Competitive Quantitative RT-PCR

A. Background

Why use competitive RT-PCR?

Steady state levels of individual RNA transcripts have traditionally been measured by Northern blotting and nuclease protection assays. These techniques, however, are limited by their sensitivity. Under optimal conditions, a minimum of about 5×10^5 copies of an RNA transcript is required for detection by ribonuclease protection assay, the most sensitive non-PCR based mRNA detection and quantitation procedure (Melton, et. al., 1984). RT-PCR is significantly more sensitive than this; it is capable of detecting moderately expressed transcripts from a single cell. Quantitation of transcription via RT-PCR can be approached using either relative RT-PCR or a competitive RT-PCR strategy. Theoretically, relative quantitation by RT-PCR can grossly estimate differences in abundance of a particular transcript between samples if the following conditions are met:

1. The amount of RNA used in the initial cDNA synthesis reaction is precisely controlled.
2. The amount of cDNA used in the PCR reaction is precisely controlled.
3. The number of PCR cycles necessary to generate enough product to detect is not past the exponential phase of the PCR.

In practice, it is very difficult to ensure that these criteria are met. Of these conditions, only the amount of input RNA must be strictly controlled to use competitive RT-PCR. The validity of the competitive amplification approach has been confirmed in numerous studies and it is the basis for several quantitative PCR and RT-PCR assays designed to quantitate human pathogens.

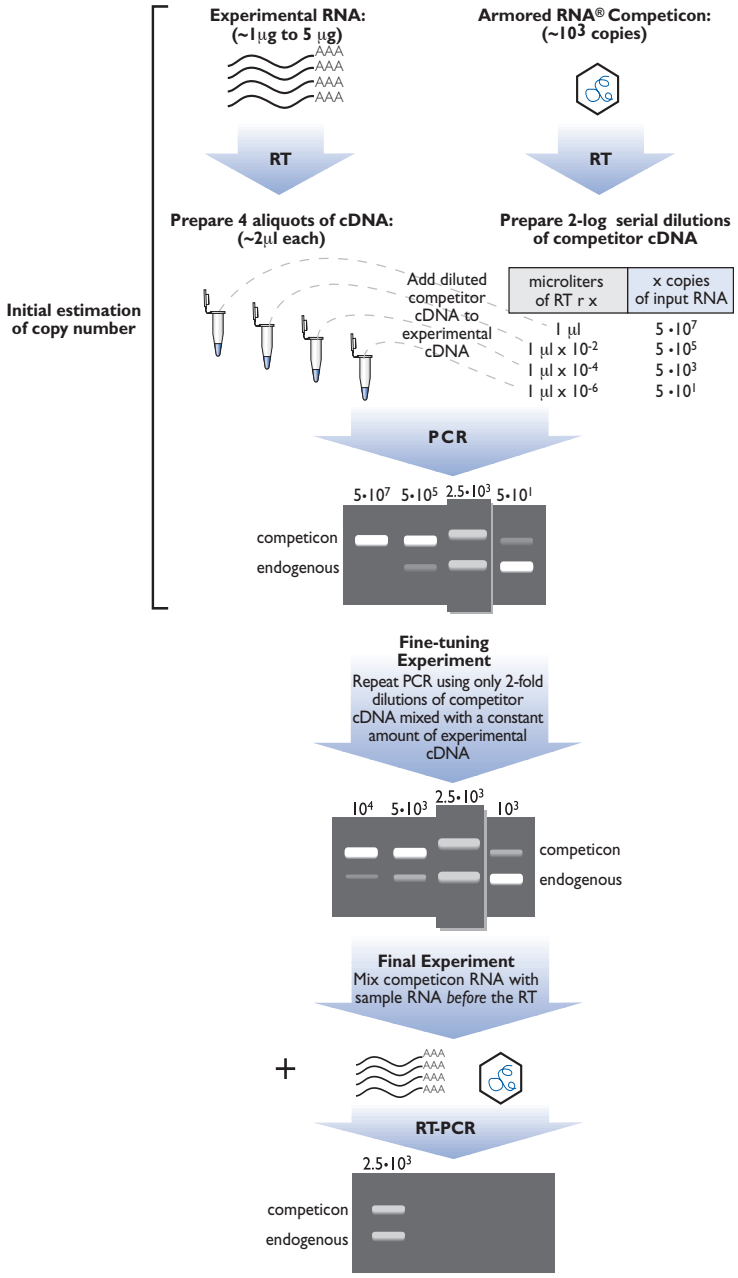
How do the Competitive Quantitative RT-PCR Kit™s work?

