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Original article

Reliable quantification of mRNA in archived formalin-fixed tissue with or without paraffin embedding

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ABSTRACT

Introduction: Formalin fixation and paraffin embedding (FFPE) is a standard method for tissue sample storage and preservation in pathology archives. The Reverse Transcriptase Quantitative Polymerase Chain Reaction (RT-qPCR) is a useful method for gene expression analysis, but its sensitivity is significantly decreased in FFPE tissue due to the fixation process. This process results in chemical modifications of RNA, cross-links proteins to RNA, and degrades RNA in these archived samples, hindering the reverse transcription step of the conventional RT-pPCR method and preventing generation of a cDNA that is long enough for the subsequent quantitative PCR step.
Methods: In this study, we used a multi-species RT-qPCR method originally developed to detect mRNA in tissue homogenate samples (Wang et al., 2011) and applied it to effectively detect a specific mRNA in formalin-fixed tissues with or without paraffin-embedding by targeting mRNA sequences as short as 24 nucleotides.
Results: Target sizes ranging from 24 to 91 nucleotides were evaluated using this multi-species RT-qPCR assay. Data generated with FFPE tissues demonstrated that use of short target sequences relieved the dependence on RNA quality and could reliably quantify mRNA. This method was highly sensitive, reproducible, and had a dynamic range of five orders of magnitude. Importantly, this method could quantify mRNA in prolonged formalin-fixed and FFPE tissue, where conventional RT-qPCR assays failed. Moreover, a similar result for small interfering RNA (siRNA)-mediated Apob mRNA knockdown was obtained from tissues fixed in formalin solution for 3 months to 4 years, and was found to be comparable to results obtained with frozen liver tissues.
Discussion: Therefore, the method presented here allows for preclinical and clinical retrospective and prospective studies on mRNA derived from archived FFPE and prolonged formalin-fixed tissue.

1. Introduction

Gene expression analysis has become an important tool to investigate the molecular mechanisms of disease and therapeutic response to a new drug. To date, frozen tissue samples have been used for the majority of mRNA-based diagnostic evaluations. However, frozen tissue samples are limited in availability, patient numbers and clinical annotation, and their long-term storage is expensive and laborious (Gnanapragasam, 2009). Fixed and FFPE tissues stored in pathology archives represent a large resource of biological information associated with histopathological diagnosis and clinical records. The ability to analyze gene expression on fixed tissue samples should allow for large retrospective and prospective studies to be performed, leading to better diagnosis and treatment of a disease (Lewis, Maughan, Smith, Hillan, & Quirke, 2001; Mittempergher et al., 2011). RT-qPCR is often used as a sensitive, reproducible, and accurate method to quantitate gene expression compared to other techniques such as immunohistochemistry, in situ hybridization, and DNA microarrays (Farragher et al., 2008; Mittempergher et al., 2011). Although formalin fixation and paraffin embedding maintain morphological features of the original tissue, this processing has a negative impact on the quality of RNA (Masuda, Ohnishi, Kawamoto, Monden, & Okubo, 1999; von Smolinski, Leverkoehne, von Samson-Himmelstjerna, & Gruber, 2005). Tissue autolysis occurs between sample collection and effective fixation. Over-fixation and prolonged storage of the embedded samples can also adversely impact RNA quality (von Ahlfen, Missel, Bendrat, & Schlumpberger, 2007). On a molecular basis, RNA isolated from such specimens is substantially degraded to fragments under 300 nucleotides in length (Cronin et al., 2004). In addition, formalin fixation crosslinks biomolecules and covalently modifies RNA by adding mono-methylol groups to nucleotides (Masuda et al., 1999). These effects significantly reduce the ability of conventional RT-qPCR methods to quantify mRNA, making it difficult to analyze gene expression using fixed and FFPE tissues.

In order to overcome these problems, some methods have been reported to improve the quality of RNA extracted from these tissues. First, proteinase K digestion was introduced into the RNA extraction protocol to ensure release of RNA from the cross-linked matrix; second, heating RNA above 70 °C for 20 min was shown to effectively reduce the amount of chemical modification that interfered with the reverse transcription step in RT-qPCR. Due to the fact that mRNA molecules

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containing poly A tails are very rare in RNA derived from fixed tissues, random hexamers or gene specific reverse transcription primers are usually used to ensure detection of a target mRNA. Some reports showed that higher levels of gene expression could be detected with an assay targeted within the 3′-UTR of an mRNA molecule (Farragher, Tanney, Kennedy, & Harkin, 2008; Godfrey et al., 2000). To increase assay sensitivity, a cDNA pre-amplification step could be added to the RT-qPCR method (Li et al., 2008). In addition, the ability to use short RNA fragments is a critical feature of RT-qPCR, making amplification of degraded RNA in the FFPE tissue possible. Previous studies suggest that a short amplicon is important to ensure that RT-qPCR can be successfully used for gene expression measurements in FFPE samples (Antonov et al., 2005).

