Quantitative mRNA Expression Analysis from Formalin-Fixed, Paraffin-Embedded Tissues Using 5' Nuclease Quantitative Reverse Transcription-Polymerase Chain Reaction

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Analysis of gene expression and correlation with clinical parameters has the potential to become an important factor in therapeutic decision making. The ability to analyze gene expression in archived tissues, for which clinical followup is already available, will greatly facilitate research in this area. A major obstacle to this approach, however, has been the uncertainty about whether gene expression analyses from routinely archived tissues accurately reflect expression before fixation. In the present study we have optimized the RNA isolation and reverse transcription steps for quantitative reverse transcription-polymerase chain reaction (RT-PCR) on archival material. Using tissue taken directly from the operating room, mRNAs with half-lives from 10 minutes to >8 hours were isolated and reverse transcribed. Subsequent real-time quantitative PCR methodology (TaqMan) on these cDNAs gives a measurement of gene expression in the fixed tissues comparable to that in the fresh tissue. In addition, we simulated routine pathology handling and demonstrate that this method of mRNA quantitation is insensitive to pre-fixation times (time from excision to fixation) of up to 12 hours. Therefore, it should be feasible to analyze gene expression in archived tissues where tissue collection procedures are largely unknown. (J Mol Diag 2000, 2:84-91)

Many genes involved in oncogenesis have been identified and abnormal expression of these genes is most likely a major determinant of the eventual tumor phenotype.¹⁻³ Currently, however, tumor pathology is the major influence on a physician's choice of treatment; unfortu-

nately, tumors with similar pathology often respond differently to identical treatment regimens. It is probable that by creating a transcription profile for a given tumor, clinicians will be better able to predict response to the various therapeutic options and thus improve clinical outcome. To develop a protocol for accurate predictions, researchers must perform a retrospective study on a large sampling of tumors for which response to therapy and clinical outcome are already known.

Although many institutions are now maintaining frozen tumor banks, which should facilitate gene expression analyses in the future, few of these now have sufficient clinical follow-up to be useful for retrospective studies correlating gene expression with clinical outcome. On the other hand, there is a vast supply of formalin-fixed, paraffin-embedded tumor tissues for which response to treatment and clinical outcome is already known. Although these archived tissues can be used for *in situ* techniques to show localization of gene expression,^{4,5} the RNA is too degraded for classical, quantitative analysis methods such as Northern blots. Reverse transcription-polymerase chain reaction (RT-PCR) has been used extensively to detect expression of genes in cultured cells and in fresh or frozen tissues, and recent technological advances now allow rapid and accurate quantitative RT-PCR analyses.^{6,7} Furthermore, the ability of RT-PCR to assay very small fragments of mRNAs makes this technique amenable to studies where the RNA is moderately or even highly degraded, as in the case of RNA from archived tissues.^{8,9}

Although it has previously been demonstrated that RNA from formalin-fixed tissues can be used for RT-PCR¹⁰⁻¹⁵ and that the RT-PCR steps can be done in a quantitative manner,^{16,17} it has not been shown that RNA levels measured in archival tissues accurately reflect expression in the tissue before fixation. Several factors may influence relative RNA representation in fixed *versus* fresh

Supported by Specialized Program of Research Excellence grant CA 58207 and by National Institutes of Health grant CA 84019. S.-H. K. was supported by the Korean Research Foundation.

Accepted for publication February 25, 2000.

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tissues and some of these, such as time in fixative and type of fixative, have already been addressed by others.^{8,9,18,19} Other important factors that have not been addressed previously include RNA half-life, time from surgical excision to fixation (pre-fixation time), and differential effect of fixation on RNA populations and the subsequent ability to extract and quantitate all RNA species equally.

The purpose of this study was to determine the effect of these variables on quantitation of gene expression as measured using TaqMan RT-PCR methodology⁷ and thus determine the feasibility of gene expression studies on archival material.

Materials and Methods

Tissue Collection and Processing

Prostate tissue blocks for use in optimizing RNA isolation were obtained from the University of California at San Francisco tissue bank. Normal liver tissue was obtained from a patient undergoing resection of a colorectal metastasis to the liver. The sample was collected in the operating room and sectioned into seven pieces. One piece was immediately placed in Trizol reagent (Life Technologies, Gaithersburg, MD) and homogenized for RNA isolation in the laboratory. A second piece was immediately placed in 10% buffered formalin (zero-hour sample) and fixed for 18 hours. The remaining five pieces were placed in phosphate buffered saline (PBS) solution and held at room temperature for 1, 2, 4, 8, and 12 hours, respectively, before beginning an 18-hour fixation incubation in formalin.

After fixation, samples were dehydrated, incubated in xylene, incubated in paraffin, and embedded in paraffin using a Leica Paraffin-Embedder (Leica Microsystems Inc., Deerfield, IL).

