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ARTICLE

Simple two-color array-based approach for mutation detection

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The ability to analyze multiple polymorphic/mutation sites rapidly and accurately is pivotal in all areas of genetic analysis. We have applied single nucleotide primer extension (SNE) for detection of multiple point mutations in a micro-array format using two-color, fluorescent dye-tagged dideoxynucleoside triphosphate terminators (ddNTPs). The oligonucleotide primer ending one nucleotide short of the mutation site being probed is bound to the slide and single-base extended in place with two different Cy5/Cy3 dye-tagged terminators using solution-phase, locus-specific, single-stranded complementary templates generated by PCR from genomic DNA. The composite fluorescence produced contains peaks of distinct wave lengths corresponding to each Cy dye-tagged terminator incorporated, resulting in a fluorescent 'fingerprint' for each DNA target. DNA polymerase-catalyzed incorporation of Cy dye-tagged dideoxynucleoside triphosphates was dependent on the particular dyes, the specific ddNTP, the DNA target concentration, sequence of the template, on-slide temperature cycling and washing conditions. Results from analysis of mutations in the human hemochromatosis and connexin 26 genes show that this approach has several advantages over existing methods and is simple, rapid, robust, cost effective and accurate with potential applications in many areas of genetic analysis. *European Journal of Human Genetics* (2000) **8**, 884–894.

Keywords: array-bound single nucleotide primer extension; minisequencing; fluorescent ddNTPs; mutation detection

Introduction

A variety of molecular alterations have been implicated in leading to the development of human genetic diseases and malignancies. Development of rapid, robust, parallel, high-throughput, cost-effective approaches to mutation detection are critical to generation of a thorough molecular diagnosis of life-threatening diseases.[1] In addition, realization of such developments will facilitate early diagnosis and evaluation of treatment strategies.

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A number of approaches has been used for mutation detection.[2] Single-nucleotide primer extension (SNE) has been used for detection of point mutations.[3] The assay is template-dependent and involves primer extension by a radioactive- or dye-labeled dideoxynucleotide terminator (ddNTP) with the tag of the incorporated base revealing the identity of the template complementary nucleotide immediately 3' to the primer. Solution-phase extensions followed by electrophoresis on gels as well as microtiter plate-based assays have been described.[4–11] A solution-phase fluorogenic PCR 5'-nuclease or TaqMan™ assay was recently reported using seven different dyes for detection of single-nucleotide polymorphisms (SNPs) in six PCR products.[12] In addition, a variety of other solution-phase mutation detection schemes

have been described which depend on fluorescent dyes, energy transfer and/or fluorescence quenching.[13–17]

Chip-based extension improves on solution-phase methods since primers can be immobilized in advance on glass. Recently, a radioactive method for multiplex detection of mutations was described in which solid-phase SNE was applied to an oligonucleotide array format.[18–20] In a comparison with hybridization and immobilized allele-specific probes (ASO), the power of discrimination between homozygotes and heterozygotes was one order of magnitude higher using the SNE method. Use of ASO requires at least wild-type and mutant probes[21,22] with annealing and washing steps requiring extensive empirical testing to define conditions for selective removal of a single-base mismatched target. Such requirements are not optimal for production of parallel arrays that simultaneously detect multiple known point mutations in different genes. Furthermore, current chip-based ASO methods utilize four probes for each SNP call and employ photolithography to generate the chips[23] which are not readily amenable to customization in a research or a clinical laboratory setting. Computer algorithms are also required to make definitive calls.

Disadvantages of current chip-based SNE approaches include use of radioisotopes or antibody conjugants for detection, and/or requirements for generation and/or purification of single-stranded target complements. In this report, we describe development of an easy, implementable, array-based assay employing single and two-color fluorescence-based detection which has advantages over existing SNE strategies.

Methods

Leukocyte DNA from normal controls and patients harboring single-base mutations in the hemochromatosis (HFE)[24,25] and connexin 26 (CX26)[26,27] genes was obtained, and all genotypes were confirmed by automated DNA sequence analysis.

Oligonucleotides

Two sets of oligonucleotides (Table 1, sets A and B) were synthesized using standard phosphoramidite chemistry and purified by HPLC. Oligonucleotides to be attached to the glass surface (primer probes) were synthesized with a 5'-amino group followed by a polyethylene glycol (PEG) spacer as described previously.[18–20] To conform to the evolving nomenclature for surface hybridization, we refer to such molecules as probes.

