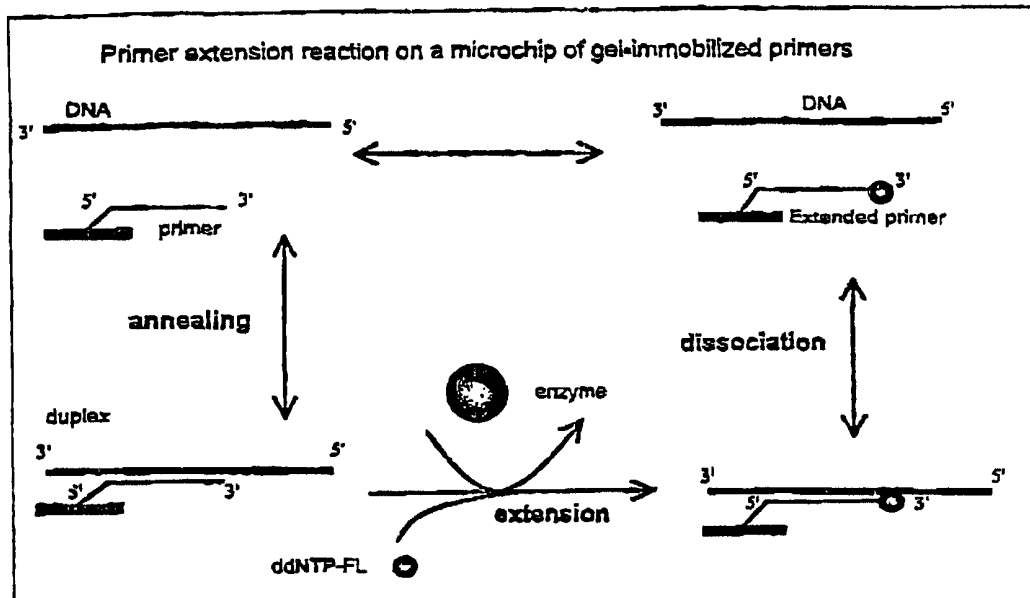




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12Q 1/68</b>	<b>A2</b>	(11) International Publication Number: <b>WO 00/65098</b> (43) International Publication Date: 2 November 2000 (02.11.00)
<p>(21) International Application Number: PCT/US00/11286</p> <p>(22) International Filing Date: 25 April 2000 (25.04.00)</p> <p>(30) Priority Data: 09/300,675 27 April 1999 (27.04.99) US</p> <p>(71) Applicant (for all designated States except US): UNIVERSITY OF CHICAGO [US/US]; 5801 S. Ellis Avenue, Chicago, IL 60637 (US).</p> <p>(72) Inventors; and (75) Inventors/Applicants (for US only): DUBILEY, Svetlana [RU/RU]; Vavilov Str., 32, Moscow, 117 984 (RU). KIRILLOV, Eugene [RU/RU]; Vavilov Str., 32, Moscow, 117984 (RU). MIRZABEKOV, Andrei [US/US]; 1005 Village Court, Darien, IL 60516 (US).</p> <p>(74) Agents: MARTIN, Alice, O.; Barnes &amp; Thornburg, 2600 Chase Plaza, 10 S. LaSalle Street, Chicago, IL 60603 (US) et al.</p>	<p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> Without international search report and to be republished upon receipt of that report.</p>	

(54) Title: NUCLEOTIDE EXTENSION ON A MICROARRAY OF GEL-IMMOBILIZED PRIMERS



(57) Abstract

Methods and compositions have been developed for nucleotide extension of primers immobilized within gel pads on a microchip using multibase primers or multiple sets of primers, or combinations thereof. Molecules or parts of molecules are identified. The single base extension was amplified by carrying out the reaction under elevated temperature.

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**NUCLEOTIDE EXTENSION ON  
A MICROARRAY OF  
GEL-IMMOBILIZED PRIMERS**

5

**INVENTORS: SVETLANA DUBILEY, EUGENE KIRILLOV,  
AND ANDREI MIRZABEKOV**

This work was supported in part by contract 9433524-3 with the Argonne  
10 National Laboratory (USA), by the Defense Advanced Research Project Agency under  
interagency agreement AO-E428, by the Russian Human Genome Program under grant  
123/97, and by the Russian Foundation for Fundamental Research under grant 96-04-  
49858.

15

**BACKGROUND OF THE INVENTION**

Methods and compositions are presented to detect and analyze molecules by  
nucleotide extension using various types of gel immobilized primers on a microchip.

