Flow Cytometric Platform for High-Throughput Single Nucleotide Polymorphism Analysis

GlaxoWellcome Research and Development, Research Triangle Park, NC, USA

ABSTRACT

We have developed a rapid, cost-effective, high-throughput readout for single nucleotide polymorphism (SNP) genotyping using flow cytometric analysis performed on a Luminex™ 100 flow cytometer. This robust technique employs a PCR-derived target DNA containing the SNP, a synthetic SNP-complementary ZipCode-bearing capture probe, a fluorescent reporter molecule, and a thermophilic DNA polymerase. An array of fluorescent microspheres, covalently coupled with complementary ZipCode sequences (cZipCodes), was hybridized to the reaction products and sequestered them for flow cytometric analysis. The single base chain extension (SBCE) reaction was used to assay 20 multiplexed SNPs for 633 patients in 96-well format. Comparison of the microsphere-based SBCE assay results to gel-based oligonucleotide ligation assay (OLA) results showed 99.3% agreement in genotype assignments. Substitution of dideoxynucleotides, thereby allowing multiplexing of all alleles of a particular SNP.

INTRODUCTION

As the sequence of the human genome approaches completion, much attention is being focused on single nucleotide polymorphisms (SNPs), the most prevalent form of genetic variation. One SNP is estimated to occur approximately every 1000 bp with a total of over three million in the human genome (4). Due to their frequency and distribution, SNPs may serve as superior or genetic markers for the assembly of a high-resolution map, aiding the identification of disease-related loci (11).

Another potential utility of SNPs is to help establish individualized medicine. The powerful, target-specific pharmaceuticals being developed today can bring profound improvements to the lives of many patients, but may have serious side effects in certain subpopulations. The genetic variation behind these differing biological responses may correlate with a small set of SNPs that could serve as a diagnostic tool to insure prescription of “the right medicine to the right patient”.

Current assays used for SNP detection and screening include restriction fragment length polymorphism (RFLP) analysis, single-strand conformation polymorphism (SSCP) analysis (17), allele-specific oligonucleotide (ASO) hybridization (19), oligonucleotide ligation assay (OLA) (12), primer extension assay (20), TaqMan® (Applied Biosystems, Foster City, CA, USA) (13), molecular beacons (21), and structure-specific flp nuclease technology (16). A variety of platforms have been used to analyze reaction products, including gel electrophoresis, fluorescence polarization (3), semiconductor chips (8), and mass spectrometry (6).

We have adapted the use of fluorescent microspheres in flow cytometric analysis (7,10,14,15) for SNP determination. We previously demonstrated the proof-of-concept for this SNP detection platform using FACSalibur™ (Becton Dickinson, Franklin Lakes, NJ, USA) instrumentation for the analysis of OLA (9) and single base chain extension (SBCE) (2) assays. In this study, we demonstrate the robustness, accuracy, and higher throughput achieved for DNA polymerase-based SNP analysis by using a less expensive flow cytometric platform with a 96-well plate reader. We also demonstrate a novel microsphere-based DNA polymerase assay that uses an allele-specific primer extension (ASPE). The ASPE assay permits multiplexed querying of different nucleotides, thereby allowing multiplexing of all alleles of a particular SNP.

MATERIALS AND METHODS

Reagents

AmpliTaq®, AmpliTaq Gold®, and AmpliTaq® FS DNA polymerases were purchased from Applied Biosystems. PLATINUM® GenoTYPE™ Tsp DNA polymerase was ordered from Life Technologies (Rockville, MD, USA). Shrimp alkaline phosphatase (SAP), E. coli exonuclease I (ExoI), and unlabeled ddNTPs were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Biotin-labeled ddNTPs, biotin-labeled dCTP, and R6G-labeled ddNTPs were obtained from NEN® Life Science Products (Boston, MA, USA). Streptavidin-phycoerythrin (SA-PE) was obtained from Molecular Probes (Eugene, OR, USA). Unmodified oligonucleotides were purchased from Keystone Biosource (Ca-
marillo, CA, USA). Oligonucleotides with 5’ amino groups were ordered from Applied Biosystems. 2-[N-morpholino]ethanesulfonic acid (MES) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma (St. Louis, MO USA) and Pierce Chemical (Rockford, IL, USA), respectively. Carboxylated fluorescent polystyrene microspheres were purchased from Luminex (Austin, TX, USA).