Table 1 Reagents for array-bound SNE

Locus	*PCR Primers (F=forward; R=reverse)*	*PCR product length (bp)*	*Array-bound SNE primer probes (S=sense strand; AS=antisense strand)*
H63D HLD-H - Set A	F: ACA TGG TTA AGG CCT GTT GC R: GCC ACA TCT GGC TTG AAA TT	208	S: XXX-CAG CTG TTC GTG TTC TAT GAT AS: XXX-GGC TCC ACA CGG CGA CTC TCA T
C282Y HLA-H - Set A	F: TGG CAA GGG TAA ACA GAT CC R: CTC AGG CAC TCC TCT CAA CC	389	S: XXX-GGG GAA GAG CAG AGA TAT ACG T AS: XXX-CAG GCC TGG GTG CTC CAC CTG G
H63D HLA-H Set B	F: CAG CTG TTC GTG TTC TAT GAT R: TCG GGG CTC CAC ACG GCG ACT	48	S: as above AS: as above
C282Y HLA-H - Set B	F: GGG GAA GAG CAG AGA TAT ACG T R: GGG CTG ATC CAG GCC TGG GTG	54	S: as above AS: as above
CX26 - SET A	F: GCATTCGTCTTTTCCAGAGC R: GGCCTACAGGGGTTTCAAAT	850	
35delG CX26			S: XXX-ACG CTG CAG ACG ATC CTG GGG G AS: XXX-GTG GAG TGT TTG TTC ACA CCC CC
167delT CX26			S: XXX-AGG CCG ACT TTG TCT GCA ACA CCC AS: XXX-CAC ACG TTC TTG CAG CCT GGC TGC
35delG CX26 - Set B	F: ACG CTG CAG ACG ATC CTG GGG G R: GTG GAG TGT TTG TTC ACA CCC CC	46	S: as above AS: as above
167delT CX26 - Set B	F: AGG CCG ACT TTG TCT GCA ACA CCC R: CAC ACG TTC TTG CAG CCT GGC TGC	49	S: as above AS: as above
Expected extension	SNE oligo targets		Array-bound SNE control probe: target (Probe)
G	SNE-C: AGC CGA CTG AGA TA		5'-XXX-AGA GAG ACT GAC TAT CTC AGT C-3'
A	SNE-T: ACG TGA CTG AGA TA		||| ||| ||| |
C	SNE-G: ACG GGA CTG AGA TA		(target) 3'-ATA GAG TCA G CGCA-5'
T	SNE-A: ACG AGA CTG AGA TA		T
A, G, C, T	SNE-N: ACG NGA CTG AGA TA*		G A N

XXX=Aminolinker; *Can be used with any ddNTP extension since a mixture of all four nucleotides (N) was used during synthesis at this site.

SNE control

Five oligonucleotide targets (14 mers) (Table 1) each complementary to 10 nt at the 3' end of a bound SNE oligonucleotide control primer probe (22 mer) were used as a positive target control for successful extensions on the arrays. The five targets contained either A, C, G, T or all four nucleotides located one nucleotide beyond the 3' nucleotide of the bound probe, thereby facilitating array-bound probe SNE with ddTTP, ddGTP, ddCTP, ddATP or all four ddNTPs, respectively. For example, sequence of the target oligonucleotide in solution contained A at this site to monitor for array-bound ddTTP extensions. Targets also contained a 3 nt overhang beyond the site of the bound primer target/directed single nucleotide extension. Target SNE controls (12 ng) are mixed with PCR products from genomic DNA prior to SNE to assess probe attachments as well as extensions with each particular terminator.

Preparation of slides Glass slides were cleaned essentially as described (http://cmgm.stanford.edu/pbrown/protocols.html).

1,4-phenylene diisothiocyanate (PDC) modification and deposition of oligonucleotide probes on slides PDC modification was as described previously.[28] Poly-l-lysine slides were modified by treatment with 0.2% (w/v) PDC (Sigma, St Louis, MO, USA) in 10% (v/v) pyridine/dimethylformamide (Fisher, Pittsburgh, PA, USA) for 2 h at room temperature and then washed with HPLC-grade methanol and acetone. After drying at 110°C for 5 min, the PDC support was ready for attachment. The amino-modified oligonucleotides at 100 pmol/μl were mixed 1:1 with Micro-Spotting solution (TeleChem International Inc, Sunnyvale, CA, USA) and arrayed manually or robotically with our in-house constructed arrayer[29] on to the glass supports. Remaining reactive groups on slides were blocked by incubation in 1 M Tris-HCl (pH 7.5) for 1 h, rinsed in 1 M NaCl and then washed three times in ddH_2O, twice at room temperature, followed by a final wash at 55°C for 15 min. Slides were dried by centrifugation at 500 rpm for 2 min in a table-top centrifuge (Sorval, Newtown, CT, USA, model RT 6000B) using a microtiter centrifuge plate holder.

Generation of PCR products (targets) and array-bound single color primer extensions

Primers were initially picked that generated PCR products of the following lengths: 208 bp for detection of H63D and 389 bp for detection of C282Y in the *HFE* gene; and 850 bp for the entire *CX26* gene which can be used as target template for detection of both 35delG and 167delT mutations in that gene. PCR was performed in 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM each of dATP, dCTP and dGTP, 80 μM dTTP, 20 μM dUTP, 100 ng genomic DNA, 100 ng each primer, 1.44 Units AmpliTaq DNA polymerase (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) and 1.44 units of TaqStart antibody (Clontech, Palo Alto, CA, USA). PCR for the two mutations in the *HFE* gene was multiplexed with the four primers for H63D and C282Y, while PCR of the *CX26* gene was done separately. Following PCR amplification, products were treated with a mixture of 0.02 U/μl shrimp alkaline phosphatase (SAP) (Amersham–Pharmacia Biotech, Arlington Heights, IL, USA), 0.4 U/μl *E. coli* exonuclease I (Exo I) (Epicenter Inc, Madison, WI, USA), and 0.02 U/μl uracylglycosylase (UDG) (Amersham–Pharmacia Biotech) for 60 min at 37°C and then heat inactivated at 95°C for 10 min. This treatment degrades dNTPs to nucleosides, digests unincorporated primers and cleaves the PCR products at dU-containing sites.[30] Fragmentation can also be accomplished by DNase I treatment (1 U for 15 min at room temperature and then heat inactivated at 65°C for 15 min) of PCR product generated in the absence of dUTP using 200 μM dTTP. Fragmented DNA (100 ng) was then mixed with ThermoSequenase buffer (Amersham–Pharmacia Biotech) and 25 ng of SNE control oligonucleotide (Table 1) in a 40 μl reaction, heated to 95°C for 10 min, centrifuged, cooled on ice and pipetted on to the array. The solution was incubated for 5 min at room temperature to facilitate probe/target annealing, and then 10 μl of a mixture containing 1 μl of 40 μM non-competitive ddNTPs, 1 μl of 40 μM Cy5 ddNTP, 6.4 units ThermoSequenase (Amersham–Pharmacia Biotech), in 1X ThermoSequenase buffer was added to the array. The array was fitted with a cover slip and incubated for 20 min at 48°C. After incubation the array was washed in 55°C ddH_2O for 5 min, dried and then scanned.

