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The authors acknowledge the DNAX Research Institute of Molecular and Cellular Biology for providing the plasmid pcDL-SRa296. *Address correspondence to Dr. Zhong Liu, Berlex Biosciences, 15049 San Pablo Avenue, P.O. Box 4099, Richmond, CA 94804-0099, USA. Internet: zhong_liu @berlex.com*

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Zhong Liu, Linda M. Cashion and Haifeng Pu
Berlex Biosciences
Richmond, CA, USA

RNA Quantitative Analysis from Fixed and Paraffin-Embedded Tissues: Membrane Hybridization and Capillary Electrophoresis

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ABSTRACT

Fixed and paraffin-embedded tissues from pathology department archives are available for RNA expression analysis. We describe a general method for quantitation of specific RNA sequence extracted from single 6–8-mm human histological tissue sections cut from paraffin blocks. For each specific mRNA, the range of linear relationship between the log of the initial total RNA concentration and the log of the specific product after reverse transcription (RT)-PCR must be established. We usually perform RT with avian myeloblastosis virus (AMV)-RT, using specific antisense primers and a variable number of cycles of PCR amplification. The number of cycles must be adjusted within the range in which a linear relationship exists between the log of the amount of amplification product and the number of cycles. The quantity of specific product is standardized relative to b-actin mRNA to normalize for the degree of RNA degradation, which can be quite different among samples. The amplification products were quantified by dot blot and ^{32}P-labeled hybridization probe or by capillary electrophoresis with a laser-induced fluorescence detector. The intratest variation range was for the dot blot mean ±10% standard deviation (SD) and for the capillary electrophoresis mean ±3% SD.

INTRODUCTION

Recent studies have shown the possibility of analyzing RNA extracted from fixed and paraffin-embedded tissues (5,14,16). A specific RNA analysis in embedded tissues facilitates the utilization of human pathological tissues. In fact, all bi-optical or surgical tissues are routinely fixed and paraffin-embedded and conserved for many years.

Some of the methods proposed (14) for the RNA extraction utilize single 6–8-μm histological sections from paraffin blocks. The RNA is first transcribed into cDNA and subsequently amplified with the polymerase chain reaction (PCR) technology. Methods to quantify RNA extracted from fresh tissues after PCR amplification have already been proposed (2,7,10). However, the quantitation of RNA from paraffin-embedded tissues presents specific problems that cannot be resolved with a quantitative competitive analysis because of the variable level of RNA degradation in the samples. We describe a relative quantitative analysis of specific RNA sequences from fixed and paraffin-embedded human tissues, comparing the membrane hybridization (MH) and capillary electrophoresis (CE) methods.

MATERIALS AND METHODS

Human tissues from surgical or autopsy origin were fixed with 10% buffered formalin and then paraffin-

embedded in the same way as routinely performed in hospital pathology departments. The samples used derived from normal liver, breast carcinoma, urinary bladder carcinoma and cervix uteri dysplasias coming from two different pathology departments.

The RNA was extracted from tissue sections 6–8-μm thick, as previously described (12,14). The total RNA was also extracted from HeLa cells.

For any gene, three oligonucleotides were synthetized: two were mRNA sense, and the other was antisense. For all the sequences studied, the first sense and the antisense oligonucleotide were in two successive exons of the gene; the second sense oligonucleotide spanned the junction over the two exons, between the sequences covered by the other two oligonucleotides. The segment of mRNA amplified is very short, between 74 and 97 nucleotides; this is because in our experience, the RNA in paraffin-embedded tissues is highly degraded, usually with fragments between 100 and 200 bases (14).

The specific gene expression studied and the corresponding oligonucleotide sequences are described in Table 1.

