Genotyping Single-Nucleotide Polymorphisms by the Invader Assay with Dual-Color Fluorescence Polarization Detection

Tony M. Hsu,1 Scott M. Law,2 Shenghui Duan,1 Bruce P. Neri,2 and Pui-Yan Kwok1*

Background: The PCR-Invader® assay is a robust, homogeneous assay that has been shown to be highly sensitive and specific in genotyping single-nucleotide polymorphism (SNP) markers. In this study, we introduce two changes to improve the assay: (a) we streamline the PCR-Invader method by assaying both alleles for each SNP in one reaction; and (b) we reduce the cost of the method by adopting fluorescence polarization (FP) as the detection method.

Methods: PCR product was incubated with Invader oligonucleotide and two primary probes at 93 °C for 5 min. Signal probes corresponding to the cleaved flaps of the primary probes [labeled with fluorescein and 6-carboxytetramethylrhodamine (TAMRA) dye] and Cleavase® VIII enzyme (a flap endonuclease) were then added to the mixture. This reaction mixture was incubated at 63 °C for 5 min. FP measurements were made with a fluorescence plate reader.

Results: Eighty-eight individuals were genotyped across a panel of 10 SNPs, using PCR product as template, for a total of 880 genotypes. An average “no call” rate of 3.2% was observed after first round of experiments. PCR products were remade in those samples that failed to produce any genotype in the first round, and all gave clear-cut genotypes. When the genotypes determined by the PCR-Invader assay and template-directed dye-terminator incorporation assay with FP were compared, they were in 100% concordance for all SNP markers and experiments.

Conclusions: The improvements introduced in this study make PCR-Invader assay simpler and more cost-effective, and therefore more suitable for high-throughput genotyping.

Single-nucleotide polymorphisms (SNPs)3 are the most frequently found polymorphisms in any genome and have been touted as the genetic markers of choice for the study of complex genetic traits (1, 2). Compared with other genetic markers, such as simple tandem repeat polymorphisms (or microsatellites), SNPs have several distinct advantages (3). The first advantage is the abundance of SNPs in the human genome [with an frequency of approximately one per kilobasepairs (kb) when any two genomes are compared], which provides the density of markers needed for any genetic mapping strategy. The second advantage is that SNPs have very low mutation rates compared with microsatellite markers. This property makes SNPs the markers of choice for association studies. The third advantage is that many of the mutations in Mendelian disorders and possibly complex diseases are attributable to single-nucleotide changes (4–6). Therefore, many SNPs may be functionally important. The final advantage is that SNPs are readily amenable to high-throughput genotyping.

Several high-throughput methods are currently in use for typing SNPs, including high-density chip arrays for allele-specific hybridization analysis (7, 8), the homogeneous 5’ nuclease (TaqMan) assay (9, 10), the dye-labeled oligonucleotide ligation (DOL) assay (11), primer extension assay with detection by fluorescence polarization (FP) (12) or mass spectrometry (13), the Invader® assay (14), and the homogeneous molecular beacon allele-specific oligonucleotide (ASO) assay (15). Many of the high-throughput SNP genotyping methods require allele-specific, dye-labeled oligonucleotides for each SNP marker,
substantially increasing the start-up cost of the assays. The PCR-Invader® assay is a robust SNP genotyping method that does not require allele-specific, dye-labeled probes for every SNP marker. For the PCR-Invader assay, two generic dye-labeled probes are sufficient for all SNP markers. The PCR-Invader assay also offers the flexibility of using the best markers as they become available without having to redesign dye-label probes or high-density DNA chips. Furthermore, the PCR-Invader assay is easy to set up and is readily scalable for large-scale genetic studies.

