

SCIENCE IMAGING SYSTEMS

Application Note No.20

QC-RT-PCR* for the Quantification of mRNA

FLA-2000 / FLA-3000

Foreword

A method for analyzing very small amounts of mRNA is indispensable for gene expression analysis. The quantitative RT-PCR method detects mRNA with high sensitivity by reverse-transcribing the mRNA to cDNA and amplifying the cDNA by PCR. Three protocols based on this methodology have been reported. In the one explained here, a varying amount of the cDNA reverse-transcribed from the mRNA is amplified by PCR together with a fixed amount of a competitor. SYBR® Green I was used for gel staining and detection was done with an FLA-2000. This methodology offers potential for greater sensitivity than conventionally used Southern blotting.

The main text of Application Note No.20 was written by Dr. Peter Ramm and Mr. Nezar Rghei, researchers at Imaging Research Inc.

*Quantitative competitive reverse transcriptase polymerase chain reaction (QC-RT-PCR)

Contents

1. Introduction
2. Materials and Methods
3. Results and Discussion
4. References

Summary

- Quantitative detection of RT-PCR fluorescence by use of direct SYBR® Green I staining was investigated.
- Axolotl (*Ambystoma mexicanum*) *Awnt-5A* gene was used as the target. The competitor (pwnt5A Δ) was prepared by treating pwnt5A1 cDNA with the restriction enzyme *PpuMI*.
- Different amounts of the cDNA (pwnt5A1) corresponding to the target mRNA were amplified by PCR together with a fixed amount of the competitor (pwnt5A Δ) to plot a calibration curve.

1 Introduction

The measurement of steady state mRNA transcript levels is used as a measure of transcriptional activity. The most common techniques used to quantitate mRNA transcript levels are Northern blots, ribonuclease protection assays, and the quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). The first two of these techniques are adequate for the study of genes expressed at moderate to low levels. However, QRT-PCR offers the potential for greater sensitivity because the combination of reverse transcription and PCR generates a logarithmic amplification of a specific mRNA-derived sequence. This technique allows quantitative measurements of low copy number mRNA, even with minimal tissue samples [1].

Within the QRT-PCR paradigm, various methods have been used to generate estimates of target transcript abundance. One approach has been to use a relatively invariant mRNA such as adolase A or β -actin as an internal standard [2]. However, the co-amplification of the endogenous, nonhomologous mRNA reflects differences in the amplification efficiencies of the target and the standard. Therefore, the abundance of the standard may not be linearly related to the abundance of the target.

Differences in amplification efficiencies, and tube-to-tube variations, can be controlled if the target and standard mRNAs are amplified with the same primer set in the same reaction tube. Quantitative competitive (QC) RT-PCR introduces an exogenous, altered internal competitor that can be co-amplified with the target mRNA in the same reaction tube, with the same primer set. The competitor and the target mRNA amplification products are distinguished either by size or by restriction enzyme cleavage pattern depending on the allelic variation that was artificially conferred on the competitor [1,3].

QC-RT-PCR analyses have used three major approaches for the quantitation of absolute amounts of mRNA.

* A serial dilution of a competitor is co-amplified with an unknown but constant amount of the target mRNA to determine the equimolar amount [4].

* The amounts of the total RNA and the internal competitor are kept constant, while the number of PCR cycles is varied [5].

* A varying amount of a cDNA transcript of the target mRNA is co-amplified with a constant amount of the competitor to generate a standard curve. The abundance of the target mRNA in an unknown sample is determined by amplifying the sample in the presence of the set amount of competitor [6].

Of these procedures, the first two require significant amounts of sample RNA and can be laborious, especially with multiple samples. In contrast, the standard curve method is quick, and minimizes the amount of RNA needed to measure transcript levels. Further, the ratio of any unknown mRNA sample amplified with the same amount of competitor can be compared to the standard curve and the number of transcripts extrapolated.

Typically, QC-RT-PCR amplification products are separated by gel electrophoresis and visualized by staining or indirect chemiluminescent or chemifluorescent reactions. The simplest method for detection is direct staining (e.g. ethidium bromide, SYBR[®] Green I). The stained products can be quantified using any scanner with appropriate wavelength characteristics.

