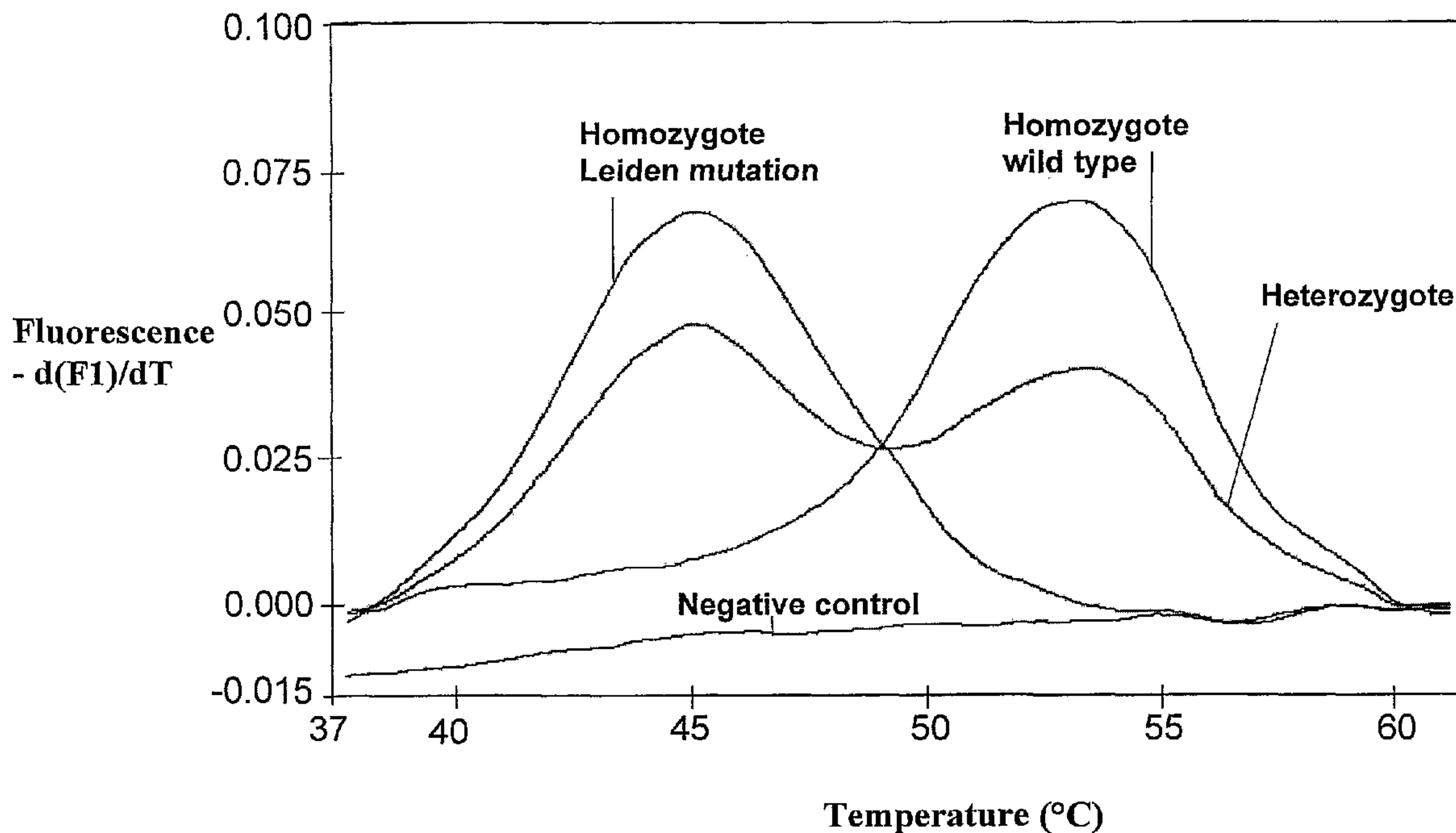




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 (72) Inventeurs/Inventors:  
 WITTWER, CARL T., US;  
 CROCKETT, ANDREW O., US;  
 CAPLIN, BRIAN E., US;  
 STEVENSON, WADE, US;  
 WAGNER, LORI A., US;  
 CHEN, JIAN, US;  
 KUSUKAWA, NORIKO, US  
 (73) Propriétaires/Owners:  
 UNIVERSITY OF UTAH RESEARCH FOUNDATION,  
 US;  
 IDAHO TECHNOLOGY, INC., US  
 (74) Agent: SMART & BIGGAR

(54) Titre : **SONDES OLIGONUCLEOTIDIQUES SIMPLES, MARQUEES**  
 (54) Title: **SINGLE-LABELED OLIGONUCLEOTIDE PROBES**



(57) **Abrégé/Abstract:**

Probes and methods are provided for detection and analysis of nucleic acid sequences. The probes are single-labeled oligonucleotide probes whose fluorescence emission changes in response to probe-target hybridization and dissociation. The methods are for analyzing one or multiple nucleic acid loci using the probes. This invention further relates to the use of fluorescence changes in single-labeled probes for melting curve analysis, genotyping, and pathogen detection, and to methods for quantification of specific sequences in real-time monitoring of nucleic acid amplification.



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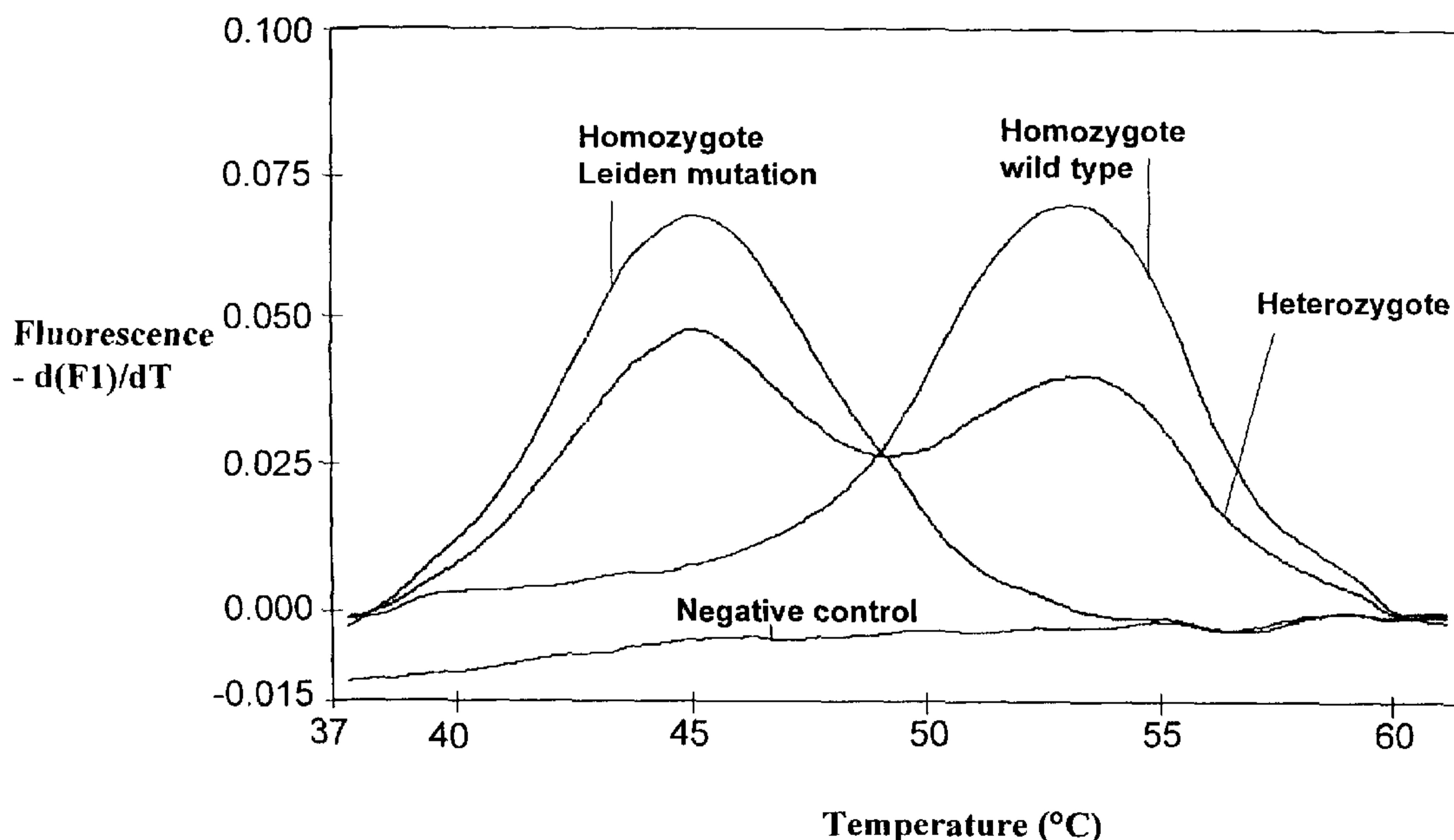
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60/240,610 16 October 2000 (16.10.2000) US(71) Applicants: UNIVERSITY OF UTAH RESEARCH  
FOUNDATION [US/US]; 615 Arapeen Drive, Suite 100,  
Salt Lake City, UT 84108 (US). IDAHO TECHNOL-  
OGY, INC. [US/US]; 390 Wakura Way, Salt Lake City,  
UT 84108 (US).(72) Inventors: WITTEWER, Carl, T.; 3479 Crestwood Drive,  
Salt Lake City, UT 84109 (US). CROCKETT, Andrew,  
O.; 4428 Walter Way, West Valley, UT 84120 (US).CAPLIN, Brian, E.; 2918 East Morningside Drive, Salt  
Lake City, UT 84124 (US). STEVENSON, Wade; 7331  
Opal Hill Drive, West Jordan, UT 84184 (US). WAGNER,  
Lori, A.; 1715 South 2000 East, Salt Lake City, UT 84108  
(US). CHEN, Jian; 9864 Geode Circle, Sandy, UT 84094  
(US). KUSUKAWA, Noriko; 3479 Crestwood Drive, Salt  
Lake City, UT 84109 (US).(74) Agent: LAMMERT, Steven, R.; Barnes & Thornburg, 11  
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(54) Title: SINGLE-LABELED OLIGONUCLEOTIDE PROBES

(57) **Abstract:** Probes and methods are provided for detection and analysis of nucleic acid sequences. The probes are single-la-  
beled oligonucleotide probes whose fluorescence emission changes in response to probe-target hybridization and dissociation. The  
methods are for analyzing one or multiple nucleic acid loci using the probes. This invention further relates to the use of fluorescence  
changes in single-labeled probes for melting curve analysis, genotyping, and pathogen detection, and to methods for quantification  
of specific sequences in real-time monitoring of nucleic acid amplification.

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## SINGLE-LABELED OLIGONUCLEOTIDE PROBES

Field of Invention

The invention relates to a method for homogeneous detection and  
5 analysis of nucleic acid sequences by use of single-labeled oligonucleotide probes  
whose fluorescence emission changes in response to probe-target hybridization and  
dissociation, and more particularly, to methods for analyzing one or multiple nucleic  
acid loci using said probes. This invention further relates to the use of fluorescence  
changes in single-labeled probes for melting curve analysis, genotyping, and pathogen  
10 detection, and to a method for quantification of specific sequences in real-time  
monitoring of nucleic acid amplification.

Background and Summary of the Invention

Probe hybridization is a widely used method for the detection, analysis,  
15 and quantification of nucleic acid sequences. Common techniques include Southern  
hybridization, dot blotting, gel-shift assays, and solution-based homogeneous assays,  
and are often coupled with polymerase chain reaction (PCR). The basic devices used  
in these techniques include electrophoresis gels, DNA arrays immobilized on surfaces  
of glass slides, beads, membranes or microtiter plates, and instrumentation for  
20 homogeneous assays such as the LightCycler<sup>\*</sup> system (Roche Molecular  
Biochemicals), the ABI PRISM7700 sequence detection system (PE Applied  
Biosystems), and the iCycler<sup>\*</sup> system (Bio-Rad Laboratories). Homogeneous assays,  
detection methods that are coupled with amplification processes, perform  
amplification and analysis in one continuous flow, eliminating or minimizing the need  
25 to transfer samples between the two processes. One key element that makes  
homogenous assays work is a reporter signal generated from probe-target  
hybridization that is detectable without the need to wash away free probe.

Current homogeneous assays either use nucleic acid-binding dyes such  
as ethidium bromide and SYBR Green I stain as reporter molecules (Higuchi, U.S.  
30 Patent No. 5,994,056 and Wittwer et al., U.S. Patent No. 6,174,670), or they use a  
minimum of two fluorophores immobilized on probes. The two fluorophores can  
either be donor-acceptor pairs individually attached to separate oligonucleotides (U.S.

\*Trade-mark

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Patent No. 6,174,670, and Di Cesare, U.S. Patent No. 5,716,784), or they can be reporter-quencher pairs attached to a single oligonucleotide (Mayrand, U.S. Patent No. 5,691,146, Pitner et al, U.S. Patent No. 5,888,739 and Livak et al, U.S. Patent No. 6,030787). Homogeneous assays using DNA binding dyes are convenient, but they  
5 provide limited sequence information. Methods based on two-dye systems can provide greater detection specificity, regardless of whether they are donor-acceptor or donor-quencher dye combinations, and are used in systems such as the Hybridization Probe assay (U.S. Patent No. 6,174,670), the Taqman\* assay (U.S. Patent No. 5,691,146), the Molecular Beacon assay (Tyagi et al, 1998. Nature Biotechnology  
10 4:359-363) and its variant, the Scorpions\* primer system (Whitcombe et al, 1999. Nature Biotechnology 17:804-807).

In hybridization probe assays, two oligonucleotide probes are used to detect the presence of a particular sequence. Reporter signal is detected when fluorescence resonance energy transfer occurs between the donor dye on one probe  
15 and the acceptor on the other by bringing the two dyes into proximity through annealing of probes to target. Once the probes are hybridized, the area under one probe can be studied for possible sequence variances. This can be done by heating the sample and monitoring the temperature at which a loss in signal occurs by dissociation (or "melting") of that probe. Sequence variances may be detected by a  
20 shift in the melting temperature ( $T_m$ ) relative to a reference sample, and such  $T_m$  shifts can be predicted using software calculations (Schütz et al, 1999. BioTechniques 27:1218-1224). However, the area under the second probe may become a "blind zone" that is not analyzed for sequence variances. The presence of blind zones may be problematic when large segments of DNA need to be analyzed for sequence  
25 variances, and multiple probe pairs need to be employed.

The Taqman and molecular beacon assays both use a single oligonucleotide probe with both a reporter and a quencher dye attached. The oligonucleotide probe hybridizes to the target sequence, and the reporter and quencher are separated either by the exonuclease activity of the polymerase or due to change in  
30 conformation upon hybridization to the target sequence. Present methods result in relative difficulty in synthesizing these dual-labeled probes. Also, Taqman\* probes

\*Trade-mark

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provide an indirect measure of hybridization, as signal continues to be generated once the reporter and quencher are separated by the exonuclease activity of the polymerase.

Changes in fluorescence efficiency of fluorophores by means other than energy transfer have been reported. Various dyes of the fluorescein family are sensitive to pH, and their emission intensities decrease at pHs lower than their pKa, and increase when the pH is close to or higher than the pKa (Sjöback et al, 1995. Spectrochim Acta A 51, L7). Also, fluorescein is quenched by more than 50% upon conjugation to biopolymers (Der-Balian et al, 1988. Analytical Biochemistry 173:9). These are general fluorescence changes that are induced by external factors. Also known is that the annealing of a fluorescent-labeled oligonucleotide and its unlabeled complementary strand may result in quenching of the probe fluorescence and a shift in the wavelength of emission upon the formation of duplex DNA (Cooper et al 1990. Biochemistry 29:9261-9268; Lee et al, 1994. Analytical Biochemistry 220:377-383; and Yguerabide et al, 1996. Analytical Biochemistry 241:238-247). Fluorescent intensity changes have also been shown using unbound dye and individual nucleotide or nucleoside molecules (Seidel et al, 1996 J. Phys Chem 100:5541-5553), RNA substrate-ribozyme interactions (Walter et al, 1997. RNA 3:392-404), and nucleic acid duplex formation using probes labeled with asymmetric cyanine dyes (Ishiguro et al 1996. Nucleic Acids Research 24:4992-4997; and Svanvik et al 2000. Analytical Biochemistry 281:26-35). However, these references do not teach the construction of probes that take advantage of sequence-dependent fluorescence.

Thus, the present invention is directed to oligonucleotide probes wherein each probe has a single fluorescent dye. The oligonucleotide probes are constructed such that hybridization of the probe to a target sequence affects the fluorescent emission of the fluorescent dye. In one embodiment of the invention, hybridization of the probe to the target sequence places the fluorescent dye in close proximity to a guanine residue, with resultant quenching of fluorescent emission. In another embodiment, the fluorescent entity replaces a base in the oligonucleotide probe structure, and upon hybridization this "virtual nucleotide" is placed in a complementary position to a G residue, with resultant quenching of fluorescence. In other embodiments, probes are constructed such that hybridization results in an increase in fluorescent emission. In one such embodiment, the fluorescent entity is

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attached to a G residue, with increased fluorescence upon hybridization. In another such embodiment, the fluorescent entity is attached to a base analog, with resultant increase in fluorescence upon hybridization. In yet another embodiment of this invention, the fluorescent entity is attached to an internal residue via a flexible linker, with resultant change in fluorescent emission upon hybridization. Finally, various examples of probe systems are provided.

In one aspect of the invention a probe is provided for analyzing a target nucleic acid, the probe comprising a fluorescent detecting entity consisting essentially of an oligonucleotide having a sequence generally complementary to a locus of the target nucleic acid and a fluorescent label linked to a terminal nucleotide of the oligonucleotide, the oligonucleotide sequence of the probe being selected so that upon hybridization of the probe to the locus of the target nucleic acid the fluorescent label is positioned in proximity to a guanine residue of the target nucleic acid with resultant quenching of the fluorescent intensity of the fluorescent label. In one embodiment, the guanine residue is located at position 0, +1, +2, +3, or +4 relative to the position of the fluorescent labeled terminal nucleotide.

In another aspect of this invention, a probe is provided for analyzing a target nucleic acid, the probe comprising an oligonucleotide having a sequence generally complementary to a locus of the target nucleic acid and further comprising a residue having a virtual nucleotide wherein a fluorescent dye is substituted for a base, and wherein the magnitude of fluorescent emission from the fluorescent dye is altered by hybridization of the probe to the target nucleic acid.

In still another aspect of this invention, a fluorescence-based probe system is provided for analyzing a target nucleic acid, the probe system consisting essentially of a single-labeled polynucleotide comprising a sequence generally complementary to a locus of the nucleic acid and a fluorescent label attached thereto, whereby upon hybridization of the single-labeled polynucleotide to the locus of the nucleic acid the fluorescent label is positioned near a residue of the target nucleic acid with a resultant increase in fluorescent intensity of the fluorescent label. Various embodiments of these augmentation probes are provided.

