

REVIEW ARTICLE

SNP Genotyping With Fluorescence Polarization Detection

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When a fluorescent molecule is excited by plane polarized light, the fluorescence emitted is also polarized. The degree of fluorescence polarization (FP) detected, under constant temperature and solvent viscosity, is proportional to the molecular weight of the dye molecule. By monitoring the FP of a fluorescent dye, one can detect significant changes in the molecular weight of the molecule without separation or purification. Because the size of the probe is altered in the course of a number of single nucleotide polymorphism (SNP) genotyping reactions, FP is therefore an excellent detection mechanism for these assays. Indeed, FP detection can be used in SNP genotyping with the primer extension TaqMan[®] and Invader[®] assays. Use of FP detection makes it possible to reduce the cost of TaqMan[®] and Invader[®] probes by abrogating the need for a fluorescence quencher. Moreover, inexpensive, unpurified, and unlabeled probes are used in the primer extension reaction with FP detection. As an end-point detection mechanism, FP detection is suitable for high-throughput SNP genotyping. *Hum Mutat* 19:315–323, 2002. © 2002 Wiley-Liss, Inc.

KEY WORDS: SNP; mutation detection; genotyping; fluorescence polarization; nuclease assay; primer extension

INTRODUCTION

The ability to determine efficiently and unambiguously the mutational status or genotype of an organism has great applications in molecular diagnostics, clinical genetic testing, population genetics, and agricultural biotechnology. High-throughput genotyping methods for single nucleotide variations currently in use discriminate between the alleles by differential hybridization, primer extension, ligation, and allele-specific probe cleavage [Kwok, 2001]. Homogeneous assays based on these allele discrimination mechanisms are conducted in aqueous solutions without separation or purification by monitoring physical changes when the reagents are turned into products. We have shown previously that fluorescence polarization (FP) is a good detection method in assays where the molecular weight of the starting fluorescent reagent changes significantly when converted into products. Indeed, FP detection has been shown to work well in the primer extension assay [Chen et al., 1999; Hsu et al., 2001b], in the 5'-nuclease (TaqMan[®]) assay [Latif et al., 2001], and in the Invader[®] assay [Hsu et al.,

2001a]. In this article, the use of FP detection and the three assays for which FP detection works well are described.

FLUORESCENCE POLARIZATION

FP is based on the observation that when a fluorescent molecule is excited by plane-polarized light, it emits polarized fluorescent light into a fixed plane relative to the molecule itself [Perrin, 1926]. The FP of a molecule is proportional to the molecule's rotational relaxation time (the time it takes to rotate through an angle of 68.5°), which is related to the viscosity of the solvent, absolute temperature, molecular volume, and the gas constant (all of which affect the molecular motion of the fluorescent dye). If

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the viscosity and temperature are held constant, FP is directly proportional to the molecular volume, which is directly proportional to the molecular weight. In other words, if the fluorescent molecule is large (with high molecular weight), it rotates and tumbles more slowly in space and FP is preserved. If the molecule is small (with low molecular weight), it rotates and tumbles faster and FP is largely lost (depolarized) (Fig. 1). The FP phenomenon has been used to study protein–DNA and protein–protein interactions [Checovich et al., 1995; Heyduk et al., 1996], DNA detection by strand displacement amplification [Walker and Linn, 1996; Walker et al., 1996], and in genotyping by hybridization [Gibson et al., 1997]. Many fluorescence polarization immunoassays (FPIA) are currently commercially available, many of which are routinely used in clinical laboratories for the measurement of therapeutics, metabolites, and drugs of abuse in biological fluids. In addition, FP detection is the basis of numerous research assays, especially those involved in ligand–receptor binding [Checovich et al., 1995].

