RNA isolation and quantitative PCR from HOPE and Formalin fixed bovine lymph node tissues

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Abbreviations: RNA, ribonucleic acid; mRNA, messenger ribonucleic acid; RT-PCR, reverse transcriptase polymerase chain reaction; qRT-PCR, quantitative RT-PCR; HOPE, Hepes glutamic acid buffer mediated organic solvent protection effect; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; bp, base pairs; CP value, crossing point value; FFPE, formalin fixed paraffin embedded.

Abstract

The use of RNA extracted from HOPE fixed tissues in quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is fairly novel. We compared qRT-PCR analysis of formalin and HOPE fixed, paraffin embedded lymph node tissues from *M.bovis* infected cattle, by extracting total RNA using a commercial kit (Ambion) and a trizol method. RNA extracted from HOPE fixed tissues showed comparable quantities between the commercial kit (82.7-107.9 μg/ml total RNA) and the trizol method (87-161.1 μg/ml total RNA), displaying a high degree of integrity when analysed by electrophoresis. RNA extracted from formalin fixed tissues using the commercial kit produced similar concentrations (80.6-145.7 μg/ml total RNA) in comparison to the HOPE tissue however the integrity was compromised. Extraction of RNA from the formalin fixed tissues using trizol was unsuccessful.

Following qRT-PCR for GAPDH, total RNA from HOPE fixed tissues showed higher levels of target mRNA ($4.05x10^{-2}$ pg/100ng total RNA using the commercial kit and $6.45x10^{-2}$ pg/100ng total RNA using trizol) in comparison to formalin fixed tissues ($5.69x10^{-4}$ pg/100ng total RNA). This could be attributed to RNA degradation by exposure to formalin fixation. In conclusion, **the** HOPE fixative proved to be a better source for RNA extraction from cattle lymph nodes and subsequent qRT-PCR.

Introduction

Molecular techniques such as quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) are rapidly becoming the preferred methods of disease prevention and diagnosis (14, 8, 17). The study of nucleic acids, in particular RNA, has provided vital information on the initial infection and advancement (18) of disease within challenged hosts. The need for extracting RNA of a high quality is integral in this process (3,4) and considerable effort has been focused on developing methods that enable tissue fixation and archival storage without the associated nucleic acid degradation (2,7). Archival storage of tissue samples has proved extremely useful in disease pathology and molecular biology due to providing a vast array of material morphologically conserved and with documented clinical backgrounds (6, 7). An example of this is rabies infected brain tissue samples that were fixed in formalin in the late 1980's and, 16 years post fixation, RT-PCR analysis of the disease was successfully performed (16).

One of the most widely used fixatives is neutral buffered formalin (13, 2) however much debate has surrounded its practical use for RNA extraction (4). Variable results have been produced on the efficacy of formalin fixation due to its ability to form methylene bridges between amino acids within the RNA molecule, thus proving extraction methods less reliable (13, 7). As an alternative to formalin, the Hepes glutamic acid buffer mediated organic solvent protection effect (HOPE) fixative has been shown to conserve RNA integrity (10,19) by avoiding amino acid cross linking. HOPE fixed paraffin embedded human tissues have been used as a source for RNA extraction, producing transcript lengths of up to 2462 nucleotides and allowing the amplification of RT-PCR products of 183 bp in length (19). More recently RNA has been extracted from laser micro dissected human lung tissue and applied in real time RT-PCR (5). However, as far as the authors are aware, HOPE fixation of animal tissues such as bovine material and its use in quantitative real time PCR (providing actual data on bovine mRNA

concentration) is largely unknown.

In addition to a high quality starting material, the extraction method itself is important in successfully procuring RNA (7). Studies on RNA extraction from HOPE fixed human tissues using the RNeasy kit (Qiagen, Germany), RNAzol B (Campro Scientific, Netherlands) and the GenoPrep mRNA kit on the GenoM- 48 Workstation (GenoVision, Norway) have provided RNA transcripts of variable quality (19, 5). The same methods were applied to formalin fixed tissues however no quantifiable RNA was produced (19). In this study we have compared two RNA extraction methods, a commercial kit Optimum^{TM} FFPE kit (Ambion, UK) that is designed for extraction from formalin fixed tissues (9) and a trizol (Invitrogen, UK) method which has had much success in non-fixed material (1). Both of these methods are new in application to HOPE fixed cattle lymph node tissues. These methods were also performed on formalin fixed cattle lymph node tissues. The extracted transcript was then analysed by agarose gel electrophoresis and qRT-PCR to determine the quantity of housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The results will provide information on the reproducibility of the fixative and extraction of RNA using different commercial sources.

