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Real-time RT-PCR for analysis of gene knockdown by RNAi — controls and calculations

Gene silencing using RNAi involves transfection of an siRNA targeting the gene of interest and subsequent analysis of gene knockdown. Quantitative, real-time RT-PCR is a commonly used method which allows accurate, sensitive analysis of knockdown at the mRNA level. In this article, a real-time, one-step RT-PCR procedure using SYBR Green detection is described to demonstrate how this technique is used for measurement of relative gene knockdown. Read more about the principle of real-time RT-PCR and SYBR Green detection.

Steps of the RNAi experiment

In this experiment, real-time, one-step RT-PCR was used for analysis of CDC2 gene knockdown. The procedure was as follows:

Step 1: siRNA transfection - HeLa S3 cells were transfected with 0.5 nM siRNA targeting the human CDC2 gene or with a nonsilencing control siRNA which has no homology to any known mammalian gene. Untransfected cells were also analyzed.

Step 2: RNA purification - after a 48-hour incubation, cells were harvested, and RNA was purified using the RNeasy Mini Kit.

Step 3: Real-time, one-step RT-PCR - purified RNA was used as template for one-step RT-PCR, in which the reverse transcription reaction and the amplification take place in the same tube. The real-time RT-PCR was performed on a Bio-Rad DNA Engine Opticon 2 Real-Time PCR Detection System using gene-specific primers (QuantiTect Primer Assays) for either CDC2 or GAPDH (a housekeeping endogenous reference gene) with the QuantiTect SYBR Green RT-PCR Kit.

For one-step RT-PCR, the following controls were used:

- **No template control**
  This is a control reaction that contains all the components of the amplification reaction except for the template. This control enables detection of any contamination that may be present in the reaction components.
- **Endogenous reference gene for normalization**
  An endogenous reference gene is a gene whose expression level should not differ between samples. Comparison of the target gene with an endogenous reference gene allows the gene expression level of the target gene to be normalized to the amount of input RNA. Normalization corrects for variation in RNA content and other differences in sample handling. In this experiment, normalization was performed using the housekeeping gene GAPDH.
- **Template from untransfected cells**
  Analysis of an untransfected control shows the gene expression levels in the absence of any treatment.
- **Template from nonsilencing control siRNA**
  Target gene expression in cells transfected with nonsilencing siRNA is compared to gene expression after transfection of gene-specific siRNA to calculate knockdown.
  The gene expression level in the untransfected control should be compared to the level observed after transfection of the negative, nonsilencing control siRNA. Gene expression should be similar in both untransfected cells and cells transfected with negative control siRNA. Any differences in gene expression between these two samples are caused by nonspecific effects.
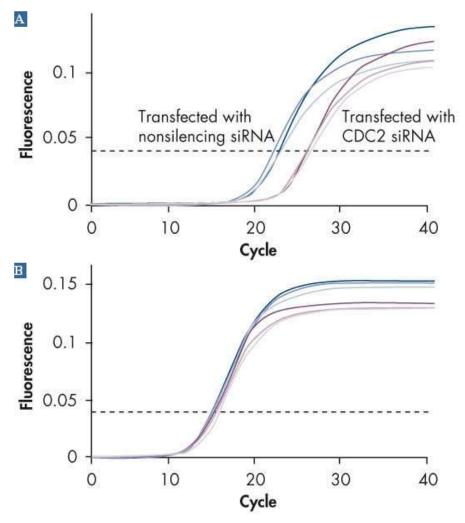- **At least 2 replicates per sample**
  Replicate experiments are important to account for sample-to-sample variation, ensuring that data are reliable and robust. A minimum of 2 replicate experiments should be performed and variation between replicates should be low. To ensure that variation is acceptably low, calculate the coefficient of variation (%Cv = (Standard deviation(replicates)/Mean C_T(replicates)) x 100). This should always be lower than 3%.

Results

Data from the real-time cycler are displayed as amplification plots with fluorescence plotted against the number of cycles (see figure 'Data from Real-Time RT-PCR Experiment').

Data from Real-Time RT-PCR Experiment

*HeLa S3 cells were transfected with siRNA targeting CDC2 or nonsilencing siRNA. After 48 hours, cells were harvested, RNA was purified, and real-time RT-PCR was performed on CDC2-siRNA–transfected samples and nonsilencing-siRNA– transfected samples using primers specific for **A** CDC2 or **B** the housekeeping gene GAPDH. Results from 3 replicates of cells transfected with CDC2 siRNA and 3 replicates of cells transfected with nonsilencing siRNA are shown.*

Threshold

The threshold is shown on the graph as a broken line. The threshold is either set manually by the user or automatically by the real-time cycler. It is the value where the fluorescence is above background, significantly below the plateau, and within the log-linear phase of the PCR. For more detailed information about data analysis, consult Critical Factors for Successful Real-Time PCR.

