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ORIGINAL PAPER

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Comparison of four flow cytometric SNP detection assays and their use in plant improvement

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Abstract Single nucleotide polymorphisms (SNPs) are attractive DNA markers due to their abundance and potential for use in automated high-throughput genotyping. Numerous SNP genotyping assays have been developed, but it is unclear which assays are best suited and most efficient for various types of plant improvement research. The objective of this study was to compare the accuracy, efficiency, and cost of four SNP genotyping assays: single-base extension (SBE), allele-specific primer extension (ASPE), oligonucleotide ligation (OL), and direct hybridization (DH). All four assay methods used the same Luminex 100 flow cytometer platform. Fifty-eight F_2-derived soybean [*Glycine max* (L.) Merr.] lines from a cross between inbred lines G99-G725 and N00-3350 were genotyped at four SNPs. SBE and ASPE clearly differentiated between the two homozygotes and the heterozygote at each SNP. Results were in agreement with those identified using the SNaPshot minisequencing assay as a control. In contrast, the OL and DH assays were unable to differentiate between genotypes at some of the SNPs. However, when the cost per data point for the four different assays was compared, the cost of OL and DH was only about 70% of that for SBE, with DH requiring the least time of the four assays. On the basis of cost and labor, ASPE is more cost-effective and simpler than SBE, and would therefore be a good method for genetic mapping and diversity studies which require a large number of markers and a high level of multiplexing. DH appears to be the most economical assay for marker-assisted selection, though optimization for DH would be required for some SNP markers.

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Introduction

Comparisons of genomic DNA sequences from different individuals within a species have revealed single DNA base substitutions or small insertions and deletions (indels) at a specific base position, collectively referred to as single nucleotide polymorphisms (SNPs) (Brookes 1999). SNPs are valuable DNA markers because of their high frequency and widespread distribution in eukaryotic genomes and because they are suitable for high-throughput, automated genotyping (Shi 2001). The potential use of SNPs for genetic mapping of complex genetic traits, pharmacogenetics, and medical diagnostics has received much attention in medical science (Kruglyak 1997; Gu et al. 1998; Nebert 1999; McCarthy and Hilfiker 2000). An abundance of SNPs has been reported in the genomes of crop plants such as maize [*Zea mays* ssp. *mays* (L.)] (Tenaillon et al. 2001), barley (*Hordeum vulgare* ssp. *vulgare*) (Kanazin et al. 2002), and soybean [*Glycine max* (L.) Merr.] (Zhu et al. 2003) and, consequently, SNPs are attractive markers to plant breeders and geneticists. With the increasing availability of public sequence data and the rapid discovery of SNPs in crop species, the development of SNP markers will accelerate.

DNA-based markers can be used to characterize genetic resources, map plant genomes, and tag genomic regions and/or specific genes (Dreher et al. 2003). Once DNA markers are shown to be associated with a target trait, plant breeders can use them for marker-assisted selection (MAS) to identify individual plants containing particular alleles of interest in large segregating

populations (Tanksley et al. 1989; Ribaut and Hoisington 1998; Morris et al. 2003). MAS is generally conducted using a small number of markers and limited multiplexing on a large number of individual plants or lines. This is different from genetic mapping and diversity studies, which require the use of a larger number of markers and a higher level of multiplexing on a more limited number of plants or lines.

High-throughput SNP genotyping requires two basic components: (1) an accurate SNP identification method, and (2) a compatible automated or semi-automated platform technology that allows rapid handling and scoring of the data. SNP genotyping assays are very diverse and have been compared in several recent reviews (Gupta et al. 2001; Gut 2001; Shi 2001; Syvänen 2001). Current methods include single-strand conformation polymorphism analysis, single-base extension (SBE), 5′ nuclease assay, allele-specific primer extension (ASPE), oligonucleotide ligation (OL), allele-specific oligonucleotide hybridization or direct hybridization (DH), and allele-specific cleavage of a flap endonuclease. SBE and ASPE both use DNA polymerase-mediated primer extension to identify the base at a SNP, and OL uses ligase to join two adjacent oligonucleotides if the appropriate base separates them, whereas direct DH does not require an enzyme for the SNP-querying step. A variety of platforms have been used with the various detection methods to facilitate SNP genotyping. These include gel electrophoresis, microarrays, mass spectrometry, fluorescence plate readers, and flow cytometry (Gut 2001).

