RT-PCR Analysis of RNA Extracted from Bouin-Fixed and Paraffin-Embedded Lymphoid Tissues

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In the present study, we have investigated whether RNA can be efficiently isolated from Bouin-fixed or formalin-fixed, paraffin-embedded lymphoid tissue specimens. To this aim, we applied a new and simple method that includes the combination of proteinase K digestion and column purification. By this method, we demonstrated that the amplification of long fragments could be accomplished after a pre-heating step before cDNA synthesis associated with the use of enzymes that work at high temperature. By means of PCR using different primers for two examined genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and CD40), we amplified segments of cDNA obtained by reverse transcription of the isolated RNA extracted from Bouin-fixed or formalin-fixed paraffin-embedded tissues. Amplified fragments of the expected sizes were obtained for both genes tested indicating that this method is suitable for the isolation of high-quality RNA. To explore the possibility for giving accurate real time quantitative RT-PCR results, cDNA obtained from matched frozen, Bouin-fixed and formalin-fixed neoplastic samples (two diffuse large cell lymphomas, one plasmacytoma) was tested for the following target genes: CD40, Aquaporin-3, BLIMP1, IRF4, Syndecan-1. Delta threshold cycle (ΔC_T) values for Bouin-fixed and formalin-fixed paraffin-embedded tissues and their correlation with those for frozen samples showed an extremely high correlation ($r > 0.90$) for all of the tested genes. These results show that the method of RNA extraction we propose is suitable for giving accurate real time quantitative RT-PCR results. (*J Mol Diagn* 2004, 6:290–296)

The continuously updated information from the Human Genome Project and new technologies are permitting researchers to comprehensively measure mRNA (mRNA) and protein levels in biological samples.^{1–5} Technologies

that are available to analyze mRNA expression in tumor tissues include Northern hybridization, subtractive hybridization, mRNA differential display, quantitative reverse transcription (RT) polymerase chain reaction (PCR), and cDNA microarrays. All of them are useful for determining differentially expressed genes in tumor tissues and for correlating these findings with the clinical outcome. However, the applications of these methods are limited by the need for fresh and unfixed tissues that allow the extraction of high-quality nucleic acid. In addition, only few tissue banks are available worldwide, which contain enough frozen material suitable for wide range genetic analyses, or which have sufficiently long-term patient follow-up and outcome data. On the other side, archival, fixed, and paraffin-embedded tissues with long-term follow-up are extensively available and easily accessible. They represent an extensive source of genetic material and proteins to be investigated for clinical usage.⁶ To exploit this potentially huge source, it is mandatory to develop methods to isolate abundant, high-quality RNA from fixed, paraffin-embedded tissue sections.

Many different RNA extraction techniques have recently been tested with varying success.^{7–17} Success or failure of RNA extraction from archival tissue mainly depends on the fixation procedure used to fix the specimens. Several researchers have demonstrated on the whole that RNA can be extracted from formalin-fixed, paraffin-embedded tissue sections and converted into cDNA.^{7–18} Moreover, several studies have provided new methods to optimize DNA and RNA extraction from archival formalin-fixed tissues,^{18–25} whereas other studies have investigated the effect of duration of fixation on quantitative RT-PCR analyses.^{10,12,13,26} There is a paucity of data in the use of other fixatives that incorporate picric acid (Bouin's solution), or mercuric chloride (B5, Zenker's, Helly's, and Ridley's solutions), or tannic acid as nucleic acid preserving agents.^{9,27,28} Only one group of researchers reported that neither DNA nor RNA could successfully be extracted from highly cross-linking fixa-

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tives such as glutaraldehyde, modified formalins containing mercuric chloride, and Bouin's fixative.⁹

There is still a need to further explore new methods and possible solutions for extracting high-quality RNA from fixed and paraffin-embedded tissue specimens. Although the results on RNA extracted from Bouin-fixed tissues are scant and scarce, it is a shared opinion that RNA extracted from Bouin-fixed, paraffin-embedded tissue section is too degraded to permit analysis.⁶ For these reasons we have explored a new method for extracting RNA from formalin- and Bouin-fixed tissues. The specific aims of this study were: 1) to demonstrate that mRNA can be reliably isolated from fresh, frozen, and fixed paraffin-embedded tissue sections; 2) to demonstrate that the amplification of relatively long fragments can be obtained; and 3) to assess the effect of Bouin fixation on the suitability of mRNA for producing reliable quantitative RT-PCR data (using real time TaqMan PCR assay).