In yet another aspect of this invention a probe for analyzing a target nucleic acid is provided, the probe comprising a fluorescent detecting entity

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consisting essentially of a single-labeled oligonucleotide having a sequence generally complementary to a locus of the target nucleic acid and a fluorescent label linked to an internal residue of the oligonucleotide, and wherein oligonucleotide sequence of the probe being selected so that upon hybridization of the probe to the locus of the target nucleic acid the magnitude of fluorescent emission from the fluorescent label is altered by hybridization of the probe to the target nucleic acid.

Additionally, an oligonucleotide probe is provided for detecting the presence of a target nucleic acid from the genus *Salmonella* the probe comprising a nucleotide sequence selected from the group consisting of

10 5'CCAAAAGGCAGCGTCTGTTCC (SEQ ID NO:3),  
5'CCAAAAGGCAGCGTCTGTTC (SEQ ID NO:4),  
5'CAAAAGGCAGCGTCTGTTCC (SEQ ID NO:5),  
5'CCAAAAGGCAGCGTCTGTT (SEQ ID NO:6), 5'CAAAAGGCAGCGTCTGTT  
(SEQ ID NO:7), 5'AAAAGGCAGCGTCTGTTC (SEQ ID NO:8),  
15 5'AAAAGGCAGCGTCTGTTCC (SEQ ID NO:9), and  
5'AAAAGGCAGCGTCTGTT (SEQ ID NO:10).

In another aspect of this invention methods are provided using the probes of this invention, in one such embodiment, a method is provided for determining the presence of a target nucleic acid sequence in a biological sample comprising combining a first single-labeled oligonucleotide probe with the sample, said first probe having an oligonucleotide sequence generally complementary to a locus of the target nucleic acid sequence and a fluorescent label linked to an end of the oligonucleotide sequence, the fluorescent label exhibiting an hybridization-dependent fluorescent emission, wherein hybridization of the first probe to the target nucleic acid sequence allows interaction of the fluorescent label with a guanine residue located on the target nucleic acid, thereby decreasing the magnitude of fluorescent emission from the label, illuminating the biological sample, and monitoring the hybridization-dependent fluorescent emission.

In a further aspect of this invention a method is provided for determining the presence of a target nucleic acid sequence in a biological sample comprising combining a single-labeled oligonucleotide probe with the sample, said probe having an oligonucleotide sequence generally complementary to a locus of the

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target nucleic acid sequence and a fluorescent label linked to a G residue of the oligonucleotide sequence, the fluorescent label exhibiting an hybridization-dependent fluorescent emission, wherein hybridization of the oligonucleotide probe to the target nucleic acid sequence alters interaction of the fluorescent label with the G residue, 5 thereby increasing the fluorescent emission from the label, illuminating the biological sample, and monitoring the hybridization-dependent fluorescent emission.

In still a further aspect of this invention a method is provided for analyzing a sample comprising a target nucleic acid sequence, comprising the steps of combining the sample and an oligonucleotide probe to create a target-probe mixture, 10 wherein the probe includes a virtual nucleotide having a fluorescent label positioned so that the magnitude of fluorescent emission from the fluorescent label is altered by hybridization of the probe to the target nucleic acid sequence, illuminating the mixture, and monitoring the fluorescent emission as a function of temperature.

In an additional aspect of this invention a method is provided for 15 determining the presence a target nucleic acid sequence in a biological sample comprising combining the biological sample with a fluorescent detecting entity consisting essentially of a single-labeled oligonucleotide probe, wherein the single-labeled probe comprises an oligonucleotide having a sequence complementary to a locus of the target nucleic acid sequence, and having a fluorescent label exhibiting an 20 hybridization-dependent emission attached thereto, wherein hybridization of the probe to a selected segment of the target nucleic acid sequence results in an increase in fluorescent emission of the fluorescent label, illuminating the biological sample, and monitoring the hybridization-dependent fluorescent emission. In one such embodiment the fluorescent label is linked to a base of the oligonucleotide probe and 25 the base is selected from the group consisting of 5-nitroindole, 4-nitroindole, 6-nitroindole, and 3-nitropyrrole deoxynucleosides. In another such embodiment, the fluorescent label is attached to a guanine residue and the monitoring step includes monitoring the increased fluorescent emission from the fluorescent label upon hybridization of the probe to the target nucleic acid. In yet another embodiment, the 30 fluorescent label is selected from the group consisting of cyanine dyes and LCRed 705.



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An additional aspect of this invention is a kit for analyzing a biological sample comprising a nucleic acid sequence, comprising a fluorescent detecting entity consisting essentially of a single-labeled oligonucleotide probe having an oligonucleotide linked to a fluorescent label, wherein said probe is  
5 configured to hybridize to a locus of the segment so that the magnitude of fluorescent emission from the fluorescent label is increased by hybridization of the probe to the locus; and components for amplification of the nucleic acid sequence.

One specific aspect of the invention relates to a fluorescence-based probe for analyzing a target nucleic acid comprising: a single-labeled polynucleotide  
10 hybridization probe comprising a sequence with at least 80% identity to the complementary sequence of a locus of the nucleic acid and a fluorescent label attached to a terminal nucleotide of the probe, wherein said nucleotide is a base analog, and wherein the base analog is selected from the group consisting of 5-nitroindole, 4-nitroindole, 6-nitroindole, 3-nitropyrrole, 5-iodo-2'-cytidine, inosine,  
15 and a nebularine deoxynucleoside.

Another aspect of the invention relates to a hybridization probe for analyzing a target nucleic acid comprising: a fluorescent detecting entity comprising a single-labeled oligonucleotide comprising a sequence with at least 80% identity to the complementary sequence of a locus of the target nucleic acid  
20 and a fluorescent label linked to an internal residue of the oligonucleotide, said internal residue comprising a base analog wherein the base analog is selected from the group consisting of 5-nitroindole, 4-nitroindole, 6-nitroindole, 3-nitropyrrole, 5-iodo-2'-cytidine, inosine, and a nebularine deoxynucleoside.

Another aspect of the invention relates to a method for determining  
25 the presence of a target nucleic acid sequence in a biological sample comprising: combining a single-labeled oligonucleotide hybridization probe with the target nucleic acid, said probe comprising an oligonucleotide sequence with at least 80% identity to the complementary sequence of a locus of the target nucleic acid sequence and a fluorescent label linked to an internal nucleotide of the  
30 oligonucleotide sequence, wherein the nucleotide is a base analog selected from 5-nitroindole, 4-nitroindole, 6-nitroindole, 5-iodo-2'-cytidine wherein hybridization of the probe to the target nucleic acid places a G residue in position +1, 0, or -1

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relative to the position of the internal residue, and the fluorescent label is linked to the internal residue by a linker sufficiently flexible to allow quenching by the G residue so the probe exhibits a hybridization-dependent increase in fluorescent emission, illuminating the probe, and monitoring the hybridization-dependent fluorescent emission, wherein increased  
5 fluorescence emission indicates the presence of the target nucleic acid sequence.

Another aspect of the invention relates to a method for determining the presence of a target nucleic acid sequence in a biological sample comprising: combining the biological sample with a pair of primers used for amplifying a selected segment of the target nucleic acid sequence and a fluorescent detecting entity comprising a single-labeled  
10 oligonucleotide hybridization probe, wherein the single-labeled probe comprises an oligonucleotide comprising a sequence with at least 80% identity to the complementary sequence of a locus of the selected segment of the target nucleic acid sequence, and comprising a fluorescent label attached to a terminal nucleotide of the probe, wherein said fluorescent label exhibits a sequence specific hybridization-dependent emission and said  
15 terminal nucleotide is a base analog selected from 4 nitroindole, 5-nitroindole, 6 nitroindole, inosine, 5-iodo-2'-cytidine, a nebularine deoxynucleoside, and a 3 nitropyrrole deoxynucleoside, and wherein hybridization of the probe to the locus results in an increase in fluorescence emission of the fluorescent label, adding a polymerase and amplifying the selected segment of the nucleic acid sequence through a plurality of amplification cycles,  
20 illuminating the biological sample, and monitoring the hybridization-dependent increase in fluorescent emission, wherein increased fluorescence emission indicates the presence of the target nucleic acid sequence.

Another aspect of the invention relates to a method for determining the presence of a target nucleic acid sequence in a biological sample comprising: combining the  
25 biological sample with a fluorescent detecting entity comprising a single-labeled oligonucleotide hybridization probe, wherein the single-labeled probe comprises an oligonucleotide sequence with at least 80% identity to the complementary sequence of a locus of the target nucleic acid sequence, and a fluorescent label attached to the 5' terminal nucleotide of the oligonucleotide, wherein said terminal nucleotide is a base analog selected  
30 from 4-nitroindole, 5 nitroindole, 6 nitroindole, inosine, 5-iodo-2'-cytidine, a nebularine deoxynucleoside, and a 3 nitropyrrole deoxynucleoside, wherein the

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fluorescent label exhibits a hybridization-dependent emission, wherein hybridization of the probe to the locus results in an increase in fluorescence emission of the fluorescent label, combining the biological sample and the probe with a second oligonucleotide and a polymerase, wherein the probe and the second oligonucleotide function as a pair of  
 5 primers for amplification, amplifying the target nucleic acid sequence, illuminating the biological sample, and monitoring the hybridization-dependent increase in fluorescence emission, wherein increased fluorescence emission indicates the presence of the target nucleic acid sequence.

Another aspect of the invention relates to a kit for analyzing a sample  
 10 comprising a nucleic acid sequence, comprising: a. a fluorescent detecting entity comprising a single-labeled oligonucleotide hybridization probe having a fluorescent label linked to a terminal nucleotide of the oligonucleotide, wherein said terminal nucleotide is a base analog and wherein the base analog is selected from 4-nitroindole, 5-nitroindole, 6-nitroindole, inosine, 5-iodo-2'-cytidine, a nebularine deoxynucleoside,  
 15 and a 3-nitropyrrole deoxynucleoside and wherein said probe hybridizes to a single-stranded locus of the nucleic acid sequence so that the magnitude of fluorescence emission from the fluorescent label increases by hybridization of the probe to the locus; and b. components for amplification of the nucleic acid sequence, wherein the components include a pair of oligonucleotide primers for amplifying a segment of  
 20 said nucleic acid sequence and a thermostable DNA polymerase.

Additional features of the present invention will become apparent to those skilled in the art upon consideration of the following detailed description of preferred embodiments exemplifying the best mode of carrying out the invention as presently perceived.

## 25 Brief Description of Drawings.

Fig. 1 shows fluorescence acquisition data for the three sets of probes and targets shown in Table 1, Set A (————), Set B (-----), Set C (— - — -).

Fig. 2 is a dF/dT plot of the data shown in Fig 1, with the melting  
 30 curves converted to melting peaks.

Fig. 3 is a plot of cycle number vs relative fluorescence quenching.

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Figs. 4A and B are plots of melting curve data for the Factor V gene, Fig. 4A shows fluorescence vs temperature and Fig. 4B shows the first derivative  $dF/dT$ .

Figs. 5A and B show melting curves for mutation analysis by probe multiplexing. Fig. 5A shows no mismatch vs mismatch under fluorescein probe, and Fig. 5B shows no mismatch vs mismatch under quencher probe or under both probes.

Fig. 6 shows homogeneous, real-time factor V Leiden (G1691A) genotyping with an internally labeled fluorescein probe, with melting curve data presented as a first derivative plot. Curves for homozygous wild type (————), homozygous mutant (-----), heterozygous genotypes (.....) are shown.

Fig. 7 shows homogeneous, real-time Factor V Leiden genotyping with a fluorescein dequenching probe. Melting curve data is presented as negative first derivative plots for homozygous wild type, homozygous mutant, heterozygous genotypes, and negative control.

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Figs. 8a and 8b are plots of peak area obtained from melting curves vs either buffer pH (fig. 8a), or cation concentration of buffer (8b). Data for the quenching probe (closed symbols) and for the dequenching probe (open symbols) are shown with buffer conditions of 10 mM Tris, 100-160 mM KCL (squares); 10 mM Tris pH8.3-8.8 (circles), and 10 mM 2-amino-2-methyl-1-propanol, 160 mM KCl (triangles).

Fig. 9 is a plot of melting curve data for the dequenching probe, in which the level and direction of fluorescence change is affected by the pH of buffer. Curves for buffer pH 7.2, pH 7.7, pH 8.2, pH 8.8 are shown.

Fig. 10 is a plot of melting peak area vs probe Tm.

#### Detailed Description of the Invention

In an illustrated embodiment, a probe of the invention is used in a homogeneous assay system wherein the detection and analysis of nucleic acid sequences are performed along with the amplification of a target polynucleotide. Alternatively, the probes of the invention may be used in end-point detection assays independent of target amplification. The binding site of the single-labeled oligonucleotide probes is generally located internally on a target nucleic acid, usually between the primers that are used to amplify the target. However, in some embodiments, hybridization of the probe to the target sequence is near or at the end of the target sequence, and in some embodiments the probe-target hybridization forms a blunt end, such as in methods wherein the probe also functions as a primer for target amplification.

The term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages including deoxyribonucleosides, ribonucleosides, protein nucleic acid nucleosides, and the like that are capable of specifically binding to a target polynucleotide by base-pairing interactions.

The term "single-labeled oligonucleotide" as used herein includes oligonucleotides having a singular fluorescent label. The label may be provided on the oligonucleotide in various ways, including linked to an end of the phosphate-sugar backbone or to a base of the oligonucleotide, or the dye may be used to replace a base as part of a "virtual nucleotide" structure. However, the term "single-labeled

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oligonucleotide” excludes constructs having multiple fluorescent dyes attached thereto, such as Taqman probes.

Whenever an oligonucleotide is represented by a sequence of letters, it will be understood that the “A” denotes deoxyadenosine, “T” denotes thymidine, “G” denotes deoxyguanosine, “C” denotes deoxycytidine, unless otherwise noted. “(F)” denotes a fluorescent label.

The term “base,” when used to indicate the position in an oligonucleotide, includes base analogs such a 5-nitroindole-2 deoxynucleoside.

The term “complementary” refers to nucleic acid sequences that form a base-paired double helix with each other. When discussing oligonucleotides, “complementary” refers to the opposing strand and when discussing individual bases of an oligonucleotide, “complementary” refers to the position or base on the opposing strand. “Generally complementary” sequences are two nucleic acid sequences that have at least 80% homology. Thus, such sequences may have mismatches but have sufficient homology to form base-paired double helix structures with each other.

According to a method of the invention, a single-labeled probe undergoes changes in fluorescence emission efficiency (or intensity) during the formation and dissociation of a probe-target duplex, the method comprising positioning the probe so that certain conditions are satisfied regarding the location of specific residues on the target strand.

In one embodiment of this invention, the specific residue is a single G residue on the target strand. In this embodiment, fluorescence change upon duplex formation and dissociation is most pronounced when the G is located as the first overhanging nucleotide relative to the fluorescent label (F) as shown diagrammatically below (vertical lines denote base pairing):

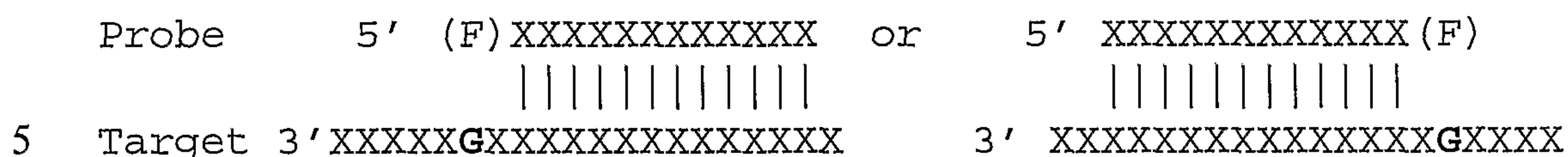
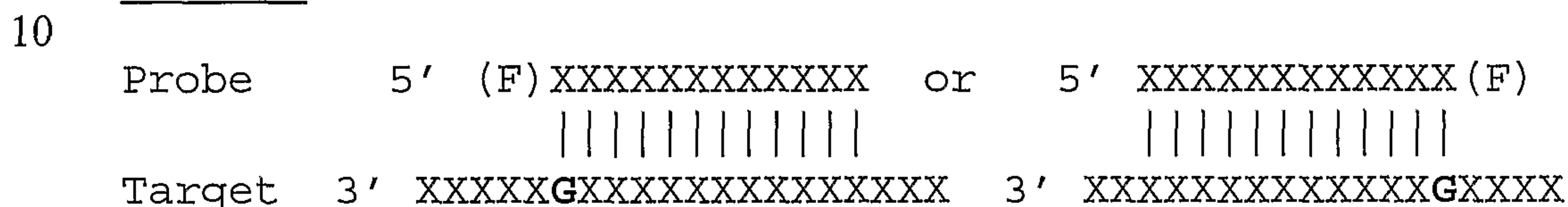
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Probe      5' (F)XXXXXXXXXXXXX   or   5' XXXXXXXXXXXXXXXX (F)
           |||
Target    3'XXXXXXGXXXXXXXXXXXXX   3' XXXXXXXXXXXXXXXXGXXXXX

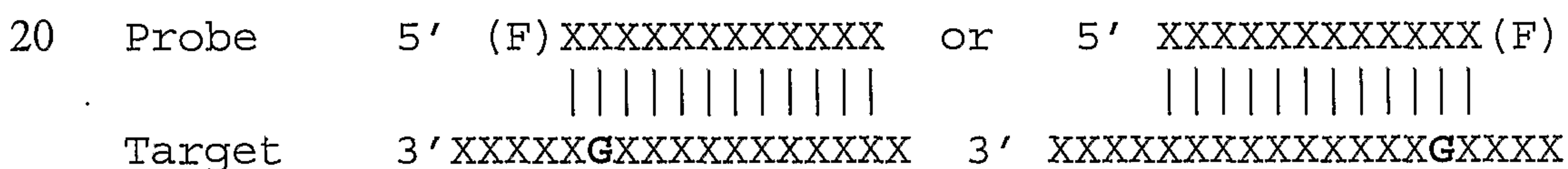
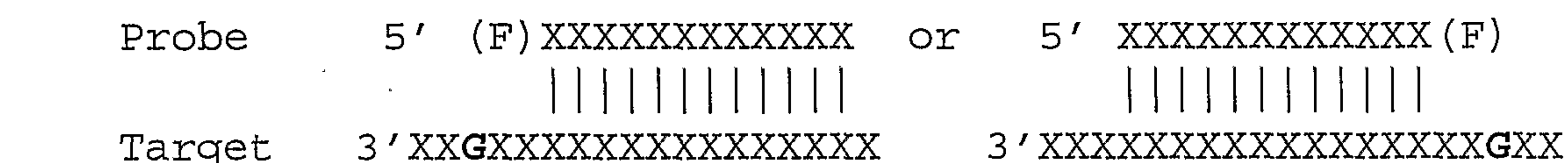
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Each of these two positions comprise “**Position +1**”. Fluorescence change, albeit smaller, is also observed when the G is at any of the following positions:

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**Position +2****Position 0**

15 whereas, single G residues in position +3 and position -1 shown below have little effect on detected fluorescence.

