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Multiplexed genotyping of ABC transporter polymorphisms with the Bioplex suspension array

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Abbreviations: ABC, ATP-binding cassette; ASPE, allele-specific primer extension; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; MES, 2[N-Morpholino] ethanesulfonic acid; MFI, median fluorescence intensity; SAP, shrimp alkaline phosphatase; SDS, sodium dodecyl sulfate; SNP, single nucleotide polymorphism.

ABSTRACT

We have developed and validated a consolidated bead-based genotyping platform, the Bioplex suspension array for simultaneous detection of multiple single nucleotide polymorphisms (SNPs) of the ATP-binding cassette transporters. Genetic polymorphisms have been known to influence therapeutic response and risk of disease pathologies. Genetic screening for therapeutic and diagnostic applications thus holds great promise in clinical management. The allele-specific primer extension (ASPE) reaction was used to assay 22 multiplexed SNPs for eight subjects. Comparison of the microsphere-based ASPE assay results to sequencing results showed complete concordance in genotype assignments. The Bioplex suspension array thus proves to be a reliable, cost-effective and high-throughput technological platform for genotyping. It can be easily adapted to customized SNP panels for specific applications involving large-scale mutation screening of clinically relevant markers.

INTRODUCTION

The advances in biomedical science have led to the discovery of genes that may influence therapeutic outcome and disease susceptibility. Inter-individual variability in drug response can often be attributed to genetic polymorphisms in genes encoding drug metabolizing enzymes, transporters, ion channels and drug target receptors (1-4). Genetic screening for therapeutic and diagnostic applications thus holds great promise though poorly realized at the present moment, owing largely to technological limitations. The factors that hinder pharmacogenetic testing and molecular diagnosis include the laborious and time-consuming nature of genotyping, the fidelity of the genotyping results, as well as the cost incurred by the test (5). Therefore, there is a need to develop a reliable, cost-effective and high-throughput technological platform for genotyping markers involved in the aetiology of variable response to drug therapy or disease pathologies. The Bioplex suspension array is a potential candidate for the development of such a genotyping tool.

The Luminex microbead system has recently been used to define single nucleotide polymorphisms (SNPs) of human leukocyte antigens (6, 7), minor histocompatibility antigens (8), drug metabolizing genes

(9, 10), disease genes (11-14), or detect microorganisms (15-18). Despite this, the use of the bead technology as a genotyping tool is currently still pretty much in infancy. Luminex/BioRad offers 100 distinct sets of color-coded tiny beads, called microspheres. The polystyrene beads are internally-dyed with differing ratios of two spectrally distinct fluorophores. The xMAP technology is a simultaneous multiplex bioassay using a rapid flow cytometer that permits detection of multiple analyses bound to the microbeads in solution of a single tube (19, 20). We describe a genotyping assay that combines an allele-specific primer extension (ASPE) with the Bioplex suspension array. The assay relies on the sequence-specific primer extension of two allele-specific capture oligonucleotide probes that differ at the last base of the 3′-end nucleotide defining the alleles. A tag sequence at the 5′-end of the capture probe allows the resulting enzymatic ASPE product to be captured by its complementary sequence (Anti-tags) which has been coupled to a specific fluorescent microsphere (Fig. 1).

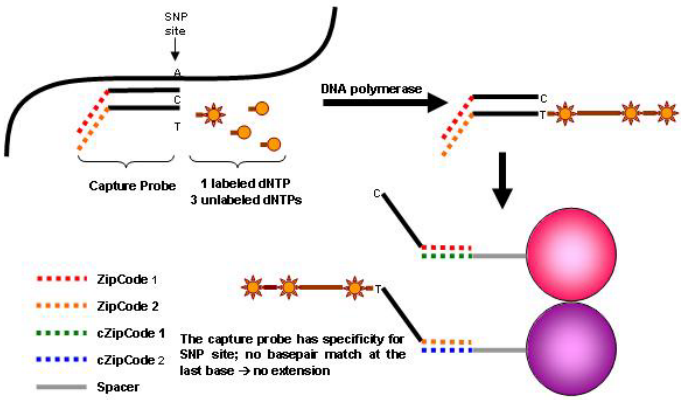

Fig. 1: Schematic diagram of ASPE reaction.

This study attempts to exploit the bead-based technological platform to rapidly screen for a basket of genetic markers that may impact on drug response. We have thus developed and validated a multiplex genotyping platform using the Bioplex suspension array for detecting a panel of ATP binding cassette (ABC) drug transporter polymorphisms. Here, we demonstrate the ability of the Bioplex suspension array to simultaneously genotype 22 SNPs in a single reaction (Table 1), with minimal cross-reactivity. This report provides detailed information including primer sequences, anti-tag/microsphere combinations and experimental conditions, hence enabling easy adaptation of the assay to customized SNP panels. This thus represents an important milestone in the development of genotyping technologies and a major step forward in ushering the realization of personalized medicine, as well as the use of molecular diagnosis in clinical management.

MATERIALS AND METHODS

Oligonucleotides

All oligonucleotides, unless otherwise stated, were synthesized by Research Biolabs, Singapore. The PCR primers used for generating the various fragments encompassing the polymorphism sites (Table 2) were unmodified and purified by standard desalting procedures. A 24-mer universal tag sequence was incorporated at the 5′-end of each ASPE sequence. The ASPE sequences (18-24mer) were designed to be matched for melting temperature at 51-56°C. The tag sequences were designed using the web-based 'Tag-IT' Oligo Design software (Tm Bioscience Corporation, Toronto, Ontario, Canada). The TAG-ASPE primers were unmodified and HPLC-purified (Table 3). The complementary oligos (Anti-tags) were amino (NH_2)-modified at 5′-end for covalent attachment to carboxylated microspheres and separated from the anti-tag sequences (Table 4) by a 6-carbon spacer to minimize potential interactions with the micropsheres. Anti-tags were purified by standard desalting procedures and obtained from Sigma-Proligo, Singapore.

Reagents

HotStar Taq 2X Master Mix was obtained from Qiagen. Platinum GenoTYPE *Tsp* DNA polymerase, individual deoxyribonucleoside triphosphates, and biotin-dCTP were purchased from Invitrogen Corporation. Shrimp Alkaline Phosphatase (SAP) was obtained from Promega, Madison, USA and Exonuclease I from New England Biolabs, Beverly, MA, USA. Bioplex coupling beads, sheath fluid, and calibration and validation kits were purchased from Bio-Rad Laboratories, Life Science Research Group, Hercules, CA. The cross-linker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Pierce, Rockford, IL. The oligonucleotide coupling and hybridization reagents: 2[N-Morpholino] ethanesulfonic acid (MES) and Tris-EDTA buffer were purchased from Sigma Aldrich, NaCl from Merck, 10% sodium dodecyl sulfate (SDS) and Tris

from 1st BASE Laboratories Sdn. Bhd, Malaysia, Triton X-100 from BioRad, and Tween 20 from USB, Cleveland, OH. The platinum grade streptavidin-conjugated phycoerythrin (1 g/L) was obtained from Molecular Probes, Eugene, OR, USA.

