# Fluorescence Polarization for Single Nucleotide Polymorphism Genotyping

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**Abstract:** Single nucleotide polymorphisms (SNPs) are the most abundant variations in the human genome and have become the primary markers for genetic studies for mapping and identifying susceptible genes for complex diseases. Methods that genotype SNPs quickly and economically are of high values for these studies because they require a large amount of genotyping. Fluorescence polarization (FP) is a robust technique that can detect products without separation and purification and it has been applied for SNP genotyping. In this article the applications of FP in SNP genotyping are reviewed and one of the methods, the FP-TDI assay, is discussed in details. It is hoped that readers could get useful information for the applications of FP in SNP genotyping and some insights of the FP-TDI assay.

## 1. INTRODUCTION

SNP is the most common form of sequence variations found in the human genome, with a density of one in 600-1000 base pairs [28,35]. An SNP is a single nucleotide substitution in a DNA sequence; although all 4 bases are possible at any given position theoretically, the majority of changes involves only 2 bases, therefore, most SNPs are biallelic. SNPs are normally identified by comparing the same DNA sequences from two or more different chromosomes. The human genome project produced millions of SNPs in the past few years and made them publicly available (http://www.ncbi.nlm.nih.gov/SNP/index.html). Because of its abundance and relatively even distribution in the genome SNP has rapidly become the most favorable marker for genetic studies.

Many studies have shown that genetic factors contribute significantly to complex diseases, examples include cardiovascular diseases, cancers, obesity, diabetes, schizophrenia and other mental disorders [2,26]. Identifying susceptible genes for these diseases has been a central topic for biomedical research for the last several decades; to fulfill the goals approaches like linkage, linkage disequilibrium and association studies are widely used [13,20,21]. In these studies a large number of subjects is genotyped for thousands or more genetic markers and tested statistically for linkage or association with disease status. Despite rapid development of genotyping technologies in the last several years the cost and throughput remain the most urgent and critical issues for such studies [14,29].

Large scale applications demand high throughput SNP genotyping. To increase throughput and to reduce cost many researchers have devised approaches to simplify genotyping protocols, the use of FP is one of such efforts [3,9,11,16,30]. FP is good at detecting changes of molecular volume and interaction and it is a homogenous method that does not require sample purification and separation. These features make it attractive for SNP genotyping, for it simplifies the protocol and makes it amenable to automation.

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## 2. FLUORESCENCE POLARIZATION FOR GENOTYPING DETECTION

### 2.1. Methods for SNP Genotyping

Genotyping is a process that identifies genotypes (alleles) for subjects at a given locus. Protocol wise, it normally consists of three steps, namely target amplification, allele discrimination and product identification. Polymerase chain reaction (PCR) is the most widely used method for target amplification although other methods are available [7,27]. The purpose of amplification is to increase the amount of target DNA because most analytical tools are not sensitive enough to assay the target directly at genomic level. Genotyping applications require PCR to amplify a unique DNA sequence in a genome. If a PCR amplifies more than one segment in a genome it can result in severe errors in genotyping because segments from unintended region may interfere with the target in subsequent procedures. After PCR a clean-up step may be needed to remove excess dNTPs and PCR primers, and this can be done enzymatically or mechanically.

The core of genotyping methods is allele specific reaction or allele discrimination. It is this reaction that the two alleles of an SNP produce distinct products; identification of the products leads to the identification of the alleles. There are several ways to generate products specific to an allele, the most commonly used approaches are primer extension [24], DNA ligation and hybridization (Figure 1). Other properties of DNA polymerases are also exploited for allele discrimination, this includes the 5' exonuclease activity [18] and the endonuclease activity [19].

The next step in the protocol is to detect products generated by allele specific reactions. There are many ways to identify the products, roughly they can be put into two different categories. One is homogeneous approach that does not require separation and purification of the products. Mechanisms for such detection include fluorescence

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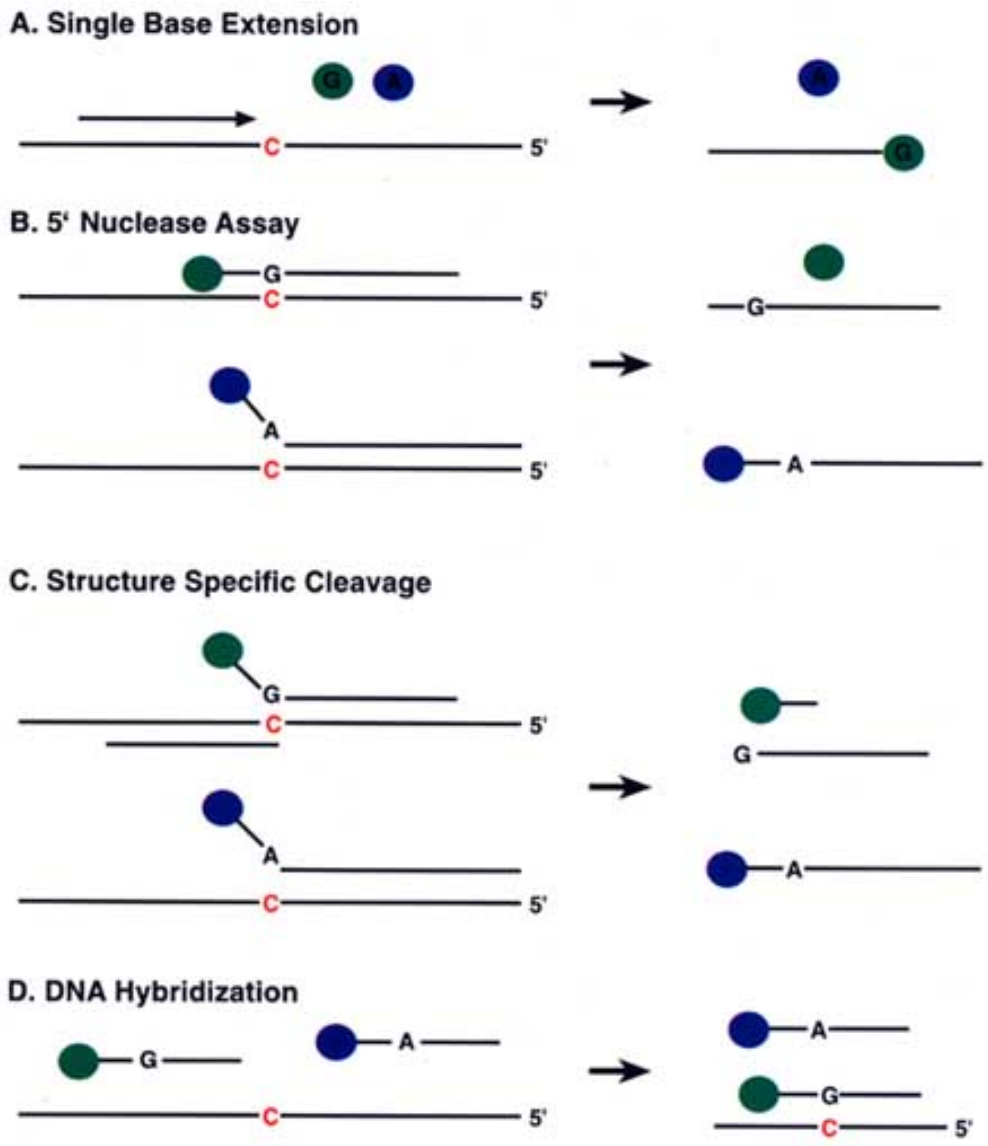


