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SNP identification in unamplified human genomic DNA with gold nanoparticle probes

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ABSTRACT

Single nucleotide polymorphisms (SNPs) comprise the most abundant source of genetic variation in the human genome. SNPs may be linked to genetic predispositions, frank disorders or adverse drug responses, or they may serve as genetic markers in linkage disequilibrium analysis. Thus far, established SNP detection techniques have utilized enzymes to meet the sensitivity and specificity requirements needed to overcome the high complexity of the human genome. Herein, we present for the first time a microarray-based method that allows multiplex SNP genotyping in total human genomic DNA without the need for target amplification or complexity reduction. This direct SNP genotyping methodology requires no enzymes and relies on the high sensitivity of the gold nanoparticle probes. Specificity is derived from two sequential oligonucleotide hybridizations to the target by allele-specific surface-immobilized capture probes and gene-specific oligonucleotide-functionalized gold nanoparticle probes. Reproducible multiplex SNP detection is demonstrated with unamplified human genomic DNA samples representing all possible genotypes for three genes involved in thrombotic disorders. The assay format is simple, rapid and robust pointing to its suitability for multiplex SNP profiling at the 'point of care'.

INTRODUCTION

The decoding of the human genome (1,2) and the resulting greater than 3 million single nucleotide polymorphisms (SNPs) present exciting avenues to study the impact of genetic variations on complex phenotypes. Moreover, the ability to associate disease states and pharmacological responses with individual SNPs and increasingly with haplotypes (3) and related linkage disequilibrium mapping studies (4–6) offer the possibility of improved therapeutic and prophylactic measures. To realize this promise at the level of the individual, highly sensitive, robust and inexpensive SNP detection methodologies are needed.

Numerous strategies have been developed for SNP discrimination with various degrees of multiplexing capability (7–10). Overcoming the greater than 1 000 000 000 bp complexity presented by the human genomic DNA remains one of the principal challenges for SNP genotyping (11,12). In allele-specific hybridization strategies, reduction in complexity is achieved by target amplification typically with PCR, which also accommodates any sensitivity limitations. The amplified targets can then be interrogated by various direct and indirect hybridization strategies (7–10). Several strategies have also been devised that rely on the high specificity of enzymes to allow SNP identification directly from genomic DNA. Thermostable ligases have been used in conjunction with two probes that bind to adjacent regions of the SNP in 'ligase chain reactions' to generate sufficient number of ligated probes that are detected by FRET (13). Related strategies combine polymerase extension and ligation of probes, which are then followed by probe amplification to allow detection (14–17). Flap endonucleases have been employed in signal amplification assays where allele-specific probes are displaced by upstream probes at the SNP site on the target (18). This three-dimensional structure is recognized as a cleavage site by the enzyme. Cleavage results in the generation of a fragment that is a substrate for secondary reactions, which generate FRET probes exponentially to provide detectable signals. Thus, overcoming genome complexity has, thus far, not been possible without enzymatic intervention.

We present an enzyme-free, allele-specific hybridization methodology for multiplex SNP profiling of total genomic DNA. The assay format employs gold nanoparticle probe-based hybridization and detection strategies in conjunction with microarrays (19,20). The SNP detection strategy relies

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on the high sensitivity of the gold nanoparticle probes and the specificity afforded by the sequential hybridizations of two sequence-specific probes that selectively 'sandwich' the allele-specific target. Significantly, this simple methodology eliminates the need for both target amplification and complexity reduction of genomic DNA.

METHODS

Materials

$HAuCl_4 \cdot 3H_2O$, trisodium citrate, Tween^{TM}-20, sodium nitrate, silver enhancer solutions (Sigma A and B), formamide and human placental DNA were purchased from Sigma-Aldrich. Additional genotyped human genomic DNA samples were obtained from the Coriell Institute. 20× SSC was from GIBCO-BRL. Gaskets used to create multiple test arrays on each slide were purchased from Grace Biolabs. CodeLink microarray substrates were purchased from Amersham.