Table 1. Specific Gene Expressions and Corresponding Oligonucleotides

β-actin gene (8)
β-ACT1: 5′-AAGGCCAACCGCGAGAAGATGA-3′ sense
β-ACT2: 5′-CCCAGATCATGTTTGAGACCTTCAACACCC-3′ probe
β-ACT3: 5′-TGGATAGCAACGTACATGGCTG-3′ antisense
α-polymerase (9,17)
POL1: 5′-AGATTCGGGACCATGGCACCTGT-3′ sense
POL2: 5′-GCACGGCGACGACTCTCTGTCAGATTCAG-3′ probe
POL3: 5′-GGCTCGAGAAGATACAAAACTCC-3′ antisense
Epidermal growth factor receptor (EGFr) (4)
EGFr1: 5′-GGCTCTGGAGGAAAAGAAAG-3′ sense
EGFr2: 5′-TTTGCCAAGGCACGAGTAACA-3′ probe
EGFr3: 5′-TCAAAAGTGCCCAACTGCTG-3′ antisense
c-erbB2 (1)
c-erbB2-1: 5′-TGTCTGAATTCTCCCGCA-3′ sense
c-erbB2-2: 5′-AGCGCTTTGTGGTCATCCAGA-3′ probe
c-erbB2-3: 5′-AAGGTGCTGTCCAAGGGACTG-3′ antisense

After the extraction, the RNA was resuspended in 50 mM Tris-HCl, pH 8.3, 50 mM KCl and 8 mM $MgCl_2$ and quantitated by spectrophotometry (11). Reverse transcription (RT) was performed with 2 U of avian myeloblastosis virus (AMV) Reverse Transcriptase (Promega, Madison, WI, USA) in 10 μL final volume containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 0.25 mM dNTPs, 4 U RNAguard® (Pharmacia Biotech, Uppsala, Sweden), 5 mM dithiothreitol (DTT) and 15 pmol of the downstream antisense oligonucleotide primer. The reaction proceeded at 42°C for 1 h (12,14).

The amplification was performed by adding 40 μL of 10 mM Tris-HCl, pH 8.3, 55 mM KCl containing 15 pmol of the upstream primer, 1.2 U of AmpliTaq® DNA Polymerase (Perkin-Elmer, Monza, Italy). The solution was denatured for 3 min at 94°C, subjected to five cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C and followed by a variable number of cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C in a thermostatic apparatus with a heated block cover system (TwinBlock™ System; Ericomp, San Diego, CA, USA) (12,14).

A quantitation is possible only if there is a linear relationship between the log of the quantity of total RNA and the log of the amplified product. These conditions must be studied with different quantities of total RNA and with a different number of amplification cycles for every sequence (13).

The amplifications were tested by dot blot with the internal oligonucleotide labeled with ^{32}P using T4 Polynucleotide Kinase (Boehringer Mannheim, Mannheim, Germany). Hybridization was performed in 5× sodium chloride sodium phosphate EDTA (SSPE), 1× Denhardt's solution and 0.5% sodium dodecyl sulfate (SDS) at 55°C. Washings were performed with 2× SSPE, 0.1% SDS two times for 10 min at room temperature and one time each at 60°C for 15 min with 1× SSPE, 0.1% SDS and with 0.1× SSPE, 0.01%

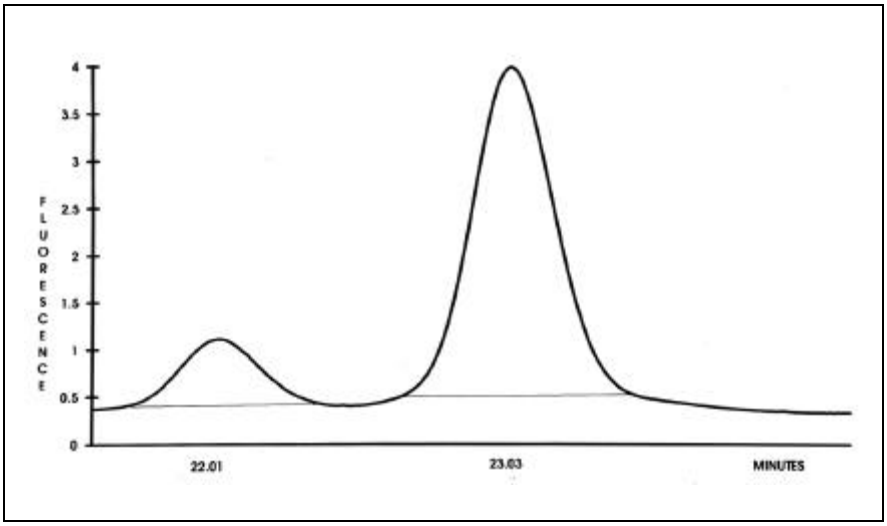

Figure 1. Chromatogram of capillary electrophoresis of β-actin (first peak at 22.01 min, 74-bp fragment) and c-erbB2 (second peak at 21.03 min, 92-bp fragment) of the same urinary bladder transitional carcinoma sample. The relative fluorescence quantities are measured as peak areas.