**Fig. (1).** Schematic drawings illustrate allele specific reactions for SNP genotyping for which FP detection format has been developed. The substrates are on the left and products are on the right. **A. Single base extension (SBE).** An extension primer is designed to anneal immediately upstream of the SNP site (red) in the template. Dye labeled ddATP (blue) and ddGTP (green) complementary to the target C/T polymorphism (only C allele shown) are added to the system along with DNA polymerases to extend the primer. DNA polymerases extend the primer selecting only the nucleotides that match the templates and form products that are one base longer than the primer with an allele specific labeling (green as shown). The increase of molecular weight/volume of the green dye causes increase of its FP value, thus scored as positive. Since the T allele is not present there is no products formed with a blue labeling. The FP value of the blue dye remains unchanged in the reaction. **B. 5' Nuclease assay.** Two allele specific probes are designed to anneal to target, with the 5' end (3-5 bases) close to the polymorphic site. The probe with green labeling matches the template perfectly and this causes it to be degraded by a moving DNA polymerase. The fluorescence labeling is thus separated from the rest of the probe. The decrease of molecular weight/volume resulted in the reaction decreases FP value of the green dye. In contrast the probe labeled with blue dye forms a structure that is partially hybridized. When DNA polymerases encounter this structure, they push off the probe without cleaving it. This does not change the weight/volume of the probe, therefore, no change of FP value is expected. **C. Structure specific cleavage.** An invader probe (drawn below the template) and an allele specific probe (labeled) form a bifurcated structure with the template at the SNP site. DNA polymerases cut off the 5' end of the allele specific probe only if the invader probe overlaps with the allele specific probe (the green probe as shown). Without the overlap the probe will not be cut (blue probe). The cleavage reaction reduces the molecular weight/volume of the probe and results in decrease of FP value. **D. DNA hybridization.** DNAs form duplex structure when they are complementary. When a probe hybridizes to a target, it forms a duplex, its molecular volume increases significantly (green probe). This change could be detected by fluorescence polarization.

resonance energy transfer (FRET) [4-6,34] and FP [3,9,16,30]. The other category is solid phase mediated approach, which, by separating and purifying the products, can accomplish higher level of multiplex and improve detection sensitivity. The FP detection is discussed in the next section, the description of FRET and solid phase mediated methods is beyond the scope of this article; readers who are interested in these subjects are referred to other reviews of genotyping methods [14,29,31].

### 2.2. Fluorescence Polarization for Genotyping Application

FP is a physical phenomenon first observed in 1926 [25]. When a fluorophore is excited by plane polarized light, its emission remains polarized if the molecule is still. In aqueous solution, however, molecules are in constant motion, while it is true that polarized light is emitted by individual molecules independently, collectively, as measured by most FP instruments, the emission appears scrambled, or depolarized. Since molecular motion, which is the cause of depolarization, is proportional to molecular mass, depolarization is also proportional to molecular mass under the conditions of constant pressure, temperature and viscosity. In other words FP can be seen as a measurement for molecular motion for fluorescence labeled molecules.

FP can be used to measure multiple species in a system. Under this condition the overall FP value of the system is the sum of weighted average of all species. In a reaction where substrates are turned into products, the sum of weighted average of molecular mass changes as the reaction proceeds, so does the FP value of the labeled fluorescence molecules [32]. The FP value would be at the minimum and the maximum when the reaction contains only substrates or products. The window between the two extremes determines how robust and realistic an assay can be developed based on FP detection. FP can also be used to monitor processes that interfere with the free motion of labeled fluorescence molecules, which may or may not chemically change the mass of the labeled molecules. Examples include ligand binding and surface interaction [23]. To apply FP principle to SNP genotyping, fluorescence labeled substrates should be used in the allele specific reaction to produce labeled products. Since most SNPs have two alleles, two different fluorophores are used, each corresponds to one allele.

Although FP has been widely used in high throughput screening its application in DNA testing and genotyping was relatively recent event. In 1997 Gibson et al [9] reported a DNA test for the cystic fibrosis ΔF508 mutation with FP detection. In the test DNA samples are first amplified by allele specific PCR and a fluorescence labeled probe is then used to hybridize to the amplified products. If the allele specific PCR is successful there would be products to hybridize to the fluorescent probe. The hybridization of the probe to the products increases FP value of the probe, thus the sample is scored positive. DNA hybridization was also used in a recent report in which locked nucleic acid (LNA) was used as probe [30](Figure 1D). The use of LNA reduces the length of the probe and improves the net change of FP values when the probes are hybridized to the products. It should be pointed out that DNA hybridization played distinct role in these two studies. For the ΔF508 mutation test, hybridization was used to detect if allele specific products were formed by the allele specific PCR, the probe was not intended to discriminate the two alleles. The discrimination of the alleles was done by allele specific PCR. In the LNA study, hybridization was designed to distinguish the two alleles. While DNA hybridization is robust to recognize specific sequences (as used in the ΔF508 mutation test) it is suboptimal to differentiate alleles (as used in the LNA study). For that reason many SNP genotyping methods do not use hybridization to separate the two alleles.

