Optimization of a specific messenger RNA extraction protocol for fresh and vitrified bovine oocytes to gene expression studies

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ABSTRACT

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To understand bovine oocytes meiotic maturation, developmental potential, gene expression is required. The gene expression studies in the preimplantation bovine oocytes has been difficult, because the procedures that are being employed for extracting total RNA are not specific for bovine oocytes and so far is not providing the required amount for further procedures. Quantification of genes generally requires large amounts of total RNA in order to overcome the problem of low amount of mRNA present, so a standardized specific protocol is recommended. These days most of the researchers are using commercial Kit protocols without knowing the significance of chemicals and how they are acting on cells. In present project a standardized protocol (modified trizol) was designed for bovine oocytes, which was specific and less expensive. The efficiency of this protocol compared with Pure Link (Kit Protocol), GNTC (Guanidinium thiocyanate) for extraction of total RNA from fresh oocytes, vitrified oocytes with PROH (1,2 propanediol) and DMSO (dimethylsulfoxide) cryoprotectans was much better. The RNA (absorbance 260/280) purity levels of the standardized protocol was ranging (1.50-2.10), whereas for GNTC protocol (1.05-1.36), Pure Link (kit protocol) (2.05-2.7). Amplification of housekeeping genes (*SDHA* and *GAPDH* gene) showed the specificity and efficiency of the standardized protocol over other protocols.

INTRODUCTION

Oocyte gene expression can be evaluated using mRNA which provides genetic functionality and the downstream effect of epigenetic influences mediating oocyte development (Rascado *et al*., 2010). In a previous study of oocytes populations at different development stages, Fair et al. (1997) identified rapid changes in mRNA profiles after oocyte maturation and subsequent fertilization. In the same way changes in mRNA transcripts levels are recently observed between GV (Germinal vesicle) and MII (Metaphase II) stage in mouse and human oocytes (Cui *et al*., 2006 and Assou *et al*., 2006). To study the gene expression in the samples with small number of cells and tissues, many different techniques have been developed. Normally a single mammalian cell contains 1×10^5 µg of total RNA out of this around 85 % is ribosomal RNA (rRNA), 15-20 % is transfer RNA (tRNA), 1-5 % messenger RNA (mRNA) and 35 % of mRNA was found in the nucleus (Schultz *et al.*, 1973 and Arjun *et al*., 2006). In this way, there are several difficulties in isolation of total RNA from embryos and oocytes because of limiting quantity of cell and consequently, RNA (Manes *et al.*, 1981). The amount of total RNA of bovine oocytes is 0.98-2.4 ng and can range from 0.7 to 5.3 ng in different stages of development (i.e. from germinal vesicle stage to embryonic development stage) (Levy *et al.*, 1978). To overcome the limitation of having low quantity of total RNA, large pools up to 300 oocytes or embryos are used to extraction. The major disadvantages of using these large pools samples are the expense and time consuming in collection of large number of oocytes and embryos (Piko *et al.*, 1982). For a standardising suitable protocol is further necessary to know about oocytes morphology, growth and development, as well as understanding the function of different other protocols for extraction of total RNA from tissue or cells like GNTC (Guanidinium thiocyanate) and commercial kit protocols. A biochemical aspect which plays a major role in the oocytes growth is the mechanisms of Ca^{2+} homoeostasis during growth and maturation (Fair *et al*., 1996a). The increased calcium (Ca^{2+}) levels helps to maintain nucleic acids in aqueous phase instead dissolving in phenol phase, by co-precipitating with nucleic acids in precipitation step of isopropanol in RNA extraction (Juliana *et al*., 2010). The quality of oocytes also has to be taken into consideration as only the good ones become an embryo which could had its gene expression studied. Bovine oocytes used for IVM (*In vitro* maturation) are selected depending on visual assessment of morphological classification schemes, which are based on the compactness and quantity of surrounding follicular cells, mainly the coverage of cumulus cells (Cetic *et al,* 1999). There are different manual and kit protocols for the extraction of total RNA, like GNTC/phenol

