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# Role of Excess Inorganic Pyrophosphate in Primer-Extension Genotyping Assays

Ming Xiao,[1] Angie Phong,[1] Kristen L. Lum,[1] Richard A. Greene,[2] Philip R. Buzby,[2,3] and Pui-Yan Kwok[1,4,5]

[1]*Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California 94143-0130, USA;*
[2]*PerkinElmer Life and Analytical Sciences, Inc., Boston, Massachusetts 02118-2512, USA*

We have developed and genotyped >15,000 SNP assays by using a primer extension genotyping assay with fluorescence polarization (FP) detection. Although the 80% success rate of this assay is similar to those of other SNP genotyping assays, we wanted to determine the reasons for the failures and find ways to improve the assay. We observed that the failed assays fell into three general patterns: PCR failure, excess of heterozygous genotypes, and loss of FP signal for one of the dye labels. After analyzing several hundred failed assays, we concluded that 5% of the assays had PCR failure and had to be redesigned. We also discovered that the other two categories of failures were due to misincorporation of one of the dye-terminators during the primer extension reaction as a result of primer shortening with a reverse reaction involving inorganic pyrophosphate, and to the quenching of R110-terminator after its incorporation onto the SNP primer. The relatively slow incorporation of R110 acycloterminators by AcycloPol compounds the problem with the R110 label. In this report, we describe the source of the problems and simple ways to correct these problems by adding pyrophosphatase, using quenching as part of the analysis, and replacing R110 by Texas red as one of the dye labels. With this new protocol, we have achieved ~95% success rate in assay development without the need for optimization.

Single-nucleotide polymorphisms (SNPs) are fast becoming the most widely used genetic markers in the study of common human diseases, and a standard set of SNPs is being assembled to construct haplotype maps of the human genome by an international consortium (International HapMap Consortium 2003). Several companies have commercialized high-throughput SNP genotyping methods capable of generating millions of SNP genotypes per day (Patil et al. 2001; De La Vega et al. 2002; Oliphant et al. 2002; Hardenbol et al. 2003; Matsuzaki et al. 2004). These methods, although very efficient when thousands of markers are assayed together in multiplex, are not very flexible, and each multiplexed set of assays takes several weeks to months to develop. Once the initial genetic studies produce a small number of regions in the human genome with evidence of association, one has to develop a custom set of SNP genotyping assays to validate the initial associations and fine-map these regions with additional patients and controls. In this setting, SNP genotyping assays that can be developed and optimized quickly and cost-effectively are highly desirable. We have demonstrated previously that the primer extension assay based on template-directed dye-terminator incorporation (TDI) and fluorescence polarization (FP) detection, a homogeneous assay using unlabeled, unpurified PCR-grade primers, is one of the most versatile assays for studies that require fast assay development and low assay development cost.

The FP-TDI assay is based on two principles, namely, that DNA polymerase catalyzes the allele-specific incorporation of a dideoxynucleotide at the polymorphic site and that the FP of a fluorescent dye increases significantly when it changes from part of a small dye-terminator to part of an extended primer at the end of the genotyping reaction (Chen et al. 1999). One can therefore infer the genotype of a DNA sample by determining the FP values of fluorescent dye-terminators at the end of the assay. Recently, a commercial reagent kit based on the FP-TDI assay has been commercialized as AcycloPrime-FP by PerkinElmer Life Sciences. This reagent kit contains dye-labeled acyclic nucleoside triphosphates (acycloterminators), instead of dideoxynucleoside triphosphates, and the AcycloPol, a thermostable DNA polymerase that incorporates acycloterminators preferentially over dideoxyterminators. The kit contains four acycloterminators: Two are dye-labeled (with R110 and TAMRA, respectively), and two are unlabeled. Six AcycloPrime-FP kits are available for use to genotype the six possible allele combinations (http://lifesciences.perkinelmer.com/products/snp.asp).

We have developed and genotyped >15,000 SNP assays during the initial phase of the HapMap Project. Although the assay performed robustly and accurately for ~80% of the assays designed without any optimization, we observed that the failed assays fell into three general patterns: PCR failure, excess of heterozygous genotypes, and loss of FP signal for one of the dye labels. Although the success rate of this assay is similar to those of other SNP genotyping assays, we wanted to determine the reasons for the failures and to find ways to improve the assay. After analyzing several hundred failed assays, we concluded that 5% of the assays failed because of PCR failure. These assays had to be redesigned. As detailed below, we also discovered that the other two categories of failures were due to misincorporation of one of the acycloterminators during the primer extension reaction, resulting in an excess in heterozygous genotype calls, and due to the quenching of R110 after its incorporation onto the SNP primer, resulting in low R110 FP values even after incorporation. The relatively slow incorporation of R110 acycloterminators by AcycloPol adds to the problem with the R110 label. In this report, we describe the source of the problems and simple ways to

**Present addresses: [3]Helicos BioSciences Corporation, Cambridge, Massachusetts 02139, USA; [4]Department of Dermatology, University of California, San Francisco, San Francisco, CA 94143-0130, USA.**
**[5]Corresponding author**
**E-MAIL Kwok@itsa.ucsf.edu; FAX (415) 476-2283.**
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correct these problems. We have achieved ~95% success rate in assay development by implementing the new protocol.