FP is expressed as the ratio of fluorescence detected in the vertical and horizontal axes and is therefore independent of the fluorescence intensity. This is a clear advantage over other fluorescence detection methods in that as long as

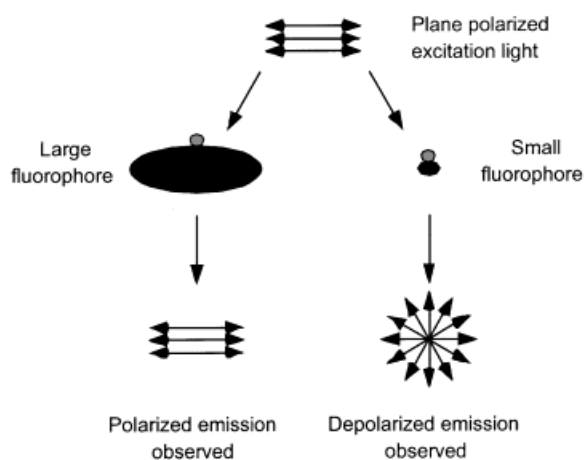


FIGURE 1. Fluorescence polarization (FP). The FP of a molecule is proportional to the molecule's rotational relaxation time, which is a function of the solvent viscosity, absolute temperature, and molecular volume. If the viscosity and temperature are held constant, FP is directly proportional to the molecular volume, which is directly proportional to the molecular weight. If the fluorescent molecule is large, it tumbles more slowly in space and FP is preserved. If the molecule is small, it tumbles faster and depolarized emission is observed.

the fluorescence is above detection limits of the instrument used, FP is a reliable measure. The degree of FP increases more or less linearly up to 10,000 Daltons in molecular weight before it levels off. Since a nucleotide bearing a fluorescent molecule has a molecular weight of about 1,000 Daltons and a fluorescent oligonucleotide with 25–30 bases is about 10,000 Daltons, FP is well suited as a detection method for any assay involving fluorescent probes of this size.

The total polarization reflects the sum of FP from all species in solution emitting at that wavelength. For example, in the primer extension reaction, where the fluorophore is attached to a low molecular weight nucleotide (with low FP) and is incorporated into the probe oligomer at the allelic site (with high FP), the polarization observed is described by the equation:

$$P = P_{\max}[\text{ddNTP}]_b + P_{\min}([\text{ddNTP}]_i - [\text{ddNTP}]_b),$$

where P_{\max} is the polarization for dye-labeled ddNTP incorporated onto the SNP probe, P_{\min} is the polarization of the unincorporated dye-labeled ddNTP, $[\text{ddNTP}]_i$ is the initial concentration of dye-labeled ddNTP, and $[\text{ddNTP}]_b$ is the concentration of incorporated dye-labeled ddNTP.

In the Taqman[®] or Invader[®] assays, where the large fluorescent probe (with high FP) is cleaved into smaller fluorescent mono-, di-, or tri-nucleotides (with low FP), the polarization observed is described by a similar equation:

$$P = P_{\max}([\text{probe}]_i - [\text{probe}]_c) + P_{\min}[\text{probe}]_c,$$

where P_{\max} is the polarization for the intact dye-labeled probe, P_{\min} is the polarization of the cleaved probe, $[\text{probe}]_i$ is the initial concentration of dye-labeled probe, and $[\text{probe}]_c$ is the concentration of cleaved probe. The maximum change in signal occurs with 100% conversion of the starting fluorescent reagent to the final product of a significantly different size. Therefore, an important aspect in experimental design is to ensure that the initial concentration of dye-labeled reagents used in the reaction is kept at a minimum and the reaction is pushed to completion.

THE FP-TDI ASSAY

The template-directed dye-terminator incorporation assay with fluorescence polarization detection (FP-TDI) assay [Chen et al., 1999] is a version of the primer extension assay that is

also called minisequencing or the single base extension assay [Syvanen, 1994]. The primer extension assay is a widely used method for SNP detection that utilizes a dideoxy chain terminating DNA sequencing protocol designed to ascertain the nature of the one base immediately 3' to the SNP-specific sequencing primer that is annealed to the target DNA immediately upstream from the polymorphic site. In the presence of DNA polymerase and the appropriate dideoxyribonucleoside triphosphate (ddNTP), the primer is extended specifically by one base as dictated by the target DNA sequence at the polymorphic site. By determining which ddNTP is incorporated, the allele(s) present in the target DNA can be inferred. This genotyping method has been widely used in many different formats and proven to be highly sensitive and specific [Nikiforov et al., 1994; Syvanen, 1998]. In the FP-TDI assay, the allele-specific dye terminator is incorporated onto an unlabeled SNP-specific primer. The genotype of the target DNA molecule can be determined simply by exciting the dye on the terminator to see if FP is observed (Fig. 2) [Chen et al., 1999].