Increasing amounts of genetic sequencing data have revealed a large number of  
nucleotide polymorphic sites and mutations in the human genome and in the genomes of  
20 other organisms. Polymorphic (variable) sites and mutations (changes) refer to a change  
in the identity of at least one nitrogen base in a nucleotide sequence as compared to some  
standard sequence. Analysis of polymorphism data has a wide application for detection  
of genetic diseases and genetic variations for population studies, for mapping of genes,  
in forensic studies, and for identification of pathogenic microorganisms. There is an  
25 urgent need for fast, inexpensive, and reliable methods to perform such analyses on a  
large scale.

A frequent application of sequencing is detection of single base changes. There  
are numerous approaches to identifying single-nucleotide mutations in a nucleic acid  
sequence, *e.g.*, single nucleotide polymorphisms (SNPs) in DNA. Methods such as  
30 restriction fragment length polymorphism (RFLP) analyses, and single-strand or double-

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strand conformational polymorphism analysis, are conventional methods used to detect such polymorphisms.

Two other methods to detect single base differences between nucleotide sequences are allele-specific hybridization of DNA molecules using microchips containing oligonucleotides, and single-base extension of a primer hybridized to a DNA molecule to be sequenced. More than single base differences can also be detected, for example, simultaneous screening for a large number of polymorphic sites, that is, changes at several positions in a nucleotide sequence or many changes at one position was performed by parallel hybridization of a DNA sample with many oligonucleotides immobilized on a microchip, where each oligonucleotide has a different capability to hybridize to certain sequences. However, the accuracy of this type of assay needs to be enhanced because often a large number of controls are needed to minimize error, and signals are often too weak to provide discrimination between matched and mismatched sequences.

In the single base extension method of primer extension, a primer is hybridized to DNA and then extended with DNA polymerase by one nucleotide with an appropriate dideoxynucleoside triphosphate, that is, one that matches the nucleotide at the (polymorphic) variable site. Separation of different extended primers according to their size by gel electrophoresis or MALDI (matrix-assisted laser desorption/ionization) mass spectrometry has also been suggested for primer extension.

There is no suggestion in previous publications to combine the two techniques, that is, to perform a single base extension method of sequencing on a gel microchip. In fact, difficulties would have been expected because resulting hybridization signals were expected to be too weak for accurate detection even in small gel elements. That is why PCR (amplification) of the target was generally required before hybridization.

### SUMMARY OF THE INVENTION

This invention relates to methods and compositions for detecting and analyzing molecules using various types of primers immobilized in gel pads in a microchip.

Immobilized oligonucleotides are used as primers for PCR amplification as well as for specific extensions by addition of one of several nucleotides. Two immobilized primers in a gel pad may inhibit the reaction due to steric hindrance. Therefore, one of

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the two primers can be released before the reaction or only one primer can be immobilized.

Detection of small amounts of nucleic acids hybridized to immobilized oligonucleotides can be difficult. In the past, the nucleic acid to be hybridized was labeled with a reporter molecule. The intensity of the hybridization signal was limited by the quantity of hybridized nucleic acids. In this invention, this drawback is overcome by amplification of nucleic acids hybridized to immobilized primers or/and enzymatic addition of the label to the primers, which are involved in specific hybridization. An elevated temperature makes it possible to have multiple hybridization events for each target molecule of nucleic acid. In addition, the enzymatic reaction can then take advantage of these multiple events, by modifying those oligonucleotides on which specific hybridization has taken place. In this way, hybridization signals are amplified by, for example, PCR with immobilized primers or by the single base primer extension assay.

A method for determining the identity of a nitrogen base in a nucleic acid molecule from a sample has been developed. The method includes:

- (a) obtaining a microchip with primers immobilized in gel pads. The primers are complementary to the nitrogen base sequence in the nucleic acid molecule that is adjacent to the nitrogen base whose identity is to be determined;
- (b) having labeled dideoxynucleoside triphosphates available for a reaction between the primers in the gel and the nucleic acids in the sample;
- (c) reacting the microchip with the sample to extend the primers; and
- (d) identifying the nitrogen base by determining the identity of the labeled dideoxynucleoside triphosphate that is incorporated into the primer by primer extension.

Using a different primer method to identify a nitrogen base in a nucleic acid molecule from a sample, steps include:

- (a) obtaining a microchip comprising a plurality of primers containing different 3' terminal nucleotides immobilized in gel pads; several primers are selected to overlap the nitrogen base when the primers are hybridized to the molecule;

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- (b) reacting the microchip with the sample to extend the primers; and
- (c) identifying the nitrogen base by determining the identity of the primer that is extended.

5 The primers include multibase primers or a multiprimer set. Suitable primers are about 10 to about 50 nucleotides in length. The concentration of primers within a gel element is from about 100 pM to about 1000 pM. The primers may be synthetic nucleic acids.

