon the efficiency of such storing process viz. relative abundance of developmentally important genes as well as on the correct timely activation of the stored molecules (Gandolfi and Gandolfi, 2001; Brevini et al., 1999). Mechanisms like extensive polyadenylation of maternal transcripts at 3' untranslated region (UTR) of mRNA determine the temporal expression and regulation of these maternal transcripts (Paynton et al., 1994; Richter et al., 1999). Timely recruitment and expression of stored molecules in the growing oocyte is thought to be essential for normal meiotic progression; for early embryogenesis until zygotic gene activation and for the control of expression later in development of the zygote for differentiation (Eichenlaub and Peschke, 2002; Duranthon et al, 2003; Barnes et al., 1991).

2.2.2 Limitations with the number of oocytes to be used for mRNA Quantification:

Studies involving expression pattern of developmentally important genes in oocytes and embryos is a relatively new research domain and has not been investigated very widely mainly due to the paucity of starting material available for the application of standard techniques for qualitative and quantitative analysis of gene expression (Lechniak, 2002; Gaudette et al.,1993). Total RNA in the oocyte is measured as 2.4ng of which rRNA is most abundant, representing 65% of the pool while polyadenylated mRNA account for only 10% (Picton et al., 1998; Bilodeau and Schultz, 1997; DeSousa et al., 1998). On and above the limitation of such minute quantity of starting material available for study, sub-categorization of available oocytes into groups of particular physiological/ functional significance further reduces the availability of material that could be studied to draw meaningful conclusions. Buffalos in this context pose a more serious challenge. This species has been traditionally described as a slow reproducer and poorly fecund species. Due to its physiological uniqueness the number of primordial follicles available in the buffalo ovary has been reported to be lower than that of its closest resemblance; cattle. The number is reported to vary from 10,000 to 19,000 (Samad and Nasseri, 1979; Danell, 1987) compared with around 150,000 in cattle (Erickson, 1996). By aspiration of 2-8mm follicles on the surface of ovaries the average recovery rate of oocytes per ovary in Buffaloes has been reported to be only 0.7 (Totey et al., 1992) which is much lower than the average recovery of around 8-12 acceptable quality oocytes per ovary in cattle (Gordon, 1994).

Development of new and sensitive molecular based techniques has provided avenues by which even the very minute quantities of stating material could be processed to derive meaningful conclusions. The northern blot analysis has been used to study gene expression during preimplantation development required about 300 oocytes or 54 blastocyst to analyze the expression of genes (Watson et al., 1992; Bilodeau and Schultz, 1997). Even large pools of oocyte (~ 200) are needed with microarray analysis where a single standard probe preparation procedure require about 20µg of total RNA and bovine oocyte has a total of 2.4ng RNA (Dalbies and Mermillod, 2003a; Robert et al., 2003).

2.2.3 Methods followed for gene expression studies in oocytes/ embryos:

To overcome the above limitations a PCR based method seem to be most promising but again the repeatability and optimization of a quantitative RT-PCR method is technically challenging. Due to high levels of amplification required for oocytes and embryos this approach demands stringent controls for variation in amplification efficiencies. As a whole it is observed that the

oocyte gene expression studies have been conducted with three main expected outcomes. They are either to isolate and characterize new genes which might be playing role in deciding oocyte competence (Velculescu et al., 1995; Natale et al., 2001), to characterize the quantitative variation of expressed genes in oocytes of different physiological status and their transcriptional control (Watson et al., 2000) or to absolutely quantify the copy number of a gene in oocytes expressed at specified time (Wrenzycki et al., 2001a; DeSousa et al., 1998). Some of the methods which have been used are mentioned below.

2.2.3.1 Semi-Quantitative RT- PCR:

Semiquantitative RT-PCR is the most frequently used approach for the analyzing the relative transcript abundance in bovine oocyte and embryos. (Lechniak, 2002). The effect of two basic culture system viz. TCM and SOF and protein supplementation on relative abundance of a set of developmentally important gene transcripts in bovine Morula and blastocyst has been studied by using α-Globin as an external control (Wrenzycki et al., 2001a). Effect of FSH on the expression of Cx-43, Cyclooxegenase-2, and FSH receptor has been studied in Equine oocytes during in vitro maturation by using this semi-quantitative approach in which G3PDH was used as a internal control (Aquila et al., 2004). Basic fibroblast growth factor receptor has been studied by Goessels et al., 2003 in in vitro mature bovine oocytes of high and low developmental competence. Similarly, the effect of various maturation protocols on the relative abundance of six marker genes Desmocollin-2, Plakophilin, Glucose transporter-1, Poly-A-Polymerase, Heat Shock protein 70.1, E-cadherin (Knijn et al.,2002) have been studied using Semiquantitative PCR technique. In an extensive study, the semi-quantitative PCR approach has been used to find out relative levels of transcripts encoding five genes viz. Na+/K+ ATPase α-1 isoform, bFGF, Cu/Zn SOD, Cyclin A and B and exogenously supplied α-globin in oocytes matured in different media combinations (Watson et al., 2000). Semi-quantitative PCR strategy has been used mostly to see the effect of media condition in correlating the expression pattern with oocyte developmental competence. Oocytes or Embryos are usually pooled into groups for analysis but single oocyte or embryo is also successfully investigated using the semi quantitative PCR approach (Wrenzycki et al., 2001b). Similarly the effect of serum or PVA on the relative abundance of different gene transcripts has been studied in preimplantation embryos (Wrenzycki et al., 1999).

2.2.3.2 Northern blot analysis:

There are only few reports on using Northern blot analysis. The change in relative abundance of various housekeeping genes such as 12s rRNA, β-Actin, Histone H3 mRNA in in vitro produced early bovine embryos has been studied using this technique. Using RNA sample

derived from in vitro matured oocytes and in vitro produced bovine embryos at different stage of early development; as stated earlier this method need significantly higher transcript concentration than the RT-PCR and therefore requires more oocytes and embryos. (Goeseels and Schultz, 1997)

2.2.3.3 Competitive RT-PCR:

Taylor et al., 2001 developed Competitive RT-PCR method to quantify as little as 2-fold difference in gene expression in individual oocytes and embryos throughout human preimplantation development. The technique was used to quantify the level of hypoxanthin phosphoribosyl transferase expression during preimplantation development. Anderson et al., 1999 used reverse transcription competitive polymerase chain reaction to determine the quantity of Cyclin B1 transcript present over the maternal to embryo transition for both in vivo and in vitro derived 4-cell porcine embryos. RT-PCR was performed on single embryo using an introduced truncated cyclin B1 DNA competitor. The levels of mRNAs for Glut-1, Glut-2 and Glut-3 were determined by competitive PCR to study the effect of maternal hyperglycemia on glucose transport and utilization in mouse preimplantation embryos (Moley et al., 1998)

2.2.3.4 Real Time PCR:

The real time RT-PCR has been successfully introduced to study the gene expression in single oocyte and embryo. Steuerwald et al., 1999 demonstrated the utility of the fluorescence monitored RT-PCR (Light cycler) for quantitative analysis of two housekeeping genes HPRT and β-Actin in single human oocyte and embryo. Similarly SYBR green approach has been used to analyze the *Xist* gene expression in a single murine embryo. Hartshon et al., 2002 used real time PCR to quantify *Xist* RNA levels in individual embryos and their blastomeres and to determine their sex. RNA levels of eight housekeeping genes were quantified by real time PCR through out the preimplantation period of the bovine embryos to find out the most suitable gene to be use as an internal standard (Robert et al., 2002).

2.2.3.5 DNA Microarray:

DNA microarray studies consist of an orderly, high density arrangement of nucleic acid spots or probes immobilized on a suitable substrate which are then exposed to a target consisting of a labeled free nucleic acid from the sample under analysis. This procedure provides a way to analyze thousands of genes at the same time and to establish general pattern of expression under specific condition (Hedge et al., 2000). Dalbies and Pascal Mermillod, 2003a analyzed gene expression in bovine oocytes before and after in vitro maturation using Heterologous hybridization on to cDNA array. Total RNA was purified on pool of over 200 oocytes. Radiolabelled cDNA probes were hybridized to Atlas human cDNA arrays. It was demonstrated

that cDNA array screening is a suitable method for analyzing the transcription pattern in oocytes. About 300 identified genes were reproducibly shown to be expressed in the bovine oocyte. The relative abundance of most messenger RNAs appeared stable during IVM, 70 transcripts were found to undergo a significant differential regulation. Similarly the cDNA microarray was found to be a useful tool to characterize gene expression pattern for sex-specific genes during early development and the result suggested the use of 'Heterologous Hybridization' technique as a useful technique in the screening of bovine genes (Nino and King, 2003). However there are again limitation regarding the amount of starting material because a standard probe preparation procedure require about 200µg of total RNA and the total RNA content of bovine oocyte has been calculated to be 2.4ng that requires a large pool of oocytes (Robert et al., 2003).

2.3 Quantitative RT- PCR (Q- RT- PCR): Importance of standards

q-RTPCR offers one of the most sensitive methods for quantitative analysis of gene expression because it combines the advantages of Reverse Transcription (RT) and PCR, which generate a logarithmic amplification of specific mRNA of even very low copy number mRNA species available from minimal tissue sample (Wang et al., 1989). Since it has been evolved (Ferre, 1992, Foley et al., 1993) this technique has been used very widely either for the relative quantification of transcripts to determine the changes in the steady state expression of genes or for the absolute quantification of the number of copies of specific RNAs present in per unit mass of tissue. However, although described as a sensitive method the quantitative RT-PCR often leads to error when employed for the relative quantification of transcripts in two or more groups of samples/ experiments due to differences in quantity and quality of initial sample amount, variation in RNA recovery, possible RNA degradation of sample material, contamination of genomic DNA in RT product, tube to tube variation in RT reaction, variation in sample loading / pipetting, errors and variation in cDNA synthesis efficiency which significantly contribute to source of variations among observed differences in the levels of transcripts (Ferre,1992; Gause and Adnovicz, 1995). To compensate for these variations requirement of a standard was felt which could be used as a benchmark to normalize the sample to sample variation and the level of product from gene of interest could be normalized against the product from the control. Grossly; depending upon the strategy adopted the standards can be of endogenous or exogenous type (Reischl et al., 1995; Zenilman et al., 1995; Diaco, 1995).

2.3.1 The internal or endogenous standard:

It involves the co-amplification of an endogenously expressed RNA that is present in the sample mixture along with the target mRNA. Ideal endogenous standards are one whose

expression does not vary in response to the experimental treatments or over the time of experimentation (temporal variation). Experimental samples are then normalized with respect to the endogenous RNA standard to allow comparison of target mRNA levels and the results are expressed as percent increase or decrease in the expression of target RNA as compared to the standard (Volkenandt et al., 1992; Ferre, 1992). But endogenous standard has problem of analyzing transcripts of widely differing abundance, necessity to use multiple primers for the same reaction and expression level of some house keeping genes have been reported to vary during experimental treatments (Foley et al.,1993; Raeymaekers,1995). In this strategy, several of the housekeeping genes have been used as standards. However, the use of endogenous standards in RT-PCR method for mRNA quantification is again limited by two factors. 1) The expression pattern of the endogenous gene decided to be used as standard may vary thereby making the comparison biased and 2) two different sets of primers used for the co-amplification of target RNA and the standard may exhibit different amplification efficiencies. Nevertheless, instead of these limitations, after properly calibrated for a particular tissue type, the endogenous control approach for mRNA quantification has been used by many workers for their genes of interest (Chelly et al., 1988) primarily for measuring differences in the relative concentration of a specific target mRNA in different RNA species but certainly not for determining absolute quantification of mRNA (De Leeuw et al., 1989).

2.3.2 The external or exogenous standards:

Second approach for using Q-RT-PCR for gene expression quantification is the use of an external or exogenous standard. As an improvement over endogenous standard, external or exogenous standards are used as synthetic nucleotide sequences, which unlike the endogenous standard are stretch of known sequences added along with the experimental RNA and are co-amplified. The initial concentration of standard and target RNA need not to be similar and can differ up to two order of magnitude (Bouaboula et al.,1992), but as its concentration is known, absolute quantification of the target RNA is possible following this method (Becker and Hahibrock,1989). The exogenous standards can be heterologous or homologous. Heterologous external standard are nucleotide sequence that are different from target sequence and homologous standards are essentially composed of the sequence of the same target gene of interest but having a different amplifiable length (Reve, 1998). For Q-RT-PCR quantification and normalization of target gene the use of external standards is more meaningful because their expression level can be controlled and could be optimized (Freeman et al., 1999). But at times exogenous standards have been reported to have an amplification

advantage over the target (Volkeondt et al., 1992) and thereby incorporating negative bias in estimating target genes.

2.3.3 Steps to standardize Q- RT- PCR:

The great sensitivity of PCR, first described by Saeki et al., 1985, have made RT-PCR as the technique of choice to quantitate low abundant mRNA transcripts and to detect mRNA in small number of cells such as in oocytes or embryos (Lechniak, 2002). However, in the context of quantification of transcripts; performing RT-PCR can be the most technically challenging method, often requiring substantial pre-experimental planning and design (Zimerrmann and Mannhalter, 1996; Grover et al., 2001). One of the principal disadvantage/ limitation of this method is that it eventually determines the level of expression of a gene in terms of end point analysis of PCR product generated by a logarithmic amplification of mRNA. The amplification and accumulation of PCR amplified products in a reaction tube are constantly affected by available ratio of essential PCR ingredients and at one point of time it becomes a limiting factor for further amplification of products. This may incorporate an error in the estimation procedure. The critical parameters, which affect the RT-PCR efficiency, can be enumerated as follows.

1. PCR cycling conditions: Number of cycles, time and temperatures of annealing, and extension.
2. Concentration of oligonucleotide primers, $Mgcl_{2,}$ reaction templates, DNA polymerase.
3. Target and control oligonucleotide composition: Length, sequence, specificity, priming efficiency. (Innis et al.,1990;Volkenandt et al.,1992)
4. Contamination: As PCR being highly sensitive, even very small amount of genomic DNA contamination in RNA preparation may serve as template for amplification and produce misleading results.

2.3.3.1 PCR cycling conditions:

The method of Q-RT-PCR principally involves detection and quantification of amplification product at the end of defined number of PCR cycles, which often represents a plateau phase. At plateau phase the product is believed to have reached to a limiting state and initial templates of varying intensity may eventually lead to the same result (Freeman et al., 1999). Thus quantification done in the exponential phase seems to be more accurate when no factor is limiting and the amplification products accumulate at the steady state. At plateau, the Taq polymerase also loose activity or cDNA begins to compete for primers and DNA amplification product concentration can increase to the point where single stranded products re-anneal with

each other rather than with primer. Optimisation of the plateau phase for a given reaction condition thus form an integral part of any Q-RT-PCR procedure.

2.3.3.2 Time and temperature of annealing and extension: The standard PCR has three steps defined by temperature of denaturation annealing and primer extension. The first step i.e. denaturation occur when reaction is heated to 92^0C-96^0C. At this step the template DNA becomes single stranded and the Taq enzyme has no measurable polymerase activity using these denatured single strands. Stability of the Taq activity also becomes a limiting factor in PCR, Gelfand, 1989, reported that Taq polymerase loose 50% of its activity at 95^0C for 40 minutes. Annealing temperature of oligonucleotide primers to template DNA varies from 45^0-65^0C depending upon the homology of the primers for the target sequence as well as the base composition of the oligonucleotide. The oligonucleotide primer is in a 10^6-10^{10} fold molar excess to target sequence and the sequence complexity of the target DNA is at least 10-fold greater than that of nucleotides. Determination of annealing temperature is critical as too high temperature may result in no product and if it is too low, non-specific products accumulate which leads to the inaccurate quantification (Volkenandt et al., 1992). Published rate of DNA synthesis by high processive Taq polymerase at 72^0C is 60 nucleotide/sec. (Innis et al., 1988). This enzyme is not completely quiescent at suboptimal temperature. In fact, elongation begins during the annealing process at 50^0C-60^0C. Taq polymerase has an extension rate of 24 nucleotide/sec. (Innis et al., 1988).

