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Short Communication

THE EFFECT OF DNA LABELING WITH THE FLUORESCENT DYES R110 AND R6G ON GENOTYPE ANALYSIS USING CAPILLARY ELECTROPHORESIS

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Abstract: We investigated the mobility of DNA fragments labeled with the fluorescent dyes R110 and R6G, specifically for use in genotyping using capillary electrophoresis. Genomic DNA was isolated from blood, and a highly polymorphic region of the HLA-C gene was amplified by PCR with the use of either [F]-dNTP-[R110] or [F]-dNTP-[R6G]. Pre-diluted (30-fold) PCR products were mixed with formamide, denatured (at 95ºC for 2 min.), and rapidly cooled on ice before being subjected to electrophoresis. The results showed that the number and mobility of allele-specific DNA fragments were independent of the two dyes used. Both dyes were equally efficient at differentiating homozygous or heterozygous allelic presentation. An additional dye-specific peak of 132-base-pair mobility was observed with the use of [F]-dNTP-[R110]; it significantly impaired the resolution of one allele-specific peak. The electropherograms obtained with [F]-dNTP-[R6G] were free from any interfering peaks within the target region, thus the [R6G]-based procedure is more preferable for genotype analysis. As this procedure does not involve any post-PCR cleanup, it is simple, rapid and cost-effective.

Key Words: Fluorescent-PCR, Genotyping, Capillary Electrophoresis

INTRODUCTION

Capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF) is a highly sensitive, rapid and reproducible technique for a wide range of applications in genetic analysis. CE-LIF has been applied for genotyping [1-3], mutation detection [4-6], paternity testing [7], bacterial identification [8] and

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Abbreviations used: R110 – 6-carboxyrhodamine; R6G – N,N′-diethyl-2′,7′-dimethyl-6-carboxyrhodamine; PCR – polymerase chain reaction; RFU – relative fluorescence unit.

gene expression analysis [9-10]. There are two basic approaches to introduce fluorescent dye into the DNA fragment to be analyzed. In the first method, one or both primers are dye-labeled at the 5′- end, and fluorescently labeled DNA fragments are synthesized during the amplification steps of PCR. The disadvantage of this method is that the labeled primers are very expensive. The second method uses dye-labeled deoxyribonucleoside triphosphates (F-dNTPs), and is more economical, flexible and convenient. Due to its simplicity and sensitivity, this approach is applied for fluorescence-based PCR single-stranded conformational polymorphism (PCR-SSCP) analysis [11].

The ready-to-use [F]-dNTPs are directly added to the PCR mix keeping the ratio of dNTPs and [F]-dNTPs between 100:1 and 1000:1, depending on the signal intensity. R110 (6-carboxyrhodamine), R6G (N,N′-diethyl-2′,7′-dimethyl-6-carboxyrhodamine), and TAMRA (N,N,N′,N′-tetramethyl-6-carboxyrhodamine) are the commonly used fluorescent dyes in [F]-dNTP reagents. Of these, TAMRA is often used in commercially available size standards. R110 and R6G have different chemical structures that may have some influence on the extent of DNA labeling and/or the mobility of labeled DNA fragments in capillary electrophoresis.

To perform a comparative evaluation of the two [F]-dNTPs (R110 and R6G), we selected the genotyping of the HLA-C locus due to its highly polymorphic presentation and its association with various diseases [12-16]. Serological typing of the HLA-C locus is difficult because of its low level of surface expression, the structural similarities of the alleles and the lack of proper typing reagents [17, 18]. On the other hand, HLA typing by PCR has been successfully applied for the accurate detection of HLA-C alleles [12, 17, 19, 20]. This study examined the relative efficiency of the two [F]-dNTPs (R110 and R6G) for HLA-C genotyping.

MATERIALS AND METHODS

Genomic DNA of an individual previously confirmed to carry the HLA-C locus was extracted from a blood sample using a GenomicPrep Blood DNA Isolation Kit (Amersham Biosciences, Piscataway, NJ, USA). Specific amplification of the HLA-C gene (residues 45 to 88) was accomplished with primer pair C133P (coding for amino-acid residues 45 to 52 of the α1 domain of the HLA-C molecules) and C243PR (residues 82 to 88), as suggested by earlier researchers [12]. To study the effect of the fluorescent dyes, two sets of PCR (one for R110 and the other for R6G) were performed in triplicate using the same DNA sample. The respective blanks had all the reagents except DNA.

