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RESEARCH

Channel Glass-based Detection of Human Short Insertion/Deletion Polymorphisms by Tandem Hybridization

Gabriel Betanzos-Cabrera · Brent W. Harker · Mitchel J. Doktycz · James L. Weber · Kenneth L. Beattie

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Abstract The development and critical evaluation of new technologies for identifying genetic polymorphisms will rapidly accelerate the discovery and diagnosis of disease-related genes. We report a novel way for distinguishing a new class of human DNA polymorphisms, short insertion/deletion polymorphisms (indels). A sensor with cylindrical pores named *channel glass* in combination with tandem hybridization, which uses a 5′-fluorescent labeled stacking probe and microarray-based short allele-specific oligonucleotide (capture probe) was investigated. This methodology allows indels to be detected individually and rapidly with small quantities of target DNA. This establishes a reliable quantitative test. Approaches for simultaneously hybridizing different targets to arrayed probes, designed to detect various indels in parallel, were examined. Five markers were consistently detected in a single hybridization. Possible factors impeding the hybridization reaction process are discussed.

Keywords Indels · Channel glass · Tandem hybridization · Arrayed probes · Detection

G. Betanzos-Cabrera (✉)
Área Académica de Nutrición, Instituto de Ciencias de la Salud, Universidad Autónoma del Estado de Hidalgo, Abasolo 600 Pachuca de Soto, Hidalgo 42000, Mexico
e-mail: gbetanzo@uaeh.reduaeh.mx

B. W. Harker
Center for Global Health and Infectious Diseases, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA

M. J. Doktycz · K. L. Beattie
Biosciences Division, Oak Ridge National Laboratory, Building 4500-S, MS 6123, Bethel Valley Road, P.O. Box 2008, Oak Ridge, TN 37831, USA

J. L. Weber
Center for Medical Genetics, Marshfield Clinical Medical Research Foundation, Marshfield, WI, USA

Introduction

Numerous genetic polymorphisms are being revealed through large-scale genomic sequencing. Whole genome polymorphism screens are widely used today in research [1–4]. They can be applied in paternity and forensic testing, linkage mapping, and will likely serve as a clinical tool in the future [5–7].

In general, common human DNA polymorphisms can be classified into two groups: single nucleotide polymorphisms (SNPs) that typically involve nucleotide substitutions, and insertion or deletion polymorphisms (indels) that involve one or more nucleotides [8]. Likewise, these polymorphisms can in turn be multiallelic or diallelic. The most common multiallelic indels are the class of short tandem repeats polymorphisms (STRPs), also called "microsatellites." The STRPs have been the markers of choice for geneticists in the 90's because they are highly informative with heterozygosities often exceeding 70% [9, 10]. However, although the SNPs are less informative, they are the most abundant with an occurrence of one in every thousand bases, and are becoming the most studied polymorphism with over 10 million SNPs reported. In contrast, diallelic indels have received very little attention. Diallelic indels can sometimes vary greatly in length between alleles, with length differences of tens or even hundreds of kilobase pairs [11]. However, by far the most common diallelic indels

(with an approximate density of one every 20 kb) are much shorter (less than 20 nucleotides) and these are stereotypical of the indels class of polymorphisms [12]. Indels have a number of important advantages over SNPs. First, it is simpler to distinguish an indel from sequencing errors among assemblies of overlapping sequences. This is especially important when the two alleles differ by two or more nucleotides in length. Second, confirmation and initial characterization of indels is simpler than base substitutions. Third, and most important, discrimination of alleles by hybridization is inherently greater for indels than SNPs. This can facilitate genotyping applications using microarray technologies.

