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Application Note

SCIENCE IMAGING SYSTEMS

Application Note No.20

基礎編：QC-RT-PCR for the Quantification of mRNA

FLA-2000 / FLA-3000G

FLA

はじめに

遺伝子の発現解析には微量なmRNAの解析手法が必須です。定量的なRT-PCR法は、mRNAを逆転写によってcDNAとし、PCR増幅を行うことにより、感度よくmRNAを検出できます。この方法論は、これまでに3つの方法が報告されていますが、今回はmRNAを逆転写したcDNAの量を変えて一定量のコンペティターとPCR増幅させる方法をご紹介します。ゲル染色にはSYBR® Green Ⅰを使用し、FLA-2000で検出しました。従来、主に使用されてきたノーザンブロッティング法よりも、この手法はさらに微量なmRNAの検出の可能性があるので注目されています。

今回はImaging Research社のDr. Peter RammおよびNezar Rghei 氏にご執筆頂きました。原文が英語ですので正確な内容をお伝えするために翻訳せず、原文のまま掲載することにしました。

Contents

Summary

- 定量的RT-PCR法の蛍光検出をSYBR® Green Ⅰ による直接染色で行うことを検討しました。
- ターゲットにaxolot1（*Ambystoma mexicanum*）*Awnt*-5A遺伝子を用いました。コンペティター（pwnt5AΔ）はpwnt5A1のcDNAを制限酵素*Ppu* MIで処理して作成しました。
- ターゲットのmRNAに対応するcDNA（pwnt5A1）の量を変えて一定量のコンペティター（pwnt5AΔ）とともにPCR増幅させたときの検量線を作成しました。

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].

mRNAの転写量の測定方法としてノーザンブロット法、リボヌクレアーゼ活性保護法や定量的RT-PCR法がよく用いられる。特にRT-PCR法はPCR増幅により特異的にmRNAの配列を増幅するので感度の高い方法と言える。

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.

定量的 RT-PCR 法では測定対象の mRNA の転写量を定量するための方法論を工夫する必要がある。定量的・競合RT-PCR法は人工的に変化させた競合物質を用いて目的の mRNA と同じ反応チューブ、同じプライマーセットで PCR 増幅する。この方法は内部標準と目的の mRNA の増幅効率の差や反応チューブ間のばらつきの問題を解決できる。増幅後の 2 つの物質を分けて検出するには分子量や分子構造の違いが引き起こす制限酵素の切断パターンの違いを用いる。

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].

具体的に本法を実施するには次のような方法がある。
* 目的の mRNA 量を一定としてコンペティターの濃度を変えて PCR 増幅する。
* コンペティターの量と total RNA 量を一定として PCR の回数を変える。
* コンペティターの量を一定としてmRNAの転写物としてのcDNA量の濃度を変えてPCR増幅し、検量線を作成する。
次にコンペティターの量を一定とし目的のmRNAとPCR増幅する。検量線より目的のmRNAを算出する。この方法は上記の2法と比べ迅速で目的のmRNA量が少なくてすむ特長がある。今回はこの方法で検討した。

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.

定量的RT-PCRの産物はゲル電気泳動で分離する。泳動後、SYBR® Green Iなどで直接ゲル染色して検出する方法がもっとも簡便である。

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].

我々はaxolot1（トラフサンショウウオ）の *Awnt*-5A遺伝子を用いてFLA-2000により定量的RT-PCRの検出が可能であることを示した。
ここで用いた *Awnt*-5A遺伝子はaxolot1の *Wnt*ファミリーに属し胎児の背面軸索の形態形成に関係している。

For the demonstration, we constructed a competitor to the pwnt5A cDNA with a deletion at the *Ppu*MI 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.

今回はモデル系としてpwnt5AのcDNAから制限酵素の*Ppu* MIで一部を取り除いたpwnt5A Δを作成した。
Awnt-5Aの遺伝子発現を評価する検量線を作成するためにpwnt5A1とpwnt5AΔの量比を変えてPCRを行い直接染色で検出した。

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* DH5α (Roche Diagnostics,). A deletion clone, pwnt5AΔ, was recovered, and the deletion was confirmed by restriction enzyme profile. Furthermore, pwnt5AΔ (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 pwnt5AΔ were purified by cesium chloride gradient centrifugation [8]. The plasmids were linearized with *Hind* III (New England Biolabs, Beverly, MA) and 1 μg 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 hr, 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 μl 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 (Perkin Elmer) according to the manufacturer's instructions. Varying amounts of native RNA were combined with 1 attomole of competitor RNA and random hexamers in a final reaction volume of 20 μl. 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 (8μl) was mixed with 1X PCR mix (10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM *Awnt*-5A primers (AGGCATACATCGTGGGG and CATTTGCAGGCG GCATC, Vetrogen, London, ON, Canada) and 1.25 U AmpliTaq DNA polymerase (Roche Molecular Systems, Inc)) to a final volume of 25 μl. 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 μm 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, pwnt5AΔ) 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.