2.3.3.3 Concentration of oligonucleotide primer and $MgCl_2$:

The effective concentration of magnesium cation is crucial to the PCR reaction. $MgCl_2$ concentration for Taq polymerase has been reported to be 1.5mM-2mM (Sambrook, 2001). Chelating molecule such as EDTA, nucleotide tri-phosphate can reduce the effective concentration of $Mg2^+$. Too high Mg^{2+} concentration may yield nonspecific PCR products whereas too low concentration will drastically reduce the efficiency of PCR. Any increase in the concentration of dNTPs will reduce the chelating effect of tri-phosphate thus, the change in dNTP concentration should be accompanied by a change in magnesium concentration in 1:1 molar ratio (Sambrook,1989). Normally $MgCl_2$ concentration is tested in range from 1.5mM to 5mM. Routinely 1.5mM $MgCl_2$ is used and a concentration of 4.5mM or 6mM has been reported to decrease nonspecific priming in some cases (Krawetz et al., 1989). Because dNTPs and oligonucleotides bind Mg^{2+}, the molar concentration of the cation must exceed the molar concentration of phosphate groups contributed by dNTPs and primers. In some cases increasing Mg^{2+} increases the specificity (Haris and Jones, 1997). Thus it has been

recommended that for any Q-RT-PCR protocol the optimum concentration of Mg^{2+} is required to be determined empirically for each combination of primer and template.

2.3.3.4 DNA Contamination:

In the context of RT-PCR the PCR primers that either do not cross intron or amplify genes which lack intron, contamination of RNA with DNA will generate a PCR product that is same size as the PCR product generated by the target cDNA. This may also occurs if the target gene is part of a large gene family or if there are large number of pseudogenes such as the case with G3PDH and β-Actin (Foss et al., 1998; Finke et al., 1993). Specific strategies are therefore required to be adopted to rule out the DNA contamination in RT-PCR product. One option is to design an intron spanning primer that generates a bigger product when amplified from genomic DNA template against a shorter cDNA amplified product. Alternatively a Reverse transcriptase negative control may be included during the cDNA synthesis. In minus RT control, Reverse transcriptase enzyme is not added during cDNA synthesis and this negative reverse transcriptase control is used as a template for PCR along with the other experimental cDNA sample. Since no cDNA can be synthesized due to lack of reverse transcriptase; the generation of PCR product from this negative control indicates genomic DNA contamination. De Sousa et al, 1998 described the similar approach for β-Actin in which primers for β-Actin amplifies a 243bp product from cDNA but also intron spanning, thus generating a larger 408bp product in the presence of bovine genomic DNA. Similarly Lequarre et al., 1997 while studying glucose metabolism during bovine preimplantation development through analysis of gene expression in single oocyte and embryos designed all primers viz. β- Actin, Hexokinase, G6PDH, Glut-1 and Glucose Phosphate Isomerase. All the primer pairs spanned at least an intron in order to differentiate RNA from DNA amplification. A last option to rule out genomic DNA contamination is to treat the RNA with RNase free DNase during isolation of RNA (Grillo and Margolis,1990). Since the housekeeping genes are conserved so there is frequent presence of corresponding intron less pseudogenes that mimic cDNA in size and sequence with 90% homology and may cause quantification error (Hanaver and Mandel, 1984). Such pseudogenes has been reported to exist for G3PDH (Foss et al., 1994) and β- Actin (Finke et al, 1993). To overcome this problem the PCR primer used to amplify the internal standard must be designed with appropriate consideration and strategy. There are also chances of genomic DNA contamination which can produce false positive results in gene expression (Kreuzer et al., 1999).

2.4 Quantitative Competitive PCR(QC- RT-PCR):

As described before in this chapter the most accurate quantification of mRNA transcripts following Q-RT-PCR is greatly hampered by a number of variables that can occur during sample preparation, or in the course of reaction, and minor variations in reaction are greatly magnified during the amplification process (Ferre, 1998; Chelly et al., 1988). These variations can be partly overcome by normalizing the amount of PCR products of specific template with respect to an internal control amplified in the same reaction tube. The most precise quantitation of the transcripts can, however, be obtained by following a strategy called competitive PCR and competitive RT-PCR (Ferre, 1992; Siebert et al., 1995; Gilliland et al., 1990, Clementi et al., 1994). This assay is based on competitive co-amplification of a target sequence together with known concentrations of an external standard/ control (competitor) in the same tube. The external standard is designed with a strategy that it has to share the primer recognition sites with the targeted template (Becker and Hahibrock, 1989). The two other a-priory assumptions that are hypothesized for validity of this test are that both the specific template and external standard are amplified with same PCR efficiency and it must be possible to analyze the PCR amplified products of target template and external standard separately (Freeman et al., 1999). Competition for amplification reaction between the target template and external standard template allows the determination of the equivalence point at which the control template and target template concentrations are assumed to be equal (Raeymaekers et al., 1995; Gilliland et al., 1995; Tsai and Wiltbank, 1996). The number of copies of the specific target template at the point of equivalence is assumed to be the same as that of added internal standard, enabling quantification of target molecule (Wang et al., 1998). During the process of competition as direct competition between the target sequence and internal standard continues throughout the PCR, so the plateau effect does not interfere with quantification.

In competitive PCR to a large extent the validity and accuracy of quantification depends on the strategy of designing the competitive standards. Lots of reports have been published describing different competitive standard designing options (Zimmermann and Mannhalter., 1996). One way to classify the competitors is based on internal sequence as Homologous and Heterologous competitors. Homologous competitors have the same nucleotide sequence as the target DNA (RNA) but containing a deletion or an insertion or has a different restriction site introduced by site specific mutagenesis (Zhang et al., 1997; Busse and Seguin, 1992; Taylor et al., 2001; Henley et al., 1996). Care should be taken to avoid the formation of heteroduplex which would interfere with subsequent quantification. Hetrologous competitors on the other hand have nucleotide sequence different from the target DNA (RNA) except for the sequence

of primer annealing sites. Heteroduplex formation can be avoided using this strategy and since the primer sequences are same the difference in amplification efficiency between target and competitor can be considered as minor (Henley et al., 1996; McCulloch et al., 1995).

Other way of classification of competitors is RNA / DNA competitors. DNA competitors are used for the quantification of DNA and cDNA from a RNA template. RNA competitors are used for direct quantification of RNA amount. RNA competition is used together with RNA target during RT reaction (Siebert et al., 1995; Raeymaekers., 1995; Zenilman et al., 1995).

2.4.1 Designing of standard template competitor:

A common strategy for designing competitor template is to inset or delete a restriction enzyme recognition site within the internal sequence of the targeted template so that the products of the target and standard amplification can be easily resolved by electrophoresis after restriction digestion (Siebert et al.,1992). Kephart, 1998 used another approach for the human β-Actin RNA. Using this approach three primers were designed. Two primers US (upstream) and DS (downstream) were designed to generate an intact 511 bp product during RT-PCR amplification of the β-Actin RNA. An additional upstream primer (USC) was required solely for construction of the competitive template by RT-PCR amplification. The USC primer was a composite of the US primer sequence and a sequence region located 132 nucleotide downstream in the RNA. When the USC and DS primers are used to amplify total RNA sequences between the composite oligonucleotide are eliminated from the amplified product to generate the delta actin cDNA. The truncated product contains binding site for the US and DS primers but is truncated relative to the native sequence. The difference in size between the full length and truncated RT-PCR products allows discrimination between product generated from endogenous RNA and competitive RNA (cDNA) template. The full length and truncated amplification products were purified and an aliquot was ligated directly into the cloning vector PGEM-T. Competent cells were transformed and resulting recombinants were screened by blue/ white color selection. Potential positive were rapidly screened by PCR using the upstream and downstream primer to check for insertion of the correct template sequence and with the downstream primer and a T7 primer to confirm the orientation of the inserted DNA. The RNA was purified and the concentration of RNA was quantified by spectrophotometric analysis. The competitive PCR has been used to measure precisely the relative expression of α, β, γ fibrinogen mRNA in rat liver lobes using the synthetic RNA as an internal control (Zhang et al., 1997).

However, the above strategies essentially require multiple step manipulations following PCR. A more simplified strategy avoiding the cloning and ligation steps have been described by

O'Connell, 1998. Following this strategy the standard is derived from the target template by PCR using the regular antisense primer and a composite primer. The composite primer is essentially composed of the original upstream primer sequence plus another stretch of ~20 bases 90-100 bases downstream from the regular sense primer falling within the amplifiable target sequence. When the target cDNA is amplified with this composite sense primer and the regular antisense primer a truncated amplicon is derived that lack the upper 5' 90-100 bp region of regular amplicon but have the sense primer recognition site intact. This shorter product has been used as standard for competition. Once the standard is prepared it can be quantified with spectrophotometer and a dilution strategy could be decided that is helpful in finding the point of equivalence between standard and target amplification. This particular strategy was used in the course of present investigation.

The quantitative competitive RT-PCR analysis has been used for the quantification of absolute amount of mRNA. A serial dilution of a competitor is co-amplified with an unknown but constant amount of the target mRNA to determine equimolar amount (Reischl and Kochanowski, 1995). The standard competes with the native for primers and enzymes thus reducing the signal of the native when the standard is in excess. As the amount of standard increases the native signal decreases (Becker and Hahibrock, 1989).

2.4.2 Mathematical considerations in quantification following Competitive RT-PCR

Q-RT-PCR being an indirect method of measurement, an understanding about the mathematical interpretation of competition result in the competitive PCR experiment is essential to reach to a most valid interpretation. Several mathematical models are described to explain the events in RT-PCR (Nedelman et al., 1992, Peccoud and Jacob, 1998, Vu et al., 2000; Wang and Spadoro, 1998). In brief, the band intensity values for the target as well as the competitor product obtained by any densitometry software forms the basic input data to interpret. From the graph of the log of standard/native signal on 'Y' axis verses the log of input RNA/cDNA standard plotted on 'x' axis, the amount of initial native RNA/ cDNA can be found at the point of equivalence i.e at the point where the ratio of input and standard becomes 1 or close to 1 and the ordinate value will be zero (Bouabovla et al, 1992). As with any standard curve, there must be standard amount above and below the equivalence point. The regression analysis of the values for standard as independent variable and the standard/ native as dependent variable must obtain a linear trend to authenticate the competition (Raeymaekers, 1995). The formulae for calculating the number of copies of cDNA present for a given amount of purified DNA and subsequent extrapolation of results to reach to a value for number of copies of RNA is mentioned by O'Connel, 1998. It has been mentioned that in performing the

competitive Q-RT-PCR when standards are added in varying concentrations in search for the point of equivalence the dilution series of the standard sometimes may not include the precise quantity to compete with the amount of target in a particular sample and as a result the exact point of equivalence is missed. Under such circumstances the target concentration can be estimated by calculating the concentrations from the target: standard ratios at either sides of the missed equivalence point and obtaining the average from these two estimates.

2.5 Housekeeping genes as standard in Q-RT-PCR

An endogenous control/ internal control to be used in a Q-RT-PCR protocol is also referred as housekeeping genes(HKG) because the nature of expression of these genes make them suitable to be used as controls. Watson et al., 1965 defined housekeeping gene as those genes that are always expressed in a cell. A more recent definition of housekeeping gene is these genes are expressed in all tissues and are highly conserved throughout evolution and usually serve basic metabolic function in the cell to successfully complete the cell cycle (Foss et al., 1994). House keeping genes are commonly used in semi-quantitative and quantitative RNA analysis such as Northern blot analysis, in situ hybridization, Ribonuclease protection assay, reverse transcription base PCR (RT-PCR), Differential Display RT-PCR and DNA microarrays. The appropriate choice of internal standard is a very important element of quantitative RNA analysis. Ideally internal RNA control should show constant expression across a wide range of physiological and experimental conditions. This is a prerequisite for a valid quantitative comparison of gene expression among different tissue types, varying developmental stages and experimentally treated cells. However, commonly used housekeeping genes do not always manifest stable expression levels under all experimental conditions (Thellin et al., 1999). Infact, expression of several widely used internal standard often varies in response to various factors hence they may not be useful for many routine applications (Zhong and Simons., 1999). The variation can be explained by the fact that housekeeping proteins are not only required in basal cell metabolism but they also participate in other functions (Petersen et al., 1990; Singh and Green., 1993; Ishiteni et al., 1996). Moreover, a consequence of conservation of house keeping genes is the frequent presence of corresponding intron less pseudogenes in mammalian genomes that mimics cDNAs in size and sequence with homology more than 90% which may cause quantification errors (Hanauer and Mandel., 1984). Such pseudogenes exist for G3PDH (Foss et al.,1994) and β-Actin (Finke et al., 1993). The expression of a test gene is described as the relative ratio over an arbitrarily selected internal control presumed to be stably expressed in any circumstances relevant to the experiment thus an ideal endogenous standard would be one whose expression does not vary

during the cell cycle, between cell types or in response to the experimental treatment that one wishes to examine. But the studies suggest that the expression of frequently used endogenous reference genes can vary substantially according to materials, tissues and conditions studied (Thellin et al., 1999; Goldsworthy et al., 1993; Lee and Costlow, 2002). There are several housekeeping genes which have been commonly used for PCR validation like Histone 2A, β-Actin, β_2-microglobin, Glyceraldehyde-3-Phospahte dehydrogenase (G3PDH), 18s ribosomal RNA, Aldolase, Glucose-6-Phospahate, Hypoxanthinphosphoribosyl transferase (HPRT), Dihydrofolatereductase (DHFR) etc. (Ullmannova and Haskovec.,2003). Suzuki et al, 2000 has reviewed the use of housekeeping genes as an internal control for the detection of gene expression in different tissues employing different methods and in different contexts of experiments. The list of their review findings has been summarized in the form of the table below.

