

Minisequencing on Functionalised Self-Assembled Monolayer as a Simple Approach for Single Nucleotide Polymorphism Analysis of Cattle

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We have developed a genetic barcode module, based on a parallel sorting facility of single nucleotide polymorphism for secure individual identification of cattle. Biotinylated allele-specific oligonucleotides were immobilized onto the predefined spots of streptavidin tethered self-assembled monolayers with long chain alkanethiols on biochips. The target DNAs for hybridization and subsequent on-chip minisequencing were produced by multiplex PCR method. After enzymatic extension, only the moiety-modified dideoxynucleotide triphosphate, when coupled to its complementary target sequence, could be detected by the corresponding antibody to the moiety in a specific and sensitive manner. The database SNPZoo was developed for storage of the sequence information consisting of cytosine/thymidine patterns. This SNP chip system can further be used in the detection of any replaceable point mutations occurring in the human and animal genes.

Key words: Microarray, Minisequencing, SNP

Introduction

Currently DNA microarrays have been extensively used in gene expressions (Fears *et al.*, 2002; Jazaeri *et al.*, 2002; Karan *et al.*, 2002; Wang *et al.*, 2002) and genotyping experiments (Ahrendt *et al.*, 1999; Conner *et al.*, 1983; Hacia *et al.*, 1996; Livak and Hainer, 1994; Lopez-Crapez *et al.*, 2001; Maskos and Southern, 1993; Nikiforov *et al.*, 1994; Syvänen, 1994) to decipher the role of various genes and their mutations in disease diagnosis and prognosis and have important clinical implications and broad practical value in pharmacogenetics. The ability to fabricate highly robust microarrays immobilized with thousand probes enables the generation of massive amounts of biologically relevant data using very low sample quantities (Hacia *et al.*, 1996; 1998; Huang *et al.*, 2001). This feature significantly increases the experimental throughput and at the same time results in substantial cost savings. Many methodologies have been developed to screen a large number of SNPs with low cost, high sensitivity and high accuracy in both basic research and clinical applications. The applications of these methods have recently been

reviewed, including direct DNA sequencing, gel electrophoresis, invader assay, 5'-nuclease Taq-Man, dynamic allele-specific hybridization, molecular beacon probes, minisequencing, silico SNP mapping, mass spectrometry, pyrosequencing, DNA chips, electric field-controlled nucleic acid hybridization, electrocatalysis and bead technology, etc (Shi, 2001; Kwok, 2001).

Technological advances in molecular biology and surface chemistry have led to the design of various microarrays utilizing a wide range of biomolecules that are immobilized on sensor substrates ranging from membrane to gold on glass. Several researches showed that the quality of self-assembled monolayers as chemical interface on planar surface (Ulman, 1996; Mrksich and Whitesides, 1996). Gold is an ideal substrate for constructing highly precise nano-scale sensor architectures, whose interfacial properties are tuned 'at-will' using different starting building blocks. Chemisorption of alkanethiols on gold gives rise to highly ordered monolayers (Bain *et al.*, 1989; Persson *et al.*, 2001). Based on the principles of self-assembly, well-defined streptavidin sensor surfaces were constructed using a biotin-terminated

thiol (Mecklenburg, 1996). Because of the high affinity between biotin and streptavidin ($K_D \sim 10^{15} \text{ M}^{-1}$), biotinylated probes are firmly immobilized onto the microarray surface and are able to withstand harsh chemical and thermal experimental protocols. Therefore, the streptavidin interface provides a versatile platform for the attachment of virtually any biotinylated molecules, including oligonucleotides, cDNA, PNA (peptide nucleic acid), carbohydrates, peptides, and proteins.

DNA marker technology is a novel technology for determining the genetic identity and kinship of an animal, which provides a fundamental material for improving livestock through selection (Heaton *et al.*, 2001a; 2002). Single nucleotide polymorphisms (SNPs) become increasingly attractive because they are abundant, genetically stable, and amenable to high-throughput automated analysis. Many genotyping researches in cattle SNPs have been carried out in different ways (Barendse *et al.*, 2001; Fries *et al.*, 2001; Heaton *et al.*, 2001a; 2001b; Konfortov *et al.*, 1999; Sauer *et al.*, 2000). Here we report the use of oligonucleotide biochips for the individual monitoring single nucleotide polymorphisms (SNPs). Using well-defined nucleotide acid probes and targets containing given SNPs, an attempt is made towards developing a barcoding model for cattle provenance. The enzymatic assay of single-base extension developed by Pastinen and coworkers (1997) was modified for effectively genotype selected cytosine and thymidine nucleotides. By using DNA polymerase enzyme, it is reliable to incorporate a base whose identity is determined by the SNP into a newly synthesized sequence of DNA. The incorporated base labeled can be detected by many read-out methods.