## RESULTS

### Acycloterminator Misincorporation During the Primer Extension Reaction

As we were looking for patterns in the failed assays, we noticed that most of the assays in which one of the homozygous clusters was missing, although the number of heterozygotes was unusually high, shared one common feature: The SNP primer ends with the same base as one of the dye-labeled terminators. It occurred to us that because our homogeneous assay did not remove any of the inorganic pyrophosphates (PPi) generated in the PCR step, dye-terminator misincorporation due to pyrophosphorolysis could explain this observation (Duetcher and Kornberg 1969; Vander Horn et al. 1997; Liu and Sommer 2002).

Figure 1 presents the proposed mechanism of pyrophosphorolysis leading to dye-terminator misincorporation. In this example, an assay for a G/A SNP uses a SNP primer with a G at the 3′-end. Once the SNP primer is hybridized to the DNA template, the AcycloPol DNA polymerase extends the primer one base allele-specifically, incorporating the complementary terminator depending on the target sequence. In the presence of a large amount of PPi generated during PCR, the DNA polymerase will also catalyze the reverse reaction and cleave the 3′-bases off the SNP primer. The forward reaction is much more efficient than is the pyrophosphorolysis reaction, and four clear clusters are formed initially. However, once the forward reaction is completed and one of the dye-terminators is exhausted, the reverse reaction becomes dominant. Because the SNP primers are in large excess, the 3′-G base of the primer is cleaved in the pyrophosphorolysis reaction. The samples with the homozygous GG or heterozygous GA genotypes are not affected because the G-dye-terminators are already used up and the FP value for the G allele is already at its maximum. For the samples with the homozygous AA genotype, however, the now shortened primer can be extended by the AcycloPol DNA polymerase with a G dye-terminator, erroneously giving rise to heterozygous GA genotypes for the homozygous AA samples. This results in three clusters of data points: negative controls in the lower left corner because there are no templates for either the forward or reverse reaction to occur, homozygous GG samples in the lower right corner where the G allele FP values are high but the A allele FP values are low, and both the homozygous AA and heterozygous GA samples in the upper right corner where FP values for both G