Control Gene	**Number of reports**	**Relative frequency of use**
G3PDH	148	33%
β-Actin	146	32%
18s-rRNA	63	14%
28s-rRNA	51	11%
36B4	8	2%
Tubulin	3	1%
Other	33	7%

Clearly the G3PDH, β-Actin and r-RNA genes (18S and 28S) predominate over others, as a eligible HKG. But at the same time it should be considered that each experimenter had a different end point in plan and had different experimental limitations/ obligations. As per Roche technical note NuLc/2001 the suitable HKG to be used as internal control must fulfill the following prerequisites:

1. It should not be regulated (induced or repressed) in the type of tissue and sample material.
2. A pseudogene free amplification (specific primers) and/or detection specific for active target (specific hybridization probes) should be selected to avoid traces of DNA contributing to the result.
3. The chosen mRNA species should be proportional to the amount of loaded or input RNA (target).
4. Should have similar expression level compared to the target to be analyzed

2.5.1 Some important house keeping genes:

1. Glyceraldehyde-3 phosphate dehydrogenase (G3PDH or GAPDH): Glyceraldehyde-3 phosphate dehydrogenase is a key enzyme in glycolysis that plays an important role in energy metabolism. The RNA encoding G3PDH is a ubiquitously expressed moderately abundant message. It is frequently used as a endogenous control for quantitative RNA analysis based on the assumption that its expression remain constant under changing cellular conditions (Edwards et al., 1985). However the use of G3PDH as an internal control may be inappropriate because this gene is known to contribute diverse cellular function unrelated to Glycolysis such as nuclear RNA export, DNA replication, DNA repair, Exocytotic membrane fusion, Cytoskeletal organization and phosphotransferase activity (Sirover, 1999). Furthermore pseudogenes cause problems when G3PDH is used as internal standard. There are many other instances where G3PDH become an inappropriate standard. G3PDH concentration has been reported to vary during different developmental stages (Pussiant et al., 1994) during the cell cycle (Mansur et al., 1993) and varies across the tissues (Warrington et al., 2000). Oxidative stress (Ito et al., 1996), Hypoxia (Zhong and Simons, 1999) also stimulate G3PDH transcription. Furthermore G3PDH is up regulated in cancer (Ripple and Wilding., 1995) and it also varies in apoptosis and neurodegenerative diseases (Tatton et al., 2000).
2. Ribosomal RNA (28s,18s): Ribosomal RNA contributes 85-90% of total cellular RNA. It has been used as a internal standard (Bustin et al., 2000). The levels of rRNA are thought less likely to vary under conditions that effects the expression of mRNAs (Barbu and Dautry.,1989). Since rRNA are transcribed by distinct RNA polymerase (Pauli and White, 2000). However, the levels of rRNA varies under several experimental conditions which has been shown in Human fibroblast (Mansur et al.,1993), Human (Zhong and Simons.,1999) and Mouse malignant cell lines (Bhatia et al.,1994). However there are drawbacks to the use of rRNA because (i) It cannot be used for normalization, when target mRNA transcripts are purified because it has no polyA tail. (ii) It is expressed at higher level than target mRNA. (iii) rRNA don't represents the mRNA pool present in the cell. Nevertheless, 18s rRNA has been suggested as a good internal standard in situations where other commonly used housekeeping genes are not found suitable (Thellin et al., 1999).
3. β-Actin: β-Actin was one of the first RNA to be used as a internal standard. β-Actin is a protein that is essential for the structure and kinetics of Cytoskeletone formation. Its mRNA is expressed moderately abundant levels in most cell types (Bustin, 2000).

However it has been reported to vary in some tissue and in some lines and in response to experimental manipulations such as under hypoxia (Zhong and Simmons., 1999) and in blastomeres (Krussell et al., 1998) and different tissues (Warrington et al., 2000). Use of β-Actin as an internal standard for quantification and with γ-Actin is problematic because of presence of pseudogenes (Finke et al., 1993).

Based on above it could be summarized that special care should be taken when choosing a housekeeping gene for the relative quantification to normalize target gene expression A specific control can be selected for each experiment. It is better to check out different HKG suitable for the experiment and that exhibit no modulation. Other approach is to use more than one control in the experiment. Similar expression for different control would lend credibility to their use and to the accuracy of the normalized data (Zhong and Simons., 1999).

2.6 Housekeeping genes as standard (studies in oocyte/embryo gene expression):

Transcriptionally the oocytes remain highly active during the secondary follicle stage and then the transcription process decreases till the germinal vesicle stage and later after maturation it ceases. After maturation and also during early cleavage stage post transcription modifications play important role (De Sousa et al., 1998). In view of this time dependent modulation of expression pattern observed as a whole it becomes important to know the status of different housekeeping gene during the course of oocyte maturation and also during the preimplantation period of development. The changes in RNA content during mouse oocyte maturation has been studied by Northern blot to quantify specific gene transcripts and mRNA degradation has been demonstrated for tissue type plasminogen activator (t-PA), actin, tubulin and hypoxanthine phosphoribosyl transferase (HPRT) (Huarte et al.,1987; Paynton et al.,1988). A semi-quantitative RT-PCR method was used to study the mRNA level for genes involved in glucose metabolism in single oocytes and embryos (Lequarre et al., 1997). Glut-1, hexokinase, glucose-6-phosphate dehydrogenase and glucose isomerase gene expression was studied. Based on the results glucose phosphate isomerase and β-Actin was chosen as reference transcripts. For actin expression pattern a small decrease from the immature oocyte up to eight cell stage was observed. Subsequently a sharp rise starting at the 16 cell stage and lasting upto morula stage was observed which finally dropped at the blastocyst stage. A similar pattern of actin expression pattern was also observed in mouse where at mid cycle phase oocyte the actin mRNA reaches the level 2 fold higher than in fully grown oocyte and then drop during oocyte maturation upto the end of two cell stage followed by sharp rise which is consistent with zygotic genome activation ((Bachvarova et al., 1989; Giebelhaus et al., 1983; Calder et al.,

2003; Goeseels et al., 1997; Taylor et al., 1990., 1990). In another study, expression pattern of β-Actin, G3PDH, ubiquitin, tubulin, Histone2A, 18srRNA was studied (Robert et al., 2002). It was found that there are relatively large amount of these transcripts in the GV oocyte and the levels decreases regularly as the embryo develop until it reaches the eight cell stage. The decrease is followed by a sharp increase at the blastocyst stage. All these studies, seriously questions the use of β-Actin as an internal standard in mRNA quantification trials during IVM and IVF.

On the contrary in the study of Robert et al.,2002 using real time Light Cycler β-Actin, GAPDH, ubiquitin, liminin and tubulin mRNA were found to be almost constant until eight cell stage. Small nuclear RNA and 18s rRNA level were also mostly constant until the 8 cell stage followed by increase in blasocyst stage and the level of Histone was particularly found to be very stable across the entire preimplantation period (Goeseels et al.,1997; Bachvarova et al.,1989). In one study to find out the variation in expression level of HAS1, HAS-2 ,HAS-3 as well as corresponding receptors CD44 and RHAMM during course of 24 hour IVM of bovine COC in response to gonadotrophins, 18s rRNA and Ubiquitin were used as a internal standard for normalization (Schoenfelder and Einspanier., 2003). In their study to specifically look into the relative abundance of developmentally important gene transcripts involved in compaction, cavitation, metabolism, heat shock protein and maternal recognition of pregnancy in bovine morulla and blastocyst stage employing semi quantitative RT-PCR assay α-globin RNA has been used as a standard (Wrenzycki et al., 2001). Similarly connexin-43, cyclooxegenase-2 and FSH receptor mRNA was detected in equine cumulus cells before and after maturation by Aquila et al., 2004 using GAPDH as normalization control and it was concluded that their level does not vary during in vivo or in vitro maturation and was neither influenced by FSH nor by precursor of hyaluronic acid synthesis.

2.7 General Conclusion of the Review of Literature

As general conclusion of the above review it could be stated that studies on gene expression profiling in oocytes and embryos is still an open topic and lots of researches are presently going on to characterize the oocyte competence, genes responsible for healthy growth of post fertilized embryos and orderly development leading to successful implantation. This information will contribute to better standardize the procedure for IVF and will help optimizing acceptable rate of offspring produced following IVF. On the other hand there is tremendous change taking place in the molecular biology methodologies to explain functional behavior and consequence of genes. As a result we are also going through a phase of continuously redefining our knowledge about the genetic control of early development processes. Studies on gene

expression in oocytes and embryos particularly in buffaloes are a completely unexplored area and almost no information is available on this topic. The present investigation is an effort in this direction to standardize a working protocol for using quantitative RT-PCR for gene expression studies in buffalo oocytes, where suitability of using a few housekeeping genes was studied with the objective of using them as internal control in future Q-RT-PCR experiments.

3. Materials and Methods

The present work was carried out with the primary objectives of evaluating the RNA extraction methods from buffalo oocytes and examining the consistency of expression pattern of some housekeeping genes in the course of in vitro maturation of follicular oocytes in buffalo. Effectiveness and accuracy of two methods of mRNA quantitation viz. RT-PCR and Competitive Quantitative RT-PCR were evaluated in the context of this study. The present section elaborates the materials used and methods followed in course of the present investigation.

3.1 General precautions observed while handling with the RNA related work:

Working with RNA is more demanding than working with DNA because of the chemical instability of the RNA and the ubiquitous presence of RNAse. Unlike DNAse, RNAse do not need metal ion co-factors to work and can maintain activity even after prolonged boiling or autoclaving. Therefore, special precautions must be taken while handling with any RNA or RNA related work. Following were the general precautions taken to prevent RNAse contamination:

1. Powder free latex gloves were used throughout the experiments and preparatory works for RNA to prevent contamination from RNAse found on most human hands.
2. Gloves were changed periodically after touching skin and common surfaces.
3. A dedicated set of pipettors was used solely for RNA work.
4. Used only the certified RNAse-free chemicals, reagents and plastic wares.
5. Designated a laminar flow hood placed in the 'low-traffic' area of the lab, away from air vents or open windows for RNA related work.

3.1.1 Preparation of RNAse free plastic wares:

RNAse free plastic wares were prepared by immersing the materials in 0.1% solution of DEPC (diethylpyrocarbonate). DEPC has been reported to destroy enzymatic activity by modifying -NH, -SH and -OH groups in RNAse (Ambion technotes). DEPC solution was prepared by adding 1 ml of DEPC (Invitrogen) in 1 liter of MiliQ water and shaking it vigorously. DEPC solution was prepared fresh every time. DEPC is a known carcinogen and adequate precautions were taken to avoid its direct contact. Plastic wares i.e. tips, tip-boxes, eppendorf tubes (0.2, 0.5, 1.5 and 2ml), and steel wares like scissors, spatula, forceps etc. were soaked in 0.1% solution of DEPC at 37^0C for overnight. The DEPC solution with immersed materials was

stirred for 3-4 times so that all tips and tubes are soaked completely in the solution. The following day DEPC solution was drained off and the treated materials were dried in an incubator at 65^0 C. Dried plastic wares were wrapped in aluminium foil and were double autoclaved at 15 lbs pressure for 45 min to remove the remaining traces of DEPC which can interfere with the reactions carried out in these DEPC treated wares.

3.1.2 Preparation of RNAse free glass wares:

Glass wares i.e. Beakers (50ml, 100ml, and 200ml), bottles (250ml, 500ml), Pasteur pipettes and measuring cylinders were cleaned and dried. Glass wares were baked at 280^0C for 4 hours to remove RNAse. RNAse free plastic wares, glass wares and other appliances were stored in a dust free and clean cabinet.

3.1.3 Preparation of RNAse free solutions:

Buffers and solutions are a common source of RNAse contamination. DEPC treatment is the most commonly used method for eliminating RNAse contamination from water, buffers, and other solutions. However, DEPC cannot be used with certain reagents containing primary amine groups (e.g., TAE Buffer), secondary or tertiary amines (e.g., HEPES). The amine groups tend to react with and 'sop up' the DEPC, making it unavailable for inactivating RNAse. To avoid the RNAse contamination, all solutions were prepared using baked glassware. And DEPC treated pyrogen free MiliQ water. RNAse free chemicals and were handled with baked and autoclaved spatulas.

3.1.4 RNAse free Surface:

Prior to using the laboratory surfaces, e.g. bench tops, laminar hood surface, centrifuges and electrophoresis tank, the surfaces were decontaminated by wiping it down with 0.1N NaOH/ 3% H_2O_2.

3.2 Source of oocytes:

Buffalo ovaries were collected from Delhi abattoir and transported to the laboratory in a thermo flask in normal saline supplemented with antibiotics (streptomycin sulphate 100 µg/ml and penicillin 100U/ ml) and maintained at 35^0C-37^0C. On an average; ovaries were subjected to oocytes collection within a time lapse of 4-6 hours following slaughter of animals. The extra tissues adhered to it were trimmed and washed three times in 0.9% saline supplemented with antibiotics (streptomycin sulphate 100 µg/ml and penicillin 100U/ ml). Cumulus oocytes

complexes (COCs) from follicles were collected by aspiration using a 18G needle attached to a 10ml syringe and collected in oocyte collection medium (Dulbecco's Phosphate Buffer Saline supplemented with 0.01% L-glutamine, 0.4% BSA, 100 µg/ml Streptomycin sulphate, 100U/ml penicillin and Sodium Pyruvate 36 µg/ ml). The collection medium was filtered through 0.22µ syringe filter prior to use. The COCs were selected according to their morphological characteristics and graded as A^+ / A^- on the basis of presence of number of cumulus layers, and homogeneity of cytoplasm (ooplasm). Briefly, Grade A^+ oocyte had a compact cumulus of more than 5 layers of cells and homogenous ooplasm; Grade A^- oocyte had slightly expanded cumulus of fewer than 5 layers of cells. Only A^+ and A^- grade oocytes (Fig. 1) were selected for RNA extraction and subsequent gene expression studies.

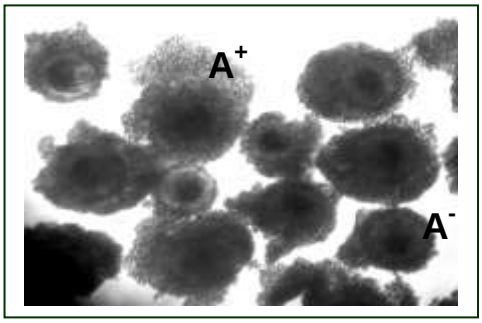

Fig.1: Buffalo oocytes of A^+ and A^- grade used in the present study.

Selected COCs were washed twice in fresh oocyte collection medium followed by washing in PBS (Calcium and Magnesium free) and were taken in an eppendorf tube (2ml) in 200 µl PBS and denuded by vortexing for 120 seconds. Denuded oocytes were again washed thrice in PBS to get rid of cumulus cells and were finally taken in counted numbers in a RNAse free eppendorf tube in minimum possible volume of PBS and in it 20 µl of 'RNA later' solution (Ambion) was added quickly. 'RNA later' (Ambion) is a tissue storage and stabilization solution that preserves RNA within tissues and cells. As per manufacturers information RNA in tissue samples stabilized in 'RNA later' is stable for one day at 37^0C, seven days at 18^0C to 25^0C, four weeks at 2^0C to 8^0C, or for extended period at -20^0C to -80^0C. Tissue samples kept in 'RNA later' can be thawed without affecting the RNA. Oocytes with RNA later, in the present case, were stored at -80^0C till RNA extraction.

3.3 RNA extraction methods:

TRIZOL Reagent is a widely used ready-to-use reagent for the isolation of total RNA from a variety of cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the single-step RNA isolation method developed by Chomczynski and Sacchi, 1987. During sample homogenization or lysis, TRIZOL Reagent

maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA was recovered by precipitation with isopropyl alcohol. 1 ml of TRI REAGENT $^{(™)}$(Sigma) is reported to be sufficient to isolate RNA, DNA and protein from 50-100 mg of tissue, 5-10×10^6 cells or 10 cm^2 of culture dish surface for cells grown in monolayer.

3.3.1 Trizol method of RNA extraction from oocytes:

In the present study the TRI^{TM} Reagent (Sigma) was used for RNA isolation from oocytes. The steps followed were as under.

1. 30 oocytes in ca $^{++}$ and Mg $^{++}$ free PBS were placed in a 2ml eppendorf tube and in it 1ml of TRI reagent at 4^0C was added and vortexed for 5 minutes to lyse the oocytes. The tube was kept at room temperature for 5 min.
2. 200 μl of chloroform (DNAse and RNAse free) was added, the tube was capped and contents were vortexed again for 5 minutes and kept at room temperature for another 5 minutes.
3. Sample was spinned at (13,000xg, 15min, and 4^0C). After centrifugation, the oocyte lysate was separated into three phases: lower organic phase of red colour (containing protein), an interphase (containing DNA) and colorless upper aqueous phase (containing RNA).
4. Carefully about approximately 500 μl of the aqueous layer was transferred to a fresh tube taking care that the interphase or the organic layers are not touched.
5. 500 μl of isopropanol (DNAse and RNAse free) was added to it and the contents were mixed few times with the help of dispenser and kept for 10 minutes at room temperature and then centrifuged (13,000xg, 10 min, 4^0C).
6. After centrifugation, RNA precipitated as a transparent pellet on the bottom of the tube.
7. The pellet was located carefully and supernatant was discarded as much as possible taking care that the pellet is not disturbed and 1 ml of 75 % ethanol was added to the RNA pellet.
8. RNA pellet was centrifuged (7500xg, 5min, 4^0C). Ethanol was decanted and the tube was left open on the laminar for 5-10 minutes to dry the pellet.
9. The pellet was not allowed to dry completely. 10 μl of nuclease free water was added to RNA pellet and heated at 65^0C for 5 min to dissolve the extracted RNA.
10. The RNA suspension was either processed for RT or was stored at –80^0C till further use.