**Design of Oligonucleotides as Capture Probes**

All capture probes were designed with a 25-nucleotide ZipCode sequence at the 5’ end and an allele-specific sequence at the 3’ end. The allele-specific sequence varied in length but always possessed a melting temperature ($T_m$) of 51°C–56°C. For each SNP analyzed by SBCE, a single capture probe was synthesized with its allele-specific portion ending immediately before the polymorphic base. For each biallelic SNP analyzed by ASPE, two capture oligonucleotides were synthesized, each probe differing in the choice of ZipCode and in the polymorphic nucleotide contained at its 3’ terminus. Thus, for a given SNP, ASPE probes were one base longer than the SBCE probe and were identical to the OLAs capture probes (9).

**Design of Oligonucleotides for Microsphere Attachment**

Oligonucleotides were designed to contain four distinct structural elements. Described from 5’ to 3’, these elements include a 5’ amino group for covalent attachment to the carboxylated microsphere surface, an 18-atom spacer ($CH_2CH_2OH)_6$ to minimize potential interaction between the oligonucleotide sequence and the microsphere surface, a 10-base LUCtag sequence (CAGGC-CAAGT) to monitor the coupling efficiency of the oligonucleotides to the microspheres, and one of a set of 25-base complementary ZipCode sequences (cZipCodes). This set of 58 cZipCodes was arbitrarily selected from the *Mycobacterium tuberculosis* genome and was checked empirically for absence of cross-hybridization between members of the set (9).

**Coupling of Oligonucleotides to Microspheres**

Carboxylated microspheres (5 x 10^6; 400 μL) were pelleted, resuspended in 50 μL 0.1 M MES buffer, and mixed with 2 nmol (2 μL 1 mM) amino-modified oligonucleotide. Ten microliters of fresh EDC (30 mg/mL) in water were added to the microsphere/oligonucleotide mixture and incubated at room temperature. After 30 min, a fresh 10-μL aliquot of EDC was added. In each case, EDC solution was made immediately before addition to the reaction. The reaction mixture was mixed and sonicated occasionally during incubation to assure microsphere separation and suspension. After total incubation for 1 h, the microspheres were washed with 1 mL 0.1% SDS, then with 1 mL 0.02% Tween® 20, and finally resuspended in 500 μL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored in the dark at 4°C.

**Coupling Reaction Quality Assurance**

Coupling reaction success was assessed by hybridizing coupled microspheres with a molar excess of biotinylated oligonucleotide complementary to the LUCtag sequence. The standard procedure was the same as that detailed below for the hybridization of reaction products to the microspheres. Effective coupling reactions produced microspheres with a mean fluorescent intensity (MFI) of 2000–4000 U. Microspheres with MFIs less than 1000 were replaced.

**PCR Amplification**

PCRs were performed in a Polyfilltrons™ 96-well plate on a PTC-100™ thermal cycler (MJ Research, Waltham, MA, USA). A typical 15-μL reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.1 mM dNTPs, 0.2 μM each primer, AmpliTaq Gold DNA polymerase (1.5 U) and 20 ng genomic DNA. The reaction was held at 95°C for 10 min to activate the DNA polymerase, followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. After a final 5-min extension at 72°C, the reaction was held at 4°C.