Ambion's Competitive Quantitative RT-PCR Kit™s provide an Armored RNA® Competicon™ to serve as an exogenous competitive template for quantitative RT-PCR experiments. This Competicon contains the same primer binding sites as the endogenous target, is amplified with equivalent efficiency, and is distinguishable from the endogenous target by agarose gel electrophoresis due to a small size difference. When this exogenous RNA is present in an RT-PCR reaction, a competition between templates for amplification is created. Each amplicon competes equally well for primers, nucleotides, and enzyme in the PCR reaction, thus the ratio of products obtained from the endogenous and exogenous targets at the end of the amplification reflects the initial ratio of target to competitor. Since the amount of Competicon added to the RT-PCR is known, the amount of endogenous target in the RNA sample can be determined. Figure 1 on page 2 shows schematically how the Competitive Quantitative RT-PCR Kit™s work.

Ambion's Armored RNA® technology makes competitive RT-PCR easy

To use competitive RT-PCR, considerable effort is required to construct and quantitate an appropriate RNA competitor; this includes in vitro transcription, accurately quantitating the RNA product, and using RNase-free technique and reagents. Using ordinary RNA competitors as standards in competitive RT-PCR is problematic because of their susceptibility to degradation by RNases, which are ubiquitous. Long term reproducibility of the assay is impossible if a percentage of the competitor is degraded by contaminating RNase each time the RNA is thawed for use. Many developers of competitive RT-PCR assays attempt to simplify the competitive RT-PCR assay by using DNA competitors, or DNA targets with primer binding sites flanking unrelated internal sequences (DNA MIMICS). While these modifications reduce the effort needed to generate competitive targets, they represent a major source of error in the reliability of the assay. DNA competitors do not address the inherent variability in the reverse transcription step of RT-PCR, nor can procedures that rely on the use of DNA mimics guarantee equivalent amplification efficiency between endogenous targets and mimics of unrelated sequence.

Figure 1. Schematic of how the Competitive Quantitative RT-PCR Kit™s work



Ambion's Armored RNA Competicons represent a breakthrough in RNA standards technology for quantitative RT-PCR. Armored RNA Competicons are RNase-proof RNAs added to experimental samples before the RT step of RT-PCR. Upon heating to 75°C for 5 minutes, the competitor RNA is released as template for RT-PCR. All of Ambion's Armored RNA Competicons share a unique 20 bp detection sequence. In this way, all can be detected and quantitated with the same oligonucleotide probe. Ambion's Competicon templates produce a PCR product 10% smaller than the corresponding endogenous target, facilitating analysis by gel electrophoresis (on 2% agarose or 5% polyacrylamide gels). Each Competitive Quantitative RT-PCR Kit™ supplies a gene-specific RNA Competicon, PCR primers, a control

target, and a protocol to quantitate a specific cellular transcript by competitive RT-PCR. Since the Competicon is an RNA, errors caused by sample to sample variation in reverse transcription efficiency are eliminated.

There are intrinsic variables in RT-PCR beyond Ambion's control. It is Ambion's intent, however, that the controls and guidelines provided in this kit will facilitate the analysis of the researcher's experimental mRNA of interest.

