PRODUCT & TECHNOLOGY REPORT

SNPstream® UHT: Ultra-High Throughput SNP Genotyping for Pharmacogenomics and Drug Discovery

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BioTechniques 32: S70-S77 (June 2002)

ABSTRACT

Single nucleotide polymorphism (SNP) genotyping is playing an increasing role in genome mapping, pharmacogenetic studies, and drug discovery. To date, genome-wide scans and studies involving thousands of SNPs and samples have been hampered by the lack of a system that can perform genotyping with cost-effective throughput, accuracy, and reliability. To address this need, Orchid has developed an automated, ultra-high throughput system, SNPstream® UHT, which uses multiplexed PCR in conjunction with our next generation SNP-IT™ tag array *single base extension genotyping technology. The system employs oligonucleotide microarrays manufactured in a 384-well format on a novel glass-bottomed plate. Multiplexed PCR and genotyping are performed in homogeneous reactions, and assay results are read by direct two-color fluorescence on the SNPstream UHT Array Imager. The system's flexibility enables large projects involving thousands of SNPs and thousands of samples as well as small projects that have hundreds of SNPs and hundreds of samples to be done cost effectively. We have successfully demonstrated this system in greater than 1 000 000 genotyping assays with >96% of samples giving genotypes with >99% accuracy.*

INTRODUCTION

Opportunities for study of genetic variation have been made possible by the recent dramatic increases in DNA sequence information available for human and other organisms. Single nucleotide polymorphisms (SNPs) represent the most common type of genetic variation, occurring every 300 to 1000 bases in humans (6) and many other organisms. Large collections of confirmed and annotated SNPs now reside in public (The SNP Consortium, National Center for Biotechnology Information, Cold Spring Harbor Laboratory) (10) and private databases. These SNP collections are beginning to be used in genome-wide association studies involving many thousands of SNPs, as well as in more focused studies of disease susceptibility and drug metabolism.

To meet the need for genotyping that enables flexible study design, we have developed a tag array platform using a 384-well microwell plate for highly multiplexed single base extension genotyping [minisequencing (8) or SNP-IT™ (7)] in an easily automated format (Figure 1). Using a Web-based primer design software tool modified to assemble primer sets into multiplex groups, 12-plex PCR sets are designed as the first step in the assay. Post-PCR cycle extension for the SNP-IT reaction is performed in solution followed by sorting on the tag array plate by hybridization capture of the tagged SNP-IT primer to the complementary tag on the array plate. Two-color fluorescence of the UHT microarray plate is detected using a SNPstream® UHT Array Imager. Flexible software tools for assay setup, data analysis, and automated genotype calling facilitate the handling of the 4608 genotypes generated per plate.

The tagged SNP-IT approach taken here has the significant advantage in that it makes use of a generic microarray that can be manufactured in large quantities. While the general concept of tag capture for sorting has been reported previously (1,2,3,5), application to arrays in microwell format has enabled very high volume and flexible genotyping experimental design.

In this study we demonstrate the utility of this approach to SNP genotyping, by the analysis of designed assays for 96 randomly selected human SNPs and blinded genotyping on 120 DNA samples. The results show 85% marker conversion at 12-plex, 96% sample call rates, and >99% concordance with reference genotypes.

MATERIALS AND METHODS

Oligonucleotides

For the purpose of this study, fifteen 20 nucleotide (nt) tag oligonucleotides were selected from among the approximately 2000 used in the GenFlex™ DNA chip (Affymetrix, Santa Clara, CA, USA) and which we previously validated for use with SNP-IT. Tags were chosen that give strong signals with minimal cross hybridization and low homology to DNA databases. These tag oligonucleotides were synthesized at the 1–10 µmol scale with a 3′-disulfide modification and purified with DMT-on by C-18 RP HPLC. Following detritylation, identity of the tag oligonucleotides was confirmed by mass spectrometry and purity was confirmed to be >90% by capillary electrophoresis (P/ACE™-MDQ; Beckman Coulter, Fullerton, CA, USA).

