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Temperature Non-homogeneity in Rapid Airflow-Based Cycler Significantly Affects Real-Time PCR

BioTechniques 33:508-512 (September 2002)

We used real-time quantitative PCR with the LightCycler™ instrument (Roche Applied Science, Mannheim, Germany) to examine p16 deletions in childhood ALL. The LightCycler instrument is a rapid thermal cycler that utilizes a temperature-controlled airflow for the heating and cooling of the samples. The PCRs are carried out in glass capillaries placed on a perimeter of a circular carousel with 32 positions. For the quantification of a particular sample, the crossing point (C_p) value is used. The C_p is the fractional cycle number at which the fluorescence signal reaches defined intensity within the early exponential phase of the reaction. Primers and hybridization probes for both p16 gene and housekeeping gene β -2-microglobulin (b2m) were designed in collaboration with TIB Molbiol (Berlin, Germany). Table 1 lists the sequences of primers and probes.

While the housekeeping gene assay we established was highly reproducible, unexpected variations in p16 genomic DNA experiments occurred. Finally, we found that the variation apparently depended on the position of the particular capillary within the sample carousel. To confirm this theory, we performed a series of experiments with one common PCR mixture (containing p16 positive DNA) divided into capillaries just before the PCR started. In such an experiment, the result (C_p ergo calculated concentration of p16 DNA) should be identical in all capillaries. Nevertheless, this was not the case, and the shape and amplitude of fluorescence curves, as well as the calculated p16 concentrations, varied markedly. Moreover, repetition of this experiment disclosed a constant pattern of the results that depended on the position of capillaries within the carousel. While the difference in results in the first eight (1–8) and last two (31 and 32) po-

Table 1. Sequences of Primers and Probes

Oligonucleotide	5'→3' Sequence	Application
p16-F	TGGACCTGGCTGAGGAGCT	primer
p16-gDNA-R	TCCTCACCTGAGGGACCTTCC	primer
p16-cDNA-R	TCAATCGGGGATGTCTGAG	primer
p16-LC	CTGCCTCTGGTGCCCCCGC	probe
p16-FL	GCGGCATCTATGCGGGCATGGTT	probe
b2m-F	CCAGCAGAGAATGGAAAGTC	primer
b2m-gDNA-R	AGTGGGGGTGAATTCAGTGTAG	primer
b2m-cDNA-R	GATGCTGCTTACATGTCTCG	primer
b2m-LC	ATGAAACCCAGACACATAGCAATTCAG	probe
b2m-FL	TTCTTCAGTAAGTCAACTTCAATGTCTGGA	probe

sitions were broadly comparable (C_p , 26.7 ± 0.5), the C_p value progressively decreased with minimum in positions 23–25 (C_p , approximately 23.2). We considered two possible explanations for this phenomenon. The decreased C_p in a part of the carousel could be either absolute, caused by temperature variations within the carousel, or rela-

tive, caused by the delayed fluorescence measurement in different capillaries. The time needed for measurement of all 32 capillaries is about 5 s, and the fluorescence intensity might be unstable during this period. We wanted to rule out the latter explanation (influence of a time needed for the measurement of all positions in

Figure 1. Relation of C_p value to the position of the sample within the carousel. Identical reactions were placed into the carousel with 32 positions, and PCR was carried out under standard conditions. While the C_p -value fluctuation was substantial in experiments with genomic DNA, only nonsignificant differences between samples were present when cDNA was used. The results of the genomic DNA experiment were markedly improved when rotation of samples during the run was enabled [curve “genomic DNA (new sw)”]. The difference of C_p between two samples ($\Delta C_p = 3.2$) results in approximately one log difference in target concentration calculation.

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carousel) definitely. Thus, we performed PCR with capillaries only in positions 1 and 23 (position previously associated with the lowest C_p), respectively. Since there were no capillaries in the remaining 30 positions, the delay between the measurement of samples 1 and 23 was less than 1 s. Remarkably, this real-time quantitative PCR showed identical C_p values in both samples, suggesting that the time between measurements might also play a role in terms of target concentration assessment when the full carousel is used. However, we repeated exactly the same experiment with just one modification: we placed capillaries containing only water into remaining 30 positions (2–22 and 24–32) of the carousel. This action had no effect on carousel movement compared to the previous experiment because the capillaries contain no fluorescence; thus, the reading apparatus of the LightCycler ignores them

and the time between the measurement of positions 1 and 23 is the same (<1 s). Astonishingly, the result was completely different with significantly lower C_p in the position 23 (C_p , 27.6 and 24.5 in positions 1 and 23, respectively). This means that the presence of capillaries in the carousel changes the temperature stability and/or the airflow inside the carousel chamber so that the results are affected demonstrably.