DNA samples

Whole blood (10 mL) was collected from normal, healthy individuals, aged 18 to 40 years old from a previous study. All volunteer subjects were recruited in accordance with local regulatory and institutional ethics requirements (Institutional Review Board, National University Hospital, Singapore) and written informed consent. Genomic DNA was isolated from whole blood using standard desalting methods. DNA samples were quantified spectrophotometrically by measuring absorbance at 260 nm, diluted to 5 ng/μL, and stored at 4°C.

Genotyping by DNA sequencing

A total of eight random samples were subjected to DNA sequencing to obtain the genotypic profile of all 22 ABC SNPs in each individual. These include both reported and novel SNPs residing in potentially functional regions of the drug transporters (21-23). For all samples used in the study, genotyping results obtained with the Bioplex assay were compared with those obtained with dideoxy dye-terminator sequencing chemistry. For each of the 22 SNPs, bidirectional (forward and reverse) sequencing was performed using the PCR primers shown in Table 2. DNA sequencing reactions were performed using BigDye Terminator 3.1 Cycle Sequencing Kit and analyzed on the automated ABI Prism Model 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Multiplex PCR (4 panels)

Multiplex PCR refers to the generation of multiple PCR products in a single reaction. Four separate multiplex PCR panels were required to generate all the fragments containing the 22 SNPs. The primer pairs were designed such that the SNP lies towards the centre of the PCR fragment. The PCR mixtures contained Qiagen Hotstar Taq 1X Master Mix, varying concentrations of primers (Table 2) and 4 ng of genomic DNA in a final volume of 20 μL. Following an initial pre-denaturation step at 95°C for 15 min, the reactions were cycled 35 times through denaturation at 94°C for 1 min, variable annealing temperatures (Table 2) for 1 min and extension at 72°C for 1 min. The reactions were terminated by an additional extension step at 72°C for 10 min. The amplification reactions were performed on the Peltier Thermal Cycler (DNA Engine Dyad; MJ Research Inc, Waltham, MA, USA). The samples were kept at 4°C until use. The PCR products were subjected to 1.6% agarose gel electrophoresis to verify successful amplification of the desired fragments.

Allele-specific primer extension

Prior to ASPE reaction, 10 μL of each PCR product was treated with 2 U of Exonuclease I and 1 U of SAP by incubating at 37°C for 15 min, followed by enzyme deactivation at 80°C for 20 min. SAP inactivates any remaining nucleotides (particularly dCTP) to allow efficient incorporation of biotin-dCTP during the primer extension reaction and Exonuclease I degrades any remaining PCR primers to avoid interference with the tagged ASPE primers during the extension reaction. The treated samples were then added directly to the ASPE reaction.

Multiplex ASPE reactions comprised 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.25 mM $MgCl_2$, 5 μM biotin-dCTP, 5 μM each of dATP, dGTP, and dTTP, 0.75 U of Platinum GenoTYPE *Tsp* DNA polymerase, 25 nM each TAG-ASPE primer and 10 μL of pooled treated PCR product in a final volume of 20 μL. The ASPE primer sequences are listed in Table 3. The ASPE reactions were incubated at 96°C for 2 min and then subjected to 35 cycles at 94°C for 30 s, 55°C for 1 min, and 74°C for 2 min. The reactions were then held at 4°C until use.

Microsphere coupling

The aminated anti-tags were coupled to their corresponding populations of carboxylated microspheres using an EDC chemistry (Table 1). Briefly, 2.5 x 10^6 microspheres of each population were resuspended in 25 μL of 0.1 M MES, pH 4.5, followed by addition of 0.2 nmole of anti-tag oligos. 2.5 μL of freshly-prepared 10 g/L EDC solution was added to each microsphere mixture and incubated for 30 min in the dark. A second 2.5 μL aliquot of freshly prepared EDC solution was added and incubated for an additional 30 min in the

dark. Following this, the coupled microspheres were washed in 500 μL of 0.2% Tween 20 and then 500 μL of 0.1% SDS. Finally, the pelleted coupled microspheres were resuspended in 50 μL of TE (10 mM Tris, 1 mM EDTA), pH 8.0. The coupled microspheres were enumerated using a hemacytometer, and stored at 4°C protected from light.

Hybridization and detection

Approximately 2500 microspheres of each of the 44 anti-tag-bearing microsphere sets (Table 1) were combined in 2X Tm Hybridization Buffer [0.4 M NaCl, 0.2 M Tris (pH 8.0), 0.16% Triton X-100]. The microsphere mixture was concentrated and resuspended to 100 of each microsphere set per μL in 2X Tm Hybridization Buffer. A 25 μL aliquot of the mixture was added to each well of a 96-well plate (MJ Research). A 10 μL aliquot of each ASPE reaction and 15 μL sterile water were then added directly to each well. 25 μL of sterile water was added to the background well. The samples were then heated to 96°C for 90 s, followed by 1 hr incubation at 37°C. After this incubation, the samples were washed twice with 1X Tm Hybridization Buffer [0.2 M NaCl, 0.1 M Tris (pH 8.0), 0.08% Triton X-100]. The microspheres were then resuspended in 120 μL of reporter solution (2 mg/L streptavidin-conjugated phycoerythrin in 1X Tm Hybridization Buffer) and incubated for 15 min at 37°C. The reactions were transferred to a flat-bottomed 96-well plate (Nunc) and 50 μL was analyzed on the Luminex xMAP/Bioplex system (Bio-Rad Laboratories, Life Science Research Group, Hercules, CA) at ambient temperature. For each sample, instrument settings were configured to read a minimum of 100 events per bead region with the gate setting being defined before the samples were run and maintained throughout the course of the study.

Data analysis

For each DNA sample subjected to the Bioplex array assay, median fluorescence intensity (MFI) values were generated for each of the 44 microsphere populations corresponding to each allele within the assay. For each allele of a given sample, the NET MFI was set to be the larger of zero and the value obtained by subtracting the no-target (water control) MFI values from the respective MFI values of the sample. The MFI units for at least one allele were required to be at least 10 times the no-target MFI for that allele and magnitude of at least 300. The genotype was then determined based on the mutant allelic ratio where:

$$\text{Mutant allele ratio} = \frac{(\text{NET MFI})_{\text{mutant allele}}}{(\text{NET MFI})_{\text{mutant allele}} + (\text{NET MFI})_{\text{wild-type allele}}}$$

The mutant allelic ratio is defined as the fraction of the total NET MFI signal for a given SNP attributed to the presence of the mutant allele. Based on pre-set threshold values, the allelic ratio was used to discriminate wild-type, mutant, and heterozygous SNP calls. Threshold values were empirically determined for each individual SNP. The genotype calling was performed with the aid of the Bioplex SNP Manager macro software provided by BioRad [http://www.bio-rad.com/snpmanager].

RESULTS

DNA sequencing

A total of eight random DNA samples were sequenced for the genotypic profile of the 22 ABC SNPs as shown in Table 5. Of the 22 SNPs, all three possible genotypes i.e. homozygous wild-type, heterozygous and homozygous mutant were present for 15 of them. Only two genotypes (homozygous wild-type or mutant and heterozygous) were present for six of the SNPs (rs1128503, rs4148557, i1-1679, rs1189437, rs1151471 and rs868853). All the screened subjects were homozygous mutant for one SNP (rs504348) (Table 5). The presence of all three genotypes for the majority (68%) of the SNPs analyzed allows a reliable assessment of the accuracy of the Bioplex suspension array assay.