RNA Extraction

RNA isolation from paraffin tissue sections was based on the methods first described by Fisher.²⁰ Paraffin-embedded tissue samples were cut into $5 \times 5 \mu\text{m}$ to $50 \times 5 \mu\text{m}$ sections, depending on the size of the embedded tissue sample, and placed in RNase-free, 2.0-ml Eppendorf tubes. Sections were deparaffinized by incubation in 1.8 ml of xylene or Americlear (Stephens Scientific, Riverdale, NJ) at 37°C for 20 minutes. The samples were then centrifuged, the supernatant was removed, and fresh xylene was added for a second incubation. After deparaffinization and centrifugation, sections were washed with 0.5 ml ethanol, air-dried for several minutes, and resuspended in 80 μl of 60 mg/ml (20 U/mg) Proteinase K (Gibco BRL, Gaithersburg, MD) plus 720 μl of a digestion buffer with the following final concentrations: 1 mol/L guanidinium thiocyanate, 25 mmol/L 2-mercaptoethanol, 0.5% Sarcosyl 20 (N-lauroylsarcosine), 20 mmol/L Tris-HCl, pH 7.5.¹⁰ Samples were vortexed and incubated overnight at 55°C. A second 80- μl aliquot of 60 mg/ml Proteinase K was then added, followed by vortexing and

a second overnight incubation at 55°C. On the third day, a final aliquot of Proteinase K was added and the samples were again vortexed, followed by overnight incubation at 55°C. RNA was obtained by extraction with an equal volume of 70% phenol (pH 4.3):30% chloroform at room temperature. Samples were centrifuged for 5 minutes at 14,000 rpm and the aqueous phase was transferred to new, RNase-free Eppendorf tubes. The RNA was precipitated by addition of an equal volume of isopropanol, along with 2 μg glycogen, at -20°C for 30 minutes. The samples were then centrifuged for 30 minutes at 14,000 rpm, the RNA pellets were washed in 70% EtOH, air-dried for several minutes on the bench, and then resuspended in 20 μl diethyl pyrocarbonate (DEPC)-treated H₂O. Trizol reagent (500 μl) was then added to each RNA sample and purification was performed according to the manufacturer's instructions with one exception: following the first Trizol treatment, the aqueous phase was not precipitated but was instead subjected to a second 500- μl Trizol extraction. The aqueous phase from this step was used for RNA precipitation with 500 μl of isopropanol and the RNA was resuspended in 20 μl deionized formamide for storage at -20°C. All RNAs were quantitated by spectrophotometer and OD 260/280 nm ratios >1.8 were obtained for all samples, indicating high purity. All solutions, including digestion buffer and ethanol/water solutions, were made using DEPC-treated water.

RNA Isolation from Fresh Tissue/Cell Lines

RNA was isolated from fresh tissues using Trizol reagent according to the manufacturer's instructions, with the exception that a second Trizol extraction was performed as described above. RNA was resuspended in 20 μl of deionized formamide for storage before a final purification step with RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was quantitated as described above.

Reverse Transcription

The optimal reverse transcription (RT) was carried out in 100- μl volumes consisting of 1 $\times$ PCR buffer II (PE Biosystems, Foster City, CA), 250 units of Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies), 40 units of RNase inhibitor (Roche Molecular Biochemicals, Indianapolis, IN), 7.5 mmol/L MgCl₂, 1 mmol/L each dNTP (Roche Molecular Biochemicals), 5 $\mu\text{mol/L}$ random hexamers (Life Technologies), and 75 to 300 ng total RNA. Reactions were incubated at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes in a Perkin-Elmer GeneAmp PCR System 9700. "No RT" controls were carried out in all cases using the same RT reaction mix but substituting DEPC-H₂O for M-MLV reverse transcriptase. For all quantitative analyses 3 RTs were carried out for each RNA sample. The RNA amounts used in each RT were 300, 150, and 75 ng per 100- μl RT reaction. All "no RT" controls were carried out with 300 ng of RNA.

Table 1. Sequences of PCR Primers and Fluorogenic Probes Used in Quantitative PCR