A great variety of solid supports for the construction of DNA microarrays have been developed and tested [13–20]. Presently, glass microscope slides are commonly used [3, 13, 15, 21]. Here, we report the use of a porous support, channel glass, for the analysis of indels. Channel glass is an advanced genosensor configuration that offers a greater surface area when compared to conventional planar supports [17, 19, 22–24]. Channel glass contains cylindrical pores, with diameters of typically 1–10 μm. The channels are arranged parallel to each other and allow for sample flow through the structure (Fig. 1). This type of sensor has

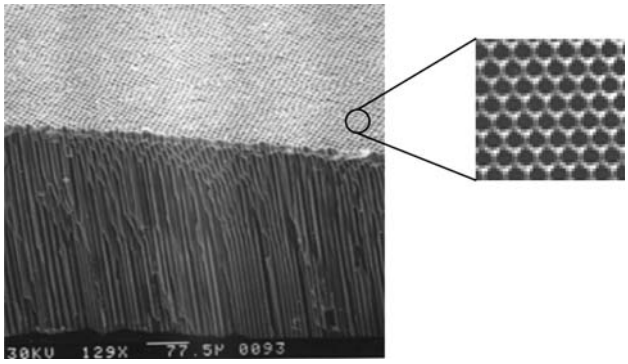

Fig. 1 Micrograph of a portion of channel glass. A cross section (left panel) showing the channels arranged parallel to each other and amplification of a region showing a regular array of densely packed pores (right panel)

shown significant advantages over flat supports [17–19, 22–24, 32].

Additionally, we used a novel tandem hybridization strategy. This strategy and its application for identification of mutations were recently reported [25–27]. Tandem hybridization has been able to detect point mutations with reliable discrimination [28]. The strategy utilizes a long 5′-fluorescent labeled stacking probe to introduce a label, and

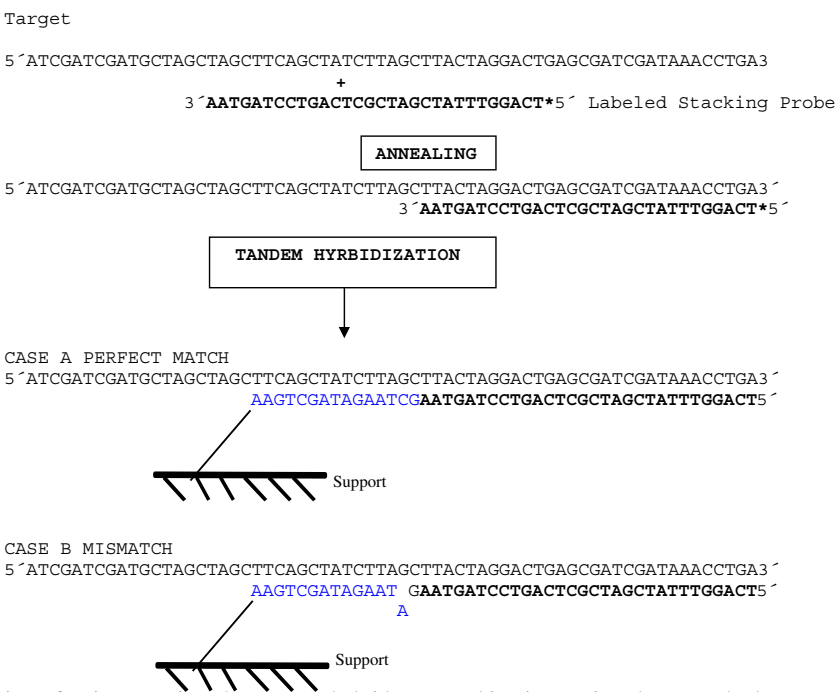

Fig. 2 Strategy for detection of point mutations by tandem hybridization. (A) The DNA target should be first annealed with a labeled stacking probe. The partially duplexed target is then hybridized to a specific short capture probe that is covalently attached to the surface of the sensor. The capture probe is designed to hybridize to the target DNA in tandem or contiguously with the longer stacking probe. Base stacking interactions between the longer stacking probe and the short capture probe provide considerable stabilization of the binding of target. (B) A mismatch near the end of the capture probe disrupts the binding of the target strand to the short probe, and prevents the stabilizing base stacking interaction between capture probe and stacking probe

to prevent secondary structures that could interfere with hybridization efficiency (Fig. 2). The PCR-amplified target DNA is first annealed to the stacking probe which hybridizes to a position adjacent to the site of interest. If a gap or mismatch occurs at the junction between the immobilized probes and stacking probe, hybridization stability is greatly reduced.

