

AcycloPrime™, a novel method for SNP analysis using fluorescence polarization

Richard A. Greene*, James J. DiMeo, Mary E. Malone, Suzanne Swartwout,
Jianzhao Liu and Philip R. Buzby
PerkinElmer Life Sciences, 549 Albany Street, Boston, MA, US 02118-2512

ABSTRACT

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation between individuals of a species and are therefore thought to be responsible for a large part of individual phenotypic variation. It has been estimated that a SNP may occur every 100-300 bases in the human genome. Research on human SNPs is expected to facilitate genetic mapping studies that may lead to a better understanding of the genetic basis for complex diseases and individual variation in drug metabolism. We have developed a novel assay for the identification of known SNPs using primer extension with the novel AcycloTerminators™ and a new thermostable polymerase, AcycloPol™, in a homogeneous fluorescence polarization (FP) format. All assay steps can be performed in the same well of either a 384- or 96-well PCR-compatible microplate. FP provides several advantages, including simplicity and low reagent cost. The homogeneous assay format eliminates any need for separation or washing steps and is amenable to automation.

1. INTRODUCTION

Single nucleotide polymorphisms (SNPs) are thought to represent approximately 70% of the genetic variability between individuals¹. The identification and study of SNPs has become an important research area because the phenotypes associated with specific SNPs include economically and personally significant attributes such as susceptibility to diseases with complex genetic causes and the ability to metabolize drugs. The large number of SNPs in the genome also suggests their utility as markers for large-scale genetic mapping. For such high-density mapping, thousands of individuals will need to be haplotyped for a large number of SNPs.

An effective method for performing this large number of assays must be inexpensive, reliable, robust and easily automated. An ideal assay platform should be amenable to testing either large numbers of individual samples for a few SNPs or a few individual samples for numerous SNPs. We have developed a homogeneous assay for the analysis of known SNPs utilizing allele-specific primer extension for discrimination between the alleles, and fluorescence polarization for detection of the result.

2. MATERIALS AND METHODS

2.1 Materials

384-well (MSP-3862) or 96-well (HSP-9666) black, skirted PCR-compatible plates were purchased from MJ Research (Waltham, MA, USA). Primers were obtained from Genosys (The Woodlands, TX, USA). Exo-SAP-IT™ reagent (78201) was purchased from USB (Cleveland, OH, USA). AcycloPrime™ kits (ACP-101, -102, -103, -104, -106, and -109) were from PerkinElmer Life Sciences (Boston, MA, USA). DNA samples from 24 individuals (PD0001-PD0024) in the National Institutes of Health (NIH) Human

DNA Polymorphism Discovery Resource were used in some of this work. Publicly available markers from the dbSNP database (<http://www.ncbi.nlm.nih.gov/SNP/>) were used for most assays (NCBI assay IDs 231, 241, 221, 245, 686, 667, 693, 701, 674, 3188, 3388, 2043, 3308, 1194, 1230, 3596, 2619, 1185, and marker D7S8). For PCR and SNP primer sequence information on these markers, see Table 1 of Hsu, *et al.*² In some studies, 40-mer synthetic oligonucleotide templates derived from reference SNP rs656 were used instead of human DNA (Table 1). Using the complements of the template sequences, it was possible to create dsDNA targets and/or evaluate the reverse primer reaction.

Table 1
Synthetic Oligo Template and Primer Sequences

Oligo	Sequence (5' – 3')
A Template	ATTGGATTATTTGTAAC TCA AAGGATAAGTGCATAAGGGG
C Template	ATTGGATTATTTGTAAC TCC AAGGATAAGTGCATAAGGGG
G Template	ATTGGATTATTTGTAAC TCG AAGGATAAGTGCATAAGGGG
T Template	ATTGGATTATTTGTAAC TCT AAGGATAAGTGCATAAGGGG
SNP Primer	CCCCTTATGCACTTATCCTT
reverse SNP Primer	ATTGGATTATTTGTAAC T C

2.2 PCR Amplification

Human genomic DNA (5 ng) was amplified in 5 μ L reaction mixtures prepared according to the manufacturer's instructions using a commercially available PCR kit (GeneAmp[®] with AmpliTaq[®] DNA polymerase [N801-0055], ABI, Foster City, CA). If evaporation during cycling seemed to be significant (more common with 96-well plates), an extra 1 μ L of water was added into each reaction. Reactions used 50–1000 nM primers and 100 μ M dNTPs. Thirty or more thermal cycles were performed in a Tetrad[™] thermal cycler (MJ Research). After an initial incubation at 95°C for 10 minutes, each cycle consisted of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 60 seconds. The reaction mixtures were incubated at 72°C for 10 minutes for the final primer extension and then held at 4°C until further use. In some work larger reactions (up to 100 μ L) were used and subsequently divided into a number of replicate 5 μ L reactions for further analysis.

