GE Healthcare

Tech Note

Demonstration of a ΔΔCq Calculation Method to Compute Relative Gene Expression from qPCR Data

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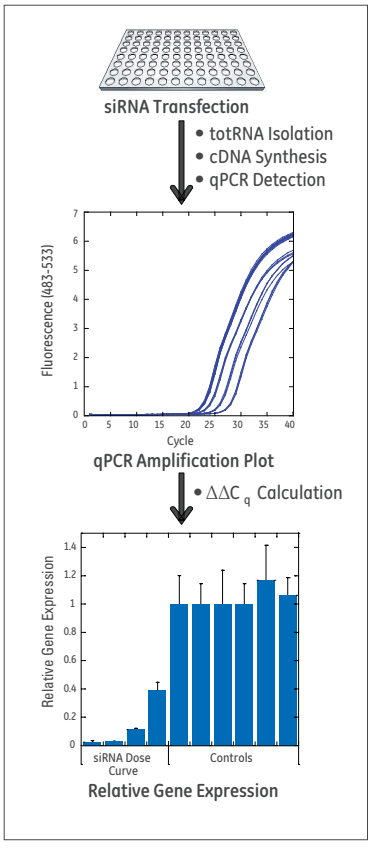
Abstract:

This technical note demonstrates the utility of a $\Delta\Delta C_q$ method for calculating relative gene expression and percent knockdown from quantification cycle (C_q) values obtained by quantitative real-time PCR (qPCR) analysis in an RNA interference (RNAi) experiment. In this study, the human aldolase A (ALDOA) message is silenced with the corresponding siGENOME™ SMARTpool™ siRNA. To determine relative gene expression, probe-based qPCR is performed using Thermo Scientific™ Solaris™ qPCR Gene Expression Assays with cDNA synthesized from total RNA harvested from cell culture. Here, a $\Delta\Delta C_q$ method is demonstrated as a normalized determination of gene knockdown, and the experimental controls it requires are described.

Introduction:



RNAi-mediated gene silencing using small interfering RNAs (siRNAs) is an effective technique used for varied applications from primary academic research to therapeutic discovery. While phenotypic observations may elucidate the effect of target-specific knockdown on biological systems, silencing efficacy should be confirmed to ensure confidence in phenotypic results. Efficacy is commonly reported as relative percent knockdown of mRNA levels compared to controls and can be determined in high throughput with easy-to-use, commercially available qPCR gene expression assays.

Solaris™ qPCR Gene Expression Assays are pre-designed, high performance gene-specific probe and primer pairs that utilize Minor Groove Binder (MGB™) and Superbase™ technologies (Epoch Biosciences Inc) to deliver reliable expression data. This technical note outlines a $\Delta\Delta C_q$ method for calculating experimental percent knockdown (%KD) from C_q values obtained by Solaris qPCR analysis in an RNAi experiment to knockdown the gene expression of ALDOA in cell culture (Figure 1).

Overall, $\Delta\Delta C_q$ yields a normalized, relative gene expression value. This is accomplished by normalization of a gene target with experimental treatment to an endogenous reference gene(s) whose expression should remain unchanged by the treatment. Subsequently, this value is normalized to the targeted gene's expression detected in a separate control sample.

Several variations on calculating relative gene expression from qPCR data exist; the method shown here is adapted for an experimental setup employing cells treated in biological replicates. For a method employing technical replicates, please see Bustin.[1]

Figure 1. Experimental workflow. siGENOME SMARTpool siRNA targeting ALDOA was transfected into HeLa cells and cDNA was synthesized from RNA isolated 48 hours post-transfection. Solaris qPCR Gene Expression reagents were used to detect ALDOA and reference genes' cDNA, and relative gene expression was calculated from C_q values using a $\Delta\Delta C_q$ method.

Materials and Methods:

Cell Culture, siRNA Transfection and RNA Isolation

HeLa cells (ATCC Cat. #CCL-2) were plated at 10,000 cells/well in a 96-well format and incubated overnight at 37 °C with 5% CO_2. Cells were treated with either siGENOME, SMARTpool siRNA targeting, ALDOA Cat. #M-010376-01) or siGENOME Non-targeting siRNA Pool #1 (Cat. #D-001206-13) complexed with the DharmaFECT™ 1 transfection reagent (Cat. #T-2001). siRNA complexes were transfected at 10, 1 and 0.1 nM final siRNA concentration in triplicate wells, creating biological replicates. Triplicates of mock transfected (MT; transfection reagent only) and untreated (UT) controls were also prepared.

Total RNA was isolated from each well simultaneously with the vacuum manifold-based Promega™ SV 96™ RNA Isolation System (Cat. #Z3500) 48 hours post-transfection. The RNA from each eluate was used in separate reverse transcription reactions. RNA concentrations were not quantified as the relative gene expression data from each sample were normalized to an endogenous reference gene for each well.