Many SNP typing methods use DNA polymerases to discriminate alleles, these include single base extension (SBE), the 5' nuclease assay and the invader assay (Figure 1A-1C). In 1999 Dr. Pui-Yan Kwok's group developed FP detection for the template-directed dye-terminator incorporation assay (FP-TDI) [4], and in 2001 the same group reported FP platform for the 5' nuclease assay [19] and the invader assay [14]. For both the 5' nuclease and the invader assays, the FP platform reduces the cost of probes by replacing doubly labeled probes with singularly labeled probes. In both assays reactions start with relatively larger probes and as the reactions proceed, these probes are degraded into much smaller nucleotides or dinucleotides. As a result, FP values for the probes decrease from 120-150 mP to about 50 mP, the change is quite robust. Dr. Pui-Yan Kwok has summarized these assays in a recent review; interested readers could get details there [15]. For the rest of this article, I will focus on the FP-TDI assay.

## 3. THE FP-TDI ASSAY

### 3.1. The Protocol

The FP-TDI protocol consists of three sequential reactions, target DNA amplification, removal of dNTPs and PCR primers and primer extension [3]. Like many other SNP typing methods FP-TDI uses PCR to amplify the targeted genomic sequence, but it optimizes PCR differently. Conventional PCR uses large excess of both primers and dNTPs and that would not cause undesired effects. But for FP-TDI too much of primers and dNTPs could compromise the protocol and result in genotyping error. The reason is that the excess primers and dNTPs have to be removed or deactivated before the SBE reaction. If there are too many primers and dNTPs left from PCR they would make the clean-up job harder and consequently the clean-up is more likely to be incomplete. Incomplete removal of PCR primers and dNTPs can cause genotype error. The amount of PCR products is important because it helps driving the SBE reaction toward completion, which has significant impact on the changes of FP value. Empirical data suggest that the amount of products that can be visualized by agarose gel and ethidium bromide staining is sufficient for the assay. The goal of optimization is to minimize the amounts of PCR primers and dNTPs without compromising the yield of products.

The second is the clean-up reaction and its goal is to remove or deactivate excess dNTPs and primers. There are many ways to clean up PCR products, the enzymatic

approach is selected mainly for its ease of use. Both shrimp alkaline phosphatase and *E. coli* exonuclease I can be inactivated easily by heat, this makes the enzymatic approach very simple and convenient: a single addition of the enzyme cocktail to the samples and two sequential incubations in PCR machine (first 37 ˚C for 45 min, and then 95 ˚C for 10 min). The two incubations can be easily programmed in a PCR machine. For the clean-up step technician's hands-on time is less than 5 min. Because there is no sample transfer involved sample lost and contamination often seen with transferring are avoided. The protocol relies on the two enzymes to deactivate dNTPs and primers, it is critical that the enzymes do a good job. To reduce the burden of the enzymes it is a good idea to optimize PCR with appropriate concentration of dNTPs and primers. If the enzyme digestions are not complete, the primers and dNTPs can cause unintended extensions. These unintended extensions increase sample's FP values just as the intended SBEs do, thus result in genotyping errors (false positives).

The third step in the protocol is allele specific extension. In the reaction primers are extended by DNA polymerases using fluorescence labeled ddNTPs. This reaction produces allele specific products, which are one base longer than the extension primer and have a fluorescence label. The products change the molecular volumes of the fluorescence label linked to the nucleotides, thus increase FP values. It is worth pointing out that the dye-terminators used in SBE are critical to the success of the assay. While the dye terminators provided by the Perkin Elmer Company (http://lifesciences.perkinelmer.com/areas/snps/solutions-text.asp) is a very good set for the assay, other dye-terminators are available (see more in the dye terminator selection section).

The FP-TDI protocol is similar to other methods that are based on SBE. What makes the method attractive is its product detection. Most methods need to do some purifications or separations before product detection, in contrast, FP-TDI reads the microtiter plate directly after SBE reaction without any further manipulation. Several factors contribute to the success of FP detection. The first is SBE reaction. As described above FP is good at monitoring changes of molecular volume. In allele specific SBE reactions, nucleotides labeled with unique fluorescence are incorporated onto extension primer. The change of molecular volume is significant. For a fluorescence labeled nucleotide, by average, is about 1000 Daltons, the primers commonly used for extension are 20-30 bases, and each base is about 330 Daltons. So in the SBE reaction, the change of molecular weight would be 5-10 folds. The dramatic change of molecular volume before and after the reaction makes it possible to use FP for detection. The second is the way in which SBE reactions are carried out. In the protocol, SBE is conducted in thermal cycler with excess extension primer and limited dye-ddNTPs. The combination of excess extension primers, limited ddNTPs and repeated cycling forces SBE reaction toward completion. When most dye-labeled nucleotides are converted into extension products, the change of FP values would approach its maximum. This makes the FP detection easier and more reliable. The third is the successful selection of a set of dye-labeled ddNTPs that can be used in the assay. While the authors listed their results of dye-ddNTP selection in the original publication they did not emphasize that not all dye-labeled ddNTPs are equal. Many users do not realize that a simple change of the dye-ddNTPs can make otherwise failed reaction successful. For that reason, testing more dye-ddNTPs and making them available to the community are important and necessary.

FP measurement is collective and quantitative. For better separation between the positives and negatives we should aim at converting most (>80%) substrates to products. The basic SBE reaction uses three substrates, the templates, which are PCR products, the dye-labeled nucleotides and the extension primers; DNA polymerases add one nucleotide to the 3' end of the primers using the templates as guidance. The products are DNAs that are one base longer than the original extension primers and are fluorescently labeled. The use of dideoxynucleotides guarantees the primer can only extend one base. Kinetics tells us that if we want to drive the reaction forward and use up one of its substrates, the dye-ddNTPs in this case, we should limit the amount of dye-ddNTPs and use more other substrates. That leaves us to increase either the amount of primers, that of the templates or both. For that reason the amount of PCR products is one of the most important factors that influence the outcome of FP measurement. Because the ways to increase the amount of PCR products are rather limited in high throughput setting, a more realistic approach is then to increase the amount of extension primer. Taking all these into consideration the protocol selects to use an amount of dye-ddNTPs similar to the molar concentration of PCR products and that of extension primer 5-10 times more. PCR normally produces about 5-10 ng or more DNA per reaction (10 μL); this would be an equivalence of 3-6 nM assuming the length of PCR product is 250 bps. This puts the amount of dye-ddNTPs at about 5-10 nM and that of primers at 50-100 nM. SBE reactions are normally performed in a PCR machine with multiple cycling. This linear amplification gives more chances to use up dye-ddNTPs and helps to normalize uneven PCR amplification between samples. Robust PCRs need only a few cycles to use up most dye-terminators, the weak ones could catch up eventually after more cycles.