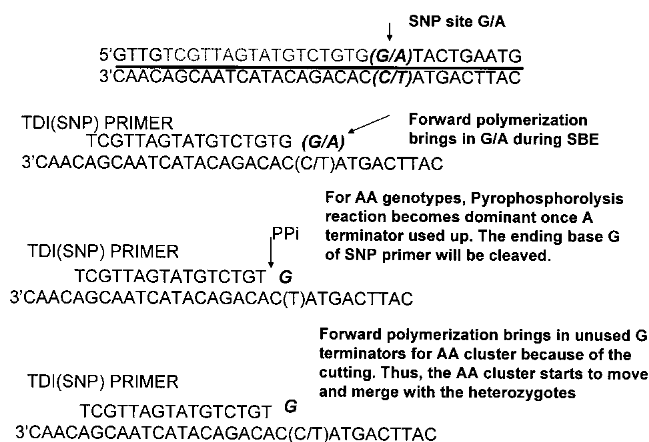


**Figure 1** Proposed mechanism for acycloterminator misincorporation.

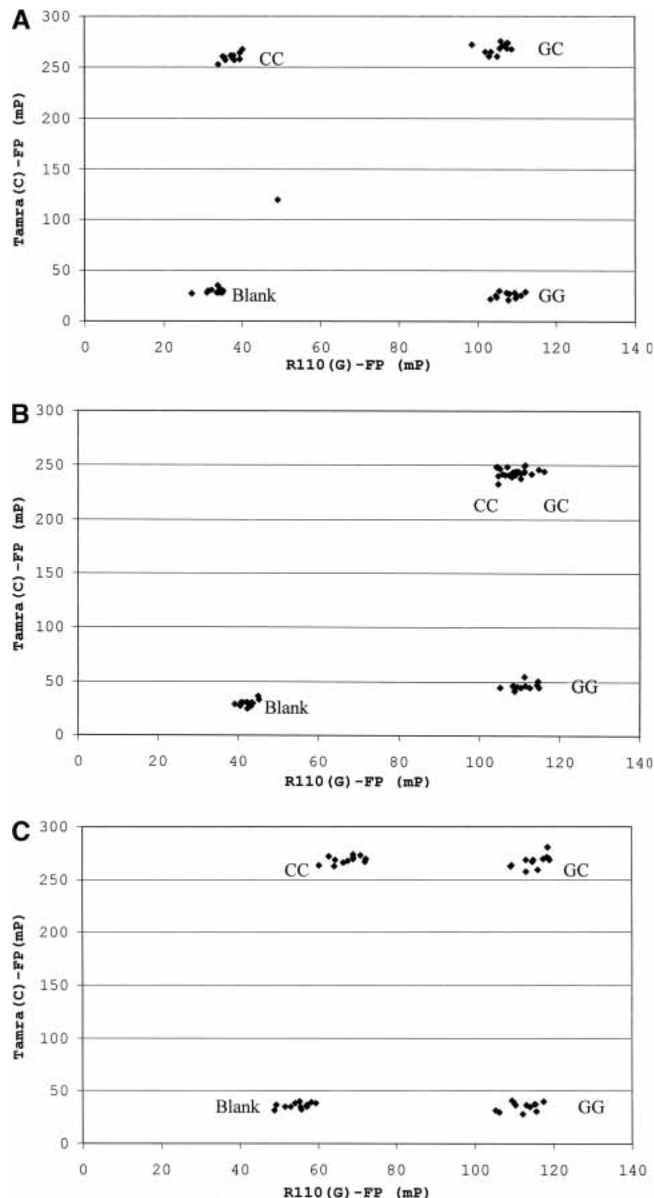


**Figure 2** Results of single base extension reaction using synthetic oligos with or without PPi and with or without pyrophosphatase. (*A*) Results of primer extension reaction in the absence of PPi. No obvious misincorporation was observed up to 70 thermal cycles. (*B*) Results of primer extension reaction in the presence of PPi. The misincorporation of R110-G for homozygotes was evident after only 10 cycles, causing the merging of the CC cluster and the GC cluster. (*C*) Results of the primer extension reaction after incubation with pyrophosphatase to remove the added PPi. No evidence of misincorporation up to 70 cycles.

and A alleles are high. When the SNP primer ends in a base that is not one of the dye-terminators in the reaction, it does not affect the FP of the dye-terminators in the reaction, although the cleaved primer is extended by the unlabeled terminator.

We first tested this hypothesis with synthetic templates. We reasoned that because the synthetic templates were not generated by PCR, no PPi was present in the reaction and no misincorporation would occur because the reverse reaction was not a factor without excess PPi. Figure 2A shows the results of a typical primer extension reaction (template 1 with (G/C) allele in Table 1) in the absence of PPi. There was no misincorporation even

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**Table 1. Sequence of Synthetic Oligos and Extension Primers**

| Synthetic oligos as extension templates | Extension primers | Misincorporation |
|---|---|---|
| Template 1 (G/C) | | |
| GTGGTGTTCCTCTTCGAAGGGCTTGCTAATCCTTGGCCCA | CCAAGGATTAGCAAGCCCTTCGAAGAG | G allele for CC cluster |
| GTGGTGTTGCTCTTCGAAGGGCTTGCTAATCCTTGGCCCA | | |
| Template 2 (G/A) | | |
| AGTGGATCCCACTGTCGATGGAGATGCCTGAGAAAGACCC | CTCAGGCATCTCCATCGACAGTGGG | G allele for AA cluster |
| AGTGGACCCCACTGTCGATGGAGATGCCTGAGAAAGACCC | CTCAGGCATCTCCATCGACAGTGGG | |
| Template 3 (A/T) | | |
| ACGAAAATTTTGCTAGTGGTTTACCCATGGTCATAGCTGT | CTATGACCATGGGTAAACCACTAGCAAAA | A allele for TT cluster |
| ACGAAATTTTTGCTAGTGGTTTACCCATGGTCATAGCTGT | | |
| Template 4 (G/A) | | |
| CAAAACTAAGATAGCTATTCCATCCCTCCATTCGTTTTTATTATC | AACGAATGGAGGGATGGAATA | A allele for GG cluster |
| CAAAACTAAGATAGTTATTCCATCCCTCCATTCGTTTTTATTATC | | |
| Template 5 (A/T) | | |
| CACTAGCAAAAATTTCGTGTCCTGTTCCCACTGGCCGTCG | GGCCAGTGGGAACAGGACACGAAA | A allele for TT cluster |
| CACTAGCAAAATTTTCGTGTCCTGTTCCCACTGGCCGTCG | | |
| Template 6 (G/A) | | |
| AGTGGATCCCACTGTCGATGGAGATGCCTGAGAAAGACCC | CTCAGGCATCTCCATCGACAGTGGG | G allele for AA cluster |
| AGTGGACCCCACTGTCGATGGAGATGCCTGAGAAAGACCC | | |

after 40 cycles of primer extension. In a second experiment, we mixed the synthetic oligo with PPi at a concentration similar to that found at the end of PCR. The results are shown in Figure 2B. The CC cluster merged with the GC cluster only after 10 cycles of the primer extension reaction. In the third experiment, the synthetic template and PPi were incubated with pyrophosphatase prior to the primer extension reaction. Once again, with the PPi degraded prior to the reaction, no misincoporation was observed (Fig. 2C).