For PCR amplification, genomic DNA (0.2 μg), primers (200 pM each), and deionized formamide (0.5 μl) were added to thin-walled PCR tubes containing 1 μM of either [F]-dNTP-[R110] or [F]-dNTP-[R6G] (Applied Biosystems, Foster City, CA, USA) and Ready-to-Go PCR beads (Amersham Biosciences). Each PCR bead contained 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of

each dNTP and 1.5 units of Taq DNA polymerase in a final reaction volume of 25 µl. The reaction mixture was overlayed with 40 µl of Nujol mineral oil (Applied Biosystems) and denatured by heating at 95ºC for 5 min. Thirty PCR cycles of denaturation at 94ºC for 30 s, annealing at 65ºC for 60 s and extension at 72ºC for 45 s were carried out followed by a final extension at 72ºC for 7 min, using a programmable Model 480 DNA thermal cycler, (Perkin Elmer, USA).
Prior to capillary electrophoresis, the PCR products were diluted (30-fold) to minimize the interference of unincorporated dye. A 47-cm long capillary was installed in the automated ABI Prism 310 genetic analyzer (Applied Biosystems) and filled with performance-optimized polymer-4 (POP-4). Pre-diluted PCR products (1 µl) were mixed with 12 µl deionized formamide and 0.5 µl TAMRA-500 size standard (Applied Biosystems), injected electrokinetically for 10 s at 7 kV, and electrophoresed for 25 min at 13 kV and 30ºC. The virtual filter was set to 'C', and the laser was run at a constant power of 9.9 mW. The raw data were analyzed for electropherogram color and fragment size using Genescan Analysis Software, Version 2.1 (Applied Biosystems).

RESULTS

The number and mobility of amplified DNA fragments were not affected by the dye type; both [F]-dNTP-[R110] and [F]-dNTP-[R6G] produced the same number of allele-specific peaks at the respective locations (Fig. 1). Both procedures were highly precise in terms of replicate concordance. An additional dye-specific peak with 132-base-pair mobility was found with the use of [F]-dNTP-[R110]; it significantly affected the quality of the genotyping results, as it partially overlapped one allele-specific peak (Fig. 1, left panel). The genotyping pattern obtained with [F]-dNTP-[R6G] was free from non-specific peaks in the target region (the vicinity of 125, spanning base pairs 115-135) (Fig. 1, right panel). Although a non-specific peak at base pair 93 was common to all the samples and blanks, its location did not interfere with the interpretation of the genotyping pattern (Fig. 1).

Tab. 1. The effect of [F]-dNTP-[R110] and [F]-dNTP-[R6G] on the intensity of the fluorescence signal for the detection of allele-specific peaks.

Peak identity	Peak height (RFU)	
	R110	R6G
1	596.26 ± 7.99	262.63 ± 41.00
2	649.72 ± 19.20	319.19 ± 51.58
3	530.62 ± 8.16	327.32 ± 63.03
4*	1200.00 ± 28.27	690.96 ± 135.10
5	595.92 ± 8.16	347.47 ± 56.56

*Homozygous allele. All the values are the means of 3 samples ± SEM. RFU stands for relative fluorescence unit, an arbitrary unit of fluorescence intensity.

The peak heights of all 5 peaks detected in the electropherograms are given in Tab. 1. The data indicate the presence of 3 alleles, 2 heterozygous (4 small peaks) and 1 homozygous (1 double-sized peak, identified as peak no. 4 in Fig. 1). The average ratio of peak heights of homozygous/heterozygous alleles was 2.02 (R110) and 2.19 (R6G). The peak heights were comparatively smaller (0.53-fold) when [F]-dNTP-[R6G] was used (Fig. 1, Tab. 1).