As described above apparently, the principle drawback with the Trizol method was that it involved separating an approximate amount of 500 μl aqueous supernatant containing RNA (step 4 as above) which by all likely incorporated a source of variation between different batches of extractions and as evident by inconsistency in the intensity of PCR product amplified for desired genes on repeated trials. Hence an alternative column based method was tried for RNA extraction from oocytes. Unlike the Trizol method in column method it involved only repeated centrifugations at sequential steps and thus reducing the chance of loosing any RNA extracted from oocytes.

3.3.2 Spin Column based method:

Total RNA was isolated from pool of 30 oocytes using a 'Nanoprep RNA Isolation Kit' (Stratagene), according to the manufacturer's instructions. The steps performed were as follows.

1. The oocyte samples stores at – 80^0C were thawed on ice.
2. 0.7μl β-mercaptoethanol was added to 100μl of lysis buffer supplied with the kit.
3. 100μl Lysis buffer as prepared above was added to the cell sample.
4. Sample was vortexed for 10 minutes to lyse the oocytes.
5. To the cell lysate, 100μl of 70% ethanol was added and vortexed for 5 seconds.
6. The mixture was transferred to RNA binding nano-spin-cup provided with the kit by taking care that the pipette tip does not touch directly to the spin cup membrane.
7. Sample was spinned at 12000g for 60 seconds.
8. Filtrate was discarded and spin cup was retained.
9. 300μl 1X high salt wash buffer (supplied with the kit) was added to the spin cup.
10. Sample was spinned at 12000g for 60 seconds.
11. Filtrate was discarded and spin cup was retained.
12. 300μl of 1X low salt wash buffer (supplied with the kit) was added and sample was spinned at 12000g for 60 seconds.
13. Filtrate was discarded again and spin cup was retained and step 12 was repeated ones more.
14. The empty spin cup was spinned at 12000g for 3 minutes to dry the matrix fiber.
15. Spin cup was transferred to a fresh 2ml tube.
16. 10 μl of elution buffer (Preheated at 65^0C) was added directly on to fiber matrix inside spin cup and incubated at room temperature for 2 minutes followed by spinning at 12000g for 5 minutes.

17. The elute (with extracted RNA) was collected in collection tube. The eluted RNA suspension was either processed for RT or was stored at –80^{0}C till further use.

Based on the experience of comparative efficacy and consistency of quality of amplifications obtained in RT-PCR products the column based Nanoprep RNA Isolation Kit (Stratagene) was used for the entire of the present study.

3.4 RT-PCR (Reverse Transcriptase- Polymerase Chain Reaction):

The 'Retroscript Kit' (Ambion) was used for cDNA synthesis from the RNA isolated from oocytes following the manufacturer's instructions. Briefly, the procedure followed was as under. The RT reaction was set on ice. To nullify the genomic DNA contamination in RNA preparations extracted by either Trizol or column based method a negative RT control reaction was always set. In the negative RT control all components were same as that of normal RT reaction except that MMLV Reverse Transcriptase was not added. After reverse transcription, PCR was done for the amplification of desired gene cDNA in which content of minus RT reaction product was used as template (RT –ve control) and also for all PCR reactions a PCR –ve control was used as quality control. The details of RT reaction were as follows.

1. 20 µl of RT reaction was prepared by adding following components in the same order.

10 X RT buffer	2 µl
Random Decamer	2 µl
10 mM dNTPs	4 µl
RNAse inhibitor	1 µl
MMLV RT	1 µl
RNA	10 µl
Total volume	**20 µl**

2. Cycling Conditions. The reaction tube was kept in a thermal cycler programmed at 44^{0}C for 1hour and at 94^{0}C for 10 minutes followed by holding the samples at 4^0 C. The prepared cDNA was stored at –20^{0}C till further use.

3.5 Primer designing for Histone, 18s rRNA and G3PDH genes:

To study the expression pattern of these housekeeping genes during in vitro oocytes maturation, primers specific for these genes were designed with a target to amplify a stretch of approximately 300 base pairs preferably from the 3' end of cDNA. Accordingly and after homology analysis of nucleotide sequences in related species available from GenBank

(http:/www.ncbi.nlm.nih.gov) primers were designed using the web-based software PRIMER-3 (http://www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi).

3.5.1 Primers for Histone:

Accession number: M 37585

Forward Primer: 5'-tggtgtcattccacacatcc-3'

Reverse Primer: 5'-tgcagaaatttggttggttg-3'

Expected Product Size: 234 bp

1..tacttgaattggcaggaaatgcatcgaaagacttgaaggtaaagcgtattacccctcgtcacttgcaacttgctattcgtggagatga
agaattggactctctcatcaaggctacaattgctggtgg**tggtgtcattccacacatcc**acaaatctctgattggaaagaaaggaca
acagaagactgtctaaaggatgcctggattccttattatctcaggactctaaatactctaacagctgtccagtgttggtgattccagtgga
ctgtatctctgtgaaaaacacaattttgccttttgtaattctatttaagcaagttggaagtttaattagctttc**caaccaaccaaatttctgc**
atttgagtcttaaccatatttaagtgttactgtggcttcaaagaagctattgatgctgaagtagtgggttttgattgagttgactgtttttaaaaa
actgtttggattttaattgtgatgcagaagttatagtaacaaacatttggttttgtacagacattatttccactctggtggataagctcaataaa
ggtcatatcccaaactgttgtgtataaaatttgcttgattatagtaggaacagctttgttgaataggtatcttacctagcaataacttaagca
catttcttccctttaaattactgttaattctgtctgtagatcacaaagttaaaaggcccaagtg..661

3.5.2 Primers for 18s rRNA:

Accession no: AF176811

Forward Primer 5'-gagaaacggctaccacatcc-3'

Reverse Primer 5'-ggacactcagctaagagcatcg-3'

Expected Product Size: 337bp

1..ccatggtgaccacgggtgacggggaatcagggttcgattccggagagggagcct**gagaaacggctaccacatcc**aaggaa
ggcagcaggcgcgcaaattacccactcccgacccggggaggtagtgacgaaaaataacaatacaggactctttcgaggccctgta
attggaatgagtccactttaaatccttccgcgaggatccattggagggcaagtctggtgccagcagccgcggtaattccagctccaata
gcgtatattaaagttgctgcagttaaaaagctcgtagttggatcttgggagcgggcgggcggtccgccgcgaggcgagccaccgccc
gtccccgccccttgctctcggcgcccccct**cgatgctcttagctgagtgtcc**cgcggggcccgaagcgtttactttg..361

3.5.3 Primers for G3PDH:

Accession number: U39091

Forward Primer: 5'-ctgacctgccgcctggagaaa-3'

Reverse Primer: 5'-gtagaagagtgagtgtcgctgtt-3'

Expected Product Size: 189bp

1..ggaagctcactggcatggccttccgcgtccccacccccaacgtgtccgttgtggatctgacctgccgcctggagaaacctgcc aagtatgatgagatcaagaaggtggtgaagcaggcgtcagagggccctctcaagggcattctaggctacactgaggaccaggtt gtctcctgcgacttcaacagcgacactcactcttctaccttcgatgctggggctggcattgccctcaacgaccactttgtcaagctcatttc ctggtacgacaatgaattcggctacagcaacagggtggtggacctcatggcccacatg.

3.5.4 Primers for β-Actin:

Primers for β-Actin were selected as per DeSousa et al., 1998. Expected amplification sizes were 408bp from bovine genomic DNA and 243bp from cDNA.

3.6 Optimization of PCR amplification conditions for respective primers

3.6.1 18s rRNA:

To ascertain the suitability of designed primers for amplifying the targeted 18s rRNA gene fragment and optimizing PCR amplification conditions the same was tested first on genomic DNA isolated from buffalo blood sample following phenol chloroform method (Sambrook et al, 2001). The 18s rRNA oligo-nucleotide primers synthesized commercially (Hysel) were diluted to get 1000 pm/µl stock and was re-diluted to a concentration of 5µM working stock. Optimal annealing temperature was established in a gradient thermal cycler (Eppendorf). PCR reaction mixture was prepared by adding following components to a sterile 0.5 ml PCR tube in the sequential manner. All the reactions were set on ice.

Reagents	Volume/25 µl reaction	Final Conc.
10 X PCR buffer	2.5 µl	1 X
18s Forward Primer	0.5 µl	0.1µM
18s Reverse Primer	0.5 µl	0.1 µM
10 mM dNTPs	0.5 µl	200 µM
Taq DNA polymerase (3U/ µl)	0.25µl	0.75U
DNAse free H_2O	19.75 µl	
Template genomic DNA	1 µl	
Total Volume	**25 µl**	

The tubes were tapped to mix, snap spinned and were placed on thermal cycler programmed for following cycling conditions:

Steps	Conditions	
First step	94^0C for 4 min	
Denaturation	94^0C for 45sec	40 cycles
Annealing	52- 62^0C for 30 sec	
Extension	72^0C for 28 sec	
Final extension	72^0C for 7 min	
Hold	4^0C	

After PCR cycling was complete, PCR tubes were removed from PCR machine and kept at – 20^0C. 10 µl of PCR product was mixed with ~2µl of loading dye and subjected to electrophoresis in 2% agarose gel with 0.5µg/ ml of EtBr and migrated at 60 Volts for 45 minutes. DNA molecular wt. markers were also run along with the samples on the same gel. The most optimized PCR amplification condition for the amplification of 18s rRNA from genomic DNA was used to amplify the 18s rRNA from cDNA prepared from oocyte isolated RNA. For this 2µl cDNA template was amplified in a total reaction volume of 25 µl for 40cycles. Annealing temperature range of 53^0C - 71^0C was tested. The $MgCl_2$ and primer concentration that gives specific amplification product from cDNA with consistent intensity was optimized with $MgCl_2$ of 1.5mM, 2mM and 2.5mM and primer concentration ranging from 0.075 – 0.3 µM. The cycling conditions as described above were used.

3.6.2 G3PDH:

Primers for G3PDH were diluted to get a working stock of 5µM as described for 18s rRNA. Optimum annealing temperature was established in a gradient thermal cycler (Eppendorff) in the range of 52^0C-60^0C. Initial optimization trials also revealed that an amount of 4µl cDNA template was required for appreciable PCR band visualization. The cycling conditions optimized were as follows.

Steps	Conditions	
First step	94^0C for 4 min	
Denaturation	94^0C for 45sec	40 cycles
Annealing	57^0C for 32 sec	
Extension	72^0C for 35 sec	
Final extension	72^0C for 7 min	
Storage	4^0C	

The $Mgcl_2$ and primer concentration optimization was carried out for the cDNA product of G3PDH in a PCR reaction volume of 25ul and 4µl cDNA template. $MgCl_2$ concentration of 1.5mM, 2mM and 2.5mM and primer concentration ranging from 0.075 – 0.15 µM was tested.

3.6.3 Histone:

As with 18s and G3PDH for histone also a working stock was 5µM primers were used. Primers were tested on genomic DNA template. Annealing temperature ranging from 51- 54^{0}C was found to yield discrete bands. For cDNA template an amount of 3µl was found to be optimum. $MgCl_2$ concentration was optimized with the primer concentration ranging from 0.1µM – 0.4 µM with 1.5mM, 2mM and 2.5mM $MgCl_2$.

Finally optimized cycling conditions for histone amplification was as follows:

Steps	**Conditions**	
First step	94^{0}C for 4 min	
Denaturation	94^{0}C for 45sec	
Annealing*	54^{0}C for 30 sec	40 cycles
Extension	72^{0}C for 28 sec	
Final extension	72^{0}C for 7 min	
Storage	4^{0}C	

3.7 Optimization of exponential phase of PCR amplification for 18s rRNA and G3PDH:

For relative quantification it is necessary to limit the cycle number to exponential phase (phase during which products amplify in exponential range). As the amplification products accumulate, the exponential phase eventually enters in to plateau phase during which products may approach similar levels irrespective of initial template concentration. Thus, for results to be meaningful quantitative comparisons must be done during exponential phase. To determine the exponential phase for G3PDH and 18s, a PCR master mixture was prepared according to previously standardized conditions for 18s and G3PDH primers, templates, $Mgcl_2$ concentration and primer concentrations. The master mixture was split into 8 tubes, which were then subjected to PCR in thermal cycler (Eppendorf) programmed at standardized cycling conditions. To ascertain the linearity of amplification at respective cycle numbers tubes were removed from the thermal cycler at the end of different cycle numbers (i.e. at 20, 23, 26, 29, 32, 35, 38 and 41 cycles). PCR products were resolved by electrophoresis (60V/45min.) on a 2% agarose gel with 0.5µg/ml EtBr and their band intensities were measured to decide the exponential phase of amplification for 18s and G3PDH products.

3.8 In vitro maturation of oocytes:

To be able to decide about an internal standard that could be used for studying expression pattern variation in candidate genes in the course of oocyte maturation it is important that the standard gene is expressed in a stable manner over the period of maturation. In the course of present investigation stability of expression pattern was studied for the 18s and G3PDH genes. Oocytes were aspirated and collected as described under materials and methods. Prior to collection of oocytes in laminar surface was cleaned with 70% alcohol and sterile conditions were maintained to avoid contamination. As described before the A^+ and A^- oocytes were washed twice in oocytes collection medium followed by washing them again in maturation media. The maturation medium used was TCM-199 with Earle's salt (Sigma), 10% FBS, 5 µg/mL of pFSH and 36 µg/ml sodium pyruvate. 50µl maturation drops were prepared and overlaid with autoclaved Mineral oil. 20 oocytes were placed in each maturation drops. Culture dish was incubated in CO_2 incubator at 38.5^0C and 5%CO_2. At the intervals of 0 (i.e. just after collection) 6, 12, 18 and 24 hour interval 20 oocytes were picked from maturation drop and vortexed to remove cumulus cells. Oocytes were washed thrice in PBS (Calcium and magnesium free) and taken in a RNAse free eppendorf in minimum volume of PBS and 20µl RNA later (Ambion) was added to oocytes and were frozen at -80^0C till RNA isolation.

3.9 Competitive RT-PCR:

3.9.1 Strategy for designing competitive primers:

Competitive RT-PCR precisely quantitates a message (mRNA to be quantified) by comparing RT-PCR product signal intensity to a concentration curve generated by a competitor RNA sequence. The competitor RNA transcript is designed for amplification by the same primers and with the same efficiency as the endogenous target. The Competitor produces a different-sized product so that it can be distinguished from the endogenous target product by gel analysis. The competitor is carefully quantitated and titrated into replicate RNA samples. Experiments are done to find the range of competitor concentration where the experimental signal is most similar. Finally, the mass of product in the experimental samples is compared to the curve to determine the amount of a specific RNA present in the sample.

