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METHODS

Novel Plexor™ SNP Genotyping Technology: Comparisons With TaqMan® and Homogenous MassEXTEND™ MALDI-TOF Mass Spectrometry

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Analysis of SNPs for association, linkage, haplotype, and pharmacogenetic studies has led to a dramatic increase in the number and evolution of medium- to high-throughput genotyping technologies. This study introduces Plexor™ as a new method for medium-throughput (single SNP) genotyping. We compare this fluorescent-based chemistry for call rate, accuracy, affordability, throughput, and overall efficiency against two commonly used technologies. These include fluorescent-based TaqMan® allelic discrimination for single SNP analysis (medium-throughput) and the homogenous MassEXTEND™ (hME™) chemistry using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for multiple SNP analysis (high-throughput). Analysis of 11 SNPs, including all six possible nucleotide substitutions, showed Plexor™ to be highly comparable for both call rate (94.7%) and accuracy (99.2%) to the TaqMan® (94.6% and 99.8%, respectively) and hME™ (91.9% and 98.1%, respectively) chemistries. We demonstrate that this novel method is an efficient, cost-effective alternative to TaqMan® genotyping commonly used in diagnostic settings. Hum Mutat 28(9), 922–927, 2007. **© 2007 Wiley-Liss, Inc.**

KEY WORDS: SNP; genotyping; Plexor; TaqMan; MassEXTEND; hME

INTRODUCTION

The most common form of human genetic variation is the dimorphic single nucleotide polymorphism (SNP) [Cargill et al., 1999]. SNPs may contribute either directly or indirectly to an individual's predisposition to complex disease, such as, cardiovascular disease, cancer, obesity, diabetes, psychiatric illness, and inflammatory disease, as well as individual differences in pharmacological responsiveness and disease progression [Evans and McLeod, 2003; Becker, 2004]. The completion of the Human Genome Project [Venter et al., 2001], followed by the introduction of the International HapMap Project in 2003 [International Hap Map Consortium, 2003], has resulted in an explosion in the identification and availability of human SNPs for genetic association, linkage, and haplotype studies. This has encouraged the development of medium- to high-throughput SNP genotyping technologies to enable rapid, reliable, accurate, and cost-effective genotyping.

There are currently a large number of genotyping technologies available, which generally employ one of two mechanisms for allelic discrimination, namely allele-specific hybridization or primer extension. Amplifluor® (Chemicon International, Inc., Temecula, CA; www.chemicon.com) [Myakishev et al., 2001], GeneChip® (Affymetrix Inc., Santa Clara, CA; www.affymetrix.com), GoldenGate® (Illumina, Inc., San Diego, CA; www.illumina.com) [Engle et al., 2006], NanoChip® (Nanogen Corp., San Diego, CA; www.nanogen.com) [Sosnowski et al., 2002], SNPlex™ and TaqMan® (Applied Biosystems [ABI], Foster City, CA; www.appliedbiosystems.com) [De La Vega et al., 2005] use an allele-specific hybridization approach to genotyping. Alternatively, SNaPShot® (ABI) [Pati et al., 2004], Pyrosequencing™ (Biotage, Uppsala, Sweden; www.biotage.com) [Fakhrai-Rad et al., 2002], homogenous MassEXTEND™ (hME™; Sequenom, San Diego, CA; www.sequenom.com) [Gut, 2004], and iPLEX™ (Sequenom) [Engle et al., 2006], including the recently released iPLEX™! Gold (Sequenom), use primer extension. A keyword search for the term "SNP genotyping" using the PubMed database (www.ncbi.nlm.nih.gov) revealed that the most commonly used genotyping technologies for the period spanning June 2004 to December 2006 included ABI TaqMan® (83/434), Affymetrix GeneChip® (60/434), and Sequenom hME™ (50/434). Although RFLP analysis was the third most commonly used SNP genotyping technology (55/434), this gel-based method lacks automation and is therefore generally not classified as medium- or high-throughput [Gut, 2001].