Gene	Amplicon size (bp)	Forward primer	Reverse primer	Probe
GAPDH	72	ATTCCACCCATGGCAAATTC	TGGGATTTCATTGATGACAAG	FAM-CAAGCTTCCCCTTCTCAGCC
β -Gus	81	CTCATTTGGAATTTTGCCGATT	CCGAGTGAAGATCCCCTTTTTA	JOE-TGAACAGTCACCGACGAGAGTGCTGG
c-Myc E2/3	86	GCCCCCTGGTGCTCCATGA	CAA CATCGATTTCCTCATCTTCT	FAM-CACCGCCGCCACCGACGAGCGAC
c-Myc 3' UTR	91	CCTGAGCAATCACCTATGAACT	TGGCTAAATCTTTCAGTCTCAAG	FAM-AAATGCAACCTCACAACCTTGGCTGA
c-Myc E2	91	GGACGACGAGACCTTCATCAA	CAGCTTCTCTGAGACGAGCTTG	FAM-CCAGGACTGTATGTGGAGCGGCTTCT
ZNF217	89	TTTTTCCGTTCAAATTATTACCTCAA	GCAGCATATTCACAAAATTCACATT	FAM-CATCTCAGACATACAGGTGAAAAACCATAC
FLT	102	TGTGGGAAATCTTCTCCTTAGGTG	TCCCTCAGGCGACTGCAAAAGTCCT	FAM-AGGACTTTTGAGTCGCTGAGGGA
KDR	102	TGGTGCACTGCAGACAGATCTAC	GCAAAACAGGTGTGGGCAAC	FAM-TTGGCCACAGCCTCTGCCAAT
Her 2	77	GTGACAGCAGAGGATGGAACAC	ATGCCAGACCATAGCACACT	FAM-CGGTGTGAGAAGTGCAGCAAGCCC
Vegf	94	CTCTACCTCCACCATGCCAAG	AGACATCCATGAATTCACCACTTC	FAM-TGGCAGAAGGAGGAGGGCAGAATCA
β -Actin	99	CCACACTGTGCCCATCTACG	AGGATCTTCATGAGGTAGTCAGTCAG	FAM-ATGCCCTCCCCATGCCATCCTGCGT
β -Actin	131	CCACACTGTGCCCATCTACG	GATGTCGAAGTGGTGGTGCC	FAM-ATGCCCTCCCCATGCCATCCTGCGT
β -Actin	175	CCACACTGTGCCCATCTACG	GTAGCACAGCTTCTCCTTAATGTAC	FAM-ATGCCCTCCCCATGCCATCCTGCGT
β -Actin	205	CCACACTGTGCCCATCTACG	CTACAGCTTCAACACACGCGCC	FAM-ATGCCCTCCCCATGCCATCCTGCGT
β -Actin	291	CCACACTGTGCCCATCTACG	CAGCGGAACCGCTCATTGCCAATGG	FAM-ATGCCCTCCCCATGCCATCCTGCGT

All probes were labeled with 3' TAMRA quencher dye.

Real Time Quantitative RT-PCR

Relative abundance of each mRNA species was assessed using the 5' fluorogenic nuclease assay to perform real time quantitative PCR.^{7,21-23} The basis for this system is to continuously measure PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe, called a TaqMan probe. This probe is composed of a short (~20–25 bases) oligodeoxynucleotide labeled with two different fluorescent dyes. The oligonucleotide probe sequence is homologous to an internal target sequence present in the PCR amplicon. When the probe is intact, energy transfer occurs between the two fluorophores and emission from the reporter is quenched by the quencher. During the extension phase of PCR, the probe is cleaved by 5' nuclease activity of *Taq* polymerase, thereby releasing the reporter from the oligonucleotide quencher and producing an increase in reporter emission intensity. The ABI Prism 7700 detects and plots this increase in fluorescence versus PCR cycle number to produce a continuous measure of PCR amplification. To provide precise quantification of initial target in each PCR reaction, the amplification plot is examined at a point during the early log phase of product accumulation. This is accomplished by assigning a fluorescence threshold above background and determining the time point at which each sample's amplification plot reaches the threshold (defined as the threshold cycle number or C_T). Differences in threshold cycle number are used to quantify the relative amount of PCR target contained within each tube as described previously.^{24,25}

The following genes were studied: glyceraldehyde phosphate dehydrogenase (GAPDH),²⁶ β -glucuronidase (β -Gus),²⁷ *c-myc*,²⁸ *erbB-2*,²⁹ β -actin,³⁰ ZNF217,²⁵ vascular endothelial growth factor (VEGF),³¹ and VEGF receptors Flt³² and KDR.³³ PCR primers and fluorogenic probe (TaqMan) were designed using Primer Express software (PE Biosystems), and the sequences are given in Table 1. Wherever possible, TaqMan primers and probes were designed to span introns in the genomic DNA, thereby minimizing signal from contaminating

genomic DNA. All probes were obtained from either PE Biosystems or Synthetic Genetics (San Diego, CA). Primers were obtained from PE Biosystems or Life Technologies. Quantitative RT-PCR was performed in duplicate in 50- μ l reaction volumes consisting of 1 $\times$ PCR buffer A (PE Biosystems), 5.5 mmol/L $MgCl_2$, 0.025 U/ μ l AmpliTaq Gold (PE Biosystems), and 5 μ l of the appropriate RT reaction. The final primer and probe concentrations used for each gene are shown in Table 2. Two-step PCR cycling was carried out as follows: 95°C 12 minutes $\times$ 1 cycle, 95°C 15 seconds, 60°C 1 minute $\times$ 40 cycles. At the end of the PCR, baseline and threshold values were set in the ABI 7700 Prism software and the calculated C_T values were exported to Microsoft Excel for analysis.