The current reaction format of the PCR-Invader assay detects the cleavage of a doubly labeled fluorescent probe (the signal probe), using fluorescence resonance energy transfer (14). Briefly, PCR product is incubated with two allele-specific oligonucleotides, termed the Invader oligonucleotide and the primary probe. The Invader oligonucleotide anneals to the downstream portion of the polymorphic site, and the 3' region of the primary probe is complementary to the upstream region of the polymorphic site. When the polymorphism is complementary to the opposing base in the primary probe, the probe overlaps the 3' end of the Invader oligonucleotide, forming a structure that is recognized and cleaved by Cleavase® enzyme at a specific site, releasing the 5' arm of the primary probe (16). This cleaved 5' arm in turn serves as an Invader oligonucleotide in a second reaction, leading to the cleavage of the doubly labeled signal probe by the Cleavase enzyme. Because the signal probe is labeled at the 5' end with a fluorophore and internally with a quencher, the cleavage event removes the 5' fluorophore and enhances fluorescence.

In this study, we aimed to improve PCR-Invader assay through two changes. (a) We used FP as an alternative detection method to reduce the cost of the PCR-Invader assay. FP is observed when a fluorophore is excited by plane-polarized light and the molecule is of sufficient size that its rate of rotation is slower than the time scale of fluorescence emission. Any significant change in the molecular weight of the fluorophore will change the polarization of the emitted light (17, 18). In the Invader assay, a signal probe that is singly labeled with a fluorophore at its 5' end is used. When the signal probe is cleaved by the Cleavase enzyme, the molecular weight of the fluorophore decreases with a corresponding decrease in FP. (b) Using primary probes with two different arms and signal probes with two different fluorophores and arms, we are able to assay each sample in one reaction. A schematic representation of the Invader assay is shown in Fig. 1.

### Materials and Methods

SOURCE OF DNA

DNA samples from 88 individuals in the NIH Polymorphism Discovery Panel (PD0002-PD0089) were used in this study.

### Table 1. Primer and probe sequences for SNPs studied.

<table>
<thead>
<tr>
<th>Marker</th>
<th>NCBI assay ID</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>PCR primer 1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR primer 2</th>
<th>Primary probe 1</th>
</tr>
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<tbody>
<tr>
<td>TSC1383</td>
<td>21949</td>
<td>A</td>
<td>T</td>
<td>ATGAAACATGAGTCCACACTCCA</td>
<td>TATGCATACCCCTTCCCTCTC</td>
<td>CGCGCAGGGATGGTGCTGTCAGG</td>
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<tr>
<td>TSC1469</td>
<td>21989</td>
<td>G</td>
<td>T</td>
<td>AATCAAGAGCGACAGCTCAAG</td>
<td>ATTTCTGGATTGCTGTCAGG</td>
<td>CGCCGAGGAGGAGAGAGAGAGG</td>
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<tr>
<td>TSC1670</td>
<td>22091</td>
<td>C</td>
<td>A</td>
<td>CAGAAAACAGGCTCAATCCCTAAG</td>
<td>AAGCCTGTATAAAGGAGTACCA</td>
<td>CGCCGAGGAGGAGAGAGAGG</td>
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<td>AC4180B</td>
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<td>SS8168A</td>
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<td>G</td>
<td>C</td>
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<td>CGCCGAGGAGGAGAGAGAGG</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sequences for primers and probes are 5’–3’.

<sup>b</sup> NA, not applicable.

![Fig. 1. Invader assay.](image-url)
PLATES
All reactions were run and read in 96-well black-skirted plates purchased from MJ Research.

SNP MARKERS
Publicly available markers from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) were used in this study. The dbSNP assay numbers are listed in Table 1.

PCR PRIMERS
All PCR primers were purchased from Life Technologies.

ENZYMES
AmpliTaq Gold polymerase was purchased from Applied Biosystems. Cleavase enzyme was a generous gift from Third Wave Technologies, Inc. (Madison, WI).

DYE-LABELLED SIGNAL PROBES
Fluorescein-labeled signal probe and 6-carboxytetramethylrhodamine (TAMRA)-labeled signal probe were designed with an optimal annealing temperature of 55 °C (Table 2) and were obtained from Genset.