Reverse Transcription - Quantitative Polymerase Chain Reaction

Total RNA from each sample (5 µL) was reverse transcribed with the Thermo Scientific™ Verso™ cDNA synthesis kit (Cat. #AB-1453) using a 3:1 (volume:volume) mixture of random hexamers to anchored oligo-dT primers in a 20 µL reaction according to the manufacturer's protocol. No reverse transcriptase controls were prepared from untreated cells' total RNA and no template controls were prepared with water in place of total RNA to indicate potential genomic DNA contamination in isolated total RNA and contamination of reagents, respectively. Each biological replicate was assayed for the siRNA-targeted gene, ALDOA, as well as the endogenous reference genes GAPDH and RPS18 using Solaris™ qPCR Gene Expression Assays (Cat. #AX-010376-00, AX-011890-00, AX-004253-00) and Solaris™ qPCR Master Mix plus ROX (Cat. #AB-4350). No reverse transcriptase and no template controls were also assayed with qPCR detection for each target. Two µL of each three-fold diluted cDNA reaction were used in 12 µL qPCR reactions and transferred into a white 384-well plate (Roche Cat. #04729749001) with the aid of a Dharmacon™ PlateMate™ 2 × 3 liquid handler. qPCR thermal cycling and fluorescent data acquisition were performed with a Roche™ LightCycler™ 480 instrument and C_q values were called using the LightCycler 480 software's 'Fit Points' algorithm yielding amplification plots (Figure 2). A $\Delta\Delta C_q$ method was then used to process these data to calculate relative gene expression for the RNAi experiment.

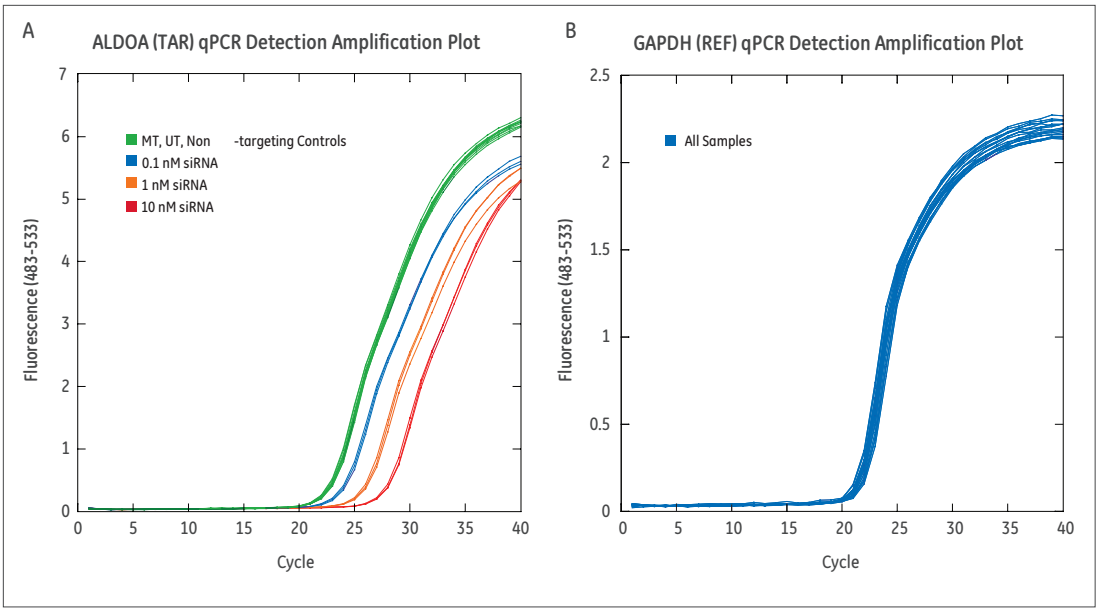


Figure 2. qPCR Amplification curves for ALDOA and GAPDH. Amplification curves represent cDNA detected in samples treated for ALDOA siRNA knockdown **(A.)** or control samples **(B.)** The ALDOA amplification curves exhibit siRNA dose-dependent message knockdown, with higher C_q values representing lower expression, or more effective silencing. For reference, a difference of one C_q value represents a 50% change in expression, while differences of 3.3 and 6.6 C_q values represent approximately 90% and 99% changes, respectively. In contrast, amplification curves for all GAPDH reference samples yield similar C_q values, indicating GADPH expression was not significantly affected by the treatment.