The Luminex 100 flow cytometer (Luminex, Austin, Tex.) combined with chemistry and software designed by MiraiBio (Alameda, Calif.) is a versatile platform (referred to hereafter as Luminex 100) that is compatible with several types of SNP detection methods. The Luminex 100 flow cytometer is a multi-analyte detection system that uses fluidics to move uniquely colored microspheres bearing attached assays through a narrow channel where a laser identifies the type of microsphere and the assay that it carries (Chen et al. 2000; Iannone et al. 2000; http://www.luminexcorp.com). A second laser quantifies the reaction, which is chemically bound to the surface of the microsphere, based on fluorescence of a reporter molecule linked to the target. Microspheres with 100 unique color codes are available, so it is theoretically possible to multiplex up to 100 reactions in a single tube. Reaction primers or probes carry a unique sequence (ZipCode) that is complementary to an oligonucleotide sequence (cZipCode) on the surface of a specific color-coded microsphere, and this provides the needed specificity.

Some of the early SNP-genotyping assays using flow cytometric analysis were based on SBE (Cai et al. 2000; Chen et al. 2000) and ASPE (Taylor et al. 2001; Ye et al. 2001). Advantages of ASPE over the SBE assay include the ability to read both alleles from a given SNP in the same tube (i.e., detection of heterozygotes) and a simpler reaction protocol which eliminates both the post-PCR cleanup and the addition of unlabeled nucleotides required for the SBE reaction. An OL assay (Iannone et al. 2000) and DH assays (Armstrong et al. 2000) have also been adapted to use a flow cytometric platform. DH was successfully used in rapid screening for mutations in the cystic fibrosis transmembrane conductance regulator gene (Dunbar and Jacobsen 2000) and for identifying β-globin variants (Colinas et al. 2000).

SNP genotyping assays that are high-throughput, accurate, and inexpensive are needed for efficiently utilizing SNP markers in plant improvement programs. The objectives of the research reported here were to compare the effectiveness and robustness of the SBE, ASPE, OL, and DH SNP genotyping assays performed on a flow cytometric platform (Luminex 100), to compare the genotyping accuracy of these assays with that of the SNaPshot assay (Applied Biosystems, Foster City, Calif.), and to evaluate the cost and time requirements for the flow cytometric assays in order to determine which methods show the most promise for plant improvement applications.

Materials and methods

Plant material

An F_2 soybean [*Glycine max* (L.) Merr.] population of 58 plants from the cross of G99-G725 × N00-3350 was used for SNP genotyping. G99-G725 was developed by backcrossing a glyphosphate tolerance transgene into ‘Boggs’ (Boerma et al. 2000), and N00-3350 is a high oleic acid breeding line which was obtained from J. W. Burton (USDA-ARS, Raleigh, N.C.). DNA was isolated from leaves of the 58 F_2 plants and the two parents using the modified CTAB procedure of Keim et al. (1988), and the final concentration was adjusted to 20 ng/μl.

SNP markers

SNP markers were obtained from Perry Cregan (USDA-ARS, Beltsville, Md.). Four SNPs, each located in an amplicon approximately 400–600 bp in length, were randomly selected for this study (Table 1). Each SNP had been obtained from an individual single-sequence repeat-selected bacterial artificial chromosome clone, so each was expected to be from a different part of the soybean genome (Cregan et al. 1999).

Coupling of cZipCode oligonucleotides to microspheres

A total of 5.0×10^6 carboxylated LabMAP microspheres (MiraiBio) per assay were pelleted, resuspended in 50 μl 0.1 *M* 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (pH 4.5), and mixed with 1 nmol amino-substituted cZipCode oligonucleotide (1 μl of a 1 m *M*)