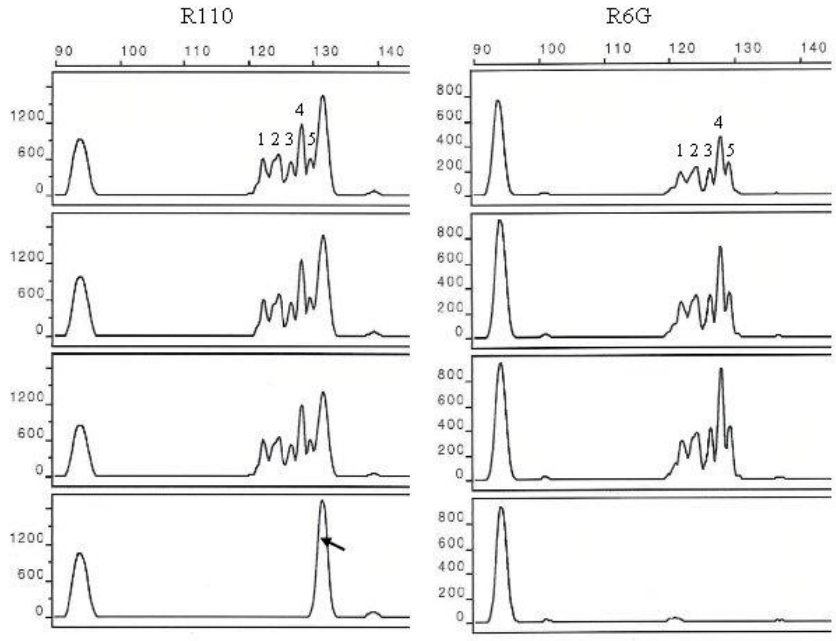

Fig. 1. Capillary gel electropherograms showing the genotyping patterns of the HLA-C region as determined using [F]-dNTP-[R110] (left panel) and [F]-dNTP-[R6G] (right panel). The top three electropherograms correspond to separate PCR reactions using the same DNA sample (triplicates). The bottom electropherograms are the respective blanks (all PCR reagents except DNA). Both R110 and R6G produced same number of allele-specific peaks, and a clear interpretation of homozygocity was possible (peak no. 4). An additional dye-specific interfering peak (arrow) was also observed with [F]-dNTP-[R110] which was absent when [F]-dNTP-[R6G] was used.

DISCUSSION

The results of this study clearly demonstrated that DNA fragments labeled with R110 and R6G possess identical mobility in denaturing gels. Iwahana ***et al.*** [11] also observed that internal- and post-labeling of DNA fragments using different [F]-dUTPs did not affect the SSCP profiles under native conditions. One of the important findings of this study was the presence of a very sharp dye-specific peak which impaired the recognition of one allele-specific peak with the use of

R110 (Fig. 1, left panel). The crucial location of this peak (132-base-pair mobility) clearly shows the limitation of [F]-dNTP-[R110] for genotype analysis using crude PCR products. It is important to note that electrokinetic injection not only allows the migration of targeted fragments but also other components like excess dNTPs, unincorporated dye and primers/dimmers. In routine use, the interference from these unwanted species can be significantly minimized by appropriately diluting the PCR products prior to sample injection. However, a 30-fold dilution of PCR products failed to rectify the interference of the R110 dye-specific peak. Hence, the PCR products of R110 labeling must be cleaned prior to electrophoresis for interference-free output [9]. However, this additional step will ultimately increase the time and cost of the assay, and may sometimes be associated with poor recovery of amplicons. Although post-PCR cleanup is an integral part of certain protocols (e.g. sequencing of DNA), this approach may not always be necessary for genotyping. Simple and economical protocols with minimal steps are usually preferred for large-scale genotype screening.

The superiority of R6G was clearly observed in the absence of any interfering peaks within the target region when it was used. Thus, amplified DNA fragments labeled with [F]-dNTP-[R6G] can be directly electrophoresed without any pre-cleanup. A common non-specific peak at base pair 93 is absolutely harmless because most of the genotype studies are designed for base-pair fragments around 100-300. Since R110 and R6G have different spectral properties, i.e. R110 absorption and emission maxima are 500 and 530 nm, while for R6G the respective values are 520 and 545 nm, their respective PCR products can be mixed and electrophoresed simultaneously in a single run, provided the PCR products resulting from [F]-dNTP-[R110] have been cleaned to get rid of the interfering peak.

The electropherograms obtained with the two dyes were highly reproducible and detected the presence of 3 alleles on the HLA-C locus, which concurs with the results of an earlier study reporting on multiple alleles of HLA-C in an individual [17]. The present methodology clearly differentiated between homozygous and heterozygous presentation of alleles (Fig. 1). Previous studies have also shown the advantage of PCR-based genotyping over serology for correct identification of homozygocity [17, 20]. The relative fluorescence unit intensity (RFU, peak height) corresponding to [F]-dNTP-[R6G] was relatively smaller than that from [F]-dNTP-[R110] (Tab. 1). However, since RFU is not an intrinsic property, it would have no analytical impact on the allelic recognition. Even the peaks obtained after a 30-fold dilution of PCR products were well resolved and above the default threshold of 50 RFU. Moreover, the intensity of RFU can easily be controlled by adjusting the injection time and voltage, and should be kept below 2000 for optimal resolution.

In conclusion, this preliminary study clearly demonstrated the superiority of [F]-dNTP-[R6G] over [F]-dNTP-[R110] for sensitive and reliable genotyping of the HLA-C gene using capillary electrophoresis without necessitating any post-PCR cleanup.

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