Materials and Methods

Preparation of oligonucleotide biochips

XNA on Gold microassays (ThermoHybaid, interactiva Division, Ulm) were used for conducting the SNP analysis. Glass slides, covered with a $0.1 \mu\text{m}$ 24-carat gold layer, were coated with a $50 \mu\text{m}$ thin hydrophobic Teflon film that creates hydrophilic micro-wells (1.5 mm in diameter) in order to circumvent problems of spot shape and array geometry. A self-assembled monolayer was formed after incubation in 16-mercaptohexadecanoic acid solution. Subsequently the monolayer

was biotinylated with Biotin-PEO-Amine (Pierce, Rockford, IL), forming a chemical interface, and then saturated with recombinant streptavidin (Roche, Basel) to biotin of the interface, further creating a biological interface so that it is feasible for any biotinylated probes to be fixed on it. The concentrations of probes and targets and wash conditions were optimized. For loading allele-specific oligonucleotides onto the streptavidin-coated micro-wells of the chips, the HPLC-purified biotinylated probes (ThermoHybaid Interactiva, Ulm) were adjusted to $1 \mu\text{M}$ in TBS-T buffer (150 mM NaCl, 10 mM tris-(hydroxymethyl)aminomethane, 0.1% Tween-20, pH 8.0, Sigma, Steinheim) and spotted $1 \mu\text{l}$ aliquots onto each micro-well. After incubation at 50°C for 1 h and then at 4°C over night, chips were rinsed, dried and stored at 4°C before use.

Selection of SNPs for genotyping of cattle

SNPs were initially detected through comparative direct sequencing of a cattle biodiversity panel composed of a variety of cattle breeds. The most informative SNPs were selected to become part of a digital DNA signature (Fries and Durstewitz, 2001). This procedure was done, based on three criteria, that is, (1) the SNP had to be polymorphic within the economically important Holstein Friesian or Simmental breeds, (2) the frequency of the minor allele had to be at least 0.2 in the Holstein Friesian breed, and (3) the genetic distance to any neighboring SNP used in the assay had to be $\geq 50 \text{ cm}$. In this study, nine mutations in three cattle (SB26, SB37, SB45) were detected: LX423 (AF440366), LXTTCR (AF440367), LX420 (AF440370), LX016 (AF440369), LX431 (AF440372), LX487 (AF440381), LX444 (AF440376), LX438 (AF440375), and LXZFX (AF032866). The allele-specific oligonucleotide probes immobilized on chips varied in 25–30 mers in length, modified with biotin at their 5'-ends. The probes will be further specifically extended at their 3' ends with labeled ddCTP or ddUTP, complementary to target reference DNA in the presence of DNA polymerase in the following minisequence reaction. In order to decrease steric interference, a 10mer-polythymidine spacer was added between allele specific sequence and biotin at 5'-ends of the probes.

Preparation of cattle genomic DNA

Genomic DNA was prepared from frozen bull semen by proteinase K digestion, followed by phenol-chloroform extraction as described (Lien *S. et al.*, 1990), with modifications according to Buitkamp's method (Buitkamp *et al.*, 1999) and quantified by gel electrophoresis and fluorometry. Genomic DNA was adjusted to a concentration of 25 ng μl^{-1} in TE buffer and then stored at 4 °C for use.