Materials and Methods

Tissues and Cell Lines

Lymph nodes were collected from seven patients with banal or reactive lymphadenopathies, from 11 patients with Hodgkin's or non-Hodgkin's lymphoma, and from two patients with plasmacytoma.

All tissues have been obtained at the time of surgical excision. The samples were subjected to either fixation or RNA isolation immediately after surgical removal. To ensure proper fixation, all tissue pieces were sectioned so that all spatial dimensions were ≤ 5 mm. In addition, a fragment from each sample was immediately snap-frozen in liquid nitrogen, and stored at -80°C .

Fresh and fixed paraffin-embedded primary effusion lymphoma (PEL) cell lines²⁹ (CRO-AP/2, CRO-AP/3, CRO-AP/5), Hodgkin's lymphoma cell line L540, and multiple myeloma cell line U266 were also included in the study.

Fixation

The tissues were placed into Bouin's solution for 4 to 5 hours or were fixed in 10% neutral-buffered formalin for 5 to 6 hours. After fixation, the samples were dehydrated, incubated in xylene, embedded in paraffin, and sectioned using standard histological protocols. The ages of the fixed tissues analyzed ranged from 88 to 6 months. All cell lines were similarly fixed before being processed to paraffin as previously described.³⁰

Deparaffinization

For this study, paraffin-embedded tissue blocks were cut with a disposable microtome blade into $1 \times 15 \mu\text{m}$ to $3 \times 15 \mu\text{m}$ sections (corresponding to 10 to 15 mg of tissue), depending on the size of the embedded tissue sample and placed in RNase-free Eppendorf tubes (Eppendorf AG, Hamburg, Germany). Tissues were deparaffinized by incubation in two consecutive baths of xylenes for 5

minutes each, then in two consecutive baths of 100% ethanol for 5 minutes each. Only for Bouin-fixed tissues another incubation with alcohol 70% saturated with Li_2CO_3 for 5 minutes was performed. After deparaffinization and centrifugation, the pellets were air-dried.

RNA Isolation from Fixed and Frozen Tissues and Fresh Cell Lines

Total RNA was isolated using the Nucleospin RNA II System (Macherey-Nagel GmbH & Co., Düren, Germany) according to the manufacturer's protocol with minor modification: 1) the pellet was resuspended in $200 \mu\text{l}$ of lysis buffer (Macherey-Nagel) containing $40 \mu\text{l}$ of proteinase K (10 mg/ml; Sigma Chemical Co., St. Louis, MO). Samples were incubated at 56°C for 2 to 3 hours with gentle agitation until complete digestion; 2) to ensure complete removal of genomic DNA, the sample was incubated for 1 hour at 37°C with Dnase I on the column. Finally the RNA was eluted with $60 \mu\text{l}$ of RNase-free water. All RNA was quantitated by spectrophotometer and optical density (OD) 260/280 nm ratios were determined.

Determination of RNA Quality

RNA isolated from frozen tissue and Bouin-fixed tissue was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano Assay according to the manufacturer's instructions and using the Agilent 2100 Bioanalyzer Software. The RNA 6000 ladder was purchased from Ambion (Austin, TX).

Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Before RT reaction the samples were heated at 70°C for 40 minutes and then cooled at 4°C .²⁰ RT-PCR reaction was performed in a two-step protocol using both oligodT and random hexamer with two different systems.

The first system used was Geneamp Gold RNA PCR Reagent Kit (PE Applied Biosystems, Inc., Foster City, CA) according to manufacturer's protocols. Reactions were incubated at 25°C for 10 minutes, 42°C for 20 minutes, and cooled at 4°C in a PE GeneAMP PCR System 2700 Thermocycler (PE Applied Biosystems).