Table 2. EGFr mRNA Quantitation in Cervix Uteri Dysplasias

Samples	cpm β-actin[a]	Norm. Factor[b]	cpm EGFr[c]	EGFr Normalized[d]
1	1069	0.90	404	363.6
2	928	1.04	375	390
3	1796	0.54	548	295.92
4	1195	0.81	369	298.89
5	557	1.73	205	354.65
6	736	1.31	358	468.98
7	850	1.13	672	759.36
8	804	1.20	192	230.4
9	1044	0.92	203	186.76
10	668	1.44	91	131.04

[a]Counts per minute of the dot blot of the RT-PCR products of the β-actin mRNA sequence.
[b]The normalization factor is calculated by ratio between the β-actin mean value and the cpm for each sample.
[c]Counts per minute of the dot blot of the RT-PCR products of the EGFr mRNA sequence.
[d]Product of the normalization factor per the cpm of EGFr product.

SDS. To control the cleanliness of the membranes from nonspecific hybridization spots after autoradiography, the dots were cut from the membranes and counted in a β-scintillation counter (Beckman Instruments, Fullerton, CA, USA). The values were subtracted from the counts per minute (cpm) of a control with no specific DNA and standardized along with the values obtained for β-actin (see Table 2).

CE with laser-induced fluorescence detection was used as an alternative to MH using a P/ACE™ 5010 apparatus with laser-induced fluorescence (Beckman Instruments). Since the amplified fragments were of different lengths, samples were injected in the same capillary for 90 s of pressure. Separation was run at 15 kV for 30 min in a 47-cm

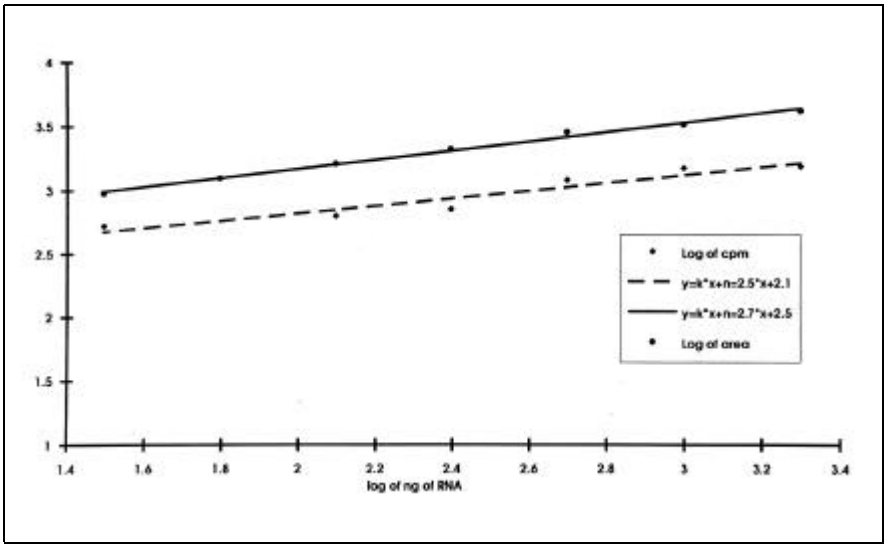

Figure 2. Regression of the log of the RT-PCR products of EGFr using the same sample (urinary bladder) with CE (upper full line) and MH (lower dotted line). The range of linearity conditions was in both cases between 31 and 2000 ng of total RNA.

length silica-gel capillary with a 100-µm diameter, filled with Tris-borate EDTA buffer containing 10% linear polyacrylamide as an anti-convective medium and 0.4 µg/mL of fluorescent EnhanCE intercalator (eCAP™ dsDNA 1000 Kit; Beckman Instruments). The chromatograms were analyzed using Gold™ Software (Beckman Instruments), which also allowed direct standardization of the data relative to β-actin values (6) that indicate the level of degradation of RNA in the tissue samples as a general index of RNA degradation.