SNP arrays, nanoparticle probes and sequences

Each SNP was represented by two allele-specific capture oligonucleotide probes and a gene-specific probe. The placement of the SNP site within the capture probes allows the detection of both alleles in the same reaction well (19). We have shown previously that placing the SNP site within the signal probe oligonucleotide also allows SNP discrimination with PCR products, but requires separate wells to evaluate the two alleles (20). Amine-modified capture oligonucleotides and thiol-modified probe oligonucleotides used in the multiplex SNP assay for the three genes were synthesized in-house (ABI 3900 DNA synthesizer) and purified by HPLC (ion-exchange). The capture probes were arrayed on CodeLink substrates in 150 mM Sodium Phosphate buffer (pH 8.5) supplemented with 0.01% SDS with an Omnigrid (GeneMachines) arraying machine. Arraying arrangements on the slide allowed multiple sub-arrays that could be physically partitioned with silicone gaskets (Grace Biolabs) to generate separate test wells. Protocols recommended by the manufacturer were followed for post-array processing of the test slides.

Gold nanoparticles (~15 nm diameter) were prepared by the citrate reduction method (21). The approximate concentration of the gold nanoparticles was deduced from equating particle size measurements obtained by transmission electron microscopy (TEM) to the gold atom concentration, which was obtained by inductive coupled plasma–atomic emission spectroscopy (ICP–AES). Optical spectra of the gold nanoparticles were recorded with an HP8453 UV-vis spectrophotomer ($\lambda_{max} = 518$ nm). The oligonucleotide-modified gold nanoparticle probes were synthesized following protocols described previously (22). Briefly, thiol-functionalized oligonucleotides (4 μM final concentration) were initially incubated with gold nanoparticles for >16 h, followed by successive additions of phosphate-buffered saline (PBS) to a final concentration of 0.8 M NaCl. After an overnight incubation, the probes were isolated by centrifugation, washed in an equivalent amount of water, and then redispersed in 0.1 M PBS, 0.01% azide at a particle concentration of 10 nM. All probes were stored at 4°C.

The sequences for oligonucleotide pairs for each SNP were designed such that the capture probe contained the mutation site and the probe oligonucleotide covered a neighboring region either 3′ or 5′ to the capture oligonucleotide site. The number of bases separating the capture and the probe oligonucleotide was typically no more than ~50 nt. The capture probes and the probe oligonucleotides for all SNPs were designed to function under isothermal assay conditions. NB: For convenience, the major and the minor alleles are referred to as wild-type (or wt) and mutant (mut) respectively.

Sequences for the capture and the probe oligonucleotides employed in the assays are listed: MTHFR wt, 5′-GATGAAATCGGCTCCCGCAGAC-3′; MTHFR mut, 5′-ATGAAATCGACTCCCGCAGACA-3′; MTHFR probe, 5′-GGAAGAATGTGTCAGCCTCAAAGAAAAGC-3′; factor V wt, 5′-TGGACAGGCGAGGAATACAGGTAT-3′; factor V mut, 5′-CTGGACAGGCAAGGAATACAGGTATT-3′; factor V probe, 5′-CCACAGAAAATGATGCCCAGTGCTTAACAAGACCATACTACAGTGA-3′; factor II wt, 5′-CTCAGCGAGCCTCAATGCTCCC-3′; factor II mut, 5′-CTCTCAGCAAGCCTCAATGCTCC-3′; factor II probe, 5′-TCCTGGAACCAATCCCGTGAAAGAATTATTTTTGTGTTTCTAAAACT-3′.

Hybridization assays

Human placenta DNA (Sigma) or patient genomic DNA samples (Coriell Institute) were independently genotyped by sequencing methods. The DNA sample was fragmented by ultrasonication (Misonix), and conditions were adjusted to yield a median DNA length of ~0.5 kb. The target hybridization mixture (5 μl) contained 4× SSC, 0.05% Tween-20, 35% formamide, and 0.5–5 μg human genomic DNA, or as indicated in the specific experiment. The hybridization mixture was added to the test well after a 3 min, 98°C heat denaturation step. Each test slide possessed several sub-arrays that could be isolated by gaskets allowing for the testing of several test samples simultaneously. The test slide was incubated at 40°C for 60 min and washed subsequently at room temperature twice (2 min each) in a wash buffer containing 0.5 M $NaNO_3$, 0.05% Tween^{TM}-20. This low stringency wash was followed by a brief high stringency wash (30 s) in a low-salt wash buffer (0.4× SSC). Each sub-array was then covered with 50 μl hybridization buffer (4× SSC, 0.05% Tween-20, 35% formamide) containing gold-nanoparticle probes (1 nM each) for 30 min at 40°C. The isolating gasket was removed and the test slide was washed again in the wash buffer for 3 min (2×) at room temperature with gentle agitation. Finally, the washed slide was stained with 2 ml of silver reagent, an admix of Silver enhancer A and B solutions (Sigma) for ~5 min, washed in ddH_2O, and dried. The dried slide was imaged with a Nanosphere Verigene ID^{TM} imaging system or with an arrayWorx biochip reader (Applied Precision).