Material and Methods

Sample preparation

Tissue samples of cervical and thoracic lymph nodes were taken at post mortem from *Mycobacterium bovis* infected cattle at the Veterinary Laboratory Agency (VLA, Surrey). Cattle lymph node tissue samples were either fixed in 10% neutral-buffered formalin for 7 days or were incubated in aqueous protection solution HOPE for 14-36 hours (0-4^{o}C) followed by incubation in a pre-mixed ice-cold acetone solution (100 ml acetone and 100 µl HOPE II solution at 0-4^{o}C)

for 2 hours and then dehydration with freshly prepared acetone (0-4^{o}C) for 3x 2 hours (as per manufacturers instructions, DCS Innovative, Germany). Both fixation methods were followed by embedding of the sections in paraffin wax. The samples were cut into 10 µm sections and placed into microcentrifuge tubes (Eppendorf, 3x 10 µm sections per tube) for subsequent RNA extraction. All tissue fixation and paraffin embedding was performed at the VLA (Surrey). This study details a representative number of samples performed for total RNA extraction using both methods (3 samples of each fixative for each method) however the isolation procedure was repeated for each fixative more then 10 times to ensure reproducibility of the results.

RNA extraction

Optimum^{TM} formalin fixed, paraffin embedded (FFPE) kit (Ambion) method:

Total RNA was extracted from both formalin and HOPE fixed, paraffin embedded sections following the manufacturer's instruction manual. Briefly, samples were deparaffinised using xylene at room temperature and dissolved in Proteinase K (60 units/µl) solution (Ambion) at 37^{o}C for 6 hours. RNA extraction buffer (Ambion, UK) was added to the supernatant and vortexed vigorously for 10 seconds. The sample was passed through a micro filter cartridge by centrifugation, followed by two washes and transferred to a micro elution tube into which 2x 10 µl volumes of pre-heated (70^{o}C) RNA elution solution (Ambion, UK) were added. This was then left at room temperature for 1 minute before centrifuging (16,500 g) for 1 minute to elute the RNA.

Trizol extraction method:

Following deparaffinisation using xylene at 57^{o}C, both formalin and HOPE fixed samples were

pulverised in liquid nitrogen and submerged in trizol (800 µl). The samples were left in a sonicating bath for intervals of 1 minute (3 xs) before adding glycogen (1mg/ml) to aid in pellet visualisation. The sample was then passed through a syringe and needle (24G) before the addition of chloroform and the tube contents mixed by vortexing for 30 seconds. After centrifuging for 10 minutes at 4^{o}C, the supernatant was transferred into a new microcentrifuge tube with ice cold isopropanol (500µl) and incubated at -20^{o}C for 4 hours. The microcentrifuge tube was then centrifuged for 15 minutes at 4^{o}C and the superntant discarded. The resultant pellet was washed in 70% ethanol and resuspended in RNase free water.

Spectrophotometry was performed to analyse the extracted total RNA for concentration and purity. Each sample was also run on agarose gel (1%) electrophoresis (using Tris Acetate EDTA (TAE) buffer) to determine the integrity of the RNA by visual analysis of the 28S and 18S rRNA bands.

Quantitative RT-PCR

Quantitative RT-PCR was performed using a dual labelled probe and primer set (amplicon of 87 bp) for the housekeeping gene GAPDH and the Quantitect^{TM} Probe RT-PCR (Qiagen, UK) kit according to the manufacturers' guidelines. Briefly, QuantiTect RT-PCR mastermix (1x), forward primer (0.4µm), reverse primer (0.4µm), probe (0.2µm) and QuantiTect RT mix were added to 100ng of total RNA to a final volume of 25ul. The RT-PCR reaction was carried out on a Quantica thermal cycler (Techne, UK) applying the following conditions: 50^{o}C for 30 minutes; 95^{o}C for 15 minutes; 50 cycles at 94^{o}C for 15 seconds, 60^{o}C for 1 minute followed by a 4^{o}C hold. The probe was dual labelled with a FAM fluorophore at the 5' end and a Black Hole

