# Application of Fluorescence Polarization to Enzyme Assays and Single Nucleotide Polymorphism Genotyping: Some Recent Developments

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**Abstract:** This article describes some recent developments in the field of fluorescence polarization (FP) as applied to enzyme assays and single nucleotide polymorphism (SNP) genotyping. First, we present our recent progress on the application of fluorescence polarization to high throughput screening (HTS). We show how the use of a 2-thiopyridinone-based, mixed disulfide biotinylation reagent can shorten the assay time of our recently reported kinase assay method based on thiophosphorylation and biotinylation from several hours to a few minutes. We also summarize our recent findings on a new approach for HTS of kinases, proteases and phosphatases based on the use of a cationic poly-amino acid such as polyarginine. We show how the careful selection of the polyarginine concentration and the ionic strength of the solution during the FP measurement allow one to expand the range of substrates that can be assayed. Both of these methods are valuable additions to the existing techniques for HTS. Most importantly, both of these methods can be applied to the assay of kinases without the need for any antibodies. In the area of genomics, we present some results from our studies on a new single nucleotide polymorphism typing approach based on the polymerase catalyzed extension of 3' fluorescein labeled primers. Contrary to our initial expectations, we observed that the enzymatic extension of these primers results in a significant decrease of the fluorescence polarization value. Possible explanations of this phenomenon are discussed.

**Keywords:** Fluorescence polarization, enzyme assays, thiophosphorylation, high throughput screening, SNP genotyping, polymerase extension.

## 1. INTRODUCTION

Fluorescence polarization is a detection technique that is finding increasing application in areas such as high-throughput screening and genomics. In fluorescence polarization experiments, the samples are excited with planar polarized light and the emitted light is detected in two planes, parallel and perpendicular to the excitation light [1]. Fluorescence polarization is particularly well suited to study biomolecular binding reactions, as it is very sensitive to changes in molecular size. One of the main advantages of this technique is that it does not require any separation of labeled molecules, such as the substrate and product of an enzymatic reaction. An excellent introduction to fluorescence polarization in high throughput screening has recently been published [2].

This article is divided in two major sections. In the first section, we focus on recently developed methods for detecting enzymatic activity by fluorescence polarization for the purposes of high throughput screening. Following a brief review of published approaches, we describe in more detail some of our recent advances in this area. In the second section of this article, we turn our attention to recently developed fluorescence polarization based techniques for single nucleotide polymorphism genotyping. Here, we present the results from our experiments directed towards the development of a novel technique for SNP genotyping based on enzymatic extension of 3' fluorescently labeled primers. It should be noted that this article is not meant to be a comprehensive review of these applications of fluorescence polarization. The present issue of this Journal contains additional important articles on these topics.

## 2. ENZYMATIC ASSAYS WITH FLUORESCENCE POLARIZATION READOUT

Enzymes such as proteases, kinases and phosphatases are important targets in high throughput screening for the identification of new inhibitory compounds and the development of new drugs. Proteases and phosphatases are usually assayed using fluorogenic substrates, i.e. molecules whose fluorescent properties change significantly upon enzymatic modification. As an example, specific peptide sequences can be modified at their C-termini by conversion to the corresponding 7-amino-4-methyl coumarin (AMC) amides. These molecules are non-fluorescent, but proteolytic cleavage at the C-terminus releases highly fluorescent free AMC. Because these assays are relatively straightforward, there has been little need to develop methods based on fluorescence polarization. In one of the few such methods published [3], a peptide substrate is fluorescently labeled at one end and biotinylated at the other end. Binding of streptavidin to such a doubly labeled peptide can be detected by a significant increase in the fluorescence polarization signal. The enzymatic cleavage of this peptide results in the separation of the fluorophore from the biotin, and no increase in the fluorescence polarization upon addition of streptavidin. The main advantage of the method is the simplified substrate design compared to the design of suitable fluorogenic energy transfer (FRET) substrates carrying both a fluorophore and a quencher moiety. The

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method has recently been used to screen natural products for inhibitors of plasmepsin II, a malarial aspartic protease [4]. An alternative, general protease assay based on fluorescence polarization has been reported [5]. The method is based on a fluorescently labeled generic protease substrate such as casein. Proteolytic cleavage of this protein releases small, fluorescently labeled peptide fragments and this process can be conveniently followed by fluorescence polarization.

Protein kinases present special challenges for assay development because the transfer of a phosphate group from ATP to the serine, threonine or tyrosine residue of a fluorescently labeled phosphate acceptor peptide in general does not result in any change in the fluorescent intensity of the fluorophore. Thus, simple fluorogenic methods have been unavailable. Recently, assay methods have been reported that measure the kinase activity indirectly, by following the ATP consumption or the ADP generation using additional enzymes such as luciferase and pyruvate kinase. However, at the present time methods based on fluorescence polarization appear to be among the most widely used techniques for screening protein kinases.

Currently, the most widely used method for assaying kinase activity using fluorescence polarization is the competitive immunoassay, originally described for tyrosine kinases [6]. In this assay, a specific peptide substrate is enzymatically phosphorylated by the kinase of interest. Then, a mixture of a fluorescently labeled phosphopeptide (referred to as tracer) and an antibody that specifically binds to this phosphopeptide is added to the reaction solution. The fluorescently labeled tracer and the enzymatically generated, unlabeled phosphopeptide compete for binding to the antibody, which is present at a limiting concentration. The binding of the antibody to the tracer is detected by fluorescence polarization. A high fluorescence polarization signal indicates that most of the fluorescent tracer is bound to the antibody, thus, no or very little unlabeled phosphopeptide has been enzymatically generated. On the other hand, a low fluorescence polarization signal is a sign that a significant concentration of unlabeled phosphopeptide has been enzymatically generated and is successfully competing for antibody binding. In high throughput screening, therefore, the presence of inhibitory compounds is revealed by wells showing a higher fluorescence polarization value than wells not containing inhibitory compounds.

Over the last few years, this approach has been used widely in high throughput screening. However, it has several limitations. The main one is the fact that while universally applicable, high affinity antiphosphotyrosine antibodies are commercially available, the same is not true for antiphosphoserine and antiphosphothreonine antibodies. The latter have to be generated on a case by case basis, using specific peptide sequences [7]. The second limitation of the method has to do with its format, that of a competitive immunoassay. It has been reported by several groups that for accurate $IC_{50}$ determination one has to convert the observed fluorescence polarization values (in mP units) to percentage of substrate converted into product. This requires the generation of standard curves of mP values observed vs. fraction of product formed (see, for example, the presentation of Jane Beebe *et al.* of the Panvera Corp., available at www.panvera.com). The need for such a conversion of the data may at least partially explain the discrepancies observed between different tyrosine kinase screening formats [8].