Multiplex PCR

Multiplex PCR conditions were established in a series of preliminary experiments (data not shown). Multiplex PCRs were optimized for annealing temperatures and individual primer concentrations. A total of 4 multiplex PCR panels were required to generate 21 fragments containing 22 SNPs analyzed in this study (Table 2).

Bioplex suspension array

Table 6 shows the raw MFI values of each individual sample (assayed in duplicates) and a non-template

background control. The results between the duplicates for all readouts were clearly consistent. The genotyping assay for the 22 SNPs was highly specific and robust; the signal-to-noise ratio (specific MFI/background) ranged from >10 to >900 and the MFI units ranged from approximately 300 to 25,000. Threshold values were empirically determined for each individual SNP. The mutant allelic ratio ranged from <0.01-0.28 for wild-type calls, 0.19–0.78 for heterozygous calls, and 0.89–1.00 for mutant calls. While the heterozygotes exhibited almost equal MFI signals for both alleles for the majority of the SNPs, the MFI signal of one allele was higher than the other for a few SNPs. This could possibly be explained by the reasoning that one allele of a SNP amplified disproportionately and obscured the presence of the other allele. Nonetheless, with prior validation using known control samples, the various genotypes are clearly distinguishable. Representative allelic ratio scatterplots are shown for two of the SNPs (Fig. 2).

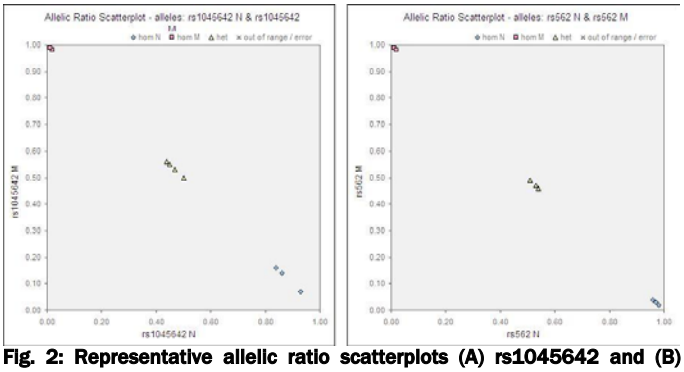

Fig. 2: Representative allelic ratio scatterplots (A) rs1045642 and (B) rs562.

Figure 3 depicts the graphical representation of allelic ratio for two of the study subjects. All the 352 genotypes (22 SNPs x 8 subjects x 2 i.e. duplicates) determined by the Bioplex suspension array were 100% concordant with results obtained using the sequencing approach.

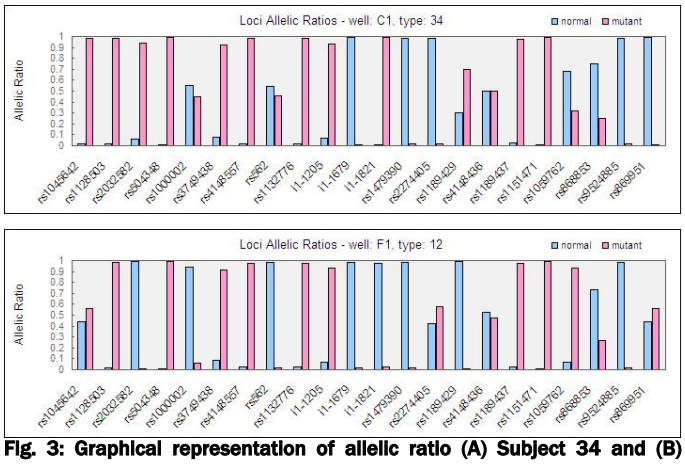

Fig. 3: Graphical representation of allelic ratio (A) Subject 34 and (B) Subject 12.

DISCUSSION

This study explores an alternative technological platform, the bead-based suspension array assay to rapidly screen a panel of genetic variants of the ABC transporters, some of which have been previously genotyped for various ethnic groups in our laboratory using minisequencing (21, 22). We have successfully developed and validated a consolidated bead-based genotyping platform for simultaneous detection of 22 SNPs in a single reaction. The results obtained using the Bioplex suspension array were unambiguous and in complete concordance with sequencing. The use of a multiplex PCR approach significantly increased the through-put and cost-savings of the assay. The ASPE method was chosen over single base chain extension and oligonucleotide ligation methods for its ability to read both alleles from a given SNP in a single tube (18, 24, 25). This study is based on a known technology performed on a small sample size. Nonetheless, we believe it is adequate for demonstrating the functionality of the assay as an initial proof-of-concept phase. A more extensive validation study involving large-scale population screening may subsequently be considered for establishing SNP genotyping panels for diagnostic applications.

The Bioplex suspension array offers several advantages over alternative genotyping methodologies. The use of direct sequencing has long been regarded as the "gold standard" for the identification of genetic variations and determination of the nucleotide arrangement in a DNA fragment. However, direct sequencing as a genotyping tool is not a commercially viable option due to its high

cost and the laborious procedures involved. Conventional genotyping methods such as allele-specific PCR and restriction fragment length polymorphism have been criticized for their poor sensitivity and specificity, are labour-intensive and time consuming. The sophisticated Affymetrix Genechip microarray, though powerful and high-throughput, is very expensive and serves more as a fishing expedition than a simple genotyping exercise catered for a specific application. The microarray is an investigative approach used widely for the search of candidate genes associated with a disease state. The Bioplex platform, on the other hand, serves as a diagnostic tool to complement microarray analysis by targeting the set of genes identified from the search. A predefined SNP genotyping assay for specific applications ensures a standardized format for rapid and cost-effective genetic analysis.

The Bioplex platform allows for the simultaneous detection of up to 50 SNPs in a single reaction well. At present, we have proven it to be a robust, reliable and reasonably high-throughput genotyping tool for multiplex analysis of 22 SNPs. The current system interface allows analysis of 2 x 96 = 192 samples in a single day, equivalent to a maximum output of 9,600 genotype designations. This technology positions itself strategically between the conventional genotyping techniques and the high-end oligonucleotide arrays. Most importantly, its versatility enables the addition of new SNPs or removal of unwanted SNPs to create customized genotyping panels for the consumer. The Bioplex platform allows for all genotyping reactions to be completed and maintained in a single multiplex assay which enables better quality assurance to be achieved. Some other appealing factors of the Bioplex platform include its requirement for minimal DNA template, true liquid phase kinetics, short running time, ease of data analysis and affordability. The disadvantages of the platform include its ability to only detect known mutations, limited throughput due to the current commercially available beads, the need for empirical determination of the threshold value for genotype designation, and the high start-up cost.

This technology offers a promising genotyping platform for developing validated multiplex genotyping kits for specific commercial and research applications involving large-scale mutation screening. There is rising awareness among pharmaceutical companies and clinicians on the importance of pharmacogenetics. The growing trend of pharmaceutical companies integrating the pharmacogenetics programme in clinical trials during new drug development is becoming evident. In view of the emerging field of pharmacogenetics, it is anticipated that there will be a high demand for rapid, reliable and economical genotyping services from pharmaceutical companies, research institutions, hospitals and clinics.