Two of the most commonly used genotyping technologies, TaqMan® and hME™, are frequently used in our laboratory.

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TaqMan® (ABI) is a medium-throughput (single SNP) method that involves the hybridization of an allele-specific probe to the SNP of interest. The probe is flanked by a reporter dye and quencher, which is cleaved during DNA amplification, enabling the reporter dye to fluoresce [McGuigan and Ralston, 2002]. Alternatively, hME™ (Sequenom) is a high-throughput (multiple SNP) genotyping chemistry, which employs matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to distinguish between the allele-specific extension products [Tang et al., 1999; Jurinke et al., 2002]. A recent study has shown call rate and reproducibility of the TaqMan® and GOOD assay with MALDI-TOF MS to be highly reliable [Giancola et al., 2006]. The GOOD assay combines the chemistry of allele-specific primer extension of chemically modified primers with a "charge tag" to increase sensitivity of detection by MALDI-TOF MS [Sauer et al., 2000].

As genotyping technologies continue to evolve, novel approaches require validation by comparison to commonly used and previously described technologies. In this study we investigate the novel genotyping chemistry from Promega Corporation (Promega, Madison, WI; www.promega.com), namely Plexor™, which, similar to TaqMan®, employs SNP-specific hybridization via allele-specific primers as opposed to allele-specific probes. In contrast to TaqMan® chemistry, Plexor™ achieves allelic discrimination by measuring a loss of fluorescence (Fig. 1). The incorporation of a modified Cystine (C) nucleotide, methylisocytosine (iso-dC), flanking the fluorescent dye on the allele-specific primer, allows for amplification specificity via a reversed pattern of hydrogen bonding, to modified Guanine (G) nucleotides, isoguanine (iso-dG) [Moser and Prudent, 2003], the latter containing a fluorescent quencher. We evaluated this new method for call rate, accuracy (reproducibility), affordability, throughput, and overall efficiency with the commonly used TaqMan® and hME™ genotyping chemistries.

MATERIALS AND METHODS

Samples and SNPs

DNA was extracted from over 2,000 stored buffy coats using QIAamp DNA blood mini kit (Qiagen Pty Ltd), 6 to 10 years prior to this study. DNA samples and reaction mixes were dispensed using liquid-handling robotics. The following SNPs, rs2228013:C>T (p.R52C), a nonsynonymous SNP in the Homeobox 3A gene (*NKX3A* or *NKX 3.1*; MIM# 602041), rs2243250:C>T, a promoter SNP 589 nucleotides upstream of translation initiation in the Interleukin 4 gene (*IL4*; MIM# 147780), rs2069762:T>G, a promoter SNP 385 nucleotides upstream of translation initiation in the Interleukin 2 gene (*IL2*; MIM# 147680), rs1800795:G>C, a promoter SNP 237 nucleotides upstream of translation initiation in the Interleukin 6 gene