Two-color array-bound primer extensions

More recently, we have generated much shorter PCR products by choosing primer pairs immediately flanking the mutation site being interrogated (Table 1, set B). This results in much smaller PCR products (45–50 bp) and eliminates the need for dU-mediated cleavage step with UDG. PCR was performed in a 50 μl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 200 μM each dNTP, 100 ng DNA, 100 ng each primer, 1.44 units AmpliTaq DNA polymerase and 1.44 units of TaqStart antibody. PCR for monitoring the two mutations in the *HFE* gene was multiplexed using four primers flanking the H63D and C282Y mutations. PCRs for CX-26 mutations were done separately because of proximity of the two mutations which, if multiplexed, could result in generation of a longer PCR product by using one forward and one reverse primer from the two sites being probed for the mutations. Conditions for PCR for HFE and CX26 mutations were 95°C for 5 min followed by 10 cycles at 94°C for 15 s, 70°C for 5 s decreasing the temperature 1°C per cycle to 60°C, then 72°C for 10 s followed by 22 cycles at 94°C for 15 s, 60°C for 5 s and 72°C for 10 s. This protocol eliminates formation of primer dimers that can co-migrate with the desired PCR products making quantitation difficult. Primer dimer formation was monitored by running a PCR reaction in the absence of genomic DNA. The PCR reaction was treated with a

mixture of SAP and *ExoI* for 60 min at 37°C and heat inactivated at 95°C for 10 min.

Two-color SNE experiments using the smaller PCR products were performed as follows: 30 ng of PCR product was mixed with ThermoSequenase buffer, 12 ng of SNE control oligonucleotide, 1 μl of 40 μM non-competitive ddNTP, 1 μl of 40 μM Cy5 ddNTP, 4 μl of 40 μM Cy3 ddNTP for ddG and ddTTP or a final concentration of 7.5 μM ddCTP or 16 μM ddATP, 1 μl of 10 μg/μl BSA, 25 μl 2 × Self-Seal Reagent (MJ Research, Inc., Waltham, MA, USA) and 32 units ThermoSequenase in a 50 μl reaction. The reaction mixture was pipetted on to the array and covered with a cover slip. The array was then placed on to the heating block of a Perkin-Elmer 9600 Thermocycler and SNE performed using a PCR cycling protocol. On-slide temperature cycling was done as follows: 96°C for 15 s, 50°C for 10 s, and 72°C for 20 min for four cycles, after which the slide was placed in a slide holder in a 62°C ddH_2O bath for 5 min. Slides were dried and then scanned.

ScanArray 3000 protocols and settings

A ScanArray 3000 (GSI Lumonics Inc, Watertown, MA, USA) was used to monitor Cy5 as well as Cy3 fluorescence using a laser power setting of 100% and PMT value of 100%. Extension signals for Cy5-tagged terminator are sometimes off-scale on the scanner necessitating a decreased setting for the laser from 100 to 75% in order to obtain data which is on the linear portion of scanner response (0–65000 gray scale units, linearity of scanner response for Cy 5-terminator is between approximately 5–5000 attomoles deposited in spot area of about 1×10^6 μ^2). Cy3 extensions were 2- to 3-fold less than Cy5 using about 1 μM terminator final concentrations. Scans of a dilution series of the 2 dye-tagged terminators at 100% settings for laser and photomultiplier as a function of attomoles of each dye deposited shows about a 2.7-fold higher reading for the same amount of terminator when comparing Cy5 to Cy3 (data not shown). This is consistent with a higher molar extinction coefficient and/or quantum yield for Cy5 compared with Cy3. In fact, the ratio of molar extinction coefficients for Cy5/Cy3-bound protein conjugates is 250 000/150 000 or 1.67, and the ratio of quantum yields is 0.28/0.15 or 1.87.[31] The product of these two ratios is 3.13 which approximates observed differences in Cy5/Cy3 fluorescence when spotting equimolar amounts of the two dyes on arrays.