Data analysis

The images captured by either system (Verigene ID^{TM} or arrayWorx biochip reader) comprise 16-bit file formats. Mean signal intensities were obtained by correcting for local background using commercially available analysis software [GenePix Pro 4.0 (Axon) or the dedicated software on the arrayWorx biochip reader]. The triplicate signals for each capture oligonucleotide were averaged to obtain mean net signals (S). Discrimination factors (DF) were calculated using the following normalizing algorithm:

$$DF = (S_{wt} - S_{mut})/(S_{wt} + S_{mut})$$

Threshold values were derived from the reproducibility studies after adding 3 SD values to the average heterozygous DF value. These threshold values allow genotype calls for unknowns with a greater than 99% confidence: DF values between +0.4 and −0.4 are called heterozygous and values below and above this range are called homozygous mutant and homozygous wild-type, respectively.

RESULTS AND DISCUSSION

The microarray-based methodology employed for SNP discrimination involves hybridization of two oligonucleotide probes, the capture and the signal probe, to the DNA target sequence (19). The allele-specific capture probe covers the SNP site and is immobilized on a glass slide surface. The signal probe, on the other hand, is covalently bound to gold nanoparticles, which hybridize to a sequence neighboring the SNP site. The basic steps for the SNP assay are outlined in Figure 1. Briefly, genomic DNA is fragmented and hybridized to a microarray containing allele-specific capture probes under conditions that are sufficiently stringent to allow allele-specific hybridization of the targets. After this hybridization step, unbound genomic DNA is removed in a wash step and followed by a second hybridization step designed to attach oligonucleotide-modified gold nanoparticle probes to the captured targets, so as to 'sandwich' the target between immobilized capture probes and gold nanoparticle probes. After removal of all unbound nanoparticle probes, a silver development step is used to precipitate elementary silver around the nanoparticle probes (23), which dramatically increases their extinction and improves their ability to scatter light by several orders of magnitude (20).

For detection, the light source, either white light or light from LEDs (λ_{max}: 630 nm), is delivered into the plane of the test slide. The resulting evanescent wave excites the silver-amplified gold nanoparticles and the scattered light is focused onto a photosensor to capture the image. This scatter-based detection method provides ultra-high sensitivity with an ability to distinguish diffraction-limited single silver-amplified gold nanoparticles (20).

As a model for SNP detection in total genomic DNA, SNPs in three coagulation genes associated with thrombotic disorders were chosen (24), factor V (1691 G→A), factor II (20210 G→A) and MTHFR (677 C→T). The arrays contained two allele-specific capture probes for each SNP (see Figure 2a), one representing the wild-type (wt) sequence and the other containing the specific nucleotide change (mutant or mut). All capture probe sequences were designed to be isothermal such that similar assay conditions could be used for SNP discrimination of the three genes. Assay optimizations revolved around establishing hybridization stringencies for reliable SNP discrimination. Salt, temperature and formamide were varied to achieve stringency conditions that allowed multiplex SNP discrimination. Genomic DNA samples, obtained from commercial sources, were sequenced by a third-party vendor to confirm the genotypes of the three genes (factor V, factor II and MTHFR). As shown in Table 1, these samples collectively represented all possible genotypes (wild-type, mutant and heterozygous) for each of the three SNPs. A typical scatter image from a multiplex SNP assay is presented in Figure 2b along with the associated quantitation. The genomic DNA sample GM 16000 was heterozygous for MTHFR (677 C→T), homozygous mutant for factor II (20210 G→A), and homozygous wild-type for factor V (1691 G→A). When hybridized to the SNP array using the optimized assay conditions, quantitation of the net signal intensities gave a relative value of >20 000 for both the wild-type and mutant MTHFR capture spots. The S_{wt}/S_{mut} ratio of 1.1 indicates a heterozygous genotype. For factor II, the signal intensities at the mutant capture spots were 10-fold greater than the wild-type capture spots to give a S_{wt}/S_{mut} ratio of <0.1 pointing to a homozygous mutant genotype. The S_{wt}/S_{mut} ratio for factor V was >5, confirming a homozygous wild-type genotype. Quantitative data for four additional genomic DNA samples that were also subjected to the genotyping assays is presented (Figure 2c–f). Each of the determinations was consistent with the data obtained from sequencing results of the genomic DNA samples, providing further evidence that the gold nanoparticle-based assay is capable of accurately identifying homozygous as well as heterozygous genotypes, from complex, unamplified genomic DNA.

