Competitive PCR Guide

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I. What is Competitive PCR ?

A. Difficulties of Quantitative Analysis in Normal PCR

The polymerase chain reaction (PCR) is a highly sensitive method for the detection of small amounts of DNA by standard PCR or RNA by reverse transcription PCR (RT-PCR). However, accurate quantitation of the target is difficult with normal PCR, as the amount of amplified product does not necessarily reflect the amount of template initially present in the reaction. This is due to the "plateau phase" of PCR, which is caused by:

- Deactivation of *Taq* DNA polymerase
- Shortage of nucleotide substrates
- Shortage of primer
- Inhibition by pyrophosphate
- Re-annealing of amplified DNAs

During the plateau phase of PCR, nearly the same amount of amplified products will be obtained after a sufficient number of PCR cycles, regardless of the initial amount of template (Figure 1). It is almost impossible to determine the PCR conditions that will yield sufficient amount of amplified products for detection by gel electrophoresis before reaching the plateau phase. Consequently, for a group of samples with different template concentrations, quantitation of the target template is difficult. Competitive PCR was developed to overcome these difficulties in quantitation.

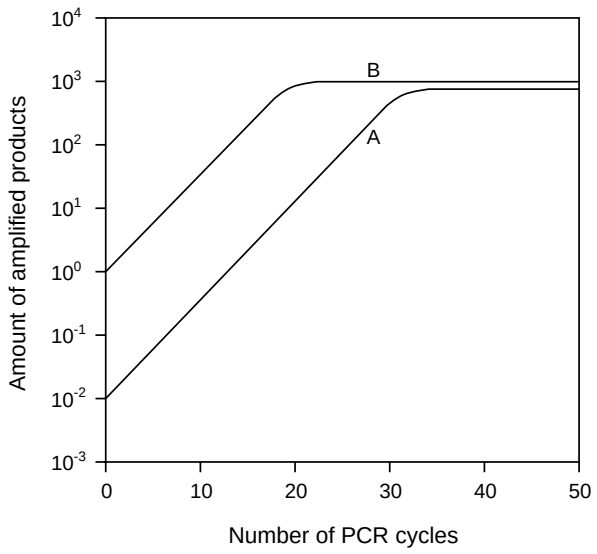

Figure 1. The plateau phase of PCR.
After approximately 30 cycles of PCR, almost the same amount of product will be obtained for samples A and B, even though they initially contain different amounts of template.

B. Principle of Competitive PCR

In competitive PCR, a known amount of a DNA fragment (competitor) is added to the sample. This competitor must contain sequences for the same primers used to amplify the target. When the target DNA and competitor are amplified together, both templates will compete for the same set of primers. Because of this competition, the ratio of the amounts of the two amplified products reflects the ratio of the amounts of the target DNA and competitor. Since the initial amount of the competitor is known, the amount of the target DNA can then be estimated according to the T:C ratio (Figure 2).

T: amount of amplified product from target DNA or RNA
C: amount of amplified product from competitor

When the T:C ratio = 1, the initial amount of target DNA or RNA will correspond to the amount of competitor.

An ideal competitor for quantitative PCR should be:

- Amplified by the same primers as the target DNA.
- Distinguishable from the target DNA (different size, different restriction fragment pattern, etc.)
- Purified and obtained at a known quantity or concentration

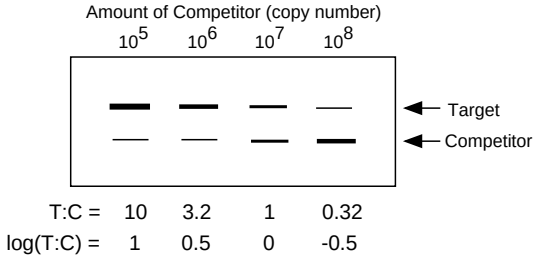

Figure 2. Principle of competitive PCR.
Samples are analyzed by agarose gel electrophoresis and the amount of competitor required to give a T:C ratio = 1 is determined. In this example, the amount of target DNA corresponds to 10^7 copies of competitor.

