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Novel competitive PCR methods for quantitation of T-cell receptor delta (*TCRD)* gene rearrangements

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Abstract. Since the invention of the polymerase chain reaction (PCR) several quantitative PCR-based approaches have been described. Recently, the real-time PCR method became a standard in quantitative PCR, although high costs of the necessary equipment and reagents make it unaffordable for many laboratories. In this paper we describe two novel competitive PCR techniques, which were used to determine the frequency of T-cell receptor delta gene (*TCRD*) rearrangements in peripheral blood leukocytes. In the reference gene competitive PCR (rgc-PCR) the rearranged *TCRD* gene competes with the reference gene (RAG1) for common reagents (dNTPs and Taq polymerase). The intensity ratio of amplification products, *TCRD*/RAG1, corresponds to the portion of cells containing a rearrangement. A series of reactions was performed, in which RAG1 primers were added to the PCR after different numbers of cycles. On the basis of the number of cycles needed to obtain equal band intensity, the frequency of cells containing a rearrangement was calculated. In the common primer competitive PCR (cpc-PCR), two gene rearrangements, Vδ1-Jδ1 and Vδ2-Jδ1, compete for the common Jδ1 primer. The competing genes are amplified from the same genomic DNA template; therefore unlike in the method using the internal competitor, the results are not affected by the quantity or quality of the analysed sample. We showed that the rgc-PCR and cpc-PCR are reliable and give reproducible results. The methods do not require any expensive equipment or reagents, and can be used to determine the frequency of gene rearrangements.

Key words: competitive PCR, gene rearrangement, gene Vδ1-Jδ1, Vδ2-Jδ1, *TCRD*.

Introduction

The T-cell receptor delta gene (*TCRD*) consists of 8 variable (V), 3 diversity (D), 4 joining (J) and a single constant (C) region (TAKIHARA et al. 1989, ARDEN et al.

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1995, BOYSEN et al. 1997). During T-lymphoid differentiation somatic VDJ rearrangements occur, thereby generating variability of the *TCRD* gene (DYER 1989, HARA et al. 1991). Productive rearrangements of the *TCRD* and *TCRG* genes lead to the expression of the γδTCR, present on a small population of mature T-cells. Rearrangements of the *TCRD* gene are analysed by Southern blotting or by polymerase chain reaction (PCR), using primers specific for the recombining segments (BREIT et al. 1993).

The PCR was originally invented as a qualitative method, although attempts were made to establish a reliable quantitative variant of this technique. The earliest quantitation was through creation of standard curves by stopping the reaction at different cycles and determining the quantity of products (LARZUL et al. 1988). The process required a radioactively labelled probe used to increase the sensitivity of the reaction. It was very laborious, involving repeated runs at different dilutions of the template. Therefore, when the quantitative competitive PCR was invented in 1990, it was considered a breakthrough (GILLILAND et al. 1990, KELLOG et al. 1990). In competitive PCR two templates are amplified in a single reaction; one is the analysed sample and the second is the competitor. The competitor is an amplified DNA fragment that was cloned and modified in order to be distinguishable from the original PCR product. It contains a short insert, causing slower migration in a gel (GILLILAND et al. 1990, BABU et al.1993, KOZBOR et al.1993), or a single-base-pair alteration, so that it includes an artificial restriction site (GILLILAND et al. 1990). The competitive PCR was carried out with scalar amounts of specific competitor DNA fragments to a fixed quantity of the cDNA or of the genomic DNA. The ratio between the amplification products was linearly correlated to the input amount of the competitor in the reaction. The competitive PCR with an internal competitor is an inexpensive and reliable method, although its accuracy depends on the quality of the DNA template. In 1992 HIGUCHI et al. showed that ethidium bromide (EtBr), when added to the reaction, binds to the double-stranded PCR product and fluorescence can be observed when excited externally by UV light. The increase in the fluorescence of EtBr, due to accumulation of the PCR product, can be continuously monitored after each PCR cycle. The kinetics of fluorescence is directly related to the starting number of copies of the DNA template; the more copies of the target, the fewer cycles are necessary to detect the fluorescence (HIGUCHI et al. 1992, 1993). This approach was convenient since no competitor was used and gel electrophoresis was no longer needed, although it did not discriminate the specific from the unspecific PCR products. In 1993 Perkin-Elmer researchers combined Higuchi's kinetic method with the "exonuclease probes", originally invented by HOLLAND et al. (1991), to develop the TaqMan method (LEE et al. 1993). The fluorescent probe, which is labelled at the 5' end with the reporter dye and with the quencher at the 3' end, hybridizes to the amplified DNA fragment. Due to the 5'→3' exonuclease activity of the Taq polymerase, the probe is then degraded, the reporter dye is separated from the quencher and a fluorescent signal is generated. The fluorescence, which

