Improved RNA quality and TaqMan® Pre-amplification method (PreAmp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials

Jinghuan Li1, Paul Smyth1, Susanne Cahill1, Karen Denning1, Richard Flavin1, Sinead Aherne1, Marco Pirotta2, Simone M Guenther2, John J O'Leary1 and Orla Sheils*1

Address: 1Deptment of Histopathology, University of Dublin, Trinity College, Dublin, Ireland and 2Applied Biosystems, Foster City, CA, USA

Email: Jinghuan Li - jinghual@tcd.ie; Paul Smyth - smythpa@tcd.ie; Susanne Cahill - sucahill@tcd.ie; Karen Denning - Denningk@tcd.ie; Richard Flavin - flavinr@tcd.ie; Sinead Aherne - ahernesi@tcd.ie; Marco Pirotta - Marco.Pirotta@eur.appliedbiosystems.com; Simone M Guenther - Simone.M.Guenther@eur.appliedbiosystems.com; John J O'Leary - olearyjj@tcd.ie; Orla Sheils* - osheils@tcd.ie

* Corresponding author

Abstract

Background: Archival formalin-fixed paraffin-embedded (FFPE) tissues represent an abundant source of clinical specimens; however their use is limited in applications involving analysis of gene expression due to RNA degradation and modification during fixation and processing. This study improved the quality of RNA extracted from FFPE by introducing a heating step into the selected extraction protocols. Further, it evaluated a novel pre-amplification system (PreAmp) designed to enhance expression analysis from tissue samples using assays with a range of amplicon size (62–164 bp).

Results: Results from the Bioanalyzer and TaqMan® data showed improvement of RNA quality extracted using the modified protocols from FFPE. Incubation at 70°C for 20 minutes was determined to be the best condition of those tested to disrupt cross-links while not compromising RNA integrity. TaqMan® detection was influenced by master mix, amplicon size and the incorporation of a pre-amplification step. TaqMan® PreAmp consistently achieved decreased C_T values in both snap frozen and FFPE aliquots compared with no pre-amplification.

Conclusion: Modification to extraction protocols has facilitated procurement of RNA that may be successfully amplified using QRT-PCR. TaqMan® PreAmp system is a robust and practical solution to limited quantities of RNA from FFPE extracts.

Background

Archival Formalin-Fixed, Paraffin-Embedded (FFPE) tissue samples represent a robust and invaluable source of human tissue for gene expression analysis. Compared to fresh and snap frozen tissue, FFPE tissue has an inherent advantage in that retrospective patient data, including survival history and treatment response etc, is readily available, allowing immediate comparison with clinical pathological parameters. Data generated can potentially highlight biomarkers useful in disease classification, diag-
nosis and prognosis, and potentially elucidate novel therapeutic targets [1,2].

However, these tissues have not been widely used in molecular biology due to the degradation and chemical modification of RNA extracted from FFPE blocks. RNA extracted from FFPE is degraded to fewer than 300 bases [3] in length because archived blocks are often stored at room temperature for long periods of time. The situation is made more complicated by the fact that RNA is modified by methylol groups to form cross-links with protein or nucleic acid during formalin fixation [4-6], which results in poor yields [1,7] and compromised extracts.

Real-time quantitative TaqMan® reverse transcriptase-polymerase chain reaction (QRT-PCR) analysis has been introduced as a sensitive, accurate, and highly reproducible method to study gene expression [8]. It has been successfully used to detect gene transcript levels from snap frozen tissue extracts and even from FFPE containing partially fragmented RNA [9-11] although the detection rate is lower as indicated for example by invariably higher Cₜ values in the latter [12-16].

In this study we examined and optimized selected RNA extraction protocols, including Stratagene Absolutely RNA® FFPE Kit and Ambion RecoverAll™ Total Nucleic Acid Isolation Kit, by comparison of parallel extracts from FFPE and snap frozen cell preparations using a cell line model (Figure 1). We further analyzed these extracts using different TaqMan® protocols, including two types of TaqMan® Master Mix (Universal PCR Master Mix (UPMM) and Gene Expression Master Mix (GEMM)) and newly developed TaqMan® with pre-amplification method (PreAmp), with a panel of assays over a range of amplicon sizes.