As described in the figure below (Fig 2), in the present study for making a composite primer a region of 20 bp was decided downstream to the original forward primer for 18s. Thus the 18s composite forward primer was comprised of 20 bases of original forward primer (A) and another 20 bases downstream. This 40 base long composite forward primer(C) was studied for its

secondary structure using the web based tool http://www.genebee.msu.su/services/rna2_reduced.html and out of many options the present primer was detected to have minimum intrastrand GC pairing. The sequence of composite forward primer decided was 5' GAG AAA CGG CTA CCA CAT CC - A ATG AGT CCA CTT TAA ATC C 3'. The primer pair A and B was already found to amplify a 337 bp product and using the primer pair C and B was hypothesized to amplify a shorter 242 bp fragment of 18s which was planned to be used as a competitor. With the present hypothesis thus, when the primer pair A and B is used to amplify a mixture of target cDNAs (i.e. the unknown cDNA we want to quantify for 18s) and the competitor cDNA the primer A is logically expected to compete for the same primer binding site on target cDNA as well as on competitor cDNA. When a fixed amount of target cDNA is mixed with serially diluted competitor cDNA and amplified with primer pair A and B that would give varying levels of competitive products of 337 bp product and 242 bp. Evaluation of a point of equivalence would reveal the exact copy number of the target present in unknown samples.

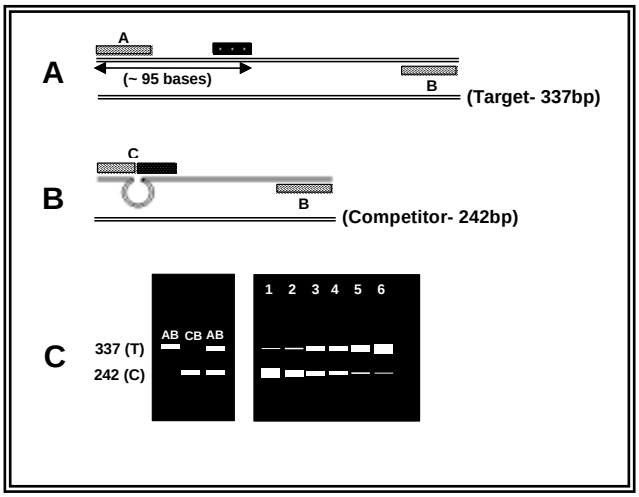

Fig. 2: Overall competitive PCR strategy to quantitate 18s rRNA transcripts. A. Selected routine sense (A) and antisense (B) primers were expected to give a 337bp PCR product. A region 95 bases down the 5' end was searched to design the composite primer (C). B. The composite sense (C) and earlier (routine) antisense (B) primers were anticipated to amplify a shorter 242 bp product. C. Thus Primers AB should amplify a 337bp product; the primers CB should yield a 242 bp product where as when the native oocyte cDNA and 242 bp products are used as templates and are amplified with AB it should yield two products which can be resolved on agarose gel based on the differences of their size and subsequently a competitive PCR can be set up with fixed volume of oocyte template and predetermined competitor template is series of gradually lowering dilutions.

Composite 18s Sense Primer: 5’ gag aaa cgg cta cca cat cc- aat gag tcc act tta aat cc 3’

Sequence of 18s rRNA cDNA and primer binding sites are shown below. The shaded portions define the binding sites for original primers and uppercase sequence defines the stretch used for making 40 bp composite competitive primers.

1 ccatggtgac cacgggtgac ggggaatcag ggttcgattc cggagaggga gcct**gagaaa**

61 **cggctaccac atcc**aaggaa ggcagcaggc gcgcaaatta cccactcccg acccggggag

121gtagtgacga aaaataacaa tacaggactc tttcgaggcc ctgtaattgg **AATGAGTCCA**

181**CTTTAAATCC** ttccgcgagg atccattgga gggcaagtct ggtgccagca gccgcggtaa

241ttccagctcc aatagcgtat attaaagttg ctgcagttaa aaagctcgta gttggatctt

301gggagcgggc gggcggtccg ccgcgaggcg agccaccgcc cgtccccgcc ccttgctctc

361ggcgcccccct **cgatgctctt agctgagtgt cc**cgcggggc ccgaagcgtt tactttg

3.9.2 Optimizing competitive primers on cDNA to generate short 18s product:

A 5µM working primer concentration was prepared for the composite forward primer and original reverse primer. cDNA amount used per 25µl reaction was 2µl. Previously, for RT-PCR the annealing temperature was optimized at 62^0C for 18s rRNA product. However, in the context of present competitive PCR experiment to encourage effective competition between target and competitor and to avoid self annealing of 40 bp forward composite primer annealing was done at slightly higher temperature of 65^0C. PCR cycling conditions were as follows:

Steps	**Conditions**	
First step	94^0C for 4 min	
Denaturation	94^0C for 45sec	44 cycles
Annealing*	65^0C for 30 sec	
Extension	72^0C for 28 sec	
Final extension	72^0C for 7 min	
Storage	4^0C	

3.9.3 Purification of competitor cDNA 18s rRNA product:

A PCR products purification system Wizard® PCR Preps DNA purification system (Promega) was used for the purification of PCR product. Briefly,

1. About 100µl Direct purification buffer was taken into a 1.5 ml eppendorf tube and 100µl of PCR product was transferred to it; Tube was vortexed briefly to mix the contents.

2. Added I ml of resin and again vortexed briefly 3 times over a 1minute period.
3. One mini-column was prepared by removing the plunger from 3ml disposable syringe and the syringe barrel was attached mini-column.
4. The PCR product-resin mixture was transferred to syringe. The entire content in the syringe was slowly passed through the mini-column. Fluid collected in eppendorf tube was discarded.
5. Syringe was detached, plunger was removed, and barrel was connected again to the mini-column. 2 ml of 80 % isopropanol was added to syringe and with the help of plunger, contents were again slowly passed through the mini-column. The solution collected in eppendorf tube was discarded. The mini-column placed in eppendorf tube was now centrifuged at 10,000g for 2 minutes to dry the resin. Solution collected in eppendorf tube was discarded.
6. Mini-column was transferred to a new micro centrifuge tube. Finally 20 µl of DNase free water was applied on top surface of the column. After standing at room temperature for one minute, mini-column was centrifuged at 10,000g for 20 seconds.
7. Mini-column was removed and discarded and the elute (20µl) contained purified DNA.
8. 2 µl of eluted sample was run on 2% agarose gel in 1X TAE buffer for ascertaining purity of DNA. The product was stored at -20^0C for future use.

3.9.4 Spectrophotometric quantification of purified product:

Purified short fragment of 18s rRNA competitor, was subjected to spectro photometric analysis for deciding the concentration of DNA. The readings obtained were as follows.

OD260	OD280	OD260/280	Dilution factor (DF)	Concentration(µg/ml) (50 x DF x OD_{260})
0.044	0.0240	1.84	35	77

3.9.5 Standardization of competitor amplification:

To ascertain the presence of binding sites for original 18s forward primer on generated short 242 bp competitor product, the PCR was performed with purified competitor as template and original forward and reverse primers. A combination of 1.5 mM $MgCl_2$ 200 µM dNTP and 0.1µM of primers was used in the same cycling conditions as described under section 3.10 for 42 cycles. PCR product was run in 2% agarose gel in 1X TAE to ascertain the product.

3.9.6 Standardization of competition range:

To optimize a viable range of competition between the competitor and native 18s target product the competitor product in different dilutions ranging from 100 to 0.3125 pg/ µl in a serial dilution of 10 and 2 were used with two different levels of oocyte derived cDNA template viz. 1 µl and 0.5 µl. Each time the PCR reactions were set up in a volume of 25µl having equal volume of oocyte cDNA template and a dilution series of competitor product. The target was to get a range of amplification products where the native and competitor precuts are visible with different degrees of competition. An equivalence point was defined as the reaction where the native and competitor products are amplified with equal intensities. It was also targeted that while searching for a range of competition the equivalence should also include competition reactions on either side of the equivalence reaction i.e. tubes where target was marginally higher than the competitor and also some competition tubes where the competitors are higher than the native. This will help in analyzing the data and will validate whether the competition range found is predictive to quantitate the unknown template concentration or not.

3.10 Densitometry and Statistical Analysis:

The quantitative data for expression analysis was obtained by resolving the amplified PCR products from different experiments on 2% agarose gel with 0.2µg/ml ethidium bromide in 1X TAE buffer. Gel images were grabbed on a 4.0 Megapixel Olympus digital camera. Densitometry data for band intensities was generated using AlphaDigiDoc^{TM} AD-1201 software under Windows^{TM} environment. For validation of competitive PCR procedure, densitometry data with respect to PCR amplified competitor and target products after log transformation were subjected to regression analysis using MSExcel programme. Similarly for temporal variation determination the hourly interval band intensity densitometry data was regressed on time interval values followed by trend analysis using MS Excel programme.

3.11 Buffers and solutions:

(I) 50 X Tris Acetate EDTA buffer (TAE)

Tris base	121 g
Glacial acetic acid	28.5 ml
Sodium EDTA	18.6 g

Total volume was made to 500 ml with double distilled and autoclaved. pH of solution was adjusted to 7.5. 50 X TAE was stored at room temperature.

(ii) 1 X TAE buffer

2 ml of 50 X TAE buffer solution was diluted to 100 ml with distilled water.

(iii) Ethidium Bromide (10 mg/ml, Stock)

One EtBr tablet (100mg) (Sigma) was dissolved in 10 ml of DEPC treated water. Concentration of resulting solution was 10mg/ml.

(iv) Ethidium Bromide (0.5µg/ml, working)

EtBr stock was diluted 10 times. (i.e. 10µl EtBr stock was added to 90µl DEPC treated water). Concentration after dilution was 1000µg/ml. 50 µl from working solution (1000µg/ml) was added to 100ml melted agarose gel to make 0.5µg/ml,

(v)Loading buffer

Loading buffer was prepared by dissolving 25 mg bromophenol blue and 25 mg xylene cyanol in 10 ml of (aqueous) 50% glycerol.

(vi) 10 X PBS (Calcium and Magnesium free) (100ml)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.24g

Total volume was made up to 80ml with MilliQ water. pH was adjusted to 7.4 with HCl. Volume was made to100ml and autoclaved. Solution was stored at room temperature.

(Vii) DPBS (Dulbecco's phosphate buffer saline)(oocytes aspiration media) (1000ml)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.150g
KH_2PO_4	0.200g
$MgCl_2.6H_2O$	0.100g
*$CaCl_2$	0.100g
Sodium pyruvate	0.036g
Glucose	1.000g
Penicillin G	0.060g
Streptomycin	0.100g
Phenol Red	0.005g

*$CaCl_2$ was first dissolved in approximately 50ml MilliQ water and added separately to the above components dissolved in 300 ml MilliQ water. pH was adjusted to 7.2 and volume was made 1000ml. Solution was filtered through 0.22µ assembly filter and stored at 4^oC. For oocyte aspiration to the filtered DPBS of 50 ml BSA 0.4% was added and kept undisturbed to allow the mixing of BSA. Finally the solution was filtered through 0.22µ filter.

(Viii) 0.1N NaOH (100ml)

Added 0.4g NaOH pellet to 100 ml milliQ water.

(ix) Oocyte Maturation media (10ml):

TCM-99 (Sigma) with Earle's salt	10ml
FBS	1ml
pFSH	0.00005g
Sodium Pyruvate	0.00036g

The medium was stored at 4^oC and on the day of use was filtered through 0.22µ filter and kept at 5% CO_2 incubator at 37^oC for at least one hour for equilibration.

(x) 2% agarose (100ml)

Weighed 2g agarose and added in 100ml of 1X TAE buffer. Heated to dissolve and allowed to cool and added EtBr in required amount. Pured on gel casting trey and allowed to polymerise.

(xi)Normal Saline (1000 ml)

Dissolved 9g NaCl (Sigma) in 1000ml MilliQ water. Solution was autoclaved.

(xii) 0.1% DEPC water

Added 1ml DEPC to 1000ml MilliQ water and mixed properly by vigorous shaking. It was incubated at 37^0C overnight and then autoclaved twice at 15 psi for 45 min.

4. Results and Discussion

The present dissertation work entitled “Quantification of expression pattern of some important housekeeping genes during *in vitro* maturation of buffalo oocytes” was taken up with two principal objectives of standardizing a method of RNA extraction from buffalo oocytes to study the expression of house keeping genes and also to establish the stability of expression pattern of these genes in curse of their in vitro maturation. From the review of literature it appears that information on gene expression profiling in oocytes and early preimplantation embryos is a recent area of research that is under intense investigation particularly to give a plausible answer to explain the poor success rate of IVF process per se (Gandolfi and Gandolfi., 2001). Most of the workers have expressed that a serious impediment to studies involved on this direction is the availability of enough number of oocytes/ embryos required as starting material for using in the standard techniques for mRNA quantification like northern blot or other nucleic acid hybridisation base techniques like RPA, ISH etc. (Lechniak et al., 2002). However, the advent of the PCR (Mullis, 1986) and hence later method of RT-PCR and quantitative RT-PCR (q-RTPCR) (Ferre., 1992; Foley et al., 1993; Raeymaekers., 1993) offered a simple and sensitive method to analyse gene expression pattern from even a very minute quantity of starting material. Considering the economic advantage of Buffalo under Indian dairy situation and also the necessity to standardise the process of IVF in this species we were interested in studying the gene expression process in buffalo oocytes which will provide vital clues in better understanding the oocyte competence and IVM process and eventually help in working out a defined IVF protocol in this species (Singh et al., 1989). Literature published on Buffalo IVF constantly reports the problem of poor availability of oocytes from ovaries in this species (Totey et al., 1992). Many probable explanations to this problem have been cited (Erickson, 1966). In practical situation when the available oocytes are further sub classified into categories based on morphology, physiological status of the follicles from where the oocytes have been obtained, the number of oocytes available per group for experimentation becomes further limiting. Thus pursuing the area of oocyte gene expression in buffalo becomes challenging and requires a potent RNA recovery system that could be employed for smaller number of oocytes to start with. Quantitative RTPCR and other mRNA quantification techniques like NB, RPA, Real time PCR etc. used either for relative or absolute quantification of transcripts always require a standard for normalisation of expression data that takes care of possible sources of error in quantification process like sample to sample variation, RNA recovery rate variation, RT as well as PCR efficiency variation, loading volume variation etc. which are steps in RTPCR experimentation (Thellin et al., 1999). The

present study was directed at searching out for a stable House Keeping Gene in oocytes which could be used as a 'standard' in quantification experiments.

Considering the above and to cater to the objectives of this project we tried to answer the following questions through our study.

1. What could be a suitable RNA extraction method that makes available analysable quantity and quality of RNA to the used in RTPCR?
2. What are the optimised PCR parameters for valid quantification of selected HKG transcripts?
3. What is the minimum number of oocytes that can be processed for quantification of selected HKG transcripts?
4. Optimising a quantitative competitive RT-PCR (qc-RTPCR) for valid (absolute) quantification of selected housekeeping genes transcripts?

Presented below are the description of results obtained and their possible explanations with respect to the experiments performed to answer the above questions.

4.1 Result of different RNA extraction method from oocytes:

In the present experiment we tried with two different RNA extraction systems viz. Trizol method and a column based centrifugation method for isolation of total cell RNA from buffalo oocytes. TRIZOL Reagent has been used widely for the isolation of total RNA from a variety of cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement over the single-step RNA isolation method developed by Chomczynski and Sacchi, 1987. During sample homogenization or lysis, TRIZOL Reagent maintains the integrity of the RNA, while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase where RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol. However, one of the drawback with the Trizol method particularly in processing smaller number of cells to be used for quantification (as in the present case) is the variability involved in pipetting out the organic phase after initial organic: aqueous phase separation. While taking the maximum of aqueous phase there is always a probability that a part of the organic phase is also taken out in this process which includes impurities and on the other hand fixing the volume of aqueous phase to be pipetted out may involve loosing some RNA, as the yield of aqueous phase varies following Trizol extraction from fixed number of cells from batch to batch. In the present study such variations were apparent in terms of variation in cDNA amplification of desired transcripts from experiment to experiment when the number of oocytes processed remained fixed (standardization data not presented in this

thesis). As an alternative, we tried with another method of RNA extraction using the 'Nanoprep RNA Isolation Kit' from Stratagene. 'Nanoprep' is a spin column based centrifugation system with advantage over the Trizol method in the way that this system involved only centrifugation steps in sequential steps and RNA is finally eluted in the last step which avoids loosing any RNA. Experience of using this method for RNA isolation from a fixed number of oocytes, their cDNA transformation following RT and subsequent PCR amplification was very consistent and for actual experimental work in the present study this method was found most suitable.