We then selected 48 SNP markers that showed patterns of misincorporation with the original protocol and ran the assays on 96 samples again with pyrophosphatase added to the Exo-Sap solution in the PCR product clean-up step. With removal of the PPi, 78% of the previously failed assays gave high-quality genotypes and showed no sign of misincorporation even after 70 TDI cycles. Results of a typical experiment are shown in Figure 3. In Figure 3, B and C, samples typed with the SNP marker rs1410064 without pyrophosphatase showed two clusters with homozygous AA samples merging with the heterozygous GA samples after only 20 cycles. With the addition of pyrophosphatase, there was no misincorporation up to 40 cycles (Fig. 3D). We also ran 20 SNP markers that showed good genotyping results with the original protocol to make sure that the addition of pyrophosphatase did not affect the results of those assays. The genotype calls from assays with pyrophosphatase were in total agreement with those obtained by using the original protocol.

## Errors Due to R110-Terminator Properties

Intrigued by the observation that some extension products of R110-acycloterminators displayed low FP values, we systematically investigated the relationship between fluorescence intensity and FP upon the incorporation of R110- and TAMRA-acycloterminators (Xiao and Kwok 2003). We found that FP values of incorporated TAMRA-acycloterminators were quite stable and all exceeded 120 mP. In addition, the degree of fluorescence quenching for TAMRA-acycloterminators seldom exceeded 30% in all the SNPs examined. In contrast, incorporated R110-acycloterminators displayed a wide range of FP values, from 30 to 150 mP, and for some assays, the fluorescence intensities were quenched as much as 90%. We further observed that the SNP primers in assays in which R110-quenching was most severe were those with a G at the 3′-end or with several Gs within 10 bases from the 3′-end. When the quenching of R110 was severe, the FP value was artificially low and became an unreliable indicator of TDI. Although the loss of FP values of R110 is found in only 5% of the SNP markers assayed, it is a limitation that needs to be addressed.

For some assays, the FP values of the samples do not cluster very well; R110 FP values are generally low, and the FP values from the heterozygotes and homozygotes are not clearly separated from each other. An example of this phenomenon is shown in Figure 4A, in which the R110 and TAMRA FP values of the reaction products for marker rs4723564 are plotted against each other. Although the homozygotes and heterozygotes are separated very well in the TAMRA direction, the homozygotes and heterozygotes do not separate well in the R110 direction. We reasoned that, in these cases, plotting the degree of quenching of R110 might be a good way to determine whether the R110-acycloterminator was incorporated in the primer extension reaction. We tested the validity of this approach by plotting the data of 50 SNP markers with TAMRA FP values against R110 quenching. The degree of R110 quenching is expressed as the ratio (R) of initial TAMRA intensity to final R110 intensity multiplied by 100 according to equation 4 in the Methods. The terminators came premixed from the manufacturer, thus the initial intensity ratio of the total intensities of TAMRA and R110-acycloterminators is very stable and shows little variation from reaction to reaction. Any deviation from the initial intensity ratio indicates that there is quenching present. In Figure 4B, the raw data from 96 individuals typed with the SNP marker rs4723564 (Fig. 4A) are plotted as the ratio R versus the corresponding TAMRA FP values. The previously overlapping data now produce four clear-cut clusters.

In the particular plate reader setting, the fluorescence intensity ratio ($R_{blank}$) of the negative control reaction is found to be 50, which indicates that the total intensity of R110-C is two times that of TAMRA-T. At the end of the primer extension reaction, the intensities of the R110-C and TAMRA-T either remain unchanged (no incorporation) or decrease due to quenching upon incorporation. For reactions in which the DNA samples came from individuals with the C/C genotype, $R_{C/C}$ values increased to ~140, because the R110-C was incorporated and its intensity was quenched to 30% of original intensity, and TAMRA-T was not