Results

RNA extraction

RNA quantity was assessed spectrophotometrically using NanoDrop® ND-1000 Spectrophotometer (Wilmington, USA), which showed that the yields from snap frozen extracts were greater than those from FFPE when RNA was extracted from identical numbers of cells using all the protocols examined (Stratagene Absolutely RNA® FFPE Kit, Ambion RecoverAll™ Total Nucleic Acid Isolation Kit, Gentra Purescript® RNA Purification Kit and Invitrogen Trizol® Reagent). In comparison of FFPE extracts (Table 1), Ambion gave the highest yields, and column based Stratagene and Ambion protocols produced clean RNA with OD 260/280 ratio greater than 1.8. RNA quality was assessed using Agilent 2100 Bioanalyzer (Agilent technologies, Waldbronn, Germany) and TaqMan® RT-PCR, which showed variations in RNA quality dependent on the protocol used. Stratagene and Ambion RecoverAll™

kits gave superior FFPE RNA results with regard to quality than the others examined.

Evaluation of modified protocols

Modification to the Stratagene and Ambion protocols generated approximately 25–40% greater yields and larger fragments of RNA (Figure 2) than the standard procedure. Adjustment to Stratagene and Ambion protocols produced decreased Cₜₜₜ (e.g. with a mean of 2.95 cycles in GEMM experiment and a mean of 3.14 cycles in PreAmp), indicating the improved quality of RNA extracted from FFPE (Figure 3).

A separate experiment using Ambion RecoverAll™ kit enabled extraction of large RNA molecules including cross-linked RNAs (Figure 4). Incubation of eluted RNA at 70°C
for 20 minutes was found to be the best condition to disrupt cross-links while not compromising RNA integrity in comparison with other modifications (70°C for 10 min, 95°C for 10 min and 95°C for 20 min).

**Comparison of TaqMan® Universal PCR Master Mix (UPMM), Gene Expression Master Mix (GEMM) and PreAmp**

C<sub>T</sub> values were also dependent on the type of TaqMan® PCR Master Mix that was used (Figure 3 and Figure 5). TaqMan® with UPMM generated higher C<sub>T</sub>s for long amplicons than GEMM. This was observed by a comparison of two GAPDH assays – one 122 bp and the other 67 bp. There was a mean threshold detection difference of 17 cycles using UPMM and a mean difference of 4 cycles using GEMM with RNA template extracted using Ambion RecoverAll™ kit. HLA_A is the largest amplicon (164 bp) which produced no product with UPMM, however, GEMM and PreAmp both allowed the detection of this amplicon size (Figure 3). GEMM improved the C<sub>T</sub> values for long amplicons (over 100 bp) compared with UPMM (Figure 5-a). When the ΔC<sub>T</sub>s were compared to Theoretical ΔC<sub>T</sub>s, PreAmp results generally correlated with GEMM for all the 8 assays analyzed (Figure 5-c).

**Evaluation of TaqMan® PreAmp using CDKN1B**

TaqMan® PreAmp analysis consistently achieved decreased C<sub>T</sub> values in both snap frozen and FFPE aliquots compared with TaqMan® without pre-amplification step (Figure 5-b, c). A good correlation between PreAmp and UPMM was observed using CDKN1B to analyze RNA extracted with different protocols (Figure 6), suggesting that PreAmp does not introduce any bias into the reaction.

**Comparison of C<sub>T</sub> difference between FFPE and snap frozen cells**

TaqMan® analysis over all assays showed C<sub>T</sub>s to be 2 to 11 cycles higher when using FFPE extracts compared to snap frozen counterparts when the amounts of input RNA were identical (Figure 3). Focussing on an analysis of GAPDH using two assays with different amplicon sizes revealed the smaller amplicon assay (67 bp) produced C<sub>T</sub>s from snap frozen and FFPE samples that were closer together (Figure 7).
Discussion
RNA analysis has generally been performed on snap-frozen or fresh materials, using variable techniques including microarray, northern blotting and RT-PCR. However, a constant challenge has been robust and reliable analysis of gene expression in archival tissues. This application has been frustrated by poor RNA yields, small sizes of extracted fragments and low levels of detectable RNA in the extracts [12]. In this study we established a cell line model to test modified extraction protocols and quantify differences in performance using a panel of QRT-PCR assays on FFPE compared with parallel snap-frozen cell preparations using TaqMan® methodology. We found that our modified extraction method and TaqMan® PreAmp enhanced expression analysis from FFPE cells optimally.