is proportional to the amount of the product, is measured after each cycle and the copy number of the target sequence is determined by comparing its threshold cycle to the known plasmid concentrations. The concurrent LightCycler method uses hybridization probes that bring two different fluorescent labels together to allow energy transfer after they hybridized to the amplification product (WITTWER et al. 1997). Yet both commercial techniques have one disadvantage: they are quite expensive and are not affordable for smaller laboratories.

In this paper we describe two novel competitive PCR methods, which were used for quantitation of *TCRD* gene rearrangements. The methods are inexpensive and reliable, and can be used by low-budget laboratories for quantitative PCR assays.

Material and methods

Material

Genomic DNA from peripheral blood leukocytes (PBL) from 6 healthy individuals was isolated by the proteinase K phenol/chloroform method.

Common primer competitive PCR (cpc-PCR)

The PCR mixture of 30 μl total volume contained: 0.1 μg genomic DNA, 5 nmol of dATP, dCTP, dGTP, dTTP, (Promega, Madison, WI); 1 U Taq polymerase, a PCR buffer (Promega, Madison, WI) and 10 pmol of each primer. Oligonucleotide primers are listed in Table 1. After the initial denaturation at 94°C for 5', 40 cycles consisting of denaturation at 94°C for 1', annealing at 62°C for 1', and extension at 72°C for 2' were performed, followed by the final extension at 72°C for 7'. In order to compare the frequencies of two rearrangements, Vδ1-Jδ1 and Vδ2-Jδ1, three primers were included in the reaction mixture. Two 5' primers, Vδ1(–275) and Vδ2(–174), were rearrangement-specific, while the third 3' primer Jδ1(+62) was used by both amplicons. In this reaction the Vδ1(–275) and the Vδ2(-174) primers compete with each other for the common Jδ1(–62)

Table 1. List of primers

Vδ1-for	5'– ACT CAA GCC CAG TCA TCA GT
Vδ2 (–174)	5'– AGG AAG ACC CAA GGT AAC ACA
Vδ2a-for	5'– GAG TCA TGT CAG CCA TTG AG
Jδ1a-bac	5'– AAA TGC TAG CTA TTT CAC CCA
J1c-bac	5'– GAG TTA CTT ACT TGG TTC CAC
RAG1-for	5'– GCC ATG AAG AGC AGT GAA TTA
RAG1-bac	5'– AGG AAT TAA CTC ACA AAC TGC

primer. Consequently, the common primer is used up by the rearrangement that is present in a higher copy number and amplification of the less frequent rearrangement is stopped. The PCR products were analysed in a 2.5% agarose gel containing EtBr. The intensity ratio of the bands was directly related to the initial copy number (frequency) of the analysed rearrangements.

Reference gene competitive PCR (rgc-PCR)

The reaction mixture of the reference gene competitive PCR (rgc-PCR) contained the same components as the cpc-PCR except for the primers. For each rgc-PCR analysis a series of 6 reactions was performed. The rearrangement-specific primers were included in the master mix and the reference primers (RAG1-for and RAG1-bac, specific for the Recombinase Activating Gene 1) were added separately to each tube after: 2, 4, 6, 8, 10 and 12 cycles of PCR. In the first cycles of rgc-PCR, *TCRD* gene rearrangements were pre-amplified. After the addition of RAG1 primers, both primer sets started to compete for the dNTPs and the Taq polymerase. The later RAG1 primers were added, the more copies of the *TCRD* gene rearrangement were pre-amplified, therefore the TCRD/RAG1 copies ratio was increasing in subsequent reactions (Figure 1). The percentage of cells with *TCRD* gene rearrangements was calculated using the formula $f = 2^{1-n}$, where n represents the number of cycles needed to achieve balance between the amplification of the rearranged *TCRD* gene and RAG1 gene. As positive controls, vectors with cloned Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements were used.