The basis for these extraordinary observations is the combined specificities of two hybridization reactions and the high sensitivity afforded by the gold nanoparticle probes. The assay design requires a sequential hybridization of the

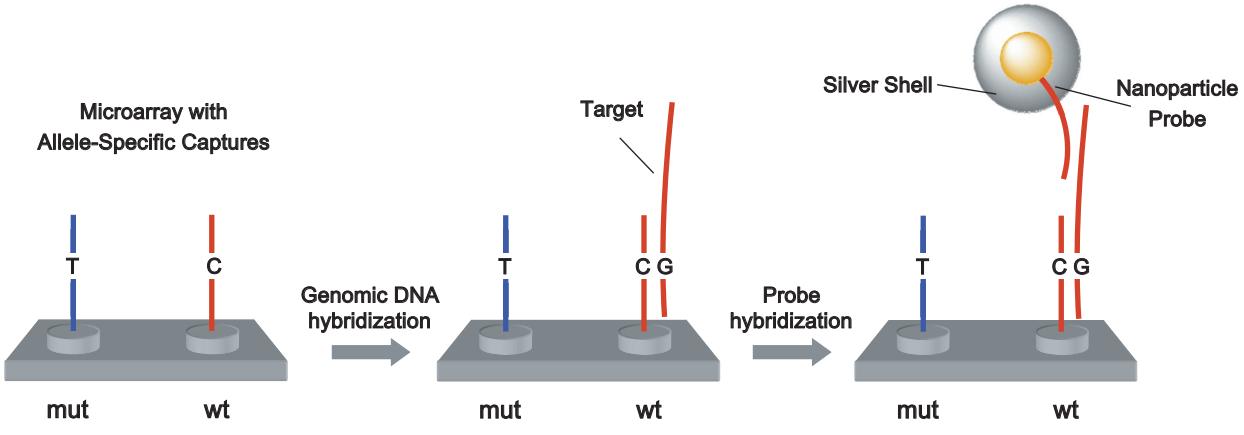

Figure 1. Fragmented unamplified genomic DNA and gold nanoparticle probes are sequentially hybridized to the SNP microarray containing capture probes representing the two possible alleles. After the two hybridization steps, the array is subjected to stringency washes and a silver development step. The scatter signal from the test sites is imaged subsequently to obtain genotyping results.