RNA and FFPE
Generally, RNA extracted from FFPE materials is fragmented and chemically modified. Fragmentation occurs possibly since tissues are surgically removed, and it continuously occurs during fixation and preservation. Experimental design was such that equal numbers of cells were processed for FFPE cell block construction and snap freezing for comparative purposes. By using cells fixed under controlled conditions possible variables due to the effects of storage on RNA degradation were negated, thus revealing the true impact of formalin fixation on RNA quality. We extracted intact RNA fragments in a separate experiment from pellets with a large number of formalin fixed paraffin embedded normal thyroid cells (Figure 4), which was consistent with a finding by Scicchitana et al [17]. In that study, intact RNA molecules were extracted from newly formalin fixed paraffin embedded human bone marrow stromal cells suggesting that RNA degradation could be a minor problem for recently fixed FFPE in some types of cells. Our study showed that the differences in RNA quality between the small and large assays using UPMM and a mean difference of 4 cycles between small and large assays using GEMM was observed.

Figure 3
TaqMan® gene expression pattern using UPMM, GEMM and PreAmp. (Str = Stratagene protocol; Am = Ambion protocol; + = modified protocols with incubation in Proteinase K buffer at 70°C for 20 minutes; and SN = snap frozen cells. Not all assays produced products, e.g. HLA_A in UPMM.) C_Ts were higher using FFPE extracts compared to snap frozen counterparts when the amount of input RNA was identical. Modification to Stratagene and Ambion protocols produced decreased C_Ts. TaqMan® with UPMM generated higher C_Ts than GEMM and PreAmp when using longer amplicon lengths. This was particularly evident when comparing two GAPDH assays – one 122 bp and the other 67 bp. A mean difference of 17 cycles between small and large assays using UPMM and a mean difference of 4 cycles between small and large assays using GEMM was observed.
Figure 4 displays the degree of RNA modification in FFPE cells caused by methylol groups during formalin fixation. Masuda and colleagues [18] suggested that formaldehyde reacts with RNA forming an N-methylol followed by an electrophilic attack to form a methylene bridge between amino groups. Interestingly, our results show a clear large RNA band, approximately 5 kb in length (Figure 4-lane 6), which is hypothetically the cross-linked RNA. The other two bands visualized were also larger than that extracted from snap frozen cells indicating the modification of RNA in FFPE. Furthermore, with incubation at high temperature, the large RNA band (~5 kb) was removed, and the sizes of the other two bands became closer to 18 s and 28 s of the snap frozen extracts. This is in agreement with the results of a mass spectrometric analysis that was carried out by Masuda group, suggesting methylol modification is reversible by heating [18,19]. However, a balance must be achieved between breaking cross-links while not contributing to degradation of labile RNA. Our results showed that incubation at 70°C for 20 minutes was the optimal condition, among those tested, to de-modify cross-linked RNA while maintaining RNA integrity (Figure 4).

**Extraction protocols**

A prerequisite for gene expression studies in formalin-fixed tissue samples was the establishment of a reliable and reproducible extraction method to provide detectable RNAs for subsequent analysis [1]. The quality of extracted RNA can be variable among different extraction methods.
Comparison of TaqMan® gene expression pattern using ΔCT method. (Am = Ambion protocol; FP = FFPE cells; + = modified protocols with incubation in Proteinase K buffer at 70°C for 20 minutes; and SN = snap frozen cells. Not all assays produced products, e.g. HLA_A in UPMM.) These ΔCT data was generated from the CTs shown in Figure 3. A Theoretical ΔCT was calculated for each chart based on equilibrating the results for any variation in input cDNA. In panel a, ΔCT = CT_UPMM - CT_GEMM. The Theoretical ΔCT of UPMM-GEMM was 0 [= Log2(20 ng/20 ng)] given identical input quantities (20 ng) were used in each system. In panel b, ΔCT = CT_UPMM - CT_PreAmp. In panel c, ΔCT = CT_GEMM - CT_PreAmp. The Theoretical ΔCT in panel b and c was 5.68 [= Log2(1024 ng/20 ng)], which was calculated based on an input of 1024 ng of cDNA for the TaqMan® real time PCR component of pre-amplification process. This quantity was generated from an initial 1 ng subjected to 10 cycles of pre-amplification with a 100% efficiency and no bias introduced from the PreAmp. The relevant Theoretical ΔCT is plotted on each chart as a reference point for measuring the actual detected ΔCT against the theoretically optimal ΔCT. The benefit of GEMM over UPMM was evident as amplicon size increased (Panel a). A comparison of UPMM and PreAmp showed a similar pattern (Panel b). However, PreAmp results generally correlated with GEMM regardless of amplicon size for the series of 8 assays analysed (Panel c).
due to several factors, such as the contamination of RNases, proteins and genomic DNA [20]. Our results showed that proteinase K digestion based protocols followed by on-column DNase digestion and RNA elution produced good detectable RNA of FFPE.