**Position -1****Position +3**

30

Small fluorescent change has also been noted when the G residue is in position +4.

When there is more than one G on the target strand, multiple G residues in any of the positions 0, +1, +2, +3, and +4 are effective to alter detected fluorescence. These are the "assigned positions." While the above representations of the assigned positions are shown with respect to G residues, the same terminology for the assigned positions is used throughout this specification with respect to other embodiments.

40 An alternative embodiment incorporates a "virtual nucleotide," in which the fluorescent dye itself is substituted for the base. In this embodiment the fluorescent entity has direct access to a guanine residue located at the complementary

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position (position 0) on the target strand. Fluorescent change may also be possible if the G is at +1 in either the 5' or 3' direction. Bases other than G in position 0 may also be useful for fluorescent change, depending on the fluorescent dye used to create the virtual nucleotide. For example, when fluorescein is used as the virtual  
5 nucleotide and an A residue is in position 0, an increase in fluorescence may be seen upon hybridization. The virtual nucleotide may be the end base or occupy an internal position of the oligonucleotide.

In another embodiment of this invention a probe with a label attached to the G residue can be used to facilitate fluorescence change upon duplex formation  
10 and dissociation. When fluorescein and fluorescein derivatives are used in this embodiment, the G to which the fluorescein is attached will itself effect quenching of the label when the probe is unbound and free. Upon formation of the probe-target duplex, the fluorescein becomes sterically sequestered from the G to which it is attached, dequenching will result, and fluorescence will be restored. A similar result  
15 is expected when the fluorophore is attached to the G residue, and a mismatching A or T is in the 0 position. In this embodiment best results have been obtained when there are no G residues in the -1 or +1 positions.

In a similar embodiment, the fluorescent entity may be attached to a residue via a flexible linker. When the residue is an A or T residue, one would expect  
20 increased fluorescence upon hybridization. Such a probe may be constructed using a C6dT nucleotide (Glen Research, Sterling, VA) to provide the suitable flexibility. This construction would be appropriate for use for both target sequence detection and mutation detection. With mutation detection, the fluorescent entity should be located sufficiently removed from the mutation so that the mismatch does not affect the steric  
25 relationship between the fluorescent entity and the base to which the fluorescent entity is linked.

A wide variety of fluorophores can be used as labels for probes in this invention. Such groups include fluorescein and its derivatives, including but not limited to JOE, FAM, rhodamines, Alexa Fluor 488, Oregon Green dyes, erythrosins,  
30 and eosins, fluorescein-cyanine conjugates, such as Big Dyes, and derivatives of the bispyrromethene boron-difluoride dyes, such as BODIPY. When these dyes are attached to oligonucleotide probes, fluorescence is usually quenched upon annealing



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of the probe with its complementary target strand if the target has G residues in the assigned position(s). However, as discussed above, in another embodiment of this invention the direction of fluorescence change may be reversed by attaching the fluorophore to a G residue on the probe, preferably wherein there is an absence of  
5 other Gs at assigned positions on the complementary strand.

Similarly, in another embodiment of this invention, a fluorescent increase is seen if the fluorophore is attached to a "base analog" such as 5-nitroindole 2'-deoxynucleoside. In general, base analogs such as 5-nitroindole, 4-nitroindole, 6-nitroindole, and 3-nitropyrrole deoxynucleosides that form relatively stable pairing  
10 with normal bases are also useful. Other base analogs such as inosine, 5-iodo-2'-cytidine, and nebularine deoxynucleosides form weak base-pairing with the normal bases, and generally require the absence of the G residue at position +1 for a fluorescence change to be observed upon duplex formation and dissociation.

In yet another embodiment of this invention, fluorophores from the  
15 group of cyanine dimers and monomers, such as TOTO, YOYO, TO-PRO, Cy3, Cy5, Cy5.5, Cy7 etc., or dyes such as LCRed 705 may be used as the fluorescent dye. It has been found that probes incorporating these fluorescent dyes exhibit fluorescence augmentation rather than quenching upon probe hybridization.

Kits of the invention contain probes labeled with a single fluorescent  
20 dye. The kits may be used to detect the presence or absence of specific nucleic acid sequences in a sample, or may be used during or after the target is prepared by an amplification process such as PCR. Multiple probes may be multiplexed by  $T_m$ , color, or by direction of change in fluorescence. Detection of multicolor reporter signals could be achieved by single-wavelength excitation or by multiple wavelength  
25 excitation. The kits may further be used for quantitative analysis of the initial concentration of analyte.

Target amplification methods of the present invention include suitable procedures known in the art for amplifying a nucleic acid, provided that the method generates one or more target nucleic acid sequences capable of hybridizing to an  
30 oligonucleotide probe. Such suitable procedures include polymerase chain reaction (PCR); strand displacement amplification (SDA); nucleic acid sequence-based amplification (NASBA); cascade rolling circle amplification (CRCA), Q beta

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replicase mediated amplification; isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN); transcription-mediated amplification (TMA), and the like. Therefore, when the term PCR is used, it should be understood to include other alternative amplification methods.

5           Analysis may occur during amplification in a homogeneous assay system. See, e.g., U.S. Patent No. 6,174,670. Alternatively, the target nucleic acid may be studied through melting curve analysis subsequent to amplification. Other end-point analysis is also within the scope of this invention, and includes use of probes that are immobilized or are used with non-fluorescent tags, such as biotin. It is  
10 also understood that nucleic acid analysis independent of amplification is within the scope of this invention. When a probe of this invention is used in a homogeneous assay with PCR, the probe may be complementary to a locus located between the primers. Alternatively, the probe itself may function as one of the primers.

          Rapid and specific detection of pathogens can be performed using  
15 single-labeled probes in real-time PCR and in post-PCR melting analysis. Pathogens include, but not limited to, *Salmonella*, pathogenic *E. coli* (such as *E. coli* O157:H7), *Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio Cholerae*, and *Clostridium botulinum*. Specimens applicable to PCR may include food samples, feces, tissue homogenate, washing fluid, and others. Single-labeled probes may also be used in  
20 mutation detection. Examples of mutations include, but are not limited to Factor V Leiden, hemoglobin C and S mutations, the thermolabile mutation of methylenetetrahydrofolate reductase, Factor II (prothrombin) G20210A mutation, hemochromatosis-associated mutations C187G and G845A, and the cystic fibrosis F508del mutation. It is understood that these lists are exemplary only, and they are  
25 not meant to be exhaustive.

**Example 1**  
**Exemplary single-labeled probes and target sequences**  
**for guanine quenching**

30

The following are examples of probes that may be used for detection of a target sequence or mutation detection. Examples of primers for use with PCR amplification are also provided.

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For Salmonella detection, a DNA fragment from the gene SpaQ (GenBank Accession # U29364) may be amplified by PCR using, for instance, primers SQF (5'TGGATGATTTAGTGTTTGC (SEQ ID NO:1)), and SQR (5'CGCCCGTAAGAGAGTAAAC (SEQ ID NO:2)), various probes may be used

5 to detect amplification. Examples include:

SQP1 5'CCAAAAGGCAGCGTCTGTTCC (SEQ ID NO:3)

SQP2 5'CCAAAAGGCAGCGTCTGTTCC (SEQ ID NO:4)

SQP3 5'CAAAAAGGCAGCGTCTGTTCC (SEQ ID NO:5)

SQP4 5'CCAAAAGGCAGCGTCTGTT (SEQ ID NO:6)

10 SQP5 5'CAAAAAGGCAGCGTCTGTT (SEQ ID NO:7)

SQP6 5'AAAAGGCAGCGTCTGTTCC (SEQ ID NO:8)

SQP7 5'AAAAGGCAGCGTCTGTTCC (SEQ ID NO:9)

SQP8 5'AAAAGGCAGCGTCTGTT (SEQ ID NO:10)

15 where the fluorescent label is attached either to the 3' or 5' end of the probe. The 5'-labeled probes may be blocked from extension by the addition of a 3' phosphate. The above probes hybridize to one of the following target sequences

SQT1 5'AGGAACAGACGCTGCCTTTTGGC (SEQ ID NO:11)

SQT2 5'AGGAACAGACGCTACCTTTTGGC (SEQ ID NO:12)

SQT3 5'AGGAACAAACGCTACCTTTTGGC (SEQ ID NO:13)

20 which is contained in the segment amplified by the primers. These designs provide G residues at positions -1 and 0, 0 and +1, or at +1 and +2 on the target strand depending on which probe is used. Fluorescence quenching or augmentation, depending on the specific fluorescent label used, may be observed by dissociation of the probe-target duplex. High selectivity and sensitivity is achieved in the detection of Salmonella  
25 subspecies using melting curve analysis. Melting curve analysis may be performed during or subsequent to PCR amplification.

Genotyping for Factor V Leiden (G1691A) mutation may be performed by PCR melting analysis by use of a single-labeled probe such as:

FVP1 5'CTGTATTCCTCGCCTGTC (SEQ ID NO:14)

30 FVP2 5'TGTATTCCTCGCCTGTC (SEQ ID NO:15)

FVP3 5'CTGTATTCCTCGCCTGT (SEQ ID NO:16)

The probe may be labeled either at the 5' end (preferably with addition

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of a 3'phosphate) or at the 3'end. These probes may be used to hybridize to a segment of the Factor V gene (Genbank Accession #L32764) having the sequence of either

FVT1 5'TGGACAGGCGAGGAATACAGGT (SEQ ID NO:17)

5 (wild type)

FVT2 5'TGGACAGGCAAGGAATACAGGT (SEQ ID NO:18)

(Leiden mutant)

or a variant of either FVT1 or FVT2 with at least about 80% homology. Hybridization of probe to target will provide G residues either at positions 0 and +1, or at +1 and +2 on the target strand. The fragment containing the target sequences of the Factor V gene may be amplified by primers such as FVF

(5'GAGAGACATCGCCTCTGGGCTA (SEQ ID NO:19)) and FVR

(5'TGTTATCACACTGGTGCTAA (SEQ ID NO:20)). The Factor V Leiden

15 mutation (a C:A mismatch) is distinguished from the normal type because of duplex destabilization leading to a decrease in T<sub>m</sub>, detectable during melting analysis.

Genotyping of hemoglobin C (HbC) and S (HbS) mutations (Genbank Accession #U01317) may be performed by post PCR melting analysis with a single-labeled probe such as:

BGP1 5'CTGACTCCTGTGGAGAAGTCTG (SEQ ID NO:21)

20 BGP2 5'TGACTCCTGTGGAGAAGTCTG (SEQ ID NO:22)

The probe may be labeled either at the 5'end (with addition of a 3'phosphate) or at the 3'end. These probes hybridize to a target sequence of either

BGT1 5'CGGCAGACTTCTCCTCAGGAGTCAGGT (SEQ ID NO 23)

(wild type)

25 BGT2 5'CGGCAGACTTCTCCACAGGAGTCAGGT (SEQ ID NO:24)

(HbS mutant)

BGT3 5'CGGCAGACTTCTCCTTAGGAGTCAGGT (SEQ ID NO:25)

(HbC mutant)

or a variant of BGT1, BGT2, or BGT3 with 80% homology. Probe-target hybridization provides G residues at positions 0 and +1, or at +1 and +2 on the target strand. The fragment containing the mutations may be amplified by primers such as BGF (5'ACACAACACTGTGTTCACTAGC (SEQ ID NO:26)) and BGR

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5'CAACTTCATCCACGTTCCACC (SEQ ID NO:27)). The HbS (complete match) and HbC genotypes (continuous G:T and T:T mismatches) can be discriminated from wild type (T:T mismatch) by differences in  $T_m$ .

5 Genotyping of the thermolabile mutation of methylenetetrahydrofolate reductase (Genbank Accession # U09806) may be performed by melting analysis with a single-labeled probe selected from the group consisting of:

MFP1 5'TGCGTGATGATGAAATCGGCTCC (SEQ ID NO:28)

MFP2 5'TGCGTGATGATGAAATCGGCTC (SEQ ID NO:29)

MFP3 5'TGCGTGATGATGAAATCGGCT (SEQ ID NO:30)

10 The probe may be labeled either at the 5' end (with addition of a 3' phosphate) or at the 3' end. These probes will hybridize to target sequences of either

MFT1 5'CGGGAGCCGATTTTCATCATCACGCAGC (SEQ ID NO:31)

(wild type)

MFT2 5'CGGGAGTCGATTTTCATCATCACGCAGC (SEQ ID NO:32)

15 (mutant)

or their variants with at least about 80% homology. Probe-target hybridization will provide a G at position +1 for the 5'-labeled probes, or G residues at positions 0 and +1, 0, +1 and +2, or +1, +2, and +3 for 3'-labeled probes. The fragment of the methylenetetrahydrofolate reductase may be amplified by primers such as MFF

20 (5'TGAAGGAGAAGGTGTCTGCGGGA (SEQ ID NO:33)) and MFR (5'AGGACGGTGCGGTGAGAGTG (SEQ ID NO:34)). The mutation results in the most stable G:T mismatch, but it can be distinguished by the destabilization of the duplex, particularly in post amplification melting analysis.

25 Genotyping of the Factor II (or prothrombin) G20210A mutation (Genbank Accession #M17262 and M33691) may be performed by post PCR melting analysis with a single-labeled probe such as F2P 5'TCTCAGCAAGCCTCAATGCT (SEQ ID NO:35). The probe may be labeled either at the 5' end (with addition of a 3' phosphate) or at the 3' end. The probe may be used to hybridize to target sequences of either

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F2T1 5'GGGAGCATTGAGGCTCGCTGAGAGT (SEQ ID NO:36)

(wild type)

F2T2 5'GGGAGCATTGAGGCTTGCTGAGAGT (SEQ ID NO:37)

(mutant)

5 or their variant with at least about 80% homology. Probe-target hybridization provides a G residue on the target strand at position +1 when the probe is 5'-labeled, and at positions +1, +2, and +3 when the probe is 3'-labeled. The fragment containing the mutation site may be amplified by primers such as F2F (5'ATTGATCAGTTTGGAGAGTAGGGG (SEQ ID NO:38)) and F2F  
10 (5'GAGCTGCCCATGAATAGCACT (SEQ ID NO:39)). The wild type duplex has a C:A mismatch and is distinguished from the mutation due to destabilization of the duplex.

Genotyping of the hemochromatosis-associated mutation C187G

(Genbank Accession #Z92910) may be performed by melting analysis using a single-

15 labeled probes such as:

HHDP1 5'CACACGGCGACTCTCATCATCATAGAAC (SEQ ID NO:40)

HHDP2 5'ACACGGCGACTCTCATCATCATAGAAC (SEQ ID NO:41)

HHDP3 5'CACACGGCGACTCTCATCATCATAGAA (SEQ ID NO:42)

The probe may be labeled either at the 5' end (with addition of a  
20 3'phosphate) or at the 3' end. These probes will hybridize to target sequences of either

HHDT1 5' TGTTCTATGATCATGAGAGTCGCCGTGTGGA (SEQ ID NO:43)

(wild type)

HHDT2 5' TGTTCTATGATGATGAGAGTCGCCGTGTGGA (SEQ ID NO:44)

(mutant)

25 or a variant with at least about 80% homology. Probe-target hybridization provides G residues on the target strand at positions 0 and +1, or +1 and +2 for the 5'-labeled probes, and a G at position 0 or +1 for 3'-labeled probes. The fragment containing the mutation site is amplified by primers such as HHDF

(5'CACATGGTTAAGGCCTGTTG (SEQ ID NO:45)) and HHDR

30 (5'GATCCCACCCTTTCAGACTC (SEQ ID NO:46)). The mutation can be

distinguished from wild type, wild type having has a C:C mismatch and a lower Tm.

Genotyping of the hemochromatosis-associated mutation G845A

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(Genbank Accession #Z92910) may be performed by post PCR melting analysis using single-labeled probes such as:

HCYP1 5'CACCTGGCACGTATATCTCTG (SEQ ID NO:47)

HCYP2 5'ACCTGGCACGTATATCTCTG (SEQ ID NO:48)

5 These probes may be labeled either at the 5' end (with addition of a 3' phosphate) or at the 3' end. These probes will hybridize to target sequences of either

HCYT1 5'AGCAGAGATATACGTGCCAGGTGGA (SEQ ID NO:49)  
(wild type)

10 HCYT2 5'AGCAGAGATATACGTACCAGGTGGA (SEQ ID NO:50)  
(mutant)

or a variant with at least about 80% homology. Probe-target hybridization provides G residues on the target strand at positions 0 and +1, or +1 and +2 for the 5'-labeled probes, and a G at position +1 for 3'-labeled probes. The fragment containing the mutation site is amplified by primers such as HCYP

15 (5'TGGCAAGGGTAAACAGATCC (SEQ ID NO:51)) and HCYR  
(5'TACCTCCTCAGGCACTCCTC (SEQ ID NO:52)). The mutation (C:A mismatch) can be distinguished from wild type by its lower T<sub>m</sub>.

20 Genotyping of the common 3 base pair deletion (F508del) associated with cystic fibrosis may be detected with a single-labeled probe selected from the group consisting of:

CFP1 5'ATAGGAAACACCAAAGATGATATTTTC (SEQ ID NO:53)

CFP2 5'ATAGGAAACACCAAAGATGATATTTT (SEQ ID NO:54)

The probe may be labeled either at the 5' end (with addition of a 3' phosphate) or at the 3' end, and hybridizes to target sequences of either

25 CFT1 5'AGAAAATATCATCTTTGGTGTTTCCTATGA (SEQ ID NO:55)  
(wild type)

CFT2 5'AGAAAATATCATTGGTGTTTCCTATGA (SEQ ID NO:56)  
(deletion mutation)

30 or their variant with at least about 80% homology. Probe-target hybridization provides a G on the target strand at position +1 with the 5' label, and a G at position 0 or +1 with the 3' label. The fragment containing the mutation site (Genbank Accession # M55115) may be amplified by primers such as CFF

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(5'GGAGGCAAGTGAATCCTGAG (SEQ ID NO:57)) and CFR (5'CCTCTTCTAGTTGGCATGCT (SEQ ID NO:58)). The mutation results in the destabilization of the duplex and a corresponding decrease in the  $T_m$ .

In all of the above examples, the fluorescent entity is provided on the 5' or 3' end nucleotide, with at least one G located in the 0, +1, or +2 position on the target strand. It is understood that the fluorescent entity may be located on a nucleotide internal to the end of the oligonucleotide probe, if the fluorescent entity has sufficient access to a G residue. For example, given appropriate linker structure, as is known in the art, the fluorescent entity may be linked one base internal to the end, and fluorescence maybe quenched by a G residue located at positions +1, 0, or -1 (relative to the position of the fluorescent entity). Other constructs wherein the linker is sufficiently flexible to allow access of the fluorescent entity to a G residue are considered to be within the scope of this invention.