Materials and Methods

Polymorphic Sequences

Sequences of human indels were provided by the laboratory of Dr. J. L. Weber [8] and were identified through analysis of overlapping genomic sequences or cDNA sequences (see Table 1). http://www.research.marshfieldclinic.org/genetics/home/index.asp

Oligonucleotides

All capture probes were synthesized by IDT, Inc. (Coralville, IA) by standard phosphoramidite procedure and then desalted. They were spectrophotometrically quantitated as previously described [29]. Stacking probes were synthesized by incorporating a 5′Cy3-fluorescent label. Capture probes to be immobilized on the channel glass surface were derivatized with a 3′-NH_2-terminal.

Capture probes were selected according to length, base composition, and the position of insertion/deletion within the hybridization sequence to yield similar T_{ms}. In general, the hybridization capture probes were selected to contain the polymorphism in the central portion of the probe. For each marker, one hybridization capture probe for each of the two different alleles was designed. A list of the capture probes, fluorescently labeled stacking probes, and primers are shown in Table 2.

Table 1 Description of the polymorphisms studied

Number of polymorphism	Sizes (bases)	Insertion/deletion sequence	GenBank accession number
1	**TCTT/...**	ACAGTAGTAAGGGTGAC TATTTAAAC	AA011444
		ACAGTAGTAAGGGTGAC**TCTT**TATTTAAAC	
2	**TTC/...**	CGTGCTGATAAACA TTCTTCTTATGGT	H06049
		CGTGCTGATAAACA**TTC**TTCTTCTTATGGT	
3	**GGTGGA/.....**	GGCTAGGGGGAG GGTGGAGGTAGG	W44558
		GGCTAGGGGGAG**GGTGGA**GGTGGAGGTAGG	
4	**AAGAT/.....**	TTCATATGTAACA AAGGACGTGTGC	N30623
		TTCATATGTAACA**AAGAT**AAGGACGTGTGC	
5	**CAAT/....**	AAGTGCACAGAATAG CAATCAATCAG	AW293998
		AAGTGCACAGAATAG**CAAT**CAATCAATCAG	
6	**AACA/....**	ATCCTCTAACA GTGTACACTCCCAGA	AI809905
		ATCCTCTAACA**AACA**GTGTACACTCCCAGA	
7	**GTT/...**	TATTTAGGCCA GTTGACAGCCACATTA	BE855824
		TATTTAGGCCA**GTT**GTTGACAGCCACATTA	
8	**TGTTT/....**	ACAACGGGTTGAATCC TGTTTTGTT	Z73420
		ACAACGGGTTGAATCC**TGTTT**TGTTTTGTT	
9	**GAA/...**	GAACTGCCTT GAAAAGGAATGGACA	Z68754
		GAACTGCCTT**GAA**GAAAAGGAATGGACA	
10	**AGA/...**	ATCCCAAATGCAACAGAATTC...AGAAGA	Z81001
		ATCCCAAATGCAACAGAATTC**AGA**AGAAGA	
11	**TAT/...**	ATCTACTCTTAATGTA TATTTCATATT	BG621581
		ATCTACTCTTAATGTA**TAT**TATTTCATATT	
12	**AGGCATGAACAAAT/.....**	CACAAAGGGA TCTTGC	AL577012
		CAAAGGGA**AGGCATGAACAAAT**TCTTGCCA	
13	**CAACAT/.....**	AAATTAACAGGA CAACATTTGTCC	AW574842
		AAATTAACAGGA**CAACAT**CAACATTTGTCC	