Calculation of Relative Expression

Relative expression of mRNA species was calculated using the comparative C_T method described previously.^{24,25} All data were controlled for quantity of RNA input by performing measurements on an endogenous reference gene β -Gus. In addition, results on RNA from fixed tissues were normalized to results obtained on RNA from

Table 2. Final Concentrations of PCR Primers and Fluorogenic Probes Used in Quantitative RT-PCR

TaqMan PCR Final Concentrations (nmol/L)			
Gene	Forward primer	Reverse primer	Probe
GAPDH	200	200	50
β -Actin (all sizes)	300	300	200
cMyc E 2	100	100	100
cMyc E2/3	100	100	100
cMyc 3' UTR	100	100	100
β -Gus	100	100	100
ZABC1/ZNF217	300	300	200
ErbB-2	900	900	200
VEGF	100	100	100
FLT	300	300	250
KDR	300	300	250

the fresh tissue. Briefly, the analysis was performed as follows:

For each RNA sample (including the fresh tissue sample), a difference in C_T values (ΔC_T) was calculated for each mRNA by taking the mean C_T of duplicate tubes and subtracting the mean C_T of the duplicate tubes for the reference RNA (β -Gus) measured on an aliquot from the same RT reaction.

$$\Delta C_T = C_T(\text{test gene}) - C_T(\beta\text{-Gus})$$

The ΔC_T for the fresh tissue sample was then subtracted from the ΔC_T for the test sample to generate a $\Delta\Delta C_T$.

$$\Delta\Delta C_T = \Delta C_T(\text{test RNA}) - \Delta C_T(\text{fresh tissue RNA})$$

Since all RNAs were reverse transcribed at 3 different RNA concentrations, 3 values of $\Delta\Delta C_T$ were calculated for each gene on each RNA sample: $\Delta\Delta C_T$ (300 ng), $\Delta\Delta C_T$ (150 ng), and $\Delta\Delta C_T$ (75 ng). The mean of these $\Delta\Delta C_T$ measurements was then used to calculate expression of the test gene relative to the reference gene and normalized to the fresh tissue sample as follows:

$$\text{Relative Expression} = 2^{-\Delta\Delta C_T}$$

This calculation assumes that all PCR reactions are working with 100% efficiency. All PCR efficiencies were measured as previously described²⁴ and were found to be >95%; therefore, this assumption introduces minimal error into the calculations.

Error bars were plotted as 80% confidence limits based on the average SD of all triplicate $\Delta\Delta C_T$ measurements within the experiment.

Results

RNA Isolation from Fixed Tissues

We first tested different RNA extraction procedures from paraffin-embedded prostate tissue. Results of RNA yield (Figure 1B) and RNA length as determined by denaturing gel electrophoresis (Figure 1A) indicated that a 3-day proteinase K incubation gave increased yield and RNA length. However, the OD260/280 ratios obtained with this procedure were relatively low (1.3–1.6), indicating impurities in the RNA. Furthermore, RT reactions were inconsistent, especially at higher RNA inputs, suggesting that these impurities were inhibiting the RT reaction in an unpredictable manner, thus making quantitative RT-PCR difficult. We believe that this is due to proteinase K carried over from the RNA extraction procedure despite heat inactivation. We have previously observed that small amounts of proteinase K activity can survive phenol extraction and heat inactivation, and will subsequently inactivate RT-PCR enzymes (Ruff DW, unpublished results). We found that two sequential Trizol extractions were sufficient to increase the OD260/280 ratios to >1.6, and made the subsequent RT steps much more consistent and quantitative. This extra purification step also has the added advantage of removing most of the genomic DNA from the RNA sample, thus making the “no RT” controls consistently negative.

A

Year	RNA Yield per 20 μ M Section	
	1 Day Incubation	3 Day Incubation
1985	3.2 μ g	7.2 μ g
1990	3.0 μ g	4.6 μ g
1993	2.5 μ g	4.7 μ g

B

Figure 1. Denaturing agarose gel of RNA isolated from 3 archival prostate tissue blocks collected in 1985, 1990, and 1993, respectively. RNA isolated using the modified 3-day protocol is longer (A) and the yield is higher (B) than with the original protocol.

Optimizing Reverse Transcription

For expression analysis by RT-PCR, the RT step must be quantitative, ie, more RNA input into the RT reaction must result in a proportional increase in cDNA product. We tested this using RNA isolated from an archived human prostate sample and found that our standard RT reaction did not give a satisfactory linear dynamic range on RNA isolated from fixed tissues. For this reason we modified the concentrations of RT reaction components to optimize the dynamic range of the reaction. The biggest single effect on RT linearity was observed by increasing the concentration of nucleotides in the reaction to 2 $\times$ that in the original reaction. RT efficiency was improved further by doubling the concentration of enzyme, nucleotides, and hexamers in combination. However, the optimal RT reaction (lowest C_T values and good linearity) was obtained by adding extra $MgCl_2$ to the 2 $\times$ enzyme, nucleotide, and hexamer reaction. This is presumably because the RT enzyme requires free Mg^{2+} for optimal activity, but the amount of free Mg^{2+} is reduced when extra nucleotides are added to the reaction. Although this 2 $\times$ + Mg protocol gives reasonable RT linearity up to

Figure 2. Amplification of different sized fragments of the β -actin mRNA from fixed tissue RNA isolates. Greatly increased sensitivity of detection is obtained with fragments less than 131 basepairs. The forward primer and TaqMan probe were kept constant and used with different reverse primers to change amplicon length (inset picture). No difference is seen between the immediate fix RNA and the 12 hour pre-fixation RNA samples (see Materials and Methods for description of tissue processing). Fresh tissue RNA was used as a high quality RNA control. Standard deviations on all points were smaller than the size of the symbols and are omitted.