The protocol that we are presenting will indeed be helpful to researchers in the field or to newcomers. This report provides the technical information necessary for establishing SNP panel testing using the Bioplex suspension array. Some relevant areas for this application include genes that impact on drug disposition/therapeutic response or disease susceptibility. These SNP panels can be classified as a combination of markers that affect response to a given drug e.g. a chemotherapeutic agent, those that confer a risk for pathologies for which prophylactic therapies are available, or those that can be used to complement clinical diagnosis of diseases. The application of this strategy in therapeutics and disease association studies will eventually lead to routine adoption of the platform in clinical molecular diagnostics.

ACKNOWLEDGMENTS

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TABLES

Table 1: Coupling of anti-tags to their corresponding Bioplex beads.

SNP ID	Bead No.	
	WT	mt
ABCB1_rs1128503	24	66
ABCB1_rs2032582	33	75
ABCB1_rs1045642	17	61
ABCC1_rs504348	19	51
ABCC5_ rs1000002	21	53
ABCC5_ rs3749438	27	54
ABCC5_ rs4148557	35	56
ABCC5_rs562	37	62
ABCC5_rs1132776	42	64
ABCC5_i1-1205	43	72
ABCC5_i1-1679	45	74
ABCC5_i1-1821	47	77
ABCC4_rs1479390	6	34
ABCC4_rs2274405	11	36
ABCC4_rs1189429	12	38
ABCC4_rs4148436	13	41
ABCC4_ rs1189437	14	44
ABCC4_ rs1151471	18	46
ABCC4_ rs1059762	20	52
ABCC4_rs868853	25	55
ABCC4_rs9524885	26	63
ABCC4_rs869951	28	65

Table 2: PCR primer sequences and amplification conditions.

Panel A (54°C)	Primer concentration (μM)	PCR Product Size (bp)
ABCB1_rs1128503F: TCTTTGTCACTTTATCCAGC	0.08	502
ABCB1_rs1128503R: TCTCACCATCCCCTCTGT	0.08	
ABCB1_ rs2032582F: TGCAGGCTATAGGTTCCAGG	0.06	284

ABCB1_ rs2032582R: TAGGGAGTAACAAAATAACAC	0.06	
ABCB1_rs1045642F: CTCACAGTAACTTGGCAG	0.14	315
ABCB1_rs1045642R: CTTACATTAGGCAGTGAC	0.14	
ABCC1_rs504348F: CAGGATGAAATGAGGGCACAG	0.12	155
ABCC1_rs504348R: GAAGCGCCTGGGATCTTTGG	0.12	
Panel B (56°C)		
ABCC5_ rs3749438F: GGGTGAAATGGAACTGACTC	0.03	390
ABCC5_ rs3749438R: GGTGCCCAGGAAACAGAG	0.03	
ABCC5_i1-1821/1679F: CAACATATATGAAGTATTTCAGCGG	0.35	770
ABCC5_i1-1821/1679R: GAAATATCTTTATGAACTTGGGAG	0.35	
ABCC5_i1-1205F: CAAGAATATGCTGCTTTACG	0.16	859
ABCC5_i1-1205R: TAACCCGTTGAGAGTCGTCA	0.16	
ABCC5_rs1132776F: AGCCCAGGGTCATATGAACAGAGA	0.02	477
ABCC5_rs1132776R: AGGCAAAAAGTCAACAACAACCAG	0.02	
ABCC5_rs562F: GCAGGTCCCAAAGCCATTCAGG	0.03	408
ABCC5_rs562R: ACCGCAGTCGTCGCACAGTCTCT	0.03	
ABCC5_rs1000002F: CTGGCCTGTCCTAGCTGGGTATG	0.05	291
ABCC5_rs1000002R: CTCTGCCTCTTCCTCTTTGCTTCC	0.05	
ABCC5_ rs4148557F: GTTGGGGCGGTGAGCAGTTTG	0.04	316
ABCC5_ rs4148557R: TTCCCCACACCCTTCATACATAGA	0.04	
Panel C (60°C)		
ABCC4_ rs1189429F: AGTGACAGTTATTGAGGTTTC	0.12	290
ABCC4_ rs1189429R: ATCTGCCTCTTTCCACTCC	0.12	
ABCC4_ rs1189437F: ACACCATCTCTACTAAAAATAC	0.20	457
ABCC4_ rs1189437R: CAGAGACCACACCAACCAC	0.20	
ABCC4_ rs868853F: CGCCCGACCAATATCTCACTTTT	0.10	230
ABCC4_ rs868853R: TCCTACAGCCATTCAACCAGCATA	0.10	
ABCC4_rs869951F: GCCTGCGCCGCTGGATGGA	0.05	393
ABCC4_rs869951R: TCGAGTTACCCGGCTTTCTTGAGG	0.05	
ABCC4_ rs4148436F: GGTGTCTGTGTGTGGGGAGT	0.04	482
ABCC4_ rs4148436R: TAAGAGTGAACCCTGCCACA	0.04	
ABCC4_ rs1479390F: CACTGCCAGGGCCTCAAAATGTA	0.07	620
ABCC4_ rs1479390R: ATGCAGCTGCCAAATGGAAGTCTA	0.07	
ABCC4_ rs1151471F: TGACTCTTGGTTCCTCTATAGC	0.15	202
ABCC4_ rs1151471R: AGGACACAATATAACATCTTGC	0.15	
Panel D (56°C)		
ABCC4_ rs1059762F: CCTCTCAGAATAAGGTGTCAC	0.10	437
ABCC4_ rs1059762R: CATTAAAACAGAAACAGGACG	0.10	
ABCC4_ rs2274405F: CACAGCCCCATACAGCGTCACT	0.10	318
ABCC4_ rs2274405R: TTTTTGTTGTTGTTGCCCAGGATG	0.10	
ABCC4_ rs9524885F: AGGAATGGAGGGAATGAGTT	0.15	637
ABCC4_ rs9524885R: CTTGTAGAACGTGATCAAAATG	0.15	

The annealing temperatures of the various multiplex PCR panels are indicated in parentheses.

Table 3: TAG-ASPE primer sequences.