Table 1 Details of SNPs used in this study

SNP name	SNP type (G99-G725/N00350)	Primer sequence ($5' \rightarrow 3'$)[a]	Amplicon size (bp)	SNP position
SNP12507	A/G	F: GCGTAATATAATGCTTTGAGTG R: GCGTTCGTTATTGAGAGTTT	513	400
SNP14049	C/T	F: GCGAGAGGATAAGTCATAAGTG R: GCCCCAATTTGTCTGTGTAATC	411	340
SNP15783	A/G	F: GCGGCTATATGTCATAAAGATAAC R: GCGGGACGTTGTAATAAAGTTGTG	574	76
SNP16289	A/G	F: GGGATGGTATCACTGTAAAGAG R: GCGGGAATAAAAAGAATTACTCAAG	472	92

[a]F, Forward primer; R, reverse primer

solution. A 2.5-µl aliquot of fresh 1-ethyl-3-3(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) solution (10 mg/ml) was added to the microspheres and incubated at room temperature in the dark. After 30 min, another 2.5-µl aliquot of fresh EDC solution was added to the microspheres and incubated for 30 min at room temperature in the dark. The microspheres were washed with 1 ml 0.02% Tween 20, then with 1 ml 0.1% sodium dodecyl sulphate (SDS), and finally resuspended in 100 µl 0.1 *M* MES (pH 4.5). Microsphere preparations were stored in the dark at 4°C until used.

PCR amplification of the SNP-containing fragment

All PCR amplifications were performed in a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, Mass.). Four different primer sets for querying the four SNP sites were multiplexed. For SBE, the components of the reaction mixture were 0.1 µl AccuPrime *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.), 0.5 µl 10× AccuPrime *Taq* DNA polymerase buffer, 200 μM of each dNTP (nucleotide triphosphate), 0.5 μM of each primer, 1.5 m *M* $MgCl_2$, and 30 ng template DNA in a total volume of 5 µl. Cycling conditions involved an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 46°C for 30 s, and extension at 68°C for 1 min. For ASPE, OL, and DH, SNP targets were pre-amplified by multiplexed PCR in 5-µl reactions using the HotStar *Taq* Master Mix (Qiagen, Valencia, Calif.). Reactions contained 1× Qiagen PCR buffer, 200 μM of each dNTP, 0.5 μM of each primer, 1.5 m *M* $MgCl_2$, 0.25 U HotStar *Taq* DNA polymerase, and 50 ng template DNA. The reactions were initially incubated at 95°C for 15 min to activate the enzyme, then cycled (35 cycles) at 94°C for 30 s (denaturation), 50°C for 1 min (annealing), and 72°C for 1 min (extension), with a final extension at 72°C for 7 min. The thermocycling conditions for specific SNP genotyping assays are shown in Table 2.

Determination of SNP genotypes using SNaPshot

SNP genotyping with the SNaPshot assay as a control was performed on DNA from the two parents and 58 F_2 plants according to the protocol provided with the ABI PRISM SNaPshot Multiplex kit (Applied Biosystems, Foster City, Calif.). The SNaPshot reaction involves SBE of an oligonucleotide probe designed to anneal adjacent to the SNP of interest, followed by extension with a fluorescent-labeled dideoxy terminator, and differs from the SBE method in that SNP alleles are determined by electrophoresis of the extension product(s).

Single-base extension

A total of 5 µl of PCR products to be multiplexed was enzymatically treated with 1 U each of shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo*I) to degrade excess PCR primers and dNTPs. The reaction solution was mixed thoroughly and incubated at 37°C for 1 h, followed by 15 min at 80°C to inactivate the enzymes. Four nearly identical reactions were set up, differing only in the choice of biotin-labeled ddNTP added. A 2.5-µl aliquot of SAP/*Exo*I-treated PCR products was added to a 2.5-µl reaction mixture containing 0.5 µl 10× Promega buffer, 0.064 U Thermo Sequenase (USB, Cleveland, Ohio), 3 m *M* $MgCl_2$, 0.12 μM of each SBE capture probe primer, 0.4 μM allele-specific biotin-la-

Table 2 Thermalcycling conditions for four SNP detection assays

Assay	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension/ligation
SBE	94°C/2 min	80	90°C/15 s	50°C/10 s	72°C/15 s
ASPE	96°C/2 min	30	94°C/30 s	55°C/60 s	74°C/120 s
OL	95°C/2 min	30	94°C/30 s	–	45°C/60 s
DH	95°C/15 min	40	94°C/30 s	55°C/30 s	72°C/30 s