$\Delta\Delta C_q$ Calculations:

In the RNAi experiment described here, expression of the siRNA-treated ALDOA gene target (TAR) was normalized to non-targeted GAPDH or RSP18 reference gene (REF) expression levels within the same sample to determine ΔC_q (Step 1, Box 1). This step serves to correct for non-treatment-related variation among wells such as potential differences in cell number. C_q values of technical replicates can be averaged at this step if they were included in the experimental design. For biological replicates in this experiment, the ΔC_q for each replicate was exponentially transformed to the ΔC_q Expression (Step 2) before averaging and determining the standard deviation (Step 3). The mean was then normalized to the expression of ALDOA (TAR) from a separate well treated with Non-targeting siRNA to find $\Delta\Delta C_q$ Expression (Step 4). This accounted for any effects associated with the experimental procedure and was expressed as the ratio of the targeted ΔC_q Expression to the non-targeted ΔC_q Expression.

Box 1. $\Delta\Delta C_q$ Overview:

Step 1. Normalize to (REF): $\Delta C_q = C_{q\,(TAR)} - C_{q\,(REF)}$

Step 2. Exponential expression transform: ΔC_q Expression $= 2^{-\Delta Cq}$

Step 3. Average replicates and calculate standard deviation

Step 4. Normalize to treatment control

Step 5. % KD = $(1 - \Delta\Delta C_q) \times 100$

	Given Values		Step 1	Step 2	Step 4	Step 5
	$C_{q(REF)}$	$C_{q\,(TAR)}$	$C_{q\,(TAR)-(REF)}$	ΔC_q Expression	$\Delta\Delta C_q$	% KD
Non-targeting Control	21.9	23.1	1.2	0.43	1.00	–
TAR_{ALDOA}	27.5	34.3	6.7	0.01	0.02	98

In an RNAi experiment, ΔC_q Expression is normalized to a corresponding Non-targeting siRNA sample. In other experimental platforms – such as a small molecule treatment – it may be appropriate to normalize to untreated or vehicle-only control samples. Percent knockdown was calculated by subtracting the normalized $\Delta\Delta C_q$ Expression from 1 (defined by the level of expression for untreated sample) and multiplying by 100 (Step 5). Table 1 illustrates a complete list of values showing how to carry multiple data points with biological replicates and mock transfected and untreated controls through this $\Delta\Delta C_q$ method.

Table 1. Detailed $\Delta\Delta C_q$ Example:

	A	B	C	D	E	F	G	H	I	J	K
	siRNA Treatment	(Conc)	C_q GAPDH	C_q ALDOA	ΔC_q	ΔC_q Expression	Mean ΔC_q Expression	ΔC_q Expression Std. Dev.	$\Delta\Delta C_q$ Expression	$\Delta\Delta C_q$ Expression Std Dev.	%KD
					$= (Cq_{ALDOA} - Cq_{GAPDH})$	$= 2^{-\Delta Cq}$	Average Replicates	Std. Dev. Replicates	Normalize to NTC		$= (1-\Delta\Delta C_q)*100$
1	**ALDOA**	10 nM	20.6	27.6	7.01	0.008	0.009	0.002	0.027	0.0057	97
2			20.8	27.3	6.54	0.011			= G1/G10	= H1/G10	
3			20.9	27.6	6.69	0.010					
4		1 nM	20.7	25.6	4.89	0.034	0.034	0.003	0.111	0.010	89
5			20.6	25.4	4.75	0.037			= G4/G13	= H4/G13	
6			20.6	25.6	5.00	0.031					
7		0.1 nM	20.7	23.5	2.82	0.142	0.123	0.016	0.394	0.052	61
8			20.6	23.7	3.10	0.117			= G7/G16	= H7/G16	
9			20.4	23.6	3.17	0.111					
10	**NTC**	10 nM	20.5	22.2	1.72	0.304	0.349	0.051	1.000	0.145	
11			21.2	22.5	1.31	0.403			= G10/G10	= H10/G10	
12			21.0	22.5	1.56	0.339					
13		1 nM	21.2	22.5	1.37	0.387	0.306	0.073	1.000	0.239	
14			20.7	22.5	1.81	0.285			= G13/G13	= H13/G13	
15			20.6	22.6	2.03	0.245					
16		0.1 nM	21.9	23.4	1.48	0.358	0.312	0.045	1.000	0.145	
17			20.7	22.6	1.90	0.268			= G16/G16	= H16/G16	
18			20.5	22.2	1.69	0.310					
19	**Mock**	N/A	20.8	22.1	1.29	0.409	0.364	0.077	1.168	0.247	
20	**Transfected**		20.2	22.1	1.86	0.275			= G19/G16	= H19/G16	
21			20.9	22.2	1.29	0.409					
22	**Untreated**	N/A	20.7	22.4	1.69	0.310	0.331	0.039	1.062	0.125	
23			21.0	22.4	1.41	0.376			= G22/G16	= H22/G16	
24			20.8	22.5	1.70	0.308					

Column A: Treatment on the cell.