Quencher (BHQ-1) at the 3' end (Biomers, Germany). Each reaction was carried out in duplicate and included two negative controls (one excluding reverse transcriptase and another excluding the template). To produce a standard curve specific to GAPDH, an oligonucleotide (107 bp) was designed and manufactured (Biomers) identical to the amplicon produced during PCR of the GAPDH transcript. Known quantities of the designed oligonucleotide were then run alongside the unknown samples to produce a standard curve of crossing point (CP) number (the point at which the amplification curve crosses the threshold line) against concentration (picograms). The results were expressed as picograms of GAPDH mRNA in 100 nanograms of total RNA (pg/100ng)

Results

Total RNA extracted from formalin and HOPE fixed, paraffin embedded tissues using the Optimum™ FFPE kit (Ambion).

Total RNA extracted from both formalin and HOPE fixed tissues using the Optimum™ FFPE kit (Ambion) produced spectrophotometer results of comparative quantification (the 3 representative formalin fixed tissue samples produced 80.6, 145.7 and 97.9 μg/ml total RNA and the HOPE fixed tissue samples produced 107.9, 82.7 and 97.3 μg/ml total RNA, Table 1A). The purity of each of these samples was also within range, between 1.7 and 1.9 (260/280 nm ratio, Table 1A). Agarose gel electrophoresis of the RNA extracted from formalin fixed tissues showed a visible 28S rRNA band (Figure 1A) and RNA extracted from HOPE fixed tissues using the same method displayed both 28S and 18S rRNA bands (Figure 1B). Extracted transcripts for both formalin and HOPE fixed tissues displayed successful mRNA expression of the housekeeping gene GAPDH after qRT-PCR **(Figure 2)** producing CP values of 34.71 and 28.19, respectively

(Table 2). This was converted to quantitative values using the standard curve **(Figure 3)** and it was found that the total RNA extracted from HOPE fixed tissues had a higher level of GAPDH mRNA at $4.05x10^{-2}$ pg/100ng of total RNA in comparison to total RNA extracted from formalin fixed tissues at $5.69x10^{-4}$ pg/100ng of total RNA (Table 2).

Total RNA extracted from formalin and HOPE fixed, paraffin embedded tissues using the trizol method.

Total RNA extracted from formalin fixed tissues using the trizol method proved unsuccessful as it could not be quantified using spectrophotometry (Table 1B) and the results were confirmed as we did not get any rRNA bands on the agarose gel (Figure 1C). It subsequently did not produce an amplifiable product above the set threshold in qRT-PCR (Table 2). The concentration of RNA extracted from HOPE fixed tissues using spectrophotometry for the three representative samples was 87, 146.7 and 161.1 µg/ml total RNA (Table 1B) and the purity was 1.8 and 1.9, respectively (260/280 nm ratio, Table 1B). The total RNA from HOPE fixed tissues also exhibited strong 28S and 18S rRNA bands when analysed by gel electrophoresis (Figure 1C). The extracted transcript displayed successful mRNA expression of the housekeeping gene GAPDH after qRT-PCR **(Figure 2)** producing a CP value of 27.39 (Table 2). This was converted to quantitative values using the standard curve **(Figure 3)** and found to contain a GAPDH mRNA concentration of $6.45x10^{-2}$ pg/100ng of total RNA (Table 2).

All controls used in qRT-PCR were negative and the standard curve produced an adequate line coefficient of 0.98 and amplification efficiency of 1.9 **(Figure 3)**.