Table 3 Oligonucleotide sequences (5′ → 3′) of capture and reporter probes for SNP

SNP name	Allele	SBE[a] (capture probe)	ASPE[b] (capture probe)	OL[c] (reporter probe)	DH[d]	
					Primer sequence	Capture probe
SNP12507	A	TTGAATGATGCAACTAGGACC	TTGAATGATGCAACTAGGACCA	TAAACTAGGTTCTATGCCTA	F: CCATCATTCAATTTAGGCATAG	CAACTAGGACCATAAACTAGG
	G		TTGAATGATGCAACTAGGACCG		R: CCTCATTATAATACGACCAGGTT	CAACTAGGACCGTAAACTAGG
SNP14049	C	GCCGCAATACTTCATTAACAG	GCCGCAATACTTCATTAACAGC	TTTAAGCAAGAATTATCGTGTT	F: GCCCCAAATTGTCTGTGTAA	CTTGCTTAAAACTGTTAATGAAG
	T		GCCGCAATACTTCATTAACAGT		R: TTCATGTGACAGGCAAAGAG	CTTGCTTAAAGCTGTTAATGAAG
SNP15783	A	GGTTCTGAAGAGTGAAGCTG	GGTTCTGAAGAGTGAAGCTGA	CATGATCAGGAAATTTTGTGC	F: AAGCGGGGAATCAATGAT	CCTGATCATGCCAGCTTCACT
	G		GGTTCTGAAGAGTGAAGCTGG		R: GGCTAGCACAAAATTTCCTG	CCTGATCATGTCAGCTTCACT
SNP16289	A	TTCAAAGTGGAGGCTATGAGT	TTCAAAGTGGAGGCTATGAGTA	TGGCTATTATGTCATGCATTG	F: TGGTTATTTGGGACTGATAATG	ATAATAGCCACACTCATAGCC
	G		TTCAAAGTGGAGGCTATGAGTG		R: CTCCTCGCTAATTATGTTTCAG	ATAATAGCCATACTCATAGCC

[a]All capture probes were designed with a 21-nt ZipCode sequence at the 5′ end and an allele-specific sequence at the 3′ end

[b]All capture probes were designed with a 21-nt ZipCode sequence at the 5′ end and an allele-specific sequence at the 3′ end. For each biallelic SNP, two capture probes were designed. Each probe differs in the choice of ZipCode and in the polymorphic nucleotide contained at its 3′ end

[c]The reporter probes were designed with a 5′ phosphate group and 3′ biotin modification, with T_m ranging from 46° to 49°C. The ASPE capture probe was also used for OL

[d]F, forward primer; R, reverse primer. PCR primers for amplifying SNP sites were designed with a 5′ biotin modification of forward primer. Capture probes were designed with a 21-nt ZipCode sequence at the 5′ end, with the allele-specific nucleotide centered within the capture probe sequence, and with a 5′-amino modification for coupling to beads

beled ddNTP, and 0.4 μM of each of the other three non-labeled ddNTPs. All capture probes for SBE were designed with a slightly modified ZipCode sequence that was one or two nucleotides shorter at the 5′-end than those used by Iannone et al. (2000), and with an allele-specific sequence at the 3′-end (Table 3). The same SBE primers were also used for the SNaPshot assay.

Allele-specific primer extension

Five microliters of multiplexed PCR products were added to a 5-μl reaction mixture containing 40 m M Tris-HCl (pH 8.4), 100 m M KCl, 2.5 m M $MgCl_2$, 50 n M of each ASPE capture probe (Table 3), 0.75 U *Tsp* DNA polymerase (Invitrogen), and 10 μM biotin dCTP. All capture probes for ASPE were designed with a slightly modified ZipCode sequence as described for SBE at the 5′-end and an allele-specific sequence at the 3′-end, one base longer than the probe for SBE (Table 3).