C. Competitive RT-PCR

Competitive RT-PCR can be performed similarly to competitive PCR. There are two different competitive RT-PCR methods.

Competition between cDNA and DNA Competitor
This method uses a DNA competitor, after cDNA synthesis from the target mRNA has been completed. The relative amounts of cDNA among different samples can be determined. Since the yield and efficiency of the RT reaction is not reflected in this method, the absolute quantity of the target mRNA cannot be determined.

Competition between mRNA and RNA Competitor
This method is based on competition between mRNA and an RNA competitor during the RT reaction. The relative amounts of target mRNA among different samples can be determined. As the yield of the RT reaction is reflected in this method, the absolute quantity of the target mRNA can be determined (Figure 3).

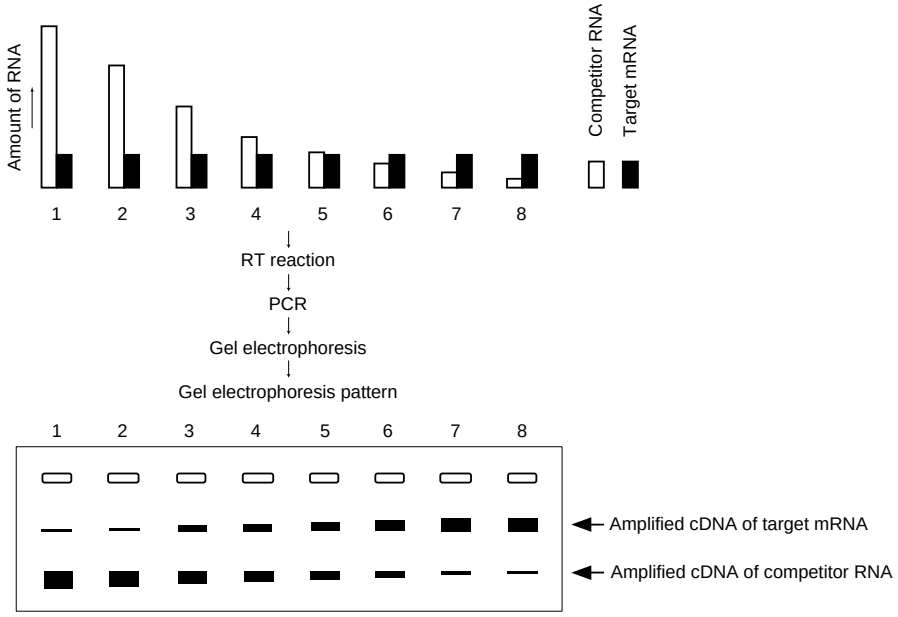

Figure 3. Competitive RT-PCR.
Fixed amounts of RNA template are mixed with increasing amounts of competitor RNA. Reverse transcription is performed, followed by PCR. The samples are then analyzed as for competitive PCR.

D. Different types of competitors

Homologous Competitor
This is a competitor that has the same nucleotide sequences as the target DNA (RNA) but contains a deletion or an insertion, or has a different restriction site introduced by site-specific mutagenesis (Figure 4). When designing this type of competitor, care should be taken to avoid the formation of a heteroduplex, which would interfere with subsequent quantitation.

Heterologous Competitor
This is a competitor that has nucleotide sequences different from the target DNA (RNA) except for the sequences of the primer annealing sites (Figure 4). A heteroduplex will not be formed. Since the primer sequences are the same, the difference in amplification efficiency between the target and heterologous competitor is usually minor.