In our search for new, antibody-free kinase assay formats, we have developed a method where fluorescently labeled peptide substrates are enzymatically phosphorylated by the

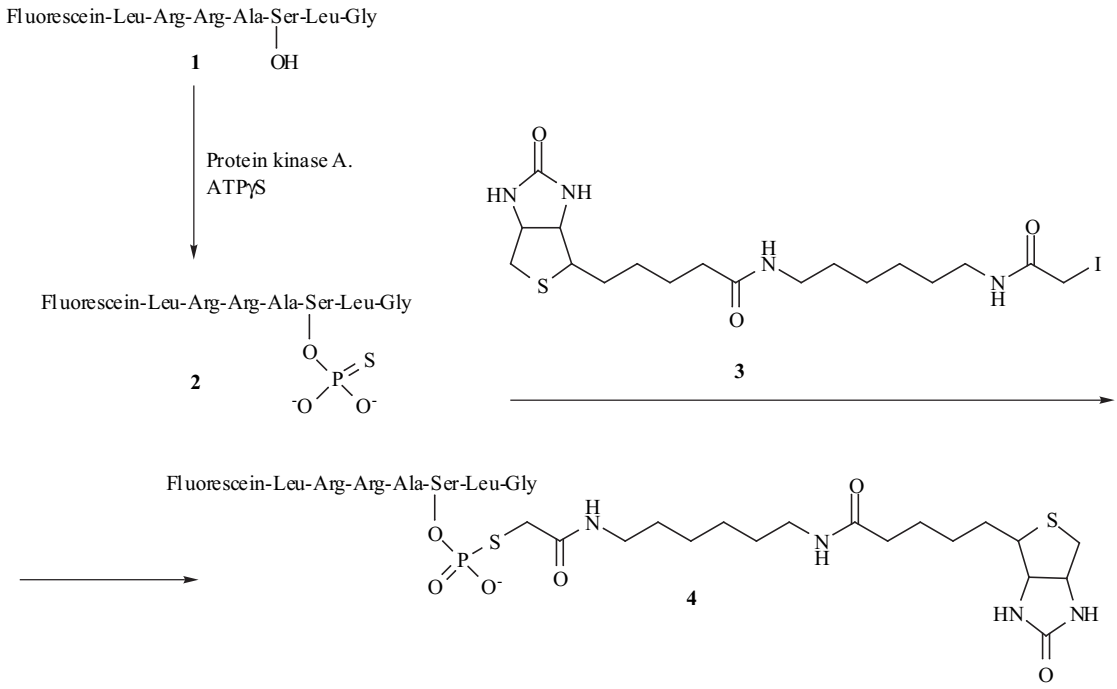


**Fig. (1).** General scheme of our original kinase assay based on thiophosphorylation and biotinylation [9]. A fluorescein-labeled peptide **1** is used as a substrate for protein kinase A (PKA). In the presence of ATPγS, the thiophosphorylated product **2** is formed, which is then reacted with the iodoacetamide derivative of biotin **3**. The formation of the biotinylated, fluorescein-labeled product **4** is detected by fluorescence polarization after addition of streptavidin.

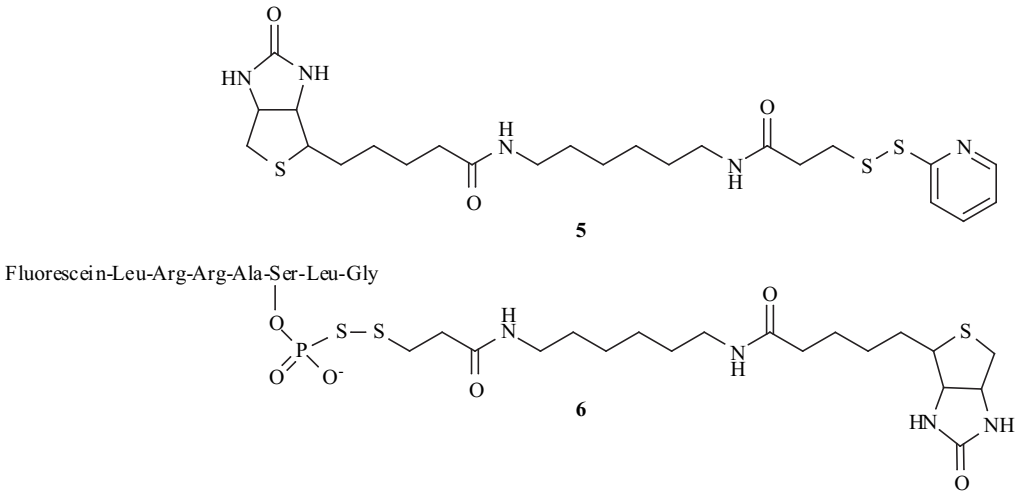


**Fig. (2).** Structure of N-((6-[biotinamido])hexyl)-3'-(2'-pyridyldithio)propionamide (biotin-HPDP, compound **5**, available from Pierce), used to rapidly biotinylate the thiophosphorylated product **2** at pH 4.2 in our modified kinase assay. The modification reaction results in the formation of compound **6**.

kinase of interest in the presence of ATPγS instead of the natural substrate ATP [9]. The use of this ATP analog results in the generation of a thiophosphorylated rather than a phosphorylated product. The sulfur atom of the thiophosphate group allows the further specific chemical modification of the thiophosphorylated product. In the originally published version of this assay, shown in Fig. (**1**), we reacted the thiophosphorylated product with an iodoacetamide derivative of biotin. This reaction results in the formation of a fluorescently labeled, biotinylated peptide product. Its formation can be conveniently detected by measuring the fluorescence polarization of the fluorescent dye attached to the peptide molecule following the addition of streptavidin (or one of its analogs, such as neutravidin and avidin). Binding of streptavidin to the biotin of the modified thiophosphorylated molecule results in a significant increase in the fluorescence polarization signal.

The main advantage of this method for measuring kinase activity is the fact that it does not require any antibodies. It is thus equally applicable to serine and threonine kinases as well as tyrosine kinases. ATPγS has been shown to be accepted as an ATP substitute by a very large number of kinases. To the best of our knowledge, there have been no

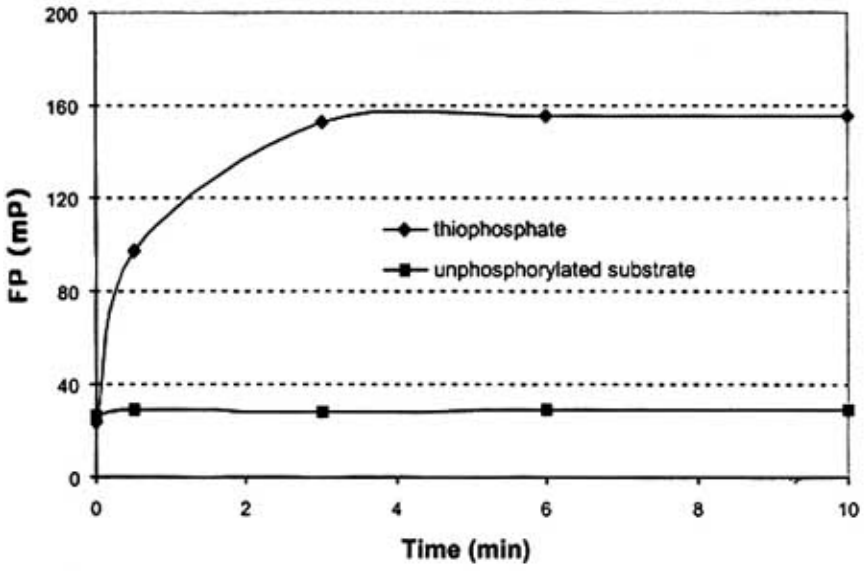


**Fig. (3).** Time course of the biotinylation reaction of the thiophosphorylated peptide **2** with biotin-HPDP **5** at pH 4.2. The unphosphorylated peptide **1** was similarly treated with biotin-HPDP as a negative control. Aliquots of the reaction mixtures were taken and mixed with an excess of the streptavidin analog neutravidin (Pierce) at pH 7.5. The fluorescence polarization values were recorded. Complete biotinylation of **2** is achieved after 3 minutes of incubation. No reaction occurs with the unphosphorylated peptide **1**, as expected.

