# Renal Biopsy Collagen I mRNA Predicts Renal Scarring in a Model of Crescentic Nephritis in Rabbits

- Application of quantitative RT-PCR method -

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## I. Quantitative RT-PCR (Reverse Transcription-Polymerase Chain Reaction) Method

### 1. Indication of quantitative RT-PCR

1) when only small amount of tissue (e.g. biopsy specimen) or cells (e.g. CSF, BAL, joint fluid) are available
2) when measuring the low abundance mRNA

### 2. Background of quantitative RT-PCR

What is the problem in measuring the level of mRNA by simple RT-PCR method ?

**1) Efficiency of PCR**

PCR amplification=$A(1+E)^n$

A : initial amount of template

E : average efficiency of a cycle

n : cycle numbers

(1) E has a theoretical maximum value of 1, but it declines during multiple cycles of amplification

(2) E is affected by multiple factors such as

① quality and concentration of reagents

② condition used for denaturation, annealing, and extension

③ rate at which temperature is changed from one step to another

④ priming efficiency of nucleotides (e.g. self-priming hair loop)

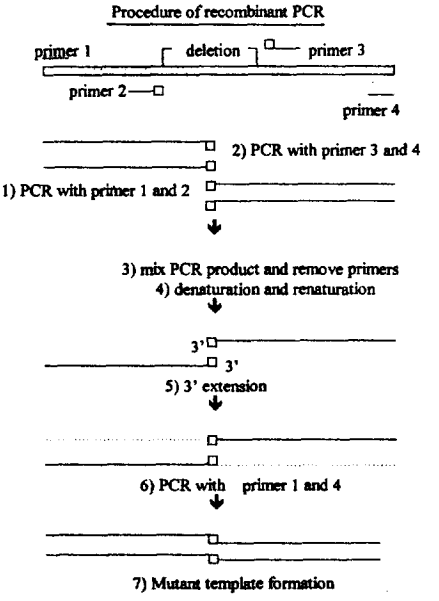
(3) Thus the yield of amplification by simple RT-PCR can vary as much as 6 fold among duplicates (Gilliland et al. : Proc Natl Acad Sci USA 87:2725, 1990)

**2) How can we solve this problem ?**

This problem has been solved by quantitative RT-PCR in which the mRNA of interest is coamplified with a reporter DNA template (mutant template) which has the same primer sites but lack naturally occurring site

**3) How can we make a reporter DNA template (mutant template), which is used as a standard ?**

(1) Recombinant PCR method is one of the simple and easy way in making mutant template.



(2) Construction of mutant template

① recombinant PCR

② subcloning into pBluescript KSM13+

③ transform in XL1 Blue

④ verify by restriction enzyme analysis and expectant size by PCR

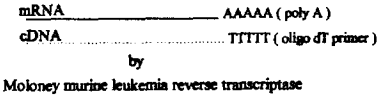
⑤ check the primer annealing sites of mutant template by DNA sequencing

⑥ large preparation of plasmid DNA

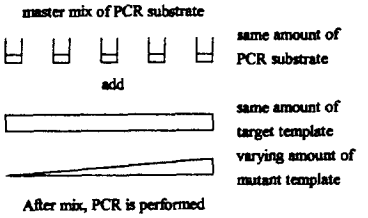
## 3. Procedure of Quantitative RT-PCR

1) RNA extraction

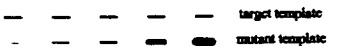
2) reverse transcription



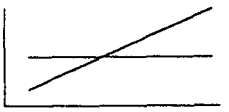

3) PCR :




4) electrophoresis :




5) quantitation :



## 4. House-keeping Gene is Needed to Correct for Variation in RNA Extraction and RT Efficiency.

Problem by using house-keeping gene

(1) assumption about the relative efficiency of RT for different mRNA molecules

(2) some kind of house keeping gene are expressed at varying levels in various human and animal disease state

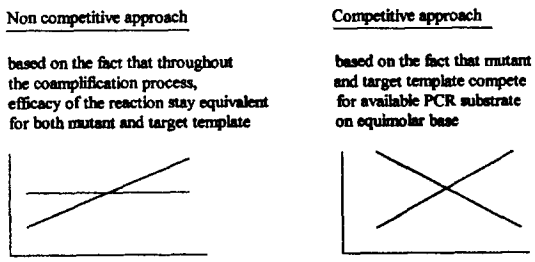
## 5. What is the Disadvantage of Quantitative RT-PCR Method ?

If efficiency of reverse transcription is less than 100%, this method will underestimate the actual amount of mRNA present in the sample

## 6. Two Quantitative Format in Quantitation of mRNA Level

1) Non competitive approach

2) Competitive approach




(1) Advantage of competitive approach : It does not require the quantitation be done in the exponential phase of amplification

(2) Disadvantage of competitive approach : It require average more than 6 amplification reactions per sample, thus it is expensive and cumbersome

## 7. Alternative Procedures in Quantitative RT-PCR Method

### 1) RNA isolation

(1) in situ RT method : RT performed in lysed cells without RNA purification
disadvantage : mRNA recovery variable depending on nuclease. cDNA prepared in nuclease rich environment tend to be unstable. Genomic DNA not separated

### 2) Construction of mutant template

(1) altered restriction enzyme site
disadvantage : heteroduplex formation which is not digested by restriction enzyme.

### 3) RNA vs. DNA Control Template

RNA template

(1) advantage : valuable technique to compare efficiency of RT

(2) disadvantage

① much less stable than DNA

② more difficult for handling

③ require quantitation of total RNA extracted from tissue

**4) PCR Product Quantification**

(1) $P^{32}$ --- scintillation counting, autoradiogram densitometry, phosphoimaging

(2) ethidium bromide staining

## II. Application of Quantitative RT-PCR Method to Animal Model of Glomerulonephritis

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Interstitial fibrosis, regardless of etiology, is associated with irrevocable loss of renal function. We therefore sought to develop a method that would predict fibrosis using a rabbit model of anti-GBM disease which progresses by day 30 to marked renal fibrosis.

We measured CI mRNA in renal biopsies on day 7 to determine whether this would predict the degree of renal fibrosis on day 30. A quantitative PCR assay, utilizing truncated cDNA internal controls, was used to quantitate CI and GAPDH cDNAs in oligo(dT)-primed cDNA prepared from total RNA from renal biopsies. The assay was linear over a range of 250-3000 molecules($R^2$>0.97, coefficient of variation=10.9%).

Since GAPDH mRNA was constant in renal cortex in normal and nephritic rabbits, the a1(I) cDNA abundance was normalized with the GAPDH cDNA to correct for differences in RNA recovery and reverse transcription efficiency (CI:G ratio).

There was considerable variation in the CI:G ratio in anti-GBM animals(range 0.21-12.07, n=16), normal animal had a CI:G ratio of 0.124±0.034(X±S.E., n=4). At day 30 renal cortex was harvested for determination of cortical hydroxyproline and blinded histologic assessment of glomerular and interstitial disease. The CI:G ratio at day 7 showed the strongest correlation with day 30 outcome parameters including cortical hydroxyproline($R^2$=0.89), interstitial fibrosis($R^2$=0.83) and % crescents($R^2$=0.83). Day 7 histology($R^2$=0.65), serum creatinine($R^2$=0.35) and urine protein: creatinine ratio($R^2$=0.65) were of much less predictive value.

In conclusion, a sensitive assay of collagen I gene expression in renal biopsies accurately predicts the development of fibrosis in crescentic nephritis in rabbits. Application of this technique to human biopsy material may prove useful for prognosis, classification of disease activity and development of novel therapeutic strategies.