B. Quantitation by Competitive RT-PCR

1. Overview

In competitive RT-PCR, increasing amounts of competitor are titrated into individual aliquots of sample RNA; these are then subjected to RT-PCR. When equal amounts of PCR product are made from competitor and endogenous RNA target, one can assume that the initial copy number of the target RNA in the sample matched the known value of the added competitor. Since the level of gene expression in an experimental sample is initially unknown, many reactions with log dilutions of competitor would have to be added to each RNA sample before performing RT-PCR to obtain an estimate of copy number. This approach would be too laborious, and would use too many expensive reagents, so we suggest that the following pilot experiment be done first.

2. RT-PCR pilot experiment

To get an initial estimation of copy number, RT reactions are performed separately on an aliquot of Armored RNA Competicon (e.g. 10^9 copies) and on a standard amount of each sample RNA (e.g. 1-5 µg). Then, instead of doing multiple ***RT*** reactions for each sample, multiple ***PCR*** reactions are performed using different dilutions of the Armored RNA RT, spiked into a constant amount of sample RT. These PCR reactions are then analyzed on an agarose gel; the dilution of Competicon RT that yields a similar amount of PCR product as the experimental samples becomes the basis for subsequent experiment(s). Figure 2 on page 3 shows an example of the results one might obtain when performing competitive RT-PCR for a moderately abundant message. This experiment does not compensate for sample to sample varaion in RT efficiency because the competitor was added *after* the RT step.

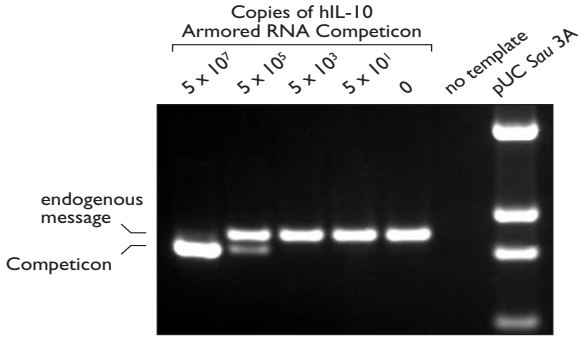


Figure 2. Example of an RT-PCR Pilot Experiment

Two micrograms of experimental RNA were reverse transcribed in a 20µl RT reaction. One microliter aliquots were prepared, and 1µl of diluted Competicon cDNA was added (the number of Competicon cDNA equivalents is shown in the figure). The mixtures of cDNA were subjected to 30 rounds of PCR, and 5 µl of the reaction products were run in an ethidium bromide stained agarose. The no template reaction contained PCR primers and reagents only.

1. Fine-tuning of the RT-PCR

Once the message has been quantitated roughly with a pilot experiment as described in the preceding section, a more precise value can be determined by repeating the experiment with a smaller range of dilution of the Competicon RT reaction (e.g.

2-fold dilutions that span a 1.5 log range). (See Figure 3). This experiment will generate data that should be quite close to the actual abundance of the endogenous target in your sample.

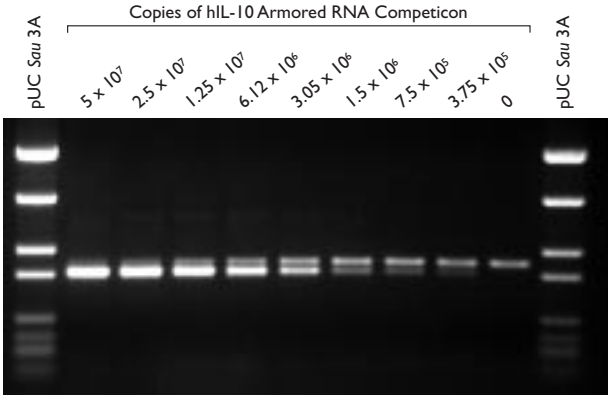


Figure 3. Fine-Tuning Experiment

This experiment was done in exactly the same way as the experiment pictured in Figure 2, except that the dilutions of Competicon cDNA cover a narrower range, with more intermediate dilution points.