(Guanidinium thiocyanate) method, Trizol, commercial kit protocols. Trizol is one of most common chemical solutions used in the extraction of RNA, DNA, and proteins included in many of the kit products, being also a brand name of Invitrogen, MRC, and TRI Reagent. Guanidinium thiocyanate and chloride are most effective protein denaturants (Nozaki *et al*., 1970). Guanidinium chloride is a strong inhibitor of ribonulcease so it is introduced as deproteinization agent for extraction of RNA by Cox (1968). Later on chloride was replaced by phenol, to extract undergrad RNA from ribonuclease rich tissues like pancreas (Chirgwin *et al*., 1979), hence GNTC/Phenol (Guanidinium thiocyanate) method is specific to large amount of tissues. The guanidinium method has been used not only in RNA isolation but also in DNA from eukaryotic cells; however the protocol to RNA differs from DNA and varies according to the type of tissues from which nucleic acids are retrieved (Cox, 1968; MacDonald *et al.*, 1987; Van Kooij and Van Oost, 1992; Kaufman *et al.*, 1995). Single step method of RNA extraction was introduced by (Chomczynski *et al*., 1986) which is known as trizol which is a chemical combination of guanidinium thiocyanate, phenol and chloroform. This method of extraction provides high yield and purity of RNA. Trizol protocol was designed to eliminate ultracentrifugation step in GNTC/phenol and chloride method and is more specific in RNA isolation because it maintains the RNA integrity during tissue homogenization and breaking down cells components (Chomczynski *et al*., 1987). In all aerobic organisms *SDHA* (Succinate dehydrogenase flavoprotein subunit A) gene functions as a membrane bound component of both citric acid cycle and respiratory chain (Mark *et al*., 1992). It is involved in complex II of mitochondrial electron transport chain and is responsible for transferring electrons from succinate to ubiquinone (Ohnishi *et al*., 1987). The physical and catalytic properties of succinate dehydrogenases are from phylogenetic sources of bovine (Ackrell *et al.*, 1992). GAPDH (glyceraldehyde 3-phosphate dehydrogenase) is also known as G3PDH which catalyses the six step of glycolysis and helps in breakdown of glucose for energy and carbon molecules (Campanella *et al*., 2005). GAPDH acts as a link between metabolic state to gene transcription by moving between cytosol and nucleus (Tisdale *et al*., 2007). The cellular location of GAPDH is cytosol since the glycolysis takes place in cytosol. *GAPDH* gene is highly stable and constitutive expressed at high levels in most of tissues and cells (Robert *et al*., 2005). For this reason *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and *SDHA* (Succinate dehydrogenase flavo protein subunit A) genes are chosen in the present experiment, which aim is to establish a standard protocol for the extraction of total RNA from a minimum number of bovine oocytes, with a good quality based on its morphology, which enables the PCR amplification with two chosen

housekeeping genes *SDHA* (Succinate dehydrogenase flavoprotein subunit A) and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase). The modifications at the trizol protocol were mainly by changing the composition of usage, centrifugation time, volume and reagents combination, in a way to be specific and efficient for RNA extraction from a minimum number of bovine oocytes.

MATERIAL AND METHODS

Chemicals

The chemicals and reagents used in reproduction experiment were obtained from Sigma-Aldrich (St.Louis, MO, USA). All the PCR reaction mixtures are from Fermentas Company USA.

Collection of ovaries

Around 80 to 100 ovaries were obtained from a local abattoir from adult animals, trimmed of adhering tissue and transported to the laboratory in Dulbecco's phosphate buffered saline (DPBS) at a temperature ranging from 34 to 37ºC within 2h post slaughtering. All ovaries were rinsed once with 70 % alcohol and followed by a wash with fresh DPBS (reference) upon arrival at laboratory.