Proteinase K digestion has also been demonstrated by many laboratories to be critical in RNA extraction protocols [1,3,21,22] possibly because of two functions. On one hand, it can degrade proteins that are covalently cross-linked with each other and nucleic acid to release RNA from the matrix, thereby allowing efficient RNA extraction from FFPE materials [21]. On the other, it can inactivate RNases that tend to be stable and do not require cofactors to function. Thus its activity avoids any potential reactivation of RNase during reversal of fixation in aqueous buffers [1].

Our data demonstrates that incubation in proteinase K buffer at 70°C for 20 minutes facilitated the disruption of cross-links, resulting in improved quantity and quality of RNA. The RNA extracted using the modified protocols (with the incubation) enhanced detection by a mean of 3 cycles (Figure 3), which is equivalent to 8 fold increased sensitivity. It is most likely that the additional incubation step removed the remaining cross-links between RNA and protein leading to the longer RNA molecules being extracted [23]. In addition, this incubation denatured proteinase K, thus avoided its further damage to RNA in the following purification procedure, which suggests the necessity of a heating (at 70°C for 20 min) application in any proteinase K based extraction of FFPE samples.

**TaqMan® PreAmp**

TaqMan® QRT-PCR technique is based on the 5’ nuclease activity of Taq DNA polymerase and involves cleavage of a specific fluorogenic hybridization probe that is flanked by PCR primers [24,25]. Because of the small target size, many laboratories have demonstrated that it is possible to measure gene expression levels using FFPE tissues as a source of mRNA [3]. Still, it seems to be problematic to perform large scale of analysis on FFPE because of the high CTs and limited concentration of extracts [14,15].

We employed a novel TaqMan® PreAmp technique which we found to be a practical solution to decrease CT values, and in particular suitable in our hands to generate realtime PCR results from limited amounts of input RNA, such as extracted from laser captured microdissected material [26]. The principle of TaqMan® PreAmp technique is to amplify target cDNA prior to real-time TaqMan® PCR analysis. Briefly, cDNA is synthesized from total RNA by use of random priming. The cDNA for the specific target assays is then amplified by pre-amplification reaction using pooled gene-specific primers to increase the number of targeted copies. The pre-amplification product is diluted and finally analyzed by real-time TaqMan® PCR using single assay containing one pair of gene-specific primers and probe.
The TaqMan® PreAmp technique addresses the challenge faced by researchers working with rare or precious samples, which only limited RNA could be extracted from, to perform gene expression analyses using real-time QRT-PCR. The simple process enables the user to perform uniform amplification from as little as one nanogram of cDNA (which was used in this study) or alternatively conduct up to 200 real-time PCR reactions per pre-amplification reaction without compromising the available sample material. Our results demonstrated that TaqMan® PreAmp overcame the difficulties usually caused by low yields of RNA extraction from FFPE.

**Influence of TaqMan® Master Mix and amplicon length**

This study demonstrated that the sensitivity of TaqMan® detection was influenced by choice of Master Mix and amplicon length in assay design. We evaluated UPMM and GEMM which showed similar sensitivity for short amplicons (less than 90 bp), while GEMM displayed better sensitivity for longer amplicons (over 100 bp) compared with UPMM in parallel snap frozen and FFPE extracts (Figure 5). This effect was dissipated when UPMM was used after pre-amplification, possibly because of the increased copy number of template available or because of the composition of the PreAmp MasterMix which is closer in components to GEMM than UPMM. Reagents can have a significant effect on assay reproducibility [27] due to some parameters such as different polymerases sensitivity [28], primer binding efficiency and the concentration of Mg²⁺ [29]. Karrer et al. described the Monte Carlo effect using plant material suggesting that the PCR reproducibility could be limited when the number of available templates is low. Increased concentrations of Mg²⁺ reduced PCR variation possibly by allowing a higher proportion of annealed primers extended by the more active polymerase [29].