15

### Example 2

#### Probe-target dissociation monitored by 3'-labeled probes

DNA oligonucleotides shown in Table 1 were prepared by standard DNA synthesis using solid-phase phosphoramidite chemistry with conventional deprotection, followed by desalting and purification steps using a Sephadex\* G-25 column and C<sub>8</sub>-reversed-phase HPLC. The probe oligonucleotides were labeled at the 3' end with a fluorescein molecule (a 5-carboxyfluorescein moiety with a C<sub>6</sub> cyclic linker) by use of fluorescein CPG column supports (Catalog number BGX-6190-1, BioGenex Inc, San Ramon, CA).

25

A real-time rapid PCR thermal cycler apparatus (LightCycler instrument, Roche Molecular Biochemicals, Indianapolis, IN) was used to monitor changes in fluorescence emission during denaturation (or dissociation) of the probe from target. The samples consisted of 0.1  $\mu\text{M}$  of probe oligonucleotide, 0.12  $\mu\text{M}$  or 0.24  $\mu\text{M}$  of target, 5 mM MgCl<sub>2</sub>, 0.25 mg/ml bovine serum albumin (BSA), and 50 mM Tris buffer (pre-set crystals, pH 8.3 at 25°C). Samples were first denatured at 95°C and cooled quickly to ensure annealing of probe to target. Then fluorescence emission intensity was measured as the temperature was changed from 40°C to 97°C at a heating ramp of 0.2°C/sec. Samples were excited at 470 nm. Fluorescence

30

\*Trade-mark



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emission was detected with a 20 nm band-pass filter centered at 530 nm using the step-acquisition mode of the instrument. For Sets A and B, an increase in fluorescence was observed as the temperature increased and transitioned through the melting temperature ( $T_m$ ) of the probe (76°C for Set A, and 66°C for Set B)(FIG 1).

5 The degree of increase in fluorescence was greater with the higher amount of target. Change in fluorescence is further clarified by plotting the first derivative of the fluorescence data against temperature (FIG 2). Set C showed very little, if any, change in fluorescence intensity.

10 **TABLE 1**

General oligonucleotide constructs (only the bases near the fluorescent label are shown)	Guanine position on target strand	Fluorescence change upon dissociation of probe from target
<u>Set A</u>		
Probe (35 ntd) 5'---ACCAC(F)3'	0, +3	Increase
15 Target(55 ntd) 3'---TGGT <u>G</u> CT <u>G</u> G--- 5'		
<u>Set B</u>		
Probe (27 ntd) 5'---AAGGG(F)3'	+1	Increase
Target(35 ntd) 3'---TTCCCG <u>T</u> CCG--- 5'		
20 (SEQ ID NO:60)		
<u>Set C</u>		
Probe (15 ntd) 5'---TAGCG(F)3'	-1	No change
Target(23 ntd) 3'---ATCG <u>C</u> ACAGC--- 5'		
25 (SEQ ID NO:61)		
Number of nucleotides (ntd); Carboxyfluorescein (F)		

### Example 3

#### Effect of base analogs on fluorescent signal

30

The G residue on the 3' end of the probe described in Set B (Example 2) was substituted with various base analogs shown in Table 2. Base analogs were obtained as phosphoramidites (Glen Research, Sterling, VA) and incorporated into the oligonucleotides during DNA synthesis. Change in fluorescence intensity as the

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probe dissociated from the target was measured as in Example 2. Fluorescence change upon hybridization of probe to target was measured by a fluorimeter. Each sample contained 0.1  $\mu\text{M}$  of probe, and 0.12  $\mu\text{M}$  of target. There was very little change in the emission wavelength of fluorescein upon probe-target annealing. The

5 samples with base analogs 5-nitroindole and 5-iodo-2'-cytidine deoxynucleosides showed fluorescence increase upon probe-target hybridization, and fluorescence decrease after probe dissociation from target. When the G at position +1 was changed to T on the target strand, increased fluorescence signal upon hybridization and fluorescence quenching upon probe dissociation were observed with these and other

10 base analogs, except for 6-methoxyaminopurine, which generated no change in fluorescence. The direction of fluorescent change was opposite of that of the original G residue (Table 2).

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TABLE 2

Set	Base substitution at 3' end of probe	G at position +1 on target strand	Fluorescence Change	
			hybridization of probe to target	dissociation of probe from target
5	Guanine (no substitution)	Yes	Decrease	Increase
		No	Increase	Decrease
	D	Nebularine	Yes	None
10	Inosine	No	Increase	Decrease
		Yes	None	None
	F	5-nitroindole	Yes	Increase
15	3-nitropyrrole	No	Increase	Decrease
		Yes	None	None
	H	5-iodo-2'-cytidine	Yes	Slight Increase
20	6-methoxy aminopurine	No	Increase	Decrease
		Yes	None	None
	I'		No	None

**Example 4**

20 **Probe-target dissociation monitored by 5'-labeled probes:  
Position, and dosage effects of guanines**

Oligonucleotides shown in Table 3 were obtained from Operon Technologies Inc (Alameda, CA). The probe oligonucleotides were 27 nucleotides in length and labeled at the 5' end with a 5-fluorescein molecule attached to a thiourea-linked C<sub>6</sub> alkyl chain, and blocked from extension with a 3-phosphate. Target oligonucleotides were complementary to probes except they had four additional overhanging nucleotides at the 3' end. Complementary pairs of probes (0.2 μM) and targets (0.4 μM) were annealed in the presence of 50 mM Tris, pH 8.3, 3 mM MgCl<sub>2</sub>,

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and 250  $\mu\text{g/ml}$  BSA, and heated at  $0.1^\circ\text{C/sec}$  to  $90^\circ\text{C}$  with continuous fluorescence acquisition to observe the change in fluorescence intensity upon dissociation of probe. The percent change in fluorescence from probe dissociation was determined by extrapolation of the linear decrease in fluorescence measured above the melting transition to values below the melting transition. The results indicated that at least one G at positions 0, +1, or +2 on the target strand is needed for significant fluorescence change to occur upon probe-target dissociation. The magnitude of fluorescence change was maximized when G residues occupied all three positions. Position +3 was marginally effective. Similar results (not shown) demonstrate that a G residue at position +4 was also marginally effective. Position -1 had very little effect, if any, as was also the case when there were no G residues at positions -1 through +3 (Table 3).

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TABLE 3

Oligonucleotide constructs		Guanine position on target	Fluorescence change upon dissociation of probe from target	Percent Change
Probe 1	5' (F)AAAGG---3'			
Probe 2	5' (F)ACAGG---3'			
5	Probe 3	5' (F) CAAGG---3'		
	Target J	3'' AAAATTTCC---5'	None	Decrease -3%
	Target K	3'AAAAT <u>G</u> TCC---5'	-1	Slight Increase +0.3%
	Target L	3'AAAAG <u>T</u> TCC---5'	0	Increase +12%
	Target M	3'AAAG <u>T</u> TTCC---5'	+1	Increase +25%
10	Target N	3'AAG <u>A</u> TTTCC---5'	+2	Increase +8%
	Target O	3'AG <u>A</u> ATTTCC---5'	+3	Increase +4%
	Target P	3'AAG <u>G</u> TTTCC---5'	+1, +2	Increase +32%
	Target Q	3'AG <u>G</u> GTTTCC---5'	+1, +2, +3	Increase +37%
	Target R	3'AAAG <u>G</u> TTCC---5'	0, +1	Increase +29%
15	Target S	3'AAG <u>G</u> GTTCC---5'	0, +1, +2	Increase +34%
	Target T	3'AG <u>G</u> GTTCC--5'	0, +1, +2, +3	Increase +38%
	No target			Unchanged 0%

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**Example 5****Probe-target dissociation monitored by 5'-labeled probes:  
Effect of the base under the label**

5                   Oligonucleotides with the same core construct as described in  
Example 4 were prepared with minor changes in their sequences as shown in Table 4.  
These oligonucleotides were used to measure changes in fluorescence emission  
intensity upon probe-target dissociation as in Example 4. The results indicated that  
the presence of a G residue at position 0 on the target strand provides significant  
10                   increase in fluorescence upon probe-target dissociation (Set W).

The C residue in position 0 caused the fluorescence change to occur in  
the opposite direction, i.e. the C residue interfered with quenching by the '5 labeled  
G residue and fluorescence signal was once again quenched upon the melting of the  
duplex (Table 4). However, this effect is best seen when there is an absence of G  
15                   residues in the -1 or +1 positions.

TABLE 4

Oligonucleotide constructs (base at position 0 is underlined)		Fluorescence change upon dissociation of probe from target	Percent Change	
5	<u>Set J</u> Probe	5' (F)AAAGG---3'	Slight decrease	-0.3%
	Target	3'AAAAT <u>TTCC</u> ---5'		
5	<u>Set U</u> Probe	5' (F)TTAAGGA---3'	Slight increase	+0.5%
	Target	3'AAAA <u>ATCC</u> ---5'		
	<u>Set V</u> Probe	5' (F)GGAGG---3'	Decrease	-16%
	Target	3'AAAAC <u>CCTCC</u> ---5'		
10	<u>Set W</u> Probe	5' (F)CCAGG---3'	Increase	+14%
	Target	3'AAAAG <u>GTCC</u> ---5'		

**Example 6****Monitoring amplification and quantification by  
fluorescence quenching using 5'-labeled probes**

15

A 5'- fluorescein-labeled 27 nucleotide oligonucleotide probe  
5'CCAGGAAAACATAGTAAAAAATGGAAT (SEQ ID NO:62) blocked at the 3'-  
end with phosphate was used to detect amplification of a fragment from the  
lipoprotein lipase gene (GenBank Accession #AF050163). The probe was positioned  
20 so that the target strand had G residues at position 0 and +1 relative to the fluorescein  
label. PCR reactions were carried out using 0.2mM each of dATP, dGTP, dCTP,  
dTTP, 0.1µM probe, 3 mM MgCl<sub>2</sub>, KlenTaq polymerase (AB Peptides, St. Louis,  
MO, 0.4 U/reaction), 50mM Tris (pH 8.3, 25°C), BSA (500 µg/ml). Primers were  
25 5'GAATCGTGGTTTATCAAGTCATTTAAAATCA (SEQ ID NO:63) (0.25 µM) and  
5'GTGTTGATACTTGAACATTATTAGCTACAA (SEQ ID NO:64) (0.5 µM).

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The starting template was purified PCR product at  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ , 100, 10, and 0 copies per reaction. Rapid cycle PCR with fluorescence monitoring was performed in 10  $\mu$ l volumes using the LightCycler instrument. Amplification was performed by denaturation at 94°C, annealing at 50°C, a transition at 1°C/sec to 54°C, a transition at 3°C/sec to 74°C, and extension at 74°C for 10 sec, producing a 169 bp product. Fluorescence was acquired once each cycle at 54°C. The amplification required 39 min for 45 cycles. Fig. 3 shows fluorescence data plotted as the relative amount of quenching beyond background vs cycle number. The original fluorescence data were adjusted by 1) inversion (taking the reciprocal of the fluorescence), 2) proportional background adjustment of each curve over the relevant cycle interval (LightCycler software), and 3) subtracting the no template control value from each sample at each cycle. The results indicated that the system can detect as little as one copy per reaction, and that reliable quantification of initial copies of template is possible.

15

### Example 7

#### Amplification, detection, and typing of *Salmonella* strains with 3'-labeled probes

Sixteen *Salmonella* serovars from the Salmonella Reference Collection C were obtained from the Salmonella Genetic Stock Centre, University of Calgary, Canada. These serovars (Centers for Disease Control and Prevention strain numbers: 151-85, 3472-64, 346-86, 409-85, 156-87, 678-94, 2584-68, 287-86, 750-72, 2703-76, 1363-65, 347-78, 2439-64, 5039-68 and strains S 6623, Institute Pasteur E88.374) represent a genetically diverse cross section of *Salmonella* as all seven subspecies of *Salmonella enterica* and *Salmonella bongorii* are represented. Five *E. coli* strains from the *E. coli* Reference Collection were also obtained as negative controls. The bacteria were cultured overnight in Luria Broth and the genomic DNA was purified using a template preparation kit (Roche Molecular Biochemicals, High Pure PCR Template Preparation kit). Oligonucleotide primers SQF (SEQ ID NO:1) and SQR (SEQ ID NO:2) were used to amplify the SpaQ gene. Probes SQP1 (SEQ ID NO:3), SQP2 (SEQ ID NO:4) and SQP3 (SEQ ID NO:5) were labeled at their 3' end with carboxyfluorescein, similar to the examples described in Example 2. Probe SQP8 (SEQ ID NO:10) was labeled at the 5' end with fluorescein, similar to the

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examples described in Example 4. PCR reactions were carried out as in Example 6, with the following exceptions: 0.5 $\mu$ M primer SQF, 0.25 $\mu$ M primer SQR, 0.6mM dUTP in place of dTTP, 0.2 $\mu$ M of one of the probes, 4 mM MgCl<sub>2</sub>, the addition of TaqStart antibody (Clontech, Palo Alto, California, 10 ng/reaction), BSA (250  $\mu$ g/ml) and 2ng of each DNA per reaction. PCR with fluorescence monitoring was performed in 10  $\mu$ l volumes in the LightCycler instrument. Amplification conditions were 94°C (0 seconds, 20°C/second transition rate); 55°C (10 seconds, 20°C/second transition rate); 74°C (10 seconds, 2°C/second transition rate). Melting curve analysis was conducted at the end of 40 PCR cycles using a ramp rate of 0.2 °C/second. All 16 *Salmonella* serovars were detected by the melting curve analysis that produced melting peaks at the appropriate Tms (64°C and 54°C for probe SQP1 (SEQ ID NO:3), and 62°C and 52°C for SQP2 (SEQ ID NO:4), 61°C and 51°C for probe SQP3 (SEQ ID NO:5), 60°C and 50°C for probe SQP8 (SEQ ID NO:10)), but none of the *E. coli* species were detected. *Salmonella* subspecies IV and VI were easily differentiated from the other subspecies on the basis of a 10°C shift in melting temperature of the probe-amplicon duplex.

### Example 8 Genotyping with 5'-labeled probes

For all of the following examples, PCR with fluorescence monitoring was performed as in Example 6, except each reaction contained 0.5  $\mu$ M of each primer, 0.4 U of Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) instead of KlenTaq polymerase, and 50 ng of purified genomic DNA. Temperature transition rates were programmed for 20°C/sec and holding times of 0 sec were used unless indicated otherwise. Melting curve analysis was performed by heating to 95°C, annealing at 40°C for 60 sec, and melting at 0.1°C/sec to 80°C with continuous acquisition of fluorescein fluorescence. Characteristic T<sub>m</sub> shifts of all of the alleles presented here are summarized in Table 5. The Table also indicates that predictive tools can be used for single-labeled probes.

**Factor V:** One hundred genomic DNA samples of unknown genotype for the factor V Leiden were obtained from clinical samples submitted to Associated Regional and University Pathologists (ARUP, Salt Lake City, UT). The factor V locus was amplified using primers FVF (SEQ ID NO:19) and FVR (SEQ ID NO:20), and

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analyzed by the single-fluorescein 5'-labeled probe FVP1 (SEQ ID NO:14). Rapid cycle PCR was performed for 45 cycles of denaturation at 94°C, annealing at 50°C for 10 sec, and a transition at 1°C/sec to 72°C, producing a 222 bp product. Melting curve analysis was automatically performed after PCR by heating to 94°C, annealing at 40°C for 2 min, and melting at 0.1°C/sec to 75°C with continuous acquisition of fluorescein fluorescence. The amplification required 41 min and the melting protocol, 9 min. Results were compared against those using conventional Hybridization probe assays with the donor-reporter dye combination (Lay et al, 1997. Clinical Chemistry 43:2262-2267). Concordance between the two methods was

100%. Eighty-seven wild-type samples, 12 heterozygous samples, and 1 homozygous mutant sample were identified by characteristic T<sub>m</sub> shifts (FIG 4).

**Beta Globin:** Rapid cycle PCR (primers BGF (SEQ ID NO:26) and BGR (SEQ ID NO:27)) was performed for 35 cycles of denaturation at 94°C, annealing at 50°C for 10 sec, and a 1°C/sec transition to 70°C, producing a 110 bp product. Melting curve analysis with the 5'-fluorescein labeled probe BGP1 (SEQ ID NO:21) was performed after PCR by heating to 95°C, annealing at 40°C for 30 sec, and melting at 0.1°C/sec to 80°C with continuous acquisition of fluorescein fluorescence. The amplification required 35 min and the melting protocol, 9 min. The genotype of all 3 alleles (wild type, HbS, HbC) was identified by characteristic T<sub>m</sub> shifts.

**Methylenetetrahydrofolate reductase:** Rapid cycle PCR (primers MFF (SEQ ID NO:33) and MFR(SEQ ID NO:34)) was performed for 40 cycles of denaturation at 94°C and annealing/extension at 60°C for 20 sec, producing a 198 bp product. TaqStart™ antibody (88 ng) was added to each reaction. The amplification required 27 min and the melting protocol, 8 min. Genotyping was performed using a 5'-fluorescein-labeled primer MFP1 (SEQ ID NO:28). The genotype of wild and mutation was identified by characteristic T<sub>m</sub> shifts.

**Factor II (prothrombin):** Rapid cycle PCR (primers F2F (SEQ ID NO:38) and F2R (SEQ ID NO:39)) was performed for 35 cycles of denaturation at 94°C, annealing at 58°C for 15 sec, and a 1°C/sec transition to 72°C, producing a 154 bp product. Melting curve analysis was performed with a 5'-fluorescein-labeled probe F2P (SEQ ID NO:35). The amplification required 29 min and the melting protocol, 8 min. The mutation and wild type alleles were distinguished by characteristic T<sub>m</sub> shifts.

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**Hereditary hemochromatosis:** Rapid cycle PCR (primers HHDF (SEQ ID NO:45) and HHDR (SEQ ID NO:46) for mutation C187G and primers HCYF and HCYR for mutation G845A) was performed for 35-50 cycles of denaturation at 94°C, annealing at 60°C for 10 sec, and a 1°C/sec transition to 72°C. Melting curve analysis was performed with 5'-fluorescein-labeled probes HHDP1 (SEQ ID NO:40) for the C187G allele, and HCYP1 (SEQ ID NO:47) for the G845A allele. Wild type and mutant alleles were identified by characteristic T<sub>m</sub> shifts.