**PCR Cleanup before SBCE**

Equal volumes of all PCR products to be simultaneously assayed were pooled together and enzymatically treated to degrade excess PCR primers and dNTPs using 1 U SAP and 2 U ExoI per 10 μL PCR products. The reaction was thoroughly mixed and incubated at 37°C for 30 min, followed by 15 min at 80°C to inactivate the enzymes. Sensitivity of the subsequent SBCE assay made concentration of the treated PCR products unnecessary.

**SBCE Reactions**

Two nearly identical reactions were set up, differing only in the choice of labeled ddNTP. Ten microliters of pooled, treated PCR products (10-20 ng each product) were added to 10 μL 2× SBCE reaction mixture containing 160 mM Tris-HCl (pH 9.0), 4 mM MgCl₂, 25 nM positive control target oligonucleotide, 25 nM positive control capture oligonucleotide, 50 nM each SNP capture oligonucleotide, 2.4 U AmpliTaq FS, 2 μM allele-specific labeled ddNTP, and 2 μM each of the other three ddNTPs. The reactions were denatured at 96°C for 2 min, followed by 30 cycles of extension at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Reactions were held at 4°C before the addition of microspheres.

**ASPE Reactions**

Ten microliters of pooled, untreated PCR products were added to 10 μL 2× ASPE reaction mixture containing 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 2.5 mM MgCl₂, 25 nM positive control target oligonucleotide, 25 nM positive control capture oligonucleotide, 50 nM each SNP capture oligonucleotide, 1.5 U Tsp DNA polymerase, and 6 μM biotin-dCTP. The reactions were denatured at 96°C for 2 min, followed by 30 cycles at 94°C for 30 s, 55°C for 1 min, and 74°C for 2 min. Reactions were held at 4°C before the addition of microspheres.

**Hybridization of Reaction Products to the Microspheres**

One thousand microspheres of each type were added to the 20 μL SBCE or ASPE reaction mixture for a final vol-
Ten samples of a 22-microsphere mixture were each hybridized to 20 pmol biotinylated oligonucleotide complementary to the LUCtag. Fluorescent signals were generated by the addition of one of the following dilutions of SA-PE: 10-fold (500 ng), 20-fold (250 ng), 40-fold (125 ng), 60-fold (83 ng), 80-fold (63 ng), 100-fold (50 ng), 125-fold (40 ng), 150-fold (33 ng), 175-fold (29 ng), or 200-fold (25 ng). The resulting MFI values from three representative microsphere types were graphed. Microsphere 39 was a negative control without oligonucleotide coupled to its surface. Microsphere 58 produced the lowest positive signal, and microsphere 39 produced the highest signal in the 22-microsphere mixture.

<table>
<thead>
<tr>
<th>Ionic Strength (mM NaCl)</th>
<th>Microspheres Counted a</th>
<th>Time (s) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3200</td>
<td>8.5</td>
</tr>
<tr>
<td>300</td>
<td>3200</td>
<td>9.8</td>
</tr>
<tr>
<td>400</td>
<td>3200</td>
<td>10.1</td>
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<tr>
<td>500</td>
<td>3200</td>
<td>57.7</td>
</tr>
<tr>
<td>600</td>
<td>3200</td>
<td>79.3</td>
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<tr>
<td>700</td>
<td>3022</td>
<td>132.8</td>
</tr>
<tr>
<td>800</td>
<td>1854</td>
<td>132.9</td>
</tr>
<tr>
<td>900</td>
<td>1247</td>
<td>132.1</td>
</tr>
<tr>
<td>1000</td>
<td>1267</td>
<td>133.4</td>
</tr>
</tbody>
</table>

a Instrument settings were configured to count 3200 microspheres total.
b Maximum time was limited to 133 s because that is the length of time required to inject 100% of the 55-µL sample volume into the flow cell.