1.25 μ g of RNA per 100- μ l reaction, all RT values for quantitative analysis were carried out at 300, 150, and 75 ng RNA inputs as described in Materials and Methods.

Effect of Amplicon Size on Quantitation

To test the effect of different PCR amplicon sizes on mRNA quantitation we assayed 99- to 291-bp fragments of the β -actin mRNA. In the first experiment we looked at absolute C_T values obtained for actin of 99, 115, 131, 175, 205, and 291 bp on high quality RNA from fresh liver tissue RNA *versus* the immediate fix and 12-hour pre-fixation RNAs (Figure 2). It can be seen that the relative abundance of quantifiable β -actin fragments is lower (higher C_T) in the fixed tissues than in the fresh tissue RNA and that this difference in abundance is greatest at the larger fragment sizes.

In a second experiment we quantitated the abundance of actin 291 bp and actin 99 bp relative to β -glucuronidase (81-bp fragment) in the pre-fixation time series RNAs (Figure 3). It can be seen that for the fixed tissues the abundance of the 291-bp actin fragment was approximately 90 times less than that of the 99-bp fragment. It should be noted, however, that the relative abundance of both the actin 99 bp and 291 bp did not change significantly over the 12-hour time series.

Effect of Pre-Fixation Time on mRNA Quantitation

To obtain meaningful RNA expression data from archived tissue banks, analysis methods must be relatively insensitive to variations in tissue fixation and collection procedures. One of the obvious variables that needs to be considered is pre-fixation time (time from excision to fixation in formalin).

Figure 3. Relative abundance of 99- and 291-bp fragments of the β -actin mRNA in fixed tissues. All points are measured relative to β -Gus and are normalized to the fresh tissue RNA.

To test the effect of pre-fixation time on relative representation of mRNA species as measured by quantitative RT-PCR, we obtained a piece of human liver tissue directly from the operating room and processed it as described in Materials and Methods. Several genes were studied and the results for GAPDH, ZNF217, *ErbB-2*, VEGF, Flt, and KDR are shown in Figure 4. All expression is relative to β -Gus and the data are normalized to the results for fresh tissue RNA to allow easy visualization of the effect of the 18-hour fixation and of pre-fixation time on mRNA quantitation. For *c-myc*, three different fragments of the mRNA were assayed (Figure 5) to study the effect of fixation and pre-fixation time on recovery and quantitation of this highly unstable mRNA species.

Discussion

The goal of this study was to determine the feasibility of obtaining meaningful quantitative gene expression data from tumor tissues collected by conventional surgical excision and with subsequent fixation. To this end, we optimized RNA isolation and reverse transcription procedures for fixed tissues, evaluated the effect of PCR amplicon size on quantitation, and studied pre-fixation times up to 12 hours for any effect on the levels of nine different mRNA species. The results indicate that it should be possible to use archival tissues for correlative studies of gene expression *versus* patient outcome.

Previously published protocols for isolating RNA from fixed tissues²⁰ were modified to optimize RNA yield and size. Reverse transcription procedures were also optimized to obtain reproducible reverse transcription efficiencies and therefore accurate quantitation of mRNA expression levels. Despite these optimized procedures, it was found that the absolute C_T values on fixed tissues were an average of ~ 5 cycles higher than on matched fresh tissues. This indicates that only one-thirtieth of the RNA in the reaction is accessible to cDNA synthesis. Presumably the remaining RNA is chemically altered by the formalin-fixation and paraffin-embedding and cannot be reverse transcribed. Furthermore, the process of formalin fixation and paraffin embedding seems to differen-

Figure 4. Effect of pre-fixation time on quantitation of six different mRNA species. All points are measured relative to β -Gus and are normalized to the fresh tissue RNA.

tially affect the ability to recover different mRNA species or fragments of those species. This difference can be seen by looking at expression measured in the fresh tissue RNA and the zero-hour fixed tissue RNA, and is especially pronounced (10-fold change) in the case of the *c-myc* exon 2/3 fragment relative to β -Gus (Figure 5). In this case formalin fixation and paraffin embedding appears to reduce the amount of this particular 86-bp fragment of *c-myc* mRNA that can be recovered, reverse transcribed, and quantitated, relative to the 81-bp fragment of β -Gus. This 10-fold change is presumably not due to biological degradation of *c-myc* message, since both tissues were processed immediately on excision from the patient and the tissue pieces were small enough ($<1 \text{ cm}^3$) for fixation to be very fast. Furthermore, when different fragments of *c-myc* mRNA were analyzed (*c-myc* exon 2 or *c-myc* 3'UTR) the relative recovery of each fragment was closer to that from fresh tissue (Figure 5).