The second system was ThermoScript RT-PCR System (Invitrogen, Paisley, UK) according to manufacturer's protocols. Reactions were incubated at 25°C for 10 minutes (only when using random hexamer), 60°C for 1 hour, and cooled at 4°C in a PE GeneAMP PCR System 2700 Thermocycler (PE Applied Biosystems).

No RT controls were carried out using the same RT reaction mix but substituting H_2O -DEPC for RT enzyme.

Primers

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and CD40 mRNA was detected in the sample by presence of 941-bp or 141-bp amplification products, respec-

tively, following PCR with specific primer. Primer pairs were from separate exons to allow distinction between products resulting from amplification of cDNA and potentially contaminating genomic DNA.

The GAPDH primer set was purchased from Maxim Biotech, Inc. (South San Francisco, CA). The CD40 primers, 5'-CGGTGAAAGCGAATTCCTAG-3' (sense) and 5'-CAGCCTTCTTCACAGGTGC-3' (antisense), were designed by using Primer Express design software (PE Applied Biosystems) and were synthesized by MWG Biotech AG (Ebersberg, Germany).

PCR amplification was performed with 43 cycles of 95°C 15 seconds, 58°C 30 seconds, 72°C 30 seconds for GAPDH and 95°C 15 seconds, 60°C 15 seconds, 72°C 25 seconds for CD40 in a PE GeneAMP PCR System 2700 Thermocycler (PE Applied Biosystems). PCR products were analyzed by electrophoresis on 2% to 3% agarose gels depending on size of the products and visualized by ethidium bromide staining.

Real Time RT-PCR

Total RNA was heated at 70°C for 40 minutes, cooled at 4°C and then converted to single-stranded cDNA using High-Capacity cDNA Archive Kit (PE Applied Biosystems).

Real time RT-PCR analyses were performed using ABI PRISM 7700 Sequence Detection System Instrument and software (PE Applied Biosystems, Inc.). Assays-on-Demand, consisting of a mix of unlabeled PCR primers and TaqMan MGB probe (FAM dye-labeled) for the target genes: CD40 (Assay ID: Hs00374176), Aquaporin-3 (AQP3) (Hs00185020), BLIMP1 (Hs00153357), IRF4 (Hs00180031), Syndecan-1 (syn-1) (Hs00174579), and Pre-Developed TaqMan Assay Reagent (FAM-MGB) for β 2 microglobulin (Product number: 4333766T) and for GAPDH (Product number: 4333764T) were purchased from PE Applied Biosystems. Assays-on-Demand gene expression products have been designed for the detection and quantitation of specific human genetic sequences in RNA samples converted to cDNA.

For evaluation of PCR efficiencies, serial dilutions of cDNA obtained from a reactive lymph node were used to construct a standard curve. PCR efficiencies were tested for β 2 microglobulin in cDNA obtained from matched frozen, Bouin-fixed, and formalin-fixed tissues.

In addition, standard curves were generated for GAPDH, CD40, AQP3, BLIMP1, IRF4, and syn-1 by using cDNA obtained from Bouin-fixed tissues.

PCR was performed with 25 μ l TaqMan Universal PCR Master Mix (PE Applied Biosystems), 2.5 μ l probe/primer mix and serial dilutions of cDNA (12.5 ng, 25 ng, 50 ng, 100 ng) in a 50- μ l final reaction mixture. After a 2-minute incubation at 50°C to allow for uracil-N-glycosylase (UNG) cleavage, AmpliTaq Gold was activated by incubation for 10 minute at 95°C. Each of the 50 PCR cycles consisted of 15 seconds of denaturation at 95°C and hybridization of probe and primers for 1 minute at 60°C. All reactions were done in triplicate and the threshold cycle (C_T) values obtained were plotted against the base

10 log of the ng cDNA. C_T is defined as the cycle at which the fluorescence is determined to be statistically significant above background and is inversely proportional to the log of the initial copy number. The higher the value, the lower the mRNA content.

Comparison of the Quantitative Results between the Fixed and Frozen Tissues

To explore the possibility of giving accurate quantitative RT-PCR results, cDNA obtained from matched frozen, Bouin-fixed, and formalin-fixed neoplastic samples (two diffuse large cell lymphomas, one plasmacytoma) was tested for target genes (CD40, AQP3, BLIMP1, IRF4, Syn-1).