Source of dye-labeled dideoxynucleotides Nucleotides labeled with Cy3 and Cy5 dyes (Amersham–Pharmacia Biotech) were prepared using C5 (pyrimidine) or C7 (purine) modified nucleotides.[32]

Results

Multiplex, single-color SNE

The basic strategy for array-bound SNE is shown in Figure 1. Expected array-bound extensions (+) with each of the four ddNTPs are seen on array-bound sense and antisense oligoprobes for the 16 possible 2-allele combinations (Figure 2, panel A). We first explored multiplexing so that simultaneous PCR and SNE using Cy5-tagged terminators could be implemented for detection of two different mutations on the same array. Expected ddNTP extensions for all nine possible genotypes at the two loci in the *HFE* gene using array-bound sense and antisense primers are shown in Figure 2, panel B. Multiplex PCR of human genomic DNA to screen for the two changes causing hemochromatosis (HFE)[24,25] is shown in Figure 2, panel C. PCR reactions contained two separate primer pairs (total of four in one reaction) which resulted in simultaneous PCR of the two different HFE regions. The PCR products were then interrogated for the two mutations (H63D and C282Y) each of which results in an amino acid change. The array contained H63D and C282Y sense and antisense bound primers as well as an SNE-bound control oligonucleotide primer. The SNE control lights up on all arrays since it represents an oligoprimer that can be extended by all four dye-tagged ddNTPs when annealed to its target complement oligonucleotide added to the PCR target solution just prior to SNE. Expected extensions (black circles) are shown in diagram form on the left in panel C. Extension on the array was done with Cy5-ddGTP providing a positive signal for H63D antisense and C282Y sense rows on the array. Genotype of the patient, determined by DNA sequence analysis was homozygous normal at the H63D (C on the sense strand) and heterozygous at the C282Y loci (G and A on the sense strand). Average florescence intensities of the row of four spots for each primer are in parentheses. These results show robust signals using Cy5-ddGTP extension for both H63D (normal/normal) as well as for C282Y (normal/mutant), consistent with presence of a normal allele at both loci. Since both sense and antisense SNE primers are array bound, heterozygote detection using the same dye for all four ddNTPs requires a second separate array extension with one of the three remaining ddNTPs.

We next typed five different genomic DNA samples for the same two mutations and compared those results with that expected from DNA analysis. Expected and observed results are shown in Table 2 for Cy5-ddGTP array-bound extensions from five different individuals (eg samples 001 to 005). Multiplex PCRs were done with two sets of primer pairs in five separate reactions, one of each of the two loci in the five individuals, and PCR products were incubated on five separate arrays to simultaneously assess these HFE genotypes. Averages for the scanning of the four quadruplicate spots representing sense and antisense primer registers at the H63D and C282Y loci are shown with results at each of the four spots. An SNE control is also included in quadruplicate on each array that extended with any dye-tagged terminator. Comparison of signals at the sense and antisense registers for the two HFE sites shows correct extensions for all five individuals characterized by a 5–10-fold signal to noise ratio (eg positive/negative average signals at sense and antisense