Immature oocytes collection

Cumulus oocytes complexes (COCs) were collected by aspiration (Fry et al., 1997) from antral follicles (2-8 mm diameter) with 18 gauge needle and transfer into a falcon tube which was maintained at 37ºC. After a few minutes clump of cells were observed down in the falcon tube as a pellet. The pellet was collected and transferred to a petri dish with washing medium TCM-199 (Tissue Culture Medium) supplemented with 2 % FBS (Foetal Bovine Serum), 0.3 mg mL^{-1} glutamine and 50 µg mL^{-1} gentamycin. The petri dishes was observed under Stereo microscope (Leica MZ75) for collecting the oocytes, which were selected, counted and transferred to a new petri dish with washing medium and were washed twice. While washing the oocytes in washing medium the granulosa cells were separated from the oocytes by mechanical process for denudation. Then the denuded oocytes and granulosa cells were washed twice again with DEPC (dietilpirocarbonate) water and collected in RNAse free tubes in a number from 5 to 60 oocytes per tube, according to the number of oocytes and quantity

of granulosa cells. The oocytes and granulosa cells collected in RNAse free tubes were stored in -80ºC for further total RNA extraction and considered as a control (fresh) group.

Frozen and thawing oocytes

After collecting and washed with the washing medium, good quality oocytes were selected based on their morphology. The selected immature oocytes were subjected to two different cryoprotectants (PROH (1,2 propanediol) and DMSO (Dimethylsulfoxide)) and initially equilibrated for 5min in holding medium (TCM 199 medium with Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), supplemented with 1.5 M (PROH or DMSO), 0.1 M sucrose, 20 % FBS and 50 µg mL^{-1} gentamycin). After equilibration, some of the oocytes were transferred to vitrification solution (2 M PROH or DMSO in TCM-199 Hepes medium with 0.1 M sucrose, 20% FBS and 50 µg mL^{-1} gentamycin) for 30 s at room temperature. The oocytes were loaded in a French mini straw (FMS) and immediately plunged into LN_2 (Liquid Nitrogen). The straws subjected to thawing, were removed from LN_2 and held in air for 10 s then transferred quickly to water bath at 37ºC for 30 s. The oocytes post thawing were washed twice with washing medium, granulosa cells were separated from vitrified oocytes and washed with DEPC water to be stored separately in RNAse free tubes based on their group of vitrification (PROH or DMSO) in the same number of the fresh group (5 to 60). Then samples were stored in -80ºC for further extraction procedures.

Extraction of total RNA

The methods of mammalian cells RNA extraction, to be compared and optimized, were chosen from protocols specific for mammalian tissues, commercial and reproductive cells in large amounts. The commercial kit was made according to the fabricant. The GNTC protocol was made as follows: 1)100µl of denaturing solution (4 M Guanidinium thiocyanate, 0.02 M of sodium citrate), 0.72 µL of 14.4 M beta-mercaptoethanol, 10 µL of 2 M sodium acetate (pH 4.0), 100 µL of phenol saturated with water (pH 5.5) and 20 µl of chloroform:isoamyl alcohol are added to the sample and vortex it for 3min vigorously; 2)the samples were centrifuge at 12,000 g for 5 min; 3) the upper phase (aqueous phase) was transfered in to the RNAse free tube; 4)1 µL of 2mg mL^{-1} of glycogen, 100 µL of isopropanol and 10 µL of 1 mg mL^{-1} carrier tRNA (Sigma Chemical Co.) was add into each sample and mix by inversion; 5)the samples were centrifuged for 12,000 g for 30 min at 4ºC; 6)the supernatant was removed to a new tube and the RNA pellet was washed twice with 200 µL of 75 % ethanol in