**Figure 1. SA-PE titration curve.** Ten samples of a 22-microsphere mixture were each hybridized to 20 pmol biotinylated oligonucleotide complementary to the LUCtag. Fluorescent signals were generated by the addition of one of the following dilutions of SA-PE: 10-fold (500 ng), 20-fold (250 ng), 40-fold (125 ng), 60-fold (83 ng), 80-fold (63 ng), 100-fold (50 ng), 125-fold (40 ng), 150-fold (33 ng), 175-fold (29 ng), or 200-fold (25 ng). The resulting MFI values from three representative microsphere types were graphed. Microsphere 39 was a negative control without oligonucleotide coupled to its surface. Microsphere 58 produced the lowest positive signal, and microsphere 39 produced the highest signal in the 22-microsphere mixture.
volume of 30 µL containing 500 mM NaCl and 13 mM EDTA. The mixture was incubated at 40°C for 1 h.

**Removal of Excess Biotin-ddNTPs**

Microspheres were washed by addition of 150 µL 1× SSC/0.02% Tween 20 at room temperature (1× SSC is 150 mM sodium chloride, 15 mM sodium citrate, pH 7.0) to remove free biotin-ddNTPs. After centrifugation at 1100×g for 5 min, most of the supernatant was removed using the HydraÒ-96 micro-dispenser (Robbins Scientific, Sunnyvale, CA, USA), typically leaving less than 10 µL (5.6%) of the volume behind. The pelleted microspheres were resuspended in 60 µL 1× SSC/0.02% Tween 20 for flow cytometric analysis.

**Addition of SA-PE to Biotin-Modified Capture Probes**

SA-PE reagent was diluted 1:10 in PBS, and 5 µL were added to the microsphere-hybridized SBCE or ASPE reaction products in 60 µL 1× SSC/0.02% Tween 20. The mixture was incubated for 30 min at room temperature and analyzed without further cleanup.

**Flow Cytometric Analysis**

Microsphere fluorescence was measured using a Luminex 100Ò cytometer (Luminex) equipped with a Luminex XYPÒ plate reader and LuminexÒ software. Data were collected from a minimum of 30 microspheres of each type.

**Data Analysis**

Data were collected from a 96-well plate containing target DNA from 96 different patient samples analyzed for one allele of a biallelic SNP. Following application of the three-step normalization calculations described below, data from the two corresponding allelic plates were merged, allowing the display of the results on a two-coordinate system in which allelic calls were made and written to a database.

**Step 1. Adjustment for Microsphere Background Fluorescence**

Each microsphere type in the 100-microsphere set was identified by its characteristic fluorescence of red and infrared wavelengths. However, each microsphere type also emitted a small amount of its own characteristic fluorescence in the orange wavelengths of the reporter channel. This inherent, analyte-independent reporter signal of each microsphere was subtracted from the MFI value of each sample on the corresponding microsphere. Microsphere corrections ranged from values of 1–100 MFI, depending on the microsphere type, and were consistent across different preparations of microspheres.

**Step 2. Negative Control Adjustment for Variability in Solution Background Fluorescence across a Plate**

Because of features incorporated into the Luminex 100 software, background contributions to fluorescence by the solution were minimal. The mean background fluorescence was 25 MFI, with a typical range of 1–50 MFI. To adjust for well-to-well variability in the wash step, every sample well contained a microsphere with no cZipCode attached. Adjusted MFI (as described in Step 1 above) from this negative control microsphere was subtracted from the MFI of every microsphere type in that particular well.

**Step 3. Positive Control Adjustment for Variability in Reaction Efficiency across a Plate**

A positive control reaction in each well was used to adjust for varying

![Figure 2. Comparison of R6G and PE reporters. Three SNPs were assayed on three microsphere types in multiplex SBCE format for 96 patients using either R6G-labeled or biotin-labeled dideoxynucleotides. The different labeling protocols were performed separately, using different preparations of PCR target DNA that had been independently amplified and treated. Normalized MFI values for the G allele are plotted on the Y-axis, while A-allele values appear on the X-axis.](image-url)
amounts of product due to variability in addition of reagents or thermal cycling efficiency of a particular well location. The mean positive control MFI of all 96 reactions across an individual plate was calculated. Wells with a positive control MFI that was three standard deviations from the mean MFI were eliminated from further SNP analysis. A new mean positive control MFI was calculated without values from the eliminated wells. MFI values for all microsphere types in the non-eliminated wells were multiplied by a well-specific normalization constant, which was calculated to adjust the positive control MFI to the new mean positive control MFI.