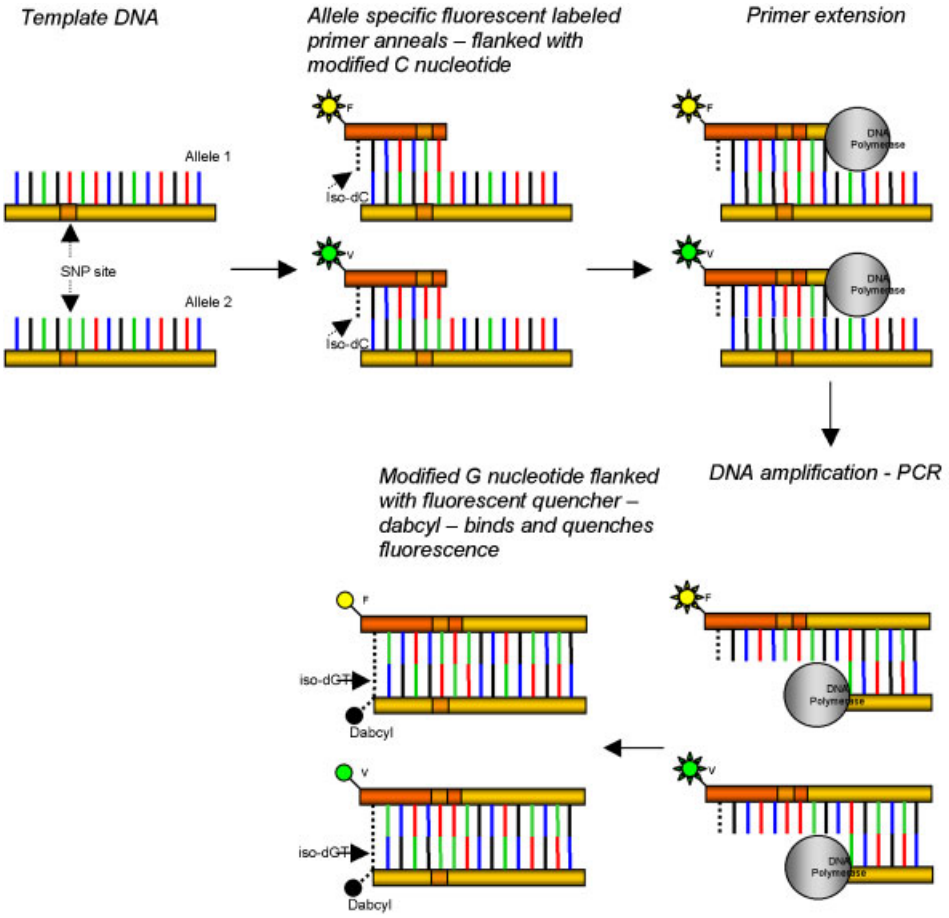

FIGURE 1. **Schematic representation of the fluorescent-based Plexor™ allele discrimination method. Allele-specific primers are labeled with fluorescing reporter dyes and flanked with a modified C nucleotide (iso-dC). Incorporation of these primers during PCR allows specific binding of a modified G nucleotide (iso-dG) complementary to iso-dC. Dabcyl-iso-dG contains dabcyl, a fluorescent quencher, which results in a loss of fluorescence.**

(*IL6*; MIM# 147620), rs753345:C>T at chromosome position 1q23.1 (no gene), and rs7260021:C>G at chromosome position 19p13.2 (no gene) were genotyped using Plexor™, TaqMan®, and hME™. In addition, rs4073:T>A, a promoter SNP 352 nucleotides upstream of translation initiation in the Interleukin 8 gene (*IL8*; MIM# 146930) was genotyped using Plexor™ and hME™, while SNPs rs1447295:A>C at chromosome position 8q24.21 (no gene), and rs878972:A>C, rs3087263:G>A, and rs315951:C>G located in noncoding regions, namely intron 1 (IVS1+2108), intron 2 (IVS2+451), and 138 nucleotides downstream of the termination codon in exon 5, respectively, of the Interleukin 1 receptor antagonist gene (*IL1RN*; MIM# 147679) were genotyped using Plexor™ and TaqMan®.

Plexor™ Genotyping

Plexor™ allele-specific and anchor primers for all 11 SNPs (Table 1) were designed with the aid of freely available primer design software in consultation with Promega, Australia (specifically Dr. A. Lai), and according to primer design parameters. Briefly, two allele-specific primers and one common, anchor primer were designed to amplify a region between 40 and 120 bases in length. In our study amplicons ranged from 46 to 95 bases in length. The polymorphism of interest is located within three nucleotides of the 3′ end of each allele-specific primer, while the 5′ end is flanked by a fluorescent reporter dye, an iso-dC residue, and a short random, noncomplementary sequence (tail). In our designs the "tails" ranged from 3 to 8 nucleotides in length and increase the melting temperature (T_m) by 10°C. Thus initial cycling at an annealing of 50°C enables amplification from the complementary portion of the allele-specific primer, after which "tail" incorporation increases the optimal annealing temperature to 60°C, allowing for increased amplification specificity.

