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Optimization of SNP Genotyping Assay with Fluorescence Polarization Detection

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ABSTRACT : Single nucleotide polymorphisms (SNPs) are valuable DNA markers due to their abundance and potential for use in automated high-throughput genotyping. Numerous SNP genotyping assays have been developed. In this report, one of effective and high throughput SNP genotyping assays which was named the template-directed dye-terminator incorporation with fluorescence polarization detection (FP-TDI) was described. Although the most of this assay succeed, the objective of this work was to determine the reasons for the failures, find ways to improve the assay and reduce the running cost. Ninety F_2-derived soybean, *Glycine max* (L.) Merr., RILs from a cross between 'Pureunkong' and 'Jinpumkong 2' were genotyped at four SNPs. FP measurement was done on Victor^3 microplate reader (Perkinelmer Inc., Boston, MA, USA). Increasing the number of thermal cycles in the single-base extension step increased the separation of the FP values between the products corresponding to different genotypes. But in some assays, excess of heterozygous genotypes was observed with increase of PCR cycles. We discovered that the excess heterozygous was due to misincorporation of one of the dye-terminators during the primer extension reaction. After pyrophosphatase incubation and thermal cycle control, misincoporation can be effectively prevented. Using long amplicons instead of short amplicons for SNP genotyping and decreasing the amount of dye terminator and Acyclopol Taq polymerase to 1/2 or 1/3 decreased the cost of the assay. With these minor adjustments, the FP-TDI assay can be used more accurately and cost-effectively.

***Keywords*:** FP-TDI, misincorporation, SNP genotyping

Single nucleotide polymorphisms (SNPs), which can be defined as single-base changes or indels at a specific nucleotide position, become the most widely used genetic markers. Since SNPs are abundant and highly stable, and often contribute directly to a phenotypic trait (Kim *et al.*, 2004), they can serve as a powerful tool for marker-assisted selection (MAS), map-based cloning, genetic mapping and association mapping of complex traits (Kruglyak, 1999). Such applications require reliable and economical methods for high-throughput SNP genotyping. Various SNP genotyping methods have been developed based on single base extension, allele-specific primer extension, allele-specific hybridization, oligonucleotide ligation, endonuclease cleavage, Invader, TaqMan and fluorescence energy transfer (reviewed in Gupta *et al.*, 2001; Gut, 2001; Syvänen, 2001; Taylor *et al.*, 2001; Ye *et al.*, 2001).

A genotyping assay based on single base extension (SBE), termed template-directed dye-terminator incorporation assay with Fluorescence Polarization (FP-TDI) detection is one of effective and high throughput SNP genotyping analyses. The FP-TDI is based on two principles: 1) catalysis of DNA polymerase for the allele-specific incorporation of a dideoxynucleotide at the polymorphic site and 2) significant increase in FP of a fluorescent dye 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). The FP-TDI assay is more cost-effective because it does not require modified probes such as dye-labeled primers and it is easy to set and readily scalable for large-scale genetic studies (Hsu *et al.*, 2001).

For genetic mapping and association analysis using SNP markers, many high throughput SNP genotyping assays must be developed for testing the genotypes of numerous samples with thousands of SNPs. Therefore, ease of optimization, low cost, and automation are very important. Even though the original FP-TDI assay was a robust and reliable assay for SNP genotyping, it did not work consistently for all the SNPs that were tested. Some assays were failed due to incomplete reactions and misincorporation of one of the dye-terminator during the primer extension reaction. In this report, we describe the source of the problems and simple ways to troubleshoot these problems. Furthermore, the alternative procedures would be suggested to make the assay more cost-effective.

MATERIALS AND METHODS

Plant Materials

An F_2-derived soybean population of 90 rombinant inbred lines (RILs) from the cross of Pureunkong and Jinpumkong 2 was used for SNP genotyping. Pureunkong produces small seeds for producing soybean sprouts with the grassy-beany flavor, while Jinpumkong 2 lacks the flavor (Kim *et al.*,

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2004). DNA was isolated from the leaves of the 90 RILs and the two parents using the modified CTAB procedure of Keim *et al.* (1988). No addition of DNA was served as a negative control.