Our data corroborated the observation that amplicon size is crucial in designing assays to analyze gene expression levels not only using FFPE extracts but also using RNAs with high integrity [12,15]. Many researchers have found that short amplicons generated lower Cₚ than longer amplicons on analysis of the same gene in FFPE [30,31]. Data generated in this study evaluating snap frozen samples using two sizes of GAPDH (Figure 3) demonstrated GAPDH-67 generated a reduction in Cₚ by 2 – 3 cycles over GAPDH-122 in both GEMM and PreAmp experiments, demonstrating that smaller amplicons give more consistent results [32].

In addition, we found Cₚ of GAPDH-122 was lower than that for GAPDH-67. For example, the analysis of GAPDH using a target amplicon of 67 bp displayed Cₚ 1 – 2.5 cycles closer between FFPE and snap frozen than the same experiment using the longer amplicon size of 122 bp. We further measured the amplification efficiency associated with these two sizes of GAPDH assays using a broad dilution range (5 Log₁₀s) which showed closed efficiencies with 99.98% for GAPDH-67 and 99.92% for GAPDH-122 (data not shown). This measurement eliminates non-specific amplification that could contribute to decreased amplification efficiency of the true target. Therefore, it seems reasonable to conclude that the shift of efficiency detected from frozen to fixed material (Figure 7) is due to degradation of RNA in FFPE, and logical to extrapolate that the longer an amplicon is, the more likely its template will be degraded in extracted RNA. As a general rule, house-keeping genes or normalising assays should have amplicon sizes that match the size of the target whose expression is to be measured [3] and amplicons less than 100 bp should be employed in gene expression studies using FFPE materials.

**Conclusion**

We evaluated the effect of modifying recommended extraction protocols to reproducibly produce RNA that may be successfully amplified using QRT-PCR. We have found the TaqMan® PreAmp system to be a robust and practical solution to limited quantities of RNA and have demonstrated comparable results in matched FFPE and snap frozen preparations providing proof of principle that this method may reliably be utilised in the context of multiple expression analyses from individual FFPE samples.

**Methods**

**Cell culture and formalin fixation**

Nthy-ori 3-1 (ECACC, Wiltshire, UK) is a normal thyroid follicular epithelial cell line derived from adult thyroid tissue that has been transfected with a plasmid encoding for the SV40 large T gene [33].

This cell line was grown to confluence in a humidified atmosphere containing 5% CO₂ at 37°C in the following plating medium: RPMI 1640 with 2 mM L-glutamine, 10% Foetal calf serum (FCS), Penicillin (100 U/ml) and Streptomycin (100 μg/ml). Tripsinized cells were counted with a hemocytometer. Approximately 1 × 10⁵ suspended cells were aliquot and were pelleted (a) snap frozen and (b) formalin fixed and paraffin embedded into a cell block. When formalin fixation was required, a cohesive solid cell pellet was constructed using 20% agar [23]. The cells were centrifuged in an eppendorf tube, and the supernatant was removed using a pipette. Approximately 30 μl of pre-warmed agar (60°C) was added to each tube. The solid cell pellet was formed within a few seconds. Cell blocks were placed in 10% buffered formalin at room temperature for 5 hours followed by tissue processing on a Tissue-Tek® V.I.P.TM tissue processor for 8 hours comprising: 10% buffered formalin fixation (4 hours at
37°C), 60% ethanol (20 minutes at 37°C), 80% ethanol (20 minutes at 37°C), 100% ethanol (20 minutes at 37°C), xylene (40 minutes at 37°C) and paraffin (80 minutes at 60°C). The pellets were subsequently paraffin embedded.

RNA extraction protocols

RNA extraction was performed using 4 protocols (Table 2): Ambion RecoverAll™ Total Nucleic Acid Isolation Kit, Stratagene Absolutely RNA® FFPE Kit, Gentra Purescript® RNA Purification Kit and Invitrogen Trizol® Reagent. (RT. = Room temperature)

RNA extraction was performed using 4 protocols (Table 2): Ambion RecoverAll™ Total Nucleic Acid Isolation Kit, Stratagene Absolutely RNA® FFPE Kit, Gentra Purescript® RNA Purification Kit and Invitrogen Trizol® Reagent. Apart from deparaffinazition, RNA was extracted from snap frozen and FFPE cells in parallel according to manufacturer's protocols including proteinase K digestion (not included in Trizol® protocol), RNA isolation and elution or hydration procedures. The modification in Stratagene and Ambion protocols involved incubation at 70°C for 20 minutes after the recommended proteinase K digestion.