RT-PCR of the studied samples was performed as described above. C_T values for β 2 microglobulin were used for normalization purposes. The averages of the normalized C_T values (ΔC_T) from the different samples were calculated. The strength of the linear association between C_T obtained from fixed and frozen samples was calculated using the Pearson correlation coefficient (r).

Results

Fixation

Evaluation of the tissue sections, after conventional and immunohistochemical staining, demonstrated high-quality of nuclear morphology, cellular morphology, tissue architecture, and good staining for lymphoid-associated antigens.

RNA Extraction

We isolated mRNA from Bouin-fixed tissues and, for comparative purposes, from formalin-fixed tissues, frozen tissues, and from fresh cell lines. Multiple samples from the same case generally gave similar results, indicating that the method of extraction was uniform. Starting from 10 to 15 mg of Bouin-fixed tissue, we obtained an average of 4 μ g (3.5 to 8) of RNA with an OD ratio ranging from 1.9 to 2.1, indicating high purity.

The quality of the total RNA preparation was assessed on an Agilent 2100 Bioanalyzer (Figure 1). As expected, the RNA sample derived from frozen tissue shows the distinct 18S and 28S ribosomal RNA (rRNA) bands. The RNA isolated from Bouin-fixed tissue displayed a broad molecular weight distribution, with an average of $\sim$ 180 nucleotides, but with a significant representation of higher molecular weights (Figure 1, middle histogram).

RT-PCR

The applicability of RNA from Bouin-fixed and formalin-fixed tissues to molecular biological analyses was assessed by RT-PCR. As targets, mRNA for CD40 (141 bases in length) and GAPDH (921 bases in length) were selected. As shown in Figure 2, CD40 could be amplified

Figure 1. Representative analysis of RNA isolated from frozen tissue and Bouin-fixed tissue using the Agilent Bioanalyzer (see Material and Methods). **Left:** Picture of the gel; the fragment sizes of the RNA ladder are indicated. **Right:** The corresponding histograms of the samples; fluorescence and time are indicated. For the size of the RNA ladder (**bottom**), see **left**. The 28S and 18S rRNA peaks are indicated in the **top** histogram.

by using cDNA obtained by RT performed both at 42°C and at 60°C while GAPDH could only be amplified by using cDNA obtained by RT performed at 60°C (Figure 3).

Real Time RT-PCR

Results of quantitative RT-PCR analysis were compared in matched frozen and fixed tissues. The RNAs were reverse-transcribed and real time amplification for the target genes was performed (see Materials and Methods).

For evaluation of PCR efficiencies, serial dilutions of cDNA obtained from a reactive lymph node were used to construct a standard curve. PCR efficiencies were tested for β 2 microglobulin in cDNA obtained from matched

Figure 2. Products of RT-PCR performed on RNA isolated from a Bouin-fixed tissue samples reverse transcribed at 42°C (**lanes 1 and 2**) or at 60°C (**lanes 3 and 4**) using primers for CD40. M, DNA marker.

Figure 3. RT-PCR for the detection of a 921-bp GAPDH fragment. **A:** RNA isolated from a Bouin-fixed (**lanes 1 to 4**, C-) reactive lymph node and reverse transcribed at 60°C by using random hexamer or oligo (dT)₂₀. C-, negative control; M, DNA marker. **B:** RNA isolated from Bouin-fixed (**lanes 1**, C1-) or formalin-fixed (**lanes 2**, C2-) reactive lymph node and reverse transcribed at 60°C. C-, negative control; M, DNA marker.

frozen, Bouin-fixed, and formalin-fixed tissues. In all cases, there was a strong linear correlation between the number of thermal cycles required to generate a significant fluorescent signal above background and the log of the input cDNA amount (correlation coefficient ≥ 0.97) (Figure 4). Even more importantly, when the resulting CA values were plotted against the log of the initial template amount and subjected to linear regression analysis, the amplification efficiencies were found to be very similar in fixed and frozen samples (Figure 4).