The denaturation temperature also had a high impact on the consistency of results. The higher the temperature, the more significant was the difference between samples in position 1 and 23, respectively. With denaturation increased to 97°C for 10 s in each cycle, the instrument showed even negative results (C_p , >40) in position 23 and clear positivity (C_p , approximately 22.5) in position 1. When the denaturation time was shortened to 1 s, the sample 23 showed positivity, but the fluorescence curve

was very flat; thus, the calculation of template concentration was not completely reliable. These results imply that, during the denaturation step, the temperature in a part of the carousel may reach even higher values than 97°C, which is set in the cycling program. This overheating may lead to a very fast degradation of the DNA polymerase enzyme, deoxynucleotides, and/or other reagents. This notion was supported by the fact that even with non-problematic target (b2m) the result of experiment with the denaturation temperature increased to 97°C/10 s was significantly worse in the 23rd position (C_p in position 1 and 23 was 14.9 and 18.7, respectively).

A similar problem with the p16 genomic DNA has been already described by Wilhelm et al. (1). The authors concluded that a region with a higher melting temperature in the vicinity of the p16 PCR product and the resulting in-

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complete denaturation of the p16 genomic DNA target in a part of the carousel might be responsible for this phenomenon. The relatively minor temperature variation during the denaturation step (mainly in the first few cycles when the genomic DNA contributes significantly to the total amount of amplifiable target) then affects the intra-assay variability of results considerably. Our further results could support this theory. We tested the reproducibility of LightCycler technology also for the p16 mRNA assessment in an real-time quantitative RT-PCR method. The probes and forward primer were identical to the genomic DNA assay, and the reverse primer was different. It overlapped the fusion of exons 2 and 3 to prevent possible amplification of potentially contaminating genomic DNA. Interestingly, the C_p variation among capillaries was much lower in the real-time quantitative RT-PCR setting than in the genomic DNA experiments. We concluded that, as the p16 mRNA transcript apparently lacks the problematic surrounding sequence described by Wilhelm et al. (1), the C_p fluctuation was only insignificant in the real-time quantitative RT-PCR experiments. Wilhelm et al. (1) overcame the problem by increasing the denaturation temperature only for the first five cy-

cles. Although our results also improved with the same approach, the C_p difference between the best and worst position remained unacceptable (>3). Moreover, the fluorescence curves were very flat for capillaries 17–28.

We wanted to verify whether the results are caused by one particular instrument, but we have experienced exactly the same problems also with another LightCycler machine using identical PCR conditions. To overcome the PCR inaccuracy, we used a new LightCycler software (LightCycler3 Run version 5.32) that, unlike the previous version 3.39, ensures a permanent rotation of the sample carousel during the PCR. Thus, the potential temperature variability between capillaries in different positions should be minimized. The use of new software improved the results significantly, and the variability in C_p was less than 0.7 cycles in this experiment. Figure 1 shows the results of experiments with genomic DNA and cDNA using the original software and genomic DNA using the new version.

In conclusion, our results suggest that, with some targets, a relatively small temperature variation between the samples, the number of samples within the carousel, and the temperature profile of the reaction can significantly affect the results of the real-time quantitative PCR assay. We uncovered this phenomenon using the LightCycler instrument, but we cannot rule out the possibility that similar problems could alter the results in other real-time quantitative PCR systems. We have tested seven different PCR systems with various targets (in addition to p16 and b2m, we tested two systems for TEL/AML1, WT1 with SYBR® Green I, WT1 with hydrolyzation probe and Asparagine synthetase gene) on the LightCycler in our laboratory. The fluctuation of C_p was detectable in all of them when the software without permanent rotation was used. The pattern was always the same with maximal deviation (either negative or positive) in positions 21–25, which is in concordance with the theory of temperature instability within the reaction chamber. Although the C_p variations between the best and worst capillaries did not reach as big a difference as in

our experiments with p16 genomic DNA, the results were affected significantly (C_p difference, >1) in two other systems. This should be generally taken into account when results from experiments performed before the introduction of the new software (even the results already published) are analyzed. For all users of real-time PCR technology, we strongly recommend evaluating the susceptibility of a particular PCR system by testing more identical reactions in different positions of the carousel or heated block before the definitive establishment of the method. In all experiments, software enabling the permanent rotation of the sample carousel during the PCR is recommended when the LightCycler instrument is to be used.

REFERENCE

1. Wilhelm, J., M. Hahn, and A. Pingoud. 2000. Influence of DNA target melting behavior on real-time PCR quantification. *Clin. Chem.* 46:1738-1743.

The work was supported by the grant no. 131/99 from Charles University and project nos. 111300001 and 111300003 from the Ministry of Education. Address correspondence to Dr. Jan Zuna, 2nd Department of Paediatrics, 2nd Medical School, Charles University Prague, V uvalu 84, 150 06 - Prague 5, Czech Republic. e-mail: jan.zuna@lfmotol.cuni.cz

Received 5 April 2002; accepted 7 May 2002.

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