2. Final experiment

To control for variations in RT efficiency, a third RT-PCR should be done, this time add the diluted Competicon RNA directly to your sample RNA using the amount of Competicon indicated by your initial experiments. Be sure to heat the sample at 75°C for 3 minutes to release the Competicon RNA prior to initiating the reverse transcription reaction. This third experiment should produce bands derived from the Competicon and from the endogenous message-of-interest that are equally intense. (See Figure 4). The amount of Competicon RNA needed to produce an RT-PCR band whose intensity matches that produced from the endogenous message represents the amount of endogenous message present in the RNA sample.

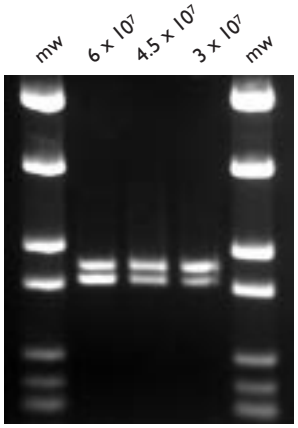


Figure 4. Final RT-PCR Experiment

The indicated amounts of Competicon were added to 1µg of sample RNA. The mixture of RNAs were subjected to RT-PCR, and 5 µl were run on a 2% agarose gel.

A word about cocktails.....

Whenever possible, cocktails of common reagents should be made when performing multiple reactions. This minimizes sample to sample variation and improves reproducibility. Because volume loss occurs in pipet tip hold up and by small errors in

pipetting, the cocktail should be made up with 10% overage to allow for loss. For example, a cocktail of dH_2O, dNTPs, decamers, and RT-PCR buffer can be made for step 1a. below.

Controls

In competitive RT-PCR, a no-template negative control reaction should be routinely run to control for contamination of the PCR components. Ideally, a minus reverse transcriptase RT reaction should be performed — one for each RNA sample. This control differentiates between PCR products amplified from genomic DNA and those amplified from cDNA.

C. Competitive Quantitative RT-PCR Kit™ Protocol

1. Reverse transcription of Competicon RNA and user samples

Initially, a Reverse Transcription reaction should be performed on a sample of Competicon (1 x 10^9 copies is a convenient starting amount). RT reactions should also be performed for each sample RNA. We have performed 20 µl reactions with as much as 5.0 µg of total sample RNA. The proper amount of input RNA will depend upon the relative abundance of the target in a given sample. A reasonable starting amount is 1-2.5 µg of total RNA.

To save reagents

- RT reactions may be scaled down to 10 µl, with an upper limit of input total RNA of ~2.5 µg
- PCR reactions can be scaled down to 25 µl.

a. Assemble RT reactions on ice in microcentrifuge tubes

(The template in one reaction is Competicon alone, additional rxns are your RNA samples, one rxn per sample)

11 µl	RNA plus dH_2O (up to 5.0 µg total RNA)
4 µl	2.5 mM dNTP mix
1 µl	random decamer 500 ng/µl
2 µl	10X RT-PCR buffer*
18 µl	final volume

* (10X RT-PCR is 0.1M Tris-Cl pH8.3, 0.5M KCl, 15 mM $MgCl_2$)

b. Mix, spin briefly in microfuge, heat 3 min at ~75˚C

This incubation denatures secondary structure that is often present in RNA, and releases Competicon RNA from its resistant form.

c. Remove tube(s) to ice; spin briefly, replace on ice

The samples must be cooled before the enzymes are added.

d. Add the following to each sample:

1 µl (25 units)	Placental RNase inhibitor
1 µl (100-200units)	M-MLV RT

Mix gently, spin briefly.

e. Incubate one hour at 42˚C

Steps 1.e.-1.f. can be done in a thermal cycler for convenience.

f. Incubate 5 min at 95˚C

This incubation inactivates the reverse transcriptase

g. Cool reactions on ice

The reactions can now be stored at -20˚C or you can proceed to the amplification step.