published reports on kinases that do not work with this ATP analog. Being a fluorescence polarization based, mix-and-read method, it is extremely well suited for high throughput screening application. It should be noted that this method is not based on a competitive antibody displacement reaction, therefore, analysis of the data (*i.e.*, the conversion of mP units to fraction substrate converted) is much more straightforward and no generation of standard curves is required. We have described a simple approach to correct for fluorescence quenching upon streptavidin binding [9]. On the negative side, the original version of our method required a rather long (up to six hours) incubation with the biotin iodoacetamide derivative to achieve complete sulfur modification. Here, we wish to report a significant improvement to our original method that shortens the required incubation time of the sulfur modification reaction to a few minutes. We have presented this version of our assay in preliminary form at the Screentech 2002 conference in San Diego, CA.

In our modified version of the assay, we have replaced the iodoacetamide derivative of biotin with a biotin derivative containing a mixed disulfide group with 2-thiopyridine (biotin-HPDP, compound 5 in Fig. (**2**)). Our use of this compound was prompted by a paper by Wu *et al.* [10], who demonstrated that the reaction rate of thiophosphates such as guanosine 5'-thiophosphate with mixed disulfide reagents containing a 2-thiopyridine leaving group is accelerated several orders of magnitude when carried out at a relatively low pH (approx. 4.5-5.0) compared to neutral pH. The reason for this rate acceleration is the increased protonation of the pyridine ring nitrogen at the lower pH. When we tested the modification reaction of a thiophosphorylated peptide with the mixed disulfide-based biotinylation reagent biotin-HPDP, we indeed observed that the reaction is complete within a few minutes when carried out at pH 4.2. The data from this experiment are shown in Fig. (**3**).

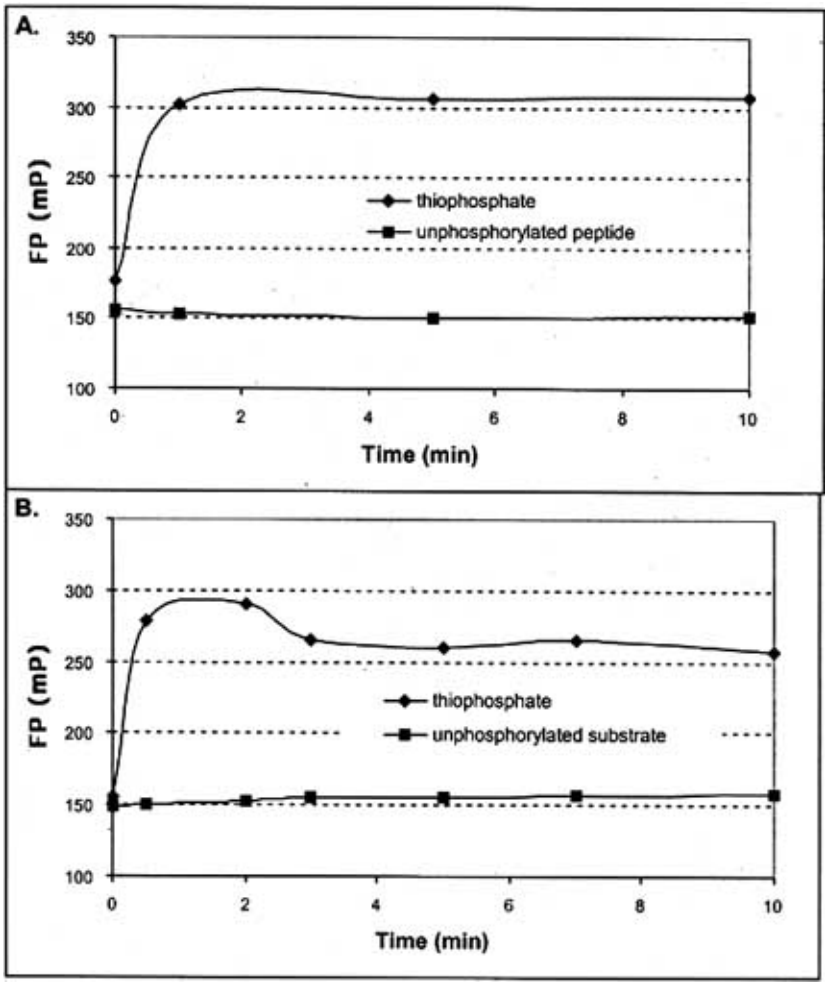


**Fig. (4).** Use of the pH-insensitive dye Alexa 647 as the fluorescent label: comparison of FP results at pH 4.2 (A) and pH 7.5 (B). The peptide used was an Alexa 647 modified analog of the PKA substrate **1**. Following complete enzymatic thiophosphorylation, the product was biotinylated with biotin-HPDP at pH 4.2. Then, neutravidin binding and FP measurements were carried out either at pH 4.2 (A) or 7.5 (B). As a negative control, an unphosphorylated version of the same peptide was used.

While we were gratified to see that the time required for the sulfur modification reaction can be shortened from a few hours to a few minutes, one problem remained. In our improved scheme the sulfur modification reaction is carried out at a pH value where the fluorescence of fluorescein, the dye that we typically use to label the peptide substrates, is significantly quenched (the pK of fluorescein is about 6.4). Thus, following the sulfur modification reaction, the addition of streptavidin had to be accompanied by the addition of a well-buffered solution of neutral or higher pH. Overall then, two pH changes were required: one to bring the pH down from the optimal value for the kinase reaction (approx. 7.0 to 7.5) to approx. 4.5, the optimal value for the sulfur conjugation chemistry; and a second one to bring the pH up again so that the fluorescein could be optimally measured. In order to simplify the method, we evaluated the use of pH insensitive dyes such as BODIPY-fluorescein and Alexa 647 (both from Molecular Probes) as fluorescent labels. The use of these fluorophores would obviate the need for an additional pH adjustment step following the sulfur modification reaction. We assumed that the binding of neutravidin to the biotinylated kinase reaction product would proceed equally well at pH 4.2 as at neutral pH. The results from such an experiment using Alexa 647 as the fluorescent label are shown in Fig. (**4**). As can be seen, the use of a pH insensitive fluorophore allows both the neutravidin binding and the fluorescence polarization measurement to be carried out at the low pH of 4.2, thus simplifying the overall assay procedure. Interestingly, in this experiment, we observed a bigger dynamic range (*i.e.*, difference between the FP signals of the fully thiophosphorylated and the unphosphorylated molecules) at the lower pH value (approx. 150 mP at pH 4.2, *vs.* approx. 100 mP at pH 7.5). We do not have an explanation for this increased dynamic range at low pH, but we obtained similar results with another pH insensitive fluorescent dye, BODIPY-fluorescein (data not shown). The only remaining drawback of this new kinase assay method is the fact that the sulfur modification reaction results in the coupling of biotin through a disulfide bond. The sensitivity of this bond to reducing reagents such as DTT precludes their addition to the assay buffer.