We have performed a demonstration of QC-RT-PCR for the axolotl (*Ambystoma mexicanum*) *Awnt-5A* gene using the fluorescent / radioisotope imaging system FLA-2000 (Fuji Photo Film Co., Ltd., Tokyo). The *Awnt-5A* gene is a member of the axolotl *Wnt* family of genes, which encode secreted proteins that act in localized cell-cell signaling for the establishment of positional information. *Awnt-5A* is involved in the establishment of the dorsal axis of the embryo [7].

For the demonstration, we constructed a competitor to the *pwnt5A* cDNA with a deletion at the *PpuMI* sites (*pwnt5AΔ*). We then used both the *pwnt5A1* and the *pwnt5AΔ* cDNAs in a QC-RT-PCR paradigm with direct staining to generate a standard curve for the assessment of *Awnt-5A* gene expression.

2 Materials and Methods

■ Construction of the Competitor

The competitor was derived from pwnt5A1, which contains a 665 bp cDNA sequence of the *Awnt-5A* gene linked to an 830 bp fragment of the hsp70b promoter sequence cloned in BlueScript™. To obtain a plasmid that would yield a distinguishable PCR product when resolved on an agarose gel, a small deletion (113 bp) was introduced by removing a *Ppu*MI restriction enzyme fragment.

The native plasmid, pwnt5A1, was digested with *Ppu*MI, and the digestion reaction was ligated with T4 DNA ligase (New England Biolabs, Beverly, MA), and subsequently transformed into competent *E. coli* DH5 α (Boehringer Mannheim, Laval PQ). A deletion clone, pwnt5A Δ , was recovered, and the deletion was confirmed by restriction enzyme profile. Furthermore, pwnt5A Δ (competitor) and pwnt5A1 (native) transcripts amplify with the same set of primers yielding distinguishable PCR products of 545 bp and 432 bp, respectively.

■ Synthesis of Native and Competitor RNA Transcripts

Both pwnt5A1 and pwnt5A Δ were purified by cesium chloride gradient centrifugation [8]. The plasmids were linearized with *Hind* III (New England Biolabs, Beverly, MA) and 1 μ g was transcribed *in vitro* using a Stratagene RNA Transcription kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The transcription reactions were incubated at 37°C for 1 hour, followed by treatment with 10 U of RNase-free DNase I at 37°C for 30 min to remove the template DNA. The synthesized RNA was precipitated with 100% ethanol and was then dissolved in 50 μ l of water treated with diethyl pyrocarbonate. RNA concentration was determined in a Beckman DU-50 spectrophotometer (Beckmann Instruments, Fullerton, CA).

■ QC-RT-PCR

Reverse transcription was carried out using a GeneAmp RNA PCR Kit (Applied BioSystems, Foster City, CA) according to the manufacturer's instructions. Varying amounts of native RNA were combined with 1 attomole (amol) of competitor RNA and random hexamers in a final reaction volume of 20 μ l. The reactions were incubated at 42°C for 15 min followed by a 95°C heating step in a programmable thermal cycler (Ericomp, San Diego, CA).

An aliquot of the reverse transcription reactions (8 μ l) was mixed with 1x PCR mix (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.4 μ M *Awnt-5A* primers (AGGCATACATCGTGGGG and CATTTCAGGCG GCATC, Vetrogen, London, ON, Canada) and 1.25 U AmpliTaq DNA polymerase (Applied BioSystems)) to a final volume of 25 μ l. The amplification conditions were; annealing at 63°C for 1 min, extension at 72°C for 30 sec, and denaturation at 95°C for 1 min, 30 cycles. The amplification cycles were preceded by an initial denaturation step at 95°C and were followed with 1 cycle of annealing at 63°C for 1 min and a final extension at 72°C for 10 min.

The RT-PCR products were separated on a 1.2% agarose gel and stained for 1 hour with 1/10,000 dilution of SYBR® Green I (Molecular Probes, Eugene, OR). The gels were scanned on a Fujifilm FLA-2000 using excitation at 473 nm, a 520 nm emission filter, a sensitivity setting of F100, and 100 μ m resolution. The gel images were analyzed using an AIS (Imaging Research Inc., St. Catharines, ON, Canada) image analysis system.