The FP-TDI assay is accomplished in four steps in the same reaction vessel (a black 96- or 384-well microtiter plate). First, the genomic DNA is amplified by PCR to generate the template needed for the primer extension reaction. Second, the excess PCR primers and deoxynucleoside triphosphates (dNTPs) are degraded enzymatically so as not to interfere with the primer extension step. Third, the exonuclease I and shrimp alkaline phosphatase used in the PCR clean-up step are heat-inactivated. Fourth, the primer extension reaction "master mix" containing DNA polymerase, SNP-specific primer, and dye-terminators are added to the reaction mixture for primer extension with thermal cycling. At the end of the assay, the reaction mixture is analyzed for FP in the plate reader [Chen et al., 1999]. After the initial PCR set up, only two reagent addition steps are needed without any separation or purification.

Recently, we described an optimized FP-TDI assay that utilized four spectrally distinct dye-terminators to achieve universal assay conditions for SNPs. The 4-dye-ddNTP mix consists of ROX-ddGTP, Bodipy-Fluorescein-ddATP, TAMRA-ddCTP, and R6G-ddUTP. Following a standard protocol, the FP readings of the samples clustered

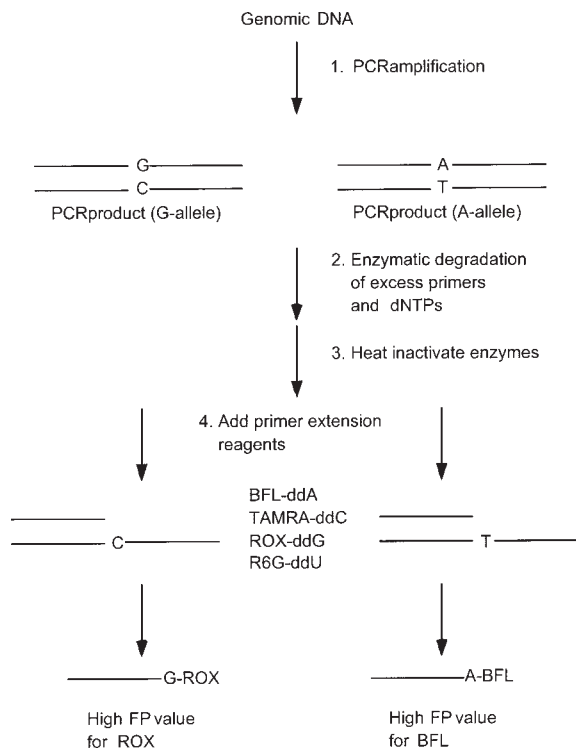


FIGURE 2. Scheme for FP-TDI Assay.

into four distinct groups without optimization for over 70% of the markers. The results of a typical assay are shown in Figure 3. As expected, the no-DNA negative controls have low FP values for both dyes analyzed and occupy the area near the origin of the plot, indicating that the small dye-terminators remain free in solution. For homozygous C individuals, the FP values for TAMRA-ddC are high and the values for R6G-ddU are low, reflecting significant incorporation of the TAMRA-ddC terminator onto the TDI primer but minimal incorporation of the R6G-ddU. Conversely, the FP values of TAMRA-ddC are low and those of R6G-ddU are high for homozygous T individuals. As for heterozygous individuals, the FP values for both TAMRA-ddC and R6G-ddU are high because of significant incorporation of both dye-terminators [Hsu et al., 2001b].

The ~30% of assays that fail initially are mostly due to reduced molecular volumes because of compact tertiary structures and misincorporation of dye-terminators when the correct terminator is used up. These assays can be salvaged by either adding *E. coli* single stranded DNA binding protein (SSB) plus a reading buffer containing ethanol and glycerol before the FP reaction mixture