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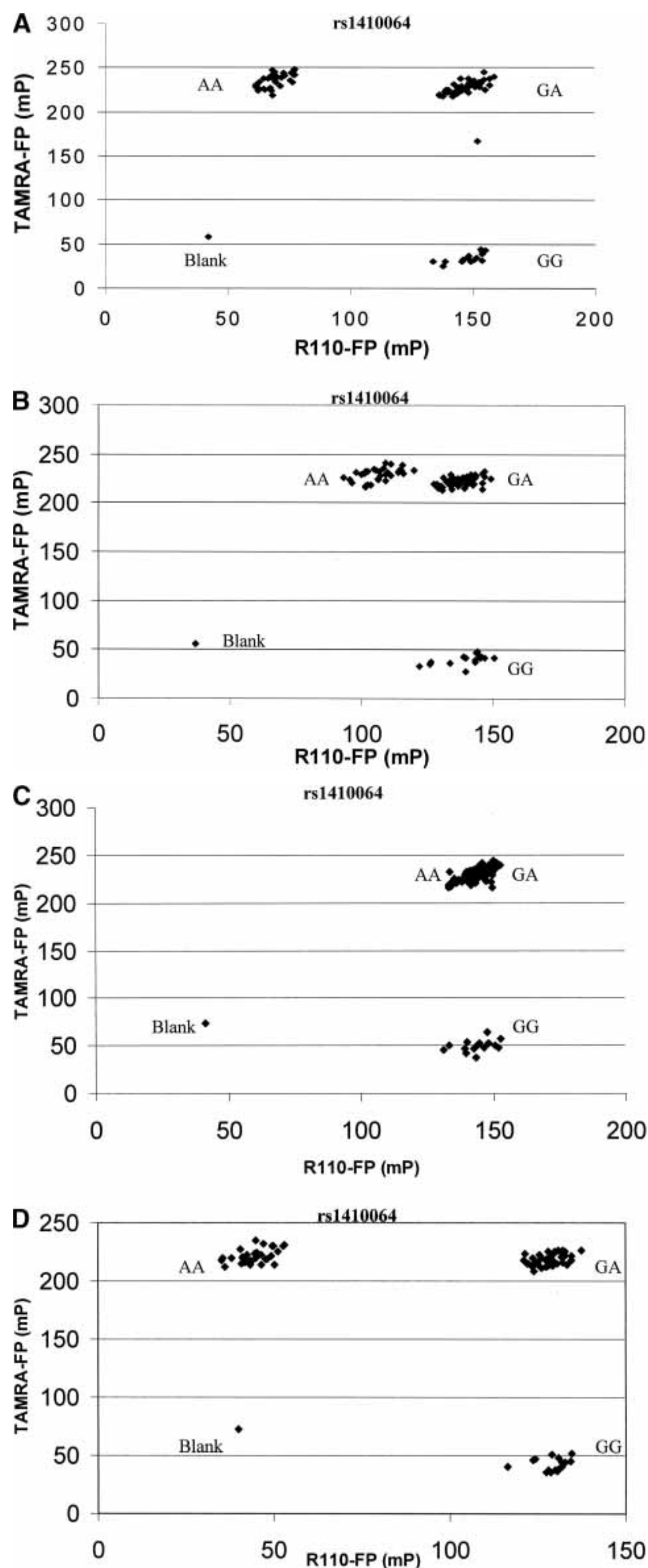


**Figure 3** Genotyping results of marker rs1410064 with or without pyrophosphatase incubation. (*A*,*B*,*C*) The genotyping results of the PCR/TDI reaction without pyrophosphatase incubation at 10 (*A*), 20 (*B*), and 40 (*C*) cycles, respectively. In all three cases, the AA cluster moved toward the GA cluster, and the two clusters merged after 20 cycles. (*D*) The same reaction was performed but with a pyrophosphatase incubation step. No misincorporation was observed at 40 cycles.

incorporated and its intensity remained unchanged. In contrast, the ratio $R_{T/T}$ decreased to ~20 for T/T homozygotes because the intensity of R110-C acycloterminators remained unchanged, and the intensity of TAMRA-T was quenched to 60% of the original intensity. For C/T heterozygotes, R110-C and TAMRA-T were both incorporated, and both intensities decreased significantly. Because R110-C was quenched more severely than was TAMRA, the ratio $R_{C/T}$ increased slightly to ~60. For the samples in which PCR failed, no incorporation occurred and $R_{failed}$ had the same value as that of $R_{blank}$, with a value of 50. The genotypes determined by the data found in Figure 4B were in complete concordance to the genotypes obtained by conducting the FP-TDI assays using the antisense DNA strand as template and its complementary SNP primer.

Combining R110 R value with its corresponding TAMRA FP value, R110 homozygotes have high R110 R values and low TAMRA FP values, and are found in the lower right corner of the scatter plot (Fig. 5). In contrast, TAMRA homozygotes have low R110 R values but high TAMRA FP values, and are found in the upper left corner of the plot. As for the heterozygotes, they have intermediate R110 R values and high TAMRA FP values and are found in the upper right corner of the plot. With this combined analytical approach, we have reanalyzed the data from >400 markers. The combined FP/quenching analysis recovered ~20% of the failed markers, increased the call rate of most markers based on FP analysis alone, and generally gave tighter clusters.

## Alternate Dye-Labeled Acycloterminators With Better Properties

For an ideal combination of dye acycloterminators, the dyes should have good fluorescence spectral separation, each dye acycloterminator should display high FP value upon incorporation, and the AcycloPol enzyme should incorporate both dye terminators with similar efficiency. We have observed that AcycloPol enzyme incorporates TAMRA-acycloterminators more efficiently than do the R110-acycloterminators (Gardner and Jack 2002). The differential incorporation efficiency of R110 and TAMRA-acycloterminators is another cause of poor separation between the heterozygous cluster and one of the homozygous clusters (data not shown).