Multiplex PCR

Each pair of primers of all the nine polymorphic sites for multiplex PCR were as follow, LX423-F: GCATAGAGCATGGGAAAGGA, LX423-R: AGAAAAGGACCCAGGTAGGG, LXTCR-F: AACCAGAATGGCAGAAAGTCTCC, LXTCR-R: GTCACCTCCATTTATAGATCAGC, LX420-F: CAAATGTGTGGATTTTG AGGTG, LX420-R: GCATCCTAGCGTCAATAAACC, LX016-F: GCTCAGTTCCCT CCTCCTA, LX016-R: GAGTCCAGGGCTGCACAGTA, LX431-F: TGACACGTGTG ACAACATGG, LX431-R: TGGCAACTGCCATTATACTTTC, LX487-F: TGGGCCAA AGCAGAAATAAC, LX487-R: TCATTTTGCTTATAGCATCAGA, LX444-F: CTGAACT TTGGCCAATGAAA, LX444-R: TCCCAGTTGGTTGGTTGGTA, LX438-F: GGGCAT CTGGCTACAGAACAA, LX438-R: GAGGTTTACAGGTTCTCCTGCATT, LXZFX-F: GAGTGTGGTAAAGGTTTTTCGTC, LXZFX-R: GATTCGCATGTGCTTTTTGA. Multiplex PCR amplifications with 4 or 5 pairs of corresponding primers were performed in one PCR reaction for obtaining relevant targets with allele-specific sequences simultaneously. Briefly, multiplex PCR was carried out in a total volume of 200 μl using 75 ng genomic DNA with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM each dNTP, 2 mM MgCl_2 , 0.5 μM each primer and 5 units AmpliTaq DNA polymerase. The sizes of PCR fragments ranged from 54 to 361 bp in length. PCR fragments were amplified in a gradient thermocycler (ThermoHybaid, UK). It started an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 30 sec denaturation at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C, and then followed by 10 min final extension at 72 °C. The DNA solution was further purified with PCR

purification kit (Qiagen, Hilden) and precipitated with ethanol (Merck, Darmstadt).

Minisequencing and fluorescent detection

DNA pellet from multiplex PCR was dissolved in 10 μl distilled water. DNA was denatured for 10 min at 99 °C, cooled on ice, mixed with 1 μl 1 M NaOH, incubated at RT for 5 min and mixed with 1 μl 1 M HCl and 1 μl TBS-T buffer. A total volume of 25 μl minisequencing reaction mix was prepared with 20 μM fluorescein isothiocyanate (FITC)-ddUTP, 20 μM Aminodigoxigenin-ddCTP (NEN, Boston, MA) and 10 U of ThermoSequenase (Amersham Pharmacia). The final concentration for each PCR fragment in minisequencing mix solution was 400 nM. The reaction mix was then pipetted onto the chip surface and distributed below a cover glass. The chips were incubated in a humid chamber at 50 °C for 2 h. After rinsing in TBS-T buffer, chips were incubated at RT for 10 min with 1:50 dilutions of Alexa488-labelled anti-FITC antibody (Alexa Fluor® 488 Signal-Amplification Kit for Fluorescein, Molecular Probes, Eugene, Oregon USA Leiden, The Netherlands) and 5-N-N'-diethyl-tetramethylindodicarbocyanine(Cy5)-labelled anti-digoxigenin antibody (ImmunoResearch, West Grove, PA). Chips were washed in TBS-T buffer, covered with a cover glass and scanned with an FLA-2000 scanner (473/520 nm and 633/675 nm, 50 μm resolution; Fujifilm).

Database generation

HGBASE (ThermoHybaid, Interactiva Division), a database of SNPs and other variations of the human genome were modified with respect to layout and further optimization for gathering SNPs of livestock species. The resulting database was called SNPZoo. Three means of access are implemented: (1), "simple search", where one enters a keyword for which all data except the sequence data in SNPZoo are queried. (2), the Sequence Retrieval System 5.1 (Etzold *et al.*, 1996), a common interface to query all kinds of life sciences related databases. (3), an interface to NCBI's BLAST program (Altschul *et al.*, 1990) for input and further analysis of sequences up to 10 kb. A set of data fields was implemented and is accessible over the URL <http://www.snpzoo.de>.

Results

Biochips of oligonucleotides may accessibly be produced by light-directed *in situ* synthesis on solid surface in a high-throughput way, but in most instances, the production of oligonucleotide chips can be reached by synthesizing the oligonucleotides individually before immobilization on solid surface. In this study, we used such a biochip that posses a biological interface of streptavidin immobilized on a chemical interface (Fig. 1), which