**Cystic Fibrosis:** Rapid cycle PCR (primers CFF (SEQ ID NO:57) and CFR (SEQ ID NO:58)) was performed for 44 cycles of denaturation at 95°C and annealing/extension at 60°C for 20 sec, producing a 256 bp product. TaqStart antibody (88 ng) was added to each reaction. Melting analysis was performed using a 5'-fluorescein labeled primer CFP1 (SEQ ID NO:53). The amplification required 25 min and the melting protocol, 8 min. The deletion allele was differentiated from the wild type allele by its characteristic T<sub>m</sub> shift.

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**TABLE 5. Measured and predicted Tms derived from single-labeled fluorescein probes.**

	Gene	Genotype	Melting Temperature (°C)	
			Measured	Predicted
5	Factor V	Wild type	59.5	58.4
		G1691A	50.0	47.0
	Hemoglobin	Wild type	60.4	57.6
		Hb S	64.7	63.1
		Hb C	55.8	N/A
10	MTHFR	Wild type	66.9	66.8
		C667T	63.1	62.2
	Cystic Fibrosis	Wild type	62.9	59.2
		F508del	53.2	N/A
	Factor II	Wild type	55.0	50.7
15		G20210A	62.5	58.7
	HFE-C282Y	Wild type	63.3	61.3
		C187G	54.7	52.6
	HFE-H63D	Wild type	63.3	61.3
		G845A	69.3	67.9
20	N/A: not available			

**Example 9****Mutation detection by probe multiplexing**

25 A 3'-fluorescein labeled oligonucleotide probe Y  
5'CTTGATGAGGATCCCAAAGACCACCCCAAGACCAC(F) (SEQ ID  
NO:65), and a second oligonucleotide labeled at its 5' end with BlackHole quencher  
dye (BH1, BioSearch Technologies, Novato, CA) probe Z  
5'(F)ACCAGCAGAATGCCAACCA (SEQ ID NO:66) were prepared. On the target  
30 strand, a G residue was located at position 0 relative to the fluorescein label. Four  
target oligonucleotides, 55 nucleotides in length, one completely complementary  
with both probes, a second with a single-base mismatch under probe Y (SEQ ID

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NO:65), the third with a single-base mismatch under probe Z (SEQ ID NO:66), and a fourth with single mismatches each under probe Y (SEQ ID NO:65) and probe Z (SEQ ID NO:66) were also prepared. The two probes were simultaneously hybridized to target, and melting analysis with fluorescence monitoring was performed as described in Example 2. An increase in fluorescence was observed as probe Z (SEQ ID NO:66)(with the quencher dye) dissociated from the template ( $T_m=70^\circ\text{C}$ ), followed by another increase in fluorescence as the fluorescein-labeled probe Y (SEQ ID NO:65) dissociated ( $T_m=77.5^\circ\text{C}$ ). The single-base mismatch under the fluorescein-labeled probe (FIG 5A) as well as that under the quencher probe (FIG 5B) were both detected by downward  $T_m$  shifts characteristic to each of the mismatches. The double-mismatch (i.e. one mismatch under each probe) was also unambiguously detected (FIG 5B).

### Example 10

#### Comparison of quenching and dequenching probes

Oligonucleotide probes (27 ntds) were synthesized with fluorescein, JOE, Cy5, and LCRed 705 dyes attached to their 5' end. A complementary strand of 38 ntds having two G residues at position 0 and +1 was also prepared. Probes were hybridized to the complementary strand, and fluorescence change was measured as the probe dissociated, as described in Example 4, except appropriate filters were used for excitation and emission detection. Percent change in fluorescence intensity was 28% for fluorescein, 20% for JOE, both indicating quenching by hybridization and dequenching by duplex dissociation, and -11% for Cy5, -12% for LCRed 705, indicating augmentation by hybridization and quenching by duplex dissociation.

### Example 11

#### Genotyping by quenching/augmentation of an oligonucleotide probe internally labeled with fluorescein as a virtual nucleotide

An oligonucleotide complementary to the factor V Leiden (G1691A) locus (Genbank Accession #L32764) was obtained from Operon (Alameda, CA) and used without further purification. A fluorescein-ON phosphoramidite (Clontech, Palo Alto, CA) was incorporated into the probe at the position complementary to the variable base and the probe was blocked from extension with a 3'-phosphate (Fig. 6).

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Hence, the fluorescein label is incorporated as a “virtual nucleotide” in the sequence CTGTATTCCTFGCCTGTCCAGG-P (SEQ ID NO:67). When hybridized to the factor V locus, the fluorescein is opposed to either a G or an A residue.

5 PCR with fluorescence monitoring was performed in 10  $\mu$ l volumes in a rapid-cycle, real-time PCR instrument (LightCycler, Roche Molecular Biochemicals, Indianapolis, IN). The probe was included in the PCR amplification mixture with primers FVF (SEQ ID NO:19) (0.5  $\mu$ M) and FVR (SEQ ID NO:20) (0.25  $\mu$ M). Each reaction included the fluorescein-labeled probe at 0.1  $\mu$ M, 200  $\mu$ M of each dNTP (dATP, dCTP, dGTP, and dTTP), 50 mM Tris, pH 8.3 (25°C), 3 mM 10  $MgCl_2$ , 500  $\mu$ g/ml bovine serum albumin, 0.4 U of Taq polymerase (Roche Molecular Biochemicals), TaqStart antibody (88 ng, Clontech) and 50 ng of purified genomic DNA.

Genomic DNA of known genotypes were obtained from a prior study (Lay MJ and CT Wittwer. Clin. Chem.43:12, 2262-2267, 1997). Rapid cycle PCR 15 was performed for 45 cycles of denaturation at 95°C, annealing at 50°C for 10 sec, and a transition at 1°C/sec to 72°C, producing a 222 bp product. Temperature transition rates not specified were programmed for 20°C/sec with holding times of 0 sec. Melting curve analysis was automatically performed after PCR by heating to 95°C, annealing at 40°C for 30 sec, and melting at 0.1°C/sec to 75°C with continuous 20 acquisition of fluorescein fluorescence.

Fluorescent melting curve analysis used commercial LightCycler software except that the positive derivative of fluorescence with respect to temperature was plotted on the Y-axis instead of the negative derivative. The temperature interval used for polynomial estimation of the derivative was 8°C and 25 the digital filter was enabled.

The homozygous wild type genotype results in F:G oppositions, quenching of fluorescein fluorescence, and a  $T_m$  of 58.4°C, while the homozygous factor V Leiden genotype results in F:A oppositions and augmentation of fluorescence with a similar  $T_m$ . A heterozygote results in both F:G and F:A 30 oppositions with an intermediate level of fluorescence. Note that genotyping is obtained by the direction of the change in fluorescence, not by characteristic  $T_m$ s of each allele as in other examples. All genotypes can be clearly distinguished from

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each other.

Another example of use of a virtual nucleotide would be for detection of Salmonella with an analog of SEQ ID NO:3, 5'CCAAAAGGNAGCGTCTGTTCC (SEQ ID NO:59), wherein N is the fluorescent-label-containing virtual nucleotide, and quenching occurs upon hybridization when the virtual nucleotide is in close proximity to the G in the 0 position on the complementary strand.

**Example 12**  
**Genotyping by dequenching of an oligonucleotide probe with a fluorescent label on a G residue**

PCR with fluorescence monitoring was performed as described in Example 11, but with the following changes: the Factor V locus was asymmetrically amplified using an 8:1 molar ratio of primer 5'AGAATAAATGTTATCACACTGGTGCTAA (SEQ ID NO:68, 0.5  $\mu$ M) and primer 5'GACATCGCCTCTGGGCTA (SEQ ID NO:69, 0.06  $\mu$ M); each reaction mixture included 200 mM Tris, pH 8.7 (25°C), without TaqStart antibody, and 0.2  $\mu$ M fluorescein-labeled probe 5'GGCGGAGGAATACAGG(F) (SEQ ID NO:70) in which the underlined G is opposed to the Leiden mutation.

Rapid cycle PCR was performed with initial incubation at 95°C for 5 s, followed by 45 to 60 cycles of denaturation (86°C), annealing (55°C, 10 sec), and extension (using a transition rate of 1°C/s from 55°C to 72°C). This produced a 226 bp product from human DNA samples. Melting curve analysis was performed after PCR by heating to 95°C, cooling to 65°C, further cooling down to 30°C at a rate of 0.25°C/s, and melting at a rate of 0.05°C/s to 65°C with continuous acquisition of fluorescein fluorescence. Temperature transition rates were programmed at 20°C/s and holding times at 0 seconds unless otherwise specified. Fluorescent melting curve analysis was performed using the commercial LightCycler software with the conventional negative first derivative of fluorescence plotted against temperature.

Probe-target hybridization resulted in an increase in fluorescence. The homozygous wild type genotype (G:C match) had a  $T_m$  of 53°C, and was easily distinguished from the homozygous Factor V Leiden genotype (G:T mismatch) which had a lower  $T_m$  of 45°C. A heterozygote genotype showed both of the  $T_m$

values (Fig. 7).

Best results are obtained with asymmetric PCR, wherein the primer of the same sense as the probe is provided in smaller amounts than the opposite primer. When 45 cycles were performed, a 1:4 primer asymmetry produced the greatest  
5 signal. At 60 cycles, a 1:8 ratio was optimal. Compared to the 1:8 ratio, the dequenching peak area at a 1:16 ratio was 62%, at 1:4, 85%, and at 1:2, 37%. With this probe system, no signal was obtained when the primer concentrations were symmetric (equal).

### 10 **Example 13** **Optimization of quenching probes**

Melting curve analyses using Probe 1 and Target P (Table 3) were performed as in Example 4, except for varying the buffer pH and cation concentration. The effect of buffer pH was studied in a 100 mM KCL, 10 mM Tris  
15 solution titrated to the various pHs with HCl (Fig. 8a, closed diamonds). Comparison of relative signal strength is possible by comparison of peak area values of the melting curve data. The optimal pH is 7.4 – 8.0, with the best signal obtained at pH 7.6 – 7.8. The effect of buffer cation content was studied by adding various concentrations of KCl to a 10 mM Tris, pH 8.3 solution (Fig. 8b, closed circles).  
20 Signal was very low at KCl concentrations of 10 or 20 mM. Good signal was observed at 50 - 200 mM KCl, with the best result obtained at about 100 mM. Similar cation effects were obtained when the cation was provided as part of the buffer such as with, but not limited to, Tris<sup>+</sup> and Tricine<sup>+</sup>. Good signal can be obtained using 100 mM Tris, pH 7.8, which is compatible with PCR.

### 25 **Example 14** **Optimization of dequenching probes, and interchangeability of quenching and dequenching by pH**

The probe and target of Set V (Table 4) were used to further study  
30 probe systems that dequench (increase signal) upon hybridization. Melting curve analysis was performed by heating the probe-target mixtures to 95°C for 0 sec, cooling to 40°C for 15 sec, followed by a ramp at 0.1°C/s to 94°C with continuous monitoring.



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Fig. 9 demonstrates the influence of buffer pH on signal strength and the direction of signal change. In this figure, the buffer was 100 mM Tris with 500  $\mu\text{g/ml}$  BSA. When the pH is more basic (alkali) than 8, the probe dequenches upon hybridization. However, when the pH is more acidic than 8, the probe quenches upon hybridization. In other words, quenching or dequenching is not entirely a property of the probe, or even of the probe/template combination, but also depends on the buffer conditions (cation concentration and pH). This is further exemplified in Fig. 8a (open diamond and open triangle symbols) in which pH ranges of 7.2 – 9.0 and 8.7 – 10.7 were studied by use of 10 mM Tris buffer, or 10 mM 2-amino-2-methyl-1-propanol buffer, respectively, in the presence of 160 mM KCL. Dequenching signal is obtained at pH 8.0 – 10.7, with best results obtained at or above pH 8.6. There was very little signal at a pH around 8.0, and slight quenching was observed at more acid pH. Buffers of 2-amino-2-methyl-1,3-propanediol can also be used to provide a basic buffer solution useful for this application.

Fig. 8b (open circles) shows the effect of cations on the dequenching signal. Signal is very low at 10 - 20 mM KCl, but strong at 50 – 320 mM KCL, with best results at 80-160 mM KCL. Similar cation effects were obtained with  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{Cs}^+$ , tetramethylammonium<sup>+</sup> ions, or if the cation was provided as part of the buffer such as with, but not limited to, Tris<sup>+</sup> and Tricine<sup>+</sup>. Good signal was also obtained using 200 mM Tris, pH 8.7 – 8.8 which is compatible with PCR and was utilized in Example 12. Compounds such as glycerol and tetrapentylammonium<sup>+</sup> inhibited the dequenching signal.

### Example 15

#### Dependence of melting peak area on probe $T_m$ in relation to the PCR annealing temperature

Single-labeled probes with various  $T_m$ s were used to study the relationship between probe  $T_m$ , PCR annealing temperature, and signal strength of fluorescence change upon probe-target melting. As in the previous examples, signal strength was assessed by relative peak area values obtained from melting curve data. The following six probes, labeled with a 5'-fluorescein and terminated with a 3' phosphate, were designed to be complementary to the region of the G845A polymorphism associated with hemochromatosis (Genbank Accession #Z92910):

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	Probe-a (21 ntd)	As probe HCYP2 plus an additional 3' C residue
	Probe-b (21 ntd)	As Probe-a, but 4 bases shifted toward the 3'-direction
	Probe-c (17 ntd)	As Probe-a, but 4 bases truncated at the 3'-end
	Probe-d (17 ntd)	As Probe-b, but 4 bases truncated at the 3'-end
5	Probe-e (13 ntd)	As Probe-c, but 4 bases truncated at the 3'-end
	Probe-f (13 ntd)	As Probe-d, but 4 bases truncated at the 3'-end

Asymmetric amplification was performed with 0.0625  $\mu$ M of primer HCYP2, and 0.5  $\mu$ M of primer 5'GGCTGGATAACCTTGGCTGTA (SEQ ID NO: 71) using the  
10 reaction mixture of Example 8 with TaqStart antibody (88 ng). Fifty PCR cycles of 94°C for 0 sec, 60°C for 10 sec and a 5°C/s ramp to 72°C were performed, followed by final heating to 94°C, cooling to 35°C, holding for 10 sec, and continuous fluorescence acquisition at 0.1°C/s to 80°C. Peak areas for each derivative melting curve were determined and normalized by the peak areas obtained with the same  
15 probes against synthetic templates without PCR. The relative peak area values for probe-a and probe-e were determined by setting the peak area of probe-c to 100. The relative peak area values for probe-b and probe-f were determined by setting the peak area of probe-d to 100. Peak area values were plotted against  $T_m$  of the probes (Fig. 10). Maximum hybridization signal was observed when the probe  $T_m$  was  
20 about 5°C below the PCR annealing temperature. In embodiments wherein it is not necessary to monitor fluorescence each cycle, the length of the probe should be adjusted so that the probe's  $T_m$  is lower than the PCR annealing temperature by 0-10°C, and most preferably lower by about 5°C. Primer  $T_m$ s are often around the annealing temperature used in PCR, so the optimal probe  $T_m$  is also often about 5°C  
25 less than primer  $T_m$ s.

The decrease in signal strength seen with probes with  $T_m$ s higher than the annealing temperature may be due to either probe hydrolysis during PCR, or to a decrease in PCR efficiency resulting from the probe blocking polymerase extension. The decrease in signal strength observed with probes with lower than optimal  $T_m$ s  
30 may result from fewer available probe binding sites resulting from annealing of product strands during cooling, or due to secondary structure formation within a strand.

## SEQUENCE LISTING

<110> University of Utah Research Foundation  
 Idaho Technology, Inc.  
 Wittwer, Carl  
 Crockett, Andrew  
 Caplin, Brian  
 Stevenson, Wade  
 Wagner, Lori  
 Chen, Jian  
 Kusakawa, Noriko

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CLAIMS:

1. A fluorescence-based probe for analyzing a target nucleic acid comprising:  
  
a single-labeled polynucleotide hybridization probe comprising a  
5 sequence with at least 80% identity to the complementary sequence of a locus of the nucleic acid and a fluorescent label attached to a terminal nucleotide of the probe, wherein said nucleotide is a base analog, and wherein the base analog is selected from the group consisting of 5-nitroindole, 4-nitroindole, 6-nitroindole, 3-nitropyrrole, 5-iodo-2'-cytidine, inosine, and nebularine deoxynucleosides.
- 10 2. The probe of claim 1 wherein the target nucleic acid has a C residue in a position complementary to the position of the base analog.
3. A hybridization probe for analyzing a target nucleic acid comprising:  
  
a fluorescent detecting entity comprising a single-labeled  
oligonucleotide comprising a sequence with at least 80% identity to the  
15 complementary sequence of a locus of the target nucleic acid and a fluorescent label linked to an internal residue of the oligonucleotide, said internal residue comprising a base analog wherein the base analog is selected from the group consisting of 5-nitroindole, 4-nitroindole, 6-nitroindole, 3-nitropyrrole, 5-iodo-2'-cytidine, inosine, and nebularine deoxynucleosides.
- 20 4. The probe of any one of claims 1 to 3 wherein the fluorescent label is selected from the group consisting of fluorescein, fluorescein derivatives, cyanine derivatives, and fluorescein-cyanine conjugates.
5. A method for determining the presence of a target nucleic acid sequence in a biological sample comprising:  
  
25 combining a single-labeled oligonucleotide hybridization probe with the target nucleic acid, said probe comprising an oligonucleotide sequence with at

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least 80% identity to the complementary sequence of a locus of the target nucleic acid sequence and a fluorescent label linked to an internal nucleotide of the oligonucleotide sequence, wherein the nucleotide is a base analog selected from 5-nitroindole, 4-nitroindole, 6-nitroindole, 5-iodo-2'-cytidine wherein hybridization of  
5 the probe to the target nucleic acid places a G residue in position +1, 0, or -1 relative to the position of the internal residue, and the fluorescent label is linked to the internal residue by a linker sufficiently flexible to allow quenching by the G residue so the probe exhibits a hybridization-dependent increase in fluorescent emission,

illuminating the probe, and

10 monitoring the hybridization-dependent fluorescent emission,

wherein increased fluorescence emission indicates the presence of the target nucleic acid sequence.

6. The method of claim 5 further comprising the steps of:

15 combining the mixture of the probe and the target nucleic acid with a pair of oligonucleotide primers, wherein the oligonucleotide primers are used for amplifying a selected segment of the target nucleic acid sequence,

adding a polymerase, and

amplifying the selected segment of the target nucleic acid sequence.

7. The method of claim 6 wherein the pair of primers has an annealing  
20 temperature, and the probe has a  $T_m$  of 0 to 10°C below the annealing temperature.

8. The method of claim 6 or 7 further comprising the step of amplifying the selected segment of the target nucleic acid sequence by a procedure selected from the group consisting of SDA, NASBA, CRCA, Q beta replicase mediated amplification, ICAN, and TMA.