SNP Markers and Primer Design

After SNPs were detected as described previously (Van *et al.*, 2004) in tentative consensus sequences (TCs) which were selected from soybean cDNA library derived from roots of 7 days old 'Bragg' supernodulating mutant available from The Institute for Genomic Research (TIGR) databases (http://www.tigr.org), four SNPs were randomly selected for this study (Table 1). PCR primers were designed using Primer3 software (Rozen *et al.*, 2000) to amplify genomic DNA that contained SNP site of 150-200 bp. In addition, a long PCR amplicon (more than 1000 bp) was also designed in SNP19 and SNP28 to investigate the effect of PCR amplicon size on genotyping data. SNP primers whose 3' ends lay immediately upstream of the polymorphic site for these fragments were designed by SBE Primer version 1.1 (Kaderarli *et al.*, 2003).

PCR Amplification

All reactions were run and read in 384-well black-skirted plates purchased from MJ research (Watertown, MA, USA). The amplification reaction contained 5 ng of genomic DNA, 100 nM of each forward and reverse primer, 100 μM of each dNTP, 1.5 mM $MgCl_2$, 10 × reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) and 0.1 U of AmpliTaq® DNA polymerase (Applied Biosystems, Fostser City, CA, USA) in a total volume of 5 μl. For the primers with long amplicons (> 1000 bp), we used 10 ng of genomic DNA, 400 nM of each forward and reverse primer, 150 μM of each dNTP to increase its quantity. PCR cycling conditions are as follows: an initial denaturation and enzyme activation step at 95 °C for 10 min, followed by 35 cycles consisting of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min. After a final primer extension step at 72 °C for 10 min, the reactions are cooled to 4 °C until further use.

Excess PCR Primer and dNTP Degradation

AcycloPrime-FP SNP Kits (PerkinElmer, Boston, MA, USA) include 10 × reaction buffer, AcycloPol Enzyme for single base extension, Exo-Sap (exonuclease I and shrimp alkaline phosphatase), and dye-labeled acycloterminators mixture, which contains equal amounts of R110 and TAMRA terminators. PCR clean-up enzyme mixture was made by dilution the PCR clean-up 10 × Reagent to 1 × Reagent (Exo-SAP) with 10 × Buffer. In addition, pyrophophatase (PPase, 1.5 μl) (Roche, IN, USA) was added to PCR clean-up enzyme mixture (10.5 μl of 10 × Buffer, 1.33

Table 1. Details of SNPs, PCR primers, and SNP primers in this study.

SNP name	SNP type	PCR primer (5' → 3')	Amplicon size (bp)	SNP position	SNP primer (5' → 3')
SNP 6	C/T	F: TATGCCACAACTGGATAAGACG R: AGACCCTGCCTTCAACAAATAC	289	146	GAGCCAAGATACTAAAGATTTAGAA[+]
SNP 18	G/A	F: CAAATGTTCTCACCCATTAGCA R: GTGTGTGCGTTGTCTGGATATT	156	60	GTTGAAAATGTTCTTGTTATTTCCA[+]
SNP19	G/A	Set I F: CTGAATGAGAATTGCTTGTCTGTC R: TTACTCACCGGAAGTGCTTTCT	191	75	CATACTCAGCAAACTTAGCATTCCA[–]
		Set II F: ACAAGGCAGAACCTTGGATG R: TGGATAGGGCAAGAATGGAG	1000	170	
SNP28	C/T	Set I F: TCTTGCAGGTGGATAGATAATAGAG R: AGTGGTGTTCGAGTAGTTATGGA	200	97	AGCTGAATTGACCAAGAAAGAATGG[+]
		Set II F: GCCACCTTCTCTCTTTGACG R: GCAAAGACGAGCTGGAATCT	1500	210	

F, forward primer; R, reverse primer.
[+], the extension primer was designed to be complementary to the 5' region from the SNP site.
[–], the extension primer was designed to be complementary to the 3' region from the SNP site.

μl of Exo-SAP). The PCR clean-up mixture (2 μl) was added to 5 μl PCR product mixture and incubated 1 h at 37 °C to degrade the excess PCR primers, dNTPs, and pyrophosphates (PPi) were generated during PCR. The enzymes were heat-inactivated for 15 min at 90 °C prior to the SBE reaction.