Table 2 List of primers, stacking probes, and capture probes used

Primers		Stacking probes	
Name	Sequence 5′→3′	Name	Sequence 5′-Cy3→3′
PCR-1F-21	TAGAATGATTTACACTTGGGA	SP1	TGTATATTACCAATGTTTTTAGTTTAAATA
PCR-1R-23	TGCTTCCTTGTCAGTATGTTGAA	SP2	TAACTAAAGTAGGGGCTGGAACCATAAGAA
PCR-2F-20	AAATTAACAGCATCTTCCAG	SP3	GTCCTCTCTGTCCCATAACCTACCTCCACC
PCR-2R-20	AGGAAAATAACTAAAGTAGG	SP4	TGCCTCTGTTTTATACCTGCACACGTCCTT
PCR-3F-22	GCAGCCAAGGAGAAAGAGGGGG	SP5	TTTATTTTTATTGACATGACTGATTGATTG
PCR-3R-21	CTTCTTGTCCTCTCTGTCCCA	SP6	AGAATATCAGCTCTGGGAGTGTACACTGTT
PCR-4F-22	TCTCTCAATATACCCGTGATAC	SP7	GCCACACCTCAAAATAATGTGGCTGTCAAC
PCR-4R-21	ATAATGAGTTCATTGCTGGGC	SP8	TGTGTCATCAGGGATGGGGACAACAAAACA
PCR-5F-20	CCTGAGAAGACTGAAGCAAC	SP9	TACAATAAAGCCACTTGTCCATTCCTTTTC
PCR-5R-23	AAATGGTGCTTTATTTAACAGAA	SP10	ATGGCCTAATCACCTCCTTAAGACTCTTCT
PCR-6F-21	CAGTATTTGAAATGGCAAAGG	SP11	AAGCTGCTTTTGTTAAACAAATATGAAATA
PCR-6R-23	AGACATGAACTAGAGGAAATGTG	SP12	CAGCTGCCCTGTGTGGGACTGAGTGGCAAG
PCR-7F-23	GCCCTAAAGTCTAACACAACTGT	SP13	TGACCATTATTCACAGGTGGACAAATGTTG
PCR-7R-20	TCAAAATAATGTGGCTGTCA		
PCR-8F-20	ATTGAAGTGCATTTGAAAGC		
PCR-8R-20	GGGGTGCCCTTTATGTAATA		
PCR-9F-20	TCCCCTCATTTTCATACTCA		
PCR-9R-20	TACAACGTGATCACTGCATC		
PCR-10F-20	TGAATGTTCGTGTCTTTTCC		
PCR-10R-20	GAGAACTCATGGCCTAATCA		
PCR-11F-22	GATGATACAAGTGAACTCTGCA		
PCR-11R-18	AAGGCATCAAGCTGCTTT		
PCR-12F-19	CATGAGGAAGAGGGTCATG		
PCR-12R-19	CTGCAATGTGAAACAGCTG		
PCR-13F-21	TCTACCCAACTACCTCCACAT		
PCR-13R-23	GATGGCTTATGTCATCAGTAAAG		

Capture probes	
Number	Sequence 5′→3′-NH_2
1d	TCACCC
1i	AAGAGTCA
2d	TGTTTATCA
2i	GAATGTTTA
3d	CTCCCC
3i	TCCACCC
4d	TGTTACATA
4i	ATCTTTGTT
5d	CTATTCTG
5i	ATTGCTATT
6d	AGAGGATG
6i	TGTTAGAG
7d	TGGCCTA
7i	AACTGGC
8d	GGATTCAA
8i	AAACAGGA
9d	AAGGCAGT
9i	TTCAAGGC