Another antibody-free kinase assay method recently developed by us is based on the use of polyarginine as a high molecular weight reagent that binds the substrate and product of a kinase reaction with different affinities and thus allows their discrimination by fluorescence polarization [11, 12]. Every phosphorylation reaction involves the transfer of

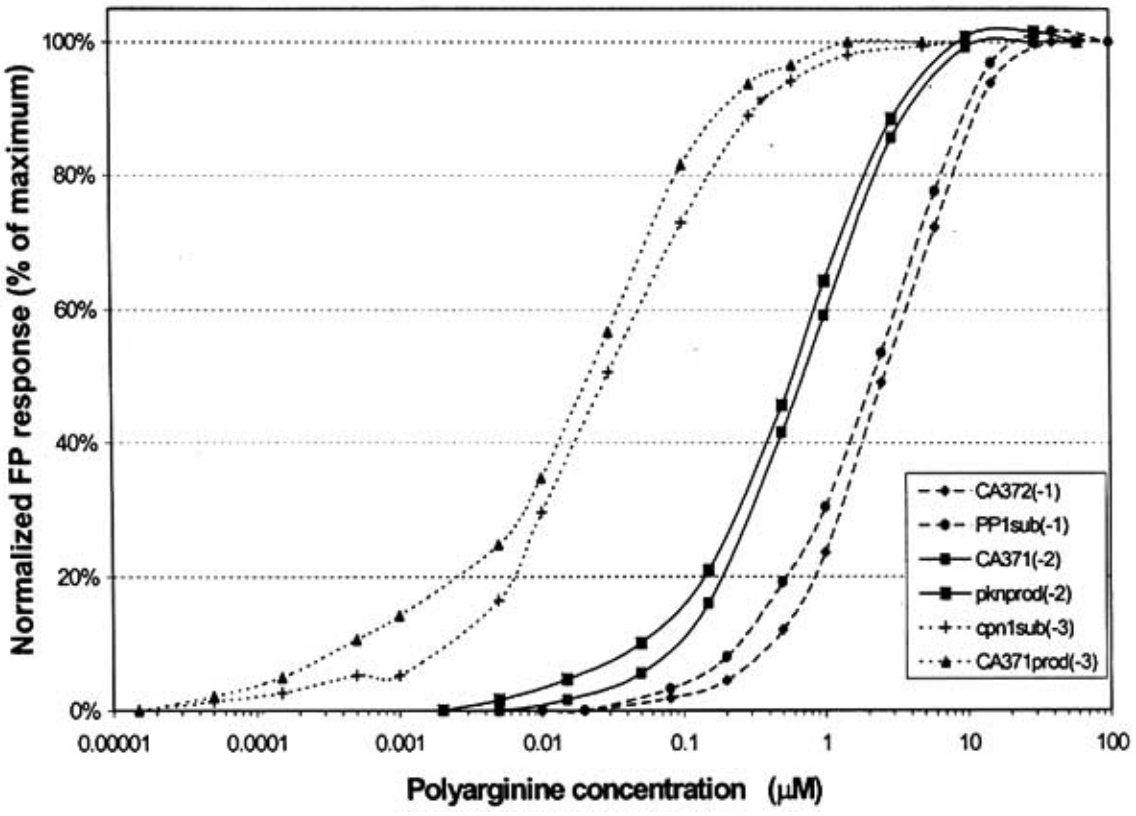


**Fig. (5).** Polyarginine titrations of six fluorescein labeled peptides with different net charges. All peptides are labeled at the N-terminus with fluorescein. The sequences of the individual peptides are (net charge at pH 7.5 given in brackets; p denotes a phosphate group): CA372 (minus 1), TPFSALK-$CONH_2$; PP1sub (minus 1), KR(pT)IRR-COOH; CA371 (minus 2), TPFSALQ-$CONH_2$; pknprod (minus 2), LRRA(pS)LG-$CONH_2$; cpn1sub (minus 3), QQEVYGMMPRD-$CONH_2$; CA371 (minus 3), TPF-COOH. All peptides were used at 500 nM. The buffer used was 50 mM HEPES, pH 7.5. The magnitude of the FP signal responses (minimum to maximum) ranged from 112 mP (peptide CA372) to 152 mP (peptide CA371).

a phosphate group from ATP to the hydroxyl group of a serine, threonine or tyrosine residue of an acceptor peptide. Thus, at neutral pH or higher, the phosphorylation products will carry two additional negative charges compared to the substrate molecules. Substrate and product can then be distinguished by methods such as gel or capillary electrophoresis and ion exchange chromatography, which are based on differences in the mass-to-charge ratios or simply the overall net charges, respectively. We found that polyarginine, a relatively large (approx. molecular weight of 100,000), positively charged molecule, binds the more highly negatively charged phosphorylation products with higher affinity than the unphosphorylated substrates. Initially [11], our choice of substrates was limited to molecules that had an overall charge (including that of the fluorescent label) close to zero, because we expected that in this situation, with the phosphorylated products having a charge of minus two, we would see the biggest differences in polyargininine binding affinities. Later on, we expanded the range of substrates to include much more highly negatively charged molecules, and found that excellent fine-tuning of the polyarginine binding affinity can be achieved by optimization of the ionic strength as well as the polyarginine concentration [12]. In this respect, Fig. (**5**) is instructive. It shows the titrations of six different, fluorescently labeled peptides with polyarginine, measured by fluorescence polarization. The six peptides include both phosphorylated and unphosphorylated sequences, and their net charges range from minus one to minus three. As can be seen, the polyarginine binding affinities are clearly determined by the overall net charges of the molecules, with the six titration curves clustering in three distinct groups based on the respective charges. From these titration curves, one can estimate the approximate $EC_{50}$ values for polyarginine binding for peptides of different charges to be, under these conditions, approximately 0.03 μM, 0.6 μM and 3 μM, for peptides of net charges of minus three, minus two, and minus one, respectively. The presence of a phosphate group does not confer additional binding energy. In other words, if two peptides have the same net charge, they bind to polyarginine with very similar affinities, even if one is phosphorylated and the other is not. It should be kept in mind when analyzing these titration results that multiple peptide molecules can bind simultaneously to polyarginine, and the binding curves can therefore not be analyzed simply in terms of a 1:1 binding stoichiometry. All of the titration curves shown in Fig. (**5**) can be shifted to the right (i.e., the affinities decrease) by increasing the ionic strength of the solution. This allowed us to successfully extend the use of this method to substrate/product pairs having charges of minus seven and minus five, respectively [12]. Moreover, we have demonstrated that the method is applicable to enzymes other than kinases and phosphatases, provided that the enzymatic reaction is accompanied by a change of the net charge of the fluorescently labeled peptide.

