Using the Fluorogenic 5' Nuclease Assay for High-Throughput Detection of (CA)_n Repeats in Radiation Hybrid Mapping

BioTechniques 28:754-765 (April 2000)

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ABSTRACT

Here, the power of the 5' nuclease assay to detect PCR products containing (CA)_n repeats was compared with that of the classical electrophoretic analysis. This assay, which relies on the use of a unique (CA)₁₀ energy transfer-labeled probe and the 5' nuclease activity of Taq DNA polymerase, was used to construct a dog radiation hybrid map consisting of microsatellite markers. Data from over 7000 PCRs were analyzed in parallel by the fluorogenic assay and the conventional ethidium bromide-stained, agarose gel-based assay. We show that the fluorogenic assay provides a sensitive, reliable and specific method for detecting (CA)_n amplimers. Moreover, as no processing is required after the PCR, the risk of carryover contamination and the time required for sample analysis are greatly reduced. All radiation hybrid (RH) assays can be performed using a single PCR protocol, and a standard analysis method has been developed that enables numerically automated data processing. On the whole, using this strategy greatly enhanced the rapidity, throughput and accuracy of the RH mapping of microsatellite markers.

INTRODUCTION

Establishing genome maps requires the positioning of dozens of markers to serve as landmarks along the chromosomes. Because all markers—genes, sequenced tag sites (STSs), expressed sequenced tags (ESTs), microsatellites and single-nucleotide polymorphisms (SNPs)—will be mapped by PCR methods (16), the construction of genomic maps demands the detection of thousands of PCR products amplified on DNA from different sources (e.g., radiation hybrid [RH] panels and YAC, BAC or cosmid clones) (3,8). Detection is commonly performed by ethidium bromide-stained agarose gel electrophoresis, a method requiring time-consuming and labor-intensive sample manipulations after PCR. However, many recently described fluorescent assays that directly detect PCR products in PCR-closed tubes bypass many steps and contamination risks that are connected with processing after PCR (6,9,11,17–19,23,27).

One of these homogeneous methods (e.g., in a closed tube), the 5' nuclease assay, is based on the use of a dual-labeled fluorogenic probe complementary to an internal segment of the target PCR product (7,9,11). During the extension phase of amplification, the 5'→3' nuclease activity of Taq DNA polymerase cleaves the probe only if the sequence complementary to the probe is present (positive sample). This discontinues the intermolecular quenching effect and enhances reporter fluorescent emission. Since no post-PCR processing is required other than fluo-

rescence monitoring, the detection of PCR products is rapid and easy. This assay, which has been successfully used in quantitative PCR experiments (10) in many fields of biology, including virology (14), bacteriology (1) and in clinical diagnoses (13), seems particularly adapted for large-scale genome mapping (12,24).

The goal of the present work is to compare this attractive detection method with the conventional agarose gels electrophoresis method, commonly used in RH mapping (2). As a first step towards the construction of a whole genome RH map of dog, 400 markers were previously typed using conventional gel-based electrophoresis methods (22) on the RHDF₅₀₀₀ panel (25,26). To increase the density of this first RH map, new markers—essentially (CA/GT)_n microsatellites—were mapped. In view of their shared (GT)_n target sequence, the detection of PCR products using the 5' nuclease strategy was particularly indicated, as only one dual labeled (CA)_n probe is required to detect all possible (CA)_n microsatellite markers.

We show that the fluorogenic assay can efficiently be used to screen RH cell lines for microsatellite markers, whatever the primers pairs. Briefly, a (CA)₁₀ fluorogenic probe with a fluorescent reporter dye and a quencher dye is introduced in typical PCRs. Direct measurement of post-PCR fluorescent emission of both reporter and quencher dyes for each PCR is performed with a classical plate fluorescence reader. Any increase in reporter dye fluorescent emission denotes the presence of the targeted PCR product.