PCR AMPLIFICATION
Human genomic DNA (20 ng) was amplified in 10-μL reaction mixtures containing 1 μL of 10× PCR buffer (Applied Biosystems), 1 μL of 25 mM MgCl₂, 0.2 μL of 2.5 mM dNTP, 0.5 μL of PCR primers (2.5 μM of each), and 0.25 U of AmpliTaq Gold DNA polymerase. The reaction mixture was held at 95 °C for 10 min for AmpliTaq Gold enzyme activation, followed by 35 amplification cycles. Each cycle consisted of denaturation at 93 °C for 30 s, primer annealing at 58 °C for 40 s, and primer extension at 72 °C for 40 s. The reaction mixtures were then incubated at 72 °C for 5 min for final primer extension. At the end of the reaction, the reaction mixtures were held at 4 °C until further use.

INVADER ASSAY
Primary probes and the Invader oligonucleotide for each SNP marker were designed and synthesized by Third Wave Technologies, Inc. (Table 1). The primary probes and Invader oligonucleotide were designed to have theoretical annealing temperatures of 63 and 77 °C, respectively, using a nearest-neighbor algorithm on the basis of final probe and target concentrations. The Invader oligonucleotides were designed so that the 3′ base overlapped with the target SNP, but was not complementary to either allele. A 3′ amine was added to each primary probe to prevent uncleaved primary probe acting as an Invader oligonucleotide in the second reaction. Primary probes were purified by ion-exchange chromatography.

At the end of the PCR assay, 5 μL of primary probe/Invader oligonucleotide mixture was added to the PCR product. The primary probe/Invader oligonucleotide mixture contained 1 μmol/L primary probe 1, 1 μmol/L primary probe 2, 0.2 μmol/L Invader oligonucleotide, 40 g/L PEG-8000, and 10 mmol/L MOPS. This mixture was incubated at 93 °C for 5 min and then kept at 63 °C until further use. For the second step of the Invader reaction, 5 μL of signal probe cocktail was added to each reaction. This signal probe cocktail contained 0.05 μmol/L fluorescein-labeled signal probe 1, 0.05 μmol/L TAMRA-labeled

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**Table 2. Signal probe sequences.**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence</th>
<th>Wavelength, nm</th>
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</thead>
<tbody>
<tr>
<td>Fluorescein-labeled signal probe</td>
<td>5′-Fluorescein-TTCGTCCTGCCTAGGGAGAGAGACCTGACCGG-3′</td>
<td>485 530</td>
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<tr>
<td>TAMRA-labeled signal probe</td>
<td>5′-TAMRA-TTCGTCCTGGAGAGAGAGACCTGACCGG-3′</td>
<td>552 575</td>
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**Table 1. Continued**

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<th>Primary probe 2</th>
<th>Invader probe</th>
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<td>ATGACGTGGCGAGCTCTCCTTTTGGCTACCAAACTCAGA</td>
<td>TGGGTACCCCTGACCAAACTTCTGCAAACTC</td>
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<tr>
<td>ATGACGTGGCGAGCCATGACCGGCTCTCCCTACCAAACT</td>
<td>TCTCCAGTAAATGCGAGCTACACTATGCAGCAG</td>
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<td>ATGACGTGGCGAGGCGATGTTCCTATTACCAAACTAC</td>
<td>CACCGCTTGGTATGGTCTCTAGTAAAGGAA</td>
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<td>ATGACGTGGCGAGCGATGCCCTGTCCTGCAAACTCAC</td>
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<td>ATGACGTGGCGAGCGGGGTGCTGTCTGCAAACTCAC</td>
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<td>ATGACGTGGCGAGCTCCTGCTCCGTCACATAGCCAGCG</td>
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<td>ATGACGTGGCGAGCTCCTGCTCCGTCACATAGCCAGCG</td>
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<td>ATGACGTGGCGAGCTCCTGCTCCGTCACATAGCCAGCG</td>
<td>GGCTGTGAGGTTAGCGGCGGTGGGGAAAGGAA</td>
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signal probe 2, 0.05 μL Cleavase enzyme (100 mg/L), and 2.95 μL of signal buffer (56 mmol/L MgCl₂ and 25 mmol/L MOPS). The reaction mixture was incubated at 63 °C for 5 min and then kept at 4 °C until FP measurements.