Figure 5. Effect of fixation and pre-fixation time on quantitation of different pieces of the *c-myc* mRNA. Primers and probe used are shown in Table 1. Fragments are affected differently by fixation but none of the fragments show a change in abundance over the 12-hour time series. All points are measured relative to β -Gus and are normalized to the fresh tissue RNA.

Thus the ability to recover and reverse transcribe different fragments of one mRNA species varies from fragment to fragment, possibly as a result of regulatory proteins bound to (and then cross-linked to) different parts of the mRNA.³⁴

Despite the improved RNA isolation protocol, RNA obtained from fixed tissues was still highly degraded, and thus one would expect smaller amplicon sizes to provide better sensitivity. When this was tested, we found that there was an increase in the C_T value as amplicon size increased and that this increase was much greater with fixed tissue RNA than with control, fresh tissue RNA. The delta C_T for the β -actin 291-bp versus the 99-bp ($\Delta C_{T291-99}$) product was 3.4 cycles on the fresh tissue RNA and 9.9 cycles on the immediate fix RNA sample. By calculating a $\Delta\Delta C_T$ for the fixed tissue RNA minus the fresh tissue RNA ($\Delta C_{T291-99}$ fresh tissue - $\Delta C_{T291-99}$ fixed tissue = 6.5) to correct for PCR efficiency differences, it can be determined that the relative abundance of the 99-bp and 291-bp fragments in the fixed tissues is 90:1 ($2^{6.5}:1$). Furthermore, the absolute C_T values on fixed tissue RNA did not change significantly with fragment sizes <131 bp, but then increased by 5 cycles for the 175-bp fragment. Thus, it seems that the best sensitivity for RT-PCR on fixed tissue RNA can be obtained with amplicon sizes less than ~ 130 bp. No large difference was seen between the zero-hour and 12-hour pre-fixation time data points, indicating that RNA degradation and cross-linking due to the fixation and embedding procedure is worse than any degradation occurring in the tissue before fixation. Therefore, comparing fragments of different sizes should give a constant ratio, as was the case when we studied 99-bp and 291-bp fragments of the β -actin gene (Figure 3), but this has not been thoroughly tested, so it seems prudent to keep both test and reference amplicons below 130 bp.

For all quantitative analyses, three separate cDNA synthesis reactions were carried out with different RNA input amounts (75–300 ng). We believe that this has several advantages over single RNA input quantitation. First, by

analyzing three points, one should see a linear decrease in C_T that corresponds to increasing RNA input in the RT reaction. If linearity is not observed, then the RT step was not quantitative, and therefore the results are questionable. Second, the slopes of RNA input *versus* C_T should be close to identical for the test and reference genes on any single RNA (and preferably on all RNAs). This similarity in slopes indicates that both the RT and PCR efficiencies were the same for both genes, a critical factor when using ΔC_T calculations for quantitation.²⁴ The main factor influencing RT slopes appeared to be the RNA purity, and therefore we found it necessary to use the rather laborious RNA isolation procedures described. Third, the use of three RT points allows one to obtain an estimate of the error associated with the measurement. The mean and SD of the $\Delta \Delta C_T$ values can be calculated and plotted as in Figure 4.

It might be expected that pre-fixation time would be the major factor influencing the ability to quantitate gene expression in fixed tissues. mRNAs with short half-lives would presumably disappear quickly, and thus pre-fixation time would affect the levels detected. Because pre-fixation time is unknown for archival tissue specimens, quantitation of expression would be impossible. Interestingly, this was not the case. Our experiments showed that times up to 12 hours in PBS before fixation and embedding did not change the relative expression of any of the genes studied using this technique. Even *c-myc* mRNA, which has a reported half-life as short as 10 minutes,³⁵ did not show a reduction with up to 12 hours pre-fixation time. This is probably due to two factors. First, the RT-PCR assay only requires a small fragment of intact RNA, so the RNA can be substantially degraded and still be detected. Second, it is reasonable to assume that the tissue is still metabolizing. mRNA half-lives are typically determined by using actinomycin D to stop RNA synthesis and then measuring decay of the pre-existing mRNA over time. *In vivo*, however, steady state levels of mRNA are controlled by both transcription and degradation rates.^{36,37} Assuming that transcription is still occurring in the tissues incubated in PBS, there is no *a priori* reason to expect steady-state levels to change. In support of this assumption, when we carried out a pre-fixation time study using a piece of tissue that had been stored at -70°C instead of collected directly from the operating room, the *c-myc* mRNA levels dropped dramatically over an 8-hour pre-fixation time course (data not shown). This implies that, once frozen, tissue metabolism is stopped and steady-state levels of mRNA are then influenced only by degradation rates, thus the *c-myc* mRNA disappears quickly. Of course, for many genes transcription and degradation rates will be affected by stresses such as, eg, hypoxia,^{38–40} temperature reduction,⁴¹ and depletion of nutrients^{42,43} imposed by excision and storage at room temperature in PBS. Indeed, in our experiments, GAPDH levels increased relative to β -Gus over the 12-hour study period, possibly due to hypoxic induction of GAPDH.^{44,45} For some genes these expression changes will be considerable and may preclude meaningful quantitation from fixed tissues collected in an uncontrolled manner. By carrying out a pre-fixation time study such as the one