RNA quantity was assessed using the NanoDrop® ND-1000 Spectrophotometer (Wilmington, USA), and the quality was measured using the RNA 6000 Pico LabChip® Kit on an Agilent 2100 Bioanalyser (Agilent technologies, Waldbronn, Germany).

TaqMan® gene expression assays

Seven TaqMan® Gene Expression Assays (P/N: 4331182, Applied Biosystems, CA, USA) and one custom designed assay (GAPDH-67) were utilised in this study with a range of amplicon sizes from 62 to 164 (Table 3). The extracted RNA was reverse transcribed into cDNA and were then quantified using TaqMan® real time PCR with or without PreAmp procedure.

Table 3: Eight of TaqMan® Gene Expression Assays. Seven of these assays were inventoried by Applied Biosystems (P/N: 4331182, Applied Biosystems, CA, USA) and one was a designed assay (GAPDH-67 Forward primer: CAT CCA TGA CAA CTT TGG TAT CGT; Reverse primer: GGG TGG CAG TGA TGG CAT; Probe: ACT CAT GAC CAC AGT CC).

| Gene Symbol – Amplicon Length | Gene Name                                           | Assay ID         |
|-------------------------------|----------------------------------------------------|------------------|
| MT4 – 62                      | metallothionein IV                                 | Hs00262914_m1    |
| GAPDH – 67                    | glyceraldehyde-3-phosphate dehydrogenase           | Designed         |
| CDKN1B – 71                   | cyclin-dependent kinase inhibitor 1B (p27, Kip1)   | Hs00153277_m1    |
| MAPK4 – 72                    | mitogen-activated protein kinase 4                 | Hs00177074_m1    |
| CD44 – 86                     | CD44 molecule (Indian blood group)                 | Hs00153304_m1    |
| SDC2 – 103                    | syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan) | Hs00299807_m1    |
| GAPDH – 122                   | glyceraldehyde-3-phosphate dehydrogenase           | Hs99999905_m1    |
| HLA-A – 164                   | major histocompatibility complex, class I, A        | Hs00740413_g1    |

Table 2: Overview of the RNA extraction protocols. Four RNA extraction protocols are Ambion RecoverAll™ Total Nucleic Acid Isolation Kit, Stratagene Absolutely RNA® FFPE Kit, Gentra Purescript® RNA Purification Kit and Invitrogen Trizol® Reagent. (RT. = Room temperature)
Reverse transcription (RT)

Applied Biosystems High-Capacity cDNA Archive Kit (P/N: 4322171, Applied Biosystems) was used following manufacturer's protocol for reverse transcription. Each RT reaction contained 50 μl of 8 ng/μl total RNA, 10 μl of 10× RT buffer, 4 μl of 25× dNTP mixture, 10 μl of 10× Random Primers, 5 μl of MultiScribe RT (50 U/μl) and 21 μl of RNase-free water. The 100 μl reactions were incubated in an Applied Biosystems Thermocycler for 10 min at 25°C, 2 hours at 37°C and then held at 4°C.

TaqMan® real time PCR

For the Real-time PCR step, amplification was carried out on the Applied Biosystems 7000 Sequence Detection System. Two types of TaqMan® Master Mix were employed in this procedure: TaqMan® Universal PCR Master Mix with UNG (P/N 4304437, Applied Biosystems) or TaqMan® Gene Expression Master Mix (P/N 4370048, Applied Biosystems). The 20 μl PCR reaction included 10 μl of 2× TaqMan® Master Mix, 5 μl of 4× TaqMan® Gene expression Assay (P/N 4331182, Applied Biosystems) and 5 μl of cDNA (RT product 4 ng/μl). The reactions were incubated in a 96-well optical plate at 50°C for 2 min, at 95°C for 10 min, following by 40 cycles of 95°C for 15 seconds and 60°C for 1 min. The real-time PCRs for each assay were run in triplicate.

TaqMan® real time PCR with PreAmp

The preamplification was performed using TaqMan® PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems). The pooled assay mix was prepared by combining 8 of 20× TaqMan® Gene Expression Assays into a Master Mix Kit protocol (P/N 4366128, Applied Biosystems). The pooled assay mix was then 1:5 diluted and analyzed by TaqMan® real time PCR with PreAmp Master Mix Kit protocol (P/N 4366128, Applied Biosystems). The preamplification was performed using TaqMan® Universal PCR Master Mix following the procedures described.