The polyarginine-based method for kinase assays has several advantages. First, it does not require any antibodies and thus is applicable to any kinase. Second, by adjustments in the polyarginine concentration and ionic strength of the solution, the range of usable peptide substrate molecules (in terms of their net charge) can be significantly expanded. Finally, we have demonstrated that the method is useful for enzymes other than kinases, such as phosphatases and proteases. We have discussed some of the limitations of the method in our previous publications [11, 12]. The method is easily miniaturizable and we believe that it will find its place among the many methods available for high throughput screening.

## 3. STUDIES TOWARDS A NEW METHOD FOR SINGLE NUCLEOTIDE POLYMORPHISM GENOTYPING BASED ON POLYMERASE EXTENSION OF 3' FLUORESCENTLY LABELED PRIMERS

Numerous methods for single nucleotide polymorphism genotyping have been described and there are several excellent reviews on the subject. Some of the more recent reviews are those of Kwok [13], Shi [14], and Landegren *et al.* [15]. Of particular interest to the readers of this article are those methods based on fluorescence polarization. In recent years, some of the most popular biochemical approaches for single nucleotide polymorphism genotyping have been modified and adapted to fluorescence polarization readouts. These include the popular primer extension method [16], the TaqMan [17] and the Invader assays [18]. A recent review by Kwok summarizes the performance of these assays by fluorescence polarization [19].

We have recently developed and published hybridization-based SNP genotyping approaches with fluorescence polarization readout that utilize nucleic acid analogs such as peptide nucleic acids (PNA) and locked nucleic acids (LNA) as probes [20, 21]. In the first of these methods, relatively short (9-13 bases) PNA probes labeled with either fluorescein or BODIPY-fluorescein were allowed to hybridize to single-stranded DNA targets in solution in the presence of polylysine [20]. This cationic polymer binds to the highly negatively charged DNA molecules with high affinity, but the uncharged PNA probes bind to the polylysine only if they are hybridized to their DNA targets. The binding of the fluorescently labeled probes to the polylysine through the DNA "bridge" can easily be detected by measuring the increase of the FP signal of the dye used to label the PNA probes. In this method, polylysine essentially acts as a fluorescence polarization signal amplifier: upon hybridization in its presence, the FP values of the PNA probes typically increased by 60-130 mP units. In its absence, on the other hand, the increases were much smaller, between 20 and 40 mP. The latter values are similar to the increases in FP observed by other authors upon hybridizing labeled DNA probes to DNA targets [22].

In another method recently developed by us, we used very short, rhodamine or hexachlorofluorescein labeled LNA probes and observed significant increases in FP upon their hybridization to DNA targets [21]. In some cases, the FP values increased by more than 140 mP units. Both PNA and LNA probes have been reported to better discriminate against targets that contain a single mismatched nucleotide compared to a fully matching target, and our results fully corroborated these observations and allowed us to achieve excellent discrimination between targets of different genotypes.

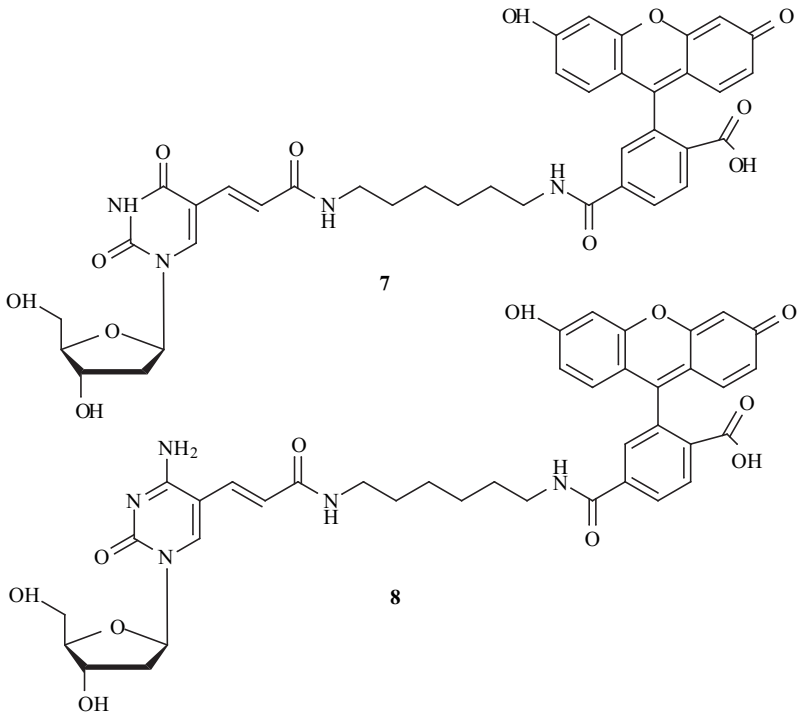

**Fig. (6).** Structures of the fluorescein-labeled thymidine **7** and deoxy-cytosine **8** introduced at the 3' ends of the genotyping primers. Molecule **7** was introduced into the synthetic DNA by initiating the synthesis from a fluorescein-T CPG. For labeling with **8**, the synthesis was initiated from amino-dC CPG and the fluorescein was attached post-synthetically.

The above-described methods based on the use of PNA or LNA probes discriminated between wild type and mutant target based on hybridization. We were interested in exploring some enzymatic genotyping approaches, such as those based on polymerases, as they can potentially provide a much higher level of discrimination than approaches based on hybridization alone [23]. We decided to explore a scheme based on the use of primers labeled at their 3' ends with a fluorescent dye. The structures of the two fluorescein labeled nucleosides that we used, a fluorescein-labeled thymidine 7 and a fluorescein-labeled 2'-deoxycytosine 8 are shown in Fig. (**6**). To introduce the former residue at the 3' end of the primer molecules, their synthesis was initiated from 3'-fluorescein-T CPG, whereas for the latter the synthesis started from amino-C6-dC CPG and the fluorescein was introduced post-synthetically by reacting the amino group with a fluorescein NHS ester. Both modified CPG materials are commercially available from Glen Research (Sterling, VA), and all primers were synthesized by Oligos, Etc. (Willsonville, OR). An important feature of the 3' labeled primers thus obtained is the fact that they have a free 3' hydroxyl group, which would allow their polymerase-catalyzed enzymatic extension. The sequences of some of the primers used in this study are shown in Figure (**7**).

| Name | Sequence (5'-3') |
|---|---|
| 425T | CTGCCATTATGTTAGGCATTA(Tfl) |
| 425C | CTGCCATTATGTTAGGCATTA(Cfl) |
| 309 | TTTGGCATGTAATGCCTAACATAATGGCAG |
| 310 | TTTGGCATATAATGCCTAACATAATGGCAG |
| 311 | ACGGTGGTCGCCTGGTCCACGTGGAAGTCCT |
| 348 | CTGCCATTATGTTAGGCATTA(Tfl)ATGCCAAAA |
| 5A | TAAATGAAACAAAACAAAATAAAAA(Tfl) |
| 6A | TAAATGAAACAAAACAAAATAAAAAA(Tfl) |

**Fig. (7).** Sequences of some of the synthetic DNA molecules used in this study. All sequences are listed in the 5' to 3' direction. The fluorescein labeled T and dC nucleosides are denoted (Tfl) and (Cfl). For the synthesis of 348, the phosphoramidite version of compound **7** was used.