Logarithms of the estimated quantity of amplified cDNA (cpm or peak area) and the logarithms of the concentrations of total RNA submitted to reverse transcription (RT)-PCR or the number of amplification cycles were compared with a linear regression, and the correlation coefficient was calculated.

In six colon adenocarcinoma cases, the tissue samples were half frozen and half formalin-fixed and paraffin-embedded. After RNA extraction, c-erbB2 mRNA was quantified in frozen samples by an RNase protection assay (RPA) using an RNA probe of 97 bases; the same sequence fragment was amplified for the paraffin-embedded tissues quantitation (3).

Table 3. Intra-Test Comparison Between MH and CE Results for EGFr in RNA from a Case of Urinary Bladder Transitional Carcinoma

Samples	Dot Blot (cpm)	Capillary Electrophoresis (Peak Area)
1	893	2677
2	663	2646
3	714	2814
4	823	2880
5	760	2685
6	678	2834
7	820	2752
8	840	2712
9	711	2696
10	812	2801
Mean	771.4	2749.7
Standard Deviation	±10%	±3%

RESULTS

The conditions for RT-PCR of RNA extracted from fixed and paraffin-embedded tissues must be adapted to the fact that the RNA is highly degraded. We used 25 cycles of amplifications when tests were performed with RNA from HeLa cells. However, with the embedded tissues, these conditions must be changed. A constant quantity of total RNA extracted from formalin-fixed and paraffin-embedded human liver (40 ng/100 µL amplification solution) was reverse-transcribed and PCR-amplified for a specific sequence of β-actin for a different number of cycles. The linearity between the number of cycles and the log of the amounts of the final product (cpm) is conserved only between 30 and 60 cycles of amplification. For this reason, we used a higher number of cycles of amplification for RNA extracted from embedded tissues than for RNA from cells or fresh tissues. The optimum number of cycles must be checked for every new sequence studied (13).

The linearity conditions are also respected for a defined number of cycles; only in a small range of the initial quantity of RNA. RNA from paraffin-embedded liver was amplified after RT for β-actin for 40 cycles with increasing quantities of RNA. The logarithms of cpm behaved linearly with an input between 0.2 and 60 ng of total RNA per 100 µL of amplification solution. This range is different for every specific mRNA, depending on the level of expression and degradation (13). For α-polymerase in breast cancer tissues, we needed 100 times more total RNA (the linearity range is between 250 and 4000 ng) to obtain the same amplification as for β-actin.

We used two methods to quantify the product of the amplification: the dot blot with a specific ^{32}P-labeled probe (MH) or the CE with a laser-induced fluorescence detector. The results of the MH in a group of cervix uteri dysplasia cases studied for the expression of EGFr mRNA are reported in Table 2. In this table, the cpm obtained for the specific sequence were corrected on the