Each self-extension control primer is 45 nt long and comprises a 5′ 20 nt sequence complementary to one of the arrayed control tags and a 3′ 25 nt sequence capable of forming 3′ self complementarity that can be extended in the SNP-IT reaction. These oligonucleotides serve as allele-specific positive controls for the SNP-IT single base extension and hybridization steps.

Primers for PCR (20–25 nt) and SNP-IT (40–45 nt) were designed using Web-based Autoprimer.com software as described below. All were synthesized at the 1 µmol scale using standard phosphoramidite chemistry (Applied Biosystems 3900, Foster City, CA, USA). Primers were desalted and solvent exchanged in water prior to use. Oligonucleotide concentrations were adjusted based on sequence-specific extinction coefficients.

Microarray Plates

Glass plates (Erie Scientific, Portsmouth, NH, USA) were immersed in Piranha solution (1:1 sulfuric acid and 30% hydrogen peroxide) for 15 minutes, and then rinsed with cascaded deionized water for 20 minutes followed by an oven dry at 120°C for 1.5 hours. Cleaned glass was then silanized with 3-mercaptopropyl trimethoxysilane (MPTS) by vapor deposition in a vacuum oven at 70°C for 2 hours followed by curing at 120°C for 2 hours. Silanized glass plates were stored in N_2 (g) purged chambers until use.

Covalent attachment of oligonucleotides onto the glass surface was achieved via a thio/disulfide exchange reaction between the mecaptosilane and 3′ disulfide modified oligonucleotide probes as previously described (4). Diluted probes were arrayed on the silanized glass surface in the 384-well plate format on PixSys 5200 Arrayers (Cartesian, Research Triangle Park, NC, USA), with ChipMaker Pins (Telechem, Sunnyvale, CA, USA). Each sub-array within a well consisted of 4 × 4 features with approximately 350-µm diameter spots with >500-µm spacing between spots (Figure 2). Reaction wells were formed by applying a compressible 384-well neoprene gasket to the arrayed plate. Arrayed plates were sealed in pouches with desiccant and were stable for greater than 9 months.

Assay Design

Design of the two PCR primers and one SNP-IT primer for each marker set was performed using Autoprimer.com (http://www.autoprimer.com), a Web-based primer design tool developed at Orchid. This Web site takes, as input, a file containing up to 200 sequences, one on each line, with the SNP marked in brackets, e.g., [G/A]. An optional field can also be used by the Web site that contains a repeat mask for the sequence. The Autoprimer.com design engine reads each sequence and designs three primers; forward and reverse PCR primers and a SNP-IT primer for the single base extension step. If a repeat mask is included in the file, Autoprimer designs around the repeat if possible. Autoprimer.com selects the best SNP-IT primer from the sequence based on T_m, secondary structure, and mispriming. SNP-IT primers may be selected from either DNA strand. PCR products are designed to fall in the range of 90 to 180 base pairs, with a strong bias toward smaller amplicon length. Once primers are picked for each sequence, they are then assembled into groups of 12 by SNP