Our original idea for a SNP genotyping approach based on the use of such 3' labeled primers is shown in Fig. (**8**). The primers were designed such that, upon hybridization to the DNA targets, their fluorescein labeled 3' ends would be placed exactly opposite the polymorphic site of the target. Thus, the resulting primer/DNA hybrids would either be fully complementary or there would be a single mismatch at the 3' end of the primer. We expected to be able to register the hybridization event by an increase in the fluorescence polarization signal over that of the free primers. Then, in the presence of dNTPs and a polymerase with proofreading (i.e., 3'-5' exonuclease) activity, we expected the fully matched primers to be enzymatically extended, whereas a mismatch would be corrected by the polymerase by removing the fluorescently labeled, mismatched nucleotide from the 3' end and then extending the resulting primer. We expected the extension reaction to result in little or no change in the fluorescence polarization signal, as the labeled base would remain part of the extension product, whereas in the case of a proofreading cleavage we expected to see a decrease in the fluorescence polarization as the relatively small, dye-labeled nucleoside is cleaved from the hybrid.

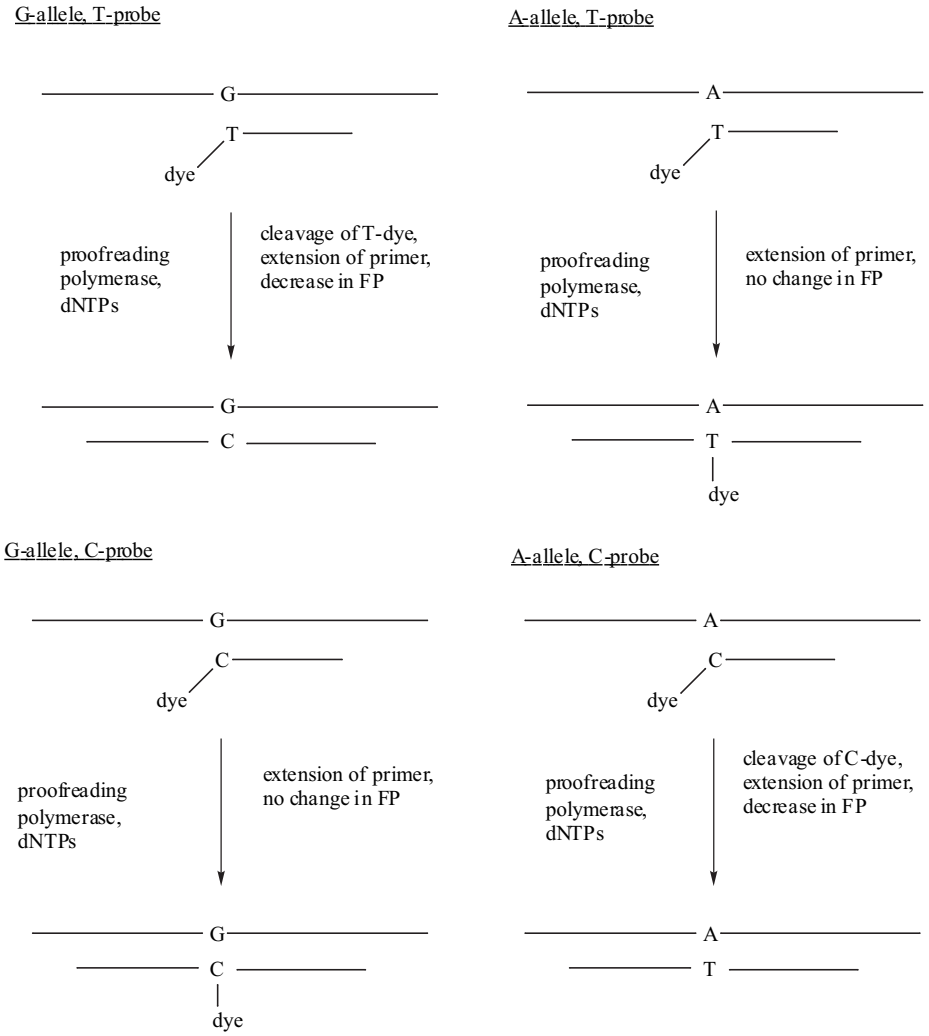


**Fig. (8).** Our original concept of a SNP genotyping method using a proofreading polymerase and 3' fluorescently labeled primers with fluorescence polarization detection. In this hypothetical case, the target contains a G/A polymorphic site and the two genotyping primers contain a labeled T or dC at their 3' ends. A fully matched hybrid was expected to be enzymatically extended, resulting in the incorporation of the dye into the extension product and no significant change in the FP value. A mismatch was expected to be corrected by the proofreading polymerase employed, resulting in cleavage of the labeled 3' base. This would result in the formation of a non-labeled extension product and a decrease in FP.

Our initial experiments with synthetic target DNA molecules and enzymes such as the Klenow polymerase, an enzyme with a 3'-5' exonuclease (proofreading) activity produced unexpected results. We observed a significant decrease in the FP signal in the case of a fully matched primer/target hybrid, and no change in the case of a mismatched hybrid, i.e., the exact opposite outcome of our expectation. The results from such an experiment are shown in Fig. (**9**). Similar results were seen with other primer/target combinations (data not shown). Using ultrafiltration, we failed to detect any fluorescently labeled, low molecular weight species that might have resulted from any exonuclease-catalyzed degradation of the labeled primers. Identical results were seen with the exo-minus version of the Klenow polymerase, an enzyme lacking the 3'-5' exonuclease activity. At this point, we became aware of a report showing that the presence of 3' fluorescently labeled nucleotides similar in structure to the ones we used inhibits the 3'-5' exonuclease activity of polymerases such as Klenow [24]. These authors exploited this fact to successfully label double stranded PCR products at their 3' ends by performing a polymerase catalyzed nucleotide exchange reaction in the presence of dye-labeled dNTPs. Clearly, then, our originally envisioned scheme as shown in Fig. (**8**) would have to be modified.