described here, one should be able to determine whether a particular mRNA of interest is subject to change with pre-fixation time and thus validate that gene for fixed tissue expression studies. We have studied >10 genes so far and only GAPDH shows a significant trend with time; even this results in a change of less than a factor of two over 12 hours. Because it is unlikely that tissue would be left more than 12 hours from surgical excision to formalin fixation, it should be possible to go back to archival fixed tissue banks and obtain meaningful gene expression data using TaqMan quantitative RT-PCR.

References

1. Fearon ER, Dang CV: Cancer genetics: tumor suppressor meets oncogene. *Curr Biol* 1999, 9:R62–R65
2. Hill JR, Kuriyama N, Kuriyama H, Israel MA: Molecular genetics of brain tumors. *Arch Neurol* 1999, 56:439–441
3. Pearson PL, Van der Luijt RB: The genetic analysis of cancer. *J Intern Med* 1998, 243:413–417
4. Steele A, Uckan D, Steele P, Chamizo W, Washington K, Koutsonikolis A, Good RA: RT in situ PCR for the detection of mRNA transcripts of Fas-L in the immune-privileged placental environment. *Cell Vis* 1998, 5:13–19
5. Gruber AD, Greiser-Wilke IM, Haas L, Hewicker-Trautwein M, Moenig V: Detection of bovine viral diarrhoea virus RNA in formalin-fixed, paraffin-embedded brain tissue by nested polymerase chain reaction. *J Virol Methods* 1993, 43:309–319
6. Lie YS, Petropoulos CJ: Advances in quantitative PCR technology: 5' nuclease assays. *Curr Opin Biotechnol* 1998, 9:43–48
7. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 1996, 6:986–994
8. Inoue T, Nabeshima K, Kataoka H, Koono M: Feasibility of archival non-buffered formalin-fixed and paraffin-embedded tissues for PCR amplification: an analysis of resected gastric carcinoma. *Pathol Int* 1996, 46:997–1004
9. Tyrrell L, Elias J, Longley J: Detection of specific mRNAs in routinely processed dermatopathology specimens. *Am J Dermatopathol* 1995, 17:476–483
10. Stanta G, Schneider C: RNA extracted from paraffin-embedded human tissues is amenable to analysis by PCR amplification. *Biotechniques* 1991, 11:304–308
11. Stanta G, Bonin S, Perin R: RNA extraction from formalin-fixed and paraffin-embedded tissues. *Methods Mol Biol* 1998, 86:23–26
12. Jackson DP, Quirke P, Lewis F, Boylston AW, Sloan JM, Robertson D, Taylor GR: Detection of measles virus RNA in paraffin-embedded tissue (letter). *Lancet* 1989, 1:1391
13. Jackson DP, Lewis FA, Taylor GR, Boylston AW, Quirke P: Tissue extraction of DNA and RNA and analysis by the polymerase chain reaction. *J Clin Pathol* 1990, 43:499–504
14. Finke J, Fritzen R, Ternes P, Lange W, Dolken G: An improved strategy and a useful housekeeping gene for RNA analysis from formalin-fixed, paraffin-embedded tissues by PCR. *Biotechniques* 1993, 14:448–453
15. Goldsworthy SM, Stockton PS, Trempus CS, Foley JF, Maronpot RR: Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinog* 1999, 25:86–91
16. Stanta G, Bonin S: RNA quantitative analysis from fixed and paraffin-embedded tissues: membrane hybridization and capillary electrophoresis. *Biotechniques* 1998, 24:271–276
17. Stanta G, Bonin S, Utrera R: RNA quantitative analysis from fixed and paraffin-embedded tissues. *Methods Mol Biol* 1998, 86:113–119
18. Foss RD, Guha-Thakurta N, Conran RM, Gutman P: Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue: comparison of two housekeeping gene mRNA controls. *Diagn Mol Pathol* 1994, 3:148–155
19. Guerrero RB, Batts KP, Brandhagen DJ, Germer JJ, Perez RG, Persing DH: Effects of formalin fixation and prolonged block storage on