A total reaction volume of 5 μl included; 5 ng of template DNA, 0.2 μl of 5 μM allele-specific primers (Integrated DNA technologies, Inc., Coalville, IA), 0.2 μl of 10 μM anchor primer and 2.5 μl of 2 × Plexor™ Master Mix (Promega) including dabcyl-iso-dG, iso-dG residues modified with the fluorescent quencher dabcyl. PCR was performed using the ABI Prism® 7900HT Sequence Detection System (SDS). Cycling conditions were as follows; 95°C for 2 minutes, 50°C for 35 seconds, followed by 40 cycles of 95°C for 5 seconds and 60°C for 35 seconds. The PCR was followed by an allelic discrimination plate read and analysis using the ABI Prism® 7900HT SDS and software version 2.2.

TaqMan® Genotyping

Predeveloped TaqMan® assays were used to genotype SNPs rs2243250:C>T, rs1447295:A>C, rs1800795:G>C, rs2069762:T>G, rs315951:C>G, rs3087263:G>A, rs878972:A>C, rs753345:C>T, and rs7260021:C>G, while rs2228013:C>T was genotyped using an ABI custom-designed assay. A total reaction volume of 5 μl included: 5 ng of template DNA, 2.5 μl 2 × TaqMan® Universal PCR Master Mix, and 0.125 μl 20 × SNP Genotyping Assay Mix (including primers and fluorescently-labeled probes). PCR cycling was performed using an ABI Prism® 7900HT SDS under the following conditions; 95°C for 10 minutes followed by 40 cycles of 92°C for 15 seconds and 60°C for 1 minute. The PCR was followed by allelic discrimination using the ABI Prism® 7900HT SDS. Results were analyzed with ABI SDS software version 2.2.

hME™ Genotyping

PCR and extension primer sequences for the four hME™ assays were designed using Sequenom® RealSNP (www.RealSNP.com; primer sequences available on request). SNPs rs2228013:C>T, rs2243250:C>T, and rs1800795:G>C were genotyped as single SNP assays, SNPs rs4073:T>A (2-plex) and rs2069762:T>G (4-plex) as "low-plex" assays (less than 6-plex), and SNPs rs753345:C>T (8-plex) and rs7260021:C>G (9-plex) as "high-plex" assays (between 6- and 12-plex). The other assays included in the multiplex reactions are not reported in this study (available on request). A 5-μl PCR contained 2.5 ng (single SNP/low-plex) or 5 ng (high-plex) of template DNA, 10 × Qiagen HotStar Taq