Table 2 continued

Capture probes	
Number	Sequence 5′→3′-NH_2
10d	GAATTCTGT
10i	TCTGAATTC
11d	TACATTAAG
11i	ATATACATTA
12d	TCCCTTTG
12i	ATTTGTTCA
13d	TCCTGTTAA
13i	TGTTGTCC

i = insertion, d = deletion

In order to clearly illustrate a perfect match and tandem hybridization on indels, two examples of annealing of the capture probe, stacking probe, and target are shown below:

```
Polymorphism 1 TCTT/....
With TCTT insertion
  5′AAGGGTGACTCTTT          Target sequence
  ATTTAAACTAAAAACATT
  GGTAATATACAAATTT3′
    3′ACTGAGAA5′            Capture probe to detect
                              insertion (1i)
      3′ATAAATTTGATTTT      Stacking probe (SP1)
  TGTAACCATTATATGT5′
With TCTT deletion
  5′AAGGGTGACTATTTA         Target sequence
  AACTAAAAACATTGGTAA
  TATACAAATTT3′
    3′CCCACTG5′             Capture probe to detect
                              deletion (id)
      3′ATAAATTTGATTTT      Stacking probe (SP1)
  TGTAACCATTATATGT5′
Polymorphism 2 TTC/...
With TTC insertion
  5′GCTGATAAACATTCTT        Target sequence
  CTTATGGTTCCAGCCCCT
  ACTTTAGTTATTT3′
    3′ATTTGTAAG5′           Capture probe to detect
                              insertion (2i)
      3′AAGAATACCAAGGT      Stacking probe (SP2)
  CGGGGATGAAATCAAT5′
With TTC/... deletion
  5′GCTGATAAACATTCTTAT      Target sequence
  GGTTCCAGCCCCTACTT
  TAGTTATTT3′
    3′ACTATTTGT5′           Capture probe to detect
                              deletion (2d)
      3′AAGAATACCAAGGTC     Stacking probe (SP2)
  GGGGATGAAATCAAT5′
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Probe Printing and Immobilization

The 3′-NH_2 capture probes were resuspended to a final concentration of 20 μM in deionized water. Aliquots of 20 nL from each probe were spotted onto channel glass obtained from Galileo Electro-Optics Corp. (Sturbridge MA). A Hamilton Microlab 2000 robot (Hamilton Company, Reno, NV) was adapted in our laboratory for solenoid ink-jet-based spotting using a sapphire dispense tip. This instrument was designed for low volume aspiration and dispensing as described by Hicks et al. [30]. The spotted chips were allowed to dry at room temperature overnight and later rinsed with deionized water before hybridization.

Target Preparation

Polymorphic regions from human genomic DNA samples were amplified by PCR as described by Weber et al. [31]. The amplification was performed in volumes of 100 μl containing 50 mM potassium chloride, 10 mM TRIS, pH 8.4, 1.5 mM magnesium chloride, 0.15 μM each primer, 200 μM each deoxyribonucleotide triphosphates (dATP, dCTP, dTTP, and dGTP), 50 ng of genomic DNA, and 0.5 units of *Taq* Polymerase (Perkin Elmer). The cycling reaction was done in a Perkin-Elmer 9600 thermocyler programmed for 1 cycle of 5 min at 95°C, 75 s at 55°C, 1 min at 72°C followed by 29 cycles of 30 s at 95°C, 75 s at 55°C, 60 s at 72°C, followed by a final 5 min hold at 72°C. Aliquots of 10 μl from each amplification reaction was resolved by electrophoresis in 2% agarose gels and detected by staining with ethidium bromide.

Hybridization of PCR Products to Arrays

In order to introduce a label into the target DNA, the target sample (PCR product) was first annealed to a long (30

bases) stacking probe that carries the label and hybridizes to a position adjacent to the polymorphism. Target DNA was annealed with five-fold molar excess of stacking probe in 100 µl 5× SSPE buffer (1× SSPE is 0.75 M NaCl, 50 mM NaH_2PO4, 5 mM EDTA, pH 7.4, 5% (w/v) polyethylene 8000). The annealing was performed in a thermocycler using a temperature program of 92°C 5 min, 65°C 30 min followed by a final cooling at 6°C. A total of 50 µl was immediately used for hybridization.