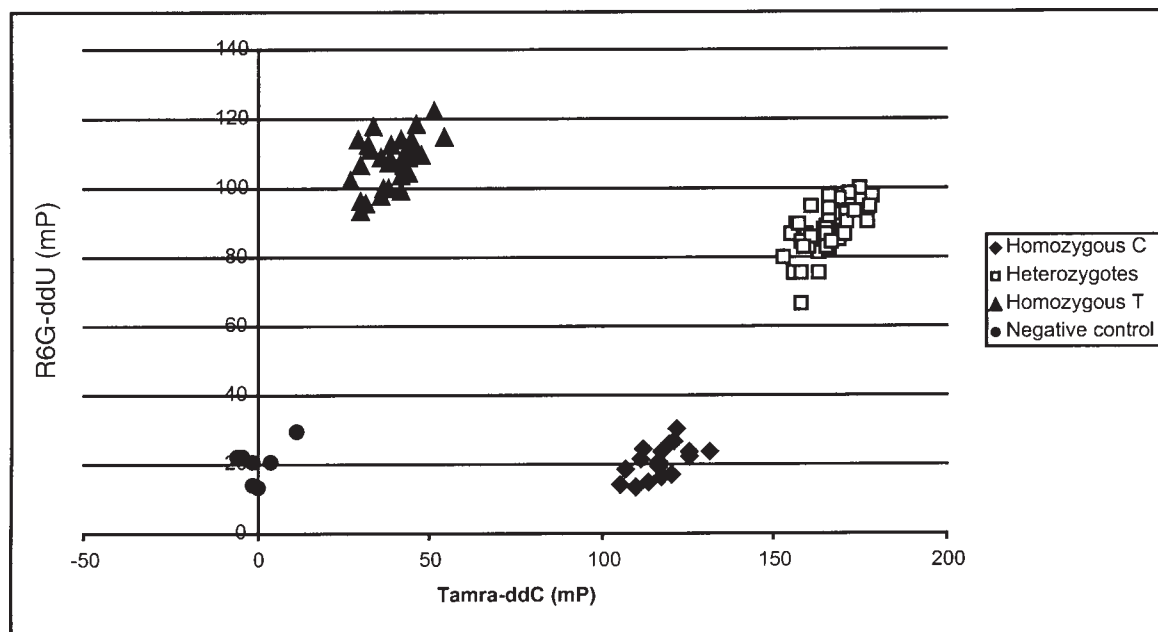


FIGURE 3. FP analysis of primer extension assay results. The homozygous C samples have high FP values for TAMRA but low FP values for R6G and occupy the lower right corner of the plot. The homozygous T samples have high FP values for R6G but low FP values for TAMRA and occupy the upper left corner of the plot. The heterozygotes are found in the upper right corner and have high FP values for both dyes. The negative controls are found near the origin, with low FP values for both dyes.

is read again or by reducing the number of cycles used in the primer extension reaction. The former measure rescues about two-thirds of the failed reactions (boosting the overall success rate to 90%) and does so by unraveling the extended SNP-specific primer to achieve a larger molecular volume while adding molecular weight to the fluorescent molecule. The latter measure rescues the remaining one-third of the failed reactions by ensuring that the correct terminator is not used up completely in the reaction. Therefore, with minimal optimization, all SNPs can be genotyped by the FP-TDI assay to-date [Hsu et al., 2001b].

Although the FP-TDI assay is one of several different methods that are currently available for automated genotyping, it has a number of advantages. First, the only instruments required are a thermal cycler and a fluorescence plate reader capable of FP measurements. A basic FP plate reader is relatively inexpensive (currently priced around U.S. \$25,000). Second, this assay is less costly to develop because it does not require any modified probes. The two PCR primers and one SNP-specific probe can be obtained quickly and very inexpensively. Third, the assays are very easy to optimize. One can literally develop a robust assay within hours of obtaining the prim-

ers. No redesigning or lengthy manufacturing of specialty probes or microarrays is necessary when a new marker is needed for a study. Finally, FP-TDI assay uses FP as a detection format, which is independent of fluorescence intensity and requires no separation of free from bound dye-terminator.

THE FP-TAQMAN[®] ASSAY

The 5'-nuclease assay is one of the simplest diagnostic assays for determining the mutational status of a DNA sample in one step [Livak, 1999]. The usual detection method in the 5'-nuclease assay relies on the increase in fluorescence intensity when a reporter fluorophore is released from its quencher as the doubly-labeled probe is cleaved during PCR in an allele-specific manner [Lee et al., 1999]. Because the 5'-nuclease assay is one where a large probe is cleaved into small molecules, it is amenable to FP detection (see Fig. 4).