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9. The method of any one of claims 6 to 8 wherein the fluorescent label is selected from the group consisting of fluorescein, fluorescein derivatives, cyanine derivatives, and fluorescein-cyanine conjugates, and hybridization of the probe to the target nucleic acid places the fluorescent label in a complementary position to a residue other than guanosine and results in increased fluorescent emission.
10. The method of claim 9 wherein the residue other than guanosine is adenosine.
11. The method of any one of claims 6 to 9 wherein the fluorescence emission is monitored as a function of temperature.
12. The method of any one of claims 5 to 11 wherein the monitoring step includes monitoring fluorescence decrease after probe dissociation from the target nucleic acid.
13. The method of any one of claims 5-12 wherein the locus of the target nucleic acid sequence has a C residue in the complementary location to the internal nucleotide.
14. The method of claim 13 wherein upon hybridization of the oligonucleotide sequence to the target nucleic acid, part of the target nucleic acid remains nonhybridized, creating an overhang adjacent to the C residue of the target nucleic acid.
15. The method of claim 14 wherein residues other than G are located at positions -1, +1, and +2 relative to the position of the fluorescent entity.
16. The method of any one of claims 14 to 15 wherein guanosine residues are absent from positions -1 and +1 on the target nucleic acid sequence relative to the position of the fluorescent entity.
17. The method of any one of claims 5 to 16 wherein the probe and sample are combined in a solution having a pH of >8.0.

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18. The method of claim 17 wherein the solution has a Tris concentration of about 200 mM.
19. The method of claim 17 or 18 wherein the fluorescent label is selected from the group consisting of fluorescein, fluorescein derivatives, and fluorescein-cyanine conjugates, and the concentration of cations is about 50-200 mM.
20. The method of claim 17 wherein the solution further comprises a buffer selected from the group consisting of Tris+, Tricine+, 2-amino-2-methyl-1-propanol, and of 2-amino-2-methyl-1,3-propanediol.
21. The method of claim 5 wherein the hybridization-dependent fluorescence emission is monitored during asymmetric PCR.
22. The method of claim 21 wherein about 45 PCR cycles are performed, and a pair of asymmetric PCR primers are provided, wherein the primers have a 1:4 primer asymmetry.
23. The method of claim 21 wherein about 60 PCR cycles are performed, and a pair of asymmetric PCR primers are provided, wherein the primers have a 1:8 primer asymmetry.
24. A method for determining the presence of a target nucleic acid sequence in a biological sample comprising:
- combining the biological sample with a pair of primers used for amplifying a selected segment of the target nucleic acid sequence and a fluorescent detecting entity comprising a single-labeled oligonucleotide hybridization probe,
- wherein the single-labeled probe comprises an oligonucleotide comprising a sequence with at least 80% identity to the complementary sequence of a locus of the selected segment of the target nucleic acid sequence, and comprising a fluorescent label attached to a terminal nucleotide of the probe, wherein said fluorescent label exhibits a sequence specific hybridization-dependent emission and

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said terminal nucleotide is a base analog selected from 4-nitroindole, 5-nitroindole, 6-nitroindole, inosine, 5-iodo-2'-cytidine, nebularine deoxynucleosides, 3-nitropyrrole deoxynucleosides, and wherein hybridization of the probe to the locus results in an increase in fluorescence emission of the fluorescent label,

5 adding a polymerase and amplifying the selected segment of the nucleic acid sequence through a plurality of amplification cycles,

illuminating the biological sample, and

10 monitoring the hybridization-dependent increase in fluorescent emission, wherein increased fluorescence emission indicates the presence of the target nucleic acid sequence.

25. The method of claim 24 further comprising the step of determining a maximum  $-dF/dT$  as the probe dissociates from the target nucleic acid sequence.

15 26. The method of claim 24 or 25 wherein the fluorescent label is linked to a base analog of the oligonucleotide probe and the base analog is selected from the group consisting of 4-nitroindole, 5-nitroindole, 6-nitroindole, and 3-nitropyrrole deoxynucleosides.

20 27. The method of claim 24 or 25 wherein the base analog is selected from the group consisting of inosine, 5-iodo-2'-cytidine, and nebularine deoxynucleosides, and wherein a residue other than guanosine is located on the target nucleic acid sequence at position +1 relative to the position of the label.

28. The method of any one of claims 24 to 27 wherein the fluorescent label is selected from the group consisting of cyanine dyes and LCRed 705.

25 29. The method of any one of claims 24 to 27 wherein the fluorescent detecting entity is immobilized on a surface and the combining step includes placing the sample in contact with the surface.

30. The method of any one of claims 24 to 29 further comprising

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providing a second fluorescent detecting entity consisting of a second single-labeled oligonucleotide probe, wherein the second single-labeled oligonucleotide probe comprises a second oligonucleotide sequence with at least 80% identity to a complementary sequence of a second selected segment of the target nucleic acid sequence and comprising a second fluorescent label linked to an end of the second oligonucleotide sequence, the second fluorescent label exhibiting a sequence specific hybridization-dependent fluorescence emission at a wavelength different from the fluorescence emission of the first probe, wherein hybridization of the second oligonucleotide probe to the second selected segment results in increased fluorescence emission from the second label and the second fluorescent signal is independent of fluorescence emission of the first fluorescent detecting entity, and

monitoring the hybridization-dependent increase in fluorescence emission of the second probe.

31. A method for determining the presence of a target nucleic acid sequence in a biological sample comprising:

combining the biological sample with a fluorescent detecting entity comprising a single-labeled oligonucleotide hybridization probe,

wherein the single-labeled probe comprises an oligonucleotide sequence with at least 80% identity to the complementary sequence of a locus of the target nucleic acid sequence, and a fluorescent label attached to the 5' terminal nucleotide of the oligonucleotide, wherein said terminal nucleotide is a base analog selected from 4-nitroindole, 5-nitroindole, 6-nitroindole, inosine, 5-iodo-2'-cytidine, nebularine deoxynucleosides, and 3-nitropyrrole deoxynucleosides, wherein the fluorescent label exhibits a hybridization-dependent emission, wherein hybridization of the probe to the locus results in an increase in fluorescence emission of the fluorescent label,

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combining the biological sample and the probe with a second oligonucleotide and a polymerase, wherein the probe and the second oligonucleotide function as a pair of primers for amplification,

amplifying the target nucleic acid sequence,

5 illuminating the biological sample, and

monitoring the hybridization-dependent increase in fluorescence emission,

wherein increased fluorescence emission indicates the presence of the target nucleic acid sequence.

10 32. A kit for analyzing a sample comprising a nucleic acid sequence, comprising:

a. a fluorescent detecting entity comprising a single-labeled oligonucleotide hybridization probe having a fluorescent label linked to a terminal nucleotide of the oligonucleotide, wherein said terminal nucleotide is a base analog  
15 and wherein the base analog is selected from 4-nitroindole, 5-nitroindole, 6-nitroindole, inosine, 5-iodo-2'-cytidine, nebularine deoxynucleosides, 3-nitropyrrole deoxynucleosides and wherein said probe hybridizes to a single-stranded locus of the nucleic acid sequence so that the magnitude of fluorescence emission from the fluorescent label increases by hybridization of the probe to the locus; and

20 b. components for amplification of the nucleic acid sequence, wherein the components include a pair of oligonucleotide primers for amplifying a segment of said nucleic acid sequence and a thermostable DNA polymerase.

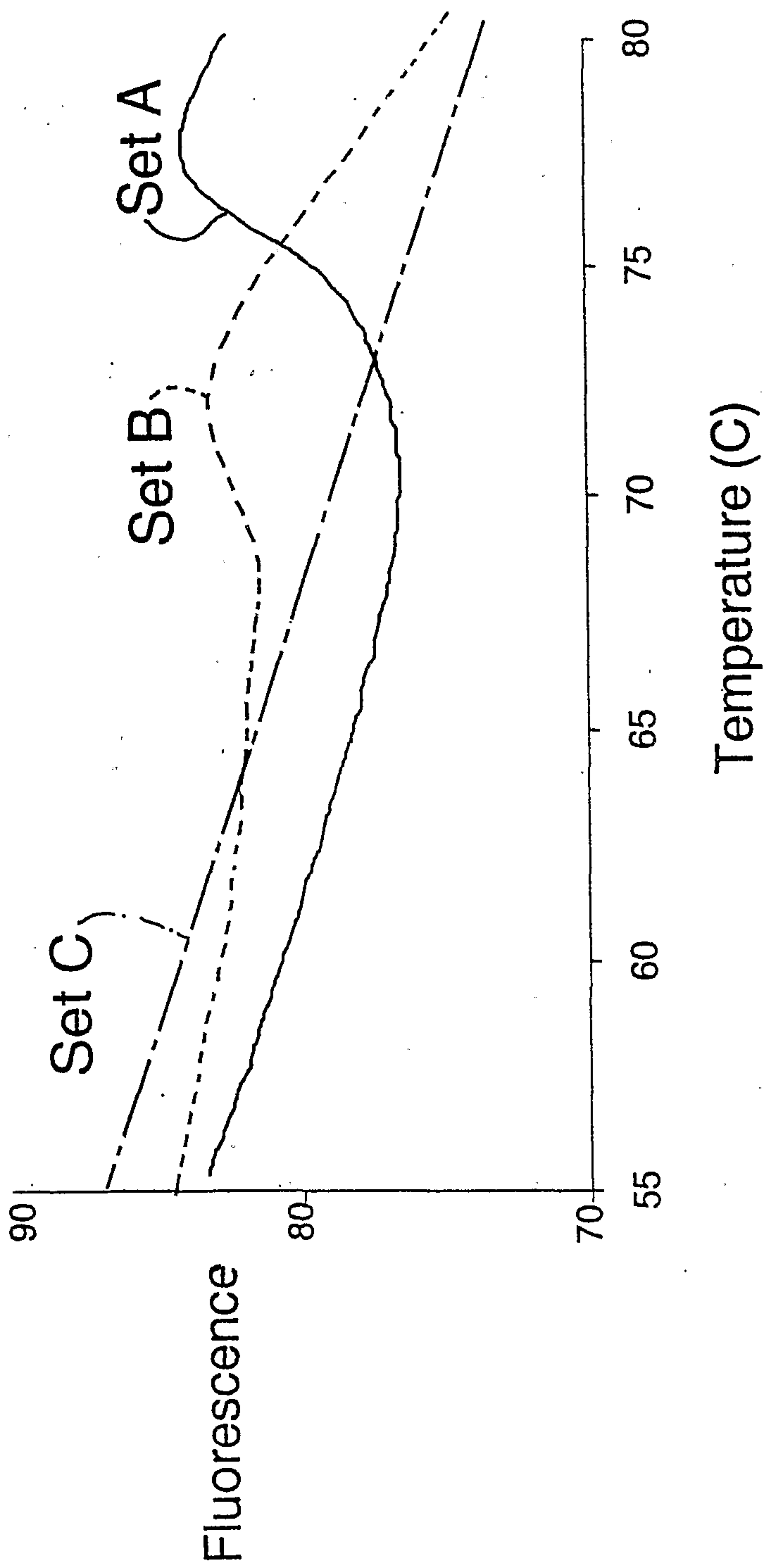
33. The kit of claim 32 further comprising

a second pair of primers for amplifying a second segment of said  
25 nucleic acid sequence comprising a second single-stranded locus; and

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a second fluorescent detecting entity comprising a second single-labeled oligonucleotide probe comprising a second fluorescent label on an internal or terminal nucleotide of the oligonucleotide, wherein said second probe hybridizes to the second locus so that the magnitude of the fluorescence emission from the second fluorescent label is increased by hybridization of the second probe to the target nucleic acid sequence.