The duplex was placed into the hybridization chamber and allowed to flow with forward and backward flow rates of 0.05 or 0.1 ml/min using a syringe pump for 20 min at 15°C. After hybridization, the glass was washed for about 30 s with cold 5× SSPE.

The flowthrough hybridization chamber was designed and built at Oak Ridge National Laboratory [32]. The hybridization solution traverses the chip and brings the fluid entry and exit points to the same face of the holder (see Fig. 3). This allows for an easy assembling and flush mounting of the chamber to the hybridization detection instruments. An optically clear acrylic window is laminated to the structure for visualizing the flow of the liquid.

Imaging

Fluorescent signals were captured by a GeneTAC 1000 analyzer system with GT Imaging & Review software (Genomic Solutions, Ann Arbor, MI). The GeneTAC imaging system has a dynamic range of 2–3 logs and displays detection linearity when the average pixel intensity is between 5,000 and 50,000. Each support was imaged for a period which yielded average pixel intensity for the brightest spots of slightly below 50,000 (typically 30,000). For each hybridization result the average pixel intensities for the six determinations were averaged, and after subtraction of background signal (average pixel intensity typically <100) the hybridization result was assigned a qualitative intensity value of 'strong' (average pixel intensity 10,000–50,000), 'weak' (average pixel intensity <10,000 but typically >5,000) or 'absent/undetected' (average pixel intensity <1,000). To statistically compare the hybridization patterns obtained with the three spotted slides in each hybridization experiment, SigmaStat version 2.03 was used for Friedman repeated measures analysis of variance on ranks. This non-parametric test consistently yielded *P*-values of 1.000 for replicate hybridization intensities, thus the differences between experimental replicates were not statistically significant, supporting the conclusion that these hybridization experiments were highly reproducible.

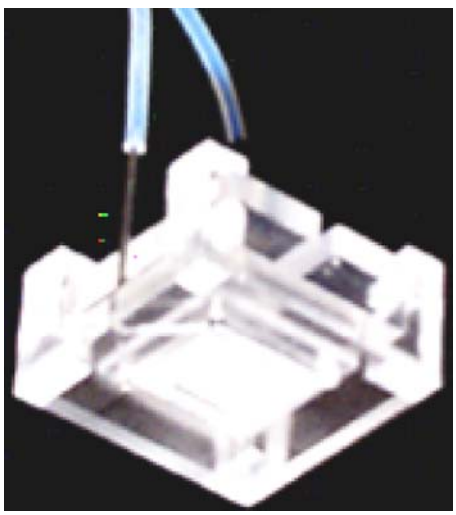


Fig. 3 Photograph of chamber used for flowthrough hybridization

Results and Discussion

With the sequencing of the human genome, identifying human polymorphisms are becoming important as a clinical tool. To take advantage of this new data, the adoption of new techniques and procedures are required [2, 4, 33, 34].

Human DNA polymorphisms, and more specifically whole genome polymorphism screens, have a number of important applications. Polymorphism screens can be used to identify individuals as, for example, in paternity and forensic testing. Polymorphism screens permit linkage mapping of genes that influence disease and other phenotypes [5–7, 9, 21, 33].