0.1 % DEPC-treated sterile water; 7)the pellet was air dried for 15 min and further dissolved in 20 µL of DEPC water. The modified trizol protocol was made by 1)adding 100 µL of trizol to the samples, pass it in the vortex and incubate for 3 min; 2)adding 50 µL of chloroform to the RNAse free tubes and invert them for 15 s incubating at room temperature for more 3 min; 3)the samples were centrifuged at 12,000 g for 30 min at 4ºC; 4)the aqueous phase was transferred in to a new tube; 5)2.5 volumes of isopropanol was added to the aqueous phase collected; 6)the tubes were centrifuged at 12,000g for 30 min at 4ºC; 7)the supernatant wasdiscarded and the pellet washed with 150 µL of 70 % ethanol and centrifuged at 7,500 g for 5 min; 8)the pellet was dried in an incubator for 30 min at 37ºC and further the pellet was dissolved 10 µL of DEPC water. All the samples from the three protocols were stored at -80. After all extractions the spectrophotometric (U.V spectrophotometer Shimadzu, U.V-1603 and NanoVeu GE Company) reading was taken from extracted total RNA samples, for the three conditions to be compared (fresh, PROH and DMSO vitrified) after preheating the samples at 60ºC for 5min. After the reading the samples were stored in -80ºC for further cDNA synthesis.

cDNA synthesis

Total RNA samples stored at -80ºC are subjected to the cDNA synthesis using 2 µg of total RNA. The cDNA reaction mixture was made according to the manufacturer's protocol to the (Revert Aid H Minus First Strand cDNA synthesis Kit) (Fermentas, 2011). All the samples were placed in PCR (Biometra alfa Gene Company) for cDNA synthesis, and subjected to RNase H (Thermo Scientific) treatment (according to Fermentas manufacture). The cDNA samples treated with Rnase H were stored in -20ºC for further polymerase chain reaction with the housekeeping genes.

Primer design and PCR

Suitable forward and reverse primers are designed by using Primer plus 3 software (http://www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi) for *GAPDH* (GenBank ref. NM_0010434034.1) and *SDHA* (GenBank ref. NM_174178.2) genes. The designed primers of these genes are shown in the Table 1.

The PCR was performed in a total volume of 20 µL, with 1 X PCR buffer with 3.0 mM of $MgCl_2$, 250 µM of dNTP mixture (adenine, cytosine, timine and guanine), 10 pmol μL^{-1} of each primer (forward and reverse), 1 unit of *Taq* DNA polymerase (Fermentas, Thermo

Scientific), 2.5 µL of cDNA and water enough to complete the total volume. The reaction was made in 35 cycles, with 30 s of denaturation at 94ºC, 30 s of annealing at 54ºC and 45 s of extension at 72ºC. The cycles had an initial denaturation at 94ºC during 3 min and were followed by a final extension at 72ºC for 10 min. The PCR products were stored in -20ºC for electrophoresis.

Gel Electrophoresis

An electrophorese was followed with 3 % of TAE (Tris acetate plus EDTA buffer) agarose gel (120 µL of 1xTAE buffer (recipe of the buffer), 3.6 g of agarose, 12 µL of SYBR safe (Invitrogen)). It was taken 8 µL of PCR products with 2 µL of loading dye solution (recipe) and loaded in to the gel. As weight molecular marker, 6 µL of 1Kb DNA ladder plus (Gene Ruler, Fermentas) was loaded and the gel was run during 30 min at 120 volts. Gel photographs were taken for further analysis with a transiluminator equipment (UVI tech,UVI Doc) and the intensity of the bands were measured.

RESULTS AND DISCUSSION

The criteria of using follicle aspiration instead of follicle dissection is due to the aspiration process is three times faster than dissection. Highest oocytes recovery rate was obtained by using 18-g needles (Hashimoto *et al*., 1999). TCM 199 medium is the washing medium to the oocytes most widely used for studies of bovine oocytes maturation. This medium including is capable of supporting the maturation of bovine oocytes even in the absence of serum (Lonergan *et al*., 1994). The selected oocytes are divided in three groups named control (the fresh one) and the vitrified with the two mentioned cryoprotectans DMSO and PROH creating a platform with three different conditions to test the protocol as these two mentioned cryoprotectans are mostly widely used agents for preservation of bovine oocytes. These cryoprotective agents have several functions during freezing process such as freezing point depression; dehydration of the cells was higher former to intracellular freezing (Farrant, J *et al*., 1980).