Oligonucleotide ligation

Reactions were carried out in 10 μl of ligase buffer, which included 2 μl of multiplexed PCR products, 0.005 μM of capture and reporter probes, and 2 U of Ampligase *Taq* DNA ligase (Epicentre, Madison, Wis.). Incubation involved heating at 95°C for 2 min, followed by 30 cycles of a two-step reaction consisting of denaturation at 94°C for 30 s and ligation at 45°C (depending on the T_m of the reporter probe). The OL reaction was performed with the same capture probe as that used for ASPE, with a ZipCode sequence at the 5′ end (Table 3). OL reporter probes of 20–22 bp in length were modified with a 5′ phosphate group and 3′-biotinylation.

Direct hybridization

Five microliters of multiplexed PCR products amplified using DH PCR primers and the HotStar *Taq* Master Mix were directly hybridized to target DNA regions and then coupled to microspheres with a 5′-amino modified capture probe (Table 3). Probes were 21–23 bp in length, complementary in sequence to the biotinylated strand of the amplicon, and designed with the specific SNP allele centered within the probe sequence.

Ethanol precipitation and hybridization

The products of the SBE, ASPE, OL, and DH assays were precipitated using 75% ethanol, for a final concentration of 60% ethanol, and were incubated in the dark at room temperature for 30 min, pelleted, and dried for hybridization. Except for slight modifications which depended on the SNP genotyping assay, hybridization procedures for binding the amplicons or ligation products to the microspheres were similar. The basic

procedure, which was carried out in a 50-µl total reaction volume, included denaturation at 90–95°C for 10 min and the addition of 1× TMAC (3 *M* tetramethylammonium chloride, 50 m *M* Tris-HCl, pH 8.0, 4 m *M* EDTA, pH 8.0, 0.1% Sarkosyl) in a mixture containing 3,000 of each type of probe-coupled microsphere from a set. The reaction was hybridized at 50–55°C (depending on the T_m of the probe) for 30 min, and labeled with 200 ng streptavidin in 10 µl 1× TMAC at 55°C for 5 min (60 µl total reaction) prior to analysis on the Luminex 100.

Flow cytometric analysis

Microsphere fluorescence was measured using a Luminex 100 cytometer equipped with a Luminex XY Platform plate reader and Luminex-compatible analysis software from MiraiBio. The fluorescence on the surface of the microspheres was measured and converted to a mean fluorescence intensity (MFI) value based on a minimum of 100 microspheres of each type, with a 50-µl sample size.

Results

Comparison of SNP genotyping methods

To validate the four flow cytometric-based SNP genotyping assays, we determined the SNP genotypes for the two parents and for 58 F_2 plants across four SNPs using the SNaPshot genotyping assay as a control. SNP genotypes were determined based on the addition of a specific fluorescently labeled ddNTP to SNaPshot oligonucleotide primers. Genotyping using the Luminex 100 depends on fluorescence signal intensity to identify the SNP allele. Therefore, SNP genotyping using the SNaPshot assay was qualitative, whereas the four flow cytometric-based assays were quantitative. χ^2 tests of the SNaPshot data revealed that segregation at all of the SNPs fit the expected ratio of 1 A1/A1:2 A1/A2:1 A2/A2.

All of the four SNPs were genotyped correctly by the SBE assay on the basis of fluorescence signal intensity (MFI values) using flow cytometric analysis, and the results were completely in agreement with those from the SNaPshot assay (100% congruence) (Fig. 1). The MFI