- detection of hepatitis C virus RNA in liver tissue. *Diagn Mol Pathol* 1997, 6:277-281
20. Fisher JA: Activity of proteinase K and RNase in guanidium thiocyanate. *FASEB J* 1988, 2:A1126 (abstr.)
21. Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K: Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl* 1995, 4:357-362
22. Gibson UE, Heid CA, Williams PM: A novel method for real time quantitative RT-PCR. *Genome Res* 1996, 6:995-1001
23. Holland PM, Abramson RD, Watson R, Gelfand DH: Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc Natl Acad Sci USA* 1991, 88:7276-7280
24. PE Applied Biosystems: ABI Prism 7700 Sequence Detection System: Relative quantitation of gene expression. User bulletin #2 Norwalk, CT, Perkin-Elmer Corp., 1997, 36 pp
25. Collins C, Rommens JM, Kowbel D, Godfrey T, Tanner M, Hwang SI, Polikoff D, Nonet G, Cochran J, Myambo K, Jay KE, Froula J, Cloutier T, Kuo WL, Yaswen P, Dairkee S, Giovanola J, Hutchinson GB, Isola J, Kallioniemi OP, Palazzolo M, Martin C, Ericsson C, Pinkel D, Gray JW: Positional cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and overexpressed in breast carcinoma. *Proc Natl Acad Sci USA* 1998, 95:8703-8708
26. Arcari P, Martinelli R, Salvatore F: The complete sequence of a full length cDNA for human liver glyceraldehyde-3-phosphate dehydrogenase: evidence for multiple mRNA species. *Nucleic Acids Res* 1984, 12:9179-9189
27. Oshima A, Kyle JW, Miller RD, Hoffmann JW, Powell PP, Grubb JH, Sly WS, Tropak M, Guise KS, Gravel RA: Cloning, sequencing, and expression of cDNA for human beta-glucuronidase. *Proc Natl Acad Sci USA* 1987, 84:685-689
28. Dalla-Favera R, Gelmann EP, Martinotti S, Franchini G, Papas TS, Gallo RC, Wong-Staal F: Cloning and characterization of different human sequences related to the onc gene (v-myc) of avian myelocytomatosis virus (MC29). *Proc Natl Acad Sci USA* 1982, 79:6497-6501
29. Padhy LC, Shih C, Cowing D, Finkelstein R, Weinberg RA: Identification of a phosphoprotein specifically induced by the transforming DNA of rat neuroblastomas. *Cell* 1982, 28:865-871
30. Vandekerckhove J, Weber K: Mammalian cytoplasmic actins are the products of at least two genes and differ in primary structure in at least 25 identified positions from skeletal muscle actins. *Proc Natl Acad Sci USA* 1978, 75:1106-1110
31. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF: Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 1983, 219:983-985
32. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushima H, Sato M: Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. *Oncogene* 1990, 5:519-524
33. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohnen P: Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun* 1992, 187:1579-1586
34. Bernstein PL, Herrick DJ, Prokipcak RD, Ross J: Control of c-myc mRNA half-life in vitro by a protein capable of binding to a coding region stability determinant. *Genes Dev* 1992, 6:642-654
35. Dani C, Blanchard JM, Piechaczyk M, El Sabouty S, Marty L, Jeanteur P: Extreme instability of myc mRNA in normal and transformed human cells. *Proc Natl Acad Sci USA* 1984, 81:7046-7050
36. Marzluff WF, Pandey NB: Multiple regulatory steps control histone mRNA concentrations. *Trends Biochem Sci* 1988, 13:49-52
37. Cleveland DW, Yen TJ: Multiple determinants of eukaryotic mRNA stability. *New Biol* 1989, 1:121-126
38. Deindl E, Schaper W: Gene expression after short periods of coronary occlusion. *Mol Cell Biochem* 1998, 186:43-51
39. Kress S, Stein A, Maurer P, Weber B, Reichert J, Buchmann A, Huppert P, Schwarz M: Expression of hypoxia-inducible genes in tumor cells. *J Cancer Res Clin Oncol* 1998, 124:315-320
40. Maxwell PH, Dachs GU, Gleadow JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 1997, 94:8104-8109
41. Cullen KE, Sarge KD: Characterization of hypothermia-induced cellular stress response in mouse tissues. *J Biol Chem* 1997, 272:1742-1746
42. Jeong JK, Huang Q, Lau SS, Monks TJ: The response of renal tubular epithelial cells to physiologically and chemically induced growth arrest. *J Biol Chem* 1997, 272:7511-7518
43. Singh JK, Chromy BA, Boyers MJ, Dawson G, Banerjee P: Induction of the serotonin1A receptor in neuronal cells during prolonged stress and degeneration. *J Neurochem* 1996, 66:2361-2372
44. Graven KK, McDonald RJ, Farber HW: Hypoxic regulation of endothelial glyceraldehyde-3-phosphate dehydrogenase. *Am J Physiol* 1998, 274:C347-C355
45. Graven KK, Troxler RF, Kornfeld H, Panchenko MV, Farber HW: Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J Biol Chem* 1994, 269:24446-24453