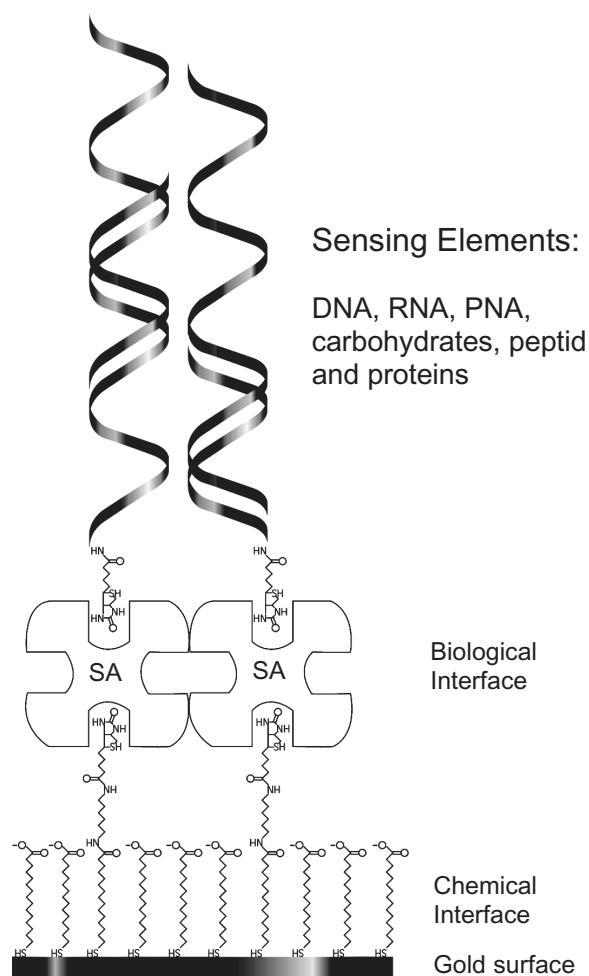


Fig. 1. Scheme of the biochip (XNA on GoldTM). Gold-coated slides printed with a Teflon-like matrix to receive predefined hydrophilic micro-wells. A chemical interface of a biotinylated long chain alkanethiol and a biological surface of streptavidin (SA) for capturing versatile biotinylated sensor molecules like DNA, PNA (peptide nucleic acid), protein or saccharides were shown on this 96 well format.

makes it possible that any chemically synthesized oligonucleotides modified with biotin may be allowed a covalent attachment to streptavidin on the surface.

In order to give quantitative information regarding oligonucleotide surface density on each spot, varying amount of Cy5-streptavidin protein were used. The amount of immobilized streptavidin (SA) per micro-well was 5 nM (2.8×10^{-13} mol cm^{-2}) (Fig. 2). Similar quantification on the SA layer revealed a capacity for 10 nM biotinylated oligonucleotide per micro-well (data not shown). This is in close accordance with the deduced molecular architecture shown in Fig. 1 and the four possible biotin-binding pockets of each SA tetramer.

Another important parameter for minisequencing and successful application is the target DNA concentration contained allele-specific loci, which affects hybridization rate and enzymatic elongation efficiency. Signal fluorescence intensity in this experiment reduced significantly below 300 nm for single strand target DNA (data not shown). However, two combinations of 200 μl multiplex PCR reactions were pooled to receive at least 10 μM for each of nine DNA PCR targets for on-chip analysis.

In the project described here, a genetic analysis of single nucleotide polymorphisms of cattle on chips was carried out. Specific cytosine (C) and

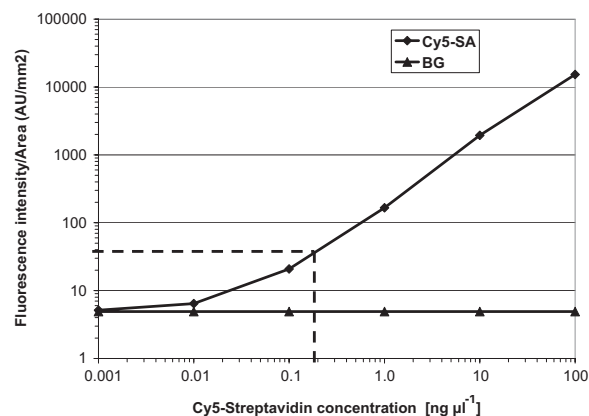


Fig. 2. Coupling efficiency for streptavidin. Fluorescence intensity of Cy5-labelled SA was estimated for different concentrations per micro-well ($n = 3$). The average coupling efficiency of $n = 7 \times 20$ micro-wells plotted into the graph revealed 0.2 ng/ μl SA per micro-well (MW approx. 40,000).