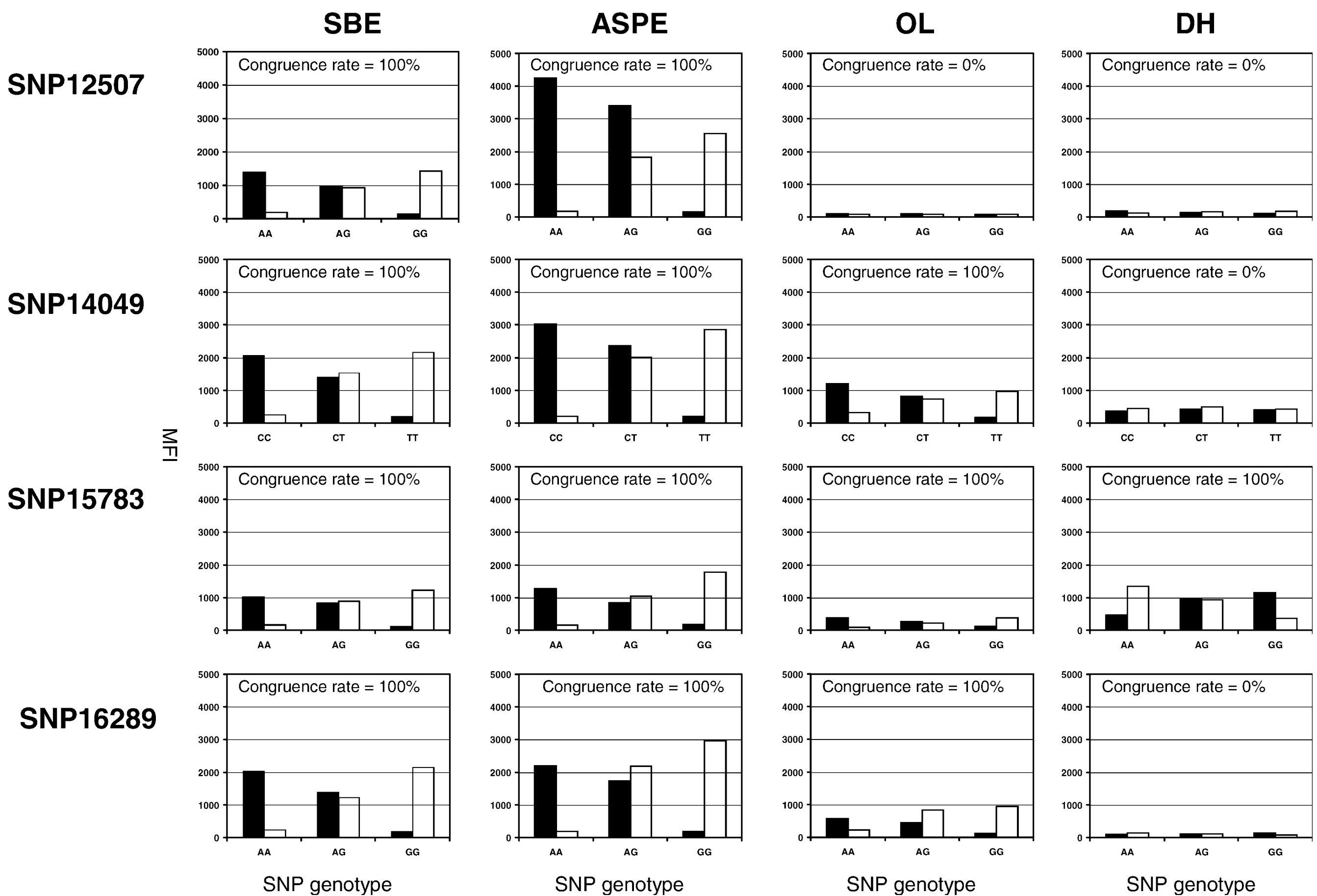

Fig. 1 Mean fluorescence intensity measurements for four SNPs by four SNP genotyping assays using a flow cytometer. The congruence rate is the percentage of correct allele calls for each SNP/assay combination compared to the SNaPshot assay

values of the four SNPs that were evaluated using the different genotyping assays are shown in Fig. 1. The background MFI values were low (less than 180.5), regardless of the SNP or the genotyping assay. For the SBE and ASPE assays, there were clear differences in signal intensity among the four SNPs. The MFI value for SNP15783 was much lower than the values for the other three SNPs. However, for all of the SNPs, including SNP15783, the signal intensity for the negative control represented only a few percentage points of the positive control, resulting in a clear differentiation between the two homozygotes and the heterozygote using these two assays. For all four SNPs, a slightly higher MFI value was observed for ASPE than for SBE.

For the OL assay, there was a large difference in MFI among the four SNPs, and this genotyping assay worked well for three out of the four SNPs (SNP14049, SNP15783, and SNP16289). For these three SNPs the congruence was 100% between OL and the SNaPshot control assay (Fig. 1). The DH assay produced a relatively low MFI compared to the other three genotyping assays and was only able to correctly genotype SNP15783 among the 58 lines. However, it is interesting to note that the DH assay successfully genotyped that SNP, even though the signal intensity of the positive control was only three- to fourfold higher than that of the negative control. For SNP15783 the allele calls from the DH assay were in 100% congruence with those of the SNaPshot assay across the 58 F_2 plants (Fig. 1).