RESULTS

Salt Effect

Among several modifications necessary to successfully transfer the SBCE protocol (2) from the FACScalibur to the Luminex 100 instrument, we found that reducing the ionic strength of the hybridization step was critical for correct microsphere classification. When microspheres were analyzed in NaCl solutions of 400 mM or less, the instrument easily counted an average of 100 events for each of 32 microsphere types within 10 s (Table 1). However, under higher salt conditions, a smaller percentage of microspheres was correctly classified. This extended the read time and, above 700 mM NaCl, also prevented the accumulation of the prescribed 3200 events. This salt phenomenon probably arises from differences between the refractive indices of the high ionic strength samples containing the microspheres and the low ionic strength sheath fluid.

Photobleaching of Microspheres

Microspheres were rigorously protected from ambient light by wrapping their containers in aluminum foil. The revision A microsphere set intended for use with the Luminex 100 contains mixtures of red and infrared dyes in varying proportions. Some of these microspheres appear to be more sensitive to photobleaching than the revision 0 microspheres, which are embedded with red and orange dyes and are used for analysis on the FACScalibur instrument. In particular, we have observed that the subset of eight 90-series, revision A microspheres (nos. 92–99) shift significantly out of their normal map positions after a 1-h exposure to ambient light.

Positive Control

Even in a complex reaction mixture in which 20 different genomic locations are being assayed for polymorphisms in a single patient sample, it is possible that the queried allele will not be present (i.e., the patient may be homozygous for the other allele at all 20 loci). In this case, the sample would generate no signal on any of the 20 microspheres. Although this may be the legitimate result of a successful assay, the same result would be observed if the polymerase reactions failed for that assay point. To minimize the occurrence of false negatives, we developed a positive control to serve as an internal standard in every assay well.

Three different pairs of synthetic target and capture probes were designed from randomly selected genomic sequence of *M. tuberculosis* H37Rv genome (GenBank® accession no. AL022001). Positions of the oligonucleotides were chosen to ensure the maximum of probe overlap.

### Table 2. Oligonucleotide Sequences

<table>
<thead>
<tr>
<th>Oligonucleotide Name</th>
<th>Probe Type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence of Oligonucleotide (5’→3’) (nucleotides)</th>
<th>Length (nucleotides)</th>
<th>T&lt;sub&gt;m&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt; (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTRL.000.NN1 target</td>
<td></td>
<td>AACCAGGGGCCAACCAACNAATCCAAGTGCAA</td>
<td>40</td>
<td>52</td>
</tr>
<tr>
<td>CTRL.023.NN1 ZipCode/capture</td>
<td></td>
<td>GTAAATTCGCCAGCAGGAAGGTCAGGATCGGCGGAGCGG</td>
<td>45</td>
<td>52</td>
</tr>
<tr>
<td>CTRL.000.NN2 target</td>
<td></td>
<td>ACCGCCGAGGCTATAGGGTGGTCGGTTAATGGCCA</td>
<td>39</td>
<td>54</td>
</tr>
<tr>
<td>CTRL.023.NN2 ZipCode/capture</td>
<td></td>
<td>GTAAATTCGCCAGCAGGAAGGTCAGGATGGGCGGCGGAGCGG</td>
<td>44</td>
<td>53</td>
</tr>
<tr>
<td>CTRL.000.NN3 target</td>
<td></td>
<td>AACCAGGGGCCAACCAACNAATCCAAGTGCAA</td>
<td>40</td>
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<tr>
<td>CTRL.023.NN3 ZipCode/capture</td>
<td></td>
<td>GTAAATTCGCCAGCAGGAAGGTCAGGATGGGCGGCGGAGCGG</td>
<td>45</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>a</sup>Target sequence was derived from the MTV406 locus of *M. tuberculosis* H37Rv genome (GenBank® accession no. AL022001).