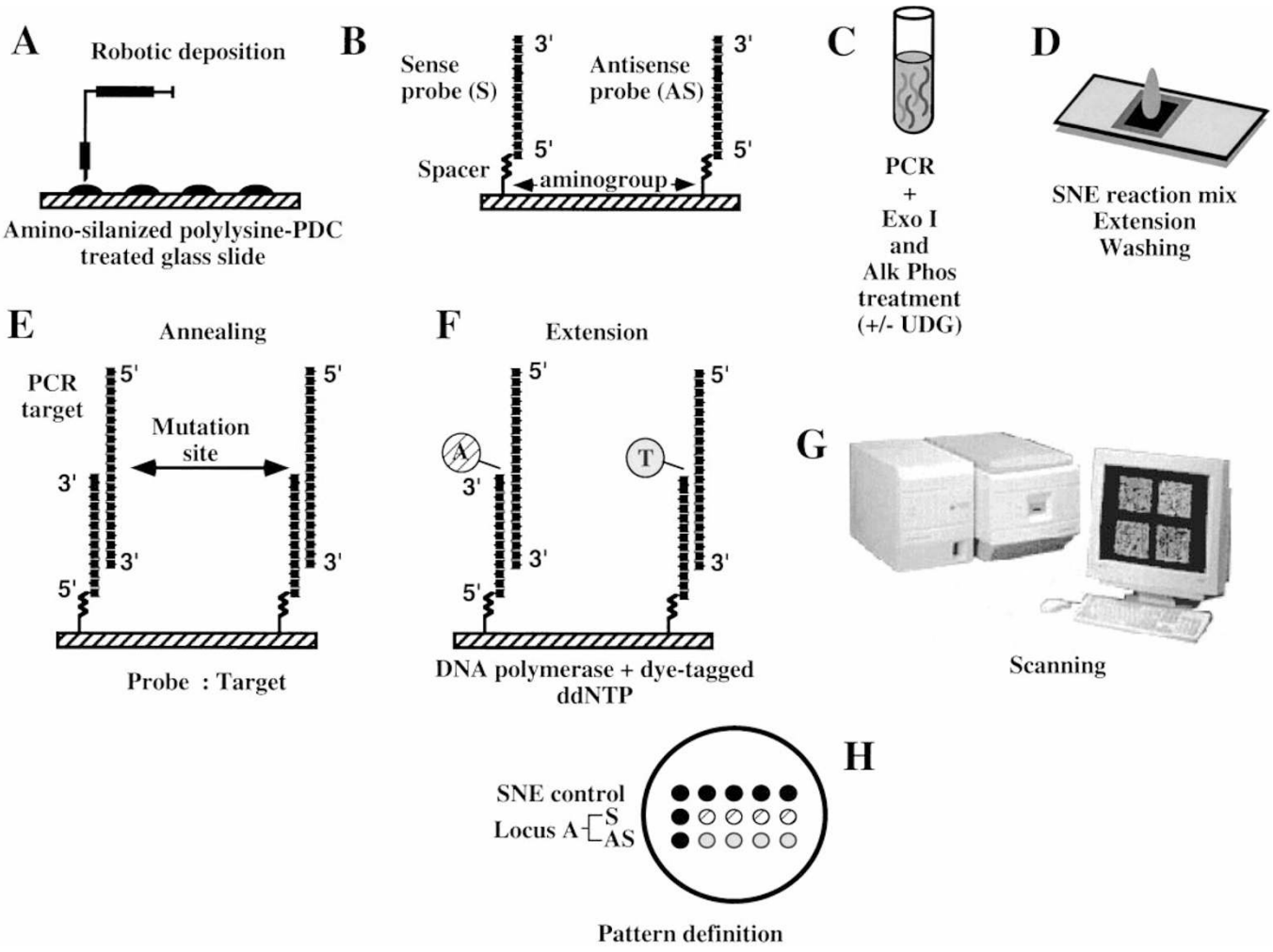

Figure 1 Strategy for array-bound SNE. Sense and antisense oligonucleotide probes containing 5' spacer and aminogroup are attached to amino-silanized polylysine-PDC treated glass slide following manual or robotic deposition (**A** and **B**). PCR targets are generated from genomic DNA and treated with Exo I and SAP (**C**). (Longer PCR products are synthesized in the presence of dUTP and subsequently cleaved with UDG). PCR targets are then placed on a slide containing the bound oligoprobes and SNE performed (**D**). After annealing of the complementary PCR target strand to the bound oligoprobes (**E**), a single dye terminator extension occurs (**F**) which is then monitored by scanning (**G**) giving rise to a specific pattern of fluorescence (**H**).

registers) for individuals with N/N genotypes at either locus.

Two-color SNE

Two-color multiplex extensions with Cy5-ddCTP and Cy3-ddTTP are shown in Figure 3. In this experiment we simultaneously probed on the same array for two common single-nucleotide deletions in the connexin 26 (CX26) gene[26,27] using a mixture of Cy5-ddCTP and Cy3-ddTTP. Again, signals are seen for both dyes at the proper array registers. Signals for Cy3 extensions were 2- to 3-fold lower than Cy5.

Two-color SNE using short PCR products, increased Cy3-ddNTPs concentration, and on-slide temperature cycling We next designed primers to generate much smaller PCR products (40–60 bp) in order to eliminate requirements for fragmentation of PCR fragments prior to array-bound SNE. In addition, concentration of the Cy3 ddNTPs was increased into an effort to maximize SNE with these terminators. Finally, an on-slide temperature cycling approach was also employed in an effort to further increase array-bound extensions for both Cy5 and Cy3 ddNTPs. Robust signals are now evident for both Cy5 and Cy3 extensions without the need for PCR fragmentation (Figure 4). In this experiment the ratio of concentrations for Cy3/Cy5 terminators was about 20/1 (16 μM compared with 0.75 μM). On-slide temperature cycling also appears to increase extensions for both dye-tagged terminators.

A

Allele		Sense oligoprobe				Antisense oligoprobe			
1	2	A	G	C	T	T	C	G	A
A	A	+	-	-	-	+	-	-	-
A	G	+	+	-	-	+	+	-	-
A	C	+	-	+	-	+	-	+	-
A	T	+	-	-	+	+	-	-	+
G	A	+	+	-	-	+	+	-	-
G	G	-	+	-	-	-	+	-	-
G	C	-	+	+	-	-	+	+	-
G	T	-	+	-	+	-	+	-	+
C	A	+	-	+	-	+	-	+	-
C	G	-	+	+	-	-	+	+	-
C	C	-	-	+	-	-	-	+	-
C	T	-	-	+	+	-	-	+	+
T	A	+	-	-	+	+	-	-	+
T	G	-	+	-	+	-	+	-	+
T	C	-	-	+	+	-	-	+	+
T	T	-	-	-	+	-	-	-	+

B

Expected ddNTP extensions for all 9 possible genotypes at two loci (C282Y and H63D) on array-bound sense (S) and antisense (AS) SNE primers

C282Y (G to A)			H63D (C to G)		
Genotype	S	AS	Genotype	S	AS
N/N	G	C	N/N	C	G
N/N	G	C	N/M	C, G	G, C
N/N	G	C	M/M	G	C
N/M	G, A	C, T	N/N	C	G
N/M	G, A	C, T	N/M	C, G	G, C
N/M	G, A	C, T	M/M	G	C
M/M	A	T	N/N	C	G
M/M	A	T	N/M	C, G	G, C
M/M	A	T	M/M	G	C

C

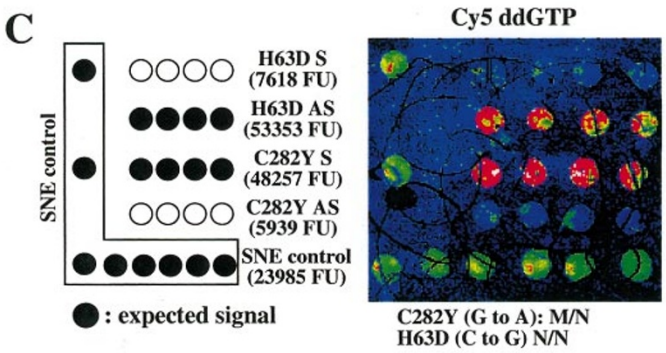

Figure 2 Detection of hemochromatosis mutations using single color SNE. Expected ddNTP extensions (+) on array-bound sense and antisense oligoprobes for each of the 16 possible nucleotide combinations for two alleles are shown in panel A. Expected extensions on sense (S) and antisense (AS) bound probes for nine possible genotypes in the *HFE* gene at the C282Y and H63D sites is shown in panel B. Arrays containing bound sense and antisense primers (Table 1) were extended as described in Methods with Cy5-ddGTP using two UDG-fragmented PCR products which encompass the sites of two mutations in the *HFE* gene (H63D and C282Y) (panel C). The two PCR reactions were multiplexed in the same tube and incubated on the same array. Expected extensions are indicated (dark circles in panel C) with experimental values shown for fluorescence (FU) representing the average of the four spots in each row shown in color on array scan.