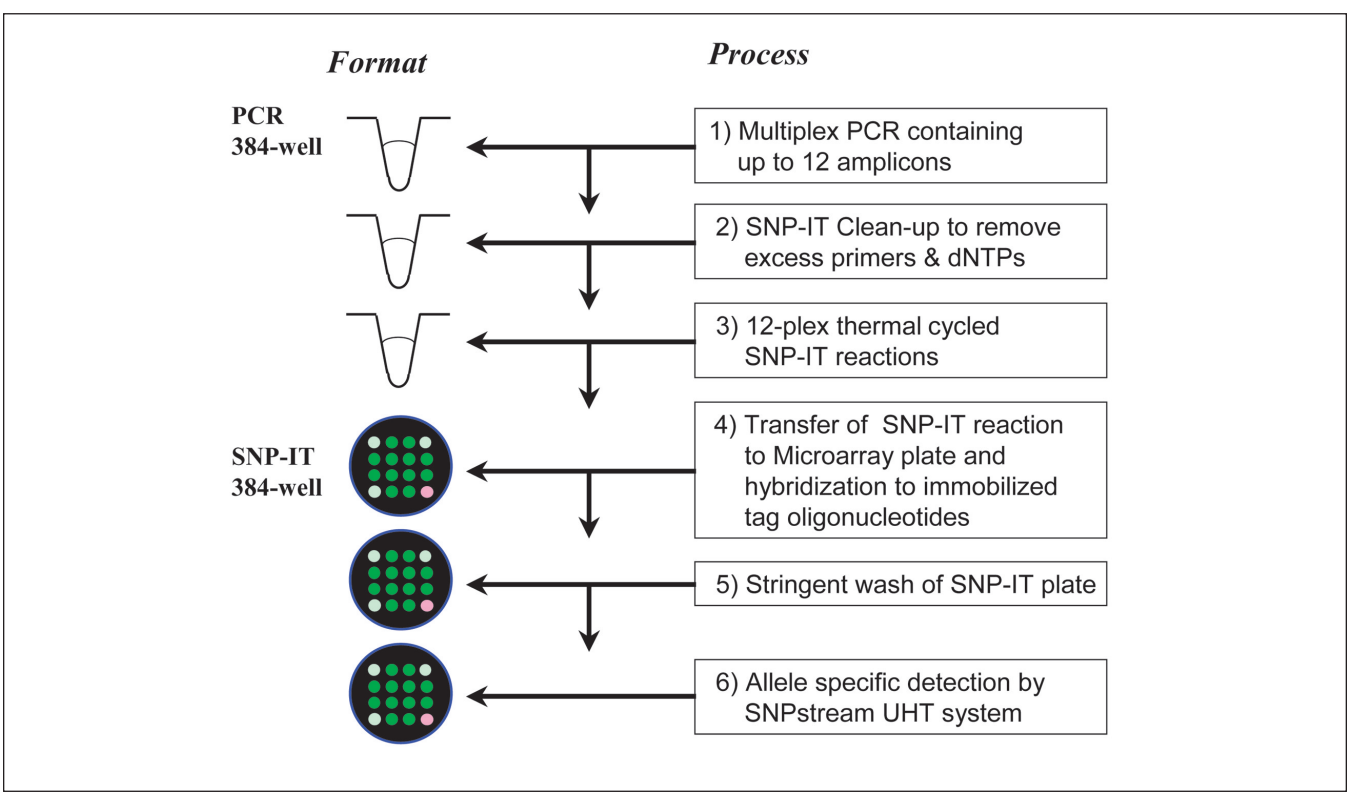

Figure 1. Tag-based SNP-IT Assay.

extension type (e.g., A/G, T/C). Each group, or panel of 12 markers, must be of the same extension type for processing on the UHT since each extension mix contains two labeled terminators (Bodipy-Fluorescein and TAMRA). Each group of twelve is referred to as a panel of markers. Autoprimer.com automatically optimizes the grouping of the markers by extension mix and appends tag sequences to the 5′ ends of the SNP-IT primers, which are complementary to the tags immobilized on the microarray plate. The software attempts to group the markers in each panel by annealing temperature, but the most important grouping criteria is by extension mix. Because of this restriction, the PCR conditions are designed to handle sequences with very different annealing temperatures (see Materials and Methods, PCR). Tags are appended to the SNP-IT primers and an output file is generated that shows the panels grouped together and the primer sequences to facilitate oligonucleotide ordering. An additional output file is generated that can be loaded into the SNPstream UHT database, indicating which markers/SNP-IT primers will assort with the 12-SNP-IT complementary probes on the UHT microarray plate.

Before designing marker panels for the SNP genotyping experiment presented here, sequences were first compared with the Golden Path database (http://genome.ucsc.edu) using BLAST. This procedure is important in identifying duplications/pseudogenes that would confound the SNP-IT assay. Also, repeat masking is highly recommended to avoid generating PCR product that is contaminated by low copy repeats.