<sup>b</sup>Melting temperature of capture/target duplex.

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sayed in a given sample well. Each capture probe was designed with a 25-nucleotide ZipCode on the 5’ end and a 19–20-nucleotide target-specific sequence on the 3’ end. All three controls produced similar SBCE assay results. The synthetic target CTRL.000.NN1 was selected for use as the standard positive control.

Validation of SBCE Reaction

To help establish high-throughput genotyping assay and analysis protocols, microsphere-based SBCE assays were performed on 633 patient samples across 20 SNPs using R6G-labeled ddNTPs. These patient samples and SNPs had previously been assayed by gel-based OLA (1.5). Of the 20 SNPs assayed, two were not successfully converted to the SBCE format. These two SNPs generated such low signal intensities that genotypic calls proved to be unreliable.

A total of 9563 genotypes were generated using both OLA and SBCE assays, which allowed direct comparison of results from the two methods. There was an average 99.3% agreement in genotype assignments. Four of the SNPs (22%) produced results with 100% agreement across all patient samples for which comparisons could be made. The poorest average agreement was 98.2% for one SNP, with a range from 95.7% across a single 96-well plate to 100% across a different plate of samples.

Indirect Labeling of SBCE Products

Phycocerythrin (PE) is a large fluorochrome with a high quantum yield that is compatible with the Luminex 100 laser system. Although this molecule generates brighter signals than R6G, it is only commercially available as a streptavidin conjugate and not as a nucleotide adduct. To employ a PE label, biotinylated ddNTPs were substituted for R6G-labeled ddNTPs in the standard SBCE reaction. An additional, post-polymerase reaction was required in which the SA-PE conjugate is allowed to bind to the biotinylated capture probe. The very strong binding of streptavidin to biotin was used for the detection of the modified capture probes. To test the signal intensity generated by PE in this assay, a titration curve was established using SA-PE (Figure 1). The observed signal decreased sharply if less than 63 ng of SA-PE were added to the reaction. We routinely add 500 ng to ensure detection of SNPs with weaker signal strength. The addition of 5000 ng SA-PE generated a background signal that could not be filtered out by the instrument software, and the negative control microsphere exhibited a strong positive signal. The threefold variation in signal observed between microsphere 58 and microsphere 79 is a function of the average number of oligonucleotides covalently attached to the microsphere surface. This is the typical range of variation observed when microsphere-saturating hybridizations are performed.

Figure 2 shows a set of scatter plots comparing PE and R6G for three representative SNPs in 96 patients. These data demonstrate that the use of SA-PE improved signal strength by up to tenfold and produced tighter data clusters, resulting in more robust and reliable genotype calls.

ASPE Assay

In an effort to simplify the SNP assay protocol, a new polymerase assay was developed that required no PCR cleanup and that used only a single labeled nucleotide. The ASPE assay was validated by genotyping a separate set of 15 SNPs in multiplex format using PCR-amplified target DNA from 96 patients. The assay signal intensity was similar to the SBCE-generated signal strength. SNP 1979 generated the strongest signals with the following average MFI values: A allele signal of 1473, A allele background of 89, G allele signal of 1386, and G allele background of 317. Although SNP 1210 displayed the weakest signals of the 15 SNPs tested, all patient genotypes for this SNP were readily determined. SNP 1210 produced average A allele signals of 361 MFI and average G allele signals of 303 MFI. The average G allele background was 68 MFI. The samples assayed contained no GG homozygous individuals, and, therefore, an A allele background could not be determined. Most importantly, comparing this set of over 1400 ASPE genotypes with OLA genotypes showed 98.7% agreement.

