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Direct quantification of picomolar concentrations of mRNAs by mathematical analysis of a reverse transcription/ exponential polymerase chain reaction assay

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Recently, we have shown that the number of target molecules (N_0) of the polymerase chain reaction (PCR) can be accurately determined by measuring the molar concentration of a product, which accumulates in consecutive cycles (n), and analyzing the equation describing product accumulation, $\log N_n = \log \text{eff} \times n + \log N_0$ by linear regression (1). Here, we extend this method to the measurement of the actual copy number of mRNAs by performing quantitative reverse transcription (RT) prior to amplification. Due to its high sensitivity, this method might become a useful tool to study gene expression, even in samples in the milligramm range, RNA transport between compartments or RNA processing.

RNA was extracted from frozen ventricles of female Sprague–Dawley rats (180 g) by the acid guanidinium isothyocyanate method (2). One μg of RNA was reverse transcribed in 50 mM Tris/HCl (pH 8.3 at 42°C), 40 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mg/ml BSA, in the presence of all four dNTPs (1 mM each), and two primers (20mers; 2.5 μM each), specific for and complementary to two isoforms of myosin heavy chain (MHC). The mixture (10 μl total volume) was heated to 70°C for 3 minutes, chilled on ice, AMV reverse transcriptase (20 units; Promega, Madison, WI) and RNAsin (20 units; Promega) were added and incubated for one hour at 42°C. Five microliters of the reaction mixture were used for amplification. Complementary primers and two, isoform specific upstream primers were added (final concentration 2.5 μM each) and amplification was performed in a reaction volume of 50 μl in the presence of alpha-^{32}P-dCTP (100 μCi) using Taq polymerase (2.5 units; AGS, Heidelberg) and the incubation buffer provided by the manufacturer. Denaturation temperature was 95°C, annealing was at 55°C and synthesis was at 72°C, each step lasting one minute. Aliquots of 1 μl were taken from the reaction after consecutive cycles and run on 15% polyacrylamide gels, which were stained with ethidium bromide. In order to facilitate localization of the two products, especially in early cycles, unlabelled, purified products generated in previous reactions were added to the gel loading buffer. The two product bands (77 bp for alpha, 99 bp for beta MHC) were isolated from the gel, slices were trimmed, dried at 80°C overnight in liquid scintillation vials and the incorporated radioactivity was determined by liquid scintillation counting. Gel slices of the same size migrating closer to the anode were used to determine background radioactivity.

For routine measurements, less radioactivity and, if more convenient, agarose gels can be used. Due to the lower specific activity of precursor and the higher background due to the presence of unincorporated label, incorporation can then be measured faithfully only at later cycles, without affecting the results significantly.

The concentration of product accumulating in consecutive cycles, N_n (moles/μl) can be calculated from the incorporated radioactivity (cpm/μl), the specific radioactivity of the precursor dCTP (cpm/mol) in the reaction mixture and the number of dCTPs which can be incorporated into the newly synthesized stretch of the product, y, according to

$$N_n \text{ (moles/}\mu\text{l)} = \frac{\text{cpm/}\mu\text{l}}{\text{cpm/mol} \times y} \qquad \text{(eq. 1)}$$

The initial concentration of a double stranded DNA template at cycle zero, N_0 (moles/μl) and the efficiency of amplification,

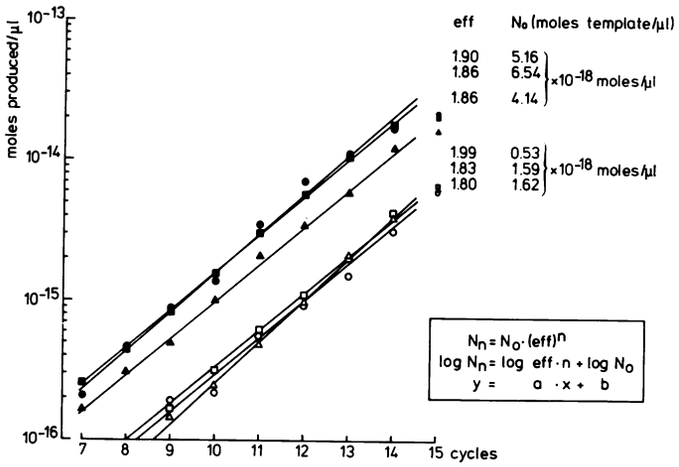

Figure 1. Accumulation of two specific products derived from alpha (filled symbols) and beta myosin heavy chain mRNA (open symbols) in the polymerase chain reaction. The figure shows results using RNA from three rat ventricles for reverse transcription. The lines fitted to the data by linear regression analysis are also shown. The inset illustrates the transformation of the equation describing product accumulation in the PCR which was used in order to facilitate mathematical treatment. Using this equation, the individual efficiencies of amplification, eff, and the number of target molecules at cycle zero, N_0, were calculated by linear regression analysis.