Table 1. Primers and Fluorogenic Probe

Marker	Ref. ^a	Forward primer sequence 5'→3'	Reverse primer sequence 5'→3'	Repeat length ^b
REN233K12	-	TGCTTACACAACCTGGCACTG	CCACAGGGAGTCTGCTTCTC	(CA) ₁₁
REN242G11	-	CCTCTGTTTCTCCCTCTCCC	GGTTAGGTGGGTATCAGCGA	(CA) ₂₁
REN256E18	-	CTGAAGGAAGCCCTCACTTG	CCCTGGATAATTCGGTGAGA	(CA) ₁₁
CO1003	28	GAAGTAAATCAACAAACAATCA	GAAGCAAAAGTATAAGAGCTGTG	(AC) ₁₁
CXX436	21	CAGCTGGATTGGGGACTC	CATCTTGCTCTCTCAAC	(TG) ₄ (GT) ₁₆
CXX373	21	TATTTAAAAAATCCCAGGCACA	AGCATCAATTAGATGTCAGCG	(TA) ₃ (CA) ₁₁
(CA) ₁₀ fluorogenic probe		[FAM]-CACACACACACACACACA-[TAMRA]		

^aMarkers indicated with REN were taken from Jouquand et al., submitted; the others were taken from the literature (21) (<http://www.cvm.msu.edu/text/res/cImm.html>).

^b(CA)_n repeats were counted in the sequence from the original clone.

Using both detection methods, mapping more than 100 (CA)_n markers on the RH panel has shown that the fluorogenic assay is more accurate and more specific than conventional agarose gel electrophoresis. Moreover, the fluorogenic assay is a high-throughput method for detecting PCR products and an efficient method for the RH mapping of microsatellite markers.

MATERIALS AND METHODS

Markers

A set of 78 (CA/GT)_n microsatellite markers (composed of more than 10 successive repeats) were isolated from a small insert canine genomic library (Jouquand et al., submitted). Fifty-one additional dog microsatellites were taken from the literature (21,28). Each microsatellite marker is characterized by a pair of PCR primers designed from unique flanking regions and is able to amplify fragments in the 150–500 bp range (Table 1).

Fluorogenic Probe

The dual-labeled (CA)₁₀ probe was synthesized from a 5' end fluorescent reporter dye [FAM (i.e., 6-carboxy-fluorescein)] and a 3' end quencher dye [TAMRA (i.e., 6-carboxy-tetramethyl-rhodamine)] (PE Biosystems, Foster

City, CA, USA). The probe is nonextensible, owing to the presence of a phosphate at the 3' end. Spectrum analysis performed with a LS-50B spectrofluorometer (PE Biosystems) showed two emission fluorescent peaks at 517 and 576 nm. Excitation was at 485 nm with a 5 nm slit width.

PCR Protocol

All PCR amplifications were performed in PTC-200™ thermocyclers (MJ Research, Waltham, MA, USA), in polypropylene PCR plates (COSTAR; Corning-Costar, Corning, NY, USA) providing low background fluorescence. Amplification was carried out in a 15 µL volume with 1.5 µL 10× Perkin Elmer buffer and 0.5 U of *Taq* Gold polymerase (PE Biosystems), 200 µM dNTPs, 5 mM MgCl₂, 50 ng of genomic DNA, 3 pM of each PCR primer and 25 nM fluorogenic (CA)₁₀ probe. The thermal PCR program was 95°C for 10 min, 20 cycles at 95°C for 15 s and 60°C for 45 s, followed by 20 cycles at 95°C for 15 s, 60°C for 15 s, 72°C for 30 s and hold at 4°C.

RH Assays

RH assays were performed on the RHDF₅₀₀₀ whole genome RH panel (25,26) for each microsatellite marker as follows. Reactions were carried out in 384-well PCR plates including DNA

samples from each of the 126 hybrid cell lines, 9 dog genomic DNAs as positive controls, 4 hamster genomic DNAs (A2H) as negative controls and 5 “no template” controls. The results were computed by the mapping software package MultiMap (15) to constitute RH linkage groups and to determine marker order and intermarker distances (cRay) into each RH linkage group.

Gel-Based Detection

Each amplification reaction was run on 1.8% agarose gels, stained with ethidium bromide in 0.5× TBE buffer (50 mM, 50 mM boric acid and 1 mM EDTA) (Hybaid electrophoresis system) at 120 V for 30 min. The PCR products were visualized under UV light, and the images were scored by a high-resolution charge-coupled device (CCD) camera system (Bioprint, Vilbert-Lourmat, France). The captured images were transferred to a UNIX workstation, and each gel was scored using a semiautomated procedure: “1” for the presence of PCR products of the expected size, “0” for the absence and “2” for an ambiguous result.