Expected two-color SNE extensions based on DNA sequence analysis were observed for the two loci interrogated allowing unambiguous typing of N/M at C282Y and N/N at H63D. For example, at the C282Y locus, extension occurred with Cy3-ddATP on the sense strand bound primer indicating presence of a mutant allele (G to A on the sense strand). In addition, extension occurred with Cy5-ddCTP on the antisense bound primer also indicating presence of a normal allele (G on the sense strand). Hence, typing at this locus shows heterozygosity for both normal and mutant alleles. Results for the H63D locus show presence of two normal alleles since extension occurred with Cy5-ddCTP on the sense strand indicating presence of one or two normal alleles (C on sense strand). Homozygosity for the normal allele is inferred since no extension with Cy5-ddCTP is detected on the antisense strand bound primer. Presence of the mutant allele (C to G on the sense strand) would have resulted in extension with ddCTP on the antisense bound primer. No extensions at the H63D locus were expected using Cy3-ddATP and none were observed.

On-slide temperature cycling increased both Cy5 and Cy3 extensions comparing one against three cycles. In these

Table 2 PCR detection of HFE mutations in five individuals using single color SNE (Cy5-ddGTP extension)

Expected signal	*Fluorescence units/spot*				*Average*	*Genotype*	*Pos/neg ratio*
Sample 001							
H63D S ○○○○○	3751	3494	3631	4407	3820	N/N	7.6
H63D AS ●●●●●	27033	30476	27253	31436	29049		
C282Y S ●●●●●	24769	24889	22439	19122	22804	N/N	12.33
C282Y AS ○○○○○	1647	1845	2249	1654	1848		
SNE CTR ●●●●●	9222	9690	6019	4342	7318		
Sample 002							
H63D S ●●●●●	16467	13488	11969	12426	13587	M/N	1.06
H63D AS ●●●●●	15776	13649	14766	15072	14815		
C282Y S ●●●●		18763	18387	14949	17366	N/N	5.5
C282Y AS ○○○○		3000	3393	3138	3177		
SNE CTR ●●●●●	36921	35505	36682	35687	36198		
Sample 003							
H63D S ○○○○○		4148	5526	5132	4935	N/N	8.19
H63D AS ●●●●●	42661	44600	40572	34031	40466		
C282Y S ●●●●●	27040	24823	20519	21992	23593	M/N	7.16
C282Y AS ○○○○		3312	3503	3068	3294		
SNE CTR ●●●●●	29189	35627	19891	16362	25267		
Sample 004							
H63D S ●●●●●	26708	22105	21716	17513	22010	M/N	1.04
H63D AS ●●●●●	22051	27415	23678	18655	22949		
C282Y S ●●●●●	21847	20953	22418	23043	22065	M/N	8.2
C282Y AS ○○○○	2539	2600	2943	2681	2690		
SNE CTR ●●●●●	25258	27587	31714	21267	26456		
Sample 005							
H63D S ○○○○○	4827	4616	5375	4815	4908	N/N	5.08
H63D AS ●●●●●	27599	26525	24890	20857	24967		
C282Y S ○○○○○	4396	4637	4302	4178	4378	M/M	1.72
C282Y AS ○○○○○	2608	2382	2708	2379	2519		
SNE CTR ●●●●●	11387	13236	12446	17929	13749		