DISCUSSION

We have modified several assays to perform accurate high-throughput genotyping on the Luminex 100 fluorimeter. This flow cytometric platform offers several major advantages over conventional flow cytometers, including several-fold reduction of initial instrument cost, an increase in sample throughput by using 96-well plates instead of single tubes, and a decrease in injected sample volume.

PE is the fluorochrome of choice for use with the Luminex 100 system. Although usable data has been generated with nucleotides directly labeled with R6G or TAMRA, the magnitude of the signal and the signal-to-noise ratio are inferior to that observed with SA-PE. When using the biotin-streptavidin indirect detection method, we have found that the reagent stoichiometries make the assay signal intensities quite sensitive to residual pools of unincorporated biotinylated ddNTP and unhybridized biotinylated capture probe. Care must be taken to consistently remove this supply of biotin before the addition of SA-PE.

We observed rather large differences in signal intensity generated between different SNPs when assaying PCR-derived targets. This variation in signal strength between SNPs was as large as fivefold and appears to be predominately dependent on the nucleotide sequence. Experiments in which different ZipCodes were employed with the same allele-specific sequence, or in which the same ZipCode was used with two opposite-strand probes for a single SNP, suggest that the variation is independent of ZipCode. It seems likely that the observed differences in signal intensities arise from DNA polymerase sequence preferences (18) and/or from probe secondary structure interference in the extension reaction. Additionally, signal intensity of the positive control was seen to be dependent on the specific fluorescent nucleotide being added to the polymerase reaction and displayed the general order of C > A > G > U in the SBCE assay.

The SBCE assay only permits SNPs with overlapping allele variants to be multiplexed. This forces segregation of SNPs into sets for analysis and can greatly complicate experimental design.
This difficulty is overcome with the ASPE assay, which allows multiplexed SNP analysis for any mixtures of allelic variants. This advantage is possible because the “query” nucleotide is part of the ASPE capture probe, while the signal-generating “labeled” nucleotide is free biotin-dCTP. In the SBCE assay, the biotin-ddNTP serves as both “query” and “labeled” nucleotide. Another advantage conferred by the ASPE assay is simplification of the reaction protocol. The residual dNTPs from the target-generating PCR are used for the primer extension, thereby eliminating both the necessity of post-PCR cleanup and the addition of unlabeled nucleotides to the ASPE reaction.

It can be difficult to establish an assay cost per SNP because that cost will vary dramatically depending on how the assay is employed. Cost parameters include the total number of assays run, the number of simultaneous assays performed (multiplex factor), and whether many SNPs are assayed on few patients (genome-wide scan) or whether few SNPs are assayed on many patients (targeted genomic region). The microspheres and coupling reagents are inexpensive compared to reagents such as enzymes or fluorescent nucleotides. We estimate our average cost is less than US $0.20 per SNP, excluding the cost of generating the PCR target. SBCE and ASPE reactions are comparable in cost. Attempts to reduce assay costs further have concentrated on minimizing reagent consumption, especially that of the enzyme and fluorochrome. Recently, we have successfully generated short target DNAs in 50-plex PCRs and have also migrated from 20-plex to 50-plex SBCE reactions. Even higher levels of complexity are theoretically possible using the complete 100-microsphere set.

In an attempt to minimize the subjectivity of genotypic calls, various data clustering algorithms are currently under development that will allow automatic assignment of genotypes to the different clusters. Observed challenges to automated allele scoring include variability in tightness of data clustering, dissimilar signal intensities between the two alleles, and the formation of extraneous data clusters due to previously undetected polymorphisms within the probe sequence. Extensive testing on large data sets will not only allow refinement of these algorithms but also may identify characteristics of problematic markers that can be avoided in future probe design.

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Address correspondence to:
J. David Taylor
Glaxo Wellcome, Inc.
5 Moore Drive
Research Triangle Park
NC 27709-3398, USA
email: jdt6451@gxowlowcome.com