Fluorogenic Detection

For each amplification reaction, fluorescence was directly detected in the polypropylene microplate, using the Fluoroskan Ascent® (Life Sciences

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Table 2. Normalization of Fluorescent Values

	Exp. No.	R/Q value (intra assay SD)	mean	SD	δR/Q value	mean	SD	ΔR/Q value	mean	SD
positive controls	1	11.22 ± 0.07			7.10			1		
dog DNA (n = 6)	2	8.42 ± 0.09			5.56			1		
	3	12.07 ± 0.14	10.57	1.91	7.90			1		
negative controls	1	4.12 ± 0.20			0			0		
hamster DNA (n = 3)	2	2.86 ± 0.05			0			0		
	3	4.17 ± 0.05	3.72	0.75	0			0		
no template controls	1	3.00 ± 0.08			-1.12					
H ₂ O (n = 3)	2	2.28 ± 0.05			-0.58					
	3	3.14 ± 0.08	2.81	0.46	-1.03					
hybrid sample no. 1	1	2.82			-0.03			-0.04		
	2	2.74			-0.12			-0.02		
	3	3.96	3.51	0.67	-0.21	-0.21	0.09	-0.03	-0.03	0.01
hybrid sample no. 2	1	10.89			6.77			0.95		
	2	8.29			5.43			0.98		
	3	11.70	10.29	1.78	7.53	6.58	1.06	0.95	0.96	0.01
hybrid sample no. 3	1	11.26			7.14			1.00		
	2	8.48			5.62			1.01		
	3	12.12	10.62	1.90	7.95	6.90	1.18	1.01	1.01	0.00
hybrid sample no. 4	1	11.16			7.04			0.99		
	2	8.40			5.54			1.00		
	3	12.08	10.54	1.91	7.91	6.83	1.20	1.00	1.00	0.01
hybrid sample no. 5	1	4.78			0.66			0.09		
	2	3.57			0.71			0.13		
	3	4.76	4.37	0.69	0.59	0.65	0.06	0.07	0.10	0.03
Three distinct RH assays were performed with the CXX373 (CA) _n marker on three different days. The mean of R/Q values and SD is given for controls within and between the RH assays. Five different hybrid cell lines are shown to illustrate the R/Q, δR/Q and ΔR/Q values (see Materials and Methods for calculations). Interassay reproducibility is improved by applying the successive normalizations as shown by the SD decrease of R/Q, δR/Q and ΔR/Q values, respectively, for each hybrid.										

International, Labsystems division, Helsinki, Finland) controlled with Ascent software. Excitation was at 485 nm with a half-bandwidth of 5 nm. Emission was measured at 538 nm for the reporter dye and at 590 nm for the quencher dye, with a half-bandwidth of 10 nm for each emission filter. Background emission from empty polypropylene PCR plate wells was close to zero. Raw emission data were directly transferred from the Ascent software to a Microsoft® Excel® spreadsheet. To normalize well-to-well variations in fluorogenic probe concentration, the

emission intensity of the reporter (R) was divided by that of the quencher (Q), which give the R/Q ratio. As a prerequisite, (CA)_n markers were considered detectable when their fluorescent signal/background ratio exceeded 2, that is, the R/Q ratio of dog DNA divided by the R/Q ratio of hamster DNA was greater than 2.

To calculate the fluorescence results in each RH assay, the R/Q ratio was normalized twice. The first normalization was applied to subtract background fluorescence: $\delta R/Q = R/Q \text{ sample} - R/Q \text{ negative}$ in which R/Q negative is the

average of R/Q values with negative controls (hamster DNA, n = 4). $\delta R/Q$ corresponds to an increase in fluorescence signal caused by specific template amplification. In a second normalization, $\delta R/Q$ was weighted by the R/Q values obtained with controls: $\Delta R/Q = \delta R/Q \text{ divided by } R/Q \text{ positive control} - R/Q \text{ negative control}$, in which R/Q positive is the average of R/Q values with positive controls (dog DNA, n = 9). By applying these two normalizations in the final calculation of the results for a given marker, the $\Delta R/Q$ value obtained with the different hybrid DNA samples was

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found to be quite reproducible even if the averaged R/Q values on the control DNAs were slightly different from one RH assay to another (Table 2).

