Quantitate sh/siRNA expression and Messenger RNA knockdown levels from a single QuantiMir RT reaction April 2007

Introduction

The use of sh/siRNAs to lower the levels of endogenous messenger RNA expression is a powerful and effective tool to study the roles of a targeted transcript in biological systems. Real-time qPCR has become the gold standard in accurately measuring the knockdown effects of specific sh/siRNAs in these experiments. The means of assessing the expression levels of sh/siRNA input has been typically achieved through laborious and nonquantitative Northern blot analyses. Here we present a new method to quantitate both the expression levels of sh/siRNA and the knockdown effect on the targeted messenger RNA transcript using a passive lysis buffer coupled with the QuantiMir kit cDNA synthesis and Real-time qPCR.

The "Cells-to-Cts" technology

Cell lysis and QuantiMir RT cDNA synthesis

After transfection of siRNA or shRNA expression constructs, the cells are removed from the transfection plate using standard trypsinization. The amount of cells tested range from 1X10⁴ to 1X10⁵. The cells are pelleted and subsequently lysed using 100 µl of the Cells-to-Cts passive lysis Buffer system. A 5 µl aliquot of the lysed cell suspension is then input directly into the QuantiMir RT reaction. The unique technologies in the QuantiMir RT kit enables the conversion of small RNA into detectable cDNA while simultaneously converting messenger RNA into cDNA. Accurate measurement of sh/siRNAs and the messenger RNA transcript in knockdown experiments can be accomplished using the cDNA created from this single RT reaction. The QuantiMir RT reaction generates enough cDNA suitable for numerous qPCR reactions.

Using the QuantiMir cDNA to measure p53 sh/siRNA levels and quantitate p53 knockdown

To measure the sh/siRNA molecule in QuantiMir cDNA, a primer was designed that was specific for the exact sequence of the p53 siRNA effector sequence. This primer was used in combination with the kit's 3' universal primer. The primer design is simple and straightforward. To quantitate p53 messenger RNA knockdown, two primer sets were designed to detect p53 mRNA. Primer Set A was designed 2 kb upstream of the siRNA target site and Primer Set B was designed such that the primers flanked the siRNA target site.



p53 shRNA expression vector



QuantiMir RT reactions anchor-tail small RNA and simultaneously convert mRNA to cDNA







Experimental Design

HT1080 cells in a suspension of 1×10^4 /ml were transduced with approximately 0.1 MOI units of Lentivirus containing the p53 shRNA expression construct. This suspension was then plated in 8 separate tissue culture plates. The media used was standard DMEM, 10% FBS, 2mM L-Glu with a 1% Pen/Strep mixture and incubated under 5% CO₂ at 37°C. Cells were collected in duplicate plates by trypsinization at the time points 0 h, 2h, 4h and 24h after transduction and plating. The cells were lysed using the Cells-to-Cts passive lysis buffer, treated with DNasel and then input into a QuantiMir RT reaction.

Real-time qPCR Analysis of QuantiMir cDNAs

Overview of entire Procedure

Three separate Real-time PCR SYBR green mastermixes were assembled to measure the p53 siRNA molecule and the p53 mRNA transcript. Primers were used at a concentration of 10 μ M each and 0.5 μ l cDNA added per 30 μ l qPCR reaction. Standard, default real-time qPCR cycling and data collection settings were used and the experiment conducted on an ABI 7300 instrument. Four transcript measurements:

- 1. p53 siRNA forward primer + universal reverse 3' primer
- 2. p53 mRNA Primer Set A (2kb upstream of siRNA target site)
- 3. p53 mRNA Primer Set B (across siRNA target site)
- 4. U6 transcript for normalization

Levels of the p53 siRNA molecule and the levels of the endogenous p53 mRNA transcript were calculated using U6 as normalization and graphed below. Successful quantitation of both the p53 siRNA increase over time as well as the decrease of endogenous p53 mRNA was achieved using the Cells-to-Cts system with the QuantiMir RT kit. Both p53 mRNA Primer Sets A and B yielded similar p53 profile data indicating that the QuantiMir RT reactions enable measuring transcripts at least 2 kb upstream of the dT first strand primer.

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Sample	p53.siRNA	U6	∆Ct		Sample	p53.mRNA	U6	∆Ct
0 hours	38.1533	24.4018	0.00007		0 hours	24.1065	24.4018	1.22714
2 hours	34.6024	28.0724	0.01082		2 hours	35.0143	28.0724	0.00813
4 hours	29.8489	28.3552	0.35510		4 hours	36.3947	28.3552	0.00380
24 hours	26.7228	27.4988	1.71238		24 hours	38.3172	27.4988	0.00055

Ct data





Summary

- 1. Cell-to-Ct lysis buffer is compatible with the QuantiMir RT kit
- QuantiMir RT kit converts siRNA and mRNA to cDNA efficiently (up to 2kb)
- 3. Enables quantitation of siRNA directly from cells
- 4. Allows measurement of mRNA knock down directly from cells

Cells-to-Cts + QuantiMir complete Kit Cat.# RA422A-1 20 rxns