4.2 Minimum number of oocytes required for RTPCR quantification of transcripts.

Based on the published literature, initially 70-80 oocytes were used to extract RNA. However, experience of poor availability of oocytes from buffalo ovaries prompted us to try the RNA extraction from lesser number of oocytes. Subsequently the procedure was standardized for extraction of the RNA from 30 oocytes following either the TRIZOL or Nanoprep method. Based on the satisfactory amplification of both 18s rRNA and G3PDH transcripts using the optimized PCR conditions (described later in this section) it was concluded that Nanoprep method was more consistent than TRIZOL. However, considering the very poor availability of acceptable quality oocytes from buffalo ovaries and also to make available enough number of oocytes required to plan for a well designed experiment, we examined further the availability of RNA and consistency of cDNA amplification from still lesser number of oocytes. Under this experiment the Nanoprep method was used to extract RNA from a batch of 5, 10, 15 and 20 oocytes. Extracted RNA was reverse transcribed into cDNA and the 18s rRNA primers were used to check cDNA amplification pattern from these experimental cDNA batches. An extra precaution was taken not to lead the PCR into plateau phase and thereby making the estimates biased. The amplified 18s rRNA products were analysed from 28 cycle PCR reactions (28 cycle falls below the optimized exponential cycle determined as 32 and explained later in this section). The resolved PCR amplified products obtained from 5, 10, 15 and 20 oocytes and their densitometric analysis is presented in the figure below. (Fig. 3 A and B)

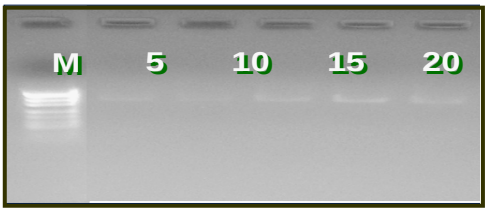

Fig. 3 A. Amplification of 337bp 18s rRNA products from cDNA generated from RNA extracted from 5, 10, 15 and 20 oocytes.

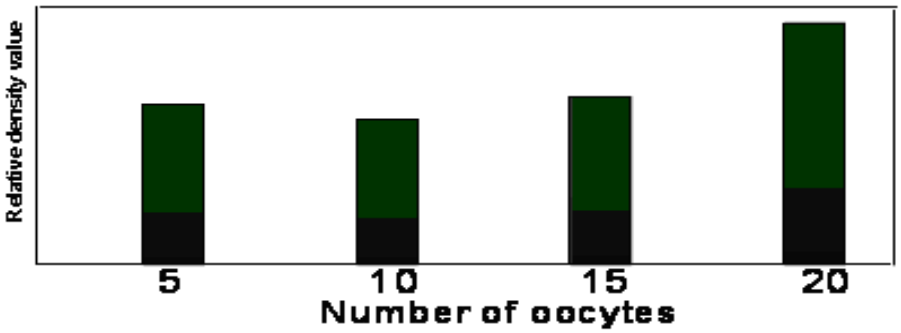

Fig. 3 B. Comparison of Relative Density Values obtained for the band intensities observed against amplified18s rRNA product from different number of oocytes.

It could be seen from the figures above that amplified 18s rRNA products could be detected even with a minimum of 5 numbers of oocytes and the amount consistently increased with the increase in oocyte number. The reduced value observed with 10 oocytes must have originated from an experimental artifact and could be ignored. Based on this experience we reduce the number of oocytes to be processed from 30 to 20 for RNA extraction in temporal variation set of experiments described later. Detectable intensity of cDNA amplification in lower number of oocyte samples was particularly encouraging in the context of experiment with buffalo oocytes in view of its poor recovery. DeSousa et al, 1998 have described a laser induced fluorescence capillary electrophoresis method which was sensitive enough to detect transcripts of Na^+-K ATPase α-subunit in bovine. Similar refinement of transcript quantification method will prove very useful for the buffalo system.

4.3 Generation of cDNA template by Reverse Transcription: The total cell RNA obtained from oocytes was reverse transcribed by MMLV RT using random decamers. The RT step has been reported as a source of variability in the kinetic of RT-PCR experiments (Sugita et al., 2001). Another source of variability is the choice of priming method used for cDNA synthesis which can be either target gene specific or non specific (oligo dT/ random decamers, hexamers etc.). Target specific gene primers work well and are especially useful to eliminate spurious transcripts (Foss et al., 1998). However, the use of gene specific primers necessitates a separate RT reaction for each gene of interest to be studied. Thus in case one is interested in studying more than one genes from oocytes it would be required to generate separate cDNA lots for separate transcripts to be studied, which may itself incorporate a source of error as it cannot be assumed that different reactions have same cDNA synthesis efficiency. The use of random decamer in present experiment was suitable in the present experiment in view of the fact that the 18s rRNA selected as a housekeeping gene presents polyA minus transcripts (Suzuki et al.,2000) and thus, oligo-dT primers was not a feasible option. The 20µl of pooled cDNA generated from a small RNA sample was used as a common source of template for quantification against different set of primers and

different experimental conditions which certainly has helped to reduce the intra assay variations.

4.4 Optimisation of PCR parameters for valid quantification of HKG transcripts:

a) Primers: Careful designing of primers is important in PCR to obtain the specificity of desired amplification and to avoid amplification of unwanted sequences. Principles of primer design for PCR amplification of DNA and RNA have been discussed in many instances (Sambrook., 2001). As thumb rule the larger an oligonucleotide the higher is the specificity for a particular target but a practice of designing primers of 18-25 nucleotides length has remained most popular. The base composition of primers should be 40% to 60% with an even distribution of all four bases along length of primers. To search for a suitable primer for amplification of a gene in a species where no sequence information is available a sequence homology search using available internet based software such as primer 3 (developed by Steve Rozen and Helen J. Skaletsky,1996,1997) available on line http://www.-genome.wi.mit.edu. helps to decide the most likely conserved regions which could be used for designing primers. In the present study primers for the amplification of 4 HKGs viz. 18s rRNA, G3PDH, Histone and β-Actin were designed using software primer-3 and the exact size and sequence of primers and expected amplification sizes are described under materials and methods section. These four HKGs were selected based on the evidence of their use in gene expression studies in varying tissues (Suzuki et al., 2000) including oocytes and embryos (Robert et al., 2002). As a strategy, an amplification length of 200 to 400 bp were desired preferably from 3' end of cDNA considering the consistent PCR efficiency (Pfaffl et al.,2002) and RT efficiency (Brooks et al.,1995). It is also mandatory in a q-RTPCR methodology to validate the fact that the ultimate PCR amplified products are representative of cDNA population and not from the genomic DNA contamination from RNA isolates. The same could be taken care of by designing primers which are intron spanning and there by yielding a bigger product when amplified from genomic DNA and a smaller product representing cDNA amplification. In the present study the primers for β-actin were designed as per this hypothesis. As an alternative approach to check he genomic DNA contamination and particularly applicable for intron less genes, like the 18s rRNA under present study, a RT negative control could be taken while PCR amplification of product and the same strategy was followed for the other three HKGs in the present study.

b) Testing efficiency of primers: In the present study before the primers were tested for their amplification efficiency on cDNA they were tested on genomic DNA mainly to optimize

the annealing temperature, and other cycling conditions. Regarding testing the primers on cDNA the amount of the template to be used for the optimization was critical. Since the determination of RNA concentration is often inaccurate and we had to work on arbitrary amount of RNA extracted from fixed number of oocytes we chose to work on fixed volume of cDNA rather than the exact quantity (Chelly et al., 1988). The figures (Fig. 4 A and B) below explain the optimized conditions for amplification of 18s rRNA in genomic DNA as well as cDNA.

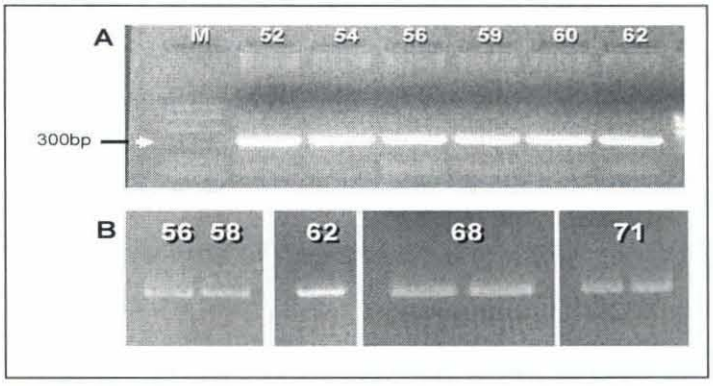

Fig. 4 A and B. Gel photographs showing the amplification optimisation results for 18s genomic DNA (A) and cDNA (B).

As described in the figures above for 18s genomic DNA amplification an annealing temperature ranging from 52°to 62°was tested. Specific products (337bp) were obtained at all temperatures. Subsequently the amplification specificity was optimised on cDNA at varying annealing temperatures ranging from 56°to 71 °C. A primer concentration of 0.1µM and cDNA template amount of 2µl (obtained from 30 oocyte RNA extract) resulted in specific amplifications at all temperatures.

Similarly for G3PDH primer optimisation experiment was conducted first for genomic DNA and then for cDNA. The results are depicted in the figure below (Fig. 5 A and B)

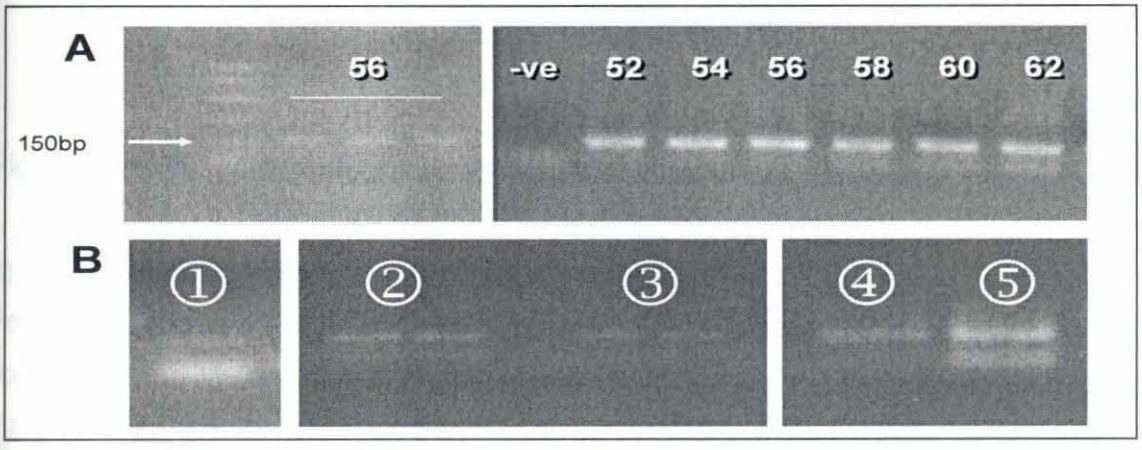

Fig. 5 A and B. Gel photographs showing the amplification optimisation results for G3PDH genomic DNA (A) and cDNA (B).

As shown in Fig 5 A annealing temperatures from 52^0C-62^0C yielded specific G3PDH 189 bp amplification products from genomic DNA. On testing the primers (0.5µM) with cDNA at temperature 56^0C and template amount of 2µl yielded a product with heavy primer dimer (Fig 5 B,1). To get rid of the dimers the primer concentration was reduced to 0.1µM and 0.075µM keeping the template amounts as same. Although the primer dimer amounts were reduced with reducing the primer concentration the band intensities were also reduced accordingly (Fig 5 B 2 & 3). Ultimately annealing temperature of 56^0C, primer at 0.1µM and template of 4 µl was found to be most ideal in terms of band intensity and reduced primer dimer (Fig 5 B, 4). With 4 µl template and a higher primer concentration again along with desired amplification intensity strong primer dimers were detected and hence this combination was not selected (Fig. 5B, 5).

Regarding histone and β-Actin the primers were found to amplify product from genomic DNA at annealing temperature from 52^0C-62^0C for histone (Fig 6 A, 1) and 52^0C-62^0C for β-Actin (Fig 6 B, 1). Although the primers were subsequently tested to yield products from cDNA (Fig. 6 A,2 & B,2) but as a whole the amplification pattern was very inconsistent due to unexplained problem of smearing in Histone (Fig.6 A, 3) and very weak signal in beta actin (Fig. 6 B, 2). The cDNA amplification from these genes could not be optimized further and the study was restricted to only two HKGs viz. 18s rRNA and G3PDH.

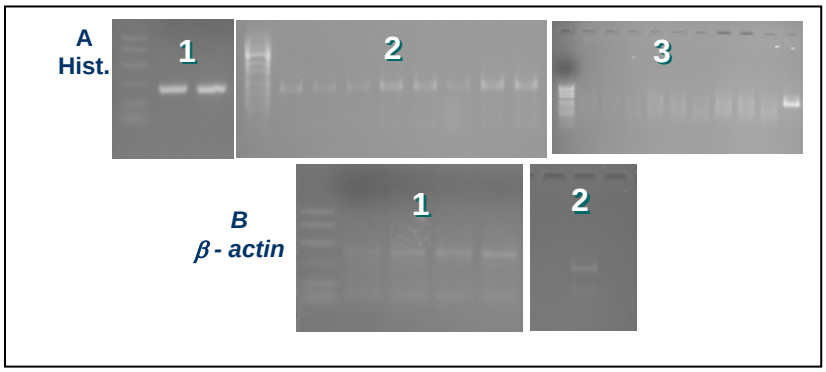

Fig. 6 A and B. Gel photographs showing the amplification optimisation results for Histone (A) and β-Actin (B) amplification.

c) Optimization of Primer and $MgCl_2$ concentration:

For performing q-RTPCR optimising for an ideal combination of primer and $MgCl_2$ concentration is important and in present experiment three different concentration of $MgCl_2$ viz.1.5, 2 and 2.5mM were used with each of the four different primer concentration viz. 0.075, 0.1, 0.2 and 0.3µM for 18s rRNA and three different concentration of primers for G3PDH viz. 0.075, 0.1 and 0.2 µM as explained in Fig. 7 A & B the ideal combinations were

selected based on their intensity and specificity of the amplified product. As a general trend increasing $MgCl_2$ under respective primer concentrations reduced amplification intensity and a higher primer concentration resulted in smearing of products along with specific amplicon. Similar trend was observed by Marone et al., 1997 while experimenting with Bcl2 and aldolase expression pattern in human erythroleukemia cell line.