Quality Controls

For both detection methods, a threshold for the number of ambiguous results in each RH assay has been established as 2.4% (3 out of 126 hybrids). In fluorogenic assays, the means of R/Q from positive and negative controls were set so that the SD from the mean is below 0.5 for the positive controls and 0.3 for the negatives.

Southern/Dot Blotting and Sequencing of PCR Products

Southern blots were prepared by transferring with a vacuum blotter (Appligene-Oncor, Gaithersburg, MD, USA) the entire PCR volume on Hybond[®]-N+ membranes. Filters were hybridized at 45°C overnight with a

(CA)₁₀ biotinylated probe and were washed for 1 h at 60°C in 6× standard saline citrate (SSC) 0.1% SDS. Detection of hybridized probes was performed by chemiluminescence and exposure to Hyperfilm[™] ECL[™] (Amersham Pharmacia Biotech, Rainham, England, UK) for 5–15 min. Sequencing of the PCR products was performed with the PCR primers according to a dye terminator protocol from PE Biosystems. Reactions were run on 6% polyacrylamide gels on a Model 377 ABI PRISM[™] DNA sequencer (PE Biosystems).

RESULTS

Characterization of the Fluorogenic Assay

The fluorogenic assay used here for detecting (CA)_n markers introduces (in a classical PCR) a fluorescent probe that was dual-labeled with a reporter and a quencher dye. The first step was

to verify that fluorescence resonance energy transfer (FRET) (4) would occur in our PCR conditions by using the designed probe as reported in the literature (11). For this, a PCR that includes a (CA)_n dog marker was terminated after a given number of cycles and examined in parallel in fluorescence and in agarose gels. Figure 1 shows that increased reporter dye (R) fluorescence and slightly decreased quencher dye (Q) fluorescence—denoting energy transfer disruption—are only significant in the case of a PCR on a (CA)_n-positive DNA sample. To standardize fluorescence in all the wells, we used the R/Q ratio (i.e., the fluorescence value obtained with the reporter divided by that obtained with the quencher) (5). Figure 1 shows that R/Q can quantify the concentration of DNA produced in an otherwise standard PCR. At the end of PCR, the R/Q differential between positives and negatives is large enough that the two sets of samples are readily discriminated in endpoint analysis.

Figure 1. Amplification plots (top) and gel analysis (bottom). Primers designed from a dog (CA)_n microsatellite (CXX436) were used in PCRs that were stopped at fixed numbers of PCR cycles (0, 20, 22, 24, 28, 34 and 40). Different templates were used. These included dog DNA (■) as positive control, hamster DNA (●) as negative control and water (▲) as a “no template” control. Cycle number is plotted vs. (A) emission intensity of the fluorescent reporter dye (R) measured at 538 nm, (B) emission intensity of the quencher dye (Q) measured at 590 nm and (C) the normalized R/Q value. Each point represents the mean of triplicate PCRs. Variation coefficients were always greater than 5% (error bars are not shown to simplify plots). For gel analysis, the same PCRs were carried out with dog (+) and hamster (-) DNA and migrated on agarose gels after completion of 0, 20, 22, 24, 28, 34 and 40 PCR cycles (lane M, 123 bp DNA ladder).

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As protocol standardization will facilitate mapping, PCR and endpoint fluorogenic detection conditions were optimized to meet those suitable for a majority of $(CA)_n$ markers. For this, several parameters were tested: PCR volume (5–50 μL), $(CA)_n$ probe size ($n = 8$, $n = 10$), final concentration of $(CA)_{10}$ probe (0.01–0.08 $\text{pmol}/\mu\text{L}$), MgCl_2 concentration (2–5 mM) and PCR programs. The selected protocol (see Materials and Methods) amplified and readily detected 85% of the $(CA)_n$ markers tested (551 out of 636).

Regarding detection sensitivity, serial dilutions of post-PCR samples obtained by the amplification of a $(CA)_n$ dog marker on dog DNA and hamster DNA as a negative control were examined in parallel by fluorogenic and gel-based assays. The results show that the fluorogenic 5' nuclease assay compares well with visualization on agarose gels after electrophoresis. No product was detected beyond 10 ng with both detection methods. The detection limit of the fluorogenic PCR assay was evaluated by amplifying a dog $(CA)_n$ marker with decreasing amounts of DNA, in the range 50 ng to 6 pg (Figure 2). The assay was capable with 100% success rate of detecting products of a PCR performed on as little as 50 pg of dog DNA (corresponding to nearly 20 copies of the target molecule, as the dog haploid genome equivalent is 3 pg). Below 50 pg, amplification becomes random.