Discussion

Studies on disease infection and progression are fast becoming reliant on modern molecular techniques such as PCR (17). The need for intact RNA extraction methods is an extremely important prelude to PCR (3, 4) and has seen vast improvements over the last decade (13). This includes the use of different tissue fixation methods to secure the integrity of RNA during archival storage. In this study we have compared two fixatives (HOPE and formalin) for their ability to maintain RNA integrity. We have also compared two different RNA extraction methods (a commercial kit (Ambion) and a trizol method) to determine which method produced the highest quantity of total RNA from tissues fixed in both HOPE and formalin. Using the commercial Optimum^{TM} FFPE kit (Ambion), total RNA extracted from HOPE fixed tissues showed comparatively similar quantities and was of a similar purity to total RNA extracted from formalin fixed tissues (statistically there was no significant difference between the concentration of total RNA extracted from the two fixation methods, Mann-Whitney test $p>0.05$). However, when using qRT-PCR to analyse the content of GAPDH mRNA, the mRNA concentration was higher in HOPE fixed tissues ($4.05x10^{-2}$ pg/100ng total RNA) compared to formalin fixed tissues ($5.69x10^{-4}$ pg/100ng total RNA) suggesting that HOPE fixation maintained the integrity of the RNA to a much higher degree (19). This was supported by the agarose gel electrophoresis, as the total RNA extracted from HOPE fixed tissues displayed both rRNA bands (18S and 28S) in comparison to the one band (28S) shown in total RNA from formalin fixed tissues. This work agrees with previously published data where it has been shown that total RNA extracted from HOPE fixed tissues displayed a stronger integrity and therefore a much higher level of detectable mRNA in RT-PCR (19, 5, 15). This ability of HOPE fixation could be due to the increased rate of acetone tissue dehydration, enabled by the diffusion of a protective

solution of amino acids into the sample, thus conserving both its morphological and nucleic acid composition (13).

Using the trizol method, the HOPE fixed tissues produced total RNA of a very similar quantity and purity to the total RNA extracted from HOPE fixed tissues using the commercial kit (statistically there was no significant difference between the concentration of total RNA using the two extraction methods, Mann-Whitney test $p>0.05$). The resultant GAPDH mRNA quantity calculated by qRT-PCR was also extremely similar, $4.05x10^{-2}$ pg/100ng total RNA extracted using the commercial kit and $6.45x10^{-2}$ pg/100ng total RNA extracted using the trizol method. As previously mentioned, the Ambion kit was successful in extracting total RNA from formalin fixed tissues however we were unable to isolate quantifiable RNA from formalin fixed tissues using the trizol method. In the Ambion Optimum^{TM} kit this is most likely attributed to the proteinase K step in the protocol, which is much more efficient at separating the RNA from its cross-linked matrix (7). However the period of sample incubation in proteinase K is extremely long (in our experience it took 6 hours to solubilise only a fraction of the tissue present) and has been reported to take up to 48 hours (11), leading to further RNA degradation. The cross-linked matrix is believed to form due to the reaction of RNA with formaldehyde, producing methylene bridges between the amino groups (13). Due to the absence of formaldehyde in the HOPE fixative method, tissues fixed in HOPE do not possess cross linkage and therefore perform equally well in RNA isolation methods with or without proteinase K solution. Similar results have been reported by Weidorn *et al* (19).

In conclusion, this study supports a strong consensus that HOPE fixative is a more appropriate fixative to use for subsequent RNA extraction and molecular techniques. This study is the first

to demonstrate the potential of HOPE fixation of *M.bovis* infected cattle lymph nodes with the subsequent quantifying of gene specific mRNA within a known amount of total RNA using qRT-PCR. This complements the work already shown on the benefits of HOPE fixative in human tissue types such as lung (5), cancer cells (15), spleen and heart (19). Due to the vastly improved nucleic acid conservation, HOPE fixative is being applied to tissues samples used in subsequent disease diagnosis, such as the possible detection and even differentiation of the *Mycobacterium tuberculosis* complex within archived infected mice tissues (12). In addition to this, this study has highlighted the need to apply, not just the more efficient fixative technique but also the appropriate extraction method specific to the fixative used, adding new methods to those previously reported (19, 5). By considering these factors and exploring the most effective combination of fixative and extraction method, the quality of molecular techniques such as PCR and their practical use in exploring genetic expression of both host and pathogen can provide a basis for future research into intervention and treatment.

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Glutamic acid buffer mediated organic solvent protection effect. Pathol Res Pract. 198 (2002) 735

Figure legends:

Figure 1A: Agarose gel electrophoresis (ethidium bromide staining) of RNA samples (1 μg) isolated with the Optimum ™ FFPE Kit (Ambion, UK) from formalin-fixed (lanes 3 and 4), paraffin embedded cattle lymph node tissue. Lane 1 is a 0.5-10 Kb RNA ladder. Lane 2 was not used. Experiment was repeated three times.

