

NORTHERN ILLINOIS UNIVERSITY

**A Novel Method for the Detection and the
Quantification of RT-PCR Products**

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of the Baccalaureate Degree with University Honors**

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Abstract:

Until recently, the measurement of mRNA levels was very time consuming and required specific parameters. With the advent of RT-PCR, mRNA fragments could be amplified and easily detected and measured. The amount of mRNA loaded into the reaction is proportional to the amount of product DNA. Here, we propose a new method for mRNA detection and quantification. The product of the amplification reaction is resolved electrophoretically on a polyacrylamide gel and then transferred to a positively charged nylon membrane. This membrane is then stained for DNA with a non-mutagenic dye. The membrane is converted to a visual image via a scanner. This image is then analyzed with the NIH Image program. The results show that this method is indeed of adequate sensitivity for comparisons of mRNA levels. Using this protocol, a linear relationship was shown between the amount of starting RNA and the amount of product DNA. This method is safer, less time consuming and at least as efficient as previously described methods.

Introduction

The importance of a method for the quantitation of RNA is unquestioned. One application of such a method is transcription levels for specific genes. Until recently the available methods for RNA quantitation were difficult, expensive and time consuming. Also, many required large amounts of RNA to function. This rules out many comparison studies of transcription levels. The advent of the polymerase chain reaction (PCR) ushered in a whole new facet of molecular biology techniques. PCR amplification following a reverse transcription (RT) step allows analysis of almost any mRNA molecule.

Due to the extremely sensitive nature of the PCR, data gathered using this method must be normalized or retrieved in an extremely controlled manner. There are two major methods that use standards to normalize the data. One calls for the construction of a DNA template that competes for the same set of primers as the sequence to be analyzed. The size of the competitor product is different than that of the experimental product. These segments can then be resolved by gel electrophoresis. This is competitive RT-PCR. The second method described by Serazin-Leroy et.al.(1998), uses the amplification of an exogenous piece of DNA in the same reaction tube as a standard. Although both these methods are specific, they are quite laborious and not necessary in comparison studies.

A separate method, described by Akiyama and colleagues (1997) and used in this study, requires no standard and is very useful in comparing relative levels of mRNA. This method requires optimization of materials and procedures to determine the time at which the PCR is in the exponential phase. During this time, the amount of signal is directly proportional to the amount of template loaded. This allows direct measurements of the amount of cDNA created in a RT reaction. Akiyama and colleagues (1997) state the two conditions to be met for accurate quantitation using this procedure: 1) tube to tube variation must be kept at an absolute minimum and, 2) all data must be retrieved

before the reaction reaches the plateau phase.

There are many methods of detection for the products of the PCR. Here, a novel method is proposed for the detection and quantitation of RT-PCR products. Resolution of the RT-PCR products is done using polyacrylamide gel electrophoresis. This product is then transferred onto a membrane in a southern blot, which is stained with methylene blue. Quantitation of the signal is done by image acquisition via a scanner and analysis using the NIH Image program.

The method described here was developed for the specific measurement of the β -actin gene. This protein plays an important role in cell structure. Chemical signaling of gene expression is quickly being linked to mechanical loading and unloading. Many studies including those done by Isei (1991) and Caiozzo and colleagues (1996) are showing the relationship to be valid. The unloading of tissue during spaceflight seems to signal cell atrophy. It is possible that this is done through downregulation of β -actin gene expression. This method will allow experimentation of this theory.

Materials and Methods

RNA PREPERATION

The RNA pellets obtained were extracted from cell culture using the TRI-Reagent protocol. The pellets were resuspended in water. Two RNA solutions were combined and phenol/chloroform extracted. The RNA was then precipitated with ethanol and resuspended in a total volume of 30 ul of DEPC treated water. Quantitation was done using spectrophotometry and a wavelength ratio of 260/230.

PRIMERS

β -actin primers were derived from Raff et.al.. The sequences are, forward: 5'-TAC AAC CTC CTT GCA GCT CC-3' and reverse: 5'-GGA TCT TCA TGA GGT AGT CTG TC-3'. These oligos code for a 630 base pair sequence that covers intron and exon segments.

cDNA SYNTHESIS

Reverse Transcription was carried out in a 42°C heat block for 30 minutes. The final concentration of the reaction mix was 1X RT buffer (Promega RT system), 5 mM MgCl₂, 1 mM equimolar dNTP mix, 1U/ul RNasin, 25 ng/ul reverse primer and 0.30 U/ul reverse transcriptase. The reaction was stopped by placing the tubes in the 4°C cooler.