We have shown recently that FP detection is indeed suitable for the 5'-nuclease assay [Latif et al., 2001]. Twenty markers, previously optimized for the 5'-nuclease assay, were used in the study. Two allele-specific probes, each labeled with either reporter dye VIC (allele 1) or FAM

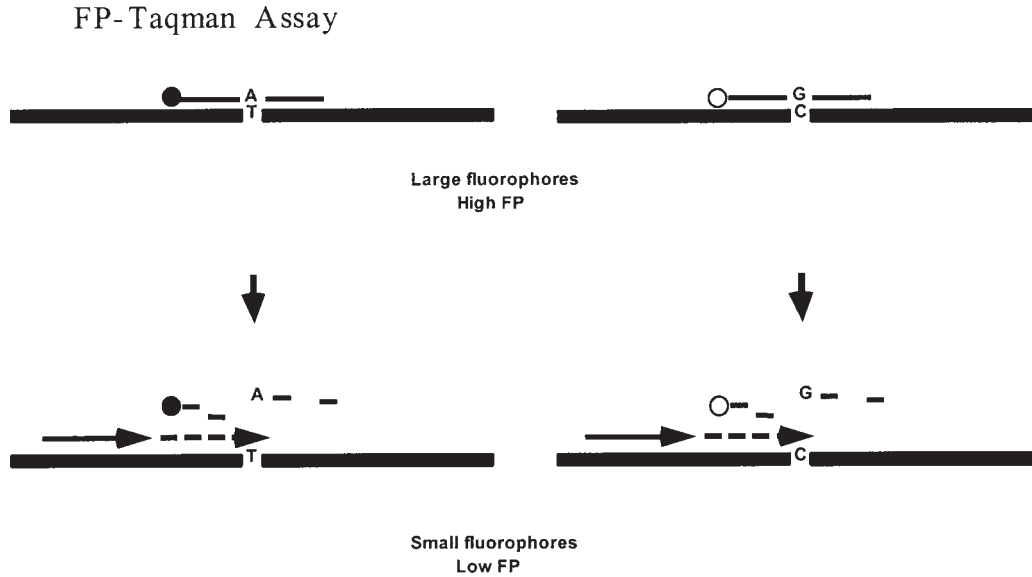


FIGURE 4. 5'-Nuclease assay with FP detection.

(allele 2), were designed for each SNP. These probes also contained a nonfluorescent quencher and fitted with a minor groove binder (which kept the probe length to a minimum and maximized the effect of the one-base mismatch) [Kutyavin et al., 1997; Afonina et al., 1997]. In the 5'-nuclease assay, the amplification and allele discrimination steps are done at the same time. Therefore, no further manipulation is required once the reaction is set up. Results of the FP analysis of a typical experiment are shown in Figure 5. The positive reactions in this case give LOW FP values (as opposed to HIGH fluorescence intensity values). For the negative controls, both probes remain intact at the end of the reaction and the FP values of both reporters remain high. Therefore, the data points for the negative controls occupy the upper right corner of the scatter plot. For the samples homozygous for allele 1, the VIC probe is cleaved but the FAM probe remains intact. They are found in the lower right corner of the plot, where VIC FP is low but FAM FP is high. Similarly, homozygotes with allele 2 occupy the upper left corner of the plot, where FAM FP is low but VIC FP is high. For the heterozygotes, both probes are cleaved and they occupy the origin of the plot, where both VIC and FAM FP values are low. The genotypes called by FP analysis were in complete concordance with those called by the conventional analysis method [Latif et al., 2001].

The use of FP as a detection method opens

up the possibility of utilizing a somewhat less costly probe, without the need for the quencher to be present. In addition, FP detection works best when the cleavage reaction is driven to completion and makes it possible to use limiting amounts of the modified probes, thereby reducing the cost of the reaction even further. If dyes compatible with FP detection can be found across the visible/infrared spectrum, a moderate level of multiplexing (perhaps up to 20 alleles, or 10 SNP markers) can be achieved.