Limiting the amount of dye-terminators does not necessarily mean the less the better. Two factors need to be considered. One is the sensitivity of instrument; the other is non-specific interaction between dye-terminators and other components of the system, especially the inner surface of the reaction vessel. Modern instruments can reliably detect fluorescence at much lower concentration (2-3 orders of magnitude), they normally do not constitute the limit of the FP assay. But precautions should be taken because instruments of different vendors vary greatly. Specifics of setting of each instrument, such as the filter bandwidth, reading duration and modes, can affect the outcome. The non-specific interactions (non-specific binding) of dye-terminators with other components in the system are often neglected by users. For a given reaction volume, the area of inner surface of the reaction vessel is a constant, this means that it would bind a certain amount of fluorescence dye regardless what concentration is used. When the concentration of the dye-terminators is sufficiently high, the fraction that binds to the surface is negligible. The relative amount of bound dye-terminators increases as the overall

concentration goes down and it can cause false positive results when it becomes significant. The reason is that non-specific binding could restrict or slow down the free motion of dye molecules, an effect that is indistinguishable from the true positives in which the dye molecules slow down because of changes of their molecular volume. From this perspective, any factors that slow down molecular motions disproportionally could cause false positives. Often seen examples are that wells on the edge of PCR plates have higher FP values. The reason is that these wells are more prone to evaporation during PCR and evaporation reduces reaction volume and increases viscosity, which affects interactions between the fluorescence dyes and other components in the system. The net results are elevated FP values for the edge wells. To prevent evaporation we use the thermal seal from MJ Research (Microseal, cat. No. MSA-5001).

There is one optional step in the protocol that can enhance the separation between the positive and negative reactions, this is to use single stranded DNA binding proteins (SSBs) after SBE reaction [10]. When the separation between the positives and negatives are not optimal, an addition of 0.1 μg of SSB to each sample followed by incubation at room temperature for 30-60 min can selectively increase the FP values for the positives. SSBs bind to single stranded DNA selectively. The binding of SSB to the single stranded products, therefore the increase of their molecular volumes, is believed to be the direct cause for selective increase of FP values for the positives. But the binding mode of SSB is complex and the exact mechanism remains unclear (see more discussion below).

### 3.2. Selection for Dye Labeled Dideoxyribonucleotides

Strictly speaking, the FP-TDI is a group of assays that applies FP detection principle to a biochemical reaction (primer extension) that uses many different substrates and produces many different products. The change of substrates, i.e. the primer, the template and the dye terminators, from one SNP to another makes the assay more dynamic. Each fluorescence dye has unique intrinsic properties and interacts with different DNA sequence differently. Before the mechanism for FP measurement of DNA molecules is fully understood there are certain probabilities that a given fluorophore may not be able to detect the products from an SBE reaction. There are at least two approaches to handle this issue. One is to investigate systematically the interaction between fluorescence dyes and DNA sequences and to establish the rules how they interact. These rules then can be applied to the design of SBE reaction. The other way is to empirically screen fluorescence dyes that produce optimal separation between different substrates and products. The latter approach was used when the FP-TDI was developed.

For the screen a set of 4 templates that have exactly same sequences except at one position which is either A, C, G or T for one of the 4 templates is designed. An extension primer annealing to the templates immediately upstream to the polymorphic position is also designed. To screen dye-terminators suitable for the assay the same templates and primer are used. It was noticed in the first few experiments that the same fluorescence dye behaved differently when it was conjugated to different nucleotides. For example, BODIPY-TMR-ddA (BTMR-ddA) had an average of 94.5 mP for the negative reactions, but that for BTMR-ddC was only about 30.5 mP (Table 1). Since the difference of molecular mass between the four nucleotides are small (<30 Daltons), the change of molecular mass along is insufficient to explain the observed difference in FP value. This suggests that other factors also affect the polarization of fluorescence dyes when they are conjugated onto nucleotides. The difference between nucleotides is their base rings, the observed difference in FP values between dye-terminators implies that the interaction of the dye moiety and the base rings influences the baseline FP value of the terminators. The effect could be direct or indirect and so far there is no evidence to suggest one way or the other. The observation that addition of organic solvent such as methanol could reduce the background of terminators indicates that the interaction between the dye moiety and base rings might have caused aggregation and the aggregation might be the direct cause for elevated FP value. In the initial report [3] 5 dye terminator groups, namely FAM-ddNTPs, BODIPY-Fluorescein (BFL)-ddNTPs, R6G-ddNTPs, TAMRA (TMR)-ddNTPs and ROX-ddNTPs, were tested for their utilities for the assay. For most dye terminator groups there are more than one nucleotides that produce significant differences between the positive and negative reactions, and for most cases, the results from base C and T are similar and

**Table 1. Dye Terminator Screen—BODIPY-Texas Red and BODIPY-TMR Labeled ddNTPs**

| | BODIPY-Texas Red | | | | BODIPY-TAMRA | | | |
|---|---|---|---|---|---|---|---|---|
| **Template** | **BTR-ddA** | **BTR-ddC** | **BTR-ddG** | **BTR-ddU** | **BTMR-ddA** | **BTMR-ddC** | **BTMR-ddG** | **BTMR-ddU** |
| CF508-48A | 171.8 | 66.2 | 173.2 | **173.4** | 100.5 | 33.9 | 42.8 | **185.5** |
| CF508-48C | 165.2 | 67.2 | **146.2** | 27.6 | 79.8 | 27.0 | **63.8** | 32.6 |
| CF508-48G | 158.2 | **188.2** | 135.6 | 24.1 | 103.1 | **190.3** | 38.5 | 54.2 |
| CF508-48T | **190.1** | 0.9 | 155.9 | 32.2 | **202.8** | 30.7 | 68.7 | 36.3 |
| Ave.Ctrl. | 165.1 | 44.8 | 167.0 | 28.0 | 94.5 | 30.5 | 57.4 | 41.0 |
| NET CHANGE | 25.1 | 143.4 | -8.7 | 145.4 | 108.3 | 159.7 | 13.8 | 144.4 |
| STAND DEV. | 7.9 | 34.5 | 20.2 | 5.0 | 14.6 | 3.9 | 18.4 | 19.5 |

that for base A and G are similar. For all tested dyes ROX-ddG is the best for the G base, but for base A, C and T there are several dyes that can be used. Since the publication of the method two more dyes, BODIPY-TAMRA (BTMR) and BODIPY-Texas Red (BTR), were tested and results are listed in Table 1. Once again, base C and T produced significant results for both dyes. For base A, the change of FP value for BTMR was acceptable but that for BTR was minimal (~25 mP). Both dyes are not good for the G base.