We then concluded that our observations are in agreement with the following working hypothesis. In the case of a fully matching primer/target hybrid, the 3' fluorescein labeled primer is successfully extended by the polymerase, as expected, but this extension results in a significant decrease of the fluorescence polarization signal of the fluorescein. On the other hand, in the case of a primer/target hybrid with a mismatch at the 3' end of the primer, no polymerase extension takes place, but the labeled mismatched nucleotide cannot be enzymatically cleaved off, thus resulting in no detectable change in the fluorescence polarization value. It is interesting to note that in some instances of mismatched primer/target hybrids we saw evidence of mismatch extension. For this to occur, the concentration of the polymerase had to be approximately ten-fold higher than that

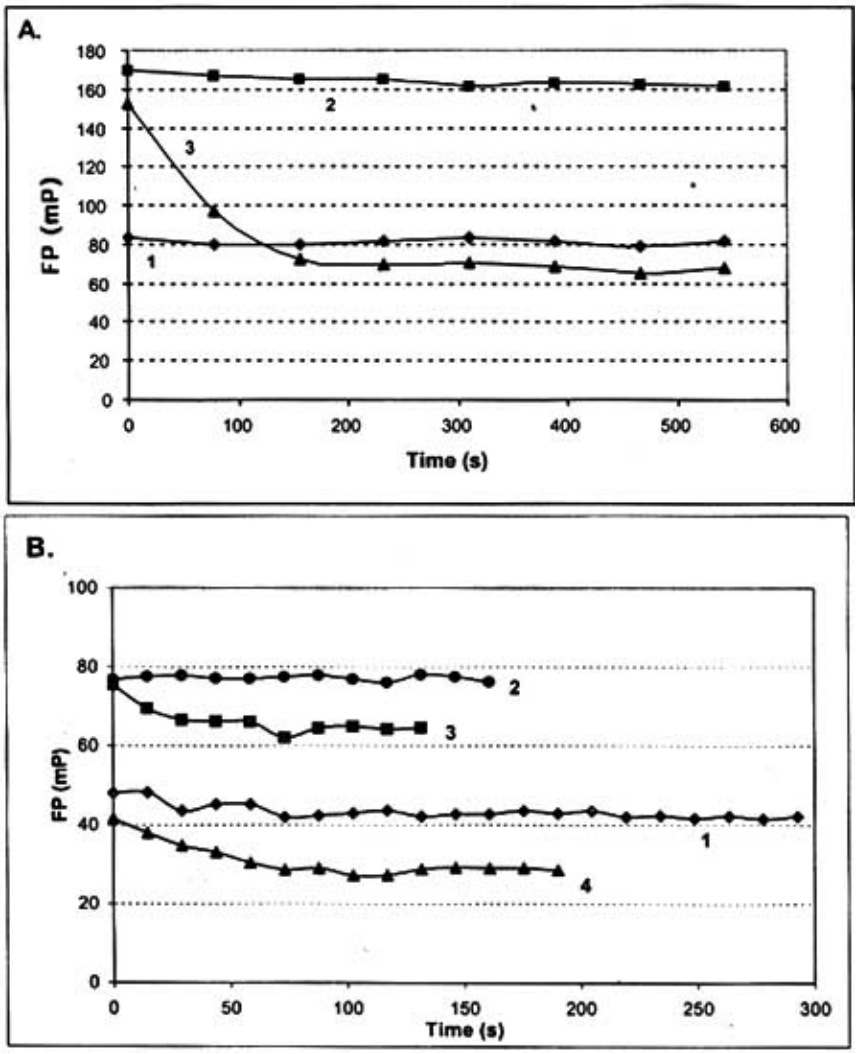


**Fig. (9).** Primer extension experiments with the 3' fluorescein labeled primers 425T (panel A) and 425C (panel B). The primers were hybridized to the synthetic targets 309 and 310, in the presence of 25 μM dNTPs, and Klenow polymerase was added to 0.01 u/μl. In panel A, the individual traces are: 1, 425T free probe; 2, 425T/309 hybrid (mismatched) plus Klenow; and 3, 425T/310 hybrid (matched) plus Klenow. In panel B: 1, 425C free probe; 2, 425C/310 hybrid (mismatched) plus Klenow; 3, 425C/309 hybrid (matched) without Klenow; and 4, 425C/309 hybrid (matched) plus Klenow. Note that the extension of primer 425C is much faster than that of 425T when both are hybridized to their fully matched targets. In both cases, the final FP values of the extended primers are lower than those of the free, unhybridized primers.

for the extension of a perfect match (0.01 units/μl of Klenow were sufficient to give a very high rate of extension of matched hybrids, whereas mismatch extension was only detectable at 0.1u/μl).

To further test the validity of this hypothesis, we looked more carefully at the FP changes that occur upon hybridizing either 3'-terminally or internally labeled probes to their corresponding targets. We observed that the hybridization of a 3' fluorescein labeled probe such as 425T to its target DNA molecules 309 and 310 results in a significant increase in fluorescence polarization. On the other hand, the hybridization of probe 348, a DNA molecule internally labeled with fluorescein, to the same target DNAs results in a decrease in the fluorescence polarization signal compared to that of the free probe. These results are shown in Fig. (**10**). It should be noted that the sequence of probe 348 is exactly the one expected to result from the enzymatic extension of the 3' fluorescein labeled primer 425T when hybridized to the same DNA target molecule 310.

A further confirmation of the validity of this hypothesis comes from a recently published study of Nazarenko *et al.* [25]. These authors have studied the changes in fluorescence intensity observed upon hybridizing 3', 5', or internally fluorescein labeled oligodeoxynucleotide probes to target DNA molecules. The authors observed significant changes in the fluorescence intensity, both increases and decreases, depending on the exact position of the fluorescein within the sequence of the probe. The most likely explanation of these findings is a hybridization-induced change in the secondary structure of the labeled probes and, as a consequence, a change in the interactions of the fluorescein molecule with DNA bases within the probe and target DNA molecules. Of

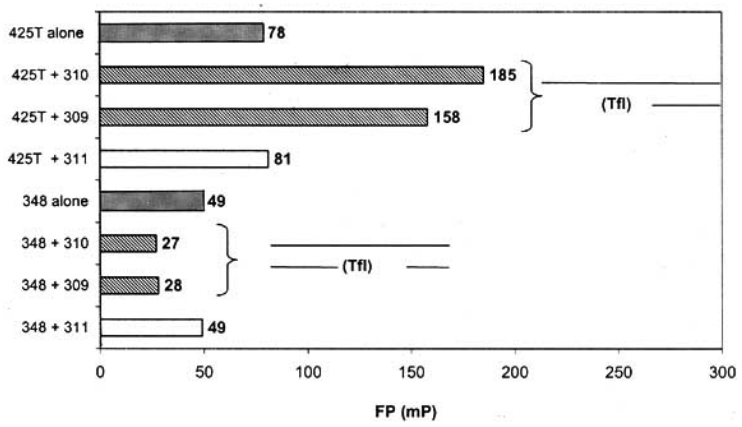


**Fig. (10).** Comparison of FP changes upon hybridization of the 3' fluorescein-labeled probe, 425T, and the internally labeled probe 348 to the same DNA targets, 309 and 310. The structures of the hybrids are shown schematically, (Tfl) denotes a fluorescein labeled T residue. For 425T, the FP signal increases significantly upon hybridization, whereas for 348 it decreases. The target 311 is a non-complementary, negative control DNA molecule. The measured FP values are shown next to the bars.

particular relevance to our findings described in the present paper is the observation of Nazarenko *et al.* of the changes in fluorescence polarization. These authors found that an increase in fluorescence intensity upon hybridization corresponds to a decrease in fluorescence polarization and vice versa. In our experiments, we have focused almost exclusively on measurements of the fluorescence polarization, but in those cases where we collected fluorescence intensity data we have made very similar observation. The ultimate reason for the observed changes in fluorescence intensity and fluorescence polarization must be sought in changes of the fluorescent dye environment and its interaction with various nucleobases, especially dG residue [26].