THE FP-INVADER® ASSAY

The Invader® assay is a robust SNP genotyping method based on cleavage of a specific structure formed by overlapping probes that are annealed to the target DNA. Because it does not require allele-specific, dye-labeled probes for every SNP marker, the assay development cost is not too high. The usual reaction format of the PCR-Invader® assay detects the cleavage of a doubly labeled fluorescent probe (the signal probe) using fluorescence resonance energy transfer (FRET) [Mein et al., 2000]. Briefly, the PCR product is incubated with two oligonucleotides, termed the Invader® oligonucleotide (which is SNP-specific) and the primary probe (which is allele-specific). The Invader® oligonucleotide anneals to the downstream portion of the polymorphic site, and the 3' region of the primary probe is complementary to the upstream region of the polymorphic site. If the polymor-

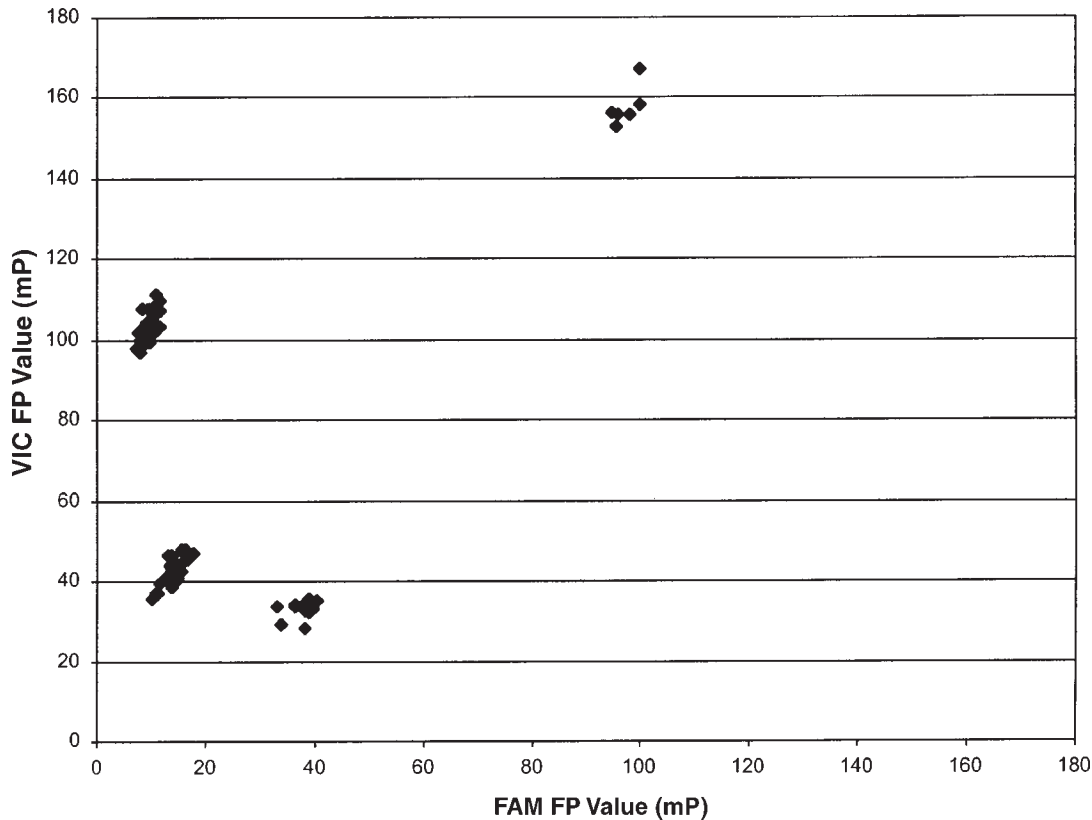


FIGURE 5. Fluorescence polarization analysis of 5'-nuclease assay with marker D2S1301 on 90 individuals. The homozygous allele 1 samples have low VIC FP values but high FAM FP values and occupy the lower right corner. The homozygous allele 2 samples have low FAM FP values but high VIC FP values and occupy the upper left corner. The heterozygotes have low FP values for both dyes and are found near the origin. The negative controls occupy the upper right corner, with high FP values for both dyes.

phism is complementary to the opposing base in the primary probe, the probe overlaps the 3' end of the Invader oligonucleotide, forming a structure that is recognized and cleaved by Cleavase enzyme at a specific site, resulting in the release of the 5' arm of the primary probe [Kaiser et al., 1999]. This cleaved 5' arm in turn serves as an Invader oligonucleotide in a second reaction, leading to the cleavage of the doubly labeled signal probe by the Cleavase enzyme. Since the signal probe is labeled at the 5' end with a fluorophore and internally with a quencher, the cleavage event removes the 5' fluorophore and enhances fluorescence (Fig. 6). In contrast, if there is mismatch between the primary probe and the target DNA, the requisite triplex structure is not present and the primary probe is not cleaved and there is no invader probe available for the secondary reaction.