It is also noticed that the amount of net change is different. For instance BTMR-ddU produces a net change of about 150 mP between the positive and negative reactions when it is tested in the synthetic templates system (Table 1). But BTMR-ddA produces a change of about 100 mP. Since there is not much difference (~ 8 Daltons) between BTMR-ddA and BTMR-ddT in terms of molecular mass, once again, molecular mass along can not explain the dramatic difference of the positive reactions. This implies that the base to which the fluorophore is linked also affects the net change. When the same fluorophores are used for different SNP markers, significant differences are also observed (Figure 2). All these seem to suggest that both the change of molecular mass (from dye-terminator to extension product) and the interactions (between the dye-moiety and the base rings/DNA sequences) contribute to the increase of FP values.

There are 6 combinations of polymorphisms when two alleles are considered for an SNP, these are A/G, A/C, A/T, G/C, G/T and C/T, of them the A/G and C/T polymorphisms are more frequent than the others. In an effort to select optimal dye-terminator pairs for these polymorphisms, different combinations of dye terminators are used to genotype the same polymorphisms for different SNPs with DNA samples of known genotypes. Examples

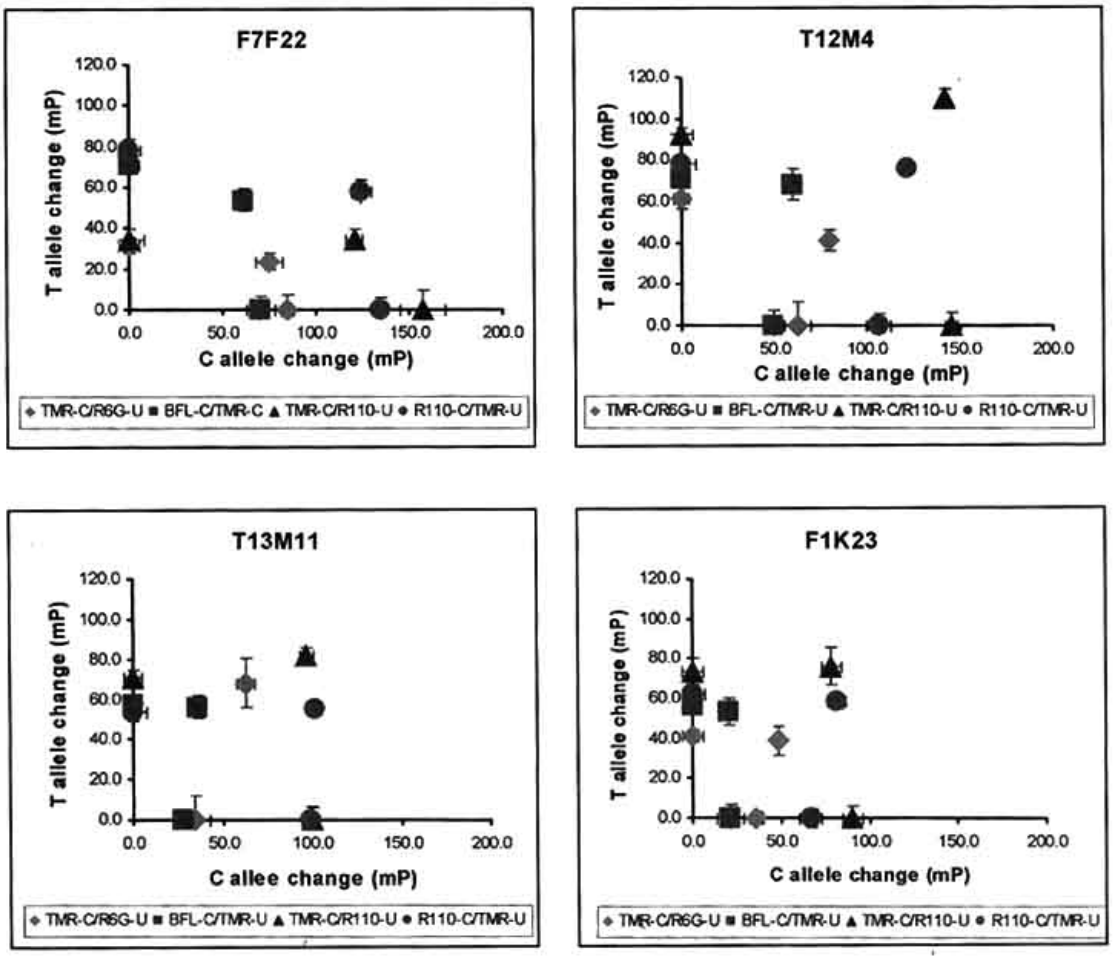


**Fig. (2).** Dye-terminators affect the separation patter of FP-TDI assay. Four dye-terminator pairs, TMR-ddC/R6G-ddU, BFL-ddC/TMR-ddU, TMR-ddC/R110-ddU and R110-ddC/TMR-ddU, are used to perform FP-TDI assay for four SNPs using DNA samples of known genotypes. For each genotype group at least 6 samples are typed, the averages of net changes between the positive and negative samples for the same genotypes are plotted; standard deviations for each genotype group are presented as error bars for both X and Y axes. For a given SNP marker the net changes vary amongst the four dye pairs, ranging from 20 to over 150 mPs. When a dye pair is compared amongst the four markers, the net changes also vary, but at a smaller range. The results imply that the fluorescence dyes interact with DNA sequences and the interactions affect the separation pattern.