PCR

The protocol for PCR was partially determined by the paper Raff et.al (1997). Optimization was done for cycle number. The final concentration of each reaction mix was 1X PCR buffer (Fisher Taq kit), 2 mM MgCl₂, 100 nM equimolar dNTP mix, 3.0 ng/ul forward primer, 3.5 ng/ul reverse primer and 0.03 U/ul Taq polymerase. The reactions were done in a Perkin Elmer 480 thermal cycler . The times and temperatures were as follows, an initial denaturation was performed at 94°C for 3 minutes, after which the reaction underwent 27 cycles of 94°C for 30s, 60°C for 1 minute and 72°C for 30s. These reactions were then held at 4°C before detection.

QUANTITATION OF PCR PRODUCTS

The products of the RT-PCR were resolved electrophoretically in an 8% polyacrylamide gel at 120 volts for 30 minutes. Optimization was done for the amount of product loaded. The DNA was then trans-blotted onto a positively charged nylon membrane (Boehinger-Manniem) at 80 volts for 30 minutes and UV crosslinked to the membrane in a UV Stratalinker 2400 (Stratagene). The membrane was stained with 0.04% methylene blue and destained with RO water. The image was acquired via a scanner using the Adobe Photoshop program. This created an image file, which was saved onto a disc until analysis was done. Quantitation was performed using the NIH Image program. The data was calibrated using the Bio-Rad molecular mass standard. The optical densities reported here are the pixel densities that were acquired by this program. A detailed manual including this procedure can be retrieved from the NIH Image website: www.rsb.info.nih.gov/nih-image/manual/contents.html.

Results

OPTIMIZATION OF RT-PCR

The reaction parameters were derived mostly from the two publications (Raff et al.(1997) and the Promega RT system website: www.promega.com/tbs/tb099/tb099.html). The only change to this protocol was limiting of the amount of RT enzyme to 6 units per reaction. The amounts of RNA used in the RT-PCR to show linearity were 0.6 ug, 0.9 ug, 1.2 ug, 1.5 ug and 1.8 ug.

ANALYSIS OF RT-PCR PRODUCTS

Clear resolution was obtained for the gel electrophoresis (Fig 1). In lanes 1-4, There is a high molecular weight band and a low molecular weight band. The sum of these two was used in the subsequent calculations. NIH Image was used to determine the optical density of both the standard (Bio-rad molecular mass standard) (Fig 2), and the amplification products (Fig 4). The measurements for the standard were used to construct a standard curve for amount of DNA (Fig 3). This curve was used to calculate the amount of DNA in each band. The amounts of product DNA were graphed versus the corresponding amounts of starting RNA (Fig 5). Using the stated parameters, the reaction was in the exponential phase between 0 and 1.5 ug of RNA.

Discussion

Gene expression may play an important role in cell atrophy during exposure to microgravity. For this and many other applications, quantitative analysis of any mRNA fragment is an important tool. Here the newest method of mRNA analysis is used, RT-PCR, along with a new protocol for detection and quantification. This method uses no radioisotopes or mutagens and has been proven to be of sufficient sensitivity for comparison of relative amounts of mRNA.

In this experiment five separate RT-PCR's were run using five different amounts of total RNA. The products of these reactions were put through the described method of

detection and quantification. The relationship between amount of RNA loaded into the RT reaction and the amount of signal DNA was linear up to 1.5 ug of RNA at 27 cycles¹. This coincides with similar RT-PCR studies using β -actin (Rogers and Mckenzie, Stratagene 1998) and other genes (Akiyama et.al. 1996).

The final outcome of this procedure is specific semi-quantitative analysis of PCR products. This method has three major advantages, 1) it uses no radioisotopes or mutagens making it much safer then previously described methods, 2) It can accommodate as many samples as can be run on two polyacrylamide gels and 3) It's very time efficient, the whole procedure can be done 3 hours after the completion of the amplification reaction. This particular reaction was developed for the amplification of β -actin RNA and is likely to prove efficient in such a study.

In summary, a novel approach to quantitation of RT-PCR products is described here. It involves no radioisotopes or mutagens and is fast and efficient. It is particularly useful in comparing relative amounts of β -actin mRNA.

¹ The products of the specific reaction described here appeared as two bands. One was a high molecular band (>1000bp) weight and one was a low molecular weight band (630 bp). This could be the result of no final annealing step. More experimentation is required to determine the origin of the high molecular weight band.