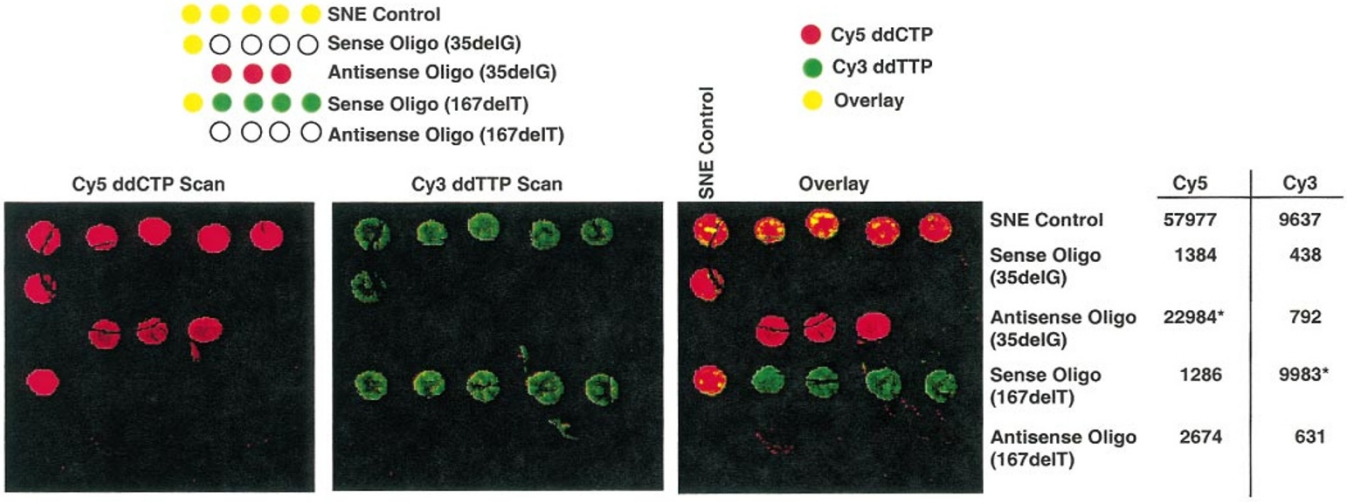

Figure 3 Detection of connexin 26 mutations using 2-color SNE. Processed images of scans are shown for Cy5 (left panel), Cy3 (center panel) and overlay of both Cy5 and Cy3 (right panel) SNE extensions on the same array. Note SNE controls (top row and three spots in left column of each panel in Cy5 and Cy3 scans) extend with both dyes generating a yellow to orange color in the overlay. Schematic diagram at top illustrates expected results for the overlay. Table on right shows averages for Cy5 and Cy3 extensions for each row; * indicates expected extensions.

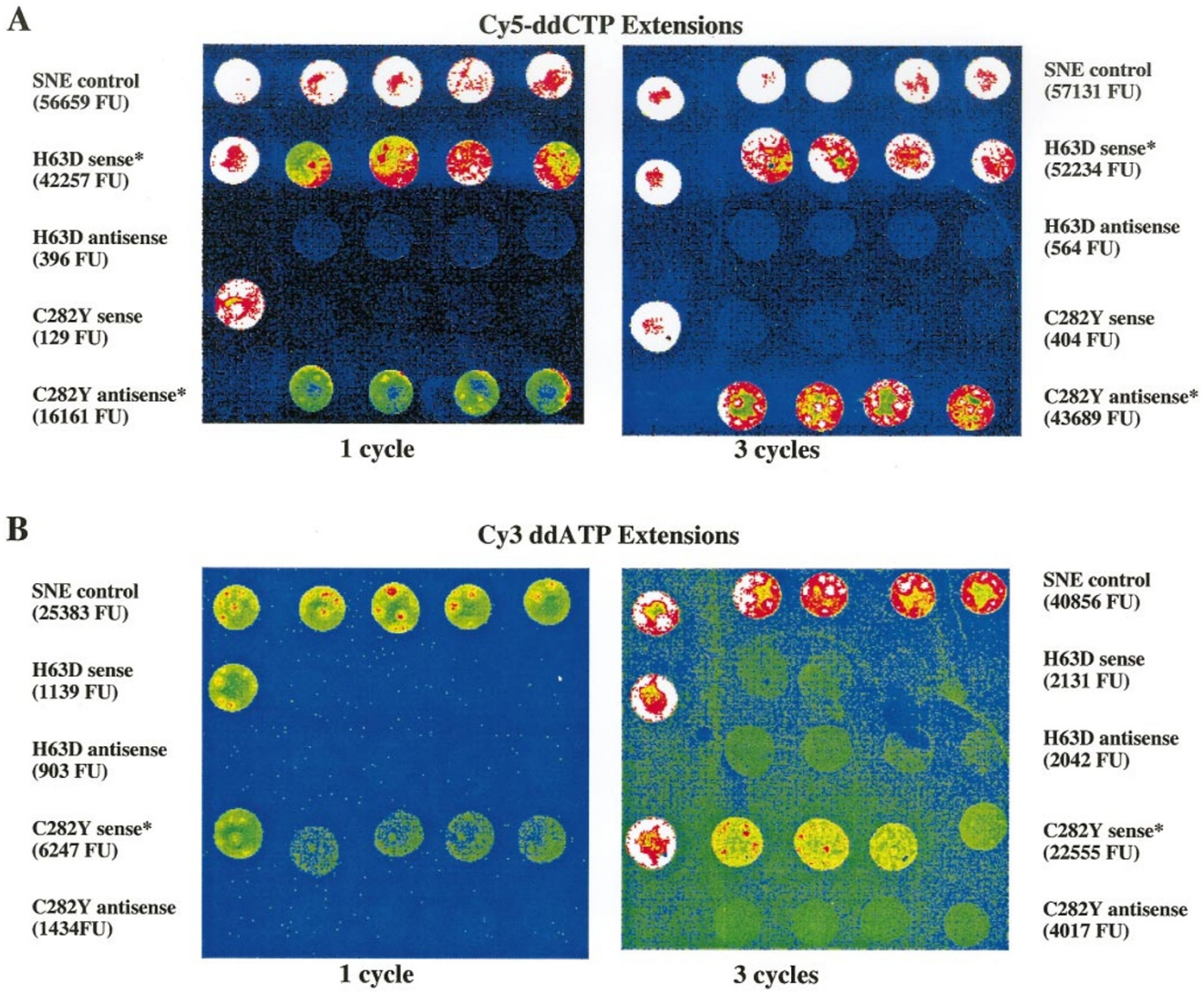

Figure 4 Detection of hemochromatosis mutations using 2-color SNE and on-slide temperature cycling. Arrays containing bound sense and antisense primers were extended as described in Methods with Cy5-ddCTP (0.75 μM) and Cy3-ddATP (16 μM) using small PCR products (40–60 bp, Table 3) which encompass the H63D and C282Y mutations in the *HFE* gene. The two PCR reactions were multiplexed in the same tube and incubated on the same array; * expected extensions with the two different dye-tagged terminators with values for fluorescence (FU) representing the average of the four spots in each row. Scans for Cy5 (panel A) and Cy3 (panel B) for one cycle (left frames) and three cycles (right frames) of on-slide temperature cycling were as described in Methods. Slides were scanned and quantitated using a ScanArray 3000. SNE controls (top row and three spots in far left column of each frame in panels A and B) extend with both ddNTPs.