In addition, standard curves were generated for GAPDH, CD40, AQP3, BLIMP1, IRF4, and syn-1 by using cDNA obtained from Bouin-fixed tissues. Table 1 shows the R^2 values and standard curve slopes calculated by the instrument for each target. As shown in Table 1, all assays resulted in a good correlation coefficient and high PCR efficiency.

Comparison of the Real Time Quantitative RT-PCR Results between Fixed and Frozen Tissues

Delta C_T values for Bouin-fixed and formalin-fixed, paraffin-embedded tissues and their correlation with those for frozen samples are shown in Table 2. In the three neoplastic samples tested, an extremely high correlation ($r > 0.90$) emerged for all of the tested genes.

Figure 4. Real time RT-PCR: standard curves for $\beta 2$ microglobulin in cDNA obtained from matched frozen, Bouin-fixed, and formalin-fixed, paraffin-embedded tissues. A strong linear correlation between the C_T values and the log of the input cDNA amount (correlation coefficients ranging from 0.97 to 1.0) was obtained. Amplification efficiency in matching frozen, Bouin-fixed, and formalin-fixed tissue samples is comparable, as indicated by similar slopes of the regression lines.

Discussion

As sequencing of the human genome is being completed, a need for techniques that permit retrospective studies on both well-known and newly discovered genes

Table 1. R^2 Values and Standard Curve Slopes Calculated for Each Target by Real Time RT-PCR on Bouin-Fixed Samples

Target gene	R^2 value	Slope
GAPDH	0.999	-3.930
$\beta 2$ microglobulin	0.984	-3.431
CD40	0.993	-3.101
IRF4	0.983	-2.937
BLIMP1	0.983	-3.349
Syndecan-1	0.977	-3.226
Aquaporin 3	0.994	-4.115

The R^2 (correlation coefficient) value calculated by the instrument is an indication of the quality of the fit of the standard curve to the standard data points plotted, with values closer to 1 indicating a better fit of the data to the line. The slope of the standard curve is directly related to the average efficiency of amplification and may be used to calculate the PCR efficiency. A reaction with 100% efficiency will produce a slope of -3.32.

Table 2. Comparison of Average Delta C_T Values between Bouin- and Formalin-Fixed versus Frozen Sample by Target Gene

Target gene	Sample	Average delta C_T values		
		Frozen	Bouin-fixed	Formalin-fixed
CD40	DLCL-CB	3.8	3.7	3.9
	DLCL-IB	5.0	5.9	4.3
	Plasmacytoma	8.6	8.7	8.6
	r^*		0.98	0.99
IRF4	DLCL-CB	6.7	5.2	5.5
	DLCL-IB	2.6	2.5	0.4
	Plasmacytoma	5.4	4.2	3.3
	r^*		1.00	0.99
BLIMP1	DLCL-CB	7.8	5.8	5.2
	DLCL-IB	5.3	4.3	1.2
	Plasmacytoma	6.7	5.3	4.1
	r^*		0.99	0.98
Syndecan-1	DLCL-CB	10.8	10.6	11.0
	DLCL-IB	10.0	13.3	11.0
	Plasmacytoma	3.7	4.4	3.8
	r^*		0.92	0.99
AQP3	DLCL-CB	8.9	5.6	7.1
	DLCL-IB	5.5	3.1	2.6
	Plasmacytoma	7.5	5.5	5.5
	r^*		0.93	1.00

* r , Pearson correlation coefficient with frozen tissue.

DLCL, diffuse large cell lymphoma; CB, centroblastic; IB, immunoblastic; AQP3, aquaporin 3.

is emerging. With the increased use of PCR and other techniques of nucleic acid analysis for clinical purposes, knowledge about the effects of fixatives on the integrity and utility of the preserved DNA and RNA is becoming increasingly important.