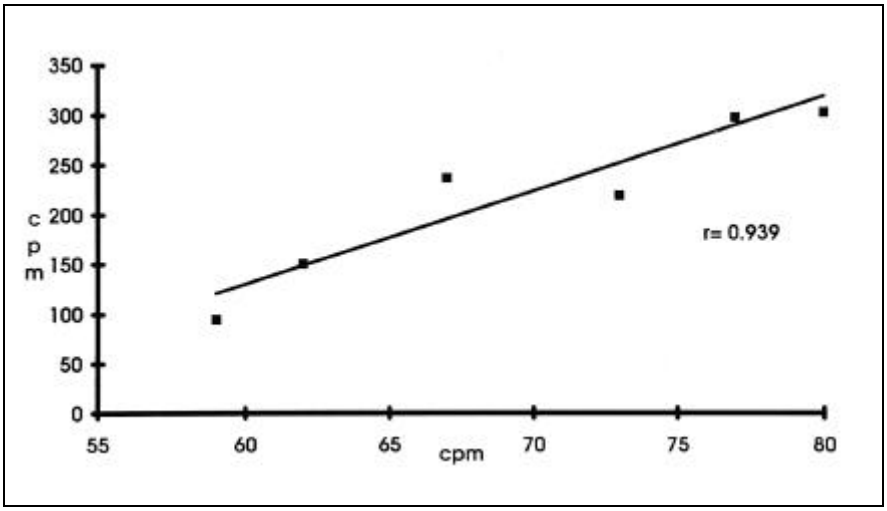

Figure 3. Comparison of c-erbB2 mRNA quantitation in fresh tissues by RPA (y) and in paraffin-embedded tissues (x).

basis of the degradation level of the RNA in the different samples, and the standardization was performed relative to the mean value of β-actin mRNA.

An example of the results obtained with the CE is reported in Figure 1; the first peak of the chromatogram corresponds to β-actin, and the second one corresponds to c-erbB2 mRNA in a urinary bladder transitional carcinoma.

Figure 2 shows the two regression lines corresponding to the same group of amplifications for c-erbB2 mRNA in a urinary bladder transitional carcinoma measured with MH and with CE. A good correlation coefficient was obtained in both cases, with coefficient values of 0.96 and 0.99 for MH and CE, respectively.

To obtain the intra-test variability of each method, we performed a series of repeated analyses of EGFr using the same RNA sample (from a urinary bladder carcinoma). As shown in Table 3, for MH, we obtained a standard deviation (SD) of ±10% of the mean value; while for CE, the SD was ±3%.

Six cases of colon adenocarcinomas were compared for c-erbB2 mRNA quantitation in fresh and paraffin-embedded tissues. For the fresh tissue samples, the RPA was used. As shown in Figure 3, the correlation between paraffin and fresh tissues was good (r = 0.94).

DISCUSSION

We have shown (14) that endogenous RNA extracted from paraffin-embedded samples is not completely degraded and that fragments averaging 100–200 bases in length are still present even in cases in which fixation was done quite late (as in autopsy material) or in RNase-rich organs, such as the pancreas. The accessibility of paraffin-embedded material for RNA analysis opens the archives of the hospital pathology departments to analysis of RNA expression or RNA virus persistence and allows the study of a large number of cases, even of rare diseases. The method could also be useful for diagnostic purposes, with the advantage of using the usual methods of storage of bi-optical or surgical tissues. To maximize success in the amplification of degraded RNA, we have chosen to amplify short sequences with lengths, including the primers, of about 70–100 bases.

We analyzed two methods to quantitate the results of the RT-PCR analysis: dot blot membrane hybridization and capillary electrophoresis.

In the quantitation of the RNA from fixed and paraffin-embedded tissues, it is not possible to use direct competitive methods, as in the case of RNA extracted from cells and fresh tissues (2). This

is because of the fact that in paraffin-extracted RNAs, there are always different levels of RNA degradation, even among samples of the same type of tissue. To obtain a reliable quantitation, the level of degradation must be standardized among samples using an internal sequence like β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, assuming that the level of degradation is comparable for any mRNA (13). Conversely, for a constant number of PCR cycles, the linearity conditions must be obtained between the log of the initial quantity of RNA and the log of the product of PCR amplification. In the paraffin-embedded tissues, in comparison with the same type of fresh tissue RNA, it is necessary to increase the quantity of initial RNA per test and/or the number of amplification cycles (13).

Of the two methods used to quantitate the results, CE was more reliable with an intra-test variation of only ±3% SD, while in MH the variation was ±10% SD around the mean value. Nevertheless, the MH is reproducible and easily performed in any molecular biology laboratory. An intra-test variation of ±10% is more than acceptable, considering that the differences in mRNA expressions investigated are usually of higher magnitude. Conversely, CE analysis is more precise and is obtained in a shorter time without the use of isotopes.

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Giorgio Stanta and Serena Bonin

International Centre for Genetic Engineering and Biotechnology
[1]University of Trieste
Trieste, Italy