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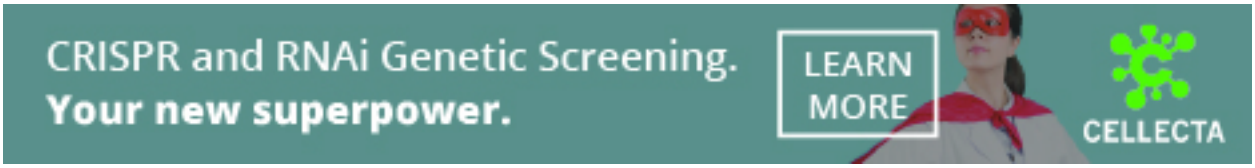

Rapid Quantitative PCR for Determination of Relative Gene Expressions in Tissue Specimens

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The quantitative measurement gene expression is of major importance in addressing many fundamental questions in biology and medicine. In recent years, extremely sensitive PCR-based methods have been developed for quantitation of specific RNA and DNA segments. Competitive PCR, the most widely used method, involves same-tube coamplification of the gene of interest and a synthetic, internal standard fragment.(1) However, competitive PCR is not very suitable for routine use because it requires gel electrophoretic resolution of two closely migrating DNA fragments after completion of the PCR. Furthermore, if normalization is required with an internal reference gene, a second competitive PCR must be performed for each sample. In our studies on the relationship between gene expressions of drug-targeted enzymes and the response of tumors to chemotherapy, we use an alternate PCR-based quantitation method that is more readily adaptable to the analysis of a large number of tumor biopsy specimens.(2,3) This method measures the relative amount (i.e., the fold difference) in mRNA contents of two or more samples with the expression of an invariantly expressed gene as an internal normalization factor for the amount of RNA isolated from the specimen. Only one reverse transcription reaction is done for each sample, and then serial dilutions of the cDNAs of the gene of interest and a reference gene (such as β-actin) representing the amount of RNA isolated are amplified separately. After PCR, the amplified DNA is transcribed to RNA with T7 DNA polymerase to further increase the sensitivity.(2,3) The data are expressed as an empirical ratio between the PCR products generated from the gene of interest and the denominator gene. Because ratios should be constant regardless of the absolute yield of RNA isolated from the specimen, this approach is well suited to the analysis of small clinical tissue specimens from which reproducible isolation of good quality RNA may be problematic and an accurate determination of total RNA for normalizing the data may not be possible. The only radiolabeled species in the tube at the end of the procedure are the transcribed RNA segment to be quantitated and unincorporated nucleotides. We have taken advantage of the latter feature to streamline PCR quantitation further by using centrifugally eluted minicolumns of Sephadex instead of time-consuming electrophoresis to isolate the final RNA product.

MATERIALS AND METHODS

The sizes of the minicolumns and the amounts of Sephadex packed into the columns were designed so that when 25 μl of solution is loaded onto the top of the columns, large molecular-weight material that would be contained in the void volume would pass entirely through the column after centrifugation but small molecules would not. To prepare the centrifugal minicolumns, microtest tubes (Sarstedt 72700) were filled to the top with Sephadex G-50 Fine (Sigma Chemical Co., St. Louis, MO) that had been swelled overnight in STE buffer (0.1 M NaCl, 10 mM Tris-HCl at pH 8.0, 1 mM EDTA). A used pipette tip rack makes a convenient holder for the minicolumns as they are being filled. This allows the easy preparation of many columns at a time. To transfer the Sephadex into the columns, pipette tips shortened to just reach the bottom of the microtubes were used and the tubes were filled starting from the bottom. After the tubes were filled with the Sephadex, the bottoms were punctured with a G23 needle (Becton-Dickinson, Rutherford, NJ). When most of the buffer had drained out, the tubes were centrifuged for 5 min at 1000*g* (Beckman J6B). (The column matrix should look semidry at this point but there should not be any cracks or air spaces.) Each column was then fitted with an adapter consisting of a 0.65- and a 1.7-ml microcentrifuge tube from which the bottoms had been cut out with a hot scalpel (Slick Seal microtubes, National Scientific, San Rafael, CA) and placed into a liquid scintillation vial (Fisher 033725). The transcription reaction (5 μl) was mixed with 20 μl of STE buffer. The 25-μl sample was then applied to the center of the gel bed's surface. (For easier observation of the column loading, 0.4% bromphenol blue may be added.) The tubes were then centrifuged for 10 min at 1000*g* (Beckman JB6) and the column and adapter removed from the scintillation vial. (The labeled RNA from the transcription reaction in the same volume as originally applied (25 μl) should now be in the scintillation vial.) After addition of 275 μl of double-distilled water and 3 ml of liquid scintillation cocktail (RIA-Solve II, RPI,

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Mount Prospect, IL) the sample was counted in a liquid scintillation counter. Commercially available Chroma Spin columns (Clontech Laboratories, Palo Alto, CA) can also be used. Product Protocol PR31063 for the Chroma Spin columns provides a useful general guide for the use of minicolumns.