The three groups of fresh, vitrified oocytes and granulosa cells were subjected to total RNA extraction with three different protocols. What could be observed in the spectrophotometry reading, the purity of the samples measured as the ratio of absorbance at 260 and 280 nm (A 260/280) to the different protocols for the control group had different ranges being GNTC from 1.05 to 1.32, the commercial kit protocol from 2.053 to 2.629, and the developed

modified trizol protocol from 1.50 to 2.11. Purity levels of modified protocol were constant (1.50-2.0) and nearest to the expected (1.5 to 2.1) when compared with GNTC protocol. When the purity levels of 8, 11 and 15 oocytes was compared between modified trizol protocol and commercial kit protocol, shows that modified trizol protocol is better than kit protocol because the purity levels were from 1.50-1.73 whereas 2.05-2.68, respectively, and also the concentration levels of modified trizol protocol had a medium value of 180 ng μL^{-1} against 24.4 ng μL^{-1} from kit protocol. Having a purity level of 2.68 means a presence of high protein contamination in the sample while below 1.70 represents more DNA contamination in the sample (Andrey *et al*., 2007).

The good purity levels of vitrified oocytes with DMSO (range from 1.92 to 2.27) were observed when compared with fresh and vitrified PROH oocytes (range from 1.47 to 2.09). Increased in purity levels with DMSO conditions may be due to dimethylsulfoxide accelerates the activity of isopropanol during the precipitation of nucleic acids extraction procedure (John *et al*.,1963), even the oocytes were thawed after vitrification.

GNTC protocol

In this total RNA extraction protocol 100 µL of denaturing solution was added to microtubes of 500 mL of volume, with 20 to 60 oocytes samples and embryos. This denaturing solution is a mixture of guanidinium thiocyanate and sodium citrate. As a chaotropic agent it helps disruption of the three dimensional structures in macro molecules such as proteins. It is also a strong inhibitor of ribonuclease (RNAse) (Mason *et al*, 2002). So this denaturing solution helps the disrupt cells walls and inhibit the RNAse. After this step 0.72 µL of beta-mercapto ethanol, 10 µL of sodium acetate, 100 µL of phenol, 20 µL of chloroform:isoamyl alcohol (24:1 proportion) was added to the microtube solution. The phenol reagent helps to dissolve the proteins, while chloroform:isoamyl alcohol is used for phase separation (aqueous phase, interphase and organic phase), and it also dissolves lipids (Chomczynski *et al*, 1986, 1987). After centrifugation at 12,000 g for 5 min the phase separation was observed and the aqueous phase of the solution was transferred into a new RNAse free tube and 1 µL of glycogen with 450 µL of isopropanol was added. After centrifugation at 12,000 g for 30 min at 4ºC, 200 µL of absolute ethanol was added to the microtube which helps in further precipitation of nucleic acids. DEPC water was added to the samples after final centrifugation of 12,000g for 10 min at 4ºC and kept for air-drying for 30 min.