Plasmid controls

Amplification products of the Vδ1-Jδ1 and Vδ2Jδ1 rearrangements were cloned into the pGEM-T Easy Vector (Promega, Madison, WI). On the basis of the DNA concentration measured by spectrophotometry and confirmed by a quantitative gel electrophoresis, the exact number of copies of vectors was determined. Vectors were diluted to concentrations of 30 000 copies/µl, 1000 copies/µl and 117 copies/µl, and used as controls in the competitive PCR.

Results

Comparison of the frequency of TCRD gene rearrangements in PBL

In humans, the repertoire of peripheral blood gd T-cells virtually consists of only two cell subsets (> 90%), expressing either the Vδ1-Jδ1 or the Vδ2-Jδ1 rearrangements. In order to compare the frequency of these two *TCRD* gene rearrangements we established a novel cpc-PCR method, which was used to analyse PBL samples from 6 healthy individuals. In the cpc-PCR the frequency of the Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements was assessed by comparing the intensity of the cor-

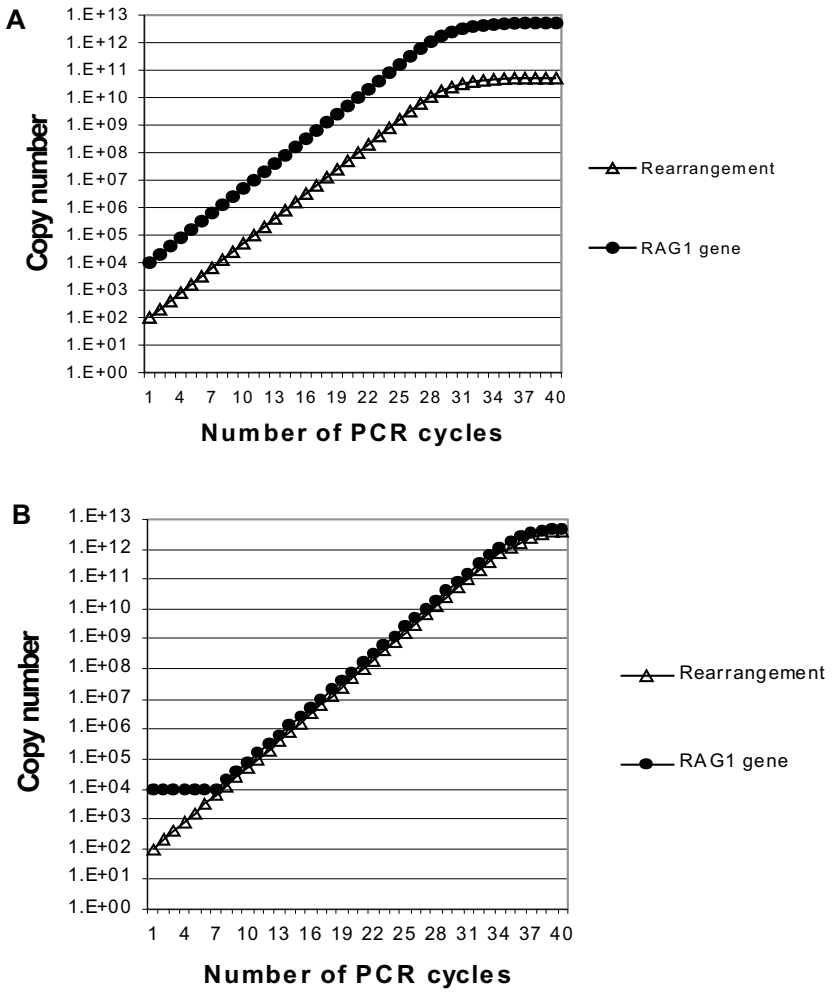

Figure 1. Theoretical model of the reference gene competitive PCR (rgc-PCR)

A = reference primers (RAG1) and *TCRD*-gene-rearrangement-specific primers were added to the reaction mixture. Both DNA templates were amplified simultaneously until the referential RAG1 gene has reached the plateau. Amplification of RAG1 and *TCRD* was stopped at the same time; therefore both fragments were amplified by the same factor and the proportion of copies of the *TCRD* and the RAG1 amplicons reflected the initial proportion of both targets.