RESULTS AND DISCUSSION

To compare the quality of the data obtained with polyacrylamide gel electro-

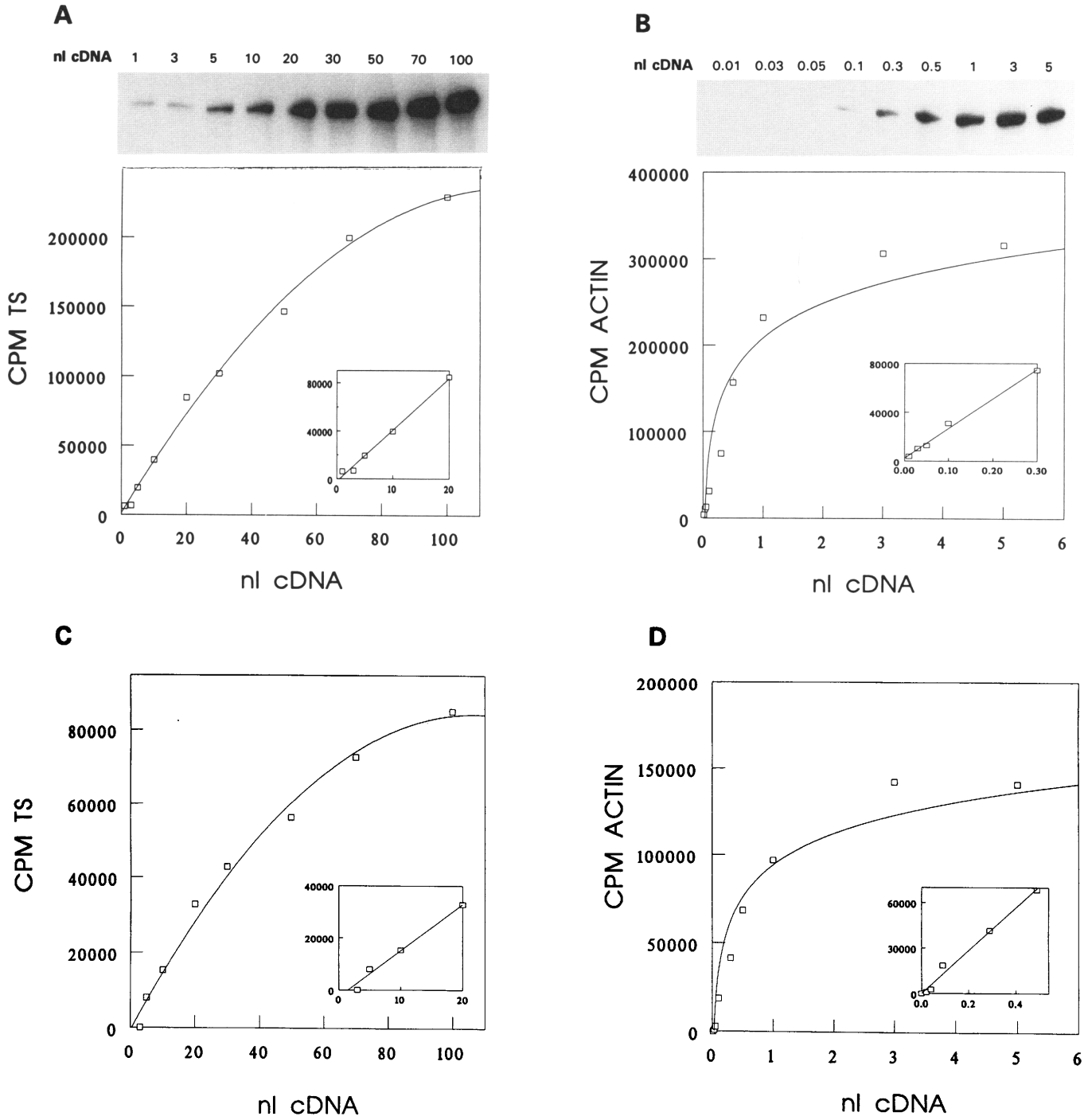

FIGURE 1 Relationship between starting amount of TS and β-actin cDNA and the amount of PCR product formed. Aliquots of a cDNA dilution from HT-29 cells were PCR amplified using the primer set BA 67 and BA 68 for β-actin and the primer set TS 60 and TS 61 for TS.[2] The PCR-amplified DNA was transcribed with T7 polymerase, and the resulting RNA was electrophoresed by PAGE (*A,B*) or loaded onto a Sephadex column (*C,D*). After PAGE of the RNA, bands were visualized by autoradiography, excised, and quantitated by liquid scintillation counting (*A,B*). After loading the RNA onto the Sephadex column, RNA was spun into a scintillation vial and counted (*C,D*). (*Insets*) The expanded linear region of the curve.

