

Quantifying RNAi-Mediated Gene Silencing by Real-Time RT-PCR Using the MasterAmp™ Real-Time RT-PCR Kit

Judith E. Meis, EPICENTRE

As reported by Pfaffl and Hageleit¹, real-time RT-PCR with SYBR® Green I dye provides a quick and sensitive method to obtain reliable results for quantitative gene expression studies. EPICENTRE's new MasterAmp™ Real-Time RT-PCR Kit uses SYBR® Green I detection and gives consistent real-time RT-PCR amplification of mRNAs from as little as 5 pg of total human cellular RNA.² The kit features RetroAmp™ RT DNA Polymerase and can be used for either one-step or two-step RT-PCR. Here we demonstrate quantification of mRNA levels from RNA interference (RNAi) experiments using the MasterAmp Real-Time RT-PCR Kit.

Methods

Preparation of short hairpin RNA for RNAi

Short hairpin RNA (shRNA), transcribed *in vitro* and transfected into cultured cells, has been shown to be easy to prepare and is as effective as short double-stranded RNA (dsRNA) for RNAi-mediated gene silencing.^{3,4,5} A 69-base shRNA specific for silencing β -actin (shRNA-actin), shown in Figure 1, was transcribed from a 92-base dsDNA oligonucleotide template in a 30-minute *in vitro* transcription reaction using EPICENTRE's AmpliScribe™ T7-Flash™ Transcription Kit (see page 8), as described previously.³

shRNA transfection of HeLa cells

HeLa cells were plated in 24-well plates and grown for 24 hours in D-MEM supplemented with 10% fetal bovine serum and 100 μ g/ml penicillin/streptomycin. Six wells of cells were transfected as previously described³ in 200 μ l serum-free D-MEM containing 4 μ l of Oligofectamine™ Reagent (Invitrogen) and 20 pmoles (100 nM) shRNA-actin. Transfected cells were incubated for 24 hours. To establish basal β -actin mRNA levels as a control, an additional 6 wells of cells were mock transfected in 200 μ l serum-free D-MEM.

Total cellular RNA was purified from 3 replicates of the shRNA-actin transfected cells and from 3 wells of mock-transfected cells using EPICENTRE's MasterPure™ RNA Purification Kit. Briefly, the culture media containing the transfection reagent was removed and the cells were washed with phosphate buffered saline. Cells were lysed directly in the wells using 300 μ l of MasterPure™ Tissue and Cell Lysis Buffer and total RNA was purified as described in the product literature.

Real-Time RT-PCR assay

Real-time RT-PCR was performed using EPICENTRE's MasterAmp Real-Time RT-PCR Kit and primers to amplify a 327-bp region of human β -actin mRNA. Amplification reactions used 100 ng of total cellular RNA from each of the 3 wells of

transfected cells or mock-transfected cells, in duplicate, for a total of 6 samples for each cell type. The 50- μ l reactions contained 0.2 μ M primers. Reverse transcription was performed for 30 minutes at 60°C. Reactions were heat denatured for 2 minutes at 95°C and real-time PCR performed and analyzed using Bio-Rad's iCycler iQ™ Real-Time PCR Detection System. Real-time PCR reactions consisted of 40 cycles of 95°C (20 seconds), 57°C (20 seconds), and 72°C (30 seconds).

A standard curve was constructed by amplifying β -actin mRNA, in triplicate, from 2-fold serial dilutions of a reference sample containing total cellular RNA from HeLa cells. Real-time RT-PCR was performed using the same primers and amplification conditions described for the experimental samples. The quantitative difference in expression levels between shRNA-actin transfected cells and mock-transfected cells was determined using this curve.

Results

In vitro transcription of shRNA-actin

AmpliScribe T7-Flash Transcription reactions yielded 60-70 μ g (approximately 2500 to 3000 pmoles) of shRNA-actin per microgram of double-stranded oligo template. As used in this study, a single AmpliScribe T7-Flash reaction produced enough shRNA-actin for more than 100 RNAi experiments.

Quantifying the silencing of β -actin by shRNA-actin

Figure 2 shows the real-time RT-PCR quantification graphs for the β -actin mRNA amplification from total RNA isolated from HeLa cells transfected with shRNA-actin and mock-transfected. The average threshold cycle (C_T) of the shRNA-actin treated samples is 15.00 ± 0.13 cycles and the average C_T of the mock-transfected samples is 13.54 ± 0.13 cycles. Figure 3 shows the real-time RT-PCR results of amplifying β -actin mRNA from 4 serial dilutions of the reference sample, total RNA from HeLa cells.

A standard curve was generated from the reference sample data by plotting the log of the dilution versus the C_T values. The relative level of β -actin mRNA from

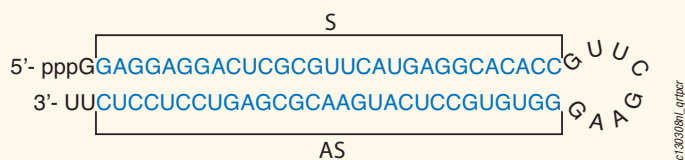


Figure 1. shRNA-actin was transcribed *in vitro* from a 92-bp double-stranded oligo DNA template containing a T7 transcription promoter. The 69-base shRNA-actin contains 1 unmatched 5'-G, a 29-base "sense" (S) sequence homologous to the targeted region of β -actin mRNA, an 8-base "loop" sequence, a 29-base "anti-sense" (AS) sequence complementary to the targeted region of β -actin mRNA and a 3'-terminal 'UU' sequence.