We tested several other dye-labeled acycloterminators, and our preliminary results indicate that Texas red acycloterminators show high and stable polarization value, less intensity quenching, and comparable incorporation efficiency compared with TAMRA acycloterminators. By using Texas red and TAMRA acycloterminators combination, we assayed 48 SNP markers, including seven markers in which incorporated R110 showed negligible increases in FP values. We observed that the FP values of incorporated Texas red acycloterminators were all >100 mP. An example of this phenomenon is shown in Figure 6B, in which Texas red/TAMRA-acycloterminators combination produced nicely separated clusters, whereas the R110/TAMRA combination did not produce clear-cut clusters due to low R110 FP values (Fig. 6A).

# DISCUSSION

Although 80% of the AcycloPrime-FP assays are successful without any optimization, and these results agree well with previous reports (Hsu et al. 2001; Akula et al. 2002) that improvements in the success rate have great impact on large-scale studies. Our studies showed that the majority of the failed assays could be salvaged with minor changes in the protocol and analysis procedure. Excluding 5% failed assays due to failed PCR amplification, the failure of the remaining 15% assays can be attributed to misincorporation of acycloterminators and/or fluorescence quench-

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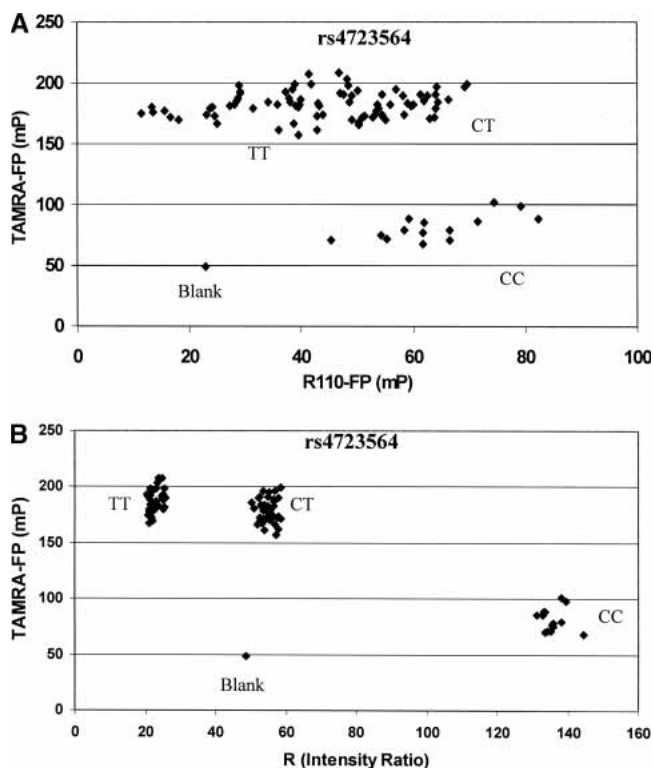


**Figure 4** Combining FP and intensity ratio (quenching) in data analysis. (*A*) Genotyping results of rs4723564 when the data were plotted as TAMRA FP versus R110 FP. Low FP values of R110 resulted in poor separation of clusters. (*B*) Same data were replotted by using TAMRA FP values versus the intensity ratio R. Four clear clusters were formed when quenching was taken into account.

ing of incorporated acycloterminators by SNP primers with certain sequence motifs.

Misincorporation of acycloterminators is hard to detect and predict because it correlates with not only the amount of PPi generated during PCR but also how far the primer extension reaction is pushed into completion. Our observation indicates that the misincorporation induced by the pyrophosphate always happens after one of the terminators is used up and forward extension reaction is completed. The enzyme starts to incorporate the wrong base if the SNP primer is shortened by the pyrophosphorolysis reaction. Once misincorporation occurs, it is impossible to undo the process, and the experiment is lost. Coupled with the occasional situation in which the yield of PCR is low, thereby rendering the primer extension reaction incomplete, the resulting data give only ambiguous genotype calls. Our study showed that the reverse pyrophosphorolysis reaction was the main cause of misincorporation during the primer-extension reaction. Incubating the PCR product mixture with pyrophosphatase during Exo-SAP clean-up step to remove the PPi generated during PCR can effectively prevent misincorporation of acycloterminators. With this approach, we can push the PCR reaction to completion and prolong the primer extension reaction up to 50 cycles without any evidence of acycloterminator misincorporation. Because the amount of PPi generated during the primer extension step is rather small, we have opted to use the inexpensive pyrophosphatase rather than the costly thermostable pyrophosphatase.