2.3 Degradation of excess PCR primers and dNTPs

After completion of the PCR reactions, excess PCR primers and dNTPs were degraded using Exo-SAP-IT reagent, which contains a mixture of exonuclease I and shrimp alkaline phosphatase. Because the glycerol in this reagent inhibited subsequent primer extension reactions, the reagent was diluted with an equal amount of water before use. 2 μ L of the diluted reagent were added to the 5 μ L PCR reaction and incubated at 37°C for 60 minutes. The enzymes were then inactivated by incubation at 80°C for 15 minutes.

Insufficient sample processing will cause assay problems. The recommended reagent and procedure can degrade 1 μ M PCR primers and 200 μ M dNTPs from the PCR reaction. Use of higher concentrations of these components during PCR may require modification of the sample processing conditions in order to obtain good results.

2.4 AcycloPrime primer design

When necessary, primers were designed using Oligo software, v6.57 (Molecular Biology Insights, Inc., Cascade, CO, USA). Primers were designed to have melting temperatures between 60°C and 80°C and lengths between 20 and 30 bases. As commonly done for PCR primers, attempts were made to minimize secondary structure and to keep the GC content to about 50% for SNP primers. Both forward and reverse primers were evaluated for each SNP.

2.5 Single-Base Extension

To the 7 µL of enzymatically-treated PCR product were added 13 µL containing components (AcycloPol, AcycloTerminator Mix, and 10X Reaction Buffer) from the AcycloPrime kit used. The reaction was incubated at 95°C for 2 minutes, followed by 10-30 cycles of 95°C for 15 seconds and 55°C for 30 seconds. To minimize warping of thin-walled plates, it was helpful to reduce the heated lid temperature to 85°C for the cycling, and to allow the heating block to cool for about 10 minutes down to 50°C – 60°C before removing the plate. If plate warping was still a problem, a plate positioner (MJ Research) was used to read the results. If necessary, samples were held at 4°C until read or reacted further.

2.6 FP Measurement

FP was measured on a Victor² V instrument (PerkinElmer). The polarization filters used were:

Fluorescent Dye	Excitation Filter	Emission Filter
R110	480 bandwidth 30	535 bandwidth 40
TAMRA	544 bandwidth 15	595 bandwidth 60

FP is defined by the formula: $mP = 1000(I_{vv} - I_{vh}) / (I_{vv} + I_{vh})$

where mP is a dimensionless millipolarization unit, I_{vv} is the emission intensity measured when the excitation and emission polarized filters are parallel and I_{vh} is the emission intensity measured when the filters are perpendicular. To facilitate comparisons between different sites and various instruments, the instrument G-factor for each dye was adjusted such that a solution containing unreacted terminators and buffer read 50 mP.

3. RESULTS

The exact combination of terminators and the polymerase used determines the properties of any primer extension method. The AcycloPrime assay method is based on an earlier published method³ called Template-directed Dye-terminator Incorporation with Fluorescence Polarization detection (TDI-FP). TDI-FP uses dideoxyterminators (ddNTPs) and either ThermoSequenase (APB) or AmpliTaq FS (ABI). These are polymerases from *Thermus aquaticus* (*Taq*), a thermophilic organism, that contain a tyrosine substitution in the active site needed to allow the enzymes to efficiently incorporate ddNTPs⁴.

During the last several years, we have screened a number of native and mutant thermostable polymerases for activity against a variety of terminators. Eventually we decided to use a thermostable polymerase, AcycloPolTM, from an Archeon not within the *Taq* family. This enzyme contains a mutation that improves its performance with AcycloTerminatorsTM (acyNTPs) a novel type of terminator, but the mutation is on the “backside” away from the nucleotide-binding site, not in the active site. Surprisingly, AcycloPol displays a strong preference for dye-acyNTPs, over not only dye-ddNTPs and unlabeled ddNTPs, but even

in some cases over normal dNTPs. This strong preference means that under optimum conditions AcycloPol is less likely to incorporate mismatched AcycloTerminators.

The differences between TDI-FP and AcycloPrime thus include the use of AcycloPol and AcycloTerminators in place of *Taq* polymerase and ddNTPs. As shown in Figure 1, ddNTPs terminate a growing nucleotide chain because they lack a hydroxyl group at the 3' position that is essential for chain elongation. AcycloTerminators have the same effect because they completely lack a 3' position.