Kit Protocol

The Pure link RNA Mini kit protocol (Invitrogen) mainly includes Lysisbuffer (with an addition of 2-mercaptoethanol), wash buffer I and wash buffer II (with addition of 100 % ethanol). Three hundred µL of Lysis buffer composed by a combination of guanidinum isothiocynate and beta-mercaptoethanol, was added to the 500 mL microtube with a range of 10 to 30 oocytes samples, granulosa cells and vitrified oocytes, for broke the membrane of the oocytes and exposing the nucleic acids and also protecting the RNA from endogenous RNAse (Chirgwin *et al*., 1979). After homogenization and centrifugation, ethanol was added to the microtube. The microtube samples was transferred into a spin cartridge which containing a clear silica based membranes where the RNA molecules binds. Then 700 µL of wash buffer I was added to the spin cartridge and centrifuged at 12,000g for 15 s at room temperature, followed by adding 500 µL of wash buffer II and centrifuged at 12,000 g for 15 s at room temperature. Wash buffer I was composed by isopropanol and wash buffer II by both isopropanol and ethanol alcohols. Centrifuging the spin cartridge with wash buffers should get rid any impurities (Vogelstin *et al*., 1979). After centrifugation the spin cartridge with wash buffers, RNAse free water was added to spin cartridge and centrifuged at 12,000 g for 2 min at room temperature.

Modified Trizol Protocol

The common trizol protocol was first developed for large amount of cells or tissue (Chomczynski *et al*, 1986). So the intention was to provide some modifications in the trizol protocol to make it specific to the minimum amount of bovine oocytes. To microtubes of 500 mL with oocytes (5 to 60 oocytes each) and vitrified oocytes (15 to 40) a 100 µL of trizol reagent was added to the microtubes instead of 700 µL. In this case, with few cells being used, the 700 µL of trizol could leads to damage in RNA and increase the chances of phenol contamination (Mason *et al*, 2002). The microtubes were vortexed and incubated at room temperature for few minutes, providing the penetration of trizol solution into the cell walls. After incubation for 3 min, 50 µL of chloroform was added to microtubes and the tubes were inverted for 15 s, following incubation for more 3 min. The microtubes were then centrifuged at 12,000 g for 30 min at 4ºC, to prevent denaturation and maintain the RNA stable. After centrifugation aqueous phase was transferred to the new RNAse free tube and 2 times the volume of isopropanol was added to the aqueous phase recovered. Isopropanol is a very good precipitating agent which helps in desalting and recovering of nucleic acids. As higher is the

concentration of isopropanol, as higher is the nucleic acids recovery (Hossain *et al*., 1997). For this reason, instead of ethanol, isopropanol was used as precipitating agent (Chomczynski *et al*, 1986). After centrifugation at 12,000 g at 4ºC for 30 min, 150 µL of ethanol was added to get rid out of RNAse from the samples. Spectrophotometry reading was taken after preheating the samples and the readings helps the chosen for samples for cDNA and PCR. The criteria of selecting these samples is to test the modified protocol specific to the minimum number of 8 oocytes to maximum number of 68 oocytes and also check three different conditions in extraction (fresh, PROH and DMSO) and PCR.

From the overall Spectrophotometry reading of RNA extraction protocols of Table 2, modified trizol protocol with stable purity ranging from 1.50 to 2.11 and concentration ranging from 10.5 to 4,978 ng μL^{-1} shows better results than GNTC and commercial kit protocols. The total RNA extracted from 8 oocytes with modified trizol protocol showing purity of 1.50 and concentration of 180 ng μL^{-1}, shows the efficiency of this protocol against the GNTC protocol, where with an extraction with 28 oocytes the results were 1.175 of A260 280 ratio and concentration of 188 ng μL^{-1}. By considering the spectrophotometry reading of all samples, can be observed that modified trizol protocol and commercial kit protocol were better than GNTC protocol.