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Figure 1: 8% polyacrylamide gel. The signals represent from left to right: 0.6, 0.9, 1.2, 1.5 and 1.8 ug of RNA.

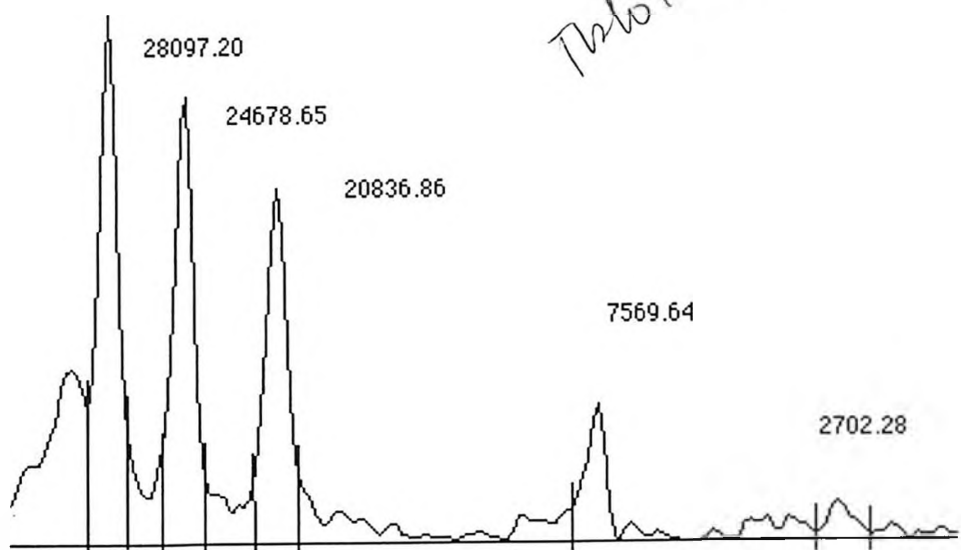


Figure 2: Quantitation of the mass ruler using NIH Image. The pixel densities can be seen above each peak. The corresponding amounts of DNA are from left to right, 100 ng, 70 ng, 50 ng, 20 ng and 10 ng.