Data analysis

Replicates were omitted if C_T standard deviation was greater than 1.5 in the triplicate. All the data were collected in Excel form. The formulas used to generate the figures are as below:

1. \[ \Delta C_T(\text{A-B}) = C_T(\text{A}) - C_T(\text{B}) \]

2. Theoretical \[ \Delta C_T = \log_2(\text{Amount cDNA in A}/\text{Amount of cDNA in B}) \]

Authors' contributions

JL performed the RNA extraction and TaqMan® analysis and wrote original and final versions of the manuscript. PS, SC helped with the extraction and TaqMan® analysis. PS, RF, KD helped draft the manuscript. KD, SA carried out cell culture. MP, SG helped with the analysis of the data. JOL and OS conceived the study and helped write the original and final versions of this manuscript. All authors read and approved the final manuscript.

Acknowledgements

This work was part-funded by Applied Biosystems and received technical support from the company. We would also like to thank staff in the Central Pathology Laboratory, St. James’s Hospital, for assistance with fixation and processing of cell blocks.

References

1. Lewis F, Maughan NJ, Smith V, Hillan K, Quirke P: Unlocking the archive–gene expression in paraffin-embedded tissue. J Pathol 2001, 195(1):667-684.
2. Srinivasan M, Sedmak D, Jewell S: Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002, 161(6):1961-1971.
3. Cronin M, Pho M, Dutta D, Stephens JC, Shah S, Kiefer MC, Esteban JM, Baker JB: Measurement of gene expression in archival paraffin-embedded tissues: development and performance of a 92-gene reverse transcriptase-polymerase chain reaction assay. Am J Pathol 2004, 164(1):35-42.
4. Haselkorn R, Doty P: The reaction of formaldehyde with poly-nucleotides. J Biol Chem 1961, 236:2738-2745.
5. Chaw YF, Crane LE, Lange P, Shapiro R: Isolation and identification of cross-links from formaldehyde-treated nucleic acids. Biochemistry 1980, 19(24):5525-5531.
6. Rait VK, Zhang Q, Fabris D, Mason JT, O'Leary TJ: Conversion of formaldehyde-modified 2'-deoxyadenosine 5'-monophosphate in conditions modeling formalin-fixed tissue dehydration. J Histochem Cytochem 2006, 54(3):301-310.
7. Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maranpot RR: Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. Mol Carcinog 1999, 25(2):86-91.
8. Mocellin S, Rossi CR, Pilati P, Nitti D, Marincola FM: Quantitative real-time PCR: a powerful ally in cancer research. Trends Mol Med 2003, 9(5):189-195.
9. Sheils OM, Sweeney EC: TSH receptor status of thyroid neoplasms--TaqMan® RT-PCR analysis of archival material. J Pathol 1999, 188(1):87-92.
10. Sheils OM, O'Leary JJ, Sweeney EC: Assessment of ret/PTC-1 rearrangements in neoplastic thyroid tissue using TaqMan RT-PCR. J Pathol 2000, 192(1):32-36.
11. Macabeo-Ong M, Ginzingger DG, Dekker N, McMillan A, Regezi JA, Wong DT, Jordan RC: Effect of duration of fixation on quantitative reverse transcription polymerase chain reaction analysis. Mod Pathol 2002, 15(9):972-987.
12. Godfrey TE, Kim SH, Chavira M, Ruff DW, Warren RS, Gray JW, Jensen RH: Quantitative mRNA expression analysis from formalin-fixed, paraffin-embedded tissues using 5' nuclease quantitative reverse transcription-polymerase chain reaction. J Mol Diagn 2000, 2(2):84-91.
13. Van Deerlin VM, Gill LH, Nelson PT: Optimizing gene expression analysis in archival brain tissue. Neurochem Res 2002, 27(10):993-1003.
14. Cohen CD, Grone HJ, Grone EF, Nelson PJ, Schliondorf D, Kretzler M: Laser microdissection and gene expression analysis on formaldehyde-fixed archival tissue. Kidney Int 2002, 61(1):125-132.
15. Abrahamsen HN, Steiniche T, Nexo E, Hamilton-Dutoit SJ, Sorensen BS: Towards quantitative mRNA analysis in paraffin-embedded tissues using real-time reverse transcriptase-polymerase chain reaction: a methodological study on lymph nodes from melanoma patients. J Mol Diagn 2003, 5(1):34-41.
16. Koch I, Slotta-Huspenina J, Hollweck R, Anastasov N, Hofler H, Quinterilla-Martinez L, Fend F: Real-time quantitative RT-PCR shows variable, assay-dependent sensitivity to formalin fixation: implications for direct comparison of transcript levels in paraffin-embedded tissues. Diagn Mol Pathol 2006, 15(3):149-156.

