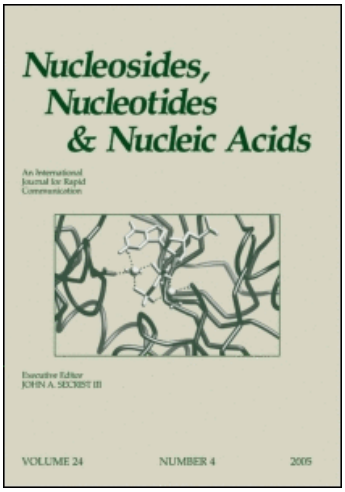


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# Quantitative Real Time PCR of Deoxycytidine Kinase mRNA by Light Cycler PCR; in Relation to Enzyme Activity

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# Quantitative Real Time PCR of Deoxycytidine Kinase mRNA by Light Cycler PCR; in Relation to Enzyme Activity

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## ABSTRACT

Deoxycytidine kinase (dCK) is essential for the phosphorylation of several deoxyribonucleosides and various analogues such as gemcitabine (2′,2′-difluorodeoxycytidine). We developed and optimized a sensitive real time Light Cycler (LC) PCR assay for dCK with SYBR green detection. The enzymatic activity measured in the same human xenografts of dCK correlated excellently with dCK mRNA expression levels measured by the LC. This assay can be used for evaluation of dCK expression in tumors.

*Key Words:* Deoxycytidine kinase; Competive template-RT-PCR; Light cycler PCR.

## INTRODUCTION

Deoxyribonucleoside analogues such as cytosine arabinoside (Ara-C) or gemcitabine (2′2′-difluorodeoxycytidine) are dependent on the enzyme deoxycytidine kinase (dCK) for its activity.[1] These drugs are widely used chemotherapeutic agents in leukemia and solid

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tumors, respectively. For evaluation of dCK mRNA expression, we earlier developed a conventional Competitive Template Reverse Transcriptase Polymerase Chain Reaction assay (CT-RT-PCR).[2] However, real time PCR enables more rapid and sensitive detection of mRNA expression using either SYBR Green or fluorescent probes using a Light Cycler (LC). We developed and optimized a sensitive real time PCR assay for dCK with SYBR green detection.

## MATERIALS AND METHODS

Tissues were immediately frozen in liquid nitrogen. RNA was isolated from pulverized tissues using RNAzol (Campro Scientific). RNA was reverse-transcribed to cDNA using random hexamers as described previously.[2] Cell pellets were suspended in an aliquot of 200 μl of RNAzol per $10^6$ cells. Twenty micrograms of the isolated RNA was used for reverse transcription into cDNA. Random hexamers were used as primers for Moloney murine leukemia virus reverse transcriptase (M-MLV-RT) at a concentration of 0.045 μg/μl. After a brief incubation at 56°C to remove secondary structures, samples were quickly cooled down on ice and annealing of the hexamers also took place on ice. The extension of the primers was at 42°C using M-MLV-RT. The reaction was terminated by heating at 95 C for 5 min. cDNA samples were stored at −20°C until further use. The assay for dCK mRNA expression was optimized for the LC real-time PCR apparatus (Roche Diagnostics). Primers for dCK were based on those described for conventional PCR:[2] forward primer: 5′-GAAGGGAACATCGCTGCAGG; reverse primer: 5′-TGGCCAAATTGGTTATTCATCC. The primers for β-Actin were similar to those described for conventional PCR: forward: 5′-GAT TCC TAT GTG GGC GAC GAG and reverse: 5′-CCA TCT CTT GCT CGA AGT CC. A Master SYBR Green I working solution was prepared by mixing 60 μl of LightCycler-FastStart Reaction Mix SYBR Green I with 4 μl of LC-FastStart Enzyme (Roche Laboratories). Two volumes of this working solution were mixed with 16 volumes of varying concentrations of $MgCl_2$, dCK or β-Actin primers and $H_2O$. Thereafter, 18 μl of this solution was pipetted into a LC capillary. The reaction was started after the addition of 2 μl cDNA of varying dilutions from tumor cells. For dCK, the final optimal concentration of $MgCl_2$ was 3 mM and that of the primer 0.7 μM. For β-Actin these concentrations were 5 mM and 0.9 μM. The dCK PCR program consisted of an initial denaturation step at 95°C for 10 minutes, followed by 45 cycles of 10 sec at 95°C, 5 sec at 60°C and 17 sec at 72°C. For β-Actin the PCR program was similar to that of dCK, except for the elongation time of 22 sec instead of 17 sec. Melting curves were produced to verify the purity of the products. In order to confirm the purity of the PCR products they were also analyzed on an agarose gel. Standard curves were produced to measure the expression of dCK which was quantified relative to β-Actin.

## RESULTS AND DISCUSSION

Optimal conditions for LC real time PCR are different from those for conventional PCR. We optimized the PCR procedure by using various $MgCl_2$ and primer concentrations, as well as various annealing temperatures for both dCK and ß-Actin.

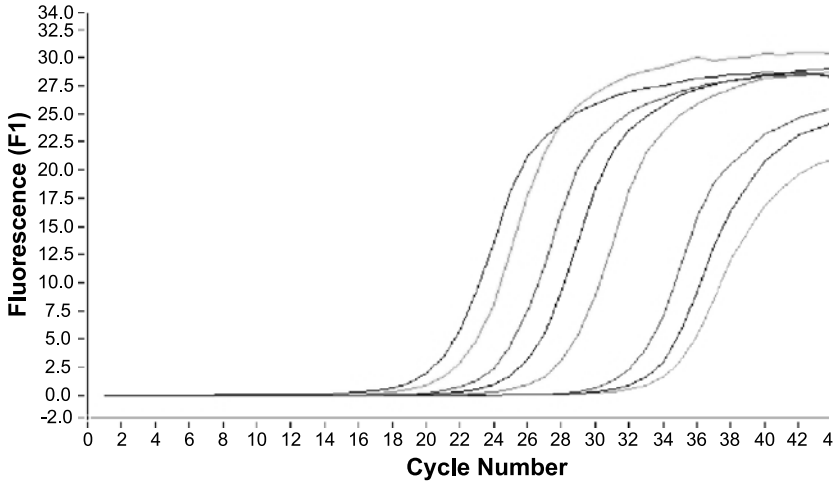


***Figure 1.*** Representative analysis of dCK gene expression using the LC Real Time PCR. Each line represents a different dilution of cDNA prepared from the leukemic CEM cell line (from left to right: 3, 10, 30, 100, 300, 1000, 3000, 10000 times diluted). Cp is calculated from the intercept of the lines with the X-axis, according to Roche software.



Crossing-points (Cp) defined by the program and the evaluation of melting curves were used for this optimization. Conditions as described in the previous section appeared to be optimal and we found linearity over a 10000-fold range (Fig. 1).

In addition, we observed a good correlation (Pearson: r = 0.972; p = 0.003) between LC-PCR and enzyme activity. Earlier reports describe real time PCR of dCK with Taqman using fluorescent probes specifically designed for the target.[3,4] However in this study, for the first time a quantitative real time PCR has been developed for dCK that uses SYBR Green fluorescent dye to detect amplification of specific PCR products. This technique provides great flexibility because no target-specific probes are required and yet specific products can be distinguished from non-specific products by studying melting peak profiles. Because Kroep et al showed that pretreatment dCK levels predict in vivo gemcitabine sensitivity[5] the SYBR Green LC-PCR for dCK is applicable in the clinic to predict chemotherapeutic agent sensitivity.

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