2. Initial Quantitation of copy number in RNA sample

To get a rough idea of the amount of message in the experimental sample, a preliminary PCR experiment is done by mixing a fixed amount of sample cDNA with serial dilutions of Competicon cDNA. This saves time and reagents compared to doing separate RT reactions with different amounts of Competicon mixed with the experimental RNA, and it gives an estimate of copy number that will form the basis for more precise experiments. Please see "Overview" on page 3 for a detailed discussion of the Competitive Quantitative RT-PCR Kit™ experimental strategy.

a. Dilute Competicon RT reaction by 2-log increments in $T_{10}E_1$ buffer

This will yield the indicated number of copy equivalents per microliter dilution(Store diluted stocks at -20°C)

1 µl of RT = 5×10^7/µl
dilute 1 µl of RT in 99 µl $T_{10}E_1$ (10^{-2} dilution) = 5×10^5/µl
dilute 1 µl of 10^{-2} in 99 µl $T_{10}E_1$ (10^{-4} dilution) = 5×10^3/µl
dilute 1 µl of 10^{-4} in 99 µl $T_{10}E_1$ (10^{-6} dilution) = 5×10^1/µl

b. Set up Competitive PCR reactions

6 reactions for each sample RNA, using a constant amount of RT from the experimental sample RNA RT reactions and 1 µl of the different dilutions of the Competicon RT reaction.

tube #	1	2	3	4	5-no competitor	6-no template
*experimental sample RT	x µl	x µl	x µl	x µl	x µl	x µl dH_20
diluted Competicon RT	1 µl	1 µl	1 µl	1 µl	1 µl dH_20	1 µl dH_20
copies of Competicon	($5x10^7$)	($5x10^5$)	($5x10^3$)	($5x10^1$)	(0)	(0)
water to final volume	36 µl	36 µl	36 µl	36 µl	36 µl	36 µl

* Depends on target abundance and primer efficiency, cDNA input may need to be adjusted. For a first attempt, try 2 µl of the RT reaction. (Generally, an input that produces a visible band on an EtBr stained 2% agarose gel after 30 cycles of PCR is adequate).

NOTE: Ambion recommends a hot start for PCR reactions. At a minimum, keep reactions on ice, preheat thermocycler to 94°C, and then place the tubes in the thermocycler.

c. To each of the six reactions, add the following:

This reaction mix can be made as a cocktail. Be sure to make enough to accommodate your experiment plus 10% overage.**:**

Amount	Component
5 µl	10X RT-PCR buffer
5 µl	dNTP Mix (2.5 mM)
4 µl (2 µl each)	PCR primers (10 µM each)
~0.25 µl (1-1.25 units)	Thermostable DNA Polymerase

This reaction mix can be made as a cocktail. Be sure to make enough to accommodate your experiment plus 10% overage.**:**

d. Perform the PCR using the following cycling profile

i. Soak
94˚C 3 minutes,

ii. Cycle
30X:
94˚C, 20 sec.
55˚C -60˚C, 30 sec.
72˚C, 30 sec.

iii. Soak
5 minutes at 72˚C

iv. Soak
4˚C - indefinitely

D. Interpretation of Results

Assess the results of the RT-PCR by electrophoresis of 10 µl PCR reaction plus loading dye. Run your samples in 2 - 2.5% agarose (with 1 µg/ml EtBr in the gel) or on a 5% acrylamide gel, post stained with 1 µg/ml ethidium bromide. Competicons correspond to inducible cellular genes, thus the abundance of the endogenous target may vary widely between samples. The expected size of the endogenous and Competicon products from Ambion's different Competitive Quantitative RT-PCR Kit™s is listed in section *I.C. Reagents Provided With the Competitive Quantitative RT-PCR Kits* on page 4 of the manual. Examples of competitive RT-PCR experiments are shown in Figures 2, 3, and 4. In Figure 4 on page 4, the center lane shows approximately equal staining intensity of the products. This gives the approximate copy number of the endogenous transcript. Since this lane contained 4.5×10^7 copies of Competicon, and the intensity of the PCR products are roughly equal, we know that the experimental sample also contained approximately 4.5×10^7 copies of the transcript.