Fluorescence Data Processing in Mapping

For RH mapping, each marker was amplified on DNA from the 126 dog/hamster hybrid cells composing the RHDF₅₀₀₀ panel (25,26). The graph in Figure 3 shows the R/Q and $\Delta R/Q$ values obtained in the RH assay for one $(CA)_n$ marker. Two groups of samples appear to be clustered around the positive ($R/Q = 15$) and negative controls ($R/Q < 5$). Parallel analysis of these samples by agarose gel electrophoresis showed the same distribution.

To precisely discriminate positive from negative samples by automating the RH assays, a threshold applicable to any $(CA)_n$ marker was established. This was determined by comparing the PCR

results obtained by fluorogenic assay with agarose gel electrophoresis. A total of 7224 samples was analyzed using both detection methods. These samples correspond to 33 $(CA)_n$ markers analyzed on the whole RHDF₅₀₀₀ panel (33×126) and another 73 analyzed on the first 42 hybrids (73×42). The distribution of the gel-positive ($1699/7224 = 23.5\%$) and gel-negative ($5505/7224 = 76.2\%$) samples as a function of their $\Delta R/Q$ is reported in the histogram in Figure 4 (gel-ambiguous results of 0.3% are not shown). Using the gel-based detection results as the sole reference and notwithstanding the existence of a few false positives and negatives, it could be concluded that: (i) The points

in the range ($\Delta R/Q < 0$) are negative, as 99.4% appear to be so in gels; (ii) the points in the range ($0 < \Delta R/Q < 0.15$) are ambiguous (noted as "2"). In fact, their status cannot be objectively decided, as the points in this range can either be negative (47%) or positive (53%) in gels; and (iii) the points in the range ($\Delta R/Q > 0.15$) are positive, as 98.9% appear to be so in gels.

Thus, for gel-positive samples 95.3%, 3% and 1.7% are positive, ambiguous and negative, respectively, according to fluorescence analysis. Likewise, for gel-negative samples 98.9%, 0.8% and 0.3% are negative, ambiguous and positive, respectively. In a total of 7224 PCRs analyzed by fluorogenic

Figure 2. Detection limit of the fluorogenic assay. Replicate PCRs (more than 4 assays) were performed with primers designed from a dog $(CA)_n$ marker (CXX436). Twofold serial dilutions of dog and hamster DNA were used as template. Each PCR was controlled on an agarose gel (data not shown). For positive and negative samples, the mean R/Q values (left y-axis) were plotted vs. initial target quantity in the range of 50 ng to 6 pg (x-axis). R/Q values of negative samples with dog DNA are superimposed on those obtained with negative control hamster DNA. Histogram shows the proportion of positive samples (right y-axis) for each dog DNA amount (x-axis). Variation coefficients (6% on average) are not shown to simplify plots.

and gel-based detection, 97.8% of the points are concordant, 1.5% are 2/+ or 2/- discrepancies and only 0.7% are $\pm$ discordant results. As these threshold values give quite concordant results (over 97%), they have been set for

mapping the $(CA)_n$ markers on the RHDF₅₀₀₀ panel (Figure 3).

Concerning the $\pm$ discrepancies, it should be noted that (i) they correspond to the weakest bands in gels and are not reproducible (75% of cases) and (ii)

Figure 3. Results of an RH assay. R/Q values and normalized $\Delta R/Q$ values obtained from PCR amplification of a $(CA)_n$ marker (c01003) on the RHDF₅₀₀₀ panel. The x-axis represents the number of each hybrid cell line (h1–h126). The left y-axis represents the R/Q value. The right y-axis gives the corresponding value expressed as $\Delta R/Q$. The means of each R/Q value for the controls—positive, negative and “no template”—are plotted. In this assay, 88 samples had $\Delta R/Q$ less than 0 and were considered as negative [0], 37 samples had $\Delta R/Q$ greater than 0.15 and were considered as positive [1] and one sample (hybrid cell no. 64) had $0 < \Delta R/Q < 0.15$ and was considered ambiguous [2].