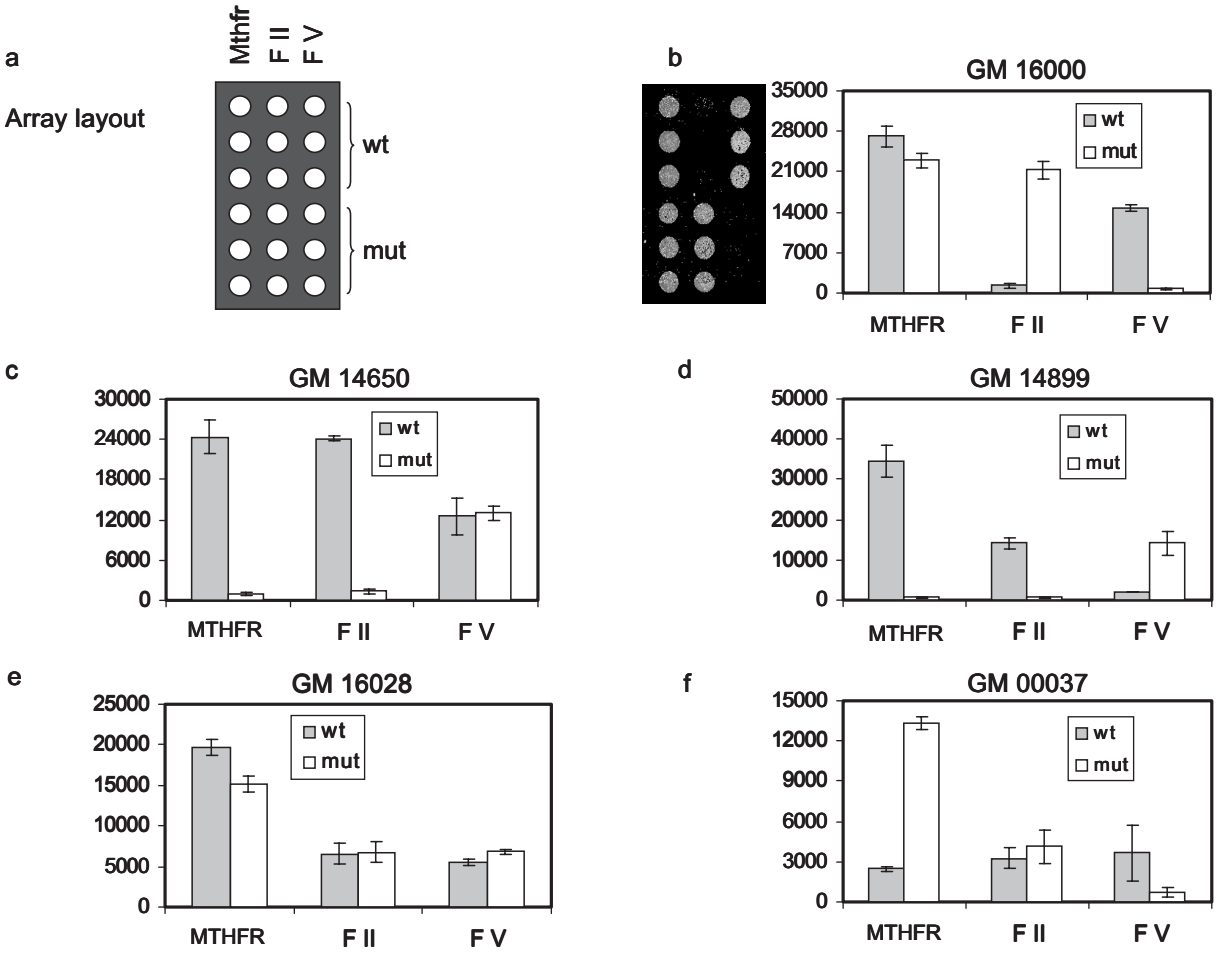

Figure 2. Multiplex detection of SNPs from unamplified genomic DNA from five different genomic DNA samples. (**a**) Allele-specific capture oligonucleotides for the hypercoagulation genes (MTHFR, FII and FV) were spotted in triplicate in the arrangement indicated. The microarrays (**b–f**) were subjected to unamplified genomic DNA from the different samples followed by gene-specific probes. The abbreviations used to identify the different DNA samples were adopted from the vendor (Coriell Institute). The samples embodied all three genotypes for the individual genes (wild-type, mutant and heterozygous) and can be identified by the presence or absence of signal at the respective captures [see image in (b)]. The graphs associated with individual images represent average net signal values from the triplicate spots for the wild-type (wt) or mutant (mut) capture spots, respectively. Error bars correspond to 1 SD.