DNA Samples

Genomic DNA samples were drawn from the Coriell Polymorphism Discovery Set (PD1-PD24; http://arginine.umdnj.edu) or were part of a blinded test set.

PCR

A five-microliter PCR was performed in 384-well plates (MJ Research, Watertown, MA, USA) using 75-µM dNTPs and 0.5 U AmpliTaq® Gold (Applied Biosystems) in 1× PCR buffer. One to two nanograms of genomic DNA was used in each reaction. At a 12-plex PCR, this represents ≤0.17 ng/genotype. The 24 PCR primers were pooled and added such that each was at a final concentration of 50 nM.

Thermal cycling was performed in DNA Engine Tetrad™ thermal cyclers (MJ Research) using the following program: 95°C for 5 seconds followed by 45 cycles of 95°C for 30 seconds; 50°–55°C for 55 seconds; 72°C for 30 seconds. The first six cycles used an annealing temperature of 50°C after which the annealing temperature was increased by 0.2°C in the subsequent cycles until the annealing temperature reached 55°C. After the last cycle, the reaction was held at 72°C for 7 minutes followed by a 4°C hold.

PCR Clean-Up

Following PCR, 384-well plates were centrifuged briefly to collect the contents and 3 µL of a cocktail containing 0.67 U exonuclease I (USB, Cleveland, OH, USA) and 0.33 U shrimp alkaline phosphatase (SAP; USB) was added. Sealed plates were incubated for 30 minutes at 37°C to degrade residual PCR primers and dNTPs, and 10 minutes at 100°C to inactivate the enzymes.

SNP-IT Reaction

To the ExoI/SAP-treated PCR, we added 7 µL of a cocktail containing one TAMRA-labeled and one Bodipy-Fluorescein-labeled nucleotide terminator (PE-NEN, Boston, MA, and Molecular Probes, Eugene, OR, USA), the two remaining unlabeled terminators, 26.6 mM $MgCl_2$, 266 mM Tris-HCl pH 9.5, two allele-specific self-extension control primers, and a thermostable, 3′ exonuclease-deficient polymerase such as Thermo Sequenase™ (Amersham Biosciences, Piscataway, NJ, USA). The total reaction volume was 15 µL. Plates were re-sealed and thermal cycled using the following program: 96°C for 3 minutes followed by 45 cycles of 94°C for 20 seconds; 40°C for 11 seconds. After the last cycle, the reaction was held at 4°C.