TABLE 1. Plexor™ Primer Sequences for 11 SNP Assays

SNP	Nucleotide change	Allele-specific primer (5′-3′)	Anchor primer (5′-3′)
rs2228013[a]	C>T	FAM-isodC-*TATCGATCC*AGCCAGAGACAG**C**G; HEX-isodC-*AGTTAGTCG*CAGCCAGAGACA**G**TG	GGTGCTCAGCTGGTCGTTCT
rs2243250[a]	C>T	Cy3-isodC-*GTACGAT*TGGGAGAACATTGT**C**C; HEX-isodC-*CCAGT*TGGGAGAACATTGT**T**CC	AGAGGCAGAATAACAGGCAGACTCT
rs1800795[b]	G>C	FAM-isodC-*GAACTAG*TGACGTCCTTTAG**C**AT**C**G; HEX-isodC-*AGTAGT*GTGACGTCCTTTAGCAT**G**G	GACGACCTAAGCTGCACTTTTCC
rs2069762[b]	T>G	FAM-isodC-*CACAG*CTCAGAAAATTTTCTTTG**T**CA**T**A; HEX-isodC-*CACTGAT*TCAGAAAATTTTCTTTGTC**C**TA	CACCACAATATGCTATTCACATGTTCA
rs753345[a]	C>T	FAM-isodC-*GTC*CAATGATGTGCTGGTAAA**C**G; HEX-isodC-*ACTGC*AATGATGTGCTGGTAAA**T**G	ACACTACAAATCACCATACTGTCTCACC
rs7260021[a]	C>G	FAM-isodC-*CGTTC*CTCACTCAGCAAC**C**G; HEX-isodC-*TAGATCAC*CTCACTCAGCAAC**G**G	CAAAATTGCCTAGTGATGCATTTCTC
rs1447295[a]	A>C	FAM-isodC-*TCAGAA*TTGGGGAGGTATGTAAAA**A**GT; HEX-isodC-*TGTC*GGGGAGGTATGTAAAA**C**GT	TTCTATCAAGGGGTTCCTGTTGC
rs315951[b]	C>G	FAM-isodC-*ATCTT*GTCCTGTGACCAGGTT**C**TT; HEX-isodC-*TCAGA*TCCTGTGACCAGGTT**G**TT	CACTGAGGACCAGCCATTGAG
rs3087263[b]	G>A	FAM-isodC-*AGAGTAC*GGCACACAAGGAG**T**TATT**G**; HEX-isodC-*CAGCTATT*AGGCACACAAGGAGTTAT**C**	CTGTGACATCTGTGACATAGAGTCA
rs878972[a]	A>C	FAM-isodC-*TAGTGC*CTGACTCAAAGGGTAAATTA**T**T; HEX-isodC-*GTGAACAG*TGACTCAAAGGGTAAATT**C**TT	AAAATTGTTTTCAAACTTGGATCCTAAA
rs4073[b]	T>A	FAM-isodC-*TGGATA*CACAATTTGGTGAATTATCAA**T**T; HEX-isodC-*TCTGAAG*ACAATTTGGTGAATTATCAA**A**T	CACCTGCCACTCTAGTACTATATCTGTCAC

[a]Allele-specific primers on the forward strand.
[b]Allele-specific primers on the reverse strand.

PCR buffer (Qiagen, Hilden, Germany; www.qiagen.com), 25 mM $MgCl_2$, 25 mM dNTPs, 200 nM of each PCR primer and 0.1 U Qiagen HotStar Taq Polymerase. PCR cycling was executed using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany; www.eppendorf.com) under the following conditions; 95°C for 15 minutes, 45 cycles of 95°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, followed by a final extension step of 3 minutes at 72°C. After exonuclease cleanup, the MassEXTEND™ reaction was performed using 1 × the appropriate termination mix (available upon request), 600 nM of each extension primer and 0.054 U (single SNP/low-plex) or 1.25 U (high-plex) of Thermo Sequenase™ (Sequenom®). The cycling conditions were as follows; 94°C for 2 minutes, 60 (single SNP/low-plex) or 100 (high-plex) cycles of 94°C for 5 seconds, 52°C for 5 seconds, and 72°C for 5 seconds. Samples were spotted onto SpectroCHIP® Bioarrays (Sequenom®) using the MassARRAY™ nanodispenser (Sequenom®) and run on a MassARRAY™ Compact system (Sequenom®). Results were analyzed using Sequenom® MassARRAY RT™ software.

Direct Sequencing

Samples showing discordance in genotype calls were amplified using sequence-specific primers (available on request) and subjected to direct sequencing using the Big Dye® Termination version 3.1 kit (ABI) and the ABI Prism® 3100 genetic analyzer.

RESULTS

Plexor™, a novel chemistry for allelic discrimination, was assessed by genotyping 11 SNPs and the data was compared to the commonly used TaqMan® and/or hME™ technologies. The SNPs assessed in this study included each of the six possible nucleotide substitutions, 3 C/T, 3 C/G, 2 C/A, 1 T/A, 1 T/G, and 1 G/A, over 13,000 genotypes.

Plexor™-specific primers were designed for each of the 11 SNPs (Table 1), and genotype calls analyzed using the ABI Prism® 7900HT SDS and ABI SDS software version 2.2. All assays (designed according to the specified requirements) worked first time and there was no further need for redesigning of primers or standardization of amplification. In contrast to TaqMan®, Plexor™ chemistry is based on a loss of fluorescence (Fig. 1), and therefore the genotype of a sample is determined by the allele present on the SNP-specific primer no longer fluorescing (Fig. 2). Thus, the ABI SDS software automatically designates the opposite allele for homozygous samples and user intervention is required to allocate the alternate genotype.