With these technical improvements, some progress has been made to measure gene expression in FFPE samples to facilitate research and drug development (Cronin et al., 2004; Kennedy et al., 2011; Specht et al., 2001; Talantov et al., 2006; Tanney & Kennedy, 2010). However, in pathology laboratories, due to the large quantity of tissues that need to be processed, some study samples are routinely stored in formalin solution for days, months, or even years. Prolonged formalin fixation (longer than 48 h) is thought to cause extensive degradation, cross-linking, and irreversible modifications to the RNA, resulting in reduction of quantifiable mRNA molecules (Macabeo-Ong et al., 2002). Thus, highly damaged mRNA is very difficult to detect and quantify using conventional RT-qPCR methods. In this study, we used a multi-species RT-qPCR method originally developed to detect mRNA in tissue homogenate samples (Wang et al., 2011) and applied it to effectively detect a specific mRNA in fixed tissues by targeting mRNA sequences as short as 24 nucleotides. This method was demonstrated to be sensitive and reproducible, with a wide dynamic range, and could reliably quantify mRNA in archived FFPE and prolonged formalin-fixed tissue.

2. Methods

2.1. Samples

Four human FFPE liver samples were purchased from Folio Biosciences. Two mouse FFPE liver samples and two mouse formalin fixed liver samples without paraffin embedding were retrieved from the archives of a pathology laboratory (Department of Safety Assessment and Laboratory Animal Resources, Merck Research Laboratories, West Point, PA). Control RNAs derived from fresh human and mouse liver were purchased from BioChain Institute, Inc. Tissue samples were also obtained from in vivo studies as described below.

2.2. In vivo studies

All experimental procedures were in accordance with established guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee and conducted in AAALAC-accredited facilities. Prolonged formalin-fixed rat liver samples were obtained from an exploratory study. Sprague-Dawley rats (5 animals per group) received 3 mg/kg of an Apob siRNA in a lipid nanoparticle (LNP) formulation by intravenous injection on Study Day 1 (Strapps et al., 2010). A phosphate buffered saline (PBS) treated control group was also included in the study. To evaluate siRNA efficacy, liver tissue was collected from rats on Study Days 2 and 8 (Wang et al., 2011). In the dissection procedure, one piece of liver was placed on dry ice and stored in a −70 °C freezer; the second piece was fixed in formalin solution for 1 to 3 days, followed by paraffin embedding for routine pathological examination; the third piece was immersed in formalin solution and stored at room temperature. In the present study, total RNA was isolated from the third piece of liver tissue that had been stored in formalin solution for 3 months and 4 years, respectively. Detailed information is provided in Table 1.

2.3. RNA extraction

Total liver RNA was isolated from three 20 micron sections of FFPE tissue or 20 to 30 mg of the fixed tissue using The Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit (Cat# AM1975) according to the manufacturer's instructions. The concentration of each purified RNA sample was measured by using a NanoDrop ND-1000™ (Nanodrop Technologies) spectrophotometer. RNA fragmentation was determined by Agilent 2100 Bioanalyzer.

2.4. Primers and probes

Primers were obtained from Integrated DNA Technologies. TaqMan MGB probes and gene expression assay mixes (mouse Apob, Mm01545154_g1; mouse Ubc, Mm01201237_m1; human Apob, Mm01545154_g1; human Ubc, Mm01201237_m1), were purchased from Applied Biosystems, Inc. Primer and probe sequences are shown in Table 2.

2.5. Multi-species RT-qPCR assay for quantification of Apob and Ubc mRNA

Standard curve RNA samples were serially diluted in Tris–EDTA (TE) buffer to final concentrations ranging from 0.01 to 100 ng RNA/μL. Reverse transcription reactions were performed in a final volume of 15 μL containing 50 nM RT primer, 1 mM dNTP mixture (containing dTTP), 1X RT buffer from the TaqMan microRNA Reverse Transcription Kit™ (Applied Biosystems), 3.3 units/μL MultiScribe™ reverse transcriptase enzyme, 0.25 units/μL RNase Inhibitor, and 2 μL of RNA using the 9700 GeneAmp™ PCR System (Applied Biosystems). The reactions were incubated in a 96-well plate for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C and held at 4 °C. Quantitative PCR reactions were carried out in a final volume of 20 μL containing 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), No AmpErase® UNG, 1.5 μM specific forward primer, 0.75 μM universal reverse primer, 0.2 μM specific probe, and 5 μL of cDNA created by the RT reaction using a standard procedure using the 7900HT™ Real-time PCR System (Applied Biosystems).

2.6. Conventional RT-qPCR assay for quantification of Apob and Ubc mRNA

RT reactions were performed in a final volume of 20 μL containing 1X RT random hexamers, 4 mM dNTP mixture (containing dTTP), 1X RT buffer from the High-Capacity cDNA Reverse Transcription Kit™ (Applied Biosystems), 2.5 units/μL MultiScribe™ reverse transcriptase enzyme, 1 unit/μL RNase Inhibitor, and 2 μL of total RNA using the 9700 GeneAmp™ PCR System (Applied Biosystems). Quantitative PCR reactions were carried out in a final volume of 20 μL containing 1X TaqMan® Universal PCR Master Mix (Applied Biosystems), No AmpErase® UNG, 1 μL of the Apob or Ubc gene expression assay mix, and 5 μL of cDNA (created by the RT reaction) using the 7900HT™ Real-time PCR System (Applied Biosystems).

2.7. Data analysis

Percent accessible RNA was calculated as follows: a normalized amount of Apob or Ubc mRNA was calculated based on a standard curve generated from control total liver RNA; accessible mRNA was calculated by dividing the normalized amount of Apob or Ubc mRNA by 20 (the amount of the input total RNA), and then multiplying by 100 to obtain a percentage.

The ΔΔCt method was used for analysis of Apob mRNA knockdown (Livak & Schmittgen, 2001). An endogenous control, Ubc (Ubiquitin C), was included in the assay. The relative percent expression of Apob

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