The genetic targets used for the genotyping developments described here are a class of diallelic human insertion/deletion polymorphisms (indels) [8]. Indels are characterized by the presence or absence of a few nucleotides. Indels markers are plentiful, with a listing of over 200,000 confirmed and candidate diallelic and multiallelic indels available. (A list is available at http://www.research.marshfieldclinic.org/genetics/home/index.asp). Over 2,000 diallelic indels have been characterized and current estimates indicate that indels comprise ~8% of all human polymorphisms. Compared to SNPs, indels are less plentiful but lend themselves to diagnostic applications. Not only is the allele length difference fairly straightforward to identify by gel electrophoresis, but also the hybridization properties of the two alleles should be easy to distinguish. Mispairing of several nucleotides is generally easier to discriminate against than that of a single nucleotide mismatch. Considering these attributes, diallelic indels were chosen for developing a genosensor-based genotyping technique.

Supplementing the advantages of indel markers, a stacking hybridization approach was investigated. In this approach, a short "capture" probe is immobilized onto the

surface of the channel glass. These immobilized probes capture the target DNA sequence that has been previously annealed to a "stacking" probe. The stacking probe binds to the region adjacent to the capture probe, allowing for contiguous base stacking between the capture and stacking probes. Previous work has demonstrated the advantages of stacking probes for hybridization-based analyses [25–28, 32].

Although currently there are numerous techniques for typing polymorphisms, these mainly focus on SNPs, and the majority use glass microscope slides [21, 34–36]. In this work a different substrate to construct microarrays was used. Channel glass has a number of practical advantages over flat glass [17–19, 22–24, 32]. The most important advantages being increased sensitivity and a faster hybridization rate.

A key benefit of stacking hybridization is an increase in hybridization specificity and stability. Compared to direct hybridization with short (<20 nucleotides) capture probes, the thermodynamic stability of a perfectly matched complement compared to a mismatched complement is improved [37–39]. The use of a stacking probe can also prevent the formation of double stranded regions in the target sequence. Intramolecular folding and intermolecular duplex formation in the target sequence can obscure hybridization sites and prevent capture of the target sequence [40]. Additionally, the use of a stacking probe presents a convenient means for introducing a label for hybridization detection [25–28].

An initial set of 13 indels containing genetic sequences was selected and used to evaluate the advantages offered by diallelic indels and stacking hybridization in genosensor-based diagnostic screens. The capture probes were arrayed on channel glass using a custom-built reagent-dispensing device [30]. Using optimized conditions, specific and strong hybridization signals were routinely obtained for each marker hybridized individually (Fig. 4). Small arrays of the capture probes were prepared and used for genotyping. Individual PCR reactions for each allele were combined and hybridized to the array. However, only 5 of the 13 markers that were evaluated were detectable consistently. The results of one such experiment is shown in Fig. 5. These results were confirmed by gel electrophoresis studies (data not shown). The evaluation of other genomic DNA samples

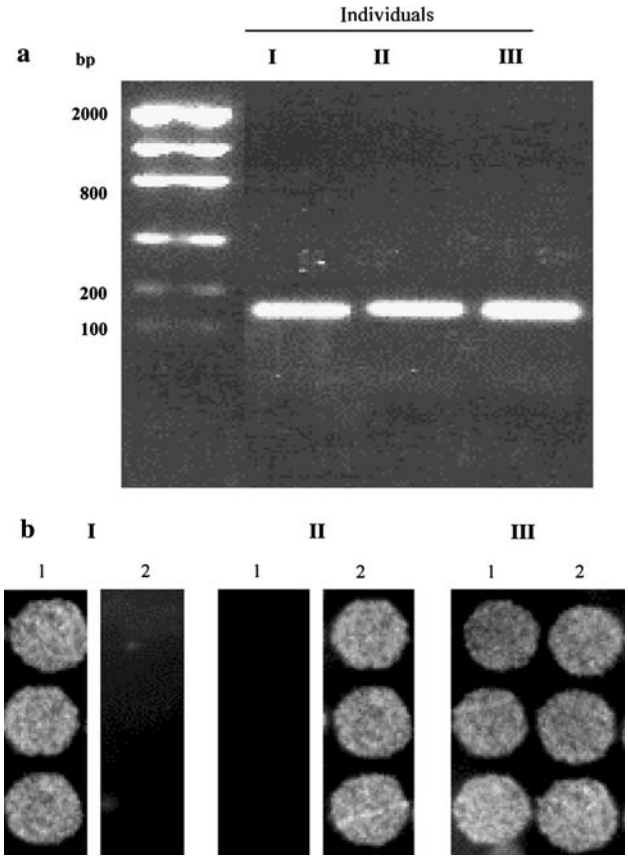
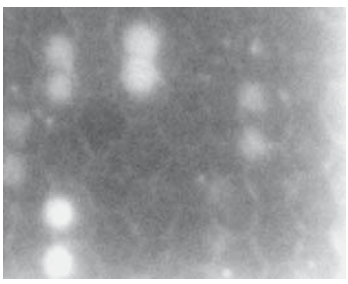