The AcycloPrime assay process is shown schematically in Figure 2. AcycloPol is used to add one of two fluorescent terminators to an oligonucleotide primer that ends immediately upstream of the SNP position to be queried, and the terminator added is identified by its increased fluorescence polarization (FP). FP is an empirical technique that measures the vertical and horizontal components of the fluorescence emission produced after excitation by plane polarized light. Polarization values (in mP units) are inversely related to the speed of molecular rotation of the fluorescent target. Since molecular rotation is inversely related to the molecular volume, incorporation of a fluorescent AcycloTerminator into a primer oligonucleotide increases its polarization. This is used to determine which of the two labeled terminators has been incorporated into the primer.

The Terminator Mix from each AcycloPrime kit contains two labeled AcycloTerminators, one labeled with R110 and one labeled with TAMRA. These dyes were chosen because instrumentation permits their measurement in the absence of significant spectral overlap, eliminating any need for data corrections. Since there are only two labeled terminators, it is necessary to be certain that the labeled terminators in the kit match the bases to be incorporated for the particular SNP being tested. During assay development, it was determined that TDI-FP and AcycloPrime gave the same results when testing the same SNPs on the same DNA samples. It appeared from this work that AcycloPrime showed greater polarization values for the same assays than TDI-FP. We interpret this to be a result of the strong preference of AcycloPol for AcycloTerminators, leading to a greater extent of reaction at completion. The larger polarization values (mP) result in greater separation between the data clusters representing homozygotes, heterozygotes and the negative controls (Figure 3), making it easier and more reliable to identify the final genotype.

For verification of assay performance, 24 DNA samples were tested for more than eight SNPs and the results were confirmed by DNA sequencing. The assay was also validated using a number of SNP laboratories as beta test sites. Results from extensive testing at several sites show that about 80% of all new SNP assays work fine with no optimization at all. In cases where optimization is required, relatively simple steps usually result in a satisfactory method.

The most common cause of assay problems is the production of too much DNA in the PCR steps. What occurs then is a large amount of primer extension in each cycle, which rapidly depletes the available supply of the "correct" terminator. For certain SNP sequences, extensive cycling beyond this point can force misincorporation of the "incorrect" terminator. The observation indicating this occurrence is the migration of the homozygotic samples into the heterozygote cluster. Because the AcycloPrime reaction is very stable, it is possible to read the result after 10 cycles or so and then run some more cycles to cause the reaction to progress farther. Since misincorporation does not occur until the supply of the correct terminator is depleted, it is possible to stop cycling before misincorporation becomes a problem.

The simplest way to prevent misincorporation from occurring is to limit the amount of PCR product that can be produced. It is therefore recommended to use 50-200 nM, preferably 100 nM, PCR primers as a way to assure the production of an optimum amount of product (approximately 200 fmoles in 5 μ L). Because limiting primers reduces the efficiency of the PCR reactions, it is recommended to use 35 or more thermal cycles to assure the production of enough PCR products. Other methods that have been found useful in fixing problem assays are using less starting DNA, fewer PCR and/or primer extension cycles, increasing the amount of terminator added, or using single-stranded DNA binding protein to fix secondary structure problems with particular SNP primer sequences².

4. DISCUSSION

Of the many methods developed for analyzing SNPs, the AcycloPrime method requires the smallest number of synthetic oligonucleotides. Since the method uses oligonucleotides that are not labeled, they are less expensive than those required for most other methods. The AcycloPrime method also needs much less of the fluorescent terminators than either fluorescence resonance energy transfer (FRET) or direct fluorescence. The expense of sample processing is being offset by including the sample-processing reagent in the kits at a minimal cost.

When using a set of SNP markers to genotype a large number of samples, issues of oligonucleotide cost and optimization effort are amortized over a large number of assays. The issues become labor cost and the variable cost of each reaction. For moving quickly from one set of markers to another and developing assays along the way, methods requiring little or no optimization are more appropriate. With the AcycloPrime method, the design and optimization of an assay are usually very straightforward and most often successful. The homogeneous assay format means that no separation or purification steps are necessary and the assay is therefore robust, flexible, not labor-intensive and easily automated for high throughput. Data handling needs have been addressed through the recent posting of simple Excel macros for free download at the following web site: <http://lifesciences.perkinelmer.com/products/snp.asp>