are shown in Figure 2, where four SNPs are tested for four dye combinations for the C/T polymorphism. The dye terminators selected are those nucleotides that have passed the nucleotide screen as described above. For each dye pair 24 samples are used, of them 8 of the samples are homozygous C allele, 8 samples are homozygous T allele and 8 samples are heterozygous. It is quite clear from the figure that the performance of the dye pairs is consistent, some dye pairs are always better than the others regardless which SNP is tested, despite that there are some variations between SNPs. For example, the R110-C/TMR-U pair has the best overall separation amongst the four tested pairs, the net change for the C allele is from 65 to 140 mP, that for the T allele is from 55 to 80 mP. In contrast the separation for TMR-C/R6G-U pair is consistently smaller, with the ranges of 40 to 80 mP and 30 to 60 mP for the C and T alleles respectively. The pair of TMR-C/R110-U has great separation, better than that of R110-C/TMR-U pair, for three of the four markers. But for the marker F7F22, the T allele has only 30 mP change, much smaller than that of the other markers. Similar trends are also observed for other polymorphisms tested (Table 2). It is worth pointing out that several polymorphisms have more than one pairs of terminators that produce optimal results. For example, all tested dye pairs of the G/C polymorphism produce good results. For the C/T polymorphisms, a total of 11 SNPs are tested for 8 pairs of dye terminators, the results should be reliable. Other polymorphisms are not tested as extensively as C/T polymorphism the optimal dye pairs may change as more and more data accumulated.

When the same dye pairs are compared across the SNP markers it is not difficult to notice that the net changes are quite different. The difference of primer sizes along is not sufficient to explain them (primer sizes for the four markers used in Figure 2 are 33 bases for F7F22, 29 bases for T12M4, 17 bases for T13M11 and 22 bases for F1K23). Because each SNP primer has unique sequence it is not unreasonable to contribute the difference to the sequences of primers. There are at least two ways that primer sequences affect FP values. One is interaction between the primer sequence and the fluorescence group. Since it is difficult to decrease polarization by increasing interaction, it would be safe to assume that a strong interaction plays favorably toward better separation. Under this assumption most sequences interact with fluorescence dyes strongly, only when the interaction is weak that a small net change be observed. The other possibility is that the configuration of the primer affects the FP values. For example some primers may fold onto each other, others may form hairpin or stem-loop structures. These configurations alter the surface energy of the molecules, therefore, can affect the FP value of the products.

In conclusion, the data demonstrated that dye-terminators selected from empirical experiments can be useful for other SNP markers and consistent results can be expected even through there are variations and fluctuations from marker to marker. The other factor that is also important is the sequence of the extension primer. Under this circumstance either the interactions between the dye moiety and the sequence or the configuration the products undertaken in solution could contribute to the difference of FP reading. So far there are no reliable ways to predict what sequences produce better results. For SBE reaction the sequences for primer selection are limited due to the restriction of SNP position and most commonly used approach is to select primers to meet certain melting temperature ($T_m$). Because fluorescence dye interacts with DNA sequence differently, and for a given SNP there is not much option to change primer sequence, a better alternative is to use different dye pairs. When the dye ddNTPs are changed the outcome is likely to be different.

### 3.3. Single Stranded DNA Binding Protein Improves FP-TDI Assay

As outlined in the protocol, the third step of the assay is primer extension. In the reaction extension primers are in large excess as compared to the templates and the products generated should be largely single stranded. Based on this rationale the single stranded DNA binding proteins (SSBs) should be able to bind to the products and increase their molecular volume, which would lead to the increase of FP values. The hypothesis is tested and indeed that SSBs

**Table 2. A Summary of Dye-Terminator Pair Testing for Different Polymorphisms**

| Polymorphism | A/G | A/C | C/T | G/C | G/T |
|---|---|---|---|---|---|
| **No. SNP tested** | **2** | **1** | **11** | **1** | **2** |
| **Dye-pair tested** | BFL-A/ROX-G, BFL-G/ROX-A, FAM-G/ROX-A, F-N6-A/ROX-G, F-12-A/ROX-G | BFL-A/TMR-C, ROX-A/TMR-C, ROX-A/R6G-C, BFL-A/BTR-C | BFL-C/TMR-U, R6G-C/TMR-U, ROX-C/TMR-U, TMR-C/BFL-U, TMR-C/R6G-U, TMR-C/BTR-U, TMR-C/R110-U, R110-C/TMR-U | ROX-G/TMR-C, ROX-G/BFL-C, ROX-G/R6G-C, ROX-G/BTMR-C | ROX-A/TMR-U, ROX-G/BFL-U, ROX-G/R6G-U, ROX-G/BTMR-U |
| **Best dye pairs** | BFL-A/ROX-G, BFL-G/ROX-A | BFL-A/TMR-C | TMR-C/BFL-U, R110-C/TMR-U, TMR-C/R110-U | ROX-G/TMR-C, ROX-G/BFL-C, ROX-G/R6G-C, ROX-G/BTMR-C | ROX-G/TMR-U, ROX-G/R6G-U |

**Abbreviations:** BFL: BODIPY fluorescein; BTR: BODIPY Texas Red; ROX: 5-(and-6)-carboxy-X-rhodamine; F: Fluorescein; TMR: 5-(and-6)-carboxytetramethylrhodamine; BTMR: BODIPY TMR; FAM: 5-(and-6)-carboxyfluorescein; R110: rhodamine 110; R6G: 5-(and-6)-carboxyrhodamine 6G.

enhance the separation between the positive and negative reactions. This was reported in the follow-up paper of the assay and the use of SSB was integrated into the protocol as an optional step after SBE reaction [10].