Assay failure due to negligible FP value changes when an acycloterminator is incorporated happens almost exclusively in R110-labeled acycloterminators, as they are heavily quenched upon incorporation under some circumstances. Fluorescence quenching due to interaction between the dyes and nucleobases has been studied extensively. It is proposed that photo-induced charge transfer between the dye and a nucleotide residue (mostly guanosine) plays a crucial role in the process (Seidel et al. 1996; Torimura et al. 2001; Nazarenko et al. 2002). Our preliminary studies on the correlation between fluorescence quenching and polarization show that different dyes behave quite differently. The FP values of all TAMRA-acycloterminators increase upon incorporation onto SNP primers, whereas their fluorescence intensities either remain constant or decrease slightly. In contrast, the fluorescence intensities of R110-acycloterminators diminish greatly upon incorporation onto SNP primers, whereas their FP values spread across a wide range. We can actually take advantage of the quenching phenomenon and reanalyze the fluorescence data by plotting FP values in the TAMRA direction versus the intensity ratio in the R110 direction to obtain good genotype calls.

In our search for the ideal combination of dye-labeled acycloterminators, we came across Texas red acycloterminators, which are hardly ever quenched by SNP primers. Our preliminary studies showed that the Texas red/TAMRA acycloterminator combination works well in the primer extension reaction, with comparable incorporation efficiencies and very little fluorescence quenching.

For the current AcycloPrime-FP kits, the assay success rate can reach as high as 95% by the small modifications outlined above without redesigning primers and adding additional expensive materials, such as single-strand binding protein (Hsu et al. 2001). Replacing R110 acycloterminators with Texas red acycloterminators will likely improve the results further.

As primer extension assays are the basis of several other genotyping methods, several of which do not remove the PPi generated by PCR in their protocol, our findings and solutions will have a positive impact on these methods also.

## METHODS

Anonymous DNA samples of 96 individuals from the National Institutes of Health (NIH) Polymorphism Discovery Panel and CEPH family and publicly available markers from dbSNP database were used in this study. All primers were designed as described previously (Vieux et al. 2002) and were obtained from IDT. All reactions were run and read in 96-well or 384-well black plates from LabSource. Liquid handling instrument Evolution 3 (Perkin-Elmer) was used for assay assembly. PlatinumTaq DNA polymerase was from Invitrogen. AcycloPrime-FP SNP Kits were from PerkinElmer, including 10× reaction buffer, AcycloPol Enzyme for single base extension, Exo-Sap (exonuclease I and shrimp alkaline phosphatase), and dye-labeled acycloterminators

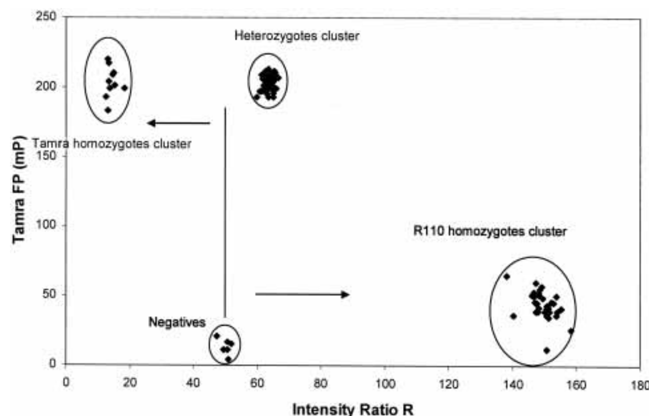


**Figure 5** Usual cluster positions in the FP/quenching plot.

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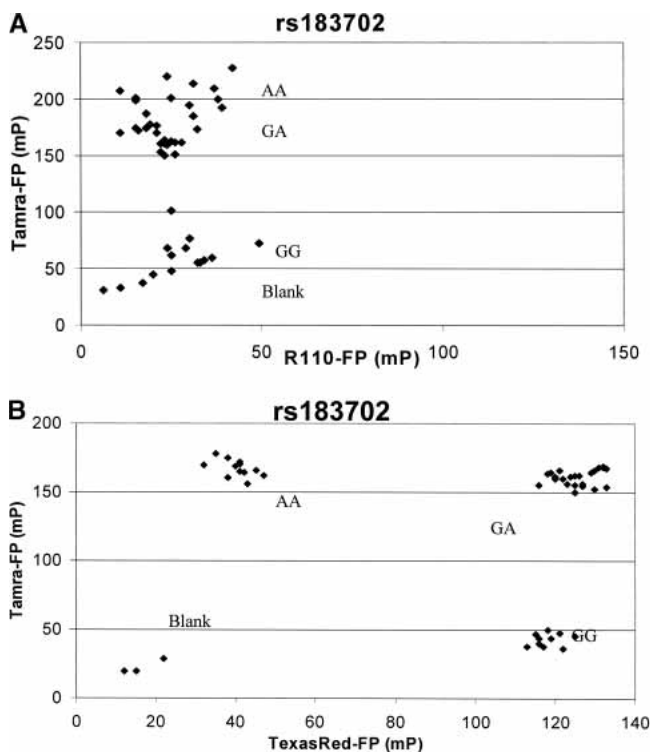