Tagged ABCB1_rs1128503wt: *TACATACACTAATAACATACTCAT* CTGGTAGATCTTGAAGGGC
Tagged ABCB1_rs1128503mt: *CAATTTACTCATATACATCACTTT* CTGGTAGATCTTGAAGGGT
Tagged ABCB1_ rs2032582wt: *ATACTACATCATAATCAAACATCA* ATAAGAAAGAACTAGAAGGTG
Tagged ABCB1_ rs2032582mt: *ATCATACATACATACAAATCTACA* ATAAGAAAGAACTAGAAGGTT
Tagged ABCB1_rs1045642wt: *CAATTAACTACATACAATACATAC* TGGTGTCACAGGAAGAGATC
Tagged ABCB1_rs1045642mt: *CTACAAACAAACAAACATTATCAA* TGGTGTCACAGGAAGAGATT
Tagged ABCC1_rs504348wt: *AATCAATCTTCATTCAAATCATCA* GGATACTGTCCTTAAACAGC
Tagged ABCC1_rs504348mt: *TCAATCAATTACTTACTCAAATAC* GGATACTGTCCTTAAACAGG
Tagged ABCC4_rs2274405wt: *CTACTATACATCTTACTATACTTT* ATTTTGCTTGCACTGAAAAAT
Tagged ABCC4_rs2274405mt: *CTATCTATCTAACTATCTATATCA* ATTTTGCTTGCACTGAAAAAC
Tagged ABCC4_rs1189429wt: *CTTTCTATCTTTCTACTCAATAAT* GGTGATCTCATGCCCTTG
Tagged ABCC4_rs1189429mt: *CTTTTACAATACTTCAATACAATC* GGTGATCTCATGCCCTTA
Tagged ABCC4_ rs1189437wt: *TCATTCATATACATACCAATTCAT* AGTATTCTTTTCAAAAATACTTG
Tagged ABCC4_ rs1189437mt: *TCAATTACTTCACTTTAATCCTTT* AGTATTCTTTTCAAAAATACTTT
Tagged ABCC4_rs868853wt: *CAATTTCATCATTCATTCATTTCA* CCATTCAAGGTTATCCTTAC
Tagged ABCC4_rs868853mt: *TTACTTCACTTTCTATTTACAATC* CCATTCAAGGTTATCCTTAT
Tagged ABCC4_rs869951wt: *TTCAATCATTCAAATCTCAACTTT* TTCTCAGGACCAAACGAC
Tagged ABCC4_rs869951mt: *AAACTAACATCAATACTTACATCA* TTCTCAGGACCAAACGAG
Tagged ABCC4_rs9524885wt: *TCATTTACCAATCTTTCTTTATAC* TGCTGATGCTGCTAATCCT
Tagged ABCC4_rs9524885mt: *CTATCTTTAAACTACAAATCTAAC* TGCTGATGCTGCTAATCCC
Tagged ABCC4_rs4148436wt: *TCATTTCAATCAATCATCAACAAT* AGCCCCATCAGTAGCACA
Tagged ABCC4_rs4148436mt: *TCAATTACCTTTTCAATACAATAC* AGCCCCATCAGTAGCACG
Tagged ABCC4_rs1479390wt: *TACACTTTCTTTCTTTCTTTCTTT* AATTGTTGTGAGTCCACTTG
Tagged ABCC4_rs1479390mt: *TTACCTTTATACCTTTCTTTTTAC* AATTGTTGTGAGTCCACTTT
Tagged ABCC4_rs1151471wt: *CAATAAACTATACTTCTTCACTAA* AAATTGTAGCTATAAGATGATC
Tagged ABCC4_rs1151471mt: *TCATTTCACAATTCAATTACTCAA* AAATTGTAGCTATAAGATGATT
Tagged ABCC4_rs1059762wt: *AATCTTACTACAAATCCTTTCTTT* ACATTTTGAATATAGCTATCG
Tagged ABCC4_rs1059762mt: *AATCCTTTTACATTCATTACTTAC* ACATTTTGAATATAGCTATCA
Tagged ABCC5_ rs1000002wt: *CTTTTCATCTTTTCATCTTTCAAT* TAGCTCTGATGGTTCTCAC
Tagged ABCC5_ rs1000002mt: *TTACTTCACTTTCTATTTACAATC* TAGCTCTGATGGTTCTCAT
Tagged ABCC5_ rs4148557wt: *TCATTTACTCAACAATTACAAATC* CGGTGAGCAGTTTGAAACA
Tagged ABCC5_ rs4148557mt: *CTTTAATCTCAATCAATACAAATC* CGGTGAGCAGTTTGAAACG
Tagged ABCC5_ rs3749438wt: *CTTTTCATCAATAATCTTACCTTT* GCACTTGGTATGTTCCCG
Tagged ABCC5_ rs3749438mt: *CTTTAATCCTTTATCACTTTATCA* GCACTTGGTATGTTCCCA
Tagged ABCC5_i1-1821wt: *TCAAAATCTCAAATACTCAAATCA* ACACCCAATCTCATTCATAA
Tagged ABCC5_i1-1821mt: *AATCTAACAAACTCATCTAAATAC* ACACCCAATCTCATTCATAG
Tagged ABCC5_i1-1679wt: *CTAACTAACAATAATCTAACTAAC* TGACAAAGTTTTTGAATAATAAA
Tagged ABCC5_i1-1679mt: *TACACTTTAAACTTACTACACTAA* TGACAAAGTTTTTGAATAATAAT
Tagged ABCC5_i1-1205wt: *TCATCAATCTTTCAATTTACTTAC* ACTTGTCCATCTTTATAACAG
Tagged ABCC5_i1-1205mt: *TCATCAATCAATCTTTTTCACTTT* ACTTGTCCATCTTTATAACAA
Tagged ABCC5_rs1132776wt: *CTATCTTCATATTTCACTATAAAC* GATGCTCTGGAAGTACCCA
Tagged ABCC5_rs1132776mt: *TCATAATCTCAACAATCTTTCTTT* GATGCTCTGGAAGTACCCG
Tagged ABCC5_rs562wt: *TATATACACTTCTCAATAACTAAC* GCAACGCTGACCATTCAAT
Tagged ABCC5_rs562mt: *AATCTACACTAACAATTTCATAAC* GCAACGCTGACCATTCAAC
The tag sequences are indicated in italics.

Table 4: Anti-tag primer sequences.