Single-base Extension Reaction (SBE)

To the reaction mixtures from the previous step, 13 μl of cocktail was added according to the manufacturer's instructions (2 μl 10 × reaction buffer, 2 μM SNP primer, 0.05 μl Acyclo Enzyme, 1 μl each of two-dye Acyclonucleotide terminators, and 9.45 μl water). In addition, the amount of dye terminators and Acyclo Enzyme was decreased to one half, one third, and one fourth. The reaction mixture was incubated at 95 °C for 2 min, 10 - 50 cycles of single-base extension at 95 °C for 15s and 55 °C for 30s. After 10 cycles, 384 well black plate was read at first, and additional cycles was made before reading the plate again.

FP Measurement

FP measurement was performed on VICTOR^3 microplate reader (PerkinElmer Inc., Boston, MA, USA). VICTOR^3 microplate reader is a multi-label and multi-task plate reader for all light-emitting and light-absorbing detection technologies including fluorescence, luminescence, absorbance, UV absorbance, time-resolved fluorometry, and fluorescence polarization.

The FP value is calculated in the instrument software by following formula:

$$mp = 1000 \times [I_{vv} - I_{vh}] / [I_{vv} + I_{vh}]$$

where I_{vv} is the emission intensity measured when the excitation and emission polarizers are parallel and I_{vh} is the emission intensity measured when the emission and excitation polarizers are oriented perpendicular to each other.

Data Analysis

After automatic data were transferred into the Microsoft Excel, the clusters were separated at least 40 mp apart to determine the genotypes, which is at least 7 times higher than standard deviation of the negative controls (> 99% significance) (Chen *et al.*, 1999).

RESULTS AND DISCUSSION

Effect of the Thermal Cycle and Pyrophosphatase

Because ESTs are relatively short sequences that are error prone and often highly redundant (Blanc *et al.*, 2004), consensus sequences were derived from assembling overlapping EST sequences using several bioinformatics algorithms (Parkinson *et al.*, 2002). A total of 700 tentative consensus (TCs) were selected and 118 TCs were detected with SNPs between 'Pureunkong' and 'Jinpumkong 2'. Four SNP markers were used in this study (Table1). Genomic DNA from 'Pureunkong' and 'Jinpumkong 2' and each of 90 RILs were typed for each marker, and two samples with no DNA served as negative controls.

Following a standard protocol, the FP reading of the samples was clustered into four groups in each SNP marker. For SNP28, increasing the number of thermal cycles in the single-base extension step increased the separation of the FP values between the clusters (Fig. 1). As expected, the negative controls without DNA have low FP values for both dyes analyzed and occupy the area near the origin of the plot, indicating that the dye terminators remain free in solution. For homozygous CC individuals, the FP values for R110-ddC are high and the values or TAMRA-ddT are low, reflecting significant incorporation of the R110-ddC terminator onto the SNP primer but minimal incorporation of the TAMRA-ddT. Conversely, the FP values of R110-ddC are low and those of TAMRA-ddT are high for homozygous TT individuals. For heterozygous individuals, the FP values for both R110-ddC and TAMRA-ddT are high.

For SNP 18, the homozygotes for one allele (AA) were clearly separated, but the homozygotes of the other allele (GG) and the heterozygotes (GA) could not be distinguished clearly (Fig.1), showing the GG cluster merged with the GA cluster after 10 cycles of the primer extension reaction. Merging of homo- and heterozygotes might be due to the misincorporation of dye-terminators when the correct terminator allele was used up prematurely. This dye-terminator misincorporation could be explained by pyrophosphorolysis, which was due to the inorganic pyrophosphates (PPi) generated in the PCR step (Duetcher & Kornberg 1969; Liu & Sommer 2002; Ming *et al.*, 2004). To correct dye-terminator misincorporation, two ways were tested. The first way involved the incubation of PCR product with pyrophosphatase (PPase) added to the Exo-Sap solution in the clean-up step. With addition of PPase to remove the PPi, the failed assay gave high-quality genotypes and no sign of misincorporation even after 50 cycles. In Fig. 2, samples typed with the SNP 18 without PPase incubation showed homozygous GG samples merging with the heterozygous GA samples at 30 cycles. With the addition of PPase, there was no misincorporation up to 50 cycles. In the second way, we reduced the number of cycles used in the single-base extension reaction. For SNP 18, reading at 10 cycles showed distinguishable clusters of FP values (Fig. 1).