Reagent cost and time analysis

Reagent cost and procedure time are important factors in selecting the genotyping method most appropriate for a particular objective. Table 4 presents the initial cost for the capture and reporter probes required for the four different methods. The probe cost for OL is currently higher than it is for the other three assays, due to the additional cost of adding a 5′ phosphate group and 3′ biotin. Biotinylation at the 5′ terminus of either the forward or the reverse primer likewise increased the cost of primers for the DH assay. Although primer and probe costs are comparatively high, the quantity purchased is sufficient for more than 20,000 reactions.

Table 5 Comparison of reagent costs per ten-plex and simplex (per data point) reactions among four SNP genotyping assays

Item	SNP assay			
	SBE	ASPE	OL	DH
Primer	$0.030	$0.032	$0.068	$0.048
DNA polymerase	$0.384	$0.314	$0.314	$0.314
SAP and ExoI	$0.152	–	–	–
SNP-detection enzyme	$0.037	$0.060	$0.001	–
Biotin d(d)NTP	$0.012	$0.026	–	–
Plate and tips	$0.186	$0.139	$0.139	$0.092
Microbeads	$0.240	$0.240	$0.240	$0.240
Total/ten-plex	$1.041	$0.811	$0.762	$0.694
Total/simplex (data point)	$0.104	$0.081	$0.076	$0.069

The reagent cost per data point varies for the four SNP assays (Table 5). Since assays can be multiplexed in a single well using the Luminex 100, the cost for SNP genotyping was calculated on a ten-plex basis. Although the initial probe investment price was lowest for SBE, the total cost was higher for this assay than that of the other three SNP genotyping assays. This is primarily due to the cost of the PCR cleanup procedure with SAP and *Exo*I, and to the cost of additional PCR plates and pipette tips required for the multiple steps in the assay. The cost per data point for OL and DH was approximately 70% of that for SBE, while the cost for ASPE was approximately 80% of that for SBE. The cost of ligation in OL is notably lower ($ 0.0001 per data point) than costs for the extension reactions used in the SBE and ASPE assays.

In addition to the cost of materials and reagents for the SNP genotyping assays, time requirements are also an important consideration. For SBE, at least 12 h were needed to complete the multiple steps of the assay, although only 2.5 h is ‘hands-on’ time (Table 6). The SBE

Table 4 Comparison of primer and probe costs (in U.S. dollars) among four SNP genotyping methods using Luminex 100. Costs were estimated on the assumption of a cost of $0.20 per base. Forward and reverse primers had 20 bases, and capture and reporter probes had 20 bases

Item	SNP assay			
	SBE	ASPE	OL	DH
PCR primers	$8.00	$8.00	$8.00	$8.00 + $40.00 (3′-B)[c]
Oligonucleotide				
Probe 1	$4.00	$4.20	$4.20	–
Probe 2	–	$4.20	$4.20	–
Reporter probe	–	–	$4.00 + $ 25.00 (5′-B)[b] + $45.00 (P)[d]	–
ZipCode	$ 4.00 + $ 45.00 (A)[a]	$4.00 + $45.00 (A)[a]	$ 4.00 + $ 45.00 (A)[a]	$4.00 + $45.00 (A)[a]
Total	$61.00	$65.40	$135.40	$97.00

[a]Cost for 5′-amino modification for coupling to beads
[b]Cost for 5′-biotinylation
[c]Cost for 3′-biotinylation
[d]Cost for 5′-phosphate modification for coupling to beads

Table 6 Comparison of time requirements for completing the four SNP genotyping assays

Item	SNP assay			
	SBE (h)	ASPE (h)	OL (h)	DH (h)
Hands-on labor	2.5	1.5	1.5	1.0
PCR	2.5	2.5	2.5	2.5
Clean-up	1.0			
SNP-detection	3.0	3.0	1.5	
Alcohol precipitation	1.0			
Hybridization	1.0	1.0	1.0	1.0
Luminex	1.0	0.5	0.5	0.5
Total	12.0	8.5	7.0	5.0

assay therefore effectively requires 2 days to complete. In contrast, genotyping with the DH assay can easily be accomplished in a single day. Time requirements for the ASPE and OL assays fall in between SBE and DH, and these two assays could potentially be completed in a single day.