ACKNOWLEDGMENTS

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Figures

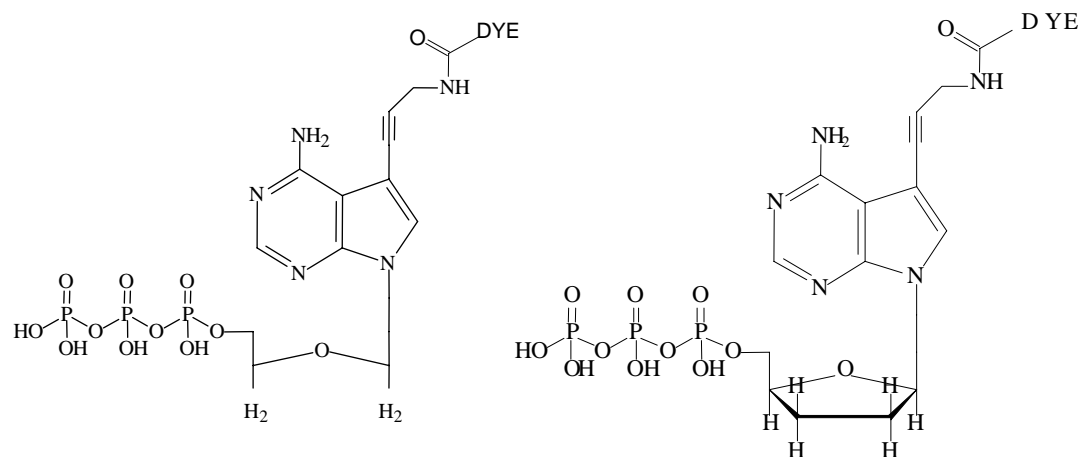


Figure 1. The structure of a dye-labeled-acyA AcycloTerminator (left) shows that the lack of a 3' position where the deoxyribose moiety would be on an ATP molecule causes this compound to behave as a chain terminator. The corresponding dye-labeled ddA dideoxyterminator structure (right) shows that the lack of a 3'-hydroxyl group in the same position causes the compound to behave as a chain terminator.