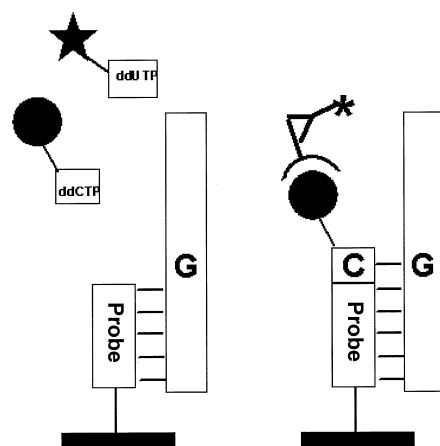


Fig. 3. On-chip C/T minisequencing assay. Immobilized oligonucleotides (probes) were subjected to target-dependent primer extension with dideoxynucleotides (ddCTP labeled with aminodigoxigenin and thymidine analogous ddUTP labeled with fluorescein) and thermostable DNA polymerase (thermoSequenase). Elongation is specific and complementary to the selected downstream single nucleotide polymorphism. After hybridization and enzymatic extension, only the digoxigenin (marked as: ●)-modified cytosine (C), when coupled to its complementary target sequence (guanine, G), can be detected by fluorescence (marked as: *)-labeled digoxigenin antibody amplification system.

thymidine (T) residues in the polymorphic sites were clearly detected on-chip with a minisequencing approach. The strategy of this study is shown in Fig. 3. The genomic DNA of three cattle (SB26, SB37, and SB45) was amplified and tested for nine polymorphic sites. The results of the minisequencing assay in this experiment are shown in Fig. 4. Values of empty micro-wells, which revealed lowest fluorescent levels, were chosen to plot the baseline. Four spots of negative control with an immobilized oligonucleotide of bacterial sequence produced a slight background. The average values of fluorescence intensity were plotted beside the results for better interpretation of positive minisequencing signals. Additional oligonucleotides on the chips were hybridized with a synthetic complementary single strand DNA and served as positive controls in the assay. The resulting nucleotide sequences of the polymorphic sites were detected as either homozygous for C or T or heterozygous for both nucleotides (C/T) and may be served as molecular barcodes in an individual-specific manner.

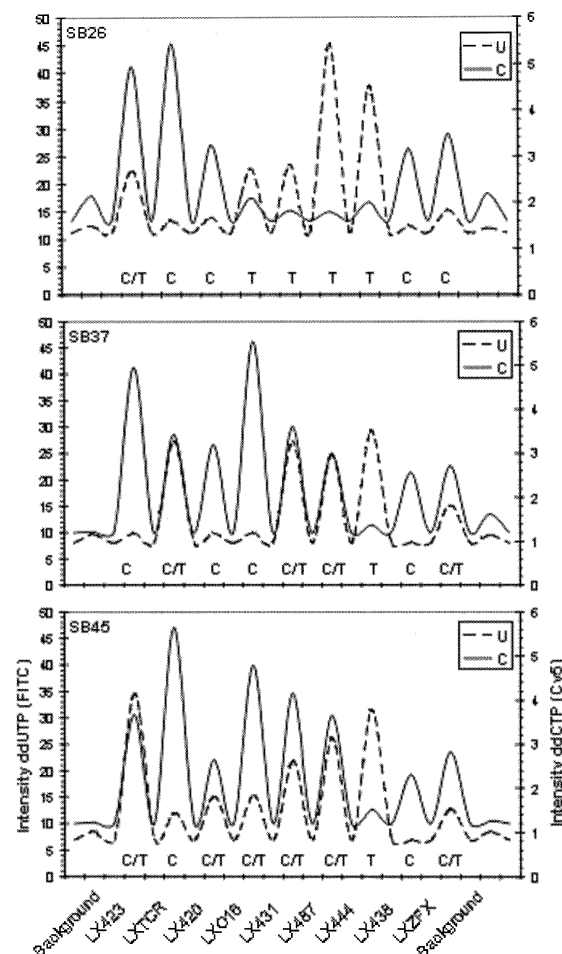


Fig. 4. Individual-specific molecular barcode of cattle. Nine different polymorphic sites (LX) of three cattle (SB26, SB37, and SB45) were screened through cytosine (C)/thymidine (T) minisequencing ($n = 2$ micro-wells). Values of empty micro-wells, which revealed lowest fluorescent levels, were chosen to plot the baseline. The average values of fluorescence intensity were plotted beside the results for better interpretation of positive minisequencing signals. The nucleotide sequences of the polymorphic sites were detected as either homozygous for C or T or heterozygous for both nucleotides (C/T) and served as molecular barcodes in an individual-specific manner.

A preliminary database was setup by modification of HGBASE (Brookes *et al.*, 2000) for storage and access to human SNPs. The resulting reference database SNPZoo provided a basis for standardized sets of SNPs for parentage control, individual identification, genetic diversity analyses and the relationship to sequences that are potentially use-

ful in candidate gene analyses (Durstewitz *et al.*, 2000). Internet information is accessible over URL <http://www.snpzoo.de>.