*FIG. 1*

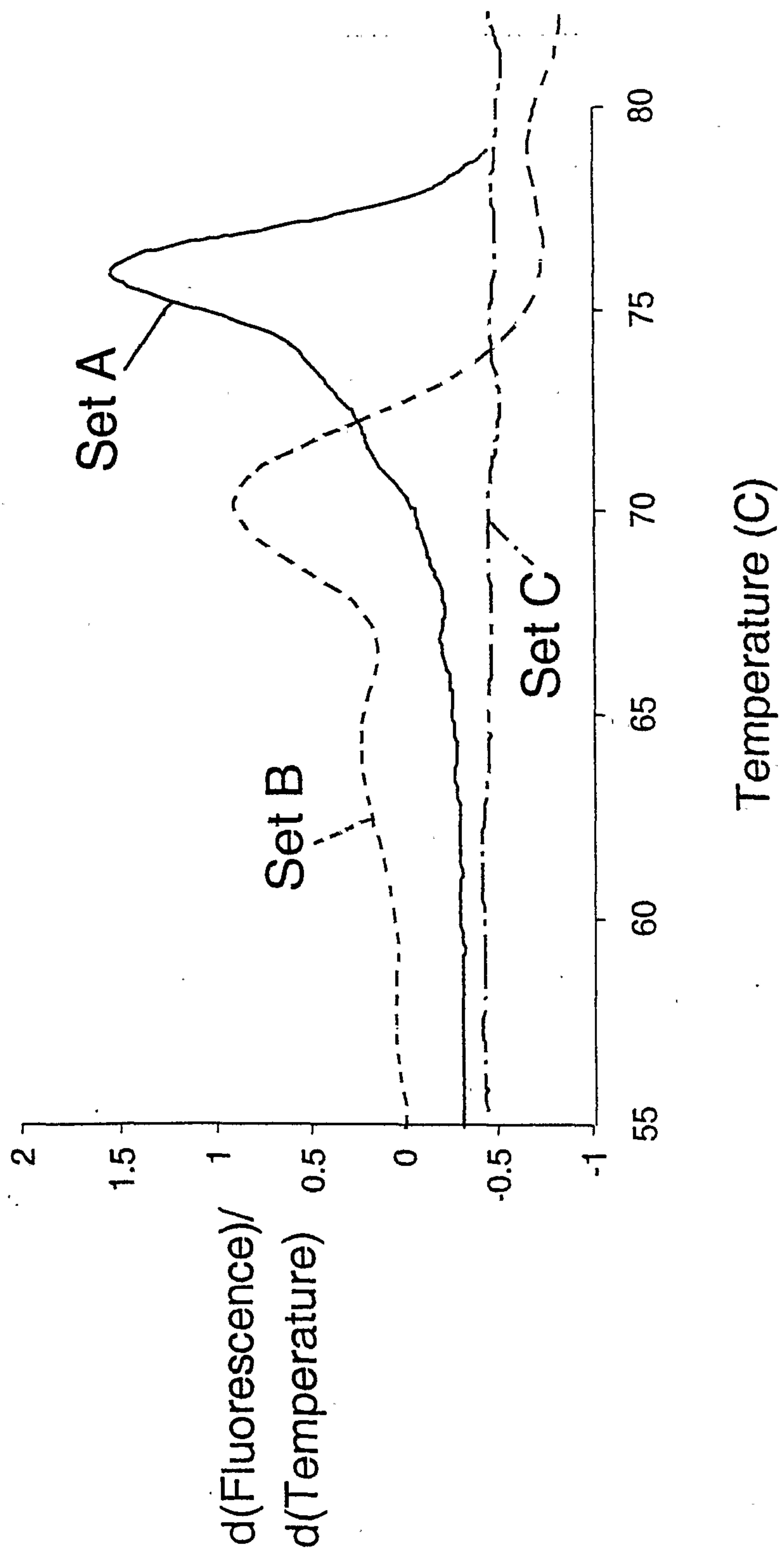


FIG. 2



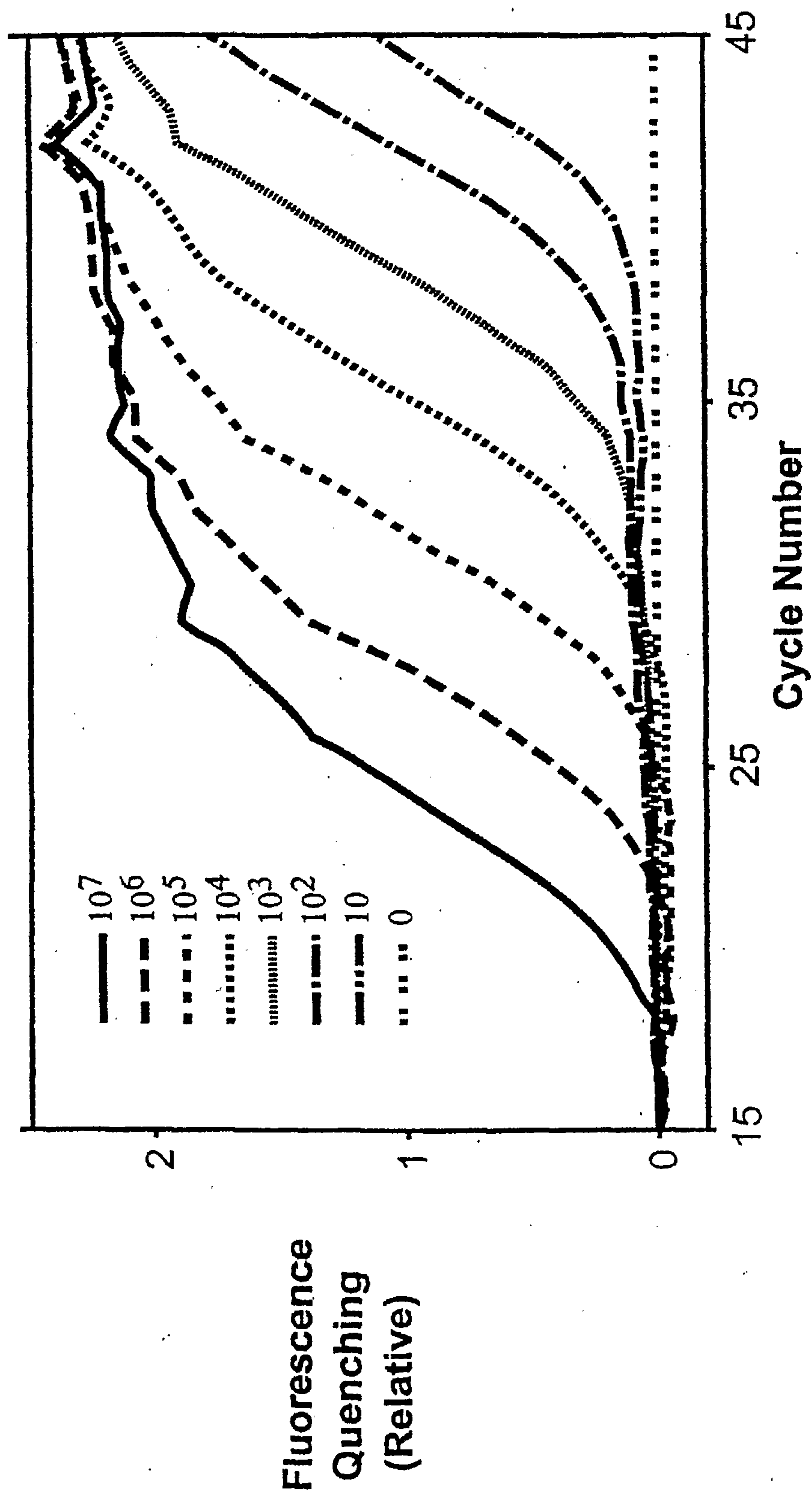


FIG. 3

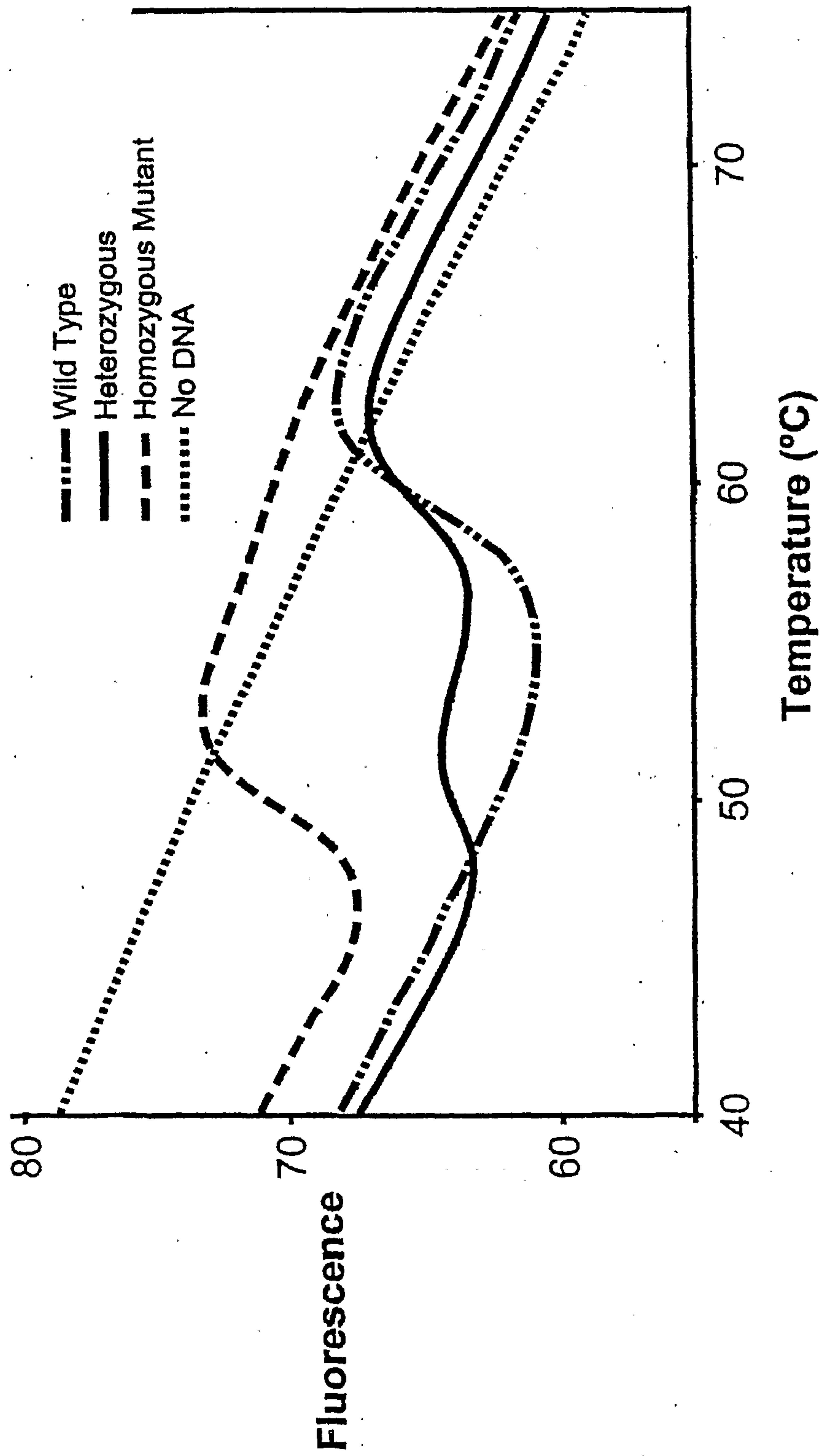


FIG. 4A

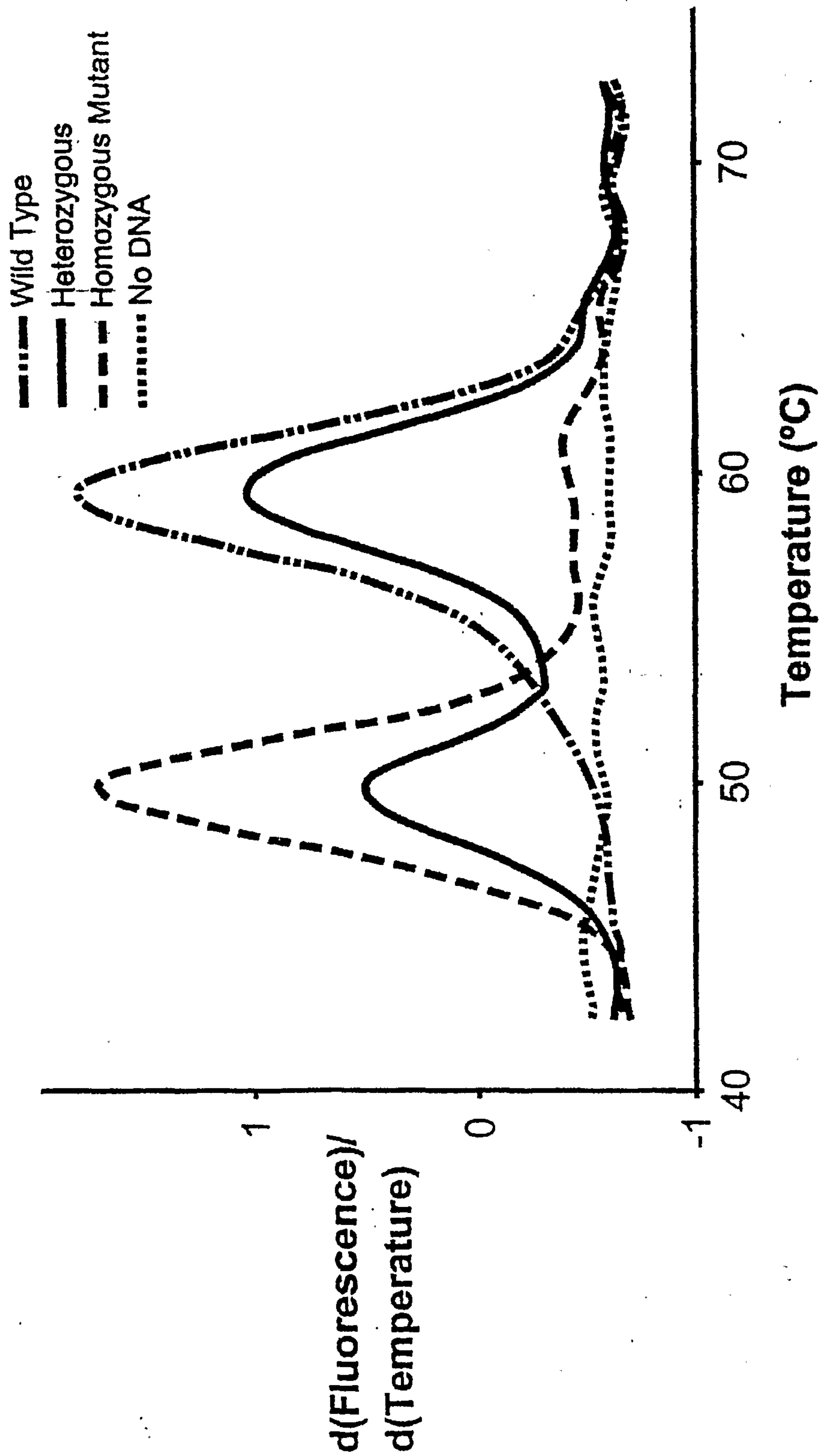


FIG. 4B

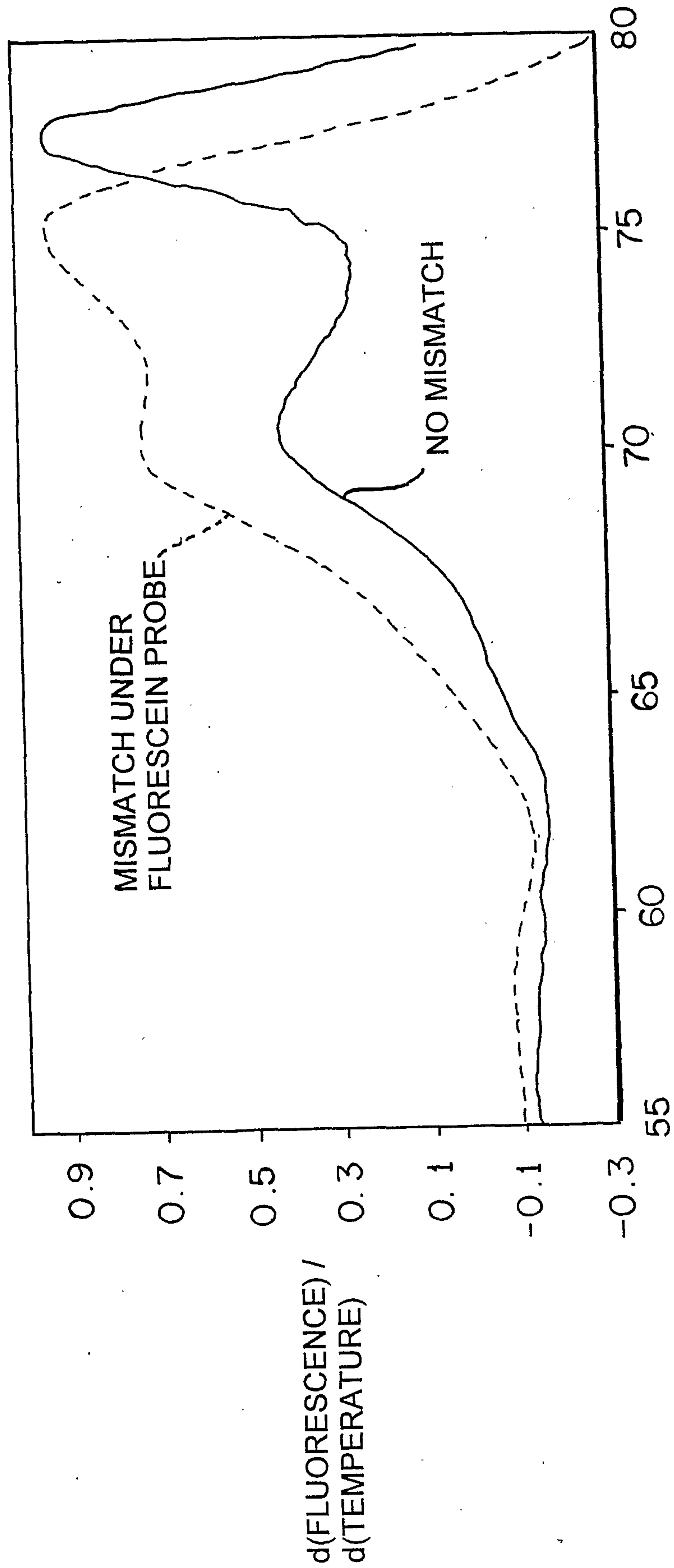


FIG. 5A

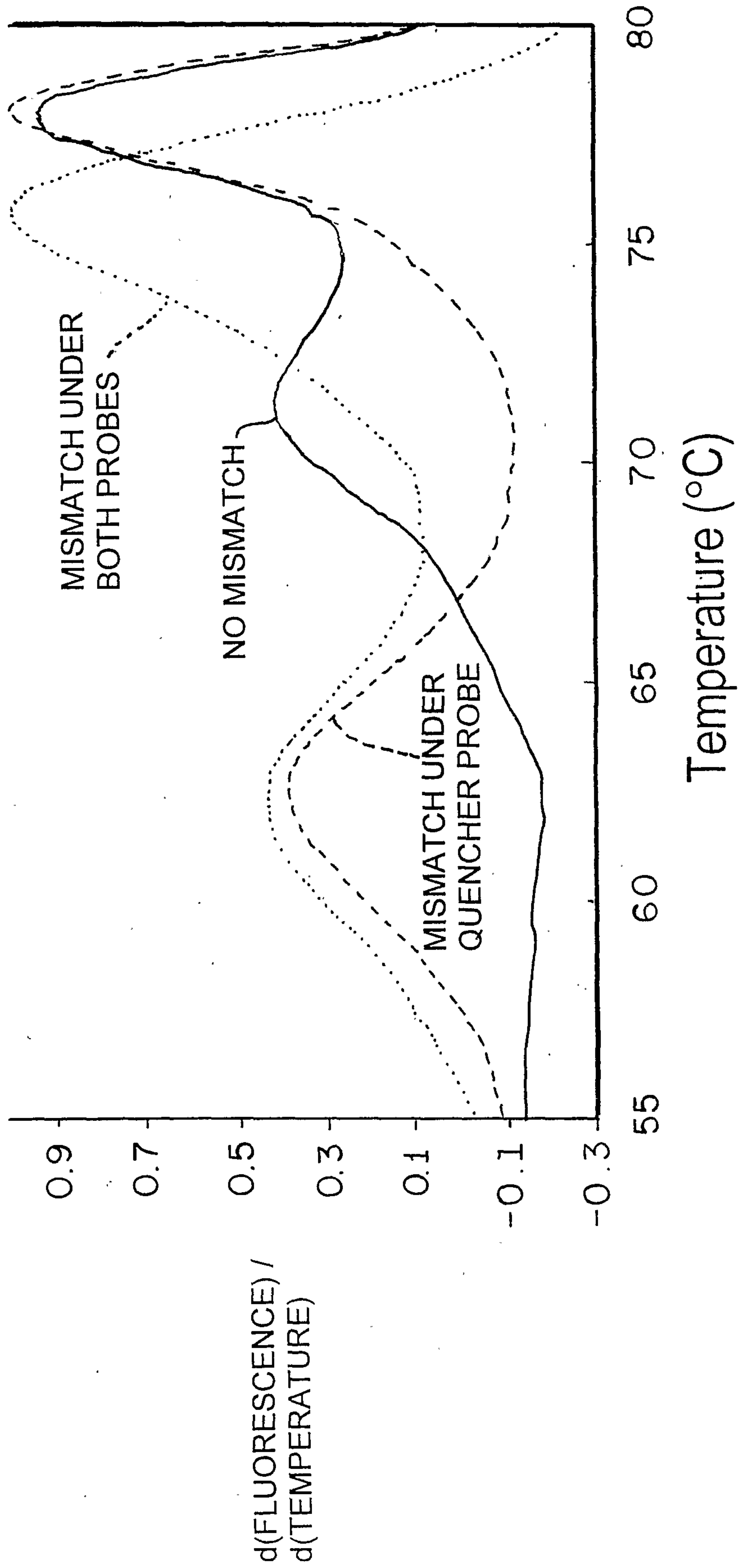


FIG. 5B