Table 1. Enzyme-free genotyping with nanoparticle probes

Gene	GM16000	GM00037	GM14899	GM14650	GM16028	Placenta DNA
MTHFR	Heterozygous	Mutant	Wild-type	Wild-type	Heterozygous	Wild-type
FII	Mutant	Heterozygous	Wild-type	Wild-type	Heterozygous	Wild-type
FV	Wild-type	Wild-type	Mutant	Heterozygous	Heterozygous	Wild-type

capture and signal probe that is critical for the assay. In the first step, fragmented genomic DNA is hybridized to the chip at high stringencies that allow allele-specific hybridization of the target molecules. But because of the extremely high complexity of human genomic DNA, it is likely that a significant number of non-target sequences are able to form stable hybrids with the capture probes. These sequences would include homologous sequences as are found by searches performed in available sequence databases. Additionally, various 'pseudo-homologous' hybrids, including bulge structures or long DNA fragments hybridized to multiple capture probes, may also form stable structures at the test sites. Indeed the number of non-target hybridization events could surpass those with true gene-specific targets even though under these conditions there is single base discrimination between the specific wild-type and the mutant targets. While the high stringency wash at the end of the first step aims to remove unbound genomic DNA and dramatically reduces overall complexity for the second step hybridization, it is not expected to eliminate the bound homologous or pseudo-homologous non-target DNA from the test sites. Thus, in the context of this assay, identifying the allele-specific targets hybridized at each test site in the midst of captured non-target DNA requires a second specific hybridization event. It is reasonable, however,

to assume that the complexity of captured DNA at the capture site is significantly lower than that of total human DNA. The second hybridization, accomplished by the gene-specific nanoparticle probe, provides the discrimination power to distinguish targets from non-targets and in the process allows SNP identification.

In order to confirm the need for two hybridizations for SNP discrimination, fragmented genomic DNA was subjected to a terminal transferase reaction in the presence of dATP to tail the ends of each DNA fragment with stretches of poly(dA) sequences. This tailed genomic DNA was then hybridized to the SNP array by using the standard protocol except that the second step of the assay was performed either with poly(dT)-modified 15 nm gold probes or with the gene-specific probes. As expected, the assay with the gene-specific probes yielded signals only at the wild-type capture spots indicative of the correct genotype (Figure 3a). In contrast, the poly(dT)-modified probes exhibited strong signals both at the wild-type and mutant test capture spots (Figure 3b). Even though the signals were strong, they did not allow proper genotyping. Striking is the inordinately high signal (10- to 40-fold) associated with the poly(dT) probe when compared with the assay performed with gene-specific probes. Since the molarity of the poly(dT) probe as well as the gene-specific probes was identical, the higher signal associated with the poly(dT) probe would suggest that a greater number of probes are bound at the test sites. While some of this may be due to the binding of multiple probes at each poly(A) tail, we have evidence that probes in close physical proximity are covered by the same silver reaction, generating only a small increase in signal. Thus, the increase in signal may be explained by the presence of non-target homologous and pseudo-homologous DNA sequences bound to the test sites along with the gene-specific target. Significant to the mechanism of this assay is the observation that gene-specific probes are able to provide accurate genotyping even in the presence of apparently a significant amount of non-target DNA. The bound non-target DNA does not interfere with the assay because the gene-specific probes are designed to hybridize to an adjacent region of the SNP site, and the probability of a non-target DNA fragment possessing also a homologous region for the nanoparticle probe is extremely low.

In order to estimate assay detection limits, the input amount of factor V mutant human genomic DNA was reduced to 2.5, 1 and 0.5 μg in a 5 μl reaction volume, corresponding to 750 000, 300 000 and 150 000 genome copies or 250, 100 and 50 fM respectively (Figure 4). As expected, attenuation of signal-to-noise ratios was observed with decreasing target concentration. Nevertheless, reliable SNP discrimination was observed at all concentrations including at 0.5 μg genomic DNA based on ~15 separate experiments; at lower amounts (0.1 μg or 30 000 genome copies) the genotyping call rates fell to ~50% primarily because target signals dropped below 3 SD of background signals. We attribute the ability to detect SNPs with 150 000 genome copies (500 ng in 5 μl; 50 fM) of human genomic DNA in a ~1 h hybridization to the high sensitivity afforded by the gold nanoparticle probes. This would be difficult to achieve by standard detection techniques using fluorophores, judging by the greater than 3 log higher detection limits of nanoparticles over fluorophores that we have observed in a particle-to-fluorophore comparison (0.001 probes/μm^2 versus 7 fluors/μm^2) (20).