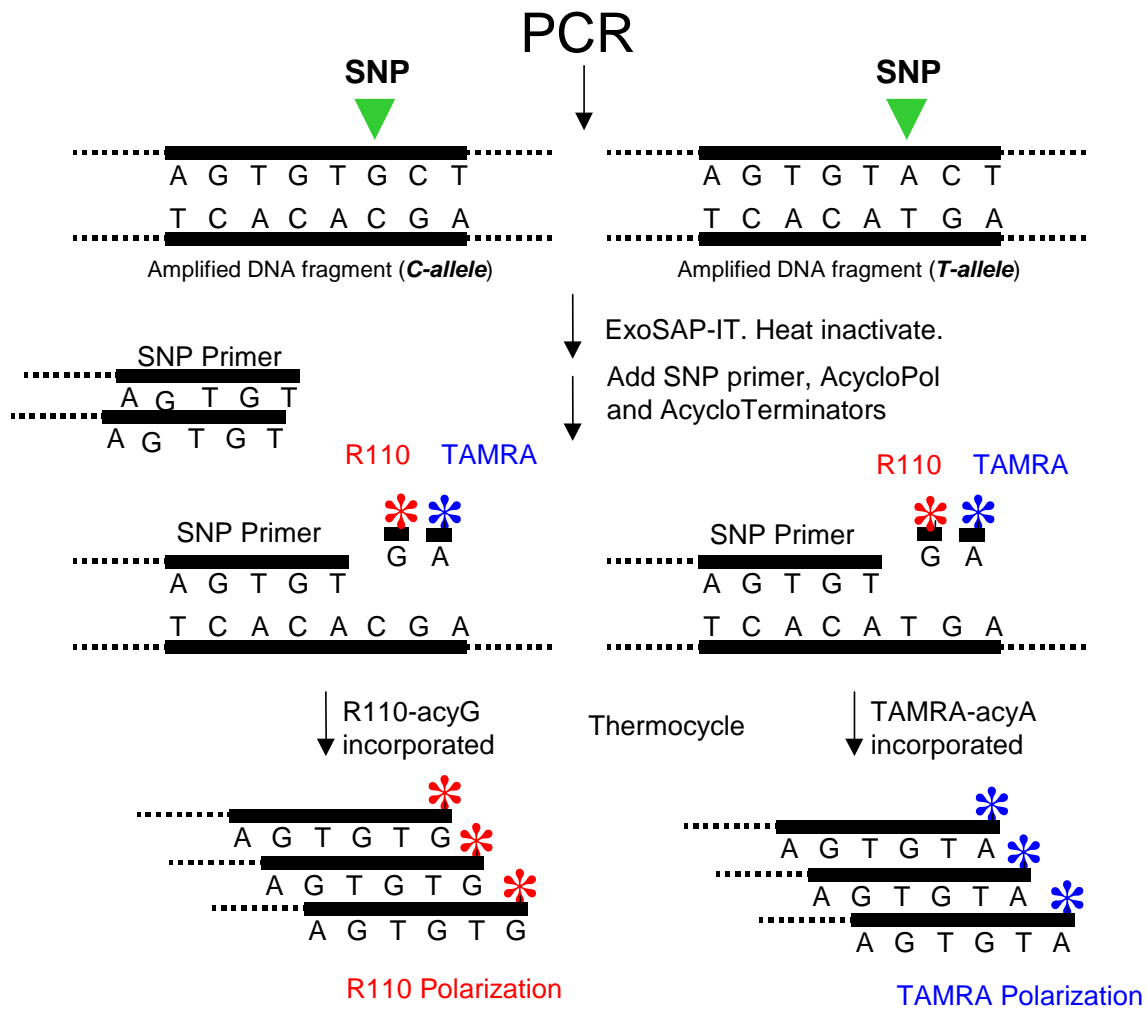


Figure 2. A schematic diagram illustrating the AcycloPrime process for a SNP assay.

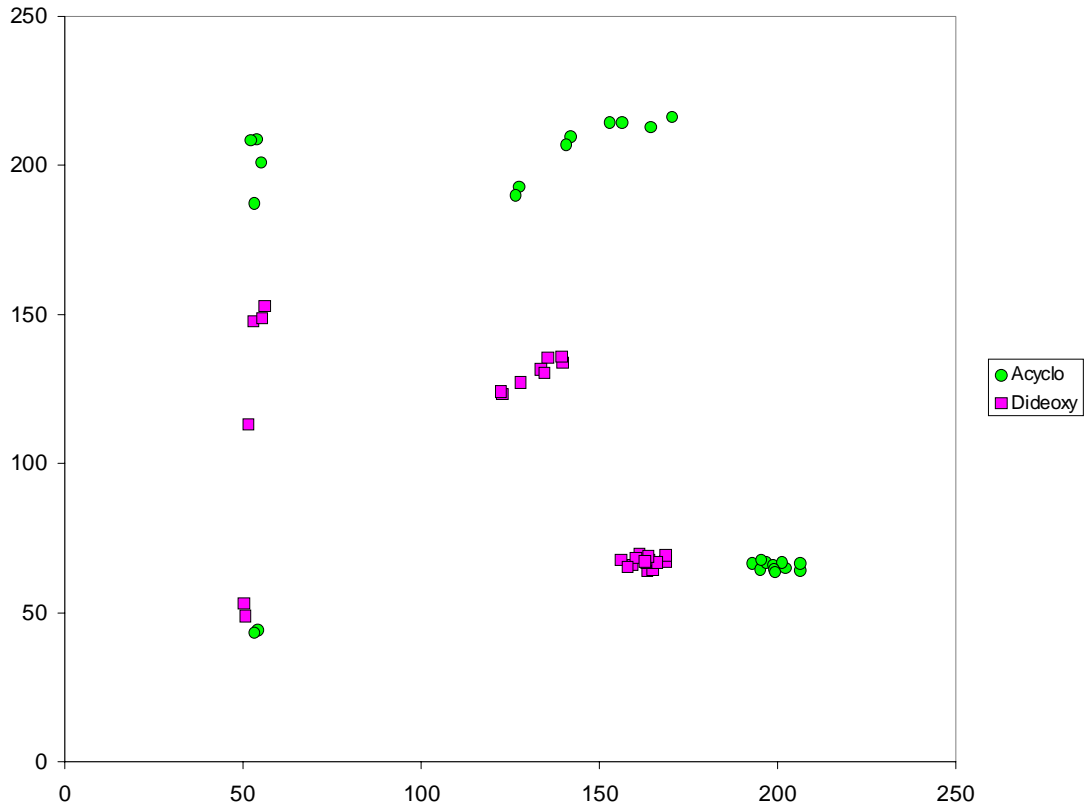


Figure 3. The same samples were assayed using the AcycloPrime process and by TDI-FP using dideoxyterminators and ThermoSequenase. The AcycloPrime process showed larger mP values for both dyes.