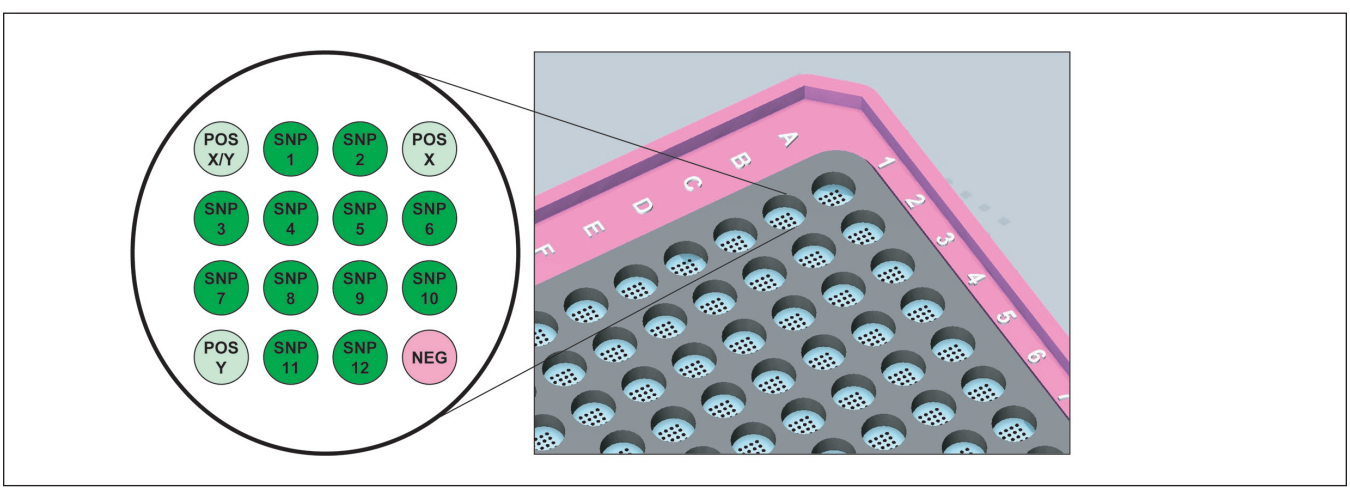

Figure 2. The layout of UHT microarray wells is shown with positions of positive (POS X, POS Y, POS X/Y) controls, negative (NEG) controls, and SNP-IT capture tags. POS X and POS Y spots contain tags to capture one of the two allele-specific self-extension control oligonucleotides that are included in each SNP-IT reaction. POS X/Y uses a mixture of the capture tags for the X and Y controls. The negative control spot contains a unique tag oligonucleotide that serves as a test for mishybridization in the assay. SNP 1 to SNP 12 indicate the positions of the specific capture tags for the tag array SNP-IT primers.

Hybridization and Washing

Following SNP-IT extension, 8 µL of hybridization buffer (5 M NaCl, 0.5 M EDTA, 580 mM morpholinoethane sulphonic acid (MES) pH 6.6, 1× Denhardt's Solution) was added and a portion of the mixture was applied to the well of a UHT microarray plate. Plates were incubated in a humidified container at 42°C for 2 hours to promote hybridization of the SNP-IT primers to their complementary immobilized tags. Plates were rinsed with UHT wash buffer using a conventional plate washer to remove unhybridized material and were then ready for imaging.

SNPstream UHT Array Imager

A custom platform was developed and optimized for high-speed, ultra-high throughput SNP image analysis. A software control interface allows the operator to select the number of 384-well SNP-IT plates (1–36) and run method to automatically capture plate images and analyze and generate genotype reports.

The SNPstream Array Imager is based upon a two-laser, two-color approach. Each sample is illuminated with a 488-nm laser beam and subsequently with a 532-nm laser beam to excite the fluorescent oligonucleotides captured on the UHT microarray plates. Laser output is delivered via fiber optic cable. Laser power density delivered to each well being imaged is approximately 8.6×10^{-3} J and 3.2×10^{-3} J for the 488-nm and 532-nm wavelengths, respectively. The system contains two emission band filters. Fluorescence emission from 488-nm excitation (Bodipy-Fluorescein) is captured in a band 50 nm wide, centered at 535 nm. Fluorescence emission from 532-nm excitation (TAMRA) is captured in a band 55 nm wide, centered at 590 nm. A color-corrected custom lens, of high numerical aperture and 100-µm depth of field, is positioned between the microarray plate and charge-coupled device (CCD) camera. The 1536- × 1024 9-µm pixel CCD is "3 × 3 binned" to give an image resolution of 27 µm. This is sufficient resolution to image a 4 × 4 spot/well array and keep image file sizes manageable. A 2 × 3 well area is imaged per frame (Figure 3). An X/Y mechanical stage assembly with a 20-µm resolution automatically positions a SNP-IT plate under the camera optics prior to imaging. Sixty-four 2 × 3 well images/color are taken per plate for a total of 384 wells. Total time required for the process is approximately seven minutes/plate, which includes plate transport and CCD camera image capture.

A robotic microarray plate handler is used to place plates into the SNPstream Array Imager to increase system throughput to 120 plates per 24 hours. The system is capable of analyzing 552 960 genotypes/24 hour in the 12-plex by 384-well format.