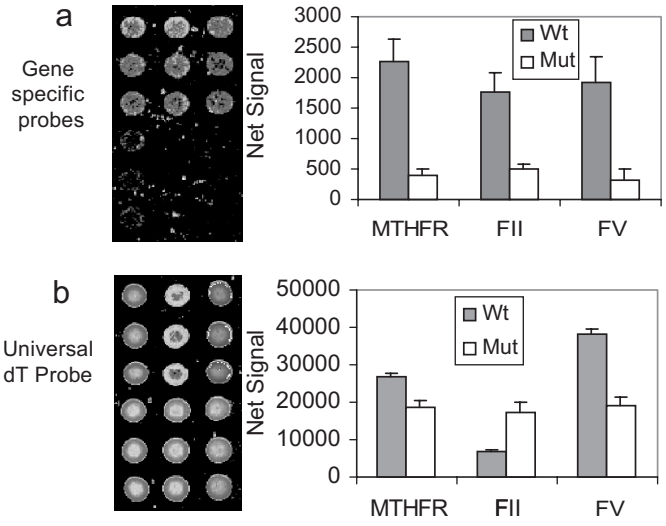

Figure 3. SNP discrimination requires the specificity provided by the sequential hybridization of target-specific capture and probes. Fragmented human placenta DNA (wild-type for FV, FII and MTHFR) was tailed with poly(dA) by terminal transferase. The poly(dA) tailed genomic DNA was hybridized to SNP arrays (5 μg/array) at 35% formamide, 4× SSC, 0.01% Tween and 0.01% SDS at 40°C for 1 h. After removing unbound genomic DNA targets, the arrays were further hybridized with either gene-specific probes (a) or poly(dT) nanoparticle probes (b) for 30 min and followed by a silver enhancing step, respectively. (**a**) Gene-specific probes employed for SNP discrimination of the poly-dA labeled genomic DNA showed clear differences between the intensities at the wt and mut captures permitting SNP discrimination even in the presence of non-target homologous DNA at the test sites. (**b**) With the poly(dT) probes, strong signals were observed both at the wt and mut capture oligonucleotides but SNP discrimination was not possible indicating that the signals derive from non-target homologous DNA.

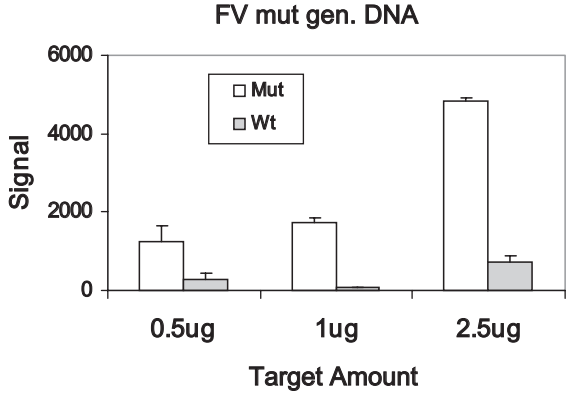

Figure 4. Detection sensitivity of the SNP discrimination assays in genomic DNA. SNP arrays for the factor V gene were hybridized with 2.5 μg (750 000 copies), 1 μg (300 000 copies) and 500 ng (150 000 copies) of unamplified human genomic DNA genotyped to be mutant for the factor V gene in a target titration experiment. Clear SNP discrimination was possible even at 500 ng total genomic DNA.

In order to test the reproducibility of this assay system, a total of 25 independent hybridization assays were performed, each with triplicate SNP testing for all three thromobosis genes. A total of six unique genomic DNA samples (Table 1) were included in the reproducibility studies. This represented 35 tests for wild-type, 30 tests for heterozygous, 10 tests for