To compare Plexor™ with TaqMan® and/or hME™ as a platform for SNP genotyping, we assessed for both call rate and accuracy (Table 2). An average call rate of 94.7% (4325/4566) using Plexor™ genotyping chemistry was highly comparable to the 94.6% (4683/4950) obtained using TaqMan® and 91.9% (3507/3818) for the hME™. Direct sequencing of discordant and undetermined genotype calls revealed a high accuracy rate averaging 99.2% (4290/4325) for Plexor™, which was once again highly comparable, with accuracy rates of 99.8% (4673/4683) and 98.1% (3440/3507) obtained for TaqMan and hME, respectively.

Further analysis of discordant genotypes across the technologies revealed a trend for heterozygous samples to be incorrectly genotyped as homozygous by Plexor (74.3%, 26/35), but to a lesser degree than observed for the TaqMan® (90%, 9/10) and hME™ (92.5%, 62/67) chemistries. Although one may suggest that either loss or favoring of one allele during hybridization (Plexor™ and TaqMan®), or loss or favoring of one allele during primer extension (hME™) or mass discrimination (MALDI-TOF MS)

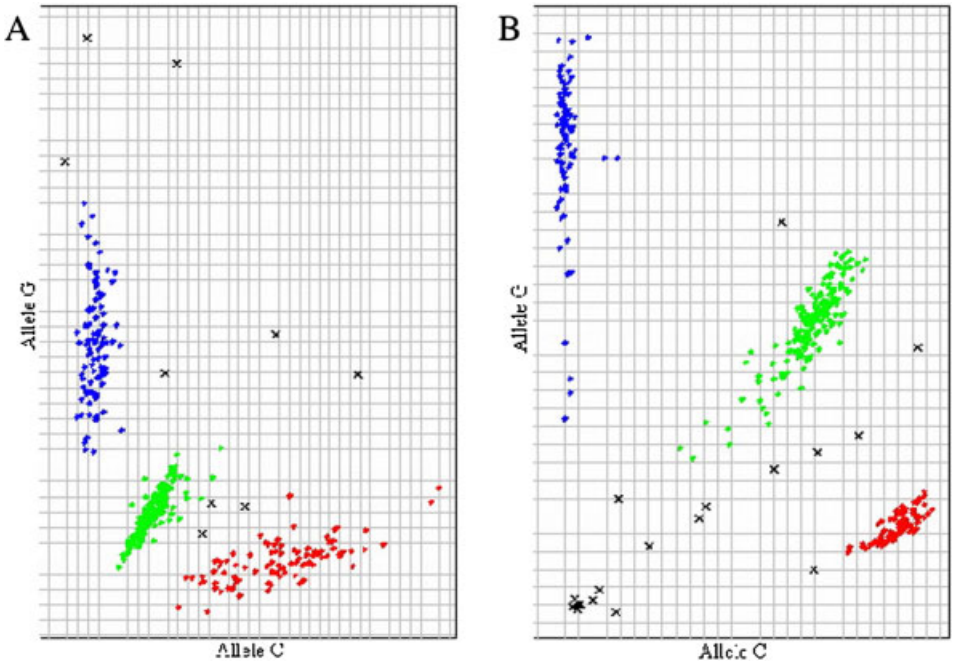

FIGURE 2. **Comparison of genotyping scatter plots for SNP rs7260021:C>G using the fluorescent-based SNP genotyping chemistries. A: Plexor™ genotyping scatter plot for SNP rs7260021:C>G, analyzed using ABI SDS software, version 2.2. Allele C samples lie along the horizontal axis, allele G samples lie along the vertical axis, and heterozygous samples lie along the diagonal axis. B: TaqMan® genotyping scatter plot analyzed using ABI SDS software version 2.2. The allele-specific probes were designed on the reverse DNA strand and therefore homozygous clusters lie along the same axis as described for the Plexor™ scatter plot (A).**