Anti-Tagged ABCB1_rs1128503wt: ATGAGTATGTTATTAGTGTATGTA
Anti-Tagged ABCB1_rs1128503mt: AAAGTGATGTATATGAGTAAATTG
Anti-Tagged ABCB1_ rs2032582wt: TGATGTTTGATTATGATGTAGTAT
Anti-Tagged ABCB1_ rs2032582mt: TGTAGATTTGTATGTATGTATGAT
Anti-Tagged ABCB1_rs1045642wt: GTATGTATTGTATGTAGTTAATTG
Anti-Tagged ABCB1_rs1045642mt: TTGATAATGTTTGTTTGTTTGTAG
Anti-Tagged ABCC1_rs504348wt: TGATGATTTGAATGAAGATTGATT
Anti-Tagged ABCC1_rs504348mt: GTATTTGAGTAAGTAATTGATTGA
Anti-Tagged ABCC4_rs2274405wt: AAAGTATAGTAAGATGTATAGTAG
Anti-Tagged ABCC4_rs2274405mt: TGATATAGATAGTTAGATAGATAG
Anti-Tagged ABCC4_rs1189429wt: ATTATTGAGTAGAAAGATAGAAAG
Anti-Tagged ABCC4_rs1189429mt: GATTGTATTGAAGTATTGTAAAAG
Anti-Tagged ABCC4_ rs1189437wt: ATGAATTGGTATGTATATGAATGA
Anti-Tagged ABCC4_ rs1189437mt: AAAGGATTAAAGTGAAGTAATTGA
Anti-Tagged ABCC4_rs868853wt: TGAAATGAATGAATGATGAAATTG
Anti-Tagged ABCC4_rs868853mt: GATTGTAAATAGAAAGTGAAGTAA
Anti-Tagged ABCC4_rs869951wt: AAAGTTGAGATTTGAATGATTGAA
Anti-Tagged ABCC4_rs869951mt: TGATGTAAGTATTGATGTTAGTTT
Anti-Tagged ABCC4_rs9524885wt: GTATAAAGAAAGATTGGTAAATGA
Anti-Tagged ABCC4_rs9524885mt: GTTAGATTTGTAGTTTAAAGATAG
Anti-Tagged ABCC4_rs4148436wt: ATTGTTGATGATTGATTGAAATGA
Anti-Tagged ABCC4_rs4148436mt: GTATTGTATTGAAAAGGTAATTGA
Anti-Tagged ABCC4_rs1479390wt: AAAGAAAGAAAGAAAGAAAGTGTA
Anti-Tagged ABCC4_rs1479390wt: GTAAAAAGAAAGGTATAAAGGTAA
Anti-Tagged ABCC4_rs1151471wt: TTAGTGAAGAAGTATAGTTTATTG
Anti-Tagged ABCC4_rs1151471mt: TTGAGTAATTGAATTGTGAAATGA
Anti-Tagged ABCC4_rs1059762wt: AAAGAAAGGATTTGTAGTAAGATT
Anti-Tagged ABCC4_rs1059762mt: GTAAGTAATGAATGTAAAAGGATT
Anti-Tagged ABCC5_ rs1000002wt: ATTGAAAGATGAAAAGATGAAAAG
Anti-Tagged ABCC5_ rs1000002mt: GATTGTAAATAGAAAGTGAAGTAA
Anti-Tagged ABCC5_ rs4148557wt: GATTTGTAATTGTTGAGTAAATGA
Anti-Tagged ABCC5_ rs4148557mt: GATTTGTATTGATTGAGATTAAAG
Anti-Tagged ABCC5_ rs3749438wt: AAAGGTAAGATTATTGATGAAAAG
Anti-Tagged ABCC5_ rs3749438mt: TGATAAAGTGATAAAGGATTAAAG
Anti-Tagged ABCC5_i1-1821wt: TGATTTGAGTATTTGAGATTTTGA
Anti-Tagged ABCC5_i1-1821mt: GTATTTAGATGAGTTTGTTAGATT
Anti-Tagged ABCC5_i1-1679wt: GTTAGTTAGATTATTGTTAGTTAG
Anti-Tagged ABCC5_i1-1679mt: TTAGTGTAGTAAGTTTAAAGTGTA
Anti-Tagged ABCC5_i1-1205wt: GTAAGTAAATTGAAAGATTGATGA
Anti-Tagged ABCC5_i1-1205mt: AAAGTGAAAAAGATTGATTGATGA
Anti-Tagged ABCC5_rs1132776wt: GTTTATAGTGAAATATGAAGATAG
Anti-Tagged ABCC5_rs1132776mt: AAAGAAAGATTGTTGAGATTATGA
Anti-Tagged ABCC5_rs562wt: GTTAGTTATTGAGAAGTGTATATA
Anti-Tagged ABCC5_rs562mt: GTTATGAAATTGTTAGTGTAGATT

Table 5: Genotypic profile of each study subject.

SNP ID	Subject ID							
	01	**02**	**34**	**35**	**11**	**12**	**13**	**14**
ABCB1_rs1128503	CT	TT	TT	TT	CT	TT	TT	CT
ABCB1_rs2032582	GT	GT	TT	TT	GT	GG	GT	GA
ABCB1_rs1045642	CT	CT	TT	TT	CT	CT	CC	CC
ABCC1_rs504348	GG	GG	GG	GG	GG	GG	GG	GG
ABCC5_ rs1000002	CC	CT	CT	TT	CC	CC	CC	TT
ABCC5_ rs3749438	GG	GG	AA	GG	GA	AA	AA	GA
ABCC5_ rs4148557	AG	AG	GG	GG	AG	GG	GG	GG
ABCC5_rs562	TT	TC	TC	CC	TT	TT	TT	CC
ABCC5_rs1132776	AA	AG	GG	GG	AG	GG	GG	GG
ABCC5_i1-1205	GA	GG	AA	GA	GA	AA	AA	AA
ABCC5_i1-1679	AT	AA	AA	AT	AA	AA	AA	AT
ABCC5_i1-1821	AA	AA	GG	AA	AA	AA	AA	AG
ABCC4_rs1479390	GT	GT	GG	TT	GG	GG	GG	GG
ABCC4_rs2274405	CC	TC	TT	TC	TT	TC	CC	TC
ABCC4_rs1189429	AA	AA	GA	AA	GA	GG	GA	GA
ABCC4_rs4148436	AG	GG	AG	AA	AG	AG	AG	AG
ABCC4_ rs1189437	TT	TT	TT	TT	GT	TT	TT	TT
ABCC4_ rs1151471	TT	TT	TT	TT	CT	TT	TT	TT
ABCC4_ rs1059762	AA	AA	GA	GA	GG	AA	AA	AA
ABCC4_rs868853	TT	TT	CT	TT	TT	CT	TT	CT
ABCC4_rs9524885	CC	TC	TT	CC	CC	TT	TC	TC
ABCC4_rs869951	GG	CG	CC	GG	GG	CG	CG	CG

Wild-type is indicated by the 1st nucleotide of the genotype nomenclature

Table 6a: MFI values of each microsphere set corresponding to each allele of ABCB1, ABCC1 and ABCC5.

Well	Sample	642N	642M	503N	503M	582N	582M	348N	348M	002N	002M	438N	438M	557N	557M	562N	562M	776N	776M	1205N	1205M	1679N	1679M	1821N	1821M
A1	1	20970	21075	11865	19202	4704	1592	75	5719	21461	2359	542	221	21654	19659	24129	995	15078	469	10269	11877	9293	3804	21108	972
B1	2	17458	19540	350	22008	6946	1853	39	5382	11142	10014	546	254	20268	18362	20732	19790	8378	10145	16181	1226	10625	149	19990	696
C1	34	432	20524	412	21905	397	5893	58	5470	8430	6921	293	3531	397	22011	13683	11576	303	15485	1758	23053	7255	101	207	14886
D1	35	413	19450	434	21423	235	4713	28	6546	486	12120	969	383	756	21800	219	18834	328	16662	21330	20663	4075	1577	13964	408
E1	11	14769	18431	19600	20609	7303	1850	49	6247	17938	1886	561	1727	20089	16835	21540	549	7686	9622	19426	16970	7458	112	16238	445
F1	12	14907	19091	466	22692	11322	155	43	6253	17092	1128	292	2959	758	22624	21716	518	375	13624	1698	22707	6917	162	15214	449
G1	13	22043	1692	761	23553	7016	1842	51	5441	17647	1919	263	2143	457	21709	22197	591	409	14252	487	9640	5713	118	13081	367
H1	14	23258	4394	16664	19975	6418	264	63	6396	450	11309	323	1082	514	21132	323	20761	408	11992	547	7792	3945	970	8721	10531
A2	1	20705	20904	12117	19497	4830	1570	59	5261	20696	2141	553	228	21627	19437	24117	964	15044	525	11915	13109	10413	3862	21317	1017
B2	2	16319	18453	325	21932	6869	1812	57	4875	9885	9300	632	262	19987	17736	20335	19163	8340	9937	18166	1353	11387	152	19986	694
C2	34	461	19756	436	21712	387	5409	53	5043	7656	6317	371	3641	421	21983	12713	11056	342	15827	1807	23578	7349	116	215	15213
D2	35	265	19055	137	15927	151	2397	117	6307	406	13300	1129	194	850	22145	271	19856	331	17732	22075	21451	4825	1821	15190	421
E2	11	8557	10252	9950	13232	3999	1221	96	5590	18759	2227	701	1828	20426	18609	21742	530	8112	10643	21719	20203	8562	126	16548	496
F2	12	14007	17367	451	22007	10548	127	13	5833	16093	1090	392	3082	741	22040	20925	511	326	14045	2036	22596	7377	123	15932	452
G2	13	22132	1635	692	23506	7171	1803	31	5191	17973	2018	333	2480	473	22048	22236	610	440	15040	652	11454	6786	101	14763	392
H2	14	24925	3984	13078	18861	6297	252	30	6308	557	15809	420	1349	543	22144	426	23154	573	14274	647	10559	6564	1915	12443	14399
A3	H20	27	58	52	64	70	75	60	74	71	48	27	52	85	46	29	57	36	68	47	74	76	82	61	33