The follow-up paper was focused on protocol and did not elaborate SSB work. To better understand the utility of SSB titration studies are performed for several SNP markers. For the testing SSBs are added after standard protocol as described [10], and an incubation of 30-60 min at room temperature is allowed before the samples are read by an FP reader. Four concentrations of SSB ranging from 0.1 to 0.4 μg/well (5.3-21.1 nM) are used and the amounts are based on the concentration of extension primers. It is reasoned that

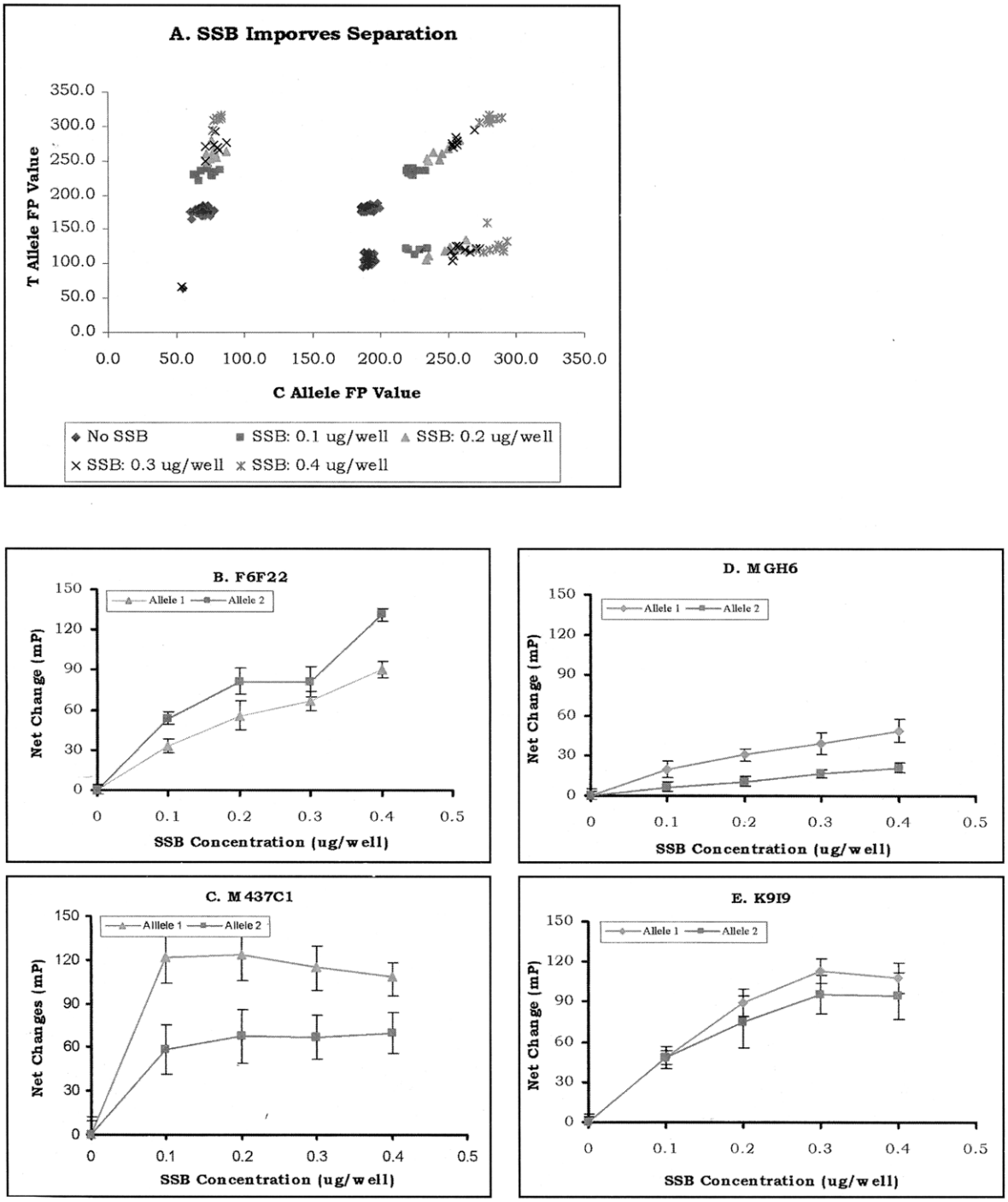


**Fig. (3).** SSBs improve the separation between the positive and negative reactions. **A. A titration of FP-TDI assay with SSBs.** As the amount of SSBs (0.1-0.4 μg/well) used increases the net change between the positive and negative reactions increases accordingly. **B-E: Net changes for four SNPs.** The net changes are plotted for the four SNP markers with the same range of SSB concentrations as shown in panel A. The results demonstrate that SSBs improve separation for all markers with no exception, but the amounts of net change vary from marker to marker, even for the same marker, the amounts of net change are different for the two alleles.

in order to achieve maximal effect, the amount of SSB needed should bind all primers. When 10 nM of primer is used in the SBE reaction, the equivalent amount of SSBs would be 0.2 μg/well. Figure 3 shows the results. Panel A is a scatter plot of FP values of the two alleles with or without the use of SSB. It is very clear that the separation between genotype groups increases as the amount of SSB used increases. SSBs specifically increase the FP values for the positive reactions. The results confirm the speculation that the extension products are single stranded. While it is true for all tested markers that SSBs increase FP values significantly, the amounts of increase are quite different from marker to marker (Figure 3, panel B-E). For one marker, M437C1, the increase reaches plateau when 0.1 μg of SSB proteins are used (Figure 3C), for others, F6F22 and MGH6, the increase continues even 4 times more SSBs are used (Figure 3B, 3D). Even for the same marker SSBs have different effect on the two alleles. Take marker M437C1 as an example (Figure 3C). For allele 1, the C allele in the C/T polymorphism, 0.1 μg of SSB protein makes a net change of 120 mP, the same amount of protein makes a change of 60 mP for the T allele. For the other three markers although there are differences between the two alleles the margin is not as wide as that of M437C1.

The results obtained suggest that the effect of SSB is more complicated than what was originally thought. SSBs form homotetramers when they bind to single stranded DNA, and the binding mode changes when different solution conditions, such as the salt type, salt concentration and protein to DNA ratio, change [12]. When divalent cations are present the four subunits of the SSB tetramer all interact with DNA molecules. Since our primers are relatively short (mostly less than 35 bases) and $Mg^{++}$ is in the buffers for all reactions, multiple primers can be bound to a homotetramer. The binding of multiple extension products to a tetramer would significantly increase their molecular volume, thus leads to the increase of FP values. Since SSB protein does not bind ddNTPs, a negative reaction would not be affected. The variation of net changes observed could largely be due to the ratio of SSB and extension primer/product, which can be considerably different between SNPs because for each SNP the PCR efficiency is different. The efficiency of PCR affects the amount of available templates for SBE reaction, which in turn affects the amount of products generated by SBE. When the total amount of primer/product is similar to that of the protein added, SSBs can bind almost all products (or to be saturated) and thus result in large net change in FP values, as seen in Figure 3C and 3E. But if the total amount of primer/product is much more than that of the protein (too many primers used in the reaction), most DNAs bound to SSBs are primers, because SSBs can not discriminate extension primer from its product. This would lead to smaller but proportional net change in FP values (Figure 3D). From this perspective if the optional step is intended the amount of extension primer should be taken into consideration when assembling SBE reaction. When a large excess of primers is used in SBE reaction moderate amount of SSB can not be much help to improve the separation.