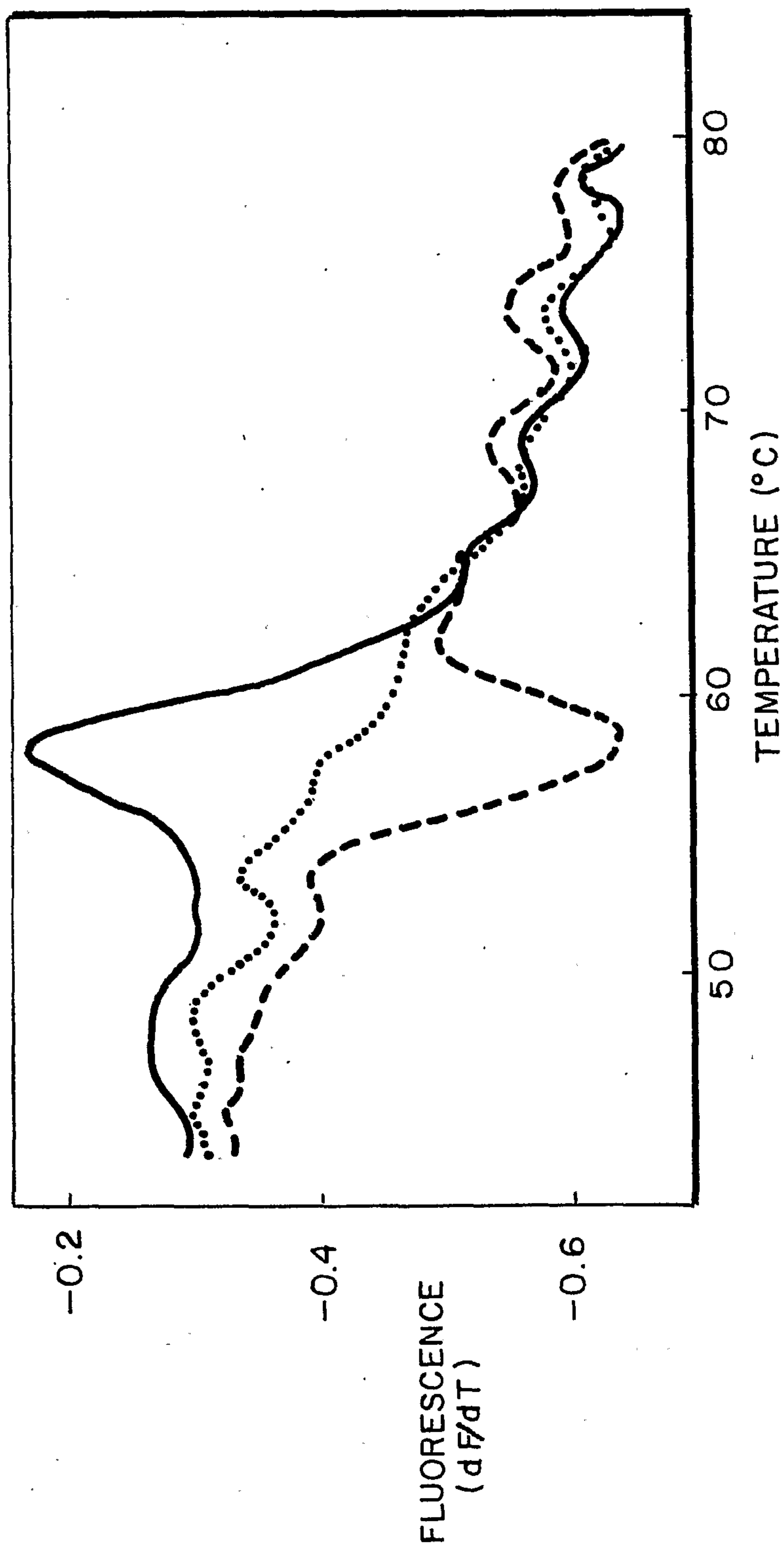


FIG. 6

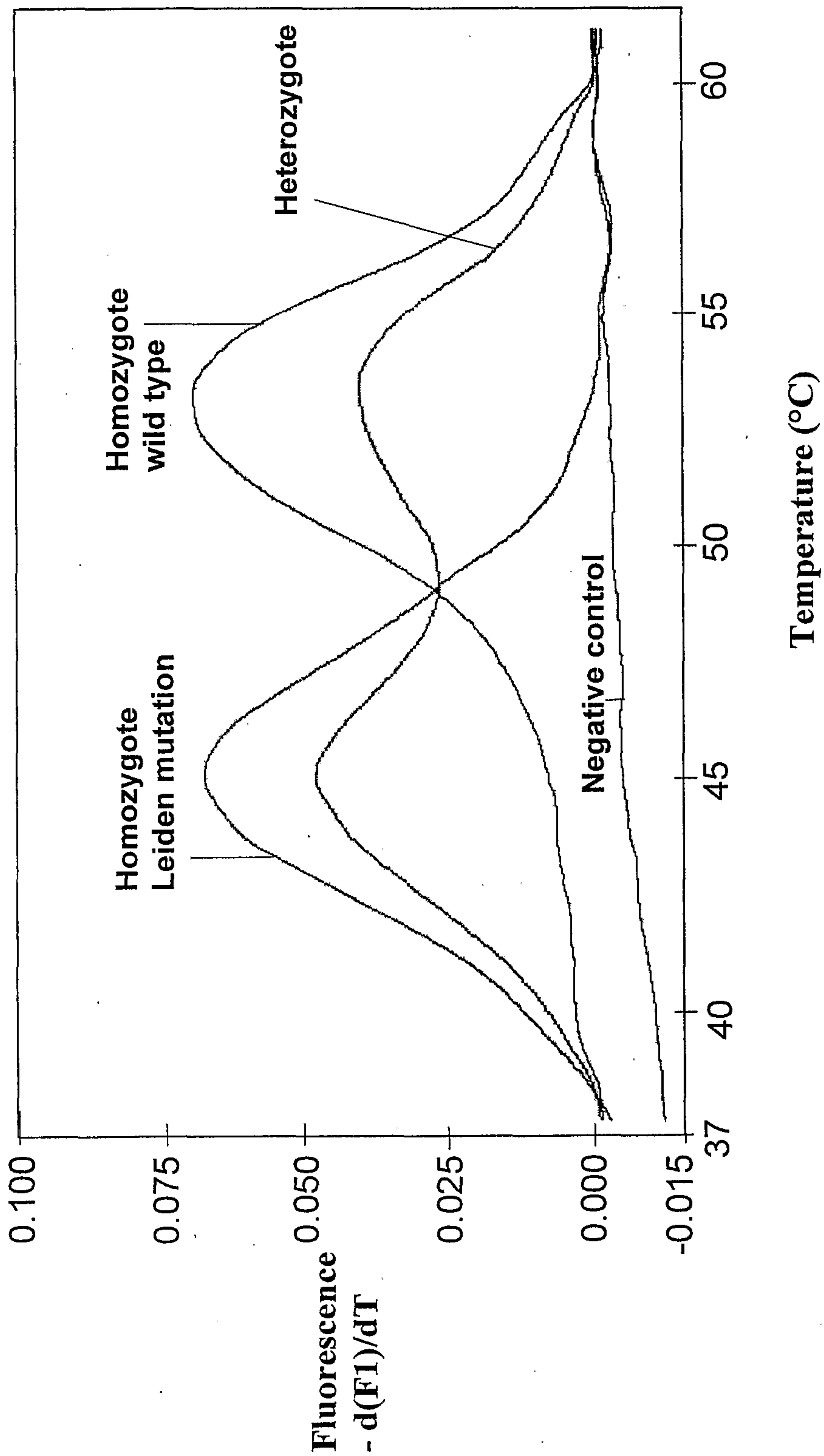
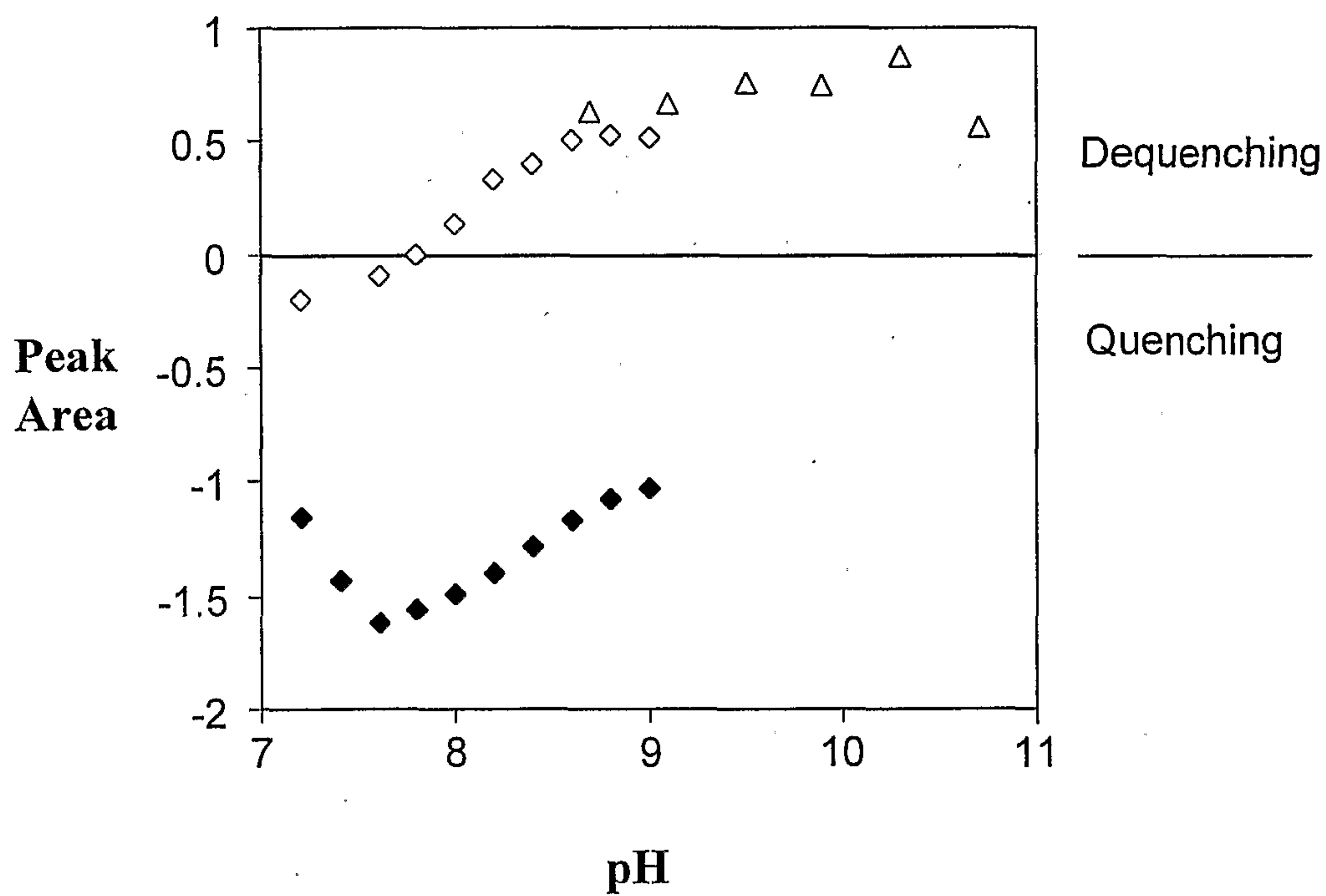
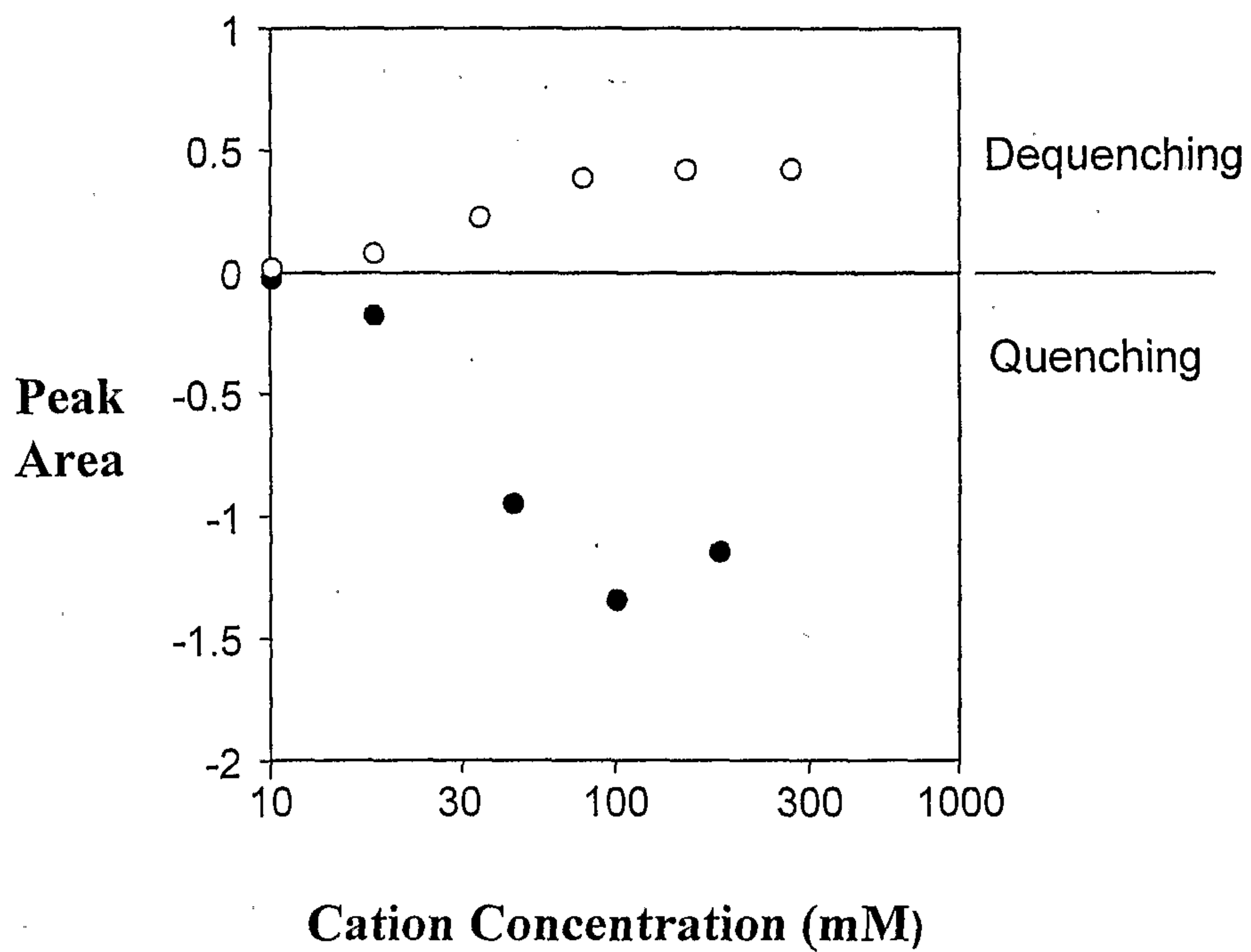


Fig 7



*Fig 8a*





*Fig 8b*

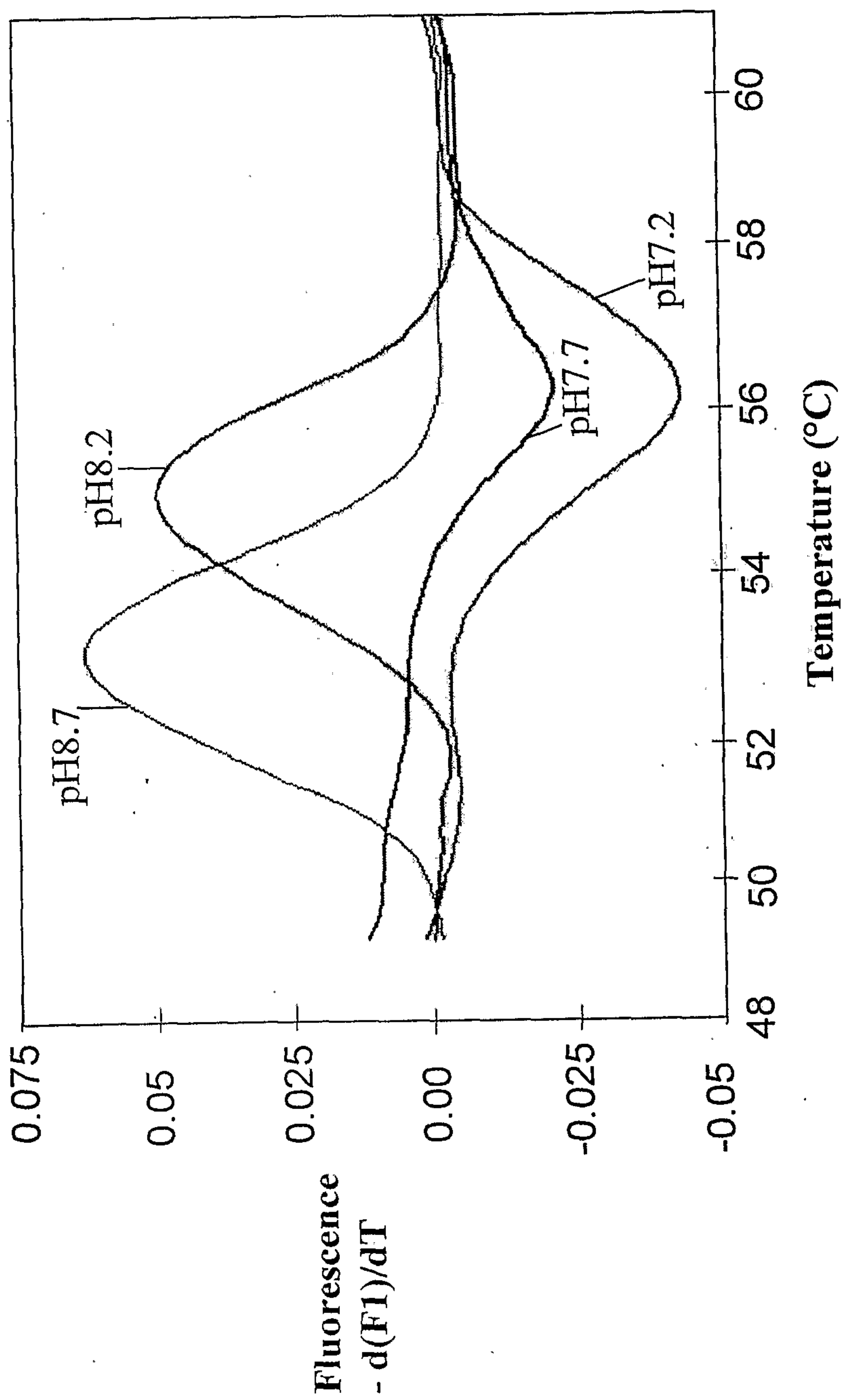
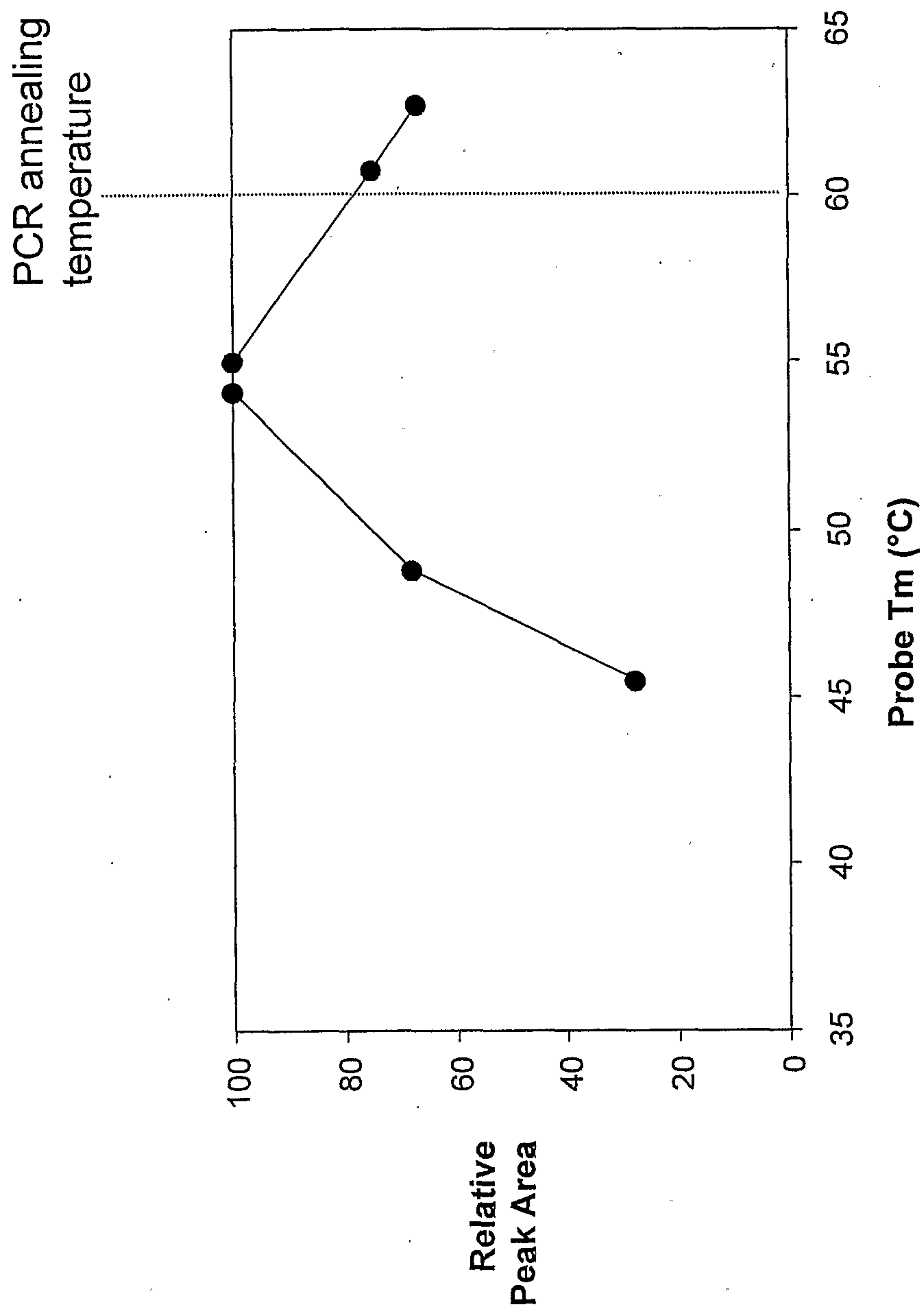


Fig 9



**Fig 10**