Fig. 4 Genotyping Analysis by tandem hybridization onto channel glass. Three genomic DNA samples of different individuals were genotyped for polymorphism 2 (TTC/...) specific stacking and capture probes were used. (**a**) Electrophoresis in agarose gel of PCR products of three different individuals. The products show the same molecular size. (**b**) The analysis by tandem hybridization onto channel glass shows the genotype of each individual is different from each other: being homozygous for insertion in case I, homozygous for deletion in case II, and heterozygous in case III. All genotypes coincide with results communicated by the laboratory of Dr. J. L. Weber



1I **1D** 2I **2D** 3I 3D 4I 4D 5I 5D
1I **1D** 2I **2D** 3I 4D 4I 4D 5I 5D
6I 6D 7I 7D 8I 8D **9I** 9D 10I 10D
6I 6D 7I 7D 8I 8D **9I** 9D 10I 10D
11I **11D** 12I 12D 13I 13D
11I **11D** 12I 12D 13I 13D

Fig. 5 Multiplex genotyping. Five polymorphisms were simultaneously genotyped. The diagram next to the figure shows in bold letters the polymorphism identified. Polymorphisms 1, 2, and 11 were homozygous for deletion, 6 and 9 for insertion. The numeric representation on the right side shows probe orientation each probe on the channel glass

also yielded consistent results between the genosensor and gel electrophoretic data. Although, melting temperatures of the chosen capture probe sequences were comparable, the lack of discernable signal for the other markers in the same hybridization reaction can be due to several factors that require additional optimization.

A factor that potentially influences the hybridization efficiency is the target sequence in the adjoining region [37–39, 41]. Although the SIDP marker is confirmed, nucleotide polymorphisms can occur in nearby sequences and affect the hybridization of the stacking probe. However, it is necessary to take into account that probe-target duplex formation is in competition with numerous other reactions occurring simultaneously, including: probe folding, target (binding site) folding, homodimer of probes, homodimer of targets, and homodimer of auxiliary probes. These other reactions can significantly inhibit the desired hybridization [40, 41]. These competing reactions will vary for each probe/target set, depending on the sequence. Finally, the sequence design of the capture and stacking probe may require further optimization for effective hybridization to the target sequence.

Conclusion

Alleles containing short insertion/deletion polymorphisms are ideal for parallel analysis using genosensors constructed from channel glass. The indel markers are easier to distinguish by hybridization-based approaches, when compared to single nucleotide polymorphisms, and are plentiful enough for performing whole genome scans. However, this feature alone is insufficient for facile analysis of polymorphisms by microarray-based approaches. The channel glass structures used here allow for a greatly increased surface area and for sample flow to occur through the substrate. The increased surface area and microscale structure allow for more sensitive detection, increased dynamic range, and faster hybridization. Stacking hybridization was employed to increase the stability and specificity of complexes formed from short capture probes. Considering the sensitive detection afforded by channel glass and stacking hybridization, lower quantities of material can be evaluated. In addition to the shorter hybridization time and smaller quantities of sample noted above, another advantage was the prevention of cross hybridization. In general, the advantages of indel markers, stacking hybridization, and channel glass combine to facilitate genetic characterizations.

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