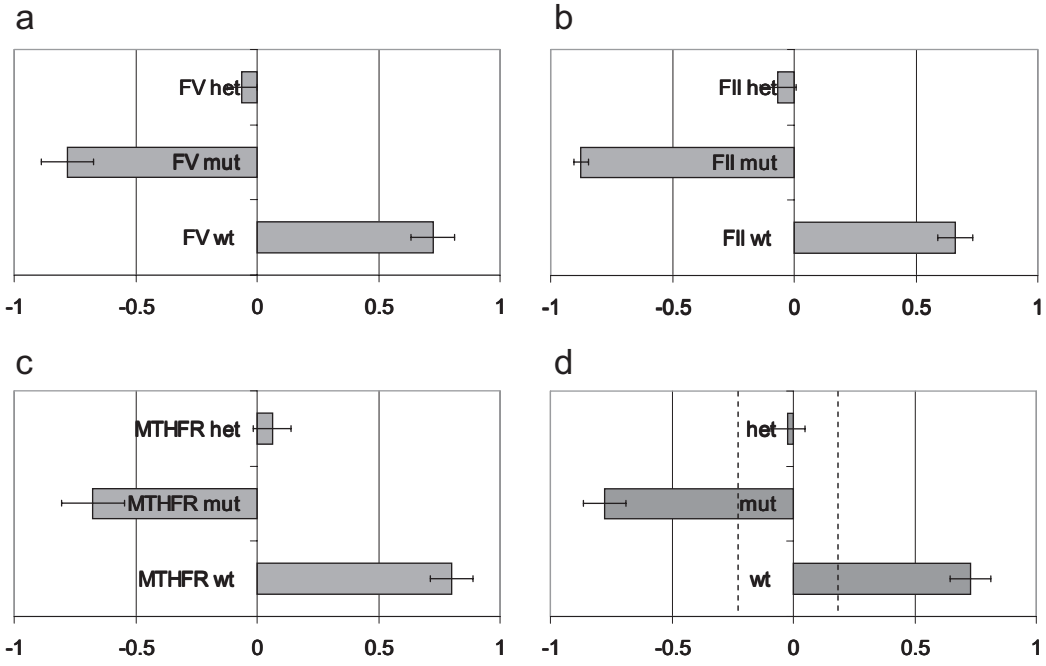

Figure 5. Discrimination factors [DF = $(S_{wt} - S_{mut})/(S_{wt} + S_{mut})$] were derived from a reproducibility study comprising 25 independent assays reflecting a total of 75 separate genotyping determinations. Of these, 35 determinations were from wild-type placenta DNA. The remaining 40 determinations (30 heterozygous and 10 mutants) came from genomic DNA samples from Coriell Institute (refer to Table 1). From the individual DF values for each gene, the average DF values were calculated for wt, mut and het, respectively (**a**–**c**). Average DF values for all three genes were plotted and a threshold to distinguish between heterozygous and homozygous genotypes was set at ±0.4 after factoring in 3 SD values above the average heterozygous signal (**d**).

mutant SNPs. Discrimination factors were calculated as described (see Materials and Methods) and averaged for each genotype (Figure 5a–c): DF factor V wt = 0.72, het = −0.06 and mut = −0.78; DF factor II wt = 0.66, het = −0.07 and mut = −0.88; DF MTHFR wt = 0.8, het = 0.06 and mut = −0.68 (Figure 5). Although the sequence context is different for the three polymorphic sites, the DF for a certain allelic status showed low variability between the different SNPs (as low as 1% for heterozygous DFs to a maximum of 20% for mutant DFs). Thus, we averaged the values for each genotype (0.72 for wild-type, −0.77 for mutant, and −0.02 for heterozygous), and set a threshold at +/− 0.4 by adding 3 SD values to the mean heterozygous DF value (Figure 5d). Since none of the homozygous or heterozygous genotypes had DF values that fell below these threshold values, it appears that this method would allow detection of an SNP in an unknown genotype with a >99% confidence level.

CONCLUSION

SNP genotyping on microarrays with total genomic DNA is possible owing to the high assay specificity derived by sandwiching the target between capture and detection probes in two sequential steps coupled with the ultra-high sensitivity of gold nanoparticle probes. The superior assay sensitivity is critical especially when hybridizations are performed within one hour with a target requirement of as little as 500 ng total genomic DNA—an amount obtained from a single drop of blood or from a buccal swab. The ability to discriminate single base changes with unamplified genomic DNA and the simplicity, low-cost and robustness of this assay points to a genotyping solution for 'point of care' diagnostic applications.

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