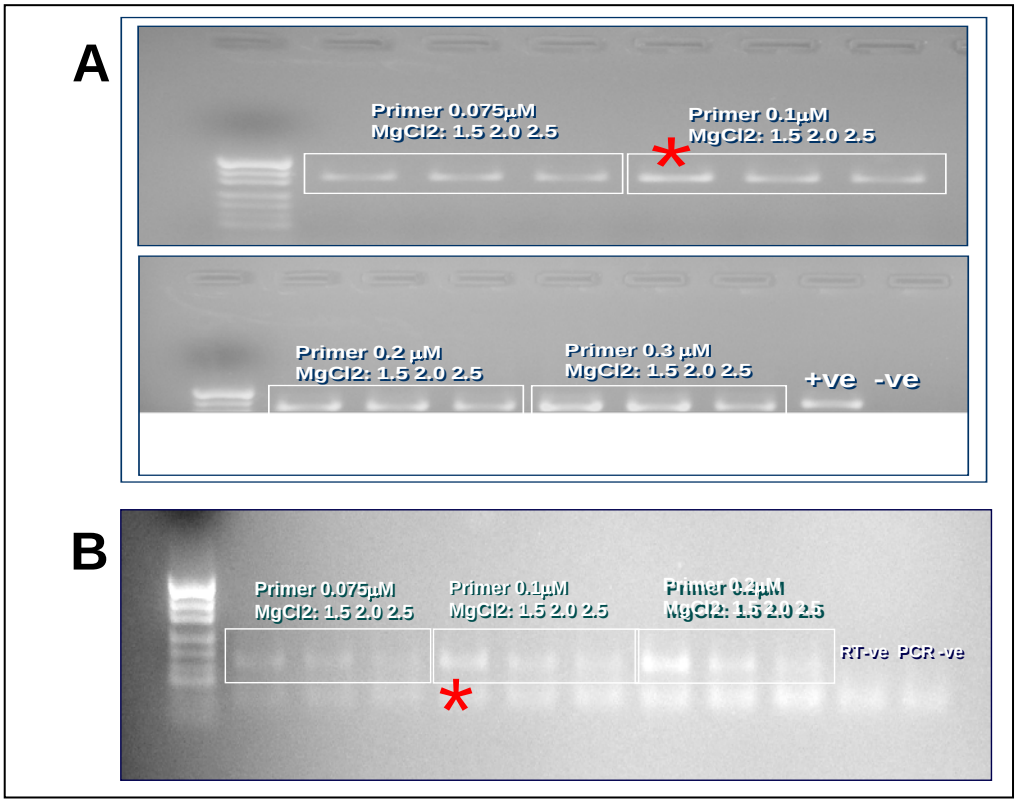

Fig. 7 Optimisation for Primer and $MgCl_2$ combination for 18s rRNA (A) and G3PDH (B) amplification. * indicates optimised combinations in either case.

Although the RT-PCR have been reported to be a highly sensitive method it may lead to a bias because any amplification error in initial phase of PCR are also amplified along with the targets. This variability can be very large and preclude accuracy and the reliability of quantification. $MgCl_2$ and primer concentrations are two such parameters which determine the specificity and efficiency of amplification process. The salt $MgCl_2$ as a component of PCR reaction buffer performs the dual job of acting as a cofactor for Taq polymerase and also making available primers at the point of annealing with template. For a given sets of primers and enzyme Taq polymerase therefore it is of utmost importance to optimize the $MgCl_2$ concentration. $MgCl_2$ concentration in the range of 1mM-5mM has been used for optimization of PCR amplification under different situation where the sequence of primer has been reported to be a determining factor to find out the best concentration (Morsetti et al., 1997). Similarly for standard PCR application a primer concentration between 0.075-

0.1µM is recommended (Rolfs et al., 1992). During PCR reaction the primers have to compete with the accumulating product in finding their target sequence which could become a limiting factor for reactions at late cycles. Thus, optimizing the ideal combination of primer concentration was required for the targeted cDNA product for both 18s rRNA and G3PDH. The optimize conditions of primer and $MgCl_2$ were used in subsequent experiments of finding out another important parameter for q-RTPCR i.e. the exponential phase of amplification for respective products.

d) The exponential phase of amplification for 18s rRNA and G3PDH products:

To find out the exponential phase of PCR amplification for 18s rRNA and G3PDH, the PCR master mixture was split into multiple tubes, which were then subjected to PCR in thermal cycler, programmed at optimised cycling conditions. Tubes were removed from the thermal cycler at 20, 23, 26, 29, 32, and 35 cycles for 18s rRNA and from 20-41 cycles at the interval of three for G3PDH. Products at end of respective cycle numbers were resolved by electrophoresis (60V/45min.) on a 2% agarose gel with 0.5µg/ml ethidium bromide and their band intensities were measured to decide the exponential phase of amplification for 18s rRNA and G3PDH products. In both the cases, no product was detectable at 20 and 23rd cycle, subsequently the intensity of the product increased with the increase in cycle number up to 32 cycles in both the cases beyond which the amplification product got more or less stable indicating the plateau phase. Results are depicted in Fig. 8 A & B.

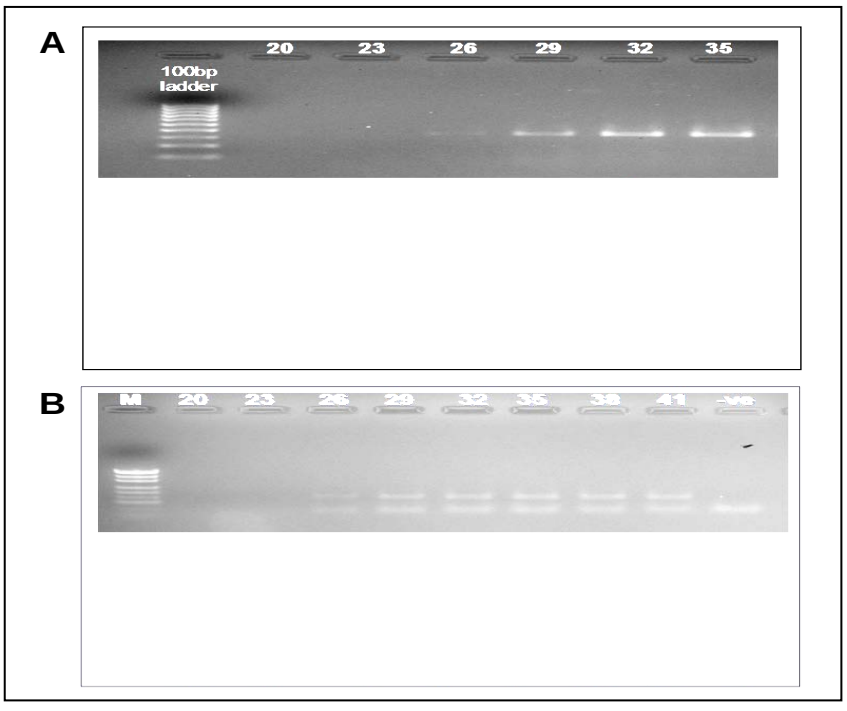

Fig. 8 Optimisation of exponential phase amplification for 18s rRNA (A) and G3PDH (B). * indicates optimised cycle number.

One of the drawbacks of using the q-RTPCR method for mRNA quantification is that following this method we try to analyse expression level of a gene based on the PCR end point product quantification. In a typical PCR reaction, the amplification of the given number of the template in PCR is dependent on availability of individual components viz. primer, dNTPs and the stability of Taq over the period of reaction. The amplified products increase by exponential number till such point that one of the components becomes limiting (Ambion technical bulletin no 1716, 1717, 1718, and 2001). Thus at plateau phase of PCR, RNA initially present at higher level may give product of equal intensity to low abundant RNA. Based on the evidence of present experiment and as described in Fig. 8, 32 cycles was optimized as the cycle number within exponential phase for both 18s rRNA and G3PDH amplification and was used for the subsequent part of the experiment.

4.5 Temporal expression pattern of housekeeping genes:

To study the temporal expression pattern, selected oocytes were put for in-vitro maturation in maturation medium droplets in a bunch of 20 oocytes per drop and were cultured for different time intervals of 0, 6, 12,18 and 24 hours. At the end of respective time intervals 20 oocytes from each group were processed for RNA extraction, RT and PCR amplification for 18s rRNA and G3PDH. The density values obtained for the intensity of amplifications were regressed over time to draw trend line curves. This set of experiment was repeated twice and the interpretations are based on duplicate values obtained against ach time interval observation over the period of 24 hours. PCR amplified products against each time interval groups are shown in Fig. 9 and Fig. 10 depicts the trend line derived from density values over the period of 24 hours for both 18s rRNA and G3PDH.

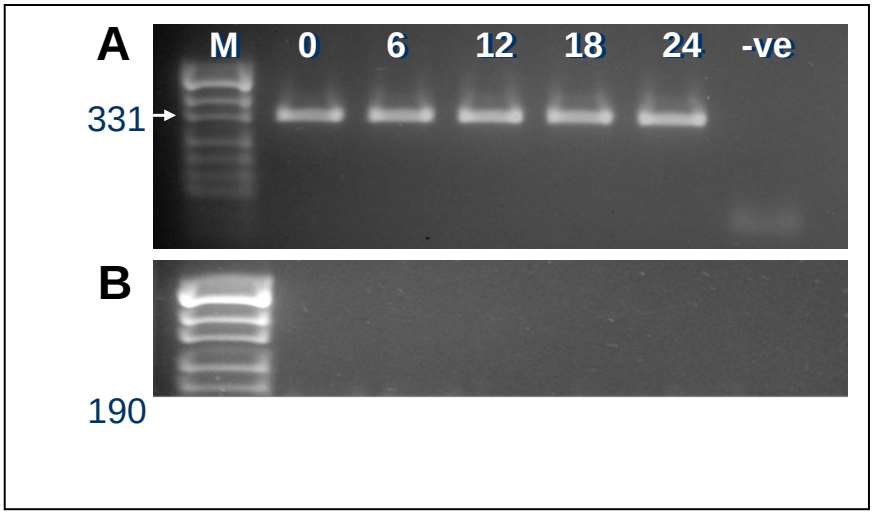

Fig. 9 Expression pattern of 18s rRNA (A) and G3PDH (B) in oocytes at 6, 12, 18, and 24 hours during of in vitro maturation.

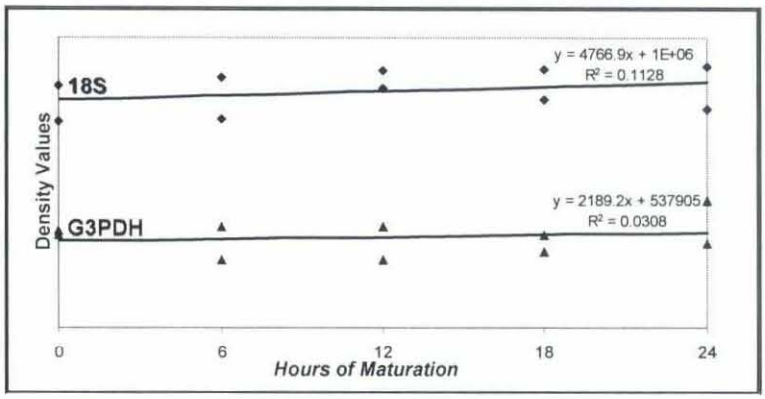

Fig 10 Trend of expression pattern of 18S and G3PDH over 24 hours of in vitro maturation in buffalo oocytes.

An overall analysis of the expression pattern of these two HKGs revealed the fact that their expression was more or less stable across different time intervals; although for G3PDH the intensity of expression was reduced slightly after 12 hours as could be seen from Fig. 9. In terms of stability the trend was better with respect to 18S than G3PDH. In either case, R^2 values for trend lines were low (0.11 for 18s, 0.03 for G3PDH) which can be attributed to independence of expression pattern over the time period of in vitro maturation. However, considering the intensity of analysable products obtained with 18S and also the fact that lower volume of cDNA template required for its amplification it could be logically concluded that 18s will be a preferred candidate for its use as internal standard for q-RTPCR quantification of other buffalo oocyte transcripts. A wide range of flexibility in annealing temperature observed for 18s (55^0C-71^0C) will be another advantageous feature for this gene to be used in standardizing a multiplex PCR with other oocyte transcripts.

Suzuki et al, 2000 based on their review on use of different housekeeping genes for quantification of gene expression in wide variety of tissues opined that 18s is a stable standard used in many contexts. In a study by Goidin et al., 2001attempting to quantitate human cytokines the 18s was interpreted as a better internal standard over G3PDH and β-Actin. Robert et al, 2002 has studied the expression pattern of a battery of eight different housekeeping genes during in vitro maturation of oocytes and up to implantation of in vitro produced embryos in bovines. They concluded that whereas the genes like beta actin, G3PDH, tubulin, lamin and ubiquitin had a tendency of increased transcript degradation after the GV stage 18s remained stable up to eight cell stage of development. However, the option of using 18s as internal standard in gene expression quantification should be considered with the background information that it is in fact a ribosomal RNA gene and not always represent the overall cellular mRNA population (Suzuki et al., 2000). Complication in using 18s as internal standard may also arise due to its high abundance in a cell and a wide difference in abundance of targeted genes transcript may not be suitable for the accurate

due to its high abundance in a cell and a wide difference in abundance of targeted genes transcript may not be suitable for the accurate quantification of target transcripts. As a way out to compensate for such wide abundance variation, amplifying !8s rRNA at lower cycle number has been suggested (Ambion Technical Bulletin Cat # 1716, 1717, 1718). Nevertheless, Thellin et al., 1999 reported that 18s rRNA could be used as an internal standard in situations where other commonly used housekeeping genes are not found suitable.

4.6 Competitive PCR for the quantification of oocyte transcripts:

In the present study apart from the conventional q-RTPCR, another method of gene quantification called competitive PCR was used for the validation of 18s rRNA expression stability during in vitro maturation of buffalo oocytes. This method originally described by Becker and Hahibrock, 1989 overcome some of the limitations of RTPCR. Most significantly the RTPCR quantification may get biased by plateau phase product analysis and thereby is unsuitable for differentiation of initial difference of transcript in different experimental groups. As an improvement over q-RTPCR method in competitive PCR the same target gene is used as a control after some modifications and the same sets of primers are used to amplify both he target and the standard. Thus the primers in the PCR reaction compete for the amplification of both target and the standard, often described as competitors. Thus under competition when the target sequence is present in higher abundance the competitor hardly gets a chance to amplify and the vice versa. The primer recognition sequence being the same between target and standard the efficiency of the PCR between them is expected to be the same.

The 18s rRNA competitor in the present case was prepared following the method of O' Connell, 2002 and is describe under the materials and method section. Principally a competitor should be of different size than the target and are designed with two basic objectives:1) the competitor has been ideally described to be ~100-200bp shorter so that it could be resolved in agarose gel and 2.) The synthesized primer being a long sequence of 40 bases may fold on itself to generate stable secondary structure and thereby interfering with the primer binding efficiency. In the present case the competitor 18s rRNA was 95 bp shorter (242bp) than the native product (337bp) and a composite primer 40bp long was designed keeping above two objectives in mind. Out of many options the present primer was decided after evaluating its secondary structure to be least complicated. The predicted secondary structure is presented in Figure 11.

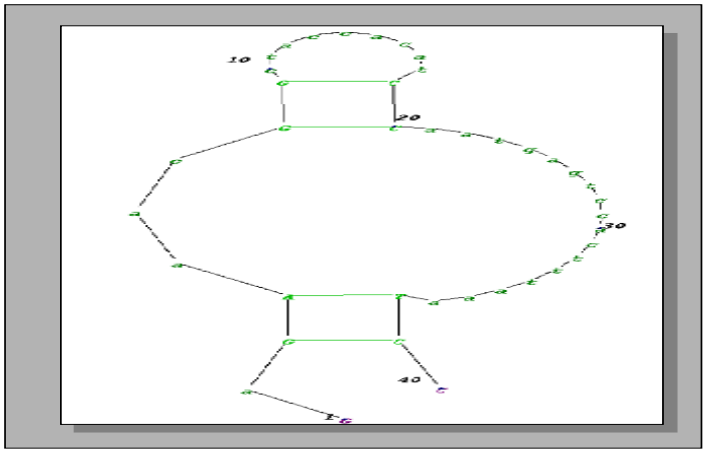

Fig. 11. Pre sense primer.