TABLE 2. Genotype Call Rate and Accuracy

SNP		Genotyping technology		
		Plexor™ % (n)	TaqMan® % (n)	hME™ % (n)
rs2228013	Call rate	98.2 (748/762)	99.0 (1510/1526)	92.4 (1421/1538)
	Accuracy	100 (748/748)	99.9 (1509/1510)	97.6 (1387/1421)
rs2243250	Call rate	99.2 (379/382)	97.6 (373/382)	89.0 (340/382)
	Accuracy	99.7 (378/379)	100 (373/373)	98.5 (335/340)
rs1800795	Call rate	95.3 (364/382)	96.6 (369/382)	87.4 (334/382)
	Accuracy	99.5 (362/364)	100 (369/369)	100 (334/334)
rs2069762	Call rate	95.5 (365/382)	83.8 (320/382)	77.0 (294/382)
	Accuracy	98.6 (360/365)	99.4 (318/320)	95.2(280/294)
rs753345	Call rate	89.1 (336/377)	96.6 (364/377)	99.5 (375/377)
	Accuracy	99.4 (334/336)	99.7 (363/364)	97.9 (367/375)
rs7260021	Call rate	97.6 (368/377)	96.0 (362/377)	97.9 (369/377)
	Accuracy	99.4 (366/368)	99.7 (361/362)	98.4 (363/369)
rs1447295	Call rate	97.6 (371/380)	97.6 (371/380)	–
	Accuracy	100 (371/371)	100 (371/371)	–
rs315951	Call rate	90.0 (342/380)	90.5 (344/380)	–
	Accuracy	97.1 (332/342)	99.4 (342/344)	–
rs3087263	Call rate	97.6 (373/382)	96.3 (368/382)	–
	Accuracy	99.2 (370/373)	99.5 (366/368)	–
rs878972	Call rate	93.5 (357/382)	79.1 (302/382)	–
	Accuracy	99.2 (354/357)	99.7 (301/302)	–
rs4073	Call rate	84.7 (322/380)	–	98.4 (374/380)
	Accuracy	97.8 (315/322)	–	100 (374/374)

TABLE 3. Assessment of Genotyping Chemistries Plexor™, TaqMan®, and hME™*

	Genotyping technology		
	Plexor™	TaqMan®	hME™
Chemistry	Fluorescence (loss)	Fluorescence (gain)	MALDI-TOF MS
Assay plexing	Single	Single	Multiplex ($\leq$12)
Assay design	Manual computer design[a]	Company or manual computer design	Automatic computer design
Assay steps	1	1	4
Assay analysis	ABI SDS software version 2.2[a]	ABI SDS software version 2.2	MassARRAY Typer Analyzer
Plate format	384-well	384-well	384-well
Reaction volume	5 μl	5 μl	5 μl
DNA quantity			
Recommended	10–100 ng	1–20 ng	2.5 ng $\leq$ 6-plex; 5 ng 7–12-plex
This study	5 ng	5 ng	2.5 ng $\leq$ 6-plex; 5 ng 7–12-plex
Price per genotype[b]			
Recommended	0.53	predeveloped 0.57; custom 0.60	1-plex 0.69; 8-plex 0.12; 9-plex 0.10
This study	0.43	predeveloped 0.41; custom 0.43	1-plex 0.69; 8-plex 0.12; 9-plex 0.10

[a]Software under development.
[b]Price in U.S. dollars (inclusive of primer costs).

may have occurred, we did not observe a trend toward allele-favoring using these technologies. The only exception was in the hME™ genotyping SNP rs2228013:C>T, which showed a trend toward incorrectly calling the rare homozygous T (low mass) allele (26/29) compared to the more common homozygous C (high mass) allele (3/29).