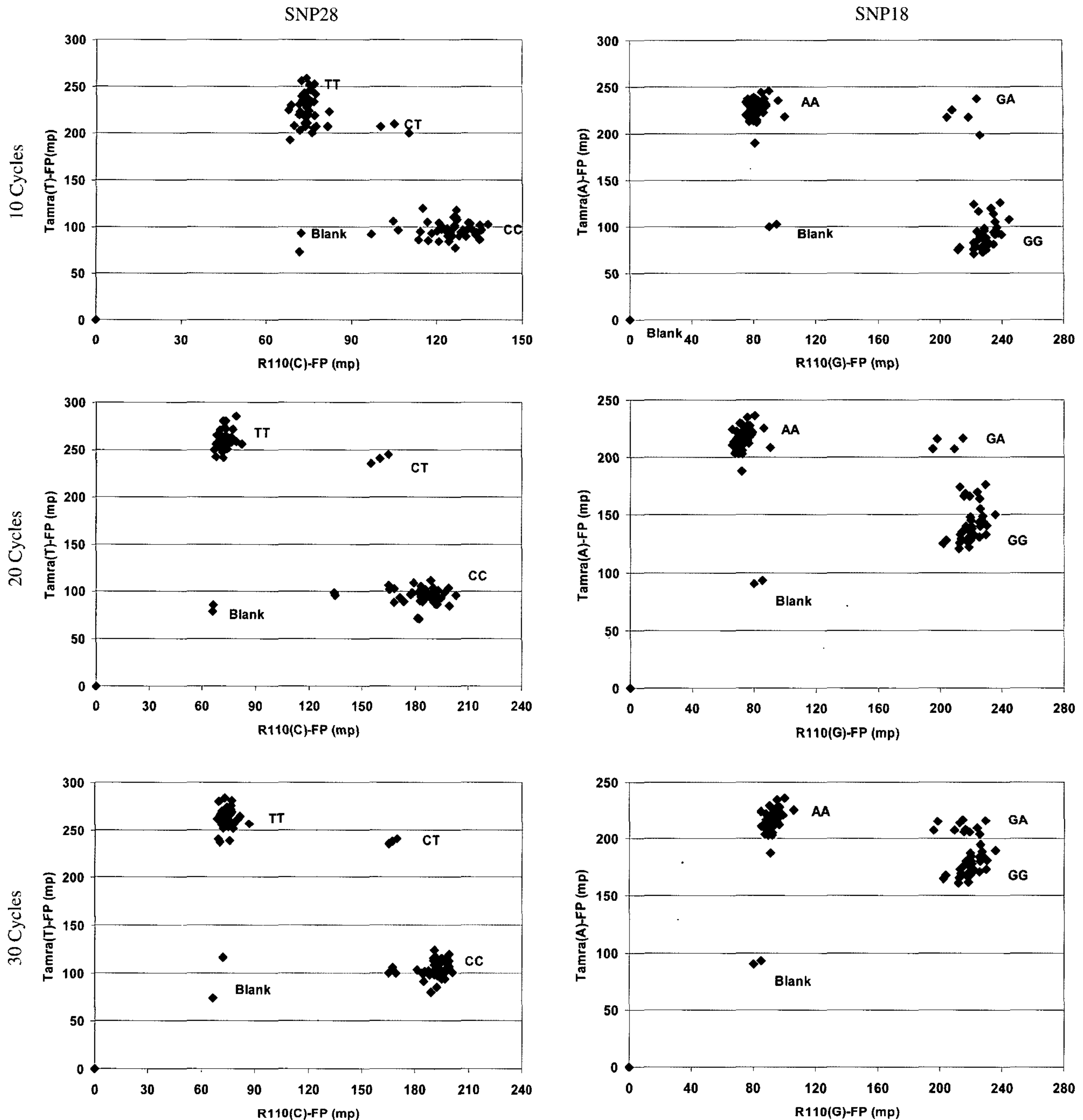

Fig. 1. Genotyping results of marker SNP28 and SNP18 at 10, 20, 30 cycles. Increasing the number of thermal cycles increased the separations between the clusters in SNP 28. The misincorporation was evident after only 10 cycles in SNP 18, causing the merging of the GG cluster and the GA cluster.