The FP-TDI assay has been used by many groups and side by side comparison studies have been done with other methods [1,8]. The overall performance of FP-TDI is comparable with other methods that use SBE, results from our lab and others show that FP-TDI has a success rate of >85% with accuracy of 99% or better. While FP-TDI is flexible and cost-effective its throughput may not be sufficient for large scale studies. Using current protocol a technician can handle 4000-6000 genotypes a day with appropriate liquid handling equipment, but this falls much short of the 50,000-100,000 genotypes/day demanded by large scale association studies. FP-TDI protocol can be treated as a module of which 90-95% of the time is spent on a PCR machine. For a 384-well plate it takes 4-5 hours (time spent on PCR machine) to finish the reactions, it takes only 5 min to read the results. To increase the throughput, therefore, the best way is to increase the number of modules which are essentially the capacity of PCR machine.

## 4. CONCLUSION AND DISCUSSION

SNPs have been received enthusiastically as a new generation of genetic markers. Its abundance and high density distribution in the human genome make it an ideal tool for genetic studies. The main applications are in the studies of pharmacogenetics, complex diseases and diagnostics and forensics. All these applications demand high throughput and cost-effective methods to genotype SNPs. Of the many methods of SNP genotyping SBE is particularly popular for its sensitivity, accuracy and relative easiness of protocol. And of the many detection formats for SBE products FP is a robust and cost-effective platform.

FP is a technique that can detect the change of molecular volume in a reaction without the need to purify or separate the products from substrates. It is a collective measure of all species in the system. For a reaction that involves a substrate and a product the FP values of the substrate and product have to be significantly different, the window between the two values determines how sensitive and robust an FP assay can be developed. For this reason selection of appropriate fluorophores is an important part of assay development. An ideal situation would be that the window between the substrate and product is greater than 100 mP. To maximize the detection power most substrates should be converted to products. When the window between the substrate and product is known the minimal conversion required for a reliable detection can be calculated. By the same rationale FP measurement is quite tolerant for minor errors. This is important for SNP typing because misincorporation does occur in SBE. For some detection format, such as mass spectrometry and capillary electrophoresis, 5-10% misincorporation is readily detectable and would affect scoring of genotypes. But the same percentage of misincorporation would not be sufficient to change the overall FP values of the sample, therefore, it should not change the outcome of genotypes.

Typically an FP assay is developed for a process with specific substrate and product, such as those developed for high throughput screen and clinical diagnostics [17,22]. Under this circumstance once an assay was successfully developed it could be applied to many different samples. But SNP genotyping is a different category. While SBE is a single reaction, the substrates (PCR products, extension primers and dye terminators) for each SNP are unique and

different, the products formed, therefore, are different for each SNP. If the primer sequences affect FP values of SBE products, different separation patterns should be expected for different SNPs. Indeed, somewhat different separation patterns, meaning the distances that separate different genotype groups, have been widely observed by several groups that have used the FP-TDI assay extensively. The data presented in Figure 2 confirm the difference from a different angle: For the same SNP separation pattern changes when different dye-terminators are used. It is important to know that both the primer sequences and dye terminators influence the separation pattern, it is equally important to know that the alternation of separation pattern does not change the outcome of genotyping results for most SNPs. For some SNPs that fail the assay, changing the dye-terminators can be an effective strategy to rescue them.

FP-TDI assay is based on SBE, like many other methods that are based on the same biochemistry its protocol includes PCR, PCR product clean-up and SBE. Because of its unique FP detection format FP-TDI has some features that users who are familiar with other SBE based methods tend to ignore. These are the characteristics of homogeneous, quantitative and collective measurements. The quantitative nature implies that FP-TDI assay is sensitive to the amount of PCR products and the concentration of dye-ddNTPs. Despite the fact that FP-TDI can work with a wide range of amount of PCR products and dye-ddNTPs, severe deviation from optimal conditions can cause deteriorative results. The homogeneous and collective features indicate that the assay does not discriminate individual components in the system and its results reflect the status of the dominant species. This rule applies to not only the fluorescent labeled components (dye-terminators, intended and unintended extension products) but also to other factors (reaction volume, buffer viscosity, temperature etc.) that potentially alter the consistency of the system. Dominant components or factors dictate FP values. Under normal conditions, the dominant component(s) are extension products for the positive samples and dye-terminators for the negative samples. When other factors, either collectively or individually, outweigh the true effect of SBE reaction, the prerequisite of the assay is violated, therefore, FP values observed do not reflect the true status of the dye-terminators or extension products any more. FP values read under these conditions are bias and misleading. Genotypes based on these FP values will be spurious. So it is critical that the assay is performed under constant and consistent conditions.

It has been noticed that several hybridization based methods do not produce robust results [30,33], the net changes between positive and negative are relatively small (20-50 mP). In contrast, single stranded DNA based assays, such as FP-TaqMan, FP-Invader and FP-TDI, produced much more significant net changes (50-150 mP). It is very interesting that the net changes between these two groups of assays vary so much. There are two main differences between the groups: one is that the fluorescence dyes used are different, the other is that one group's products are single stranded while the other group's products are double stranded. If the use of different fluorophores is largely responsible for the difference then hybridization based methods could benefit greatly by changing the labeling fluorophores; or if the configurations of the products are largely responsible then DNA hybridization may not be a robust test for FP. Many factors influence the FP readings of labeled DNA molecules, the most obvious one is the sequences of the DNAs. This effect can be direct or indirect. For example, we know DNA sequences affect their folding (configuration) in solution, we also know the sequences affect hybridization behavior. Both of them can affect FP values. But we do not know if the fluorophores interact directly with the sequences. To improve the assays that use FP to detect DNAs it is necessary to further investigate these questions and understand the underlying mechanisms.

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