Threshold cycle (C_T)

The C_T is the cycle (read from the x-axis) at which the amplification plot crosses the threshold. This is the cycle at which there is a significant detectable increase in fluorescence. A low C_T value indicates high expression, as the template was detected after a low number of cycles. A high C_T value indicates low expression, as the template was only detected after a high number of cycles. The C_T values which can be read from the amplification plots in this experiment are detailed in the table below:

	CDC2-specific PCR primers, 3 replicates	**Average CDC2-specific PCR primers**	GAPDH-specific PCR primers, 3 replicates	**Average GAPDH-specific PCR primers**
Transfected with CDC2 siRNA	26.04, 26.2, 26.42	**26.22**	15.27, 15.38, 15.81	**15.49**
Transfected with nonsilencing siRNA	22.9, 23.13, 22.34	**22.79**	15.18, 15.41, 15.25	**15.28**

Calculation of knockdown using the $\Delta\Delta C_T$ method

The $\Delta\Delta C_T$ method assumes that the amplification efficiencies of the target gene (CDC2) and the endogenous reference gene (GAPDH) are very similar. Using the $\Delta\Delta C_T$ method, knockdown is calculated as shown below.

In the formulae, "sample" refers to the transfections with gene-specific siRNA (CDC2 siRNA) and "calibrator" refers to the transfections with nonsilencing siRNA.

Formula*	Result
ΔC_T (sample) = C_T target gene − C_T reference gene ΔC_T (calibrator) = C_T target gene − C_T reference gene	ΔC_T (sample) = 26.22 − 15.49 = 10.73 ΔC_T (calibrator) = 22.79 − 15.28 = 7.51
$\Delta\Delta C_T = \Delta C_T$ (sample) − ΔC_T (calibrator)	$\Delta\Delta C_T$ = 10.73 − 7.51 = 3.22
Normalized target gene expression in sample = $2^{-\Delta\Delta C_T}$	Normalized target gene expression in sample = $2^{-3.22}$ = 0.107

* "sample" is the gene-specific–siRNA transfection.
"calibrator" is the nonsilencing-siRNA transfection.

The normalized level of CDC2 gene expression in cells transfected with nonsilencing siRNA (calibrator) is always 1 or 100%. From the calculation above, the normalized level of CDC2 gene expression in CDC2-siRNA–transfected cells (sample) is 0.107 or 10.7%. **Therefore the level of knockdown achieved is 100% − 10.7% = 89.3%**.

In general, knockdown of 70% or greater is considered to be significantly high. However, depending on the cell type, target gene, and downstream assay, lower knockdown levels may also be sufficient for RNAi studies.
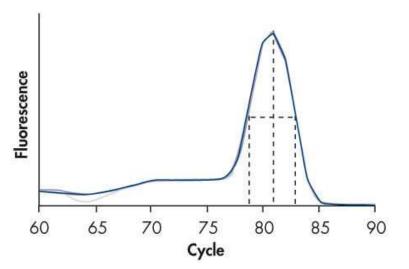
Melting curve analysis for quality control

When DNA-binding dyes such as SYBR Green are used for real-time RT-PCR, melting curve analysis of the RT-PCR products can be performed on the real-time cycler for the purpose of quality control.

Double-stranded DNA has a melting point (T_m) which is the temperature at which 50% of the DNA molecules are single stranded. In melting curve analysis, RT-PCR products are heated and a sudden decrease in fluorescence is detected when the T_m is reached due to the dissociation of DNA strands and release of SYBR Green dye. Typically, the negative first derivative of the melting curves are plotted and the melting point is the maximum of the resulting melting peak.

A single, clear peak demonstrates that the specific PCR product is detectable and that primer-dimers are absent (see figure 'Melting Curve Analysis'). This is important, as primer-dimers or nonspecific products would contribute to the fluorescent signal and may cause inaccurate results.

Melting Curve Analysis

Melting curve analysis was performed on RT-PCR products achieved using GAPDH primers and template from
untransfected N2a mouse neuroblastoma cells. Analysis was performed in triplicate. All 3 replicates overlap in a single
peak, showing the specificity of the PCR and the absence of primer-dimers.

As well as the $\Delta\Delta C_T$ method described in detail above, knockdown can alternatively be calculated using the standard curve method. For more detailed information about the standard curve method, descriptions of different real-time RT-PCR methods, a glossary of terms, and application data, consult Critical Factors for Successful Real-Time PCR.