B = PCR was started with *TCRD*-specific primers only, and RAG1 primers were added after the 6th cycle of PCR. After the initial pre-amplification of the *TCRD* gene rearrangement (2^6=64), both templates were amplified until the plateau was reached. Equal number of copies (similar intensity of PCR bands) indicated that the Vδ1-Jδ1 rearrangement was present in $1/2^{(6-1)}$=1/32 cells.

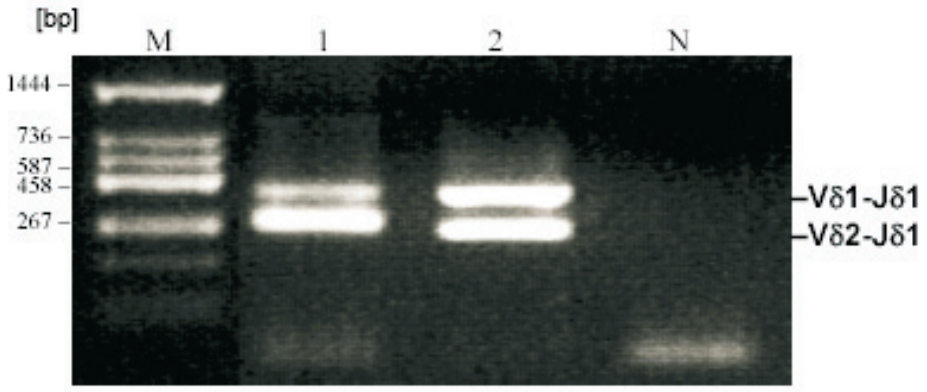

Figure 2. Frequency comparison of Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements in peripheral blood leukocytes by the common primer competitive PCR (cpc-PCR)
M = molecular weight marker, 1 = HD-21, 2 = vector control, N = no template control

responding PCR bands. The intensity of bands was estimated on a scale from 0 to 3, where 0 = no band visible, 1 = week band, 2 = strong band and 3 = very strong band. To validate this approach we mixed equal copy numbers of two vectors (10^3 copies each), one with the Vδ1-Jδ1 and the other with the Vδ2-Jδ1 rearrangement, and used them as a control. A representative analysis was shown in Figure 2. In the control both bands showed similar intensity, confirming that the Vδ1-Jδ1 and the Vδ2-Jδ1 rearrangements are amplified with equal efficiency, which is crucial for the analysis. In the analysed PBL sample the lower band was stronger than the upper band, indicating that Vδ2-Jδ1 rearrangement was present in more cells than the Vδ1-Jd1. Altogether, in 4 of 6 individuals the V2-J1 rearrangements were more frequent (HD-21: 3/1; HD-22: 2/1; HD-23: 2/1; HD-42: 2/1) and in 2 individuals the analysed rearrangements showed similar frequency (HD-24: 1/1; HD-41: 1/1).

Quantitation of cells containing TCRD gene rearrangements

The cpc-PCR allows to compare the frequency of two rearrangements, but it does not provide information about the percentage of cells containing them. For this purpose we established another PCR-based method, the rgc-PCR. In this method we compared the intensity of the amplification product of a rearranged *TCRD* gene with the RAG1 reference gene. For the Vδ2Jδ1 rearrangement an equal intensity of PCR bands was achieved when RAG1 specific primers were added to the reaction after 6 cycles (Figure 3) and for the Vδ1Jδ1 after 8 cycles (data not shown). Using the formula $f = 2^{1-n}$ the frequency of cells containing the Vδ2Jδ1 rearrangement was estimated at 1/32 cells and the frequency of Vδ1Jδ1

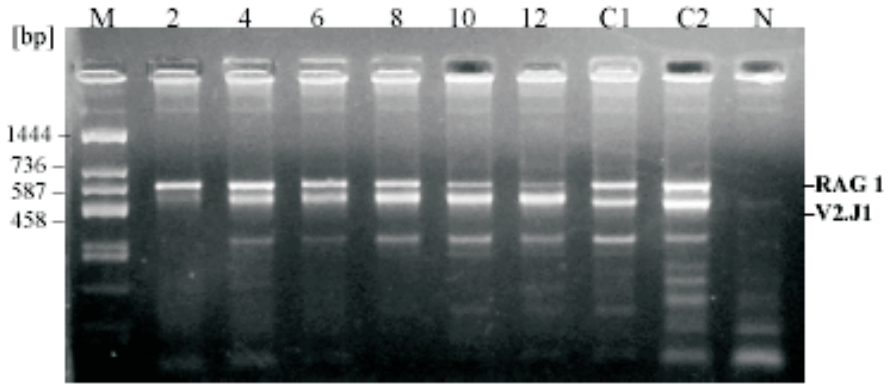

Figure 3. Reference gene competitive PCR (rgc-PCR) analysis of the frequency of the Vδ2-Jδ1 rearrangement in peripheral blood leukocytes