We have shown recently that the Invader assay, in which a large fluorescent probe is cleaved into a much smaller flap, is suitable for FP de-

tection [Hsu et al., 2001a]. Moreover, we designed two signal probes, each with a different fluorescent dye label and a unique recognition sequence corresponding to the two 5' arms released when the primary probes are cleaved. These modifications reduce the cost of genotyping assays based on FP detection significantly while retaining the flexibility, sensibility, and specificity of the assay.

Following a standard protocol as described previously, the FP readings of the samples were found to cluster into four distinct groups for all SNP markers. The results of a typical assay are shown in Figure 7. The no-DNA negative controls, with both signal probes intact, have high FP values for both dyes analyzed and occupy the right upper corner of the plot, indicating that both dyes are part of high molecular weight species. For homozygous T individuals, the FP values for Fluorescein are low and the values for Tamra are high, reflecting significant cleavage of the Fluorescein signal probe but minimal

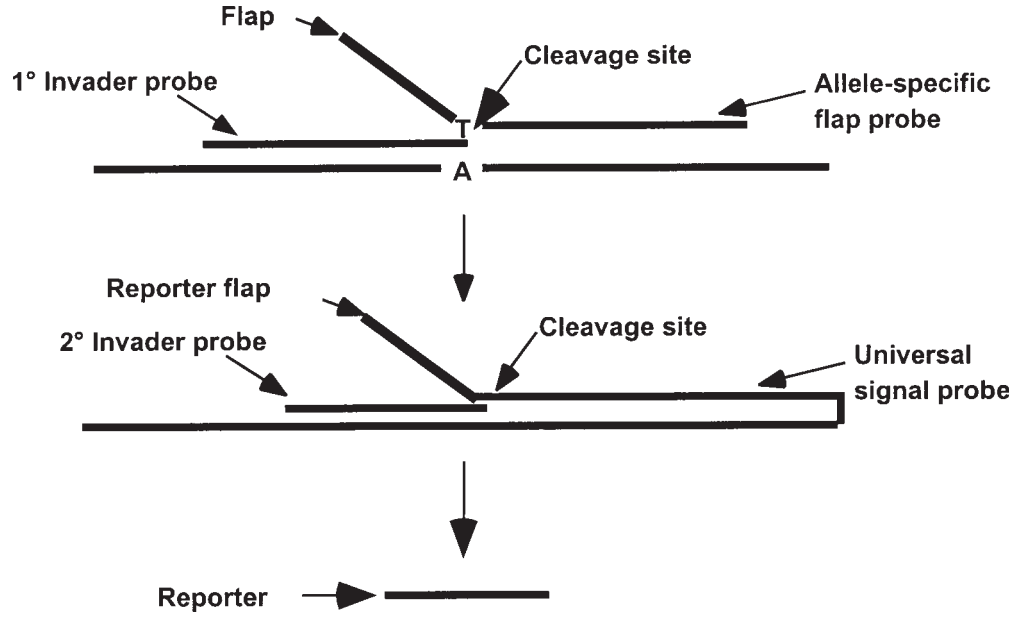


FIGURE 6. Invader Assay. When there is a match between allele-specific flap probe (primary probe) and DNA target at the polymorphic site, an overlapping structure between the 1° Invader probe and the primary probe is formed. A thermostable Cleavase enzyme recognizes this structure and cleaves the allele-specific probe, which releases the 5' flap. This 5' flap in turn serves as the 2° Invader probe in the second reaction where the signal probe was cleaved and the fluorescent molecule is released.