experiments, arrays were done in parallel on the same day using Cy5 and Cy3 extensions on the same array. The SNE FU controls for Cy5 extensions were almost identical for one (56659) and three (57131) cycles, whilst values for Cy5 extensions at one against three cycles increased from 42257 to 52234 for one locus (H63D) and from 16161 to 43689 for the second locus (C282Y). Values for Cy3 extensions at C282Y were 6247 and 22555 with SNE FU controls for Cy3 extensions of 25383 and 40856 at one against three cycles, respectively.

Discussion

Our strategy for two-color array-based detection of mutations by single-nucleotide primer extension offers numerous advantages over existing strategies:

(1) Cy5/Cy3 dye-tagged ddNTPs are employed instead of fluorescein, which is subject to photobleaching;[32]

(2) on-slide temperature cycling increases signal intensity maximizing extensions for robust SNE;

(3) use of an SNE control which extends with any of the terminators represents an internal control for attachment, annealing and SNE on each array;

(4) primers designed to generate 40 to 60 bp PCR products eliminate the need for fragmentation prior to SNE;

(5) the assay is robust and straightforward and does not require generation or purification of single-stranded targets or antibody conjugants for detection;

(6) the entire assay is greatly simplified since PCR reactions followed by alkaline phosphatase and exonuclease I digestion are done in the same tube, and then the entire tube contents are directly applied to the array;

(7) if long PCR products are needed, dUTP can be used during PCR followed by UDG cleavage to generate optimal targets for SNE;

(8) the use of short PCR products may eliminate misincorporation due to generation of secondary structures in templates which can form in long PCR products;[33]

(9) a direct read-out of Cy5/Cy3 fluorescence is obtained on a commercially available fluorescence scanner, since computer-based algorithms like those for evaluation of ASO-based arrays are not required because direct read-out is apparent for normal and mutant alleles without use of antibody conjugants or radioisotopes; and

(10) the system is readily implementable in research and clinical laboratories.

Our results suggest Cy3 extensions are less efficient than Cy5, and that different dyes may inhibit polymerase-catalyzed extension to different degrees. This conclusion is further substantiated by the much lower level of Cy3 extensions compared with Cy5 for the C282Y locus (ie see Figure 3) when using similar ddNTPs concentrations on the same array. For example, robust Cy5 extensions were evident using 0.75 μM ddNTPs, whilst robust Cy3 extensions require a much higher concentration of ddNTPs. Furthermore, for Cy3 extensions there was also a preference for incorporation of the different ddNTPs (G > T > C > A, data not shown). In addition, our results suggest that extension efficiencies vary with the same dye-tagged terminator depending on what locus is being interrogated, suggesting a preference for certain primer-template configurations.

Although this approach is ideal for typing known SNPs or mutations, its utility defining new mutations in continuous DNA sequence analysis remains unexplored. One of the major cost implications of array-based SNE for sequencing is the cost of the array-bound oligonucleotide primers which traditionally contain a spacer arm and an aminolink group and have required HPLC purification. Our recent experiences indicate that HPLC purification may not be required for capture of complementary targets by array-bound probes (S McKenzie *et al*, 1999 unpublished observations). In addition, spacer-containing sense and antisense primers ending one nucleotide short of the base being interrogated and which are used for array-bound SNE can also be used as forward and reverse primers in solution-phase to generate the short PCR products (data not shown), thereby minimizing oligonucleotide cost. Costs could be decreased further by direct attachment of spacers to the array surface instead of the more commonly used costly spacer synthesis directly on the oligonucleotide primers.[34] A two-color array-based approach decreases the number of arrays from four to two for typing unknown nucleotide changes, in addition to the elimination of the need for use of radioisotopes. In addition, obvious savings are realized when scale up is achieved, since maximization of the number of loci to be interrogated simultaneously using a multicolor fluorescence approach will minimize the cost of having to use multiple arrays to obtain the same information.

The development of a robust four-color SNE protocol would eliminate the need to do two independent arrays now required with a two-color approach we describe to type each allele, if the base change is not known. This would require use of four different dyes the spectral properties of which for excitation and emission were appropriate to accomplish the four-color simultaneous SNE. Such dye-labeled terminators are currently used for automated DNA sequence analysis but are not commercially sold separately. Existing dyes can be chemically attached to terminators to generate these substrates but are very costly, therefore limiting their use. In addition, with a simultaneous four-color approach only one bound oligonucleotide primer would be required to interrogate each locus. In fact, preliminary studies show feasibility for four-color detection on arrays using arrayed primer extension (APEX) methodology.[35–40] However, in cases where the normal and mutant polymorphic site are known, then only one two-color array needs to be done to type unambiguously the particular site.

In summary, we describe a two-color array-based approach to mutation detection using Cy5-/Cy3-dye labeled ddNTPs which has a number of important advantages over existing SNE-based approaches. Multicolor array-bound SNE will be of major advantage provided that the dye-labeled terminators become commercially available at a reasonable cost and that the number of sites which can be interrogated on a single array is high, thereby maximizing allele calls and minimizing cost.

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