DISCUSSION

We have described and successfully utilized a novel genotyping technology, namely Plexor™, which combines the advantages of a highly sensitive fluorescence-based chemistry with the specific interaction of two modified nucleotides iso-dG and iso-dC. This chemistry was tested against the commonly used TaqMan® and hME™ genotyping technologies for 11 SNPs including all six possible combinations of nucleotide substitutions. In addition, one SNP rs2228013:C>T within the *NKX3.1* gene, lies within a GC-rich region (152/200 bases, 100 bases either side of the SNP) lacking Thymine (T) nucleotides (10/200 bases) and was selected for its sequence complexity—usually associated with genotyping/mutation detection failure. Using Plexor™ we were able to genotype this SNP for over 700 samples with 100% accuracy. Overall, our results provide a strong basis for concluding Plexor™ as not only a reliable approach to genotyping (even for SNPs within a complex sequence), but also highly comparable to the commonly used medium-to-high-throughput TaqMan® and hME™ chemistries for both call rate and accuracy.

Choosing a genotyping technology is also dependent on factors such as cost and throughput (Table 3). In addition to call rate and accuracy, we found Plexor™ to be highly comparable to TaqMan® with respect to cost, throughput, and DNA quantity required per

genotype reaction. Primer costs for the Plexor™ assays, including fluorescent label and iso-C modified base, were factored into the genotype call costs based on an average of 8,444 reactions per primer mix (equating to U.S. $0.026 per genotype). The Plexor™ and TaqMan® technologies are currently distinguishable with regard to assay design. TaqMan® is well-established, has a user-friendly approach to assay design, and includes 4.5 million predeveloped and over 180,000 predeveloped and validated SNP assays (as of January 2007). For SNPs not currently available in the predeveloped range, ABI provides a web-based service to custom design assays (www.appliedbiosystems.com). For the purpose of this study, the Plexor™ assay design was termed user-dependant following parameters outlined by Promega. Promega is currently adapting a software package (Plexor™ Analysis Software) to facilitate assay design and data analysis. Although the Plexor™ assay design was deemed user-dependent, all assays in this study worked on first attempt. In addition, the primer design of the Plexor™ technology may be classified as distinctly advantageous due to its simplicity over probe-based designs. The use of the modified iso-dC and iso-dG nucleotides has been shown to increase amplification specificity [Johnson et al., 2004], while the incorporation of a short random, noncomplementary "tail" during first round amplification increases the annealing temperature, thus further increasing amplification specificity.

Both Plexor™ and TaqMan®, with their simple one-step procedure, are ideally suited for use in diagnostic applications. Although in theory it is feasible to use the Plexor™ chemistry to perform genotyping in duplex (via four-color discrimination and depending on the instrument of detection used), this potential capability was not investigated in this study. Thus the inability of technologies such as Plexor™ and TaqMan® to multiplex makes them less applicable for larger studies as required for genomewide scans. These disadvantages include cost per genotype and conservation of valuable DNA resources required in the research setting. In comparison, hME™ chemistry, with one of the lowest ranges of multiplexing capability of the "truly" high-throughput genotyping technologies available (including the iPLEX™ and iPLEX™ Gold chemistries from Sequenom® with 24- to 40-plex capabilities, respectively), is calculated at approximately 4.5 times cheaper per genotype for high-plex genotyping than the average cost of a genotype using either Plexor™ or TaqMan® technologies. Although hME™ requires multiple steps, expanding the sample preparation time prior to analysis almost three-fold and increasing the potential for human error and reducing reproducibility, the employment of liquid handling robotics has largely overcome this limitation.

In conclusion, we demonstrate that Plexor™, a novel SNP genotyping technology, is highly comparable in terms of reliability, throughput, and cost to the commonly used TaqMan® allelic discrimination method for single SNP analysis. We also demonstrate that this method is highly comparable with regard to call rate and accuracy to one of the higher-throughput technologies currently utilized, namely hME™ Mass Spectrometry. The availability of appropriate software for primer design will therefore enable Plexor™ to be developed as a true competitor for single dinucleotide genotyping studies typically used in the diagnostic setting.

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