N: normal, M: mutant, blue: homozygous wild-type, yellow: heterozygous, pink: homozygous mutant
The SNPs are denoted by the last 3-4 digits of their respective full ID.

Table 6b: MFI values of each microsphere set corresponding to each allele of ABCC4.

Well	Sample	390N	390M	405N	405M	429N	429M	436N	436M	437N	437M	471N	471M	762N	762M	853N	853M	885N	885M	951N	951M
A1	1	12080	10290	229	22742	141	22800	21500	21086	70	1829	156	16885	134	4339	96	1046	179	19795	267	22097
B1	2	10333	8874	11081	18194	110	22145	800	23212	57	1660	170	12810	159	2976	96	1151	14417	12198	11925	19626
C1	34	12914	231	13958	214	6746	15488	17082	16746	54	1571	113	10308	2507	1165	2923	998	19927	460	20732	309
D1	35	196	12028	7417	10928	71	20937	21563	216	54	1046	133	9141	2424	1059	121	1203	137	19741	205	20221
E1	11	12883	222	13930	227	7044	14989	17803	17001	564	717	7922	5418	4134	148	94	1108	119	20302	325	21460
F1	12	13669	246	8703	12022	12549	187	17825	16051	31	1118	144	9819	173	2211	3330	1253	19331	453	12787	16057
G1	13	13703	259	185	20122	7303	16029	18552	18032	49	1623	170	10071	154	2090	126	1089	14730	12506	15064	18382
H1	14	14723	357	8738	13397	6393	14139	17211	16335	44	1226	181	8609	166	1534	2741	1275	13819	13319	15887	17372
A2	1	11761	9858	238	22954	114	22860	21271	20776	33	1997	152	15836	157	3764	91	981	145	20490	267	22592
B2	2	9792	8207	11620	18072	126	22114	846	23257	53	1795	136	12039	181	2757	83	1156	15203	12903	12727	20363
C2	34	12148	204	12917	251	6331	14670	15367	16470	64	1577	141	9352	2338	1051	2829	992	20209	476	20576	291
D2	35	177	13197	8150	12488	105	21051	21766	274	24	1234	133	10426	2677	1167	120	1203	150	20376	236	20689
E2	11	13703	240	15370	233	7560	16431	18559	17268	595	859	8343	5657	4764	148	87	1299	141	20729	305	21582
F2	12	13554	275	8243	12358	12756	198	17332	16342	42	1217	155	9212	171	2137	3052	1385	19840	464	12809	16720
G2	13	14149	251	212	20738	7391	16105	18231	18000	65	1978	150	11013	167	2275	111	1068	16339	13603	15957	18972
H2	14	3122	80	11287	16853	6967	15739	6929	5515	63	1773	210	15364	126	2597	3866	1299	14734	13302	6868	9658
A3	H20	33	70	39	38	64	46	25	43	54	42	49	48	26	39	8	75	27	61	48	52

N: normal, M: mutant, blue: homozygous wild-type, yellow: heterozygous, pink: homozygous mutant
The SNPs are denoted by the last 3 digits of their respective full ID.

REFERENCES

1. Evans WE, Johnson JA. Pharmacogenomics: the inherited basis for interindividual differences in drug response. *Annu Rev Genomics Hum Genet* 2001; 2:9-39.
2. Evans WE, Relling MV. Pharmacogenomics: translating functional genomics into rational therapeutics. *Science* 1999; 286:487-491.
3. Kleyn PW, Vesell ES. Genetic variation as a guide to drug development. *Science* 1998; 281:1820-1821.
4. Severino G, Chillotti C, Stochino ME, Del Zompo M. Pharmacogenomics: state of the research and perspectives in clinical application. *Neurol Sci* 2003; 24:S146-148.
5. Koo SH, Lee EJ. Pharmacogenetics approach to therapeutics. *Clin Exp Pharmacol Physiol* 2006; 33:525-532.
6. Itoh Y, Inoko H, Kulski JK, Sasaki S, Meguro A, Takiyama N, Nishida T, Yuasa T, Ohno S, Mizuki N. Four-digit allele genotyping of the HLA-A and HLA-B genes in Japanese patients with Behcet's disease by a PCR-SSOP-Luminex method. *Tissue Antigens* 2006; 67:390-394.
7. Itoh Y, Mizuki N, Shimada T, Azuma F, Itakura M, Kashiwase K, Kikkawa E, Kulski JK, Satake M, Inoko H. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-Luminex method in the Japanese population. *Immunogenetics* 2005; 57:717-729.
8. Pietz BC, Warden MB, DuChateau BK, Ellis TM. Multiplex genotyping of human minor histocompatibility antigens. *Hum Immunol* 2005; 66:1174-1182.
9. Pickering JW, McMillin GA, Gedge F, Hill HR, Lyon E. Flow cytometric assay for genotyping cytochrome p450 2C9 and 2C19: comparison with a microelectronic DNA array. *Am J Pharmacogenomics* 2004; 4:199-207.
10. Zhu Y, Hein DW, Doll MA, Reynolds KK, Abudu N, Valdes Jr R, Linder MW. Simultaneous determination of 7 N-acetyltransferase-2 single-nucleotide variations by allele-specific primer extension assay. *Clin Chem* 2006; 52:1033-1039.
11. Bortolin S, Black M, Modi H, Boszko I, Kobler D, Fieldhouse D, Lopes E, Lacroix JM, Grimwood R, Wells P, Janeczko R, Zastawny R. Analytical validation of the tag-it high-throughput microsphere-based universal array genotyping platform: application to the multiplex detection of a panel of thrombophilia-associated single-nucleotide polymorphisms. *Clin Chem* 2004; 50:2028-2036.
12. Colinas RJ, Bellisario R, Pass KA. Multiplexed genotyping of beta-globin variants from PCR-amplified newborn blood spot DNA by hybridization with allele-specific oligodeoxynucleotides coupled to an array of fluorescent microspheres. *Clin Chem* 2000; 46:996-998.
13. Diaz MR, Fell JW. High-throughput detection of pathogenic yeasts of the genus trichosporon. *J Clin Microbiol* 2004; 42:3696-3706.
14. Strom CM, Janeczko RA, Anderson B, Redman J, Quan F, Buller A, McGinniss MJ, Sun WM. Technical validation of a multiplex platform to detect thirty mutations in eight genetic diseases prevalent in individuals of Ashkenazi Jewish descent. *Genet Med* 2005; 7:633-639.
15. Cowan LS, Diem L, Brake MC, Crawford JT. Transfer of a Mycobacterium tuberculosis genotyping method, Spoligotyping, from a reverse line-blot hybridization, membrane-based assay to the Luminex multianalyte profiling system. *J Clin Microbiol* 2004; 42:474-477.
16. Jiang HL, Zhu HH, Zhou LF, Chen F, Chen Z. Genotyping of human papillomavirus in cervical lesions by L1 consensus PCR and the Luminex xMAP system. *J Med Microbiol* 2006; 55:715-720.
17. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T. Bead-based multiplex genotyping of human papillomaviruses. *J Clin Microbiol* 2006; 44:504-512.
18. Ye F, Li MS, Taylor JD, Nguyen Q, Colton HM, Casey WM, Wagner M, Weiner MP, Chen J. Fluorescent microsphere-based readout technology for multiplexed human single nucleotide polymorphism analysis and bacterial identification. *Hum Mutat* 2001; 17:305-316.
19. Dunbar SA, Vander Zee CA, Oliver KG, Karem KL, Jacobson JW. Quantitative, multiplexed detection of bacterial pathogens: DNA and protein applications of the Luminex LabMAP system. *J Microbiol Methods* 2003; 53:245-252.
20. Spiro A, Lowe M, Brown D. A bead-based method for multiplexed identification and quantitation of DNA sequences using flow cytometry. *Appl Environ Microbiol* 2000; 66:4258-4265.