Misincorporation of acycloterminators correlates with how far the primer extension reaction is pushed into completion and the amount of PPi generated during PCR (Ming *et al.*, 2004). The misincorporation induced by the PPi always happens after one of the terminators is used up. The enzyme starts to incorporate the wrong base if the SNP primer is shortened by the PPi reaction. Our results showed that incubation of the PCR product mixture with PPase during Exo-SAP clean-up step to remove the PPi generated during PCR can effectively prevent misincorporation. With this approach, we can push the PCR reaction to completion and obtain the high-quality data up to 50 cycles without any evidence of acycloterminator misincorporation.

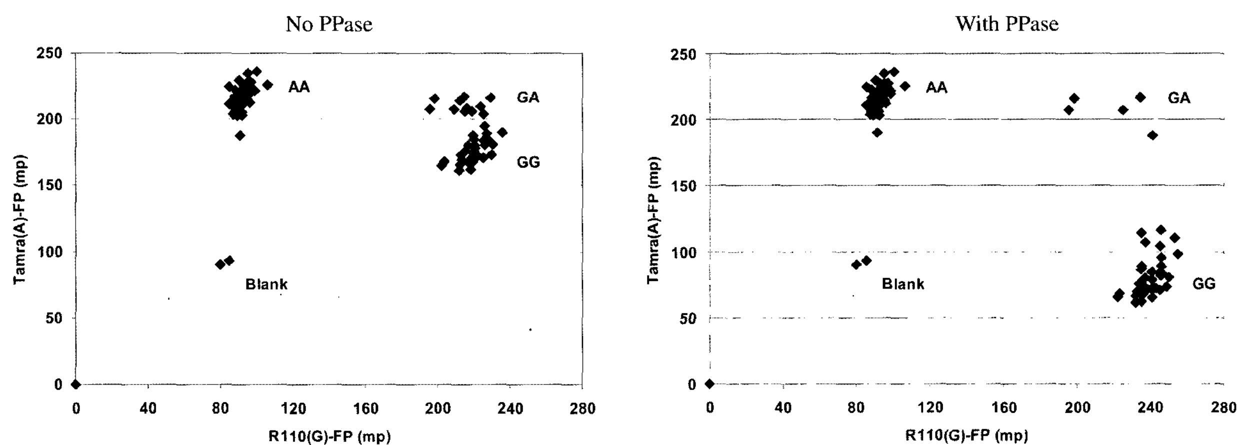

Fig. 2. Comparison of genotyping results of marker SNP 18 with or without pyrophosphatase incubation. No misincorporation was observed at 50 cycles after PPase incubation.