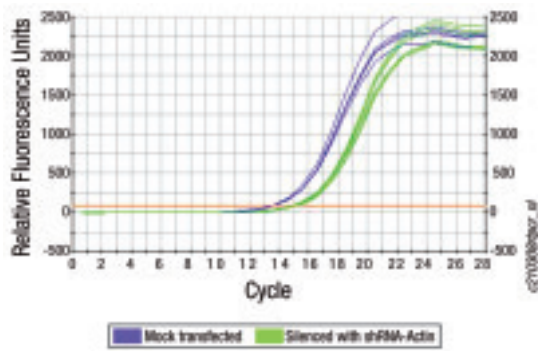


Figure 2. Real-time RT-PCR amplification of β -actin mRNA from shRNA-actin transfected (green line) and mock-transfected (purple line) HeLa cells. Total cellular RNA (100 ng) from 6 replicates each of shRNA-actin transfected cells and mock-transfected cells was amplified using the MasterAmp™ Real-Time RT-PCR Kit. The average C_T value of shRNA-actin transfected cells was 15.00 ± 0.13 , and the average C_T value of mock-transfected cells was 13.54 ± 0.13 .

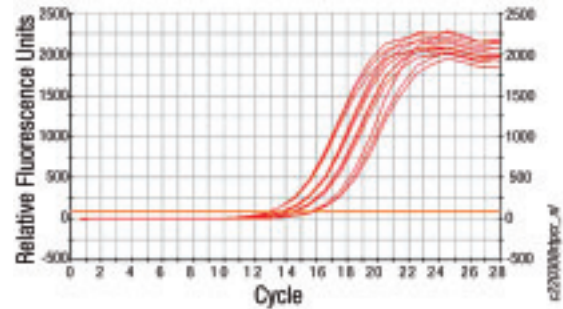


Figure 3. Real-time RT-PCR amplification of β -actin mRNA from serial dilutions of a reference sample containing total cellular RNA from HeLa cells. Total cellular RNA from 3 replicates each of 4 serial dilutions of a reference sample from HeLa cells was amplified using the MasterAmp™ Real-Time RT-PCR Kit. The average C_T from each dilution series was used to construct the standard curve in Figure 4.

shRNA-actin transfected cells and from mock-transfected cells was determined by plotting the respective C_T values on the standard curve (Figure 4). When compared to the standard curve, these values represent a 3-fold decrease (as determined by the iCycler iQ software) in β -actin levels in the shRNA-actin transfected cells compared to the mock-transfected cells. Therefore, the shRNA-actin reduced expression of β -actin by 3-fold or 67%.

Conclusion

Using the MasterAmp Real-Time RT-PCR Kit we detected and compared the mRNA levels of human β -actin using 100 ng of total RNA. Results show a 67% reduction

in β -actin mRNA in the HeLa cells that were transfected with the shRNA-actin. The results also demonstrate a sensitive method of analyzing RNAi-mediated gene silencing using the MasterAmp Real-Time RT-PCR Kit.

References

1. Pfaffl, M.W. and Hageleit, M. (2001) *Biotechnology Letters* **23**, 275.
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3. Meis, J.E. (2003) *EPICENTRE Forum* **10**(2),1.
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5. Yu, J. et al. (2002) *Proc. Natl. Acad. Sci. USA* **99**(9), 6047.

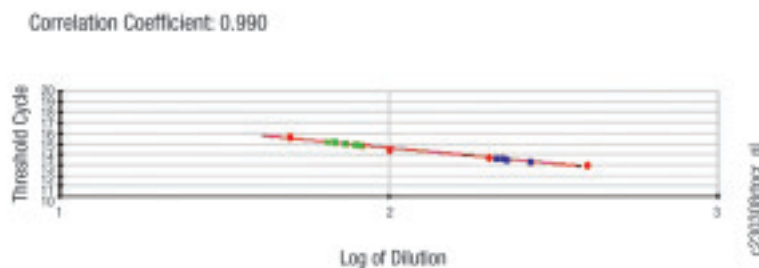


Figure 4. shRNA-actin reduced the level of β -actin mRNA by 67% in transfected HeLa cells. The average C_T values from dilutions of the reference RNA sample (●) and the average C_T values of the shRNA-actin transfected (■) cells and the mock-transfected (■) cells were plotted using the iCycler iQ software. The shRNA-actin produced a 3-fold reduction (67% “knockdown”) in the level of β -actin mRNA compared to mock-transfected cells.

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MasterAmp™ Real-Time RT-PCR Kit

MAR03825	25 Reactions
MAR03100	100 Reactions

Contents:

RetroAmp™ RT DNA Polymerase
2X Green RT-PCR PreMix
MasterAmp™ 10X PCR Enhancer
25 mM MgCl₂
25 mM MnSO₄
Sterile Water

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MasterPure™ RNA Purification Kit

MCR85102	100 Purifications
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Contents:

Red Cell Lysis Solution
Tissue and Cell Lysis Solution
MPC Protein Precipitation Reagent
2X T&C Lysis Solution
TE Buffer
DNase I, RNase-Free
Proteinase K
1X DNase Buffer

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