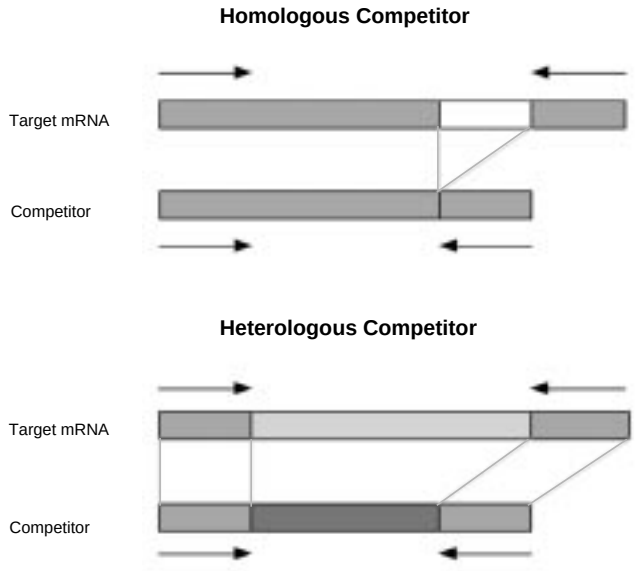

Figure 4. Types of competitors.

E. Competitive RT-PCR Using an Internal Reference Template

(Normalization of the amount of RNA in different samples using the Human β-actin Competitive PCR Set)

When dealing with highly purified, intact RNA samples, total RNA can be estimated by absorbance (A_{260}) values. However, crude RNA samples are often used for RT-PCR. In crude samples, the actual amount of intact RNA is less than the total RNA amount estimated by absorbance values due to DNA contaminants and/or degraded RNA. Therefore, A_{260} values cannot be relied upon to accurately compare the amount of RNA among crude RNA samples.

To precisely measure the expression level of a target gene in cells, the RNA amounts applied in the assay should be normalized to a fixed amount. This can be achieved by performing competitive RT-PCR and amplifying an internal reference template such as a housekeeping gene. Takara's Human β-Actin Competitive PCR Set uses the human β-actin mRNA as the internal reference template. The β-actin mRNA is widely used as a standard, since it is a housekeeping gene that is expressed at a constant level in most cells and tissues. The Human β-Actin Competitive PCR Set can therefore be used with any kind of sample derived from human cell lines, while providing more precise correction than conventional methods.

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3. Gause, W.C. and Admovicz, J. (1995) Use of PCR to quantitate relative differences in gene expression. *PCR PRIMER,* 293-311.
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II. Analyzing the Results of Competitive PCR

A. Considerations

From the results of competitive PCR, the initial amount of target DNA (RNA) template can be estimated from the ratio of T to C (T = amount of amplified target template; C= amount of amplified competitor). By determining the amount of competitor which gives a T:C ratio of 1 for each sample, the relative amounts of target DNA (RNA) can be obtained. This method assumes, however, that the amplification efficiency of the target template and the competitor are equal.

For the amplification efficiencies of target DNA (RNA) and competitor to be equal, the size and the sequence of each should be identical. However, this is not possible in practice, since the target DNA (RNA) and competitor must be easily distinguished by size or by internal sequence. These differences may also affect amplification efficiencies. Due to these differences, it is difficult to accurately estimate the initial amount of the template DNA or RNA. This is usually not a limitation, since most experiments do not require calculation of the absolute amount of target template in a given sample. Instead, the relative amounts of target in a group of samples are calculated.

To facilitate analysis of competitive PCR, a competitor with a different size from the target is often used, as its amplification product is easy to distinguish from that of the target. This is known as a heterologous competitor. A heterologous competitor has the advantage that it does not form a heteroduplex with the target template; however, it may exhibit a different amplification efficiency from the target. This difference is usually minor if the size of the competitor is not substantially different from that of the competitor.

To minimize the difference in amplification efficiency, a homologous competitor can be used, which has almost the same sequence and size as the target, but has a different restriction site introduced by mutagenesis. This type of competitor can be distinguished from the target by restriction digestion of the amplified products, but has the disadvantage of forming a heteroduplex with target DNA (RNA).

B. Examples

A series of diluted competitors is added to a fixed amount of sample to perform competitive PCR. After completion of the reaction, equal aliquots from each sample are analyzed by agarose gel electrophoresis.

1. Visual Estimation Method

By comparing the intensity of the bands, the concentration of competitor is noted for each sample when T:C = 1, i.e., when the two bands of amplified product appear to be of equal intensity. The relative amounts of RNA in each sample can then be calculated, based on the amount of competitor (Figure 5).