3 Results and Discussion

The competitor we used (Fig.3-1, pwnt5A Δ) differs from pwnt5A1 by a 113 bp deletion, and should be distinguishable from the native after amplification with the same set of primers. To confirm that the native and competitor PCR products are distinguishable from each other, both species were run in adjacent lanes of a test gel (Fig.3-2). The estimated band sizes (native 538 bp, competitor 419 bp) correlate well with the actual species sizes (native 545 bp, competitor 432 bp), indicating that our constructs were distinguishable.

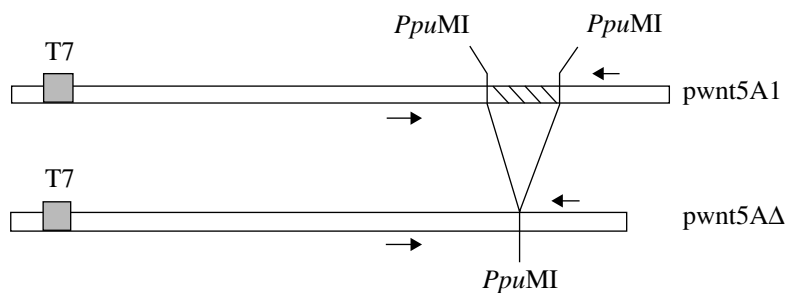


Fig.3-1

Fig.3-1: Schematic of pwnt5A1, and the deleted competitor pwnt5A Δ , showing the site of the deletion lying between the two restriction sites for *Ppu* MI. The specific *Awnt*-5A primer sites are indicated with arrows. Transcription is under control of the T7 promoter, lying upstream.

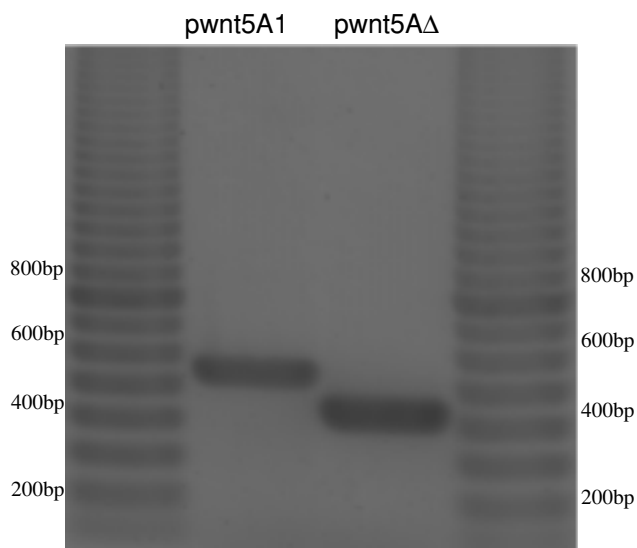


Fig.3-2

Fig.3-2: 1.2% agarose gel of RT-PCR products of pwnt5A1 and pwnt5A Δ amplified at 30 cycles, and stained with SYBR[®] Green I. Both transcripts were amplified with *Awnt*-5A-specific primers, and yielded products differing in size by 113 base pairs.

Twenty cycles of amplification produced an easily detected signal (Fig.3-3). However, all amplifications were carried out using 25 or 30 cycles, to ensure that both increases and decreases in transcription could be quantified.