M = molecular weight marker; 2, 4, 6, 8, 10, 12 = number of PCR cycles after which RAG1 primers were added to the reaction; C1 = control, in which plasmid containing rearrangement Vδ2-Jδ1 was mixed with placental DNA in molar ratio 1: 256, and RAG1 primers were added after 8 cycles; C2 = control, in which placental DNA was mixed with plasmid containing Vδ2-Jδ1 rearrangement in molar ratio 1:1 and all primers were added simultaneously; N = no template control

at 1/128 cells. To check this method we included two controls of placental DNA, which does not contain *TCRD* gene rearrangements, supplemented with a known number of vectors with *TCRD* gene rearrangements. When 30 000 copies of vectors with the Vδ2Jδ1 rearrangement were added to 100 ng of the placental DNA, corresponding to an equal number of RAG1 genes, and the reaction was started with both primer sets simultaneously, the bands showed similar intensity (C2; Figure 3). When the vector was diluted 1/256, and the RAG1 specific primers were added after $\log_2 256 = 8$ cycles the balance between the PCR bands was restored (C1; Figure 3).

Discussion

Lymphoid cells differ on the DNA level in antigen receptor gene rearrangements, which can be detected by PCR with primers specific for the rearranging segments. Different methods, including the competitive PCR and the real-time PCR were used for quantitation of cells containing gene rearrangements. Recently, the real-time PCR method (LightCycler or TaqMan) became a standard in quantitative PCR, although high costs of the necessary equipment and reagents make it unaffordable for many laboratories. On the other hand, the establishing of a classical competitive PCR is quite time-consuming since it requires construction and cloning of an internal competitor. Moreover, several reactions with different

concentrations of the competitor are needed and the results can be influenced by the quality and quantity of the genomic DNA. Here, we described two novel competitive PCR methods, which were used to determine the frequency of *TCRD* gene rearrangements, Vδ1-Jδ1 and Vδ2-Jδ1, in PBL of healthy individuals. In the cpc-PCR method two 5' primers specific for the Vδ regions compete for a common 3' primer specific for the Jδ1 region used by both rearrangements. Since both reactions use the same genomic DNA as a template, the results are not affected either by the quality or quantity of the analysed sample. The method does not require preparation of a competitor and the frequency of two rearrangements can be compared in a single reaction. Furthermore, the cpc-PCR, which detects two-fold differences in the frequency of *TCRD* gene rearrangements, is more precise than the competitive PCR with a internal competitor, which is performed in ten-fold intervals.

To determine the portion of cells containing *TCRD* gene rearrangements we established the rgc-PCR, which was derived from the method we used in the analysis of *TCRD* gene rearrangements in γδ T-cell lymphomas. We made an observation that in the case of clonal cell expansion (all cells with the same *TCRD* gene rearrangements) inclusion of *TCRD* and RAG1-specific primers resulted in co-amplification of both bands, while in normal polyclonal cells (*TCRD* gene rearrangement present in a small cell fraction) only the reference band was visible (SALHANY et al. 1998, PRZYBYLSKI et al. 2000). In the rgc-PCR the rearranged gene was pre-amplified for different numbers of cycles and then the reference primers were added to the reactions. The frequency of *TCRD* gene rearrangements was calculated on the basis of the number of pre-amplification cycles necessary to achieve equal intensity of the PCR bands of the analysed rearrangement and the RAG1 reference. Similarly to the competitive PCR with the internal competitor, the rgc-PCR requires a series of reactions to be performed, but the results are obtained in four-fold intervals and are not affected by the quality or the quantity of the analysed sample.

In conclusion, we established two novel competitive PCR methods for quantitation of *TCRD* gene rearrangements. The methods do not require any expensive equipment or reagents. We showed that the rgc-PCR and cpc-PCR are reliable, give reproducible results and can be used for quantitation of gene rearrangements.

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