Data Analysis

Generation of genotype calls from spots detected using the SNPstream UHT Array Imager involves two discrete steps, both of which are fully automated. First, the location and intensity of a spot within the well and plate is determined for each wavelength; second, a genotype call is made based on the relative fluorescent intensities of each spot. Once a genotype call has been

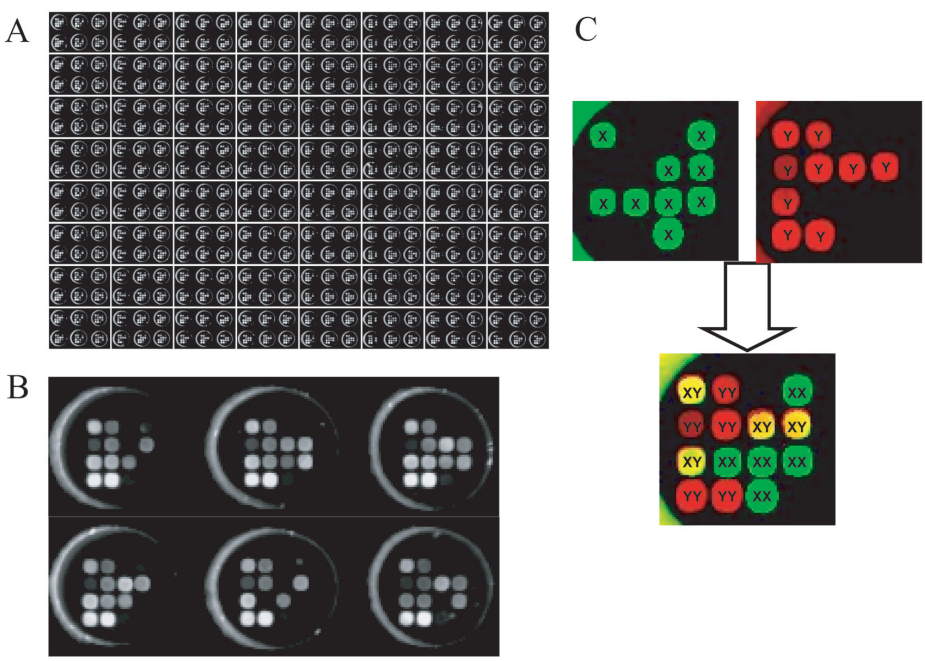

Figure 3. Microarray images. (A) Montage of sixty-four 2 × 3-well images to recreate a single 384-well plate. A single channel image (532 nm λex) of a representative plate is shown. (B) Enlargement of a 2 × 3-well image from (A) to show detail. Note the positions of the X/Y control in the upper left position of each well; X control in the upper right, Y control in the lower left, and NEG at lower right. (C) False-color images of a single well excited with 488-nm (left-green) and 532-nm (right-red) lasers. Superposition of the two images illustrates genotype calls for individual spots.

made, results are written to an Oracle® database where the data can easily be retrieved for viewing.

Spot detection is an automatic process performed by UHTImage software using an advanced grid drawing algorithm developed at Orchid. Positive controls in each well are used to align the grids around the 4 × 4 element array. Once a grid is drawn, each spot is analyzed for morphology (i.e., circular shape and regular pixel intensity across each spot). Spots with low intensity or unusual morphology are marked as empty or fail. For each spot that passes the morphology test, an intensity value is generated and loaded into the UHT database. Failed spots are carried through the analysis but are flagged for the user to review.

Genotype calling is performed once all spot intensities are in the database for each sample within a plate. Each SNP marker is analyzed separately using UHT GetGenos™ software. This software automatically creates genotype calls based on the intensity value of each spot at each wavelength for a given sample. These calls are based on how the sample points cluster when plotted on a X,Y graph where X corresponds to the intensity in the 488-nm channel and Y to that of the 532-nm channel. If a point falls between clusters or the intensity of the point is too low, the sample is failed. Otherwise the point is called as XX, XY, or YY with the X's and Y's being replaced by the actual allele calls (A,C,G,T). UHT GetGenos uses a proprietary algorithm to determine the clusters and the genotypes for each sample. After the genotype calling, the results are stored in the database by microarray plate number, well, and spot location. Most of the allelograms can be passed or failed easily. A small percentage require visual review and some manual adjustment of the genotype clusters. This flexibility is added to the system to maximize accuracy and throughput and reduce the potential need to re-run assays.

