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BRIEF METHOD

Intrinsic Deoxyguanosine Quenching of Fluorescein-Labeled Hybridization Probes: A Simple Method for Real-Time PCR Detection and Genotyping

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Real-time polymerase chain reaction (PCR) technologies using fluorescence quenching schemes for product detection have used dual-labeled oligonucleotide probes in the exonuclease (TaqMan) (Holland et al, 1991) or hairpin (Molecular Beacon) (Tyagi and Kramer, 1996) configuration. It is desirable to use a probe labeled with a single fluorophore for real-time PCR detection because dual labeling of oligonucleotides is technically difficult and relatively expensive. Using the Kaposi sarcoma herpesvirus/human herpesvirus-8 (KSHV/HHV-8) as a model, we have exploited the inherent quenching capacity of deoxyguanosine nucleotides on fluorescein (Crockett and Wittwer, 2001) for the design of a real-time PCR assay using a single fluorescein-labeled probe. We demonstrate a 100% concordance between the results of this assay and that of a conventional PCR and Southern hybridization-based assay for the detection of KSHV/HHV-8. The simplicity of this format will permit applications directed toward the detection, quantification, and genotyping of other specific nucleic acid targets.

DNA was extracted from fixed paraffin-embedded tissue sections, frozen tissues, or cell line pellets using standard methods. Thirty DNA samples, previously assessed for the presence of KSHV/HHV-8 by conventional PCR (Pan et al, 2001), were obtained from the molecular diagnostics laboratory of Focus Technologies (Cypress, California) and from the Department of Pathology of the Sunnybrook and Women's College Health Sciences Centre (Toronto, Canada). We tested DNA from 9 known KSHV/HHV-8–positive and 21 KSHV/HHV-8–negative samples. The KSHV/HHV-8–negative specimens included one sample each that was known to be positive for Epstein-Barr virus, Herpes Simplex type I, Herpes Simplex type II, Cytomegalovirus, or Varicella-Zoster virus. We performed fluorescence PCR analysis for KSHV/HHV-8 using primers specific for the ORF26 highly conserved region of the KSHV/HHV-8 genome (Pan et al, 2001). The forward primer sequence was 5'-AGCCGAAAGGATTCCACCAT-3' (base pairs 47287 to 47306, Genbank accession U75698). The reverse primer sequence was 5'-GGATCCGTGTTGTCTACGTC-3' (base pairs 47522 to 47503, GenBank accession U75698). The 3' fluorescein-labeled hybridization probe sequence was 5'-ACGGATTTGACCTCGTGTTCCC-3' (base pairs 47321 to 47342, GenBank accession U75698). All oligonucleotides were obtained from Genset Corporation (La Jolla, California).

Single-probe fluorescence PCR for KSHV/HHV-8 was performed using the LightCycler (Roche Molecular Biochemicals, Indianapolis, Indiana). Fifty nanograms of DNA was amplified in a 10-μl reaction containing 2 μl Mastermix (LightCycler DNA Master Hybridization Probes; Roche Diagnostics, Mannheim, Germany; containing buffer, dATP, dCTP, dGTP, dUTP, and *Taq* polymerase), 3.0 mM $MgCl_2$, 0.5 μM of each primer, the fluorescein-labeled probe at 0.1 μM, and 0.1 units AmpErase uracil N-glycosylase (Applied Biosystems, Foster City, California). All assays included an initial incubation (50° C for 3 minutes and then 95° C for 2 minutes) followed by 45 cycles of denaturation (95° C for 0 seconds), annealing (55° C for 10 seconds), and extension (72° C for 10 seconds). The ramp rate during transition from one stage of PCR to another was 20° C/second. After amplification, the products were cooled to 45° C and heated to 90° C at a rate of 0.2° C/second. For comparison, a fluorescence PCR assay for KSHV/HHV-8 using a dual linear hybridization format was performed using a slight modification of the protocol described above for the single probe system,

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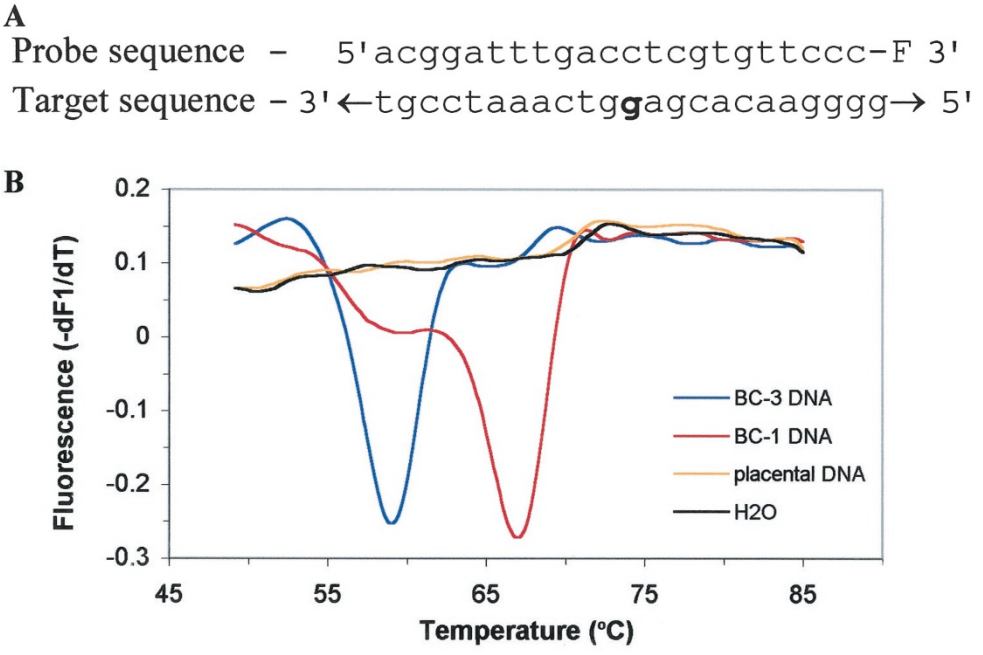

Figure 1.