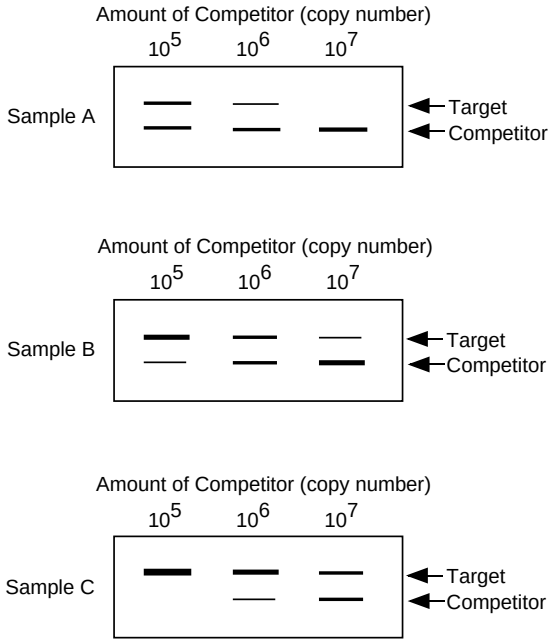

Figure 5. Visual estimation of the results of competitive PCR.
For each sample (A, B, and C) the amount of competitor required to give a T:C ratio is determined after agarose gel electrophoresis.

	Sample	Amount of Competitor (copy number)
1	Sample A	10^5
2	Sample B	10^6
3	Sample C	10^7

2. Graphical method

The T and C values for each set of samples are determined quantitatively after electrophoresis, using a densitometer or image analyzer. For each set of samples, a graph is plotted of log(T:C) against the initial amount of competitor. The initial amount of competitor corresponding to log(T:C) = 0 can be determined for each sample. This method is more precise than visual estimation, but requires specialized equipment. Both methods are affected by smearing of PCR products in the gel, which interferes with precise quantitation.

	Sample	Amount of Competitor (copy number)	T:C	log(T:C)
1	Sample A	10^5	1	0
2	Sample A	10^6	0.1	-1
3	Sample A	10^7	0.01	-2
4	Sample B	10^5	10	1
5	Sample B	10^6	1	0
6	Sample B	10^7	0.1	-1
7	Sample C	10^5	100	2
8	Sample C	10^6	10	1
9	Sample C	10^7	1	0

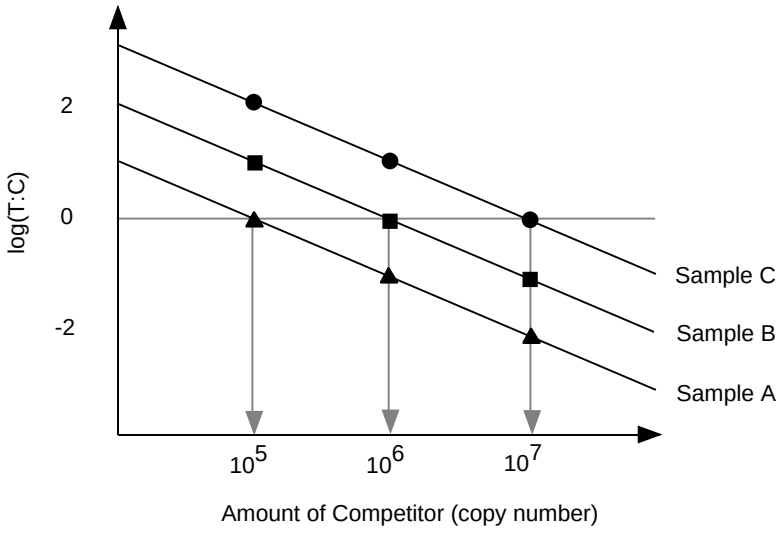

Figure 6. Graphical analysis of competitive PCR.
The band intensities of amplified products are determined, and T:C ratios are calculated. From the plot above, the target is quantitated by determining the value for which log(T:C) = 0.