**FP MEASUREMENT**

FP measurements were done on an LJI Analyst fluorescence reader (Molecular Devices). The FP value is defined by the formula:

\[ P = \frac{(I_{vv} - I_{vh})}{(I_{vv} + I_{vh})} \]

Where \( I_{vv} \) is the emission intensity measured when the excitation and emission polarized filters are parallel, and \( I_{vh} \) is the emission intensity measured when these filters are perpendicular. Polarization (P) is closely related to anisotropy, an alternative system for relating these measurements. In essence, polarization is twice the fraction of light that is polarized. It is useful to express the measurements as millipolarization (\( mP \)):

\[ mP = 1000 \times \frac{I_{vv} - (G \times I_{vh})}{I_{vv} + (G \times I_{vh})} \]

where \( G \) is a gating factor near a value of 1.0 used to adjust for slight differences in the two optical paths used (manufacturer’s information). \( G \) was estimated for each of the four dyes, using an assumed \( mP \) value for the dye of 30 \( mP \) and measured values for \( I_{vv} \) and \( I_{vh} \).

**Results**

Ten SNP markers previously genotyped in our laboratory were used in this study (Table 1). Genomic DNA from each of 88 individuals in the NIH Polymorphism Discovery Panel was typed for each marker, for a total of 880 genotypes. For each experiment, two signal probes were used, one labeled with fluorescein and the other labeled with TAMRA (Table 2).

After a standard protocol as described in Materials and Methods, the FP readings of the samples were found to cluster into four distinct groups for all 10 SNP markers. The results of a typical assay are shown in Fig. 2. The no-DNA negative controls, with both signal probes intact, had high FP values for both dyes analyzed and occupy the top right corner of the plot in Fig. 2 (with high fluorescein and TAMRA FP values) and most likely represent PCR failure. The PCR-Invader assay was repeated for all of these failed samples, and all of them gave unambiguous genotypes. The genotypes called based on the PCR-Invader assay were compared with the previously obtained results, using the template-directed dye-terminator incorporation assay with FP for all 10 SNP markers. The concordance rate of these experiments was 100% for all markers (880 genotypes).

**Discussion**

Several properties of PCR-Invader technology make it suitable for high-throughput genotyping. The PCR-Invader assay is a homogeneous assay requiring no gel electrophoresis, purification, or manual data entry. The assay can be performed in a single microtiter plate with no need for transferring or separation. Another advantage of this technology is its flexibility and robustness. No redesigning or lengthy manufacturing of specialty probes or microarrays is necessary when a new marker is needed for a study; in addition, two universal, fluorophore-labeled oligonucleotides are sufficient for all SNP markers.

One major disadvantage of the current technology is
the need to assay the two alleles of each SNP in separate reaction wells. This reaction format makes this assay more time-consuming and labor-intensive. Furthermore, genotype miscalling can occur when one of the two reactions of the sample does not work, leading to a heterozygous individual being mistyped as homozygous. With FP detection, we used two signal probes with two different fluorophores, fluorescein and TAMRA. Consequently, we were able to assay each sample in one reaction, thereby saving time and reducing genotype miscalls. The use of more than two fluorophores would also allow multiplexing of assays in the future.

In this study, we also showed that FP is a simple, cost-effective, and accurate detection method for the PCR-Invader assay. Because FP is independent of fluorescence intensity, it requires no separation of cleaved fluorescent probes from the intact fluorescent probes. The use of FP as a detection method also opens up the possibility of using a somewhat less costly probe, without the need for the quencher to be present. In addition, FP detection works best when the cleavage reaction is driven to completion and makes it possible to use a limiting amount of the modified probes, further reducing the cost of the reaction. As more complex diseases are being studied and more SNPs are made available, flexible and robust genotyping methods such as the PCR-Invader assay discussed here will be of great utility.

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References