SNP Genotyping

To demonstrate the UHT system, 96 unique SNPs not previously tested were chosen for development. All sequences were checked for repeats and multiple hits in GenBank prior to design with AutoPrimer. Assortment of these into 12-plexes resulted in 3 GA panels (36 SNPs), 3 TC panels (36 SNPs), 1 GC panel (12 SNPs), and 1 GT panel (12 SNPs).

RESULTS AND DISCUSSION

SNP Assay

SNP-IT involves annealing an oligonucleotide primer immediately adjacent to a SNP site and single base extension by DNA polymerase in the presence of labeled terminating nucleotides (4,7). We adapted the SNP-IT assay to use fluorescent terminators in a microarray format. We have also adopted a tag-based approach (1,2,3,5), which allows us to create a generic microarray containing a total of sixteen oligonucleotide spots. Four of these spots contain sites for positive and negative assay controls, twelve spots are intended for capture of tagged SNP-IT primers (Figure 2). Several thousand such generic UHT microarray plates have been manufactured at Orchid, and we have achieved a very low defect rate (<0.5%). Since the microarray plates are prepared with a well-forming gasket in a 384-well format, direct liquid transfer from 384-well SNP-IT reaction plates to the microarray plate is straightforward, and plates are compatible with common laboratory robotics. We have routinely used TECAN Genesis (Medford, MA, USA) and Beckman Coulter Multimek (Fullerton, CA, USA) for reaction setup and liquid transfer steps, though reactions have also been prepared using standard 8- and 12-channel pipets.

Assay Conversion

To demonstrate the UHT system, SNP-IT *tag array* assays were designed for 96 human SNPs using AutoPrimer.com as described under Materials and Methods. SNPs were tested with 96 DNA samples of blinded genotype, and data was subjected to automated image analysis and allele calling using UHT MegaGetGenos software. Of the 96 designed SNP assays, 82 were found to meet stringent criteria for call rate, signal intensity, and genotype clustering. Of the 14 marker designs that did not pass our criteria, 11 showed insufficient fluorescent signal intensity (below 1000) in both fluorescent channels, suggesting either PCR failure, a failure of the SNP-IT primer to extend, or incorrect starting sequence data. We did not determine the source of failure for these SNPs. The remaining three SNPs failed due to poor clustering of genotypes or low sample call rates. Multiplex PCRs were analyzed using an Agilent Bioanalyzer, but due to the tight distribution of the multiplexed PCR products at approximately 100 bp, individual bands could not be resolved or identified in this case. In parallel work, we have found that when single PCRs are done on SNPs that have apparently failed in multiplexing, many show no PCR product, suggesting that some of the SNP-IT reaction failures can be attributed to poor quality input sequences or failed PCR (data not shown). Despite this, our conditions give a very high success rate for 12-plex