Fluorescent PCR detection of Kaposi sarcoma herpesvirus/human herpesvirus-8 (KSHV/HHV-8) by single fluorescein-labeled probe quenching. A, The oligonucleotide probe is labeled at the 3' end with fluorescein. The amplicon is a 236 bp segment of the KSHV/HHV-8 gene (GenBank Accession No. U75698). Note that the fluorescein label is located in proximity to a run of deoxyguanosines present in the target strand. The bolded type "**g**" in the target sequence represents the KSHV/HHV-8 sequence present in the BC-1 cell line. The BC-3 cell line harbors virus with a "**t**" in the same nucleotide position. This sequence variation is reflected in the different melting temperatures (Tms) obtained by probe-melting curve analysis using the single fluorescein-labeled probe (see Panel B). B, Fluorescence melting curve for KSHV/HHV-8 amplification product. Melting curves were acquired after 45 cycles of amplification with primers to the KSHV/HHV-8 genome as described in the text. After PCR, the products were cooled to 45° C and gradually heated to 90° C at 0.2° C/second. Fluorescence melting peaks were obtained by plotting the negative derivative of fluorescence over temperature versus temperature (*-dF/dT vs T*). The blue line represents the inverted melting peak of BC-3 (a KSHV/HHV-8–positive cell line) showing a Tm of ~59° C. The red line shows the inverted melting peak of BC-1, another KSHV/HHV-8–positive cell line, with a Tm of ~67° C. The difference in the Tms of the two cell lines reflects differences in the KSHV/HHV-8 gene sequence in the region interrogated by the fluorescein-labeled probe. The black line represents the fluorescence profile of H_20, and the orange line represents placental DNA. No inverted melting peak is discernible in either the placental DNA or H_20.

with the important exception that a 5' LCRed 640–labeled probe (5'-ATGGTCGTGCCGCAGCAACTGGG-3', base pairs 47344 to 47366 of GenBank accession U75698) at 0.2 μM was included. Probe melting peak analysis was performed using *-dF/dT versus T* plots as previously described (Elenitoba-Johnson et al, 2001).

Table 1. Comparison of Results of Conventional, Dual-Hybridization Probe and Single Fluorescein-Labeled Probe PCR for KSHV/HHV-8 Detection[a]

Sample	Conventional PCR and gel electrophoresis	Dual hybridization probe FRET-based PCR	Single fluorescein-labeled probe quenching-based PCR
BC-1 cell line	+	+	+
BC-3 cell line	+	+	+
BCP-1 cell line	+	+	+
Clinical sample 1	+	+	+
Clinical sample 2	N/A	+	+
Clinical sample 3	+	+	+
Clinical sample 4	+	+	+
Clinical sample 5	+	–	+
Clinical sample 6	+	–	+
Placenta	–	–	–
CMV +	–	–	–
EBV +	–	–	–
HSV-1 +	–	–	–
HSV-2 +	–	–	–
VZV +	–	–	–

KSHV/HHV-8, Kaposi sarcoma herpesvirus/human herpesvirus-8; FRET, fluorescence resonance energy transfer; CMV, cytomegalovirus; EBV, Epstein-Barr virus; HSV, herpes simplex virus; VZV, varicella-zoster virus; N/A, not assessed; +, positive; –, represents negative.

[a] Fifteen additional samples that tested negative for KSHV/HHV-8 using all three assays have been excluded from Table 1 for brevity.

The *-dF/dT versus T* plots were visualized as inverted peaks in positive reactions (Fig. 1B). All runs included a negative DNA control (placenta), two positive controls (BC-1, BC-3 cell line DNA, ATCC Nos. CRL-2230 and CRL-2277, respectively), and a template-free (H_2O) control. Conventional PCR for KSHV/HHV-8 was performed as described (Pan et al, 2001).

The results of the conventional, dual labeled hybridization probe and single-fluorescein-labeled probe reactions for KSHV/HHV-8 are summarized in Table 1. Positive samples for KSHV/HHV-8 using the single fluorophore labeled probe displayed an inverted melting peak at 58.9 $\pm$ 0.2° C or 66.5 $\pm$ 0.4° C, depending on sequence variations in the region of probe coverage (Fig. 1, A and B). There was a 93% concordance between the results of the single fluorescein-labeled and dual-labeled probe-based formats, and 100% between the single fluorescein-labeled, probe-based assay and conventional PCR. In all cases, a single-probe assay for β-globin revealed positive signals indicative of intact DNA from the host sample. Dilutional analysis revealed unambiguous detection of 0.05 pg viral DNA from a KSHV-infected cell line (10–20 copies of virus per cell in the BC-3 cell line) (Pan et al, 2001) diluted in 50 ng of placental DNA. The probe melting temperatures obtained using this approach were comparable to those using a dual-labeled linear hybridization probe assay ($R^2 = 0.98$).

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