コンペティターとネイティブの分子量には113 bpの差がある。これらが電気泳動で分離できることを示した。

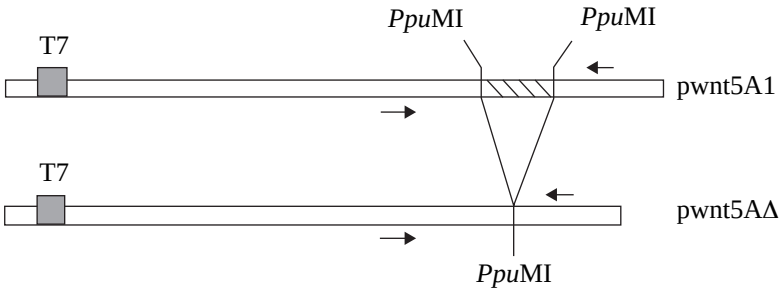

Fig.3-1

Fig.3-1: Schematic of pwnt5A1, and the deleted competitor pwnt5AΔ, 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.3-1: ネイティブのpwnt-5A1とコンペティターのpwnt5AΔを*Ppu* MIで処理した場合の概念図
除去された部分は*Ppu* MIの切断部位に挟まれたところである。特異的なAwnt-5Aのプライマーの働く部位は矢印で示した。転写活性はより上流にあるT7プロモーターで制御されている。

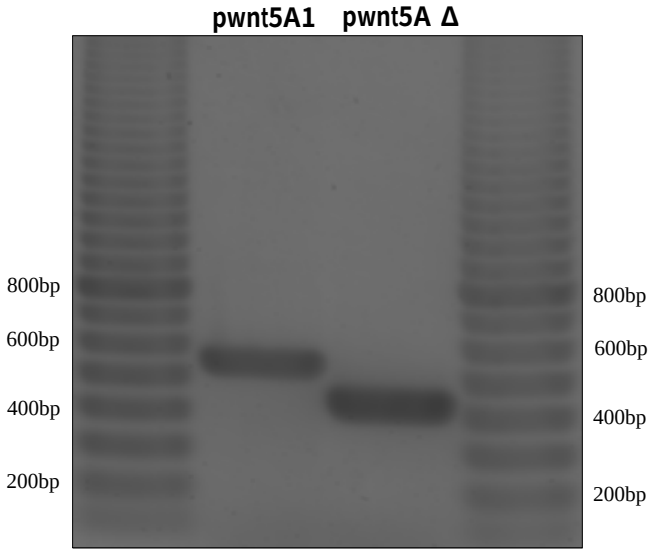

Fig.3-2

Fig.3-2: 1.2% agarose gel of RT-PCR products of pwnt5A1 and pwnt5AΔ 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.

Fig.3-2: pwnt5A1とpwnt-5AΔのRT-PCR産物の検出
1.2%アガロース
PCR　30サイクル
SYBR® GreenI染色
Awnt-5A特異的プライマーにてPCR増幅
分子量の差は113 bp

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.

定量的に取り扱える増幅サイクル数を求めた。

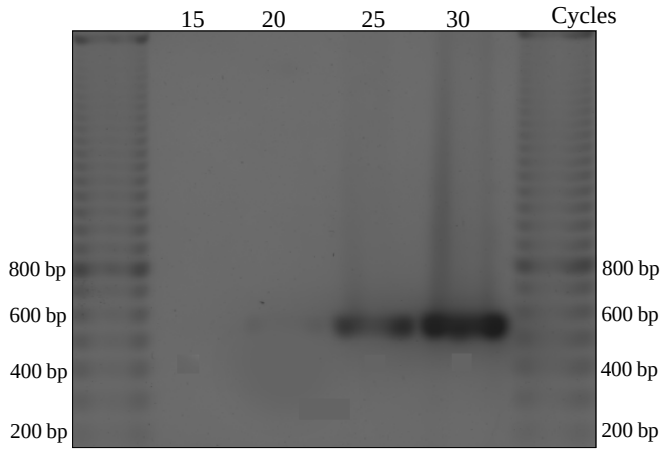

Fig.3-3-a

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.

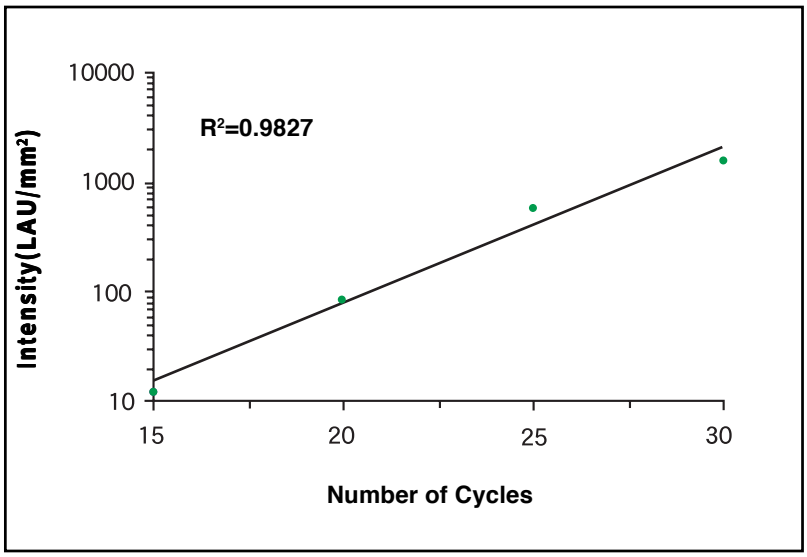

Fig.3-3-b

Fig.3-3: 増幅サイクル数とシグナル強度

a: 1.2%アガロースゲル電気泳動でpwnt5A1のRT-PCR産物を分離後SYBR® Green Iで染色。外側のレーンは100 bp分子量マーカーである。

b: サイクル数とシグナル強度のグラフ。サイクル数が25回または30回のいずれでもシグナルの増減を測定可能であった。その時に使われたpwnt5A1量は4 amol。

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.