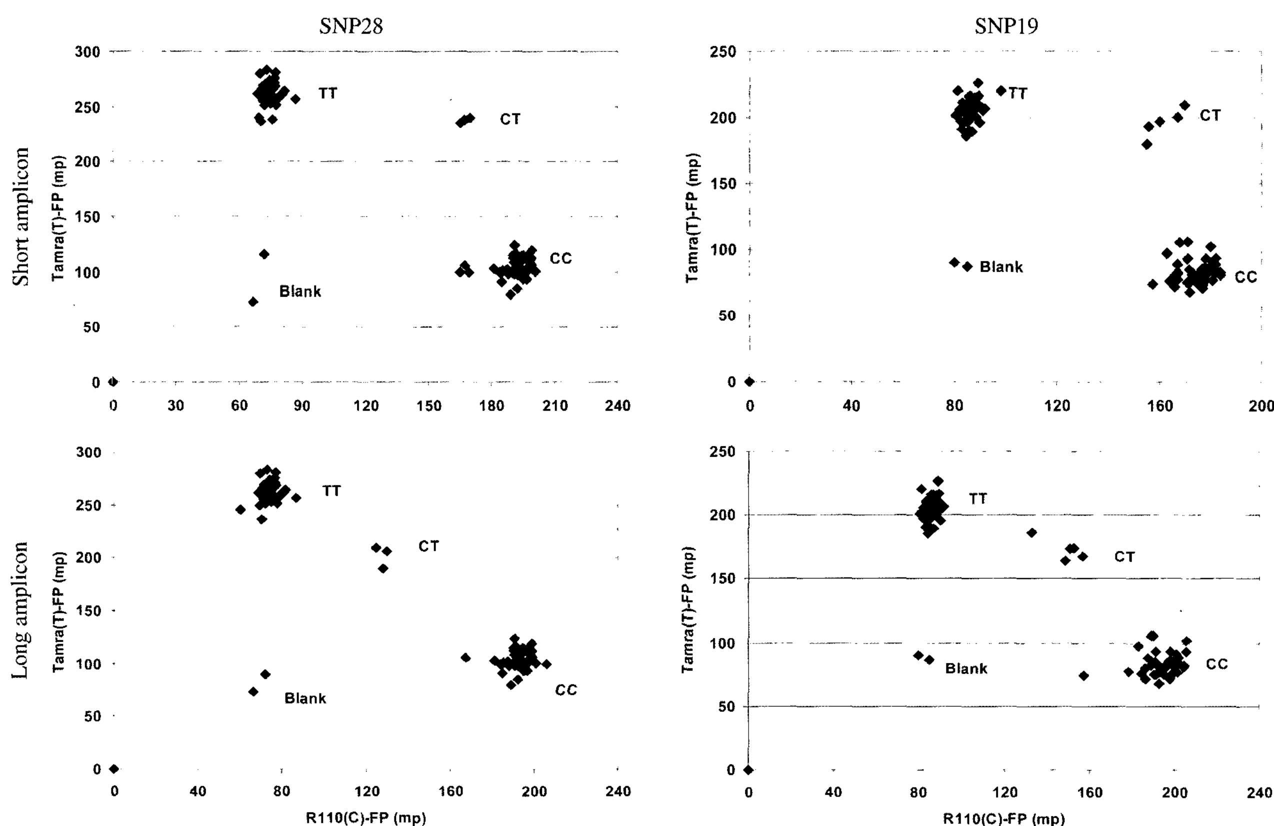

Fig. 3. Comparison of genotyping results between long amplicons and short amplicons.

Effect of PCR Amplicon Size

As templates, short amplicons of 200 bp or less are recommended in the standard protocol. Long amplicon may have high possibility to interact with capture probe, which can result in failure of SNP genotyping. But in most cases, the primers used for SNP discovery amplified long product more than 500 bp. It means the PCR primers should be redesigned to amplify short amplicons for SNP genotyping. So we genotyped the same SNP site with short amplicon and long amplicon as template to compare the typing difference between amplicons.

Two sets of primers with short amplicons (set I) and long amplicons (set II) were selected to genotype SNP 19 and SNP 28. Although the long amplicons are more than 1000 bp, it worked as well as short amplicons in both SNP mark-