Column B: Final concentration (Conc) of siRNA [ALDOA or Non-targeting Control (NTC)] or Not Applicable (N/A) and for Mock Transfected and Untreated control samples.

Column C: C_q value reported by software for GAPDH (REF)

Column D: C_q value reported by software for ALDOA (TAR)

Column E: Normalize C_q values for all TAR samples to the REF gene of its corresponding sample. This is expressed as the difference in C_q values for target (Column D) and reference (Column C) messages, ΔC_q.

Column F: Exponentially transform ΔC_q to ΔC_q Expression for each biological replicate; 2 raised to the $-\Delta C_q$ (Column E) yields ΔC_q Expression. Note the base of 2 assumes 100% qPCR amplification efficiency for all reactions, or a doubling of amplicon with each subsequent qPCR cycle.

Column G: Mean of ΔC_q Expression replicates (Column F).

Column H: Standard deviation of the mean for ΔC_q Expression replicates.

Column I: Normalize the TAR Mean ΔC_q Expression to that of the Non-targeting Control to obtain $\Delta\Delta C_q$ Expression. This is expressed as the ratio of the targeted Mean ΔC_q Expression to that of the non-targeted for samples of corresponding concentration. For MT and UT controls – that do not have association with concentration – normalize to the lowest concentration NTC Mean ΔC_q Expression.

Column J: To find the standard deviation of $\Delta\Delta C_q$ Expression, divide the standard deviation of the targeted sample's Mean ΔC_q Expression (Column H) by that of the Non-targeting Control sample of corresponding concentration (Column G). The standard deviation of $\Delta\Delta C_q$ Expression for MT and UT controls is found by dividing their Mean ΔC_q Expression standard deviation's (Column H) by the Mean ΔC_q Expression of the lowest concentration for the non-targeting group (Column G).

Column K: Percent knockdown (%KD) is calculated by subtracting the normalized $\Delta\Delta C_q$ Expression from 1 (defined by the level of expression for untreated sample) and multiplying by 100.

Results and Conclusions:

Calculations using the $\Delta\Delta C_q$ method described here revealed dose-dependent silencing of ALDOA message with 61%, 89% and 97% knockdown when cells were treated with 0.1, 1 and 10 nM final concentration of the targeting SMARTpool siRNA, respectively [normalized to GAPDH REF (Figure 3)]. Similar results were obtained with the REF gene, RPS18 (data not shown). Comparison of mock transfected and Non-targeting siRNA samples to untreated samples indicated no significant impact of the transfection reagent or siRNA treatment, respectively, on expression of the REF genes detected in this experiment.

In summary, the utility of this $\Delta\Delta C_q$ method has been demonstrated in the context of an RNAi experiment for calculating relative gene expression from C_q values obtained from qPCR analysis. By normalizing changes between the target and reference genes within wells, and by normalizing this ΔC_q Expression to that of a control sample for the experimental treatment, the method described here yields relative gene expression values that account for both experimental and non-experimental variation that may otherwise introduce bias in results.

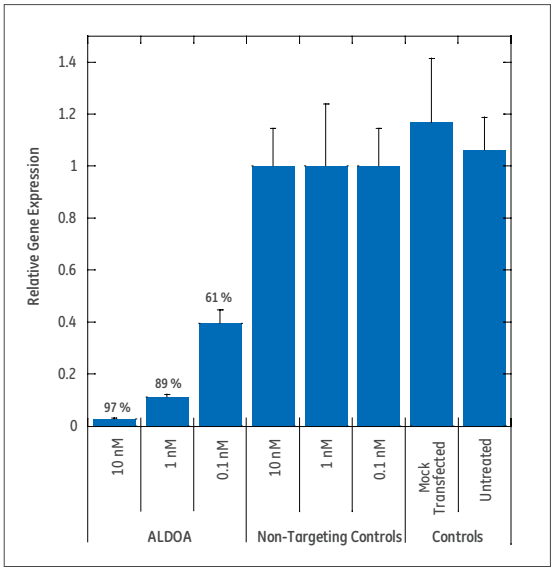


Figure 3. siRNA-mediated silencing of ALDOA was assessed using a $\Delta\Delta C_q$ method to determine relative gene expression from qPCR data with GAPDH as an endogenous REF gene. The cells exhibited siRNA dose-dependent knockdown of ALDOA message, with mRNA reduction by 61%, 89% and 97% when cells were treated with 0.1, 1 and 10 nM final concentration of the targeting SMARTpool siRNA, respectively. Comparison of mock transfected and Non-targeting siRNA to Untreated samples suggests that there is no significant effect of transfection reagent or transfection reagent plus siRNA, respectively, on the cell.

Reference:

1. Bustin, Stephen A., ed. A-Z of Quantitative PCR. La Jolla, CA: International University Line, 2004-2006.

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