定量的RT-PCRの実用的範囲の決定の為にコンペティターの一定量と種々の濃度のネイティブを同時増幅した。元のネイティブ濃度を増やすとPCR産物のネイティブ量が増え、コンペティター量が減少した。

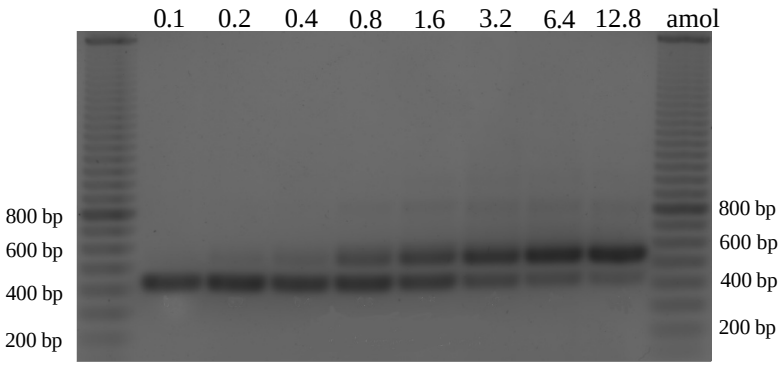

Fig.3-4

Fig.3-4: Amplification products of QC-RT-PCR of pwnt5A1 and pwnt5AΔ 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 pwnt5AΔ competitor for 30 cycles. Ladders in the outside lanes are 100 bp markers.

Fig.3-4: 定量的RT-PCRによるpwnt5A1とpwnt5AΔの増幅産物の検出

1.2%ゲルで分離後SYBR® Green I染色にて検出した。
Pwnt5A1(ネイティブ)0.1~12.8 amolを1 amolのpwnt5AΔ(コンペティター)と一緒に30サイクルでPCR増幅した。外側のレーンのラダーは100 bp分子量マーカーである。

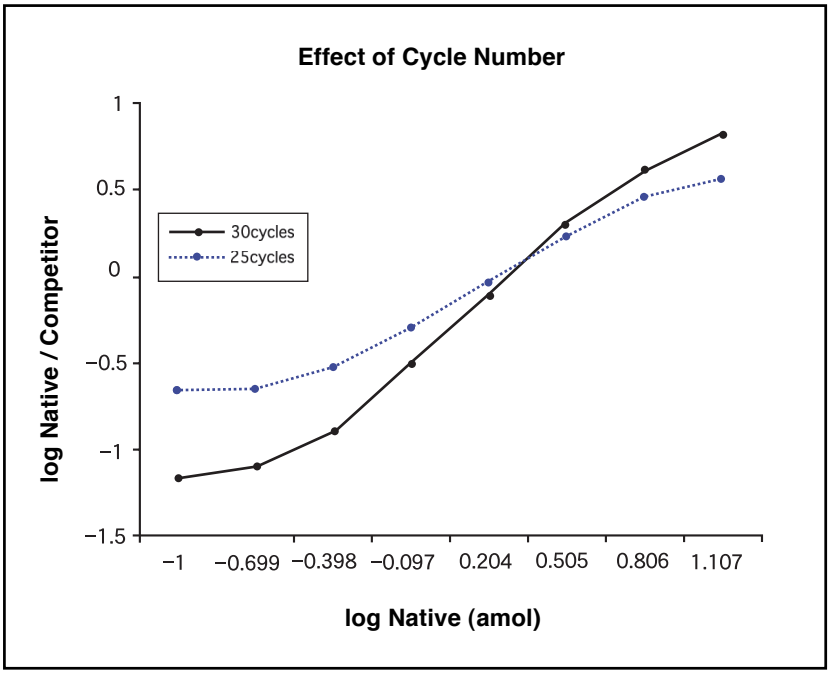

Fig.3-5

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: Log-Logスケールで表示した検量線

横軸にネイティブのpwnt5A1の量をとり縦軸にネイティブ/コンペティターの比を取った。ネイティブの量が少ないときはコンペティター量の変化を検出できない。ネイティブ量が0.4 amol(log(ネイティブ)=-0.398)以上でこのアッセイは有効となる。

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.

FLA-2000の定量的RT-PCRにおける有用性が示された。すなわち感度が十分に高いので比較的ダイナミックレンジが狭いRT-PCRも十分にカバーしていた。増幅が30サイクルの時は25サイクルの時よりもダイナミックレンジが広くなる。

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