Figure 1B: Agarose gel electrophoresis (ethidium bromide staining) of RNA samples (1 μg) isolated with the Optimum ™ FFPE Kit (Ambion, UK) from HOPE fixed (lanes 2, 3 and 4) paraffin embedded cattle lymph node tissue. Lane 1 is a 0.5-10 Kb RNA ladder. Experiment was repeated three times.

Figure 1C: Agarose gel electrophoresis (ethidium bromide staining) of RNA samples (1 μg) isolated with a trizol method from HOPE fixed (lanes 3 and 5) and formalin-fixed (lanes 2 and 4), paraffin embedded cattle lymph node tissue. Lane 1 is a 0.5-10 Kb RNA ladder. Lane 6 was not used. Experiment was repeated three times.

Figure 2: Real time RT-PCR of total RNA extracted from both the formalin fixed and HOPE fixed paraffin embedded tissues using the Optimum ™ FFPE kit (brown and red lines, respectively) and the trizol method (blue and green lines, respectively). The target sequence was

an 87 bp fragment of the RNA of bovine Glyceraldehyde-3-phosphate dehydrogenase and each reaction was performed in duplicate.

Figure 3: Real time RT-PCR of the manufactured standard template (Biomers.net), designed to be identical in sequence to the amplicon produced during the PCR of the GAPDH transcript. Known concentrations of the standard template (50 pg, 5 pg, $5x10^{-3}$ pg and $5x10^{-7}$ pg) were run on qRT-PCR alongside the unknown samples to produce a standard curve of crossing point (CP) value against log concentration (pg). The equation of the plotted line (above the graph) was then used to quantify the GAPDH mRNA concentration within the unknown samples (Table 2).