Figure 4. Distribution of 1699 PCR samples scored as positive on agarose gels and of 5505 PCR samples scored as negative on agarose gels, according to their $\Delta R/Q$ values (x-axis). $\Delta R/Q$ value is 1 for the positive controls and 0 for the negatives. The y-axis represents the percentage of PCR samples.

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they are randomly distributed among the hybrids of the RHDF₅₀₀₀ panel and among the markers. In terms of mapping results, the impact of a 2/+ or 2/- discrepancy is less important than that of a $\pm$ discrepancy. Indeed, in view of the low levels of $\pm$ (0.7%) and 2/+ or 2/- discrepancies (1.5%) reported between fluorogenic and gel-based detection, the incidence on marker positioning on the map is small. For one marker detected by both methods, the respective position on the RH map only differs by 2.5 cRay₅₀₀₀ (about 400 kb), a figure similar to the theoretical limit of mapping resolution of the RH panel (22) and, as shown below, smaller than that from PCR per se.

Reproducibility of PCR Results and Incidence of the Method Used for Mapping Accuracy

When markers are typed on the RHDF₅₀₀₀ panel in duplicates—same marker, two PCRs—any discrepancy between the two independent experimental results can be ascribed to problems with PCR product detection or to PCR failures that occur regardless of the detection method used. In 49 duplicate PCRs run on agarose gels, the averaged position deviation of the markers on the RH map was 11 cRay₅₀₀₀. In 15 duplicate PCRs analyzed by the fluorogenic assay, the averaged position deviation was 6 cRay₅₀₀₀. In 45 duplicate PCRs, one of which was analyzed on gels and the other by fluorescence, the markers differed by 7 cRay₅₀₀₀. These deviations show that the results of the fluorogenic

assay are more readily reproducible than the gel-based assay. Consequently, the former is more accurate.

Specificity of Detection

To assess the feasibility and robustness of the fluorogenic 5' nuclease assay, 100 (CA)_n markers taken at random were tested by PCR/fluorogenic assay on the 126 hybrids of the RH panel. The number of markers meeting the quality criteria set for RH assays (i.e., below the threshold for the number of ambiguous results [2.4%]; see Materials and Methods) was 90% with the fluorogenic detection method and 76% with the gel-based assay. When comparing the two detection methods, 73% gave identical patterns by gel and by fluorogenic assays with one exception, 16% had a satisfactory pattern in fluorescence only and 2% on gels only and 8% gave noninterpretable patterns, whatever the detection method used. Noninterpretable gel patterns are due to the presence of nonspecific spurious bands—usually varying in number, size and intensity in different hybrid DNAs—that most probably originate from hamster DNA. To assess the specificity of the fluorogenic method, Southern and/or dot blots from six different markers that met the quality criteria in fluorescence but were noninterpretable on gels were hybridized to a labeled (CA)₁₀ probe. In Figure 5, A and B, only the positive samples in fluorescence were also positive in Southern, indicating that the fluorogenic assay, as it discriminates between the true amplicon and the

parasite DNA, is highly specific.

However, in Figure 5C, marker REN256E18 (Table 1) gives a clear pattern on agarose gel, with PCR products of equal size and brightness. Some amplicons (lanes 8 and 9) were reproducibly undetected in fluorescence. After sequencing, those products showed an imperfect motif [(CA)₄TA(CA)₅TA(CA)₆], whereas the amplicons found to be positive using both detection methods (positive sample, lanes 5 and 12) exhibited a perfect (CA)₂₀ motif. Thus, some hybrids have retained allele (CA)₂₀, others retained allele [(CA)₄TA₅CA)(TA(CA)₆] and some retained both. It seems that under the stringency conditions of the PCR, the two TA insertions prevent the (CA)₁₀ probe from hybridizing, although they let the probe reach the target DNA in Southern blots. This is the only instance of allelic discrimination encountered in this study.