**Figure 6** Results of genotyping assay using the Texas red/TAMRA acycloterminator combination. (*A*) Genotyping results of rs183702 on 48 DNA samples using the TAMRA/R110 combination. The G/A heterozygous and G/G homozygous clusters collapsed into A/A homozygous and blanks, respectively, because there were no difference in FP value between free R110 acycloterminators and primer extension products. (*B*) Genotyping results of rs183702 using the Texas red/TAMRA acycloterminator combination. Four clusters are clearly seen.

mixture, which contains equal amounts of R110 and TAMRA terminators. PPi was purchased from Sigma. Pyrophosphatase was from Roche. Texas red–labeled acycloterminators are from Perkin-Elmer. The assays were read in an Envison or Victor2 microplate reader (Perkin-Elmer) when R110 and TAMRA acycloterminators were used. For the combination of Texas red and TAMRA acycloterminators, the assays were read in an Analyst microplate reader (Molecular Devices).

## Primer Extension Reaction Using Synthetic Oligos

For each primer extension reaction using synthetic oligos, two template oligos were synthesized each with one of the two allelic bases at the target site. The heterozygous templates were made by mixing equal amounts of the two synthetic templates. The template sequences of four such reactions were summarized in Table 1. Forty-eight samples were prepared for each primer extension reaction, and each genotype has 12 samples; 100 nM synthetic templates were prepared in PCR buffer with or without PPi (100 µM).

Seven microliters of synthetic template with or without PPi and 13 µL TDI cocktail (containing 2 µL 10× TDI reaction buffer, 0.5 µL SNP primer [final concentration 384 nM], 0.05 µL AcycloPol enzyme, 1 µL dye acycloterminator mix, and 8.95 µL water) were mixed and incubated from 2 min at 95°C, up to 70 cycles of single base extension for 15 sec at 95°C and 30 sec at 55°C. After every five cycles, the product mixtures were read on an Envision fluorescence plate reader (PerkinElmer).

To a mixture containing PPi and synthetic templates, 2 µL Exo-SAP/pyrophosphatase mixture (1.8 µL Exo-Sap buffer and 0.15 µL pyrophosphatase) was added, and the reaction mixture was incubated for 1 h at 37°C. TDI cocktail (13 µL) was then added, and the extension reaction was performed as above.

## PCR/TDI Reaction With or Without Pyrophosphatase

All reactions were performed according to the manufacture's manual. Briefly, DNA (3 ng) was amplified in 5 µL reaction mixtures containing PCR primers and PCR reagents by using the following thermal cycling protocol: The mixture was held at 95°C for 2 min followed by 40 cycles of 10 sec at 92°C, 20 sec at 58°C, and 30 sec at 68°C. The reaction mixtures were then incubated for 10 min at 68°C before they were held at 4°C until further use.

Pyrophosphatase (1.5 µL) was added to a stock solution of PCR clean-up enzyme mixture (10.5 µL of 10× buffer, 1.33 µL Exo-SAP). The PCR clean-up mixture (2 µL) was added to 5 µL PCR product mixture and incubated from 1 h at 37°C to degrade the excess PCR primer, excess dNTP, and PPi generated during PCR. The enzymes were heat-inactivated for 15 min at 80°C prior to the TDI reaction.

TDI cocktail (13 µL) was added to the reaction mixtures (7 µL) from previous step. The reaction mixture was incubated for 2 min at 95°C, five to 70 cycles of 15 sec at 95°C and 30 sec at 55°C. The final product mixtures were read on a fluorescence plate reader (PerkinElmer).

## FP, Fluorescence Quenching Measurements, and the Intensity Ratio of TAMRA Versus R110

FP measurements were done on an Envision (Perkin-Elmer). The FP (P) and total intensities ($I_{total}$) were calculated according to the following formulae:

$$P = (I_{hh} - I_{vh})/(I_{hh} + I_{vh}) \quad (1)$$

$$I_{total} = I_{hh} + 2 * I_{vh} \quad (2)$$

where $I_{hh}$ is the emission intensity measured when excitation and emission polarizer are parallel, and $I_{vh}$ is the emission intensity when these filters are perpendicular. In general, FP is expressed as millipolarization (mp) as

$$mP = 1000 * P \quad (3)$$

The intensity ratio (R) of TAMRA versus R110 is defined as

$$R = 100 * I_{total\ of\ TAMRA} / I_{total\ of\ R110} \quad (4)$$

## Measurement of the Relative Incorporation Efficiency of Dye-Terminators

The estimation of the incorporation efficiency of dye-terminators has been described previously (Xiao and Kwok 2003; Xiao et al. 2003). FP of a heterozygous sample was read every two cycles and converted to anisotropy (A). Anisotropies of two dyes were then plotted against cycle numbers, respectively, and the plots will be linear for initial linear incorporation stage. The slopes of the linear lines were calculated, and the ratio of the two lines will be the relative incorporation efficiency for the two dye terminators.

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