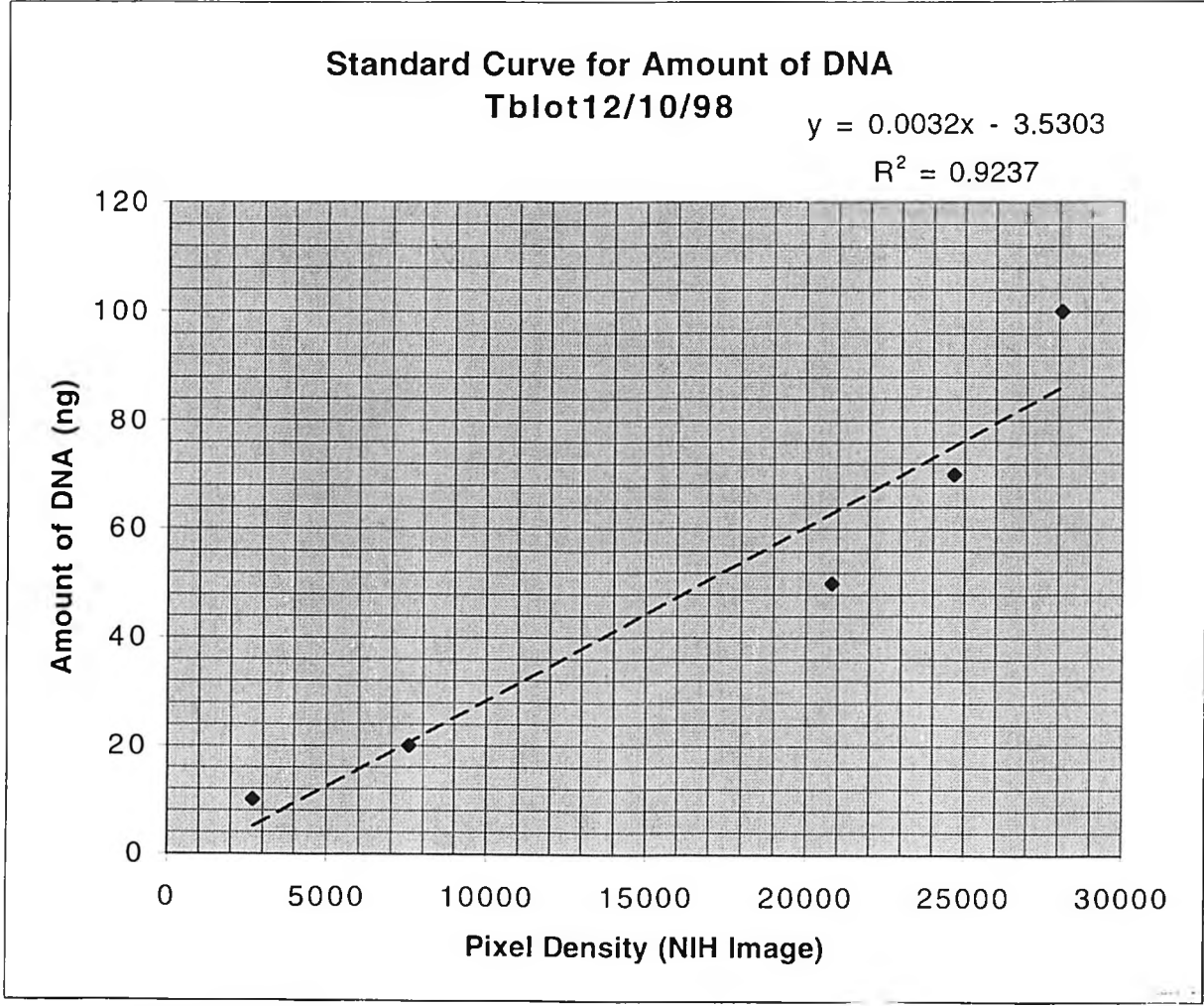


Figure 3: Standard curve based on the quantitation of the biorad molecular mass standard (Figure 2).