In the present study, we have investigated whether RNA can be efficiently isolated from Bouin-fixed, paraffin-embedded lymphoid tissue specimens, in a form suitable for gene expression analysis using RT-PCR and real time RT-PCR. To this aim, we applied a new and simple method that allows RNA extraction from fresh, frozen, and formalin-fixed paraffin-embedded tissue sections, as also shown in this study. This method includes previously reported as well as newly adopted conditions for RNA isolation. We found that the combination of proteinase K digestion and column purification permits recovery of high-quality RNA from Bouin-fixed, paraffin-embedded lymphoid tissue that can be successfully used for RT-PCR studies. In addition, we have demonstrated that the amplification of relatively long fragments can be accomplished after a pre-heating step before c-DNA synthesis associated with the use of enzymes that work at high temperature.

It is known that RNA is readily extracted from fresh frozen samples using a guanidinium thiocyanate-caesium chloride gradient³¹ or a combined guanidinium thiocyanate acid-phenol chloroform procedure.³² These methods produce high-quality total RNA that can be used for further investigation. Simplified methods for RNA ex-

traction have become available from numerous commercial suppliers to speed up the process without loss of quality of the extracted RNA. The application of these methods to formalin-fixed, paraffin-embedded tissues, however, generally results in failure to extract RNA in sufficient quantity to enable further investigation. To date, the most successful method for extraction of total RNA from formalin-fixed, paraffin-embedded tissues utilizes a proteinase K digestion before acid-phenol chloroform extraction and carrier precipitation.^{20,33,34} However, the RNA extracted from formalin-fixed, paraffin-embedded tissue is significantly degraded.^{34,35} Previous attempts to amplify fragments longer than 200 bp were usually unsuccessful and only amplification of fragments in a range of 60 to 120 bp generally resulted in a very high success rate that approached 100%.⁶

Our results provide an advance in this area by showing, for the first time, that high-quality RNA can be extracted with a simple method from Bouin-fixed and paraffin-embedded tissues of reactive and neoplastic lymphoid disorders. We have also demonstrated that, by this method, RNA can be successfully extracted from formalin-fixed, paraffin-embedded lymphoid tissue section. Our method has been proven valid using lymph node tissues, which are very fragile, even when RNA isolation is performed on fresh material. Although not shown here, results from our group corroborate that the new method could be readily adopted to the analysis of many other, less fragile, tissue types (for example, carcinomas).

Through the isolation process described here, sufficient RNA for RT-PCR was isolated from a single section of paraffin-embedded lymph node tissue sample. According to our experience, a single 15- μ m section corresponds to 10 to 15 mg of tissue. By means of PCR, using different primers for two examined genes (GAPDH and CD40), we amplified segments of cDNA gained by reverse transcription of the isolated RNA extracted from Bouin-fixed or formalin-fixed, paraffin-embedded tissues. The sizes of the amplified fragments were as expected 921 and 141 bp for GAPDH and CD40, respectively, which provides evidence of good-quality mRNA, despite overall degradation. Furthermore, to explore the possibility of giving accurate real time quantitative RT-PCR results, cDNA obtained from matched frozen, Bouin-fixed and formalin-fixed neoplastic samples (two diffuse large cell lymphomas, one plasmacytoma) was tested for several target genes. Delta threshold cycle (ΔC_T) values for Bouin-fixed and formalin-fixed paraffin-embedded tissues and their correlation with those for frozen samples showed an extremely high correlation ($r > 0.90$) for all of the tested genes. Therefore, these results show that RNA isolated from Bouin-fixed, paraffin-embedded tissue sections can produce reliable quantitative RT-PCR data. Conversely, preliminary studies have shown that the amounts of total RNA that can be reverse-transcribed following Bouin-isolation are too low to achieve sufficient generation of the amounts of labeled cRNA currently required for gene chip hybridization (data not shown). A reasonable explanation for this apparent failure may be that only a fraction of the RNA in the reaction is accessi-

ble to cDNA synthesis. Presumably, the remaining RNA is chemically altered by the fixation and paraffin-embedding and cannot be reverse transcribed.

In conclusion, the importance of this study lies in the application of a simple method that permits high-quality RNA isolation from archival fixed and paraffin-embedded lymphoid tissues, thus allowing genetic and proteomic investigations of lymphoproliferative disorders using RT-PCR and real time quantitative RT-PCR. This method can be automated using commercial robotic systems, allowing its easy and rapid application to large-scale studies.

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