phoresis (PAGE) and the minicolumns, we performed PCR quantitation of thymidylate synthase (TS) mRNA from HT-29 cells and 10 tumor tissue samples[2,3] using both methods. Total RNA was isolated[4] and reverse-transcribed. Serial dilutions of the corresponding cDNA solutions were PCR amplified with the appropriate primers for either β-actin or TS, described in Ref. 2. The 5′ primers for each gene had a T7 polymerase promoter sequence attached for subsequent transcription of the PCR product RNA.[5] The transcription reactions were divided into two portions, and the RNA fragments were isolated both by PAGE, as described previously,[2,3] and by the minicolumns as described above. The curves obtained using PAGE (Fig. 1A,B) represent typical, not optimal, ones and demonstrate the excellent tube-to-tube reproducibility of the PCR reaction ($r=0.98$ for the linear region). The data obtained from the minicolumns (Fig. 1C,D) were just as good as the PAGE method ($r=0.98$). The quantitation factor for each sample is the ratio between the slopes of the linear regions of the target gene (TS) and the reference gene (β-actin) (shown in the insets). From Figure 1, the value of this ratio obtained by the PAGE method was 11.7 ± 1.0 compared with 12.1 ± 1.9 by the minicolumn method. Dividing this ratio with that from another sample will give the relative TS expression in the two samples provided that the expression of the reference gene is constant. More data points are shown in Figure 1 than are normally taken for routine analysis to demonstrate the extent of the linear amplification region. Table 1 compares the TS/β-actin ratios in 10 tumor tissue specimens obtained by using PAGE and minicolumns, and in all cases the values as well as the standard errors of the data obtained by the two methods were in good agreement.

The main pitfall with the use of minicolumns is overloading them with the transcription mixture. When using PAGE, one can load the entire 25 μl of the transcription mixture onto the gels if necessary. However, when volumes of 10–25 μl were loaded onto the columns, the amount of radioactivity eluting through the column in the void volume was not proportional to the volume loaded, and large nonzero intercepts on the *y*-axis were obtained when, as in Figure 1, the amount of radioactivity in the void volume was plotted against the volume of cDNA used in the PCR reaction. A high background elution indicates that the separation between the large and the small molecules was not complete when too much of the transcription reaction was loaded. The Clontech product protocol referred to above suggests that the viscosity of the solution placed on the column can have more of an effect on elution than the amount of nucleotide material placed on the column. To find the optimal volume of the transcription mixture to load onto the columns, the amount of the transcription mixture loaded was decreased until the background elution (i.e., the nonzero intercept of the aforementioned plot) decreased to an acceptably low value. We found that loading 5 μl of the transcription mixture diluted with 20 μl of 0.1 M STE buffer onto the Sephadex columns gave a background of 2–5000 cpm (compared with ~500 cpm with PAGE) and reducing the volume further did not appreciably lower the amount of background elution calculated as a percentage of the total radioactivity in the void volume. The points on the curves in Figure 1, C and D, represent the radioactivity eluted through the columns corrected by subtracting the backgrounds.

TABLE 1 Relative Thymidylate Synthase Gene Expression in Gastrointestinal Tumors and HT-29 Cells as Measured by Quantitative PCR Using either PAGE or Sephadex to Isolate the RNA Fragment from Transcription of the PCR Products

	TS/β-actin ($\times 10^{-3}$)[a]	
Tumor sample	PAGE	Sephadex
L-164	1.6 ± 0.1	2.5 ± 0.2
L-173	8.2 ± 0.6	7.0 ± 1.5
L-182	2.9 ± 0.3	3.6 ± 0.5
L-192	4.5 ± 0.2	2.6 ± 1.3
L-196	8.0 ± 0.8	6.1 ± 1.3
L-201	16.2 ± 4.3	11.8 ± 1.3
L-202	8.8 ± 2.5	8.8 ± 2.4
L-210	4.5 ± 0.1	2.9 ± 0.3
L-217	7.2 ± 1.0	6.5 ± 0.4
L-219	3.5 ± 0.6	4.3 ± 0.4
HT-29	11.7 ± 1.0	12.1 ± 1.9

[a]Each value represents the average ± S.D. of at least three measurements.

The major requirement for successful use of the minicolumn procedure is that the RNA product of the transcription of the PCR reaction be reasonably homogeneous without appreciable amounts of nonspecific amplification products or spurious products such as primer–dimers. The primers that we developed for TS amplification gave a PCR product that appeared to be >90% homogeneous; thus, we chose TS as the test system for demonstrating the use of Sephadex minicolumns in PCR quantitation. In our experience, relatively clean PCR products can be obtained from most target DNA segments either by manipulating the PCR conditions (e.g., raising the annealing temperature by a few degrees) or by changing one of the primers. In addition, the T7 transcription step often eliminates some of the spurious nonspecific bands. If one does not wish to perform the T7 transcription step, one can adapt the minicolumn procedure for separating the amplified DNA from the PCR primers.

By using the minicolumn purification of transcribed PCR products rather than PAGE, ~4 hr of time and many manual manipulations are saved, thereby often permitting PCR quantitations to be performed in one working day. This may be an important consideration when it is necessary to quantitate the same gene expression in a large number of specimens on a routine basis. The background radioactivity with the minicolumns is higher than with PAGE, but our experience has shown that if the tissue sample is in the range of 10 mg, the radioactivity in the RNA band is usually ⩾10-fold greater than the background even when measuring nonabundant mRNAs such as TS. However, PAGE must still be used when the target concentration is extremely low or the sample size is very small.

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