Discussion

In order to make as objective of a comparison as possible between the four SNP genotyping assays, we evaluated all of them using the Luminex 100 flow cytometry platform. The Luminex 100 flow cytometer uses a microsphere-based genotyping system which offers many attractive features. Multiplex analyses are made possible through the use of reaction-specific microspheres that fluoresce at different frequencies, thereby permitting multiple discrete assays in a single tube with the same sample at the same time (Fulton et al. 1997). According to Chen et al. (2000), up to 120,000 genotypes per machine in an 8-h day could be determined through the combination of a set of 100 microspheres, automation, and decreasing the number of the microspheres to be read. High-throughput analysis with multiplexing ability permits SNP genotyping at a relatively low cost. Another attractive feature is that the Luminex 100 platform can be used with a variety of SNP genotyping assays.

Although the robustness and high-throughput capacity of SBE and ASPE with a flow cytometer have recently been compared (Taylor et al. 2001; Ye et al. 2001), this is, to our knowledge, the first comparison of four assays. We were interested in both the ability of the different assays to accurately distinguish between the genotypes at four random SNPs and the relative differences in reagent cost and technical time required for the four assays. We wanted to compare the SBE, ASPE, OL, and DH assays because each has attractive features, and all can be genotyped with the Luminex 100. The SNaPshot assay was used as a control because minisequencing methods have proven to be robust and highly accurate (Syvänen 2001). However, this assay is not a viable option for many plant breeding programs because of its expense.

The four different SNPs were successfully genotyped using SBE and ASPE, which produced a stronger MFI than did OLA and DH, and were more robust across the four SNPs. An advantage of ASPE over SBE is the ability to read both alleles from the same SNP in one ASPE reaction tube (Taylor et al. 2001; Ye et al. 2001). For SBE, each allele requires analysis in a separate reaction tube when using ddNTP terminators labeled with one fluorochrome. In addition, post-PCR cleanup and the addition of unlabeled nucleotides are unnecessary with ASPE. The residual dNTPs from the target-amplification PCR reaction are subsequently used for primer extension in ASPE. Another consideration is that the ASPE capture probe can also be used for OL. Reporter probe design permits a rapid switch to the OL assay if some SNPs cannot be genotyped by ASPE, or if one wants to take advantage of the cost savings of the OL assay for MAS.

In contrast to SBE and ASPE, the DH assay failed in genotyping three of the four SNPs. The success of DH in the microsphere-based system depends on the sequence quality and nucleotide content surrounding the SNP sites. Extensive experience is required in designing an effective capture probe specific for a particular SNP allele. The DH assay differs from the other three methods in that allele detection and hybridization to the microspheres occur simultaneously, and this may contribute to the failure of DH to accurately genotype some SNPs. Therefore, optimization procedures are likely to be needed in DH capture probe design for the successful genotyping of some SNPs. Although DH is less robust than the other methods, it is the most attractive of the four methods in terms of reagent costs, simplicity, and speed.

There are many considerations in selecting a SNP genotyping assay, and the choice depends partly on the purpose of the research. Two of the main considerations are cost per data point and simplicity of data acquisition. For genetic mapping and diversity studies requiring a large number of markers and a high level of multiplexing, ASPE is more cost-effective and simpler than SBE. Another alternative would be OL, due to the low cost for genotyping and the fairly high robustness of this method. One must consider the greater initial investment in OL probes compared to the ASPE probes, however. MAS generally involves a comparatively small number of markers being used to screen large numbers of samples. DH should therefore be considered for MAS due to its low cost, shorter time requirement, and simplicity, even though some additional time and effort may be required to optimize the probe design and assay conditions. The OL assay would be a potential alternative to DH for MAS. Although its cost per data point is somewhat higher, and the assay takes somewhat longer than DH, it is less expensive than SBE and was more robust than DH in correctly genotyping certain SNPs.

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