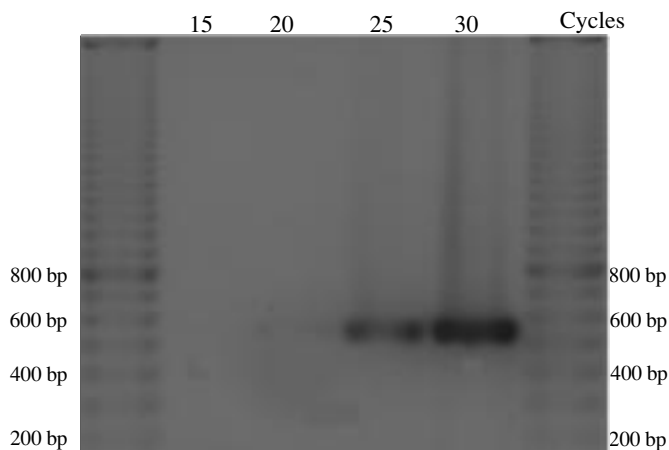
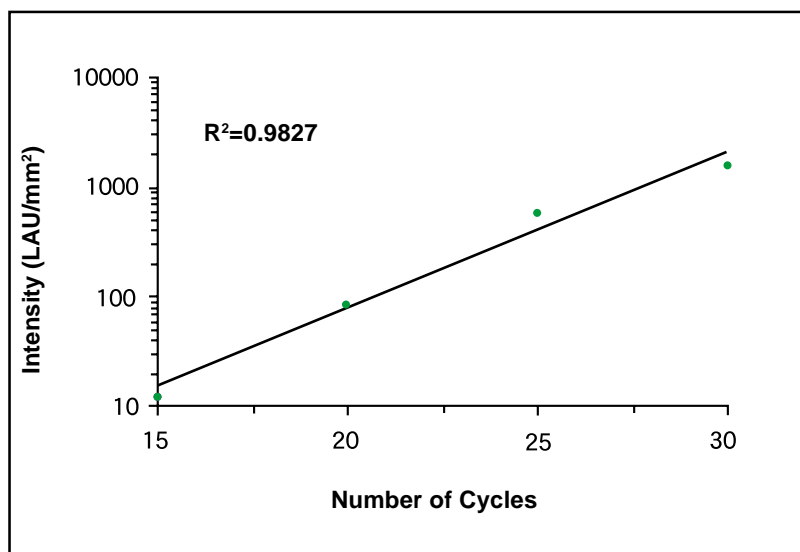


Fig.3-3: Effects of amplification cycles on signal strength.

a: A 1.2% agarose gel of RT-PCR products of pwnt5A1 was stained with SYBR® Green I. Outside lanes are 100 bp markers.

b: Graphical representation of effects of cycle number on signal strength (4 amol pwnt5A1). Both 25 and 30 cycles yield sufficient fluorescence to measure an increase or decrease in signal.

Fig.3-3-a



LAU: Linear Arbitrary Unit

Fig.3-3-b

To demonstrate a useful range for the QC-RT-PCR assay, a constant amount of competitor transcript was co-amplified with varying amounts of native transcript. We observed increased staining in the native, as the amount of input RNA increased (Fig.3-4). There was also a decrease of the competitor PCR products as the amount of the native increased.

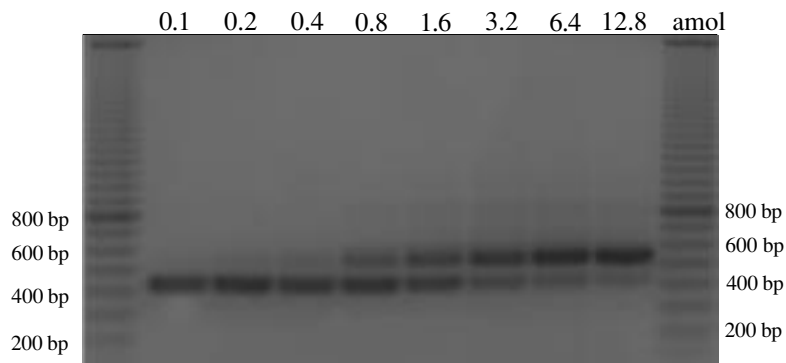


Fig.3-4: Amplification products of QC-RT-PCR of pwnt5A1 and pwnt5A Δ resolved on a 1.2% agarose gel, and stained with SYBR[®]Green I. Varying amounts of the pwnt5A1 native (0.1 to 12.8 amol) were co-amplified with 1 amol of the pwnt5A Δ competitor for 30 cycles. Ladders in the outside lanes are 100 bp markers.