DISCUSSION

This report demonstrates through a thorough comparison of the fluorogenic and the gel-based detection methods that the 5' nuclease assay offers clear advantages over gel-based procedures for detecting PCR products containing (CA)_n microsatellite repeats in large-scale DNA projects such as building RH maps. Here, all RH assays were performed using a single PCR protocol because the latter works with 85% of the microsatellite marker. Furthermore, an entirely automated procedure for collecting and analyzing fluorescent data has been developed. As a single (CA)₁₀ fluorogenic probe is required to detect a large number of individual amplicons, the larger the number of samples, the cheaper is the fluorogenic assay. Comparing the two detection methods showed that the fluorogenic assay is as sensitive as the ethidium bromide-stained gel electrophoresis method because both methods detect the same minimal amount of amplified DNA fragments (about 10 ng). Furthermore, the detection limit of PCR guarantees that low target molecule concentrations in hybrid cells (as few as 20 starting copies) will be detected by both methods. The high correlation of

Figure 5. PCRs were carried out with dog-specific primers of (CA)_n markers for REN233K12 (A), REN242G11 (B) and REN256E18 (C). Different templates were used. T+ is dog DNA as positive control, T- is water as a "no template" control and lanes 1–12 are DNA from 12 different hybrid cell lines. PCRs were analyzed on the left panel by agarose gel and on the right panel by Southern blotting. The "+" sign superimposed on the autoradiogram indicates a positive result obtained by fluorogenic assay. No sign indicates a negative fluorogenic result.

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97.8% recorded by comparing 7224 gel/fluorescent data advocated confident use of the fluorogenic assay for routine RH mapping.

By performing RH assays in duplicates, the fluorogenic assay proved more accurate than the gel-based assay. With regard to mapping, increased accuracy in marker localization and ultimately in mapping precision resulted. There are two reasons for this. First, fluorescence PCR data are numerically recorded, whereas, on agarose gels, the visualization of PCR products remains highly subjective. Second, with regard to crossover post-PCR contamination and sample leaks in gel lanes, loading agarose gels can also cause inaccuracy.

The power of this fluorescent method lies in its specificity. The fluorogenic assay only detects PCR products that contain the target sequence complementary to the fluorogenic probe. For interpreting agarose gel pat-

terns, parasite DNA may be misleading because nonspecific amplification products are often indistinguishable from the true amplicon. In the present study, 25% of the dog markers typed on the RHDF₅₀₀₀ panel were not interpretable on agarose gels. More than half could be mapped by means of the fluorogenic assay, and all markers but one gave reliable results following verification by Southern/dot blotting or sequencing.

In this study, cases of allelic discrimination were rare. Out of 129 (CA)_n markers, only one (including an allele that exhibited several disruptions in the microsatellite sequence) was not detected. No other instance of allelic discrimination was noted in another set of 98 random (CA)_n markers (data not shown). This method has also been reported to specifically pinpoint allelic variations (9), but it seems that in most instances, mismatches between linear probes and target can be tolerated, al-

though they are a disadvantage in allelic discrimination studies (11). As 20% of dog (CA)_n microsatellites are imperfect (20), the tolerance of mismatches turned out to be advantageous for their detection using the fluorogenic assay. Using this method for detecting (CA)_n repeats—same probe for all assays—overcomes the general drawback of the 5' nuclease assay, namely the need to use a unique probe for each amplicon. While other methods have been recently described (18,27), the requirement of a specific oligonucleotide tail attached to one of the PCR primers and complementary to a universal energy transfer-labeled probe, complicates the protocol. Moreover, the fluorescence signal can be nonspecific, as primer incorporation can occur in specific as well as nonspecific PCR products, in contrast to hybridization methods that use a probe that specifically recognizes the amplicon under investigation (5,23).

In conclusion, this fluorogenic method for the detection of amplimers containing a (CA/GT)_n microsatellite is sensitive, accurate, specific and can be used to analyze many samples in a very short time. Such a high-throughput and reliable method is particularly adapted for the RH mapping of microsatellite markers.

ACKNOWLEDGMENTS

We thank Cyril Cueff and Nicolas Soriano for computing assistance. We are grateful to Patrice Allard (Perkin Elmer) for his help and advice and to Frédérique Hubler and Yvon Cavaloc for comments and discussions and for carefully reading the manuscript. This work was supported by Centre National de la Recherche Scientifique, Université Rennes1 and Conseil Régional de Bretagne.

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Received 23 August 1999; accepted 13 December 1999.

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