The 18s rRNA cc rom oocyte cDNA template using the composite sense and normal antisense primer and in a parallel reaction tube the same template was amplified using normal sense and antisense primers. On electrophoresis of PCR products, the discrete 242 and 337 bp products could be identified (Fig. 12)

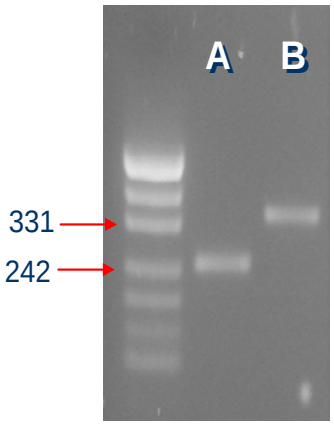

Fig. 12 Generation of 18s rRNA shorter cDNA product used as a competitor. In tube 'A' the oocyte cDNA was amplified with 18s rRNA composite sense primer and routine antisense primer. For tube 'B' primers used were 18s rRNA routine sense and antisense primers.

The 242 bp PCR product (18s rRNA competitor) was purified and quantified spectrophotometrically and the OD values obtained were as under.

OD260	OD280	OD260/280	Dilution Factor (DF)	Conc. (µg/ml) OD260 X 50 X DF
0.044	0.0240	1.84	1:35	77

Before setting the competition reaction, we had to optimize a viable range of competition between the competitor and native 18s rRNA target product. This was particularly important because the concentration of cDNA in our oocyte extracted samples was unknown. In present case, the cDNA product experimented in competition range finding experiment was

derived from 30 oocytes. To start with we prepared the competitor standard in different dilutions ranging from 100 to 0.1pg/ml in a serial dilution of 10. PCR reactions were set up in a volume of 25µl and each reaction had 1 µl of oocyte cDNA template (unknown concentration) and equal volume of competitor cDNA (known concentration). The PCR was performed under optimized conditions for 18s rRNA for 42 cycles. The products obtained were resolved on 2% agarose gel and presented in Figure 13.

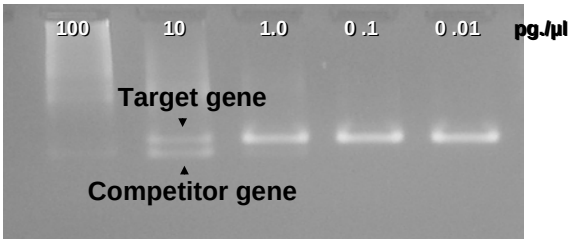

Fig. 13 Result of amplification of target and competitor cDNA in dilution series of 100 to 0.01 pg./ µl.

As could be seen in above figure a 100pg standard template was probably too high to amplify any product. We could see both the competitor and native products in 10pg tube and in tubes 1pg and further downwards the native product was principally amplified signifying that in all these tubes the competitor template was too low to participate in the competition process. From this we could interpret that an effective competition started some where between 100 and 10pg and continued up to a range between 10 and 1pg tubes. To further experiment on this and in an effort to find out the exact range of competition in an next set of experiment the standards were prepared in concentrations from 20 to 0.3125 pg/ µl with serial dilution of 2 and as a modification the native cDNA template in each competition reaction was reduced from 1 µl to 0.5 µl. The competitive PCR was set as above and the result is described in Fig. 14.

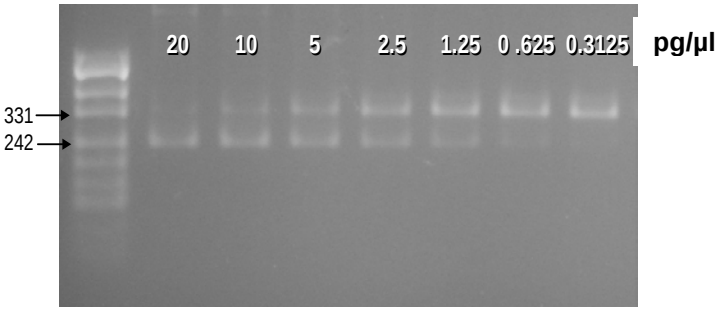

Fig. 14 Result of amplification of target and competitor cDNA in dilution series of 20 to 0.3125 pg/ µl.

As we could see this time a competitive reaction could be seen in almost all the tubes and as hypothesised the target product (337bp) was seen to be amplified in increasing order of intensity when amplified in the presence of decreasing concentration of competitors (242bp). The nature of competition was ideal in 5 and 2.5 pg tubes which could be described as points close to equivalence. In fact this was considered to be an ideal situation where we could see different levels of competitions on either side of the equivalence point. The validity of competition was ascertained with regression analysis of densitometric data derived from band intensities of both competitor and native product. In the regression analysis the log values obtained for ratios of competitor : target (C/T) was regressed over log values of competitor (C). The regression curve as depicted in Figure 15 revealed a best fit equation which had a R^2 value of 0.987 indicating validity of competition over the range of different standards used.

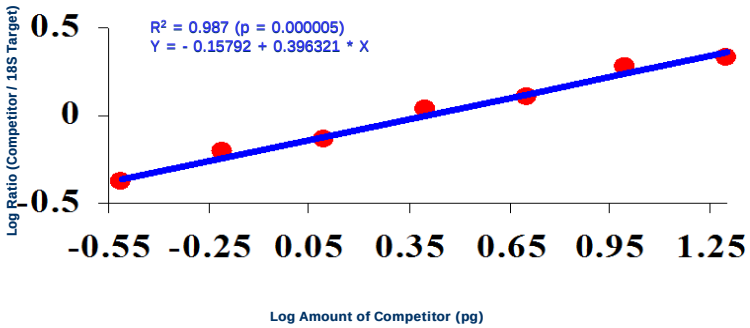

Fig. 15 Regression curve and best fit equation obtained to validate the range of competition between the native and competitor 18s rRNA products.

4.7 Validation of 18s rRNA temporal expression pattern using competitive PCR during in vitro maturation:

With optimized competitive PCR conditions as described above, 0.5 µl cDNA templates obtained from each of the hourly interval samples along with competitors ranging from 20 to 0.156 pg were used to quantitate 18s rRNA transcripts. Fig. 16 describes the nature of competitions observed in respective cases.

As a whole following conclusion could be drawn from the figure.

1. A range of competition was observed over all the reaction tubes at respective hourly interval samples.
2. There is a strong indication that a perfect competition indicating the point of equivalence or a point closest to equivalence was consistently observes at the reaction tube having 2.5pg of standard.

As described before, regression analysis of respective hourly interval data showed validity of competition and the temporal variation trend line data presented in Figure 17 indicates approximately stable expression pattern of 18s rRNA over the period of in vitro maturation of oocytes.

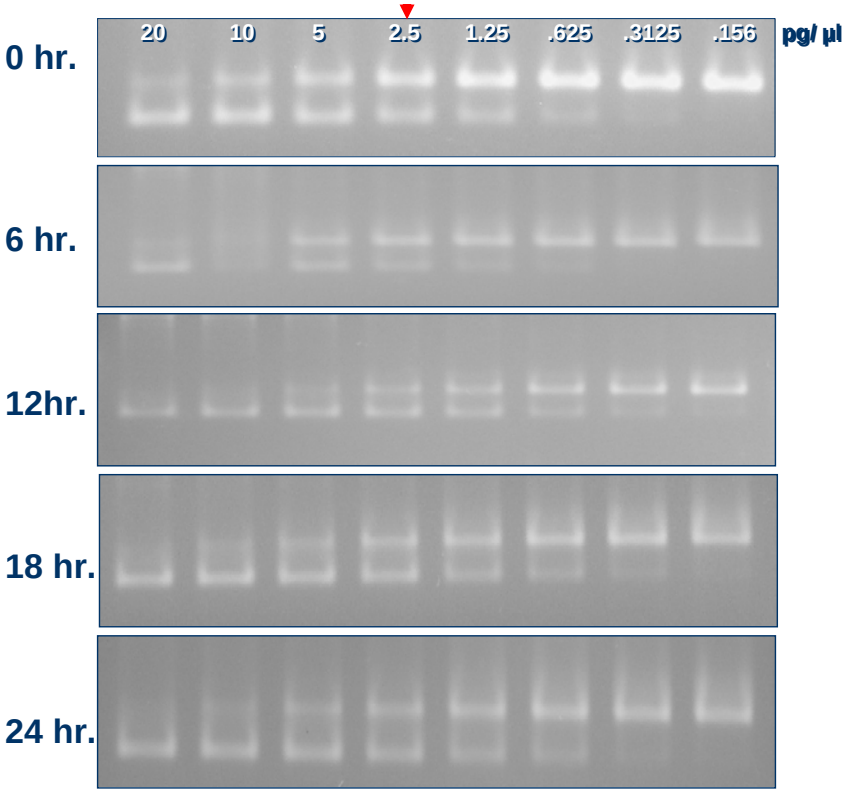

Fig. 16 18s rRNA competitive PCR products obtained from cDNA samples of oocytes during 6, 12, 18 and 24 hours of maturation of buffalo oocytes.

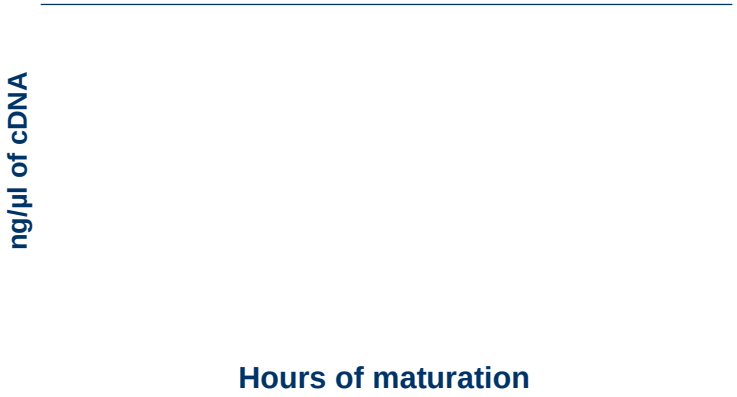

Fig. 17 Trend line of expression pattern of 18s rRNA over 24 hours of IVM of buffalo oocytes.

The competitive PCR method has been used for the quantification of the gene expression in several contexts including that in oocytes and embryos (Taylor et al.,2000; Krussel et al.,1998; Anderson et al.,1999; Moley et al.,1998; Navarrete et al.,2000). Robert et al.,

2002 in this regard remarked that the competitive PCR procedure is a truly quantitative method for quantifying mRNA transcripts. In the present experiment the competitor used was a PCR amplified DNA product generated from oocyte cDNA. The assumption behind using a cDNA amplified competitor was that the RT efficiency is same for the target as well as the competitor product. This assumption however has been questioned and variations in RT efficiency has been cited as a limitation for the sensitivity of this method (Freeman et al., 1999; TaKaRa technical notes,1995). Nevertheless, O Connell et al., 2002 opined that the approach of using a composite primer based cDNA competitor has advantage considering the simple experimental procedure involved and has used it successfully for expression quantification of caspase-1 in colonic epithelial cell lines. Goode et al., 2002 in this connection has reported that although such preformed cDNA competitors is logically can't be used as a control for variation in RT step this should not be considered a serious limitation as the efficiency of RT does not vary greatly between RNA samples prepared by same isolation protocols. As per their experience in most cases for which the efficiency of RT reaction had been measured by incorporation of p^{32} labelled nucleotides into cDNA it was usually found that RT catalysed conversion of mRNA to cDNA occurs with an stable efficiency of around 10%. In this light our observations can be interpreted as valid.

Regarding the PCR cycle number to be followed for performing quantitative PCR the opinion seems to be divided. Freeman et al., 1999 reported that the quantification of competition should be done at exponential phase because different amounts of initial templates may yield similar amplification product signal at plateau phase. However, in the present experiment the competition was evaluated at 42 cycles with the logic that during qc-RTPCR, because the two templates i.e. native and competitor competes for PCR amplification, it may be necessary to perform more cycles to achieve good amplification of both the targets. Similar reports of successful quantification of transcripts following qc-RTPCR at higher PCR cycle number are available (Clementi etal., 1995; Cottrez et al., 1994; Pannetier et al., 1993).

To conclude the competitive PCR approach followed in the present study could be considered as an important step towards transcriptome analysis particularly with reference to oocyte biology. However the present set of competitive PCR data was based on a single set of observation. Adding more replicates will contribute to the validation of 18s rRNA expression stability in the course of in vitro maturation of buffalo oocytes. Scope also remains open for further narrowing down the range of standards to get closer to the exact point of equivalence during competition. Exponential phase validation of the present approach may also be considered for increasing the authenticity of results obtained.

5. Summary and Conclusions

The present dissertation work entitled "Quantification of expression pattern of some important housekeeping genes during *in vitro* maturation of buffalo oocytes" was taken up with two principal objectives of standardizing a method of RNA extraction from buffalo oocytes to study the expression pattern variation of house keeping genes and also to establish the stability of expression pattern of these genes in course of their in vitro maturation. Buffalo oocytes were collected from the ovaries of slaughtered animals and were subjected to two different oocyte recovery methods to judge their suitability and consistency in extracting RNA required as a starting material for transcript analysis. Standard reverse transcription using random decamer priming was used to generate cDNA and designed oligonucleotide primers were used to amplify four House Keeping Genes viz. 18S rRNA, G3PDH, β-Actin and Histone 2A from cDNA derived from fixed number of oocytes to assess the stability of their expression pattern during in vitro maturation of buffalo oocytes. To assess the trend of their expression over the maturation period fixed amount of cDNA samples obtained from fixed number of oocytes were amplified at 0, 6, 12, 18 and 24 hours of maturation in standard oocyte maturation medium comprising of TCM-199 supplemented with Pyruvate, FBS, FSH-p and antibiotics. Apart from RT-PCR a competitive quantitative RT-PCR (QC-RT-PCR) procedure was adopted to verify the expression stability of oocyte transcript particularly for the 18s rRNA. To generate the quantitative data for expression pattern assessment the PCR product band intensities were measured by densitometric software and values were processed for regression analysis and trend analysis. Following conclusions were derived from the study.

1. A Spin column method was found to be more consistent than Trizol method for the isolation of RNA from buffalo oocytes that yielded consistent RT-PCR amplifications on repeated attempt from fixed number of oocytes.
2. Amplification of 18s rRNA and G3PDH was optimized from cDNA prepared from buffalo oocyte extracted RNA using orthologous primers designed mainly from the reported cattle sequences.
3. Essential parameters for a RT-PCR based relative quantification assay for gene expression viz. the exponential phase, primer and $MgCl_2$ combination was optimized for 18s rRNA and G3PDH.
4. Amplification of β-Actin and Histone transcripts were problematic and not yielded consistent result in the present study.

5. A composite primer strategy was used to generate homologous cDNA competitor for 18s rRNA and used in a competitive RT-PCR approach to quantitate expression pattern variability of 18s rRNA.
6. The template amounts of 20 to 0.3125 pg of competitor with 0.5 oocyte equivalent of cDNA displayed an effective range of competition in QC-RT-PCR for 18s rRNA.
7. The efficiency of the competitive PCR protocol developed for the quantitation of 18s rRNA was found to be valid with high level of confidence (R^2 = 0.987)
8. The temporal expression pattern of 18s rRNA and G3PDH genes were experimented during IVM of buffalo oocytes and 18s rRNA was found to be a better candidate than G3PDH for its use as internal standard for quantification of gene expression in buffalo oocytes in terms of its stability of expression and quality of RT-PCR amplification.
9. The competitive PCR strategy used was found valid for absolute quantification of 18s rRNA from buffalo oocytes.
10. The competitive quantitative RT-PCR method applied also confirmed the stability of expression of 18s rRNA in the course of in vitro maturation of oocytes in buffalo.

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