21. Gwee PC, Tang K, Chua JM, Lee EJ, Chong SS, Lee CG. Simultaneous genotyping of seven single-nucleotide polymorphisms in the MDR1 gene by single-tube multiplex minisequencing. *Clin Chem* 2003; 49:672-676.
22. Gwee PC, Tang K, Sew PH, Lee EJ, Chong SS, Lee CG. Strong linkage disequilibrium at the nucleotide analogue transporter ABCC5 gene locus. *Pharmacogenet Genomics* 2005; 15:91-104.
23. Wang Z, Wang B, Tang K, Lee EJ, Chong SS, Lee CG. A functional polymorphism within the MRP1 gene locus identified through its genomic signature of positive selection. *Hum Mol Genet* 2005; 14:2075-2087.
24. Lee SH, Walker DR, Cregan PB, Boerma HR. Comparison of four flow cytometric SNP detection assays and their use in plant improvement. *Theor Appl Genet* 2004; 110:167-174.
25. Taylor JD, Briley D, Nguyen Q, Long K, Iannone MA, Li MS, Ye F, Afshari A, Lai E, Wagner M, Chen J, Weiner MP. Flow cytometric platform for high-throughput single nucleotide polymorphism analysis. *Biotechniques* 2001; 30:661-666, 668-669.

PROTOCOLS

Microspheres should be protected from prolonged exposure to light throughout the following procedures.

Microsphere coupling

1. Bring a fresh aliquot of -20°C, desiccated EDC powder to room temperature.
2. Resuspend the amine-substituted oligonucleotide ("probe" or "capture" oligo) to 100 μM in sterile water.
3. Resuspend the stock microspheres by vortexing for approximately 20 seconds.
4. Transfer 2.5 x 10^6 of the stock microspheres to a microfuge tube.
5. Pellet the stock microspheres by microcentrifugation at 10,000 x g for 3 minutes.
6. Remove the supernatant and resuspend the pelleted microspheres in 25 μL of 0.1 M MES, pH 4.5 by vortexing for approximately 20 seconds.
7. Add 2 μL of the capture oligo (100 μM) to the resuspended microspheres and mix by vortexing.
8. Prepare a fresh solution of 10 g/L EDC in sterile water. (Note: Return the EDC powder to desiccant to re-use for the second EDC addition.)
9. One by one for each reaction, add 2.5 μL of fresh 10 g/L EDC to the microspheres and mix by vortexing.
10. Incubate for 30 minutes at room temperature in the dark.
11. Prepare a second fresh solution of 10 g/L EDC in sterile water.
12. One by one for each reaction, add 2.5 μL of fresh 10 g/L EDC to the microspheres and mix by vortexing.
13. Incubate for 30 minutes at room temperature in the dark.
14. Add 500 μL of 0.02% Tween-20 to the coupled microspheres.
15. Pellet the coupled microspheres by microcentrifugation at 10,000 x g for 3 minutes.
16. Remove the supernatant and resuspend the coupled microspheres in 500 μL of 0.1% SDS by vortexing.
17. Pellet the coupled microspheres by microcentrifugation at 10,000 x g for 3 minutes.
18. Remove the supernatant and resuspend the coupled microspheres in 50 μL of TE, pH 8.0 by vortexing for approximately 20 seconds.
19. Enumerate the coupled microspheres by hemacytometer:
    a) Dilute the resuspended, coupled microspheres 1:100 in sterile water.
    b) Mix thoroughly by vortexing.
    c) Transfer 10 μL to the hemacytometer.
    d) Count the microspheres within the 4 large corners of the hemacytometer grid.
    e) Microspheres/μL = (Sum of microspheres in 4 large corners) x 2.5 x 100 (dilution factor). (Note: maximum is 50,000 microspheres/μL.)
20. Store coupled microspheres refrigerated at 4°C in the dark.

Hybridisation and detection

1. Select the appropriate microsphere sets and resuspend by vortexing for approximately 20 seconds.
2. Combine 2500 microspheres of each set per reaction.
3. Concentrate the microsphere mixture by centrifugation at 10,000 x g for 3 minutes.
4. Remove the supernatant and resuspend to 100 of each microsphere set per μL in 2X Tm Hybridization Buffer by vortexing for approximately 20 seconds.
5. Aliquot 25 μL of the microsphere mixture to each well.
6. Add 25 μL of sterile water to each background well.
7. Add 10 μL of each ASPE reaction to appropriate wells. (Note: 5 μL is usually sufficient.)
8. Adjust the total volume to 50 μL by adding 15 μL of sterile water to each sample well.

9. Cover the plate to prevent evaporation and denature at 96°C for 90 seconds.
10. Hybridize at 37°C for 1 hour.
11. Pellet the microspheres by centrifugation at 5000 x g for 3 minutes and remove the supernatant.
12. Resuspend the pelleted microspheres in 70 μL of 1X Tm Hybridization Buffer.
13. Pellet the microspheres by centrifugation at 5000 x g for 3 minutes and remove the supernatant.
14. Repeat steps 11 and 12 for a total of two washes.
15. Resuspend microspheres in 120 μL of 1X Tm Hybridization Buffer containing 2 mg/L streptavidin-R-phycoerythrin.
16. Incubate at 37°C for 15 minutes.
17. Analyze 50 μL at room temperature on the Luminex xMAP/Bioplex analyzer.