Discussion

One of the most important applications in biochips technologies currently is the genomic DNA analysis of single nucleotide polymorphism, because detection of individual-specific differences is of importance for the development of pharmacogenomics and medical diagnostics (Ahrendt *et al.*, 1999; Baron *et al.*, 1996; Conner *et al.*, 1983; Hacia *et al.*, 1996; Livak and Hainer, 1994; Lopez-Crapez *et al.*, 2001; Maskos and Southern, 1993; Nikiforov *et al.*, 1994; Syvänen, 1994), even in population survey (Raitio *et al.*, 2001). Moreover, it can serve as individual molecular barcode for unequivocal identification of cattle (Barendse *et al.*, 2001; Fries *et al.*, 2001; Heaton *et al.*, 2001; Konfortov *et al.*, 1999; Sauer *et al.*, 2000). The problem of slow emerging diseases such as BSE has raised the interest to fulfill a legal requirement for secure, individual identification of cattle provenance. Increasing demands of consumer protection will enclose further livestock species in future. Therefore, a strategy was developed in this study so that a molecular barcode could qualitatively be estimated even from a sample of a newborn animal collected simultaneously with the first ear-marking event.

Minisequencing on biochips was proven to be one of the suitable and efficient tools to monitor a number of SNPs. The genotypes were identified previously through DNA sequencing and have been confirmed by oligonucleotide ligation assays (Baron *et al.*, 1996). In this study, the sequenced probes on chips are immediately upstream from the polymorphic site. When incubated in the presence of dideoxynucleotide triphosphates labeled with various moieties (FITC or aminodigoxigenin), the allele-specific moiety-labeled dideoxynucleotide triphosphate is linked to the probe in the presence of DNA polymerase and target DNA. The genotype of the target DNA molecule can be detected by the corresponding antibody amplification systems and different variations can be analyzed in a single array. Like any other biochip applications, it is essential to amplify the genomic DNA of interest before the analysis.

However, this methodology has some advantages. Firstly, it is not necessary to modify primers for PCR amplification of targets, which is much more facilitated to design and synthesize the oligonucleotides. And the DNA targets do not be modified with haptens or labeled with fluorophores, which is a reliable and cost-effective approach and simplify the target preparation for high throughput applications. Secondly, the real amount for each target per extension is less, which will lead to a minimal fluorescence background. Thirdly, using different moiety modifications and fluorescence detection systems, it is reliable to screen all four base variants simultaneously. The specificity of this assay originates from the specific binding of the moiety-incorporated dideoxynucleotide triphosphate to the complementary target sequence. After enzymatic extension, only the moiety modified dideoxynucleotide triphosphate, when coupled to its complementary target sequence, could be detected by the corresponding antibody to the moiety in a specific and sensitive manner. With the signal amplifications, fluorescence intensity from each mutation can further be enlarged. Fourthly, all variable mutations can be detected at the same experiment, time-saving and conventionally. And finally, quantification of minisequencing results is less complicated, which is suitable for any routine readout softwares. However, problems such as weak primer extension or false positive elongation may occur occasionally, all sensor oligonucleotides of the biochips have to be confirmed empirically after selection of conventional SNPs.

Parallel sequencing of SNPs on a biochip provides the individual-specific molecular barcode. It may be stored as digital signature in an established reference database (SNPZoo). The primary data set also can be verified at any points during breeding, manipulation, even further transport and processing of beef and animal products containing genomic DNA. Three allele-specific possibilities (homozygous AA or BB and heterozygous AB) have to be extracted from various signal intensities for each SNP. Reliable processing of data within a high dynamic range above two orders of magnitude is not necessary. A standardized individual-specific set of SNPs needs to be established and verified, similar to commercial primer sets for microsatellite analysis (Applied Biosystems). It was calculated in this study that 40 SNPs yield in-

dividual specific DNA signatures. The probability of identity will be 10^{-15} if the frequency of the minor allele is > 0.3 . A low-density oligonucleotide biochip is sufficient enough for the application. The custom-design biotinylated oligonucleotides can be obtained through large-scale chemical synthesis. This is a notable advantage because large quantities of sensor molecules have been available for high throughput applications in human disease (Warrington *et al.*, 2002). This methodology will be

further extended into genotyping diagnosis of other human and animal diseases.

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