Fig.3-4

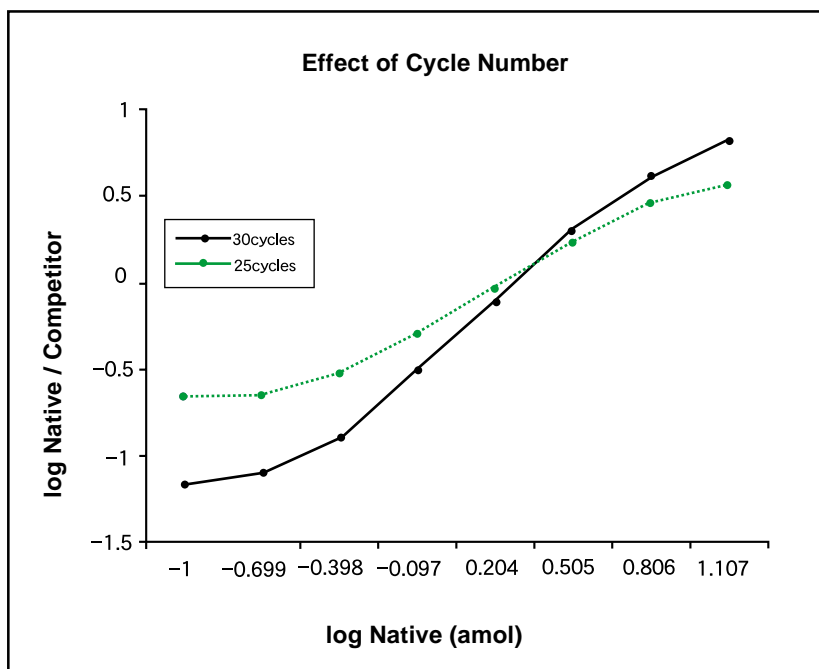


Fig.3-5: Log-log standard curve plotting native/competitor ratios as a function of varying the concentration of the native. At low native concentrations, the assay is unable to detect small changes in competitor concentration. At concentrations above 0.4 amol (log native = -0.398) of native, the assay becomes useful. Amplification for 30 cycles yields a broader dynamic range than 25 cycles.

Fig.3-5

In summary, the FLA-2000 was used for detection of a typical QC-RT-PCR assay. The sensitivity of the instrument is sufficient so that analyses may be conducted within a small part of the instrumental dynamic range. This is critical to assays such as QC-RT-PCR, which exhibit a narrow range of response.

4 References

- 1) Wang, A.M., Doyle, M.V., and Mark, D.F., Quantitation of mRNA by the polymerase chain reaction, *Proc. Natl. Acad. Sci. USA* 86, 9717-9721 (1989).
- 2) Chelly, J., Kaplan, J.-C., Maire, P., Gautron, S., and Kahn, A., Transcription of the dystrophin gene in human muscle and non-muscle tissues, *Nature* 333, 858-860 (1988).
- 3) Zenilman, M. E., Graham, W., Tanner, K., and Shuldiner, A. R., Competitive reverse-transcriptase polymerase chain reaction without an artificial internal standard, *Analytical Biochemistry* 224, 339-346 (1995).
- 4) Reischl, U., and Kochanowski, B., Quantitative PCR: A survey of the present technology, *Molecular Biotechnology* 3, 55-71 (1995).
- 5) Zhang, J., Desai, M., Ozanne, S. E., Doherty, C., Hales, C. N., and Byrne, C. D., Two variants of quantitative reverse transcriptase PCR used to show differential expression of α -, β - and γ -fibrinogen genes in rat liver lobes, *Biochem. J.* 321, 769-775 (1997).
- 6) Tsai, S. -J., and Wiltbank, M. C., Quantification of mRNA using competitive RT-PCR with standard-curve methodology, *Biotechniques* 21, 862-866 (1996).
- 7) Busse, U., and Sèguin, C., Isolation of cDNAs for two closely related members of the axolotl *Wnt* family, *Awnt-5A* and *Awnt-5B*, and analysis of their expression during development, *Mechanisms of Development* 40, 63-72 (1992).
- 8) Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning, A Laboratory Manual*. Second edition. Cold Spring Harbor, Lab., Cold Spring Harbor, NY (1989).

Writers

Nezar Rghei, M.Sc.
Peter Ramm, Ph.D.
(Imaging Research Inc.)

Editors

Kenji Miura, Ph.D.
Makiko Nagashima
Ruriko Ooka
Miyuki Ishii
Chieri Teruya
(Fuji Photo Film Co., Ltd.)

SYBR® is a registered trademark of Molecular Probes Inc.

February 2001



FUJI PHOTO FILM CO., LTD.

SCIENCE SYSTEMS, INDUSTRIAL MATERIALS & PRODUCTS DIVISION

26-30, NISHIAZABU 2-CHOME, MINATO-KU, TOKYO 106-8620, JAPAN

Telephone: +81-3-3406-2201

Facsimile: +81-3-3406-2158

E-mail: sginfo@tokyo.fujifilm.co.jp

<http://home.fujifilm.com/products/science/index.html>

©1997-2001 Fuji Photo Film Co., Ltd.