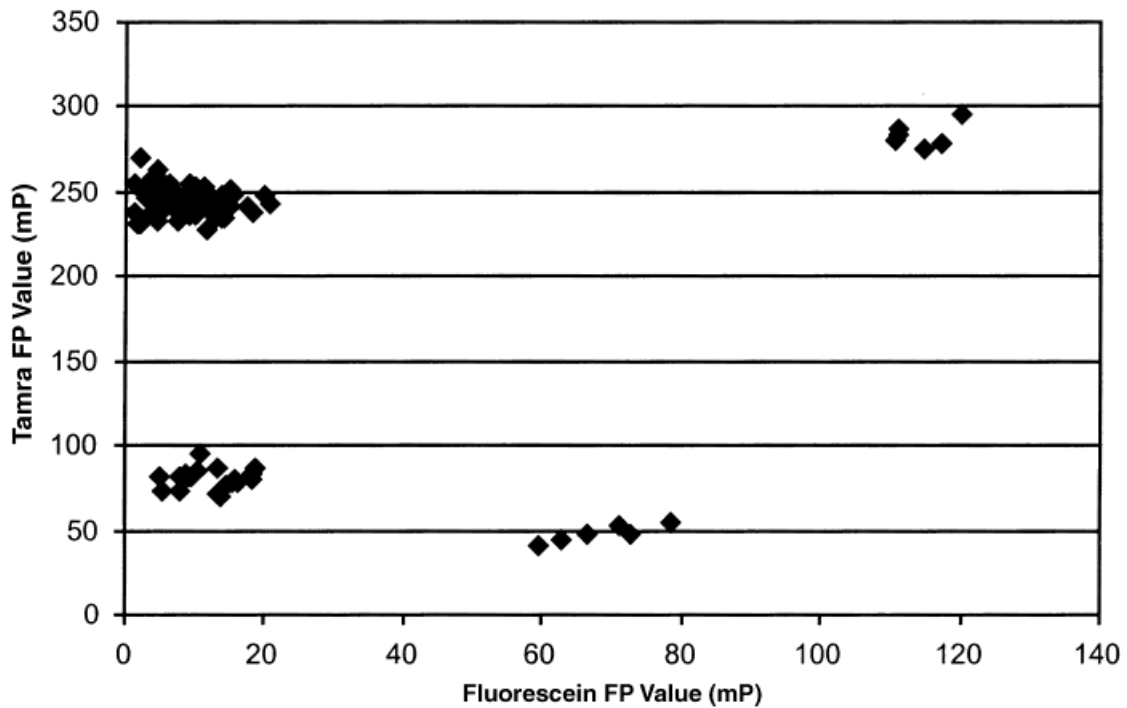


FIGURE 7. PCR-Invader genotyping data for TSC 1469. Homozygous T individuals form a cluster in the upper left corner with high Tamra FP values and low Fluorescein FP values. Homozygous G samples form a cluster in the lower right corner with high Fluorescein FP values and low Tamra FP values. Heterozygotes have low Tamra and Fluorescein FP values, and they cluster in the lower left corner. The no-DNA negative controls, with both signal probes intact, have high FP values for both dyes analyzed and occupy the right upper corner of the plot, indicating that both dyes are part of high molecular weight species.

cleavage of the Tamra signal probe, and the data points occupy the upper left corner of the plot. Conversely, the FP values of Tamra are low and those of Fluorescein are high for homozygous G individuals, and the data points are found in the lower right corner of the plot. As for heterozygous individuals, the FP values of both Fluorescein and Tamra are low because of significant cleavage of both signal probes and the data points are near the origin [Hsu et al., 2001a].

With two signal probes using two different fluorophores (Fluorescein and Tamra), both alleles of an SNP can be assayed in the same reaction, thereby saving time and reducing genotype miscalls. The use of more than two fluorophores would also allow moderate multiplexing of assays in the future.

DISCUSSION

For association studies using SNP markers, many new genotyping assays must be developed quickly for testing hundreds of samples. Therefore, ease of optimization and assay implementation are very important. With these requirements in mind, primer extension assays are the most versatile and cheapest to develop. When a set of SNPs is eventually selected for whole genome association studies or candidate gene association studies, it will consist of some 100,000 SNPs that will be used for multiple studies. High-density SNP mapping will call for robust assays with very low operating costs. The Taqman[®] assay and the Invader[®] assay are two of the simplest assays to operate, once the assays are developed.

FP is a simple, cost-effective, and accurate detection method for the primer extension, Taqman[®], and Invader[®] assays. Because FP is independent of fluorescence intensity, it requires no separation of the starting materials from the products. The use of FP as a detection method also opens up the possibility of utilizing a somewhat less costly probe in the Taqman[®] and Invader[®] assays (without quenchers), and unlabeled primers in the TDI assay. In addition, FP detection works best when the conversion from starting materials to products is driven to completion and makes it possible to use a limiting amount of the expensive fluorescent reagents, thereby reducing the cost of the reaction even further. FP detection may prove to be the most versatile method in homogeneous SNP genotyping.

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