17. Sicchitano MS, Dalmas DA, Bertiaux MA, Anderson SM, Turner LR, Thomas RA, Mirlab R, Boyce RW: Preliminary comparison of quantities, quality, and microarray performance of RNA extracted from formalin-fixed, paraffin-embedded, and unfixed frozen tissue samples. J Histochem Cytochem 2006, 54(11):1229-1237.

18. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K: Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999, 27(22):4436-4443.

19. Hamatani K, Eguchi H, Takahashi K, Koyama K, Mukai M, Ito R, Taka M, Yasui W, Nakachi K: Improved RT-PCR amplification for molecular analyses with long-term preserved formalin-fixed, paraffin-embedded tissue specimens. J Histochem Cytochem 2006, 54(7):773-780.

20. Fleige S, Pfaffl MW: RNA integrity and the effect on the real-time qRT-PCR performance. Mol Aspects Med 2006, 27(2-3):126-139.

21. Specht K, Richter T, Muller U, Walch A, Werner M, Hofler H: Quantitative gene expression analysis in microdissected archival formalin-fixed and paraffin-embedded tumor tissue. Am J Pathol 2001, 158(2):419-429.

22. Korblar T, Grskovic M, Dominis M, Antica M: A simple method for RNA isolation from formalin-fixed and paraffin-embedded lymphatic tissues. Exp Mol Pathol 2003, 74(3):336-340.

23. Li J, Smyth P, Flavin R, Cahill S, Denning K, Ahern S, Guenther SM, O'Leary J J, Shells O: Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. BMC Biotechnol 2007, 7:36.

24. Heid CA, Stevens J, Livak KJ, Williams PM: Real-time quantitative PCR. Genome Res 1996, 6(10):986-994.

25. Gibson UE, Heid CA, Williams PM: A novel method for real time quantitative RT-PCR. Genome Res 1996, 6(10):995-1001.

26. Denning KM, Smyth PC, Cahill SF, Finn SP, Conlon E, Li J, Flavin RJ, Ahern S, Guenther SM, Ferlinz A, O'Leary J J, Shells OM: A molecular expression signature distinguishing follicular lesions in thyroid carcinoma using preamplification RT-PCR in archival samples. Mod Pathol 2007, 20(10):1095-1102.

27. Burgos JS, Ramirez C, Tenorio R, Sastre I, Bullido MJ: Influence of reagents formulation on real-time PCR parameters. Mol Cell Probes 2002, 16(4):257-260.

28. Bustin SA, Mueller R: Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin Sci (Lond) 2005, 109(4):365-379.

29. Karrer EE, Lincoln JE, Hogenhout S, Bennett AB, Bostock RM, Martineau B, Lucas WJ, Gilchrist DG, Alexander D: In situ isolation of mRNA from individual plant cells: creation of cell-specific cDNA libraries. Proc Natl Acad Sci U S A 1995, 92(9):3814-3818.

30. Lehmann U, Kreipe H: Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies. Methods 2001, 25(4):409-418.

31. Antonov J, Goldstein DR, Oberli A, Baltzer A, Pirotta M, Fleischmann A, Altermatt HJ, Jäger R: Reliable gene expression measurements from degraded RNA by quantitative real-time PCR depend on short amplicons and a proper normalization. Lab Invest 2002, 85(8):1040-1050.

32. Fleige S, Walf V, Huch S, Prigomet C, Sehm J, Pfaffl MW: Comparison of relative mRNA quantitation models and the impact of RNA integrity in quantitative real-time RT-PCR. Biotechnol Lett 2006, 28(19):1601-1613.

33. Cahill S, Smyth P, Finn SP, Denning K, Flavin R, O'Regan EM, Li J, Potraz A, Guenther SM, Henfrey R, O'Leary JJ, Shells O: Effect of ret/PTC1 rearrangement on transcription and post-transcriptional regulation in a papillary thyroid carcinoma model. Mol Cancer 2006, 5:70.