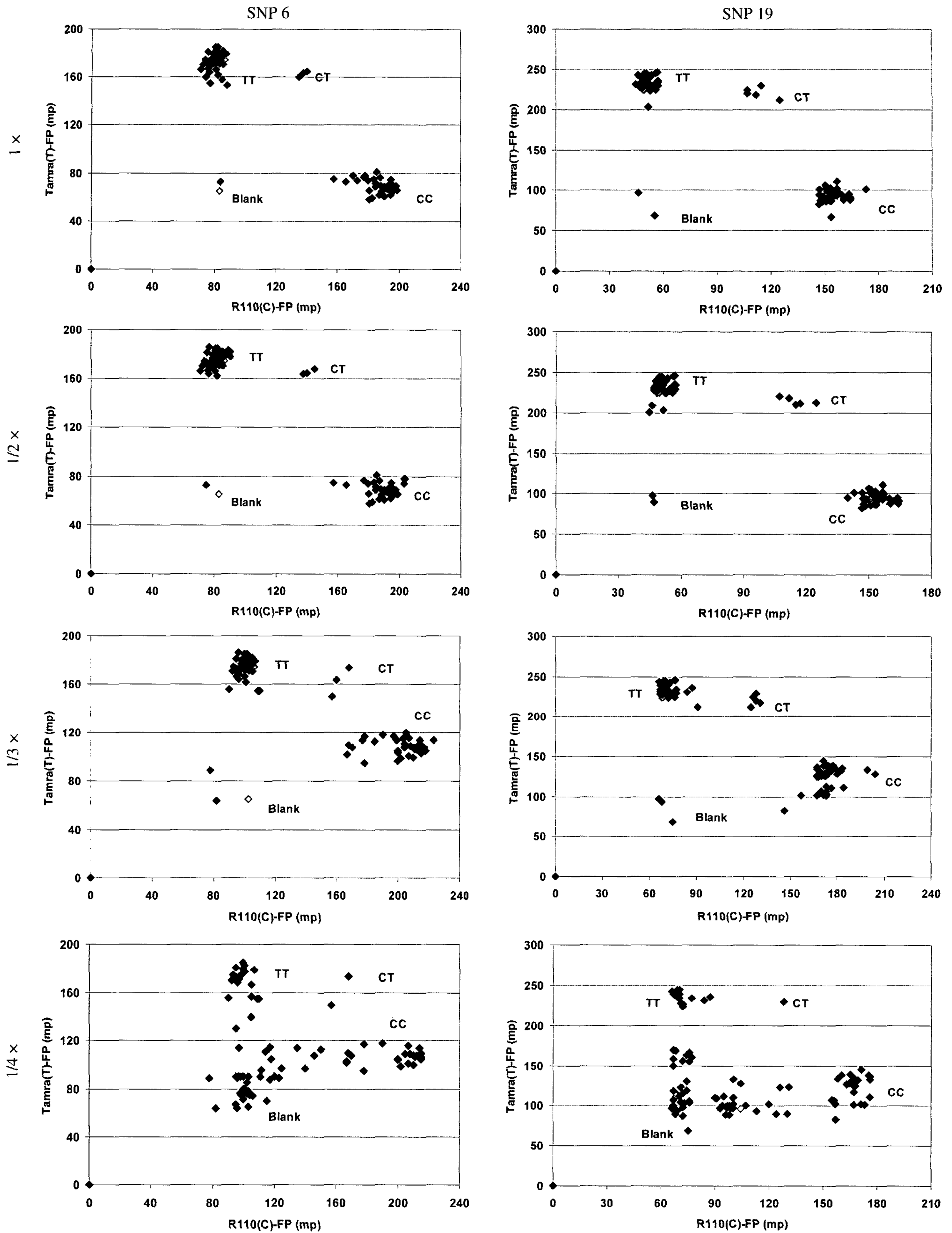

Fig. 4. Genotyping results of SNP6 and SNP19 with 1 ×, 1/2 ×, 1/3 ×, 1/4 × dye terminators and Acyclopol.

ers (Fig. 3). Therefore SNP fragment can be reamplifed with the same primers that were used for SNP discovery, and no redesign of PCR primer for SNP genotyping is needed.

Effect of the Amount of Dye Terminators and Acyclo Enzyme

SNP genotyping ability was tested in SNP 6 and SNP 19 by reducing the amount of dye terminators and Acyclo enzyme by 1/2, 1/3 and 1/4 to reduce the cost for this assay. Compared with the genotyping results of 1 × dye terminators and Acyclo enzyme as requested in protocol, there are no evident difference in 1/2 × (Fig. 4). The clusters of samples were a little lower in 1/3 × than 1 × and 1/2 ×, but the genotypes can be called correctly (Fig. 4). However, when dye terminator and Acyclo enzyme are decreased to 1/4, there are many missing samples in the reaction (Fig. 4). So, the dye terminator and Acyclo enzyme can be reduced by 1/2 × and 1/3 ×. Thus, the FP-TDI assay is used more cost-effectively.

Although the FP-TDI assay is one of several different methods that are currently available for automated genotyping, it has a number of advantages. First, this assay is cost-effective because it does not require modified probes such as dye-labeled probes. Second, the FP-TDI assay is simple to set up, (by adding the standard reagent minute to the DNA template), the results are obtained in electronic form minutes after the reaction is performed. Finally, FP-TDI assay uses FP as a detection format, which is independent of fluorescence intensity and requires no separation of free from bound dye ddNTP (Hsu *et al.*, 2001). It is a simple, highly sensitive, and specific detection method in a homogeneous primer extension reaction for single base-pair changes.

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