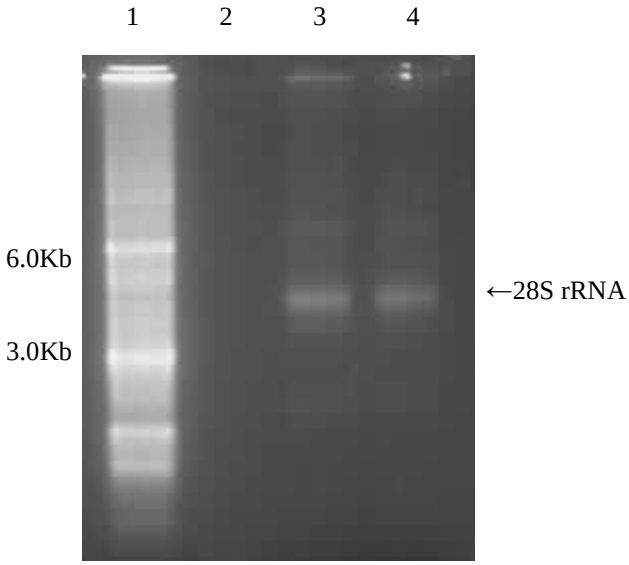


Figure 1A

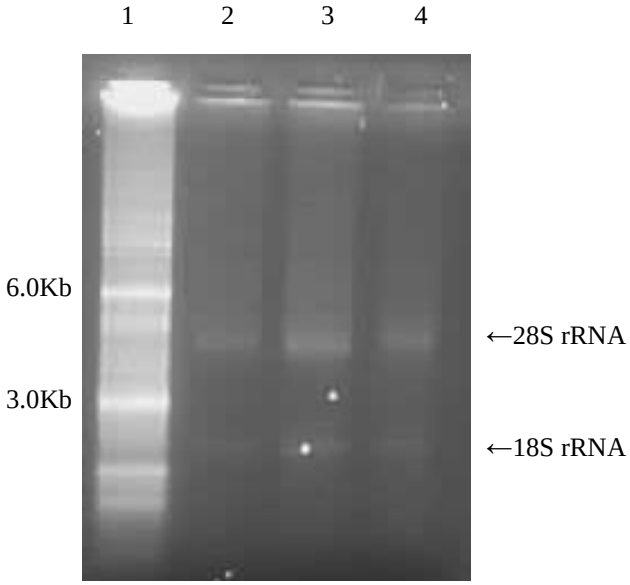


Figure 1B

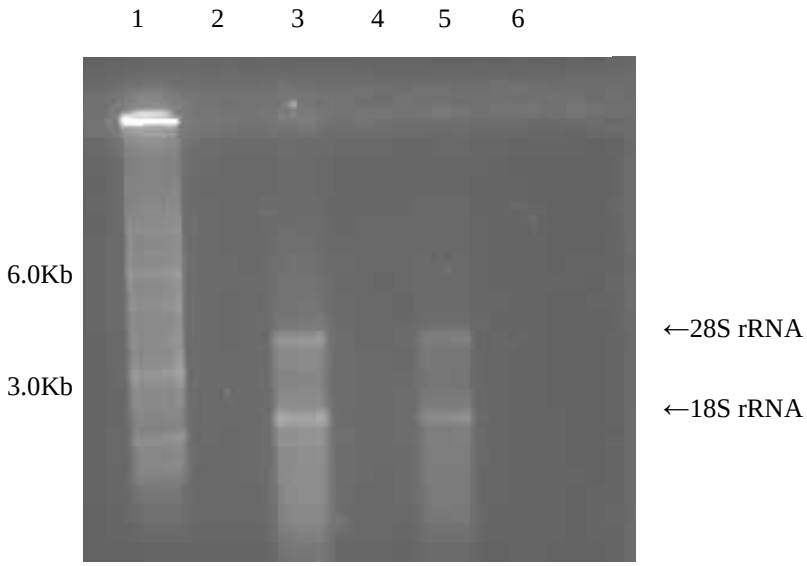
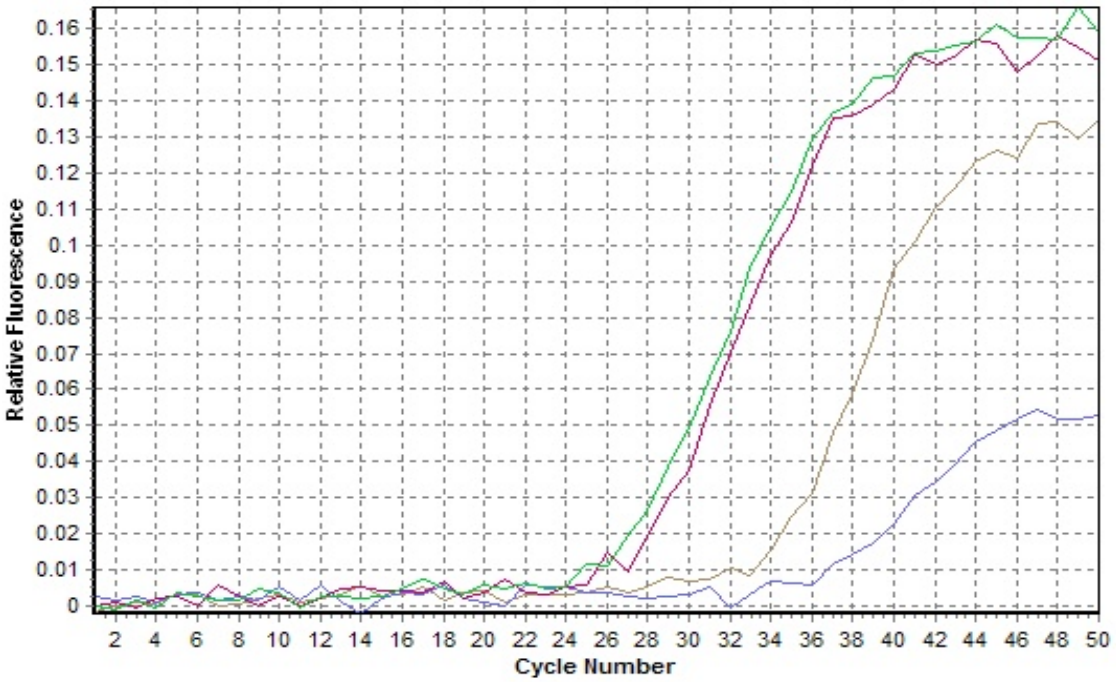


Figure 1C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0.15
0.14
0.13
0.12
0.11
0.1
0.09
0.08
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0.04
0.03
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2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Cycle Number