eff, can then be calculated by linear regression analysis of the transformed equation describing product accumulation in the PCR,

$$\log N_n = \log \text{eff} \times n + \log N_0 \qquad \text{(eq. 2, Figure 1).}$$

In the first cycle of the PCR, only one strand complementary to the single stranded cDNA generated by reverse transcription will be synthetized, since Taq polymerase has no reverse transcriptase activity under these conditions (3). The concentration of this double stranded product at cycle one, however, is equimolar to the concentration of single stranded cDNA at cycle zero, and also target mRNA, as will be discussed below. Thus, the concentration of double stranded target at cycle zero, N_0, which is obtained by linear regression analysis, has to be multiplied by eff in order to obtain the concentration of double stranded DNA at cycle one equivalent to the initial concentration of mRNA template. Using Avogadro's number, the initial amount of mRNA (moles per reaction) can be converted to molecules per reaction or molecules per μg of RNA used as substrate.

In the experiment shown, one μg of RNA from three ventricles was subjected to RT/PCR (Figure 1). Incorporation of radioactivity into the two products derived from alpha and beta MHC mRNA was measurable at cycle 7 or 9, respectively, and increased exponentially up to cycle 14 under these conditions. Then, a plateau was reached. Lines were fitted to the data points by linear regression analysis; the coefficient of correlation was always > 0.99, and the level of significance was always $p >> 0.001$, showing that product accumulation is indeed exponential. The efficiency of amplification differed considerably between tubes, lending support to previous reports which came to the conclusion that it is imperative to use an internal standard when using protocols comparing accumulation of products derived from a target and a standard (4, 5). From our measurements, it can be calculated that rat ventricles contain $6.0 \pm 1.4 \times 10^8$ molecules of alpha MHC mRNA per μg substrate RNA and $1.4 \pm 0.7 \times 10^8$ molecules of beta MHC mRNA.

Since rat ventricle contains about 3.5 mg of total RNA per g wet weight (6), and about 8×10^7 myocytes per g wet weight (7), it can be calculated that individual rat myocytes contain approximately 26,000 and 6,000 molecules of alpha and beta MHC mRNA, respectively. The alpha/beta ratio agrees well with data reported previously showing that in ventricle from rats of this age, beta MHC mRNA amounts to approximately 20% of total MHC mRNA, as measured by comparison of the accumulation of specific PCR products (8) or S1 nuclease protection analysis (9). Also, it can be calculated that total ventricular RNA contains approximately 2.5 ng/μg of the 6 kb mRNA coding for MHC. Since about 8% of muscle total RNA is mRNA based on weight (6), mRNA coding for MHC amounts to 3% of total mRNA, which is probably a reasonable number for a high abundance protein like MHC in muscle. These results also make significant systematic errors inherent in our assumption on the exponential growth of product accumulation over the total range of PCR cycles very unlikely, since this would lead to erroneously wrong numbers when calculating N_0 by extrapolation.

However, the method presented here measures true mRNA molecule numbers only if all mRNA templates are reverse transcribed into cDNA. Amplification for 12 cycles of samples taken from the reverse transcription reaction at different time points yielded the same incorporation of radioactivity from 15 minutes up to one hour (data not shown). In addition, decreasing the concentration of substrate RNA from 1 μg down to 30 ng by two-fold dilution steps yielded the same incorporation of radioactivity after amplification, however one cycle later for each dilution step (data not shown). Thus, cDNA synthesis is completed under these conditions and does not depend on the primer-to-template ratio, as would have been expected if the efficiency of reverse transcription were less than 100%. Moreover, Berger *et al.* (10) have shown that 50% of input mRNA was reverse transcribed into full-length cDNA under comparable conditions, but using only a 2–3-fold excess of primer over template. Since primers were present in our standard assay in at least 50,000-fold excess, and the cDNAs which are generated are less than 100 bp long, we conclude that all template mRNAs were reverse transcribed and that the method presented here is indeed a quantitative one.

The advantage over methods published previously (4, 5, 11) is that it bypasses the laborious *in vitro* synthesis of an RNA standard, which 1) has to be calibrated exactly; 2) should be full length in order to form a secondary structure comparable to the target sequence, and 3) should be slightly different from the target in order to be used as an internal standard.

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