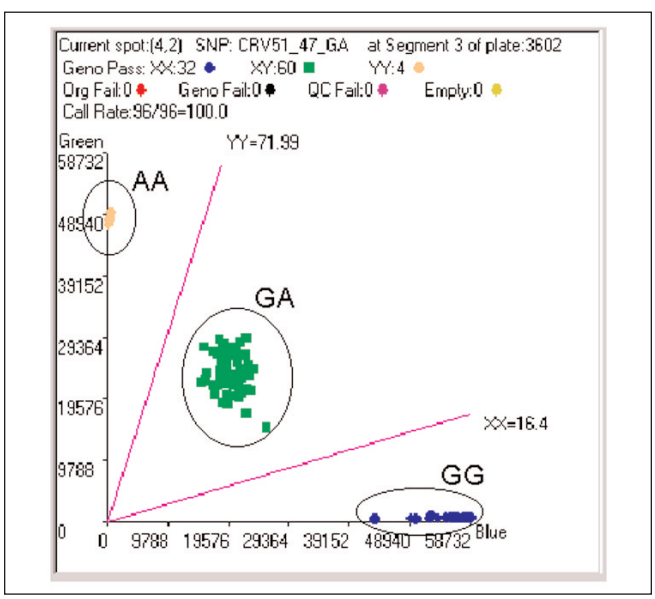

Figure 4. SNPstream UHT Scatter Plot. A representative graphical output for a GA SNP. The header shows the SNP#, plate location, and count for each genotype. The Y-axis shows the TAMRA-A fluorescent intensity; the X-axis shows the Bodipy-Fluorescein-G intensity. Rays that define the three genotype groups are labeled with the angle relative to the X-axis, and each cluster is shown circled.

Table 1. SNP-IT Results

Parameter	Result
Assay Conversion	85% 82 of 96 SNPs yield working assays
Genotyping Success Rate	96% 7556 of 7872 possible genotypes
Concordance	99.5% Relative to DNA sequencing

PCR, and we frequently use 24 and higher plexing with similar results. Taken together, with 82 of 96 SNPs converting in this study to yield high-quality SNP assays, we calculated a first-pass success rate of 85%. This rate corresponds to 10 of the 12 multiplexed SNPs converting on average in the first trial and is comparable to the best success rates of other reported multiplex reactions (2,5,9). We also found no apparent bias in conversion rate by extension type. All extensions tested (G/A, T/C, C/A, G/T, T/A and G/C) gave between 8 and 12 acceptable assays. A representative scatter plot is shown in Figure 4.

Call Rate

In order for an assay to be acceptable, we set a threshold of 95% for the sample call rate. Of 100 DNA samples, 95 must give a clear genotype call. We have found that genomic DNA purified by commercially available ion exchange methods performs well in the multiplexed PCR and SNP-IT *tag array* assay. In addition, relatively crude DNA preparations from blood and buccal swabs have been used in several hundred thousand SNPstream UHT genotyping reactions and give >95% sample call rate (J. Baisch, personal communication). In the current study, a call rate of 96% for both test samples and Coriell genomic DNAs was observed (Table 1).

Concordance and Accuracy

In a blinded experiment, we compared approximately 7800 UHT genotypes with those determined by DNA sequencing. The results showed 99.5% concordance to the reference genotypes. We were unable to confirm the DNA sequencing data to resolve conflicts, but of the 50 discordant genotypes, we found high consistency in the UHT data such that in the majority of cases, the discordant genotypes could be attributed to errors in DNA sequencing.

SUMMARY

We have developed and demonstrated the SNPstream UHT, a scalable system capable of running 120 384-well plates, for over 550 000 genotyping tests per 24-hour period. The tag-based approach makes use of a generic microarray plate with the advantage of higher volume and lower cost manufacturing than would be obtained with plates customized for each SNP panel to be tested. PCR and SNP-IT *tag array* multiplexing combined with reaction volume reductions have also given significant improvements in cost and genotype, and preliminary work suggests that further increases in multiplexing and expanded microarrays will make genome and population-wide SNP genotyping increasingly practical.

ACKNOWLEDGMENTS

We would like to thank members of the SNPstream UHT project team: Valery Bogdanov, Paula Carpenter, Kunal Chokshi, Cathryn Crego; Mike DiCola, Fugui Dong, Michael Forrest, Emily Huang, Francine Jones, Joanne Corbo, Eric Friedman, Fan Kang, Feng-Shen Kuo, Yi Liu, Monica Mody, Shirish Parekh, Sunita Patel, Vitaly Pozharov, Mark Ruben, Ali Recber, Gary Schnerr, James Seaman, Cindy Smith, Lisa Spangler, Jeff Thompson, Ray Wu, Azzam Yasseen, and Kaijiang Zhang for their essential contributions to the development of the UHT system. We would also like to thank Mike Pirrung and Steve Anderson for useful technical discussions. This work was funded in part by NIST Advanced Technology Program Grant No. 7ONANB8H 4001.

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