Figure 2

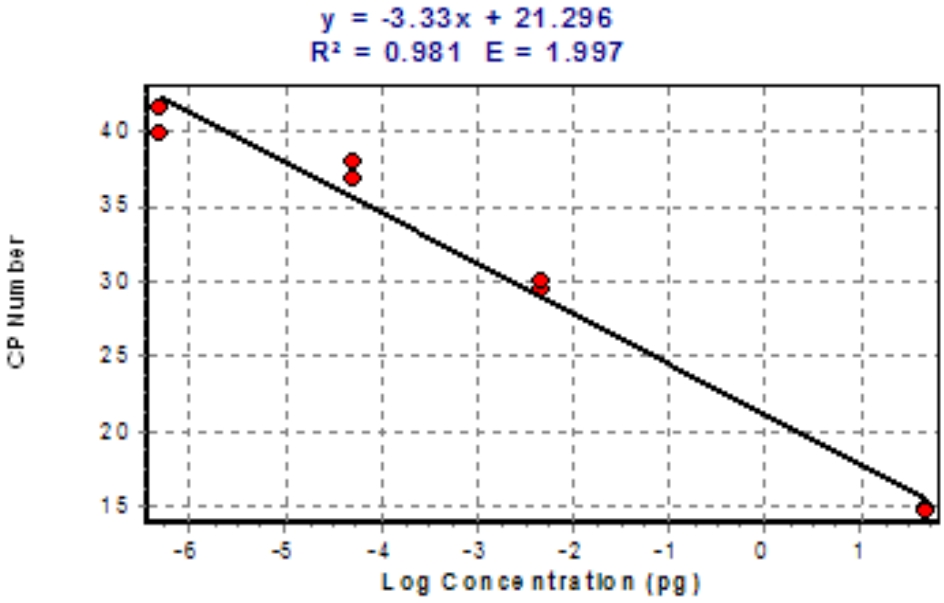
y = -3.33x + 21.296
R² = 0.981 E = 1.997
CP Number
40
35
30
25
20
15
-6
-5
-4
-3
-2
-1
0
1
Log Concentration (pg)

Figure 3

Table 1A: Quantification and purity (as determined by 260/280nm ratio) of total RNA determined by spectrophotometery isolated from three formalin-fixed (FORM) and three HOPE fixed, paraffin embedded cattle lymph node tissue sections using the Optimum^{TM} FFPE kit (Ambion). The three tissue sections from each fixative are a representative group of the isolation procedure that was repeated more then 10 times.

Fixation method and sample number	Total RNA quantification (μg/ml)	260/280 nm ratio (purity)
HOPE 1	107.9	1.8
HOPE 2	82.7	1.9
HOPE 3	97.3	1.9
FORM 1	80.6	1.7
FORM 2	145.7	1.8
FORM 3	97.9	1.9

Table 1B: Quantification and purity (as determined by 260/280nm ratio) of total RNA determined by spectrophotometery isolated from three formalin fixed (FORM) and three HOPE fixed, paraffin embedded cattle lymph node tissues using the Trizol method. The three tissue sections from each fixative are a representative group of the isolation procedure that was repeated more then 10 times.

Fixation method and Sample number	Total RNA quantification (μg/ml)	260/280 nm ratio (purity)
HOPE 1	146.7	1.8
HOPE 2	87.0	1.8
HOPE 3	161.1	1.9
FORM 1	Negative	Negative
FORM 2	Negative	Negative
FORM 3	Negative	Negative

Table 2: QRT-PCR crossing point (CP) values for the expression of GAPDH mRNA in total RNA (100ng) extracted from formalin fixed and HOPE fixed, paraffin embedded samples using two methods, the Optimum™ Kit (Ambion) and a trizol method. Each reaction was performed in duplicate and the average displayed in the table. The average CP values were then converted into quantitative values using the standard curve (data not shown) and expressed in picograms (pg) of GAPDH mRNA in 100ng total RNA.

Total RNA extraction method	Sample fixation method	Average CP value	GAPDH mRNA concentration (pg/100ng total RNA)
Optimum™ Kit (Ambion)	Formalin	34.71	5.69×10^{-4}
	HOPE	28.19	4.05×10^{-2}
Trizol	Formalin	Negative	Negative
	HOPE	27.39	6.45×10^{-2}