PCR Results

All the RNA samples extracted from the three different protocols were subjected to cDNA synthesis followed by PCR as described in material and methods. After the electrophoresis the gel pictures of *GAPDH* and *SDHA* genes were obtained as shown in the Figures 1 and 2. Samples 1 to 7 represent RNA extraction by GNTC protocol, samples 8 to 14 represent RNA extraction by modified trizol protocol, and samples 15 to 18 represent RNA extraction by commercial kit protocol. No negative control amplification was observed, showing none contamination. The samples 1 to 7 had light bands even though more number of oocytes and embryos were used during the extraction. The embryo used was in different stages, being in 2 cell stage to 32 cells. Possible reason for light bands in PCR seeing in Figure 1 may be due to poor purity levels of the samples (ratio from 1.05 to 1.36), indicating more protein and phenol contamination (Sambrook *et al*., 2001). The major disadvantage of GNTC protocol is the use of larger of amounts toxic components like phenol, beta-mercaptoethanol, which may leads to the contamination of samples and affects purity levels. In GNTC protocol higher amount of phenol was used compared to guanidium isothiocyanate reagent. The use of higher amount of

phenol leads to less adsorption of RNA to aqueous phase during the phase separation process of the RNA extraction. Its happens because less amount of guanidium isothiocyanate leads to the increase strong repulsion forces between the negatively charged nucleic acids and the hydroxyl groups of phenol which cause loss of nucleic acids in phenol (i.e. more number of nucleic acids resides in phenol phase). Guanidium isothiocyanate acts as bridge between phenols, nucleic acids and also decrease the repulsion forces between them (Lei Xu *et al*., 2011). The other disadvantages of GNTC protocol was time consuming method preparation of chemicals components, bio hazardous and expensive.

The PCR result from commercial kit protocol observed at the Figure 2 shows more non-specific bands for all the samples than in the PCR from other protocols. These non-specific bands are may be due to the required RNA binding to the walls of spin cartage while washing the samples with wash buffers. The purity levels of some of the samples were more than 2.0 (i.e. 2.27 and 2.7) showing protein contamination in the samples which may leads to the non-specific bands. There may be loss of total RNA material while transferring the total RNA to new vials. Also some molecules of total RNA can remain in the silica cartridge with a low volume of washing buffered supplied. In the other way round if a high amount of washing buffer is used can lead to a high diluted final sample. Loss of total RNA leads to reduced concentration of mRNA; hence the concentration levels of kit protocol samples were low. The major disadvantage of this kit protocol is being expensive when the total RNA is not a routine in the laboratory.

The modification in the trizol protocol shows good results to PCR reaction as observed in the Figure 2, except to the sample 12 which did not amplified may be due to the less concentration of total RNA (79.2ug/ml). Two samples had shown non-specific bands, 11 and 14 probably because the lower purity (1.50) and high RNA concentration 119 ng μL^{-1} in the first and high concentration (4978 ng μL^{-1} in the second. The most are the chance of protein contamination in both samples the most probability of having the non-specific bands. But in the case of kit protocol even though the purity, concentration are good more non-specific bands were observed. In this case non-specific bands are seen in at 188 bp.

Figure 2 shows the vitrified sample 3 (PROH) with a purity of 1.50, concentration of 48.8 ng μL^{-1} was not amplified may be due to the less purity. The remaining samples with high concentration had showed bright bands and samples with low concentration had shown light brand. The concentration levels of vitrified samples were low maybe damage of cells due to

cryprotents (Victoria *et al*., 2005). To prove the efficacy of the modified trizol protocol further PCR was performed with *SDHA* and *GAPDH* gene the results are shown in Figure 3.From the Figure 3(a) and (b) the gene amplification of the two genes are exactly on the specific size expected. This shows the modified trizol protocol was more specific to the bovine oocytes by obtaining specific bands with minimum number (i.e 20~25 oocytes).

In conclusion by analysing all the spectrophotometry and PCR results it was possible to develop a manual, time costless and not expensive tissue-specific protocol to mRNA extraction, and further obtaining good end-point PCR amplification. The efficiency of a manual protocol over using a kit protocol is the manual protocol can be developed to specific cells or tissues whereas kit protocol is not so specific because they are not designed for a particular cells or tissues. By considering the positive aspects and negative ones from results obtained a protocol providing the aims and objectives was optimized. In the Table 3 is possible to see the advantages and disadvantages of the three different protocols to the three groups' studies.

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