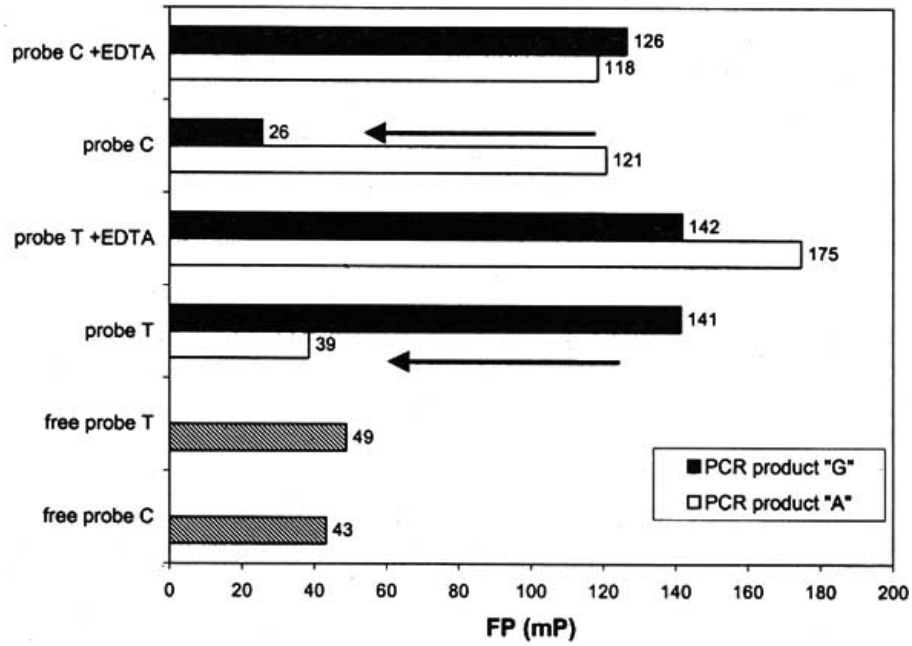


**Fig. (11).** Genotyping of two PCR products by enzymatic extension of probes 425C and 425T. Genomic DNA samples of known genotypes A and G at this locus were amplified by PCR, the resulting products rendered single stranded and hybridized to the 3' fluorescein labeled primers 425T and 425C, in the presence or absence of EDTA. The concentration of the primers was 50 nM, and that of the PCR products approximately 75 nM. The enzymatic extension was catalyzed by residual Taq polymerase activity. Successful primer extension resulted in a decrease of the FP signals in the case of perfectly matched primer/template hybrids in the absence of EDTA (indicated by the arrows). In the presence of EDTA or mismatched primer/template hybrids, no extension occurred and the FP values remained high.

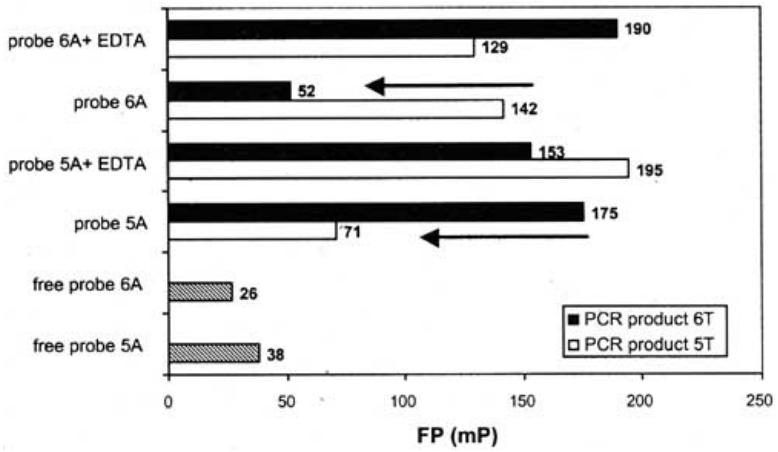


**Fig. (12).** Genotyping of the insertion/deletion polymorphism of locus "5A/6A" by the 3' primer extension method. Human DNA samples of known genotypes at this locus were PCR amplified and genotyped as described in the legend of Fig. (**11**), using 50 nM of the probes 5A and 6A. A decrease of the fluorescence polarization values was observed only in the case of the fully matched primer/target hybrids (indicated by the arrows).

Finally, we tested the possibility of performing SNP genotyping of PCR products obtained from genomic DNA. We tested two polymorphic loci, designated "425" and "5A/6A". The former one is a G/A polymorphism, whereas the latter is an insertion/deletion polymorphism where one of the genotypes contains a stretch of five consecutive dA residues and the other six such residues. To perform the experiment, human genomic DNA samples of known genotypes at the two loci were PCR amplified with one phosphorothioated and one non-modified primer, followed by treatment with T7 gene 6 exonuclease to generate single stranded products [27]. The exonuclease was heat inactivated, and the products were then allowed to hybridize, in separate reactions, to the different 3' fluorescein labeled primers, either in the presence or absence of EDTA. The experiments with EDTA served as hybridization controls, as no polymerase extension could occur under these conditions. We found that no addition of polymerase was required, as there was sufficient active Taq polymerase left from the PCR step to successfully extend the fully matched primers in the absence of EDTA. The results from these genotyping experiments are shown in Fig. (**11**) and Fig. (**12**). As can be seen, in the presence of EDTA probe/target hybridization occurred and was detected by an increase of the FP signals over those of the free probes. In the absence of EDTA, the residual Taq polymerase successfully extended the 3' ends of the fully matching primers, and this was detected by a decrease in the FP signals. In the case of probe/target hybrids containing a mismatch at the 3' end of the primers, no extension occurred and the FP signals remained high.

## 4. CONCLUSION

In this paper, we have presented some of the most recent results of our studies on the application of fluorescence polarization in high throughput screening and single nucleotide polymorphism genotyping. Our improvements in the approaches for antibody-independent kinase assays based on thiophosphorylation and biotinylation and on the use of cationic polyamino acids such as polyarginine should make them very attractive for real world applications. In the area of single nucleotide polymorphism genotyping, our findings on FP changes upon enzymatic extension of 3' fluorescein labeled primers are both novel and unexpected, and, with more studies, especially with primers containing 3' fluorescently labeled dA and dG residues, could be developed into a practically useful method that complements the plethora of already existing approaches.

## ABBREVIATIONS

| | | |
|---|---|---|
| SNP | = | Single-nucleotide polymorphism |
| FP | = | Fluorescence polarization |
| ATP | = | Adenosine triphosphate |
| dNTPs | = | Deoxyribonucleoside triphosphates |
| biotin-HPDP | = | N-((6-[Biotinamido])hexyl)-3'-(2'-pyridyldithio) propionamide. |

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