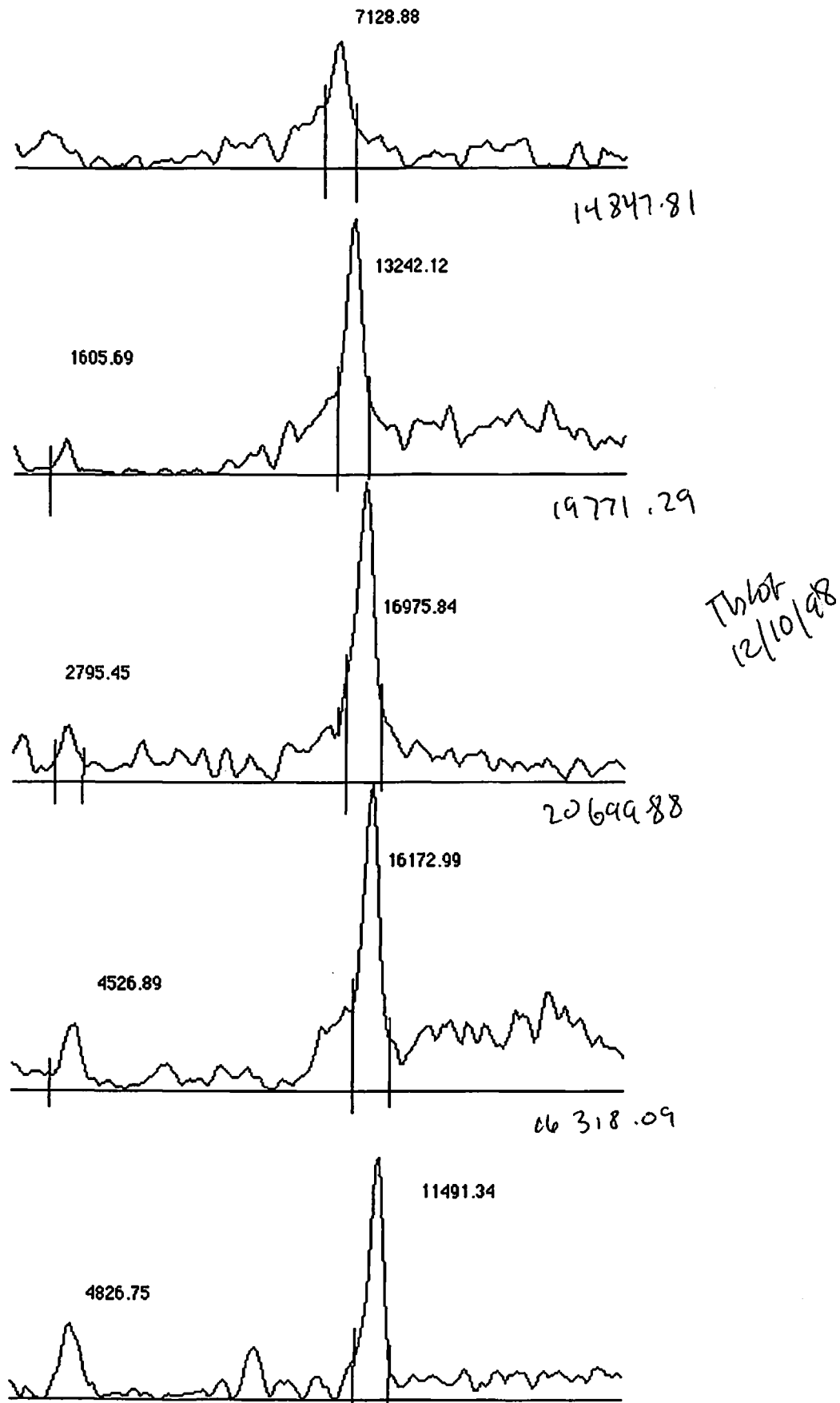


Figure 4: Quantitation of the DNA products of amplification. The pixel densities can be seen above each peak. The corresponding amounts of startin RNA are from top to bottom, 0.6, 0.9, 1.2, 1.5 and 1.8 ug of RNA.

Amount of DNA from 5 RT-PCR with Varying Amounts of Starting RNA

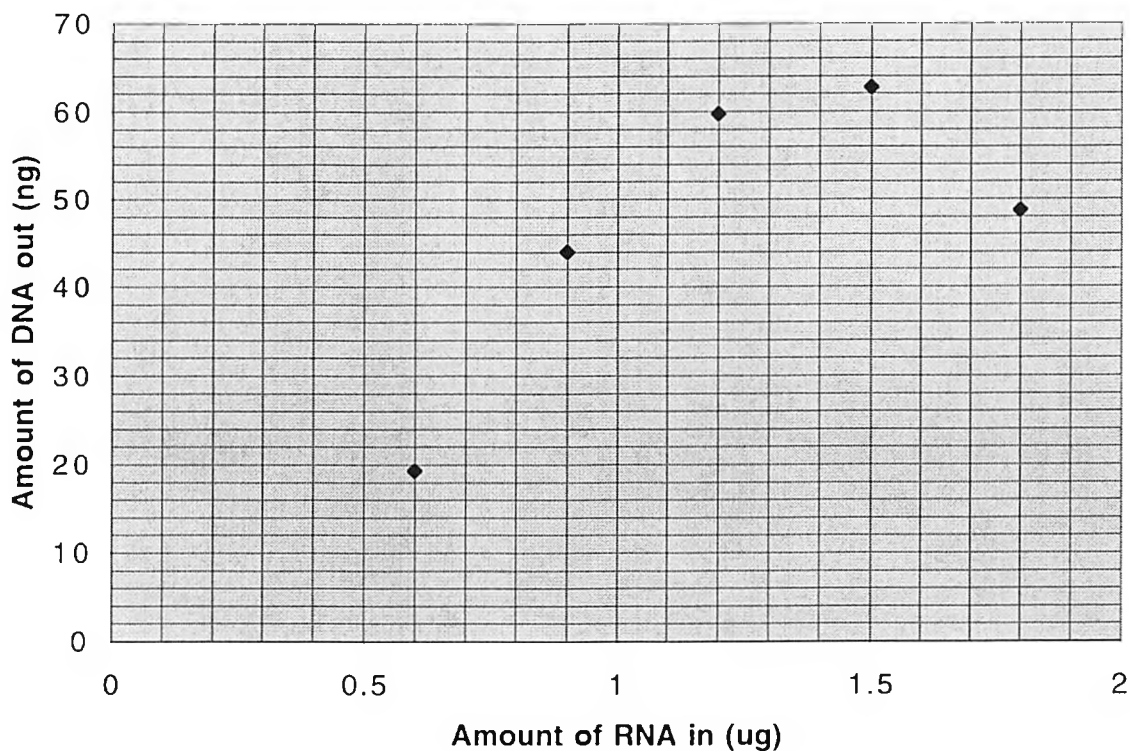


Figure 5: Linear relationship between amount of RNA loaded in the RT-PCR and amount of DNA produced. The linear relationship is present up to 1.5 ug of RNA, where the reaction is in the plateau phase.