The invention also includes a method for detection of a small quantity of a nucleic acid target by single base primer extension on a gel microchip. The method includes the  
10 following steps:

- (a) obtaining a microchip containing immobilized primers for the nucleic acid target;
- (b) introducing a label into the 3' terminus of those primers which are complementary to the nucleic acid target by incubation of the microchip with a  
15 plurality of target nucleic acid fragments, a DNA- or RNA-dependent polymerase and a plurality of dideoxynucleotide triphosphates conjugated with any detectable label; and
- (c) detecting the label coupled to the immobilized primers from which presence of the target is inferred.

20 This method is useful for detection in biological samples of specific nucleic acids of interest, for which at least a partial sequence is known; gene expression studies, detection of genes (see paragraph "toxin gene detection"), pathogenic microorganisms, viruses, and so forth.

25 Target nucleic acids may be single or double stranded. The target nucleic acid may be RNA or DNA. Other types of molecules -- for example, proteins such as antibodies or enzymes -- may also be targets. The nucleic acid target may be a plurality of nucleic acids that differ in one nucleotide from each other and are represented in different proportions from each other.

30 Suitable dideoxynucleotide triphosphates conjugated with the label are a plurality of ddNTPs selected from A, T, C, G triphosphates and universal substitutes. The label is different for each triphosphate.

The target nucleic acid can include a plurality of RNA fragments, total RNA, fractionated mRNA, ribosomal RNA, low molecular weight RNA, as well as RNA transcripts of DNA.

5 The primer extension reaction is carried out at a constant temperature. In an embodiment of the invention, the reaction is carried out first at a lower temperature to allow the target nucleic acid to hybridize with a primer and enzyme to extend the immobilized primer, then the temperature is raised to achieve denaturing conditions, and then the temperature is lowered to allow another round of hybridization to occur. Elevated temperature is needed for amplification of single base extensions.

10 The method of the present invention for detection of a nucleic acid target by a polymerase reaction on a gel microchip includes the steps of:

- (a) preparing a microchip of independently 5' immobilized primers;
- (b) extending the primers which are complementary to target nucleic acids by exposing the microchip to a mixture of target nucleic acid fragments, enzymes and deoxynucleotide triphosphates; and
- 15 (c) detecting the extended primers from which presence of the target is inferred.

In another embodiment, wherein the molecule being detected and analyzed is not a nucleic acid, e.g. is an antibody, (a) a specific target nucleic acid is coupled to the molecule, and (b) the nucleic acid is identified in accord with methods of the invention.

20

Another aspect of the invention is gel elements of a microchip that have primers immobilized in the gel pads.

The invention is useful for detecting rare target DNAs/RNAs, quantifying DNAs/RNAs, making nucleotide-tagged target molecules, using nucleotide-tagged molecules for identification of non-nucleotide molecules, making releasable nucleotide tags, and using releasable nucleotide tags for identification of other molecules. Rare molecules are concentrated on the microchips to enhance sensitivity of detection. All variable nucleotides in a sample may be identified in one reaction.

25

An aspect of the invention is a method to perform minisequencing that allows the sequencing of a short run of bases in a DNA or RNA polymer, providing not only single nucleotide sequencing. A series of single nucleotide sequencing steps may be combined to achieve sequencing of more than one base.

30

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagrammatic representation of a primer extension reaction on a microchip of gel-immobilized primers.

FIGS. 2a and 2b present DNA sequences and primers used for embodiments of the present invention; for FIG. 2a, the oligonucleotide, D50ntN, was synthesized DNA, and the microchip-immobilized multibase primer is designated as "PN"; multiprimer sets are primers containing one of the four bases A, G, T, or C at the polymorphic site, N with the length of sequences (nt) incorporated into primer names; in FIG. 2b, the DNA gene sequence is represented as the upper, bold sequence; the multibase primers are those designated with names that end in an Arabic numeral; multiprimer sets are those designated with names that end with an "N".

FIG. 3 shows time dependence of the fluorescence signal amplification in the microchip multiprimer set method; the method was implemented at 66°C with the nucleotide designated D50ntG DNA (2 nM), matched primers P20ntC, P19ntC, P18ntC, P17ntC, and 3' terminally mismatched primers P20ntA, P20ntG, and P20ntT.

FIG. 4 shows the effect of DNA concentration on the reaction yield in a microchip multiprimer set using the single oligonucleotide extension method; the method was implemented with an oligonucleotide designated D50ntA as a target, and with a primer designated P25ntT and fluorescently labeled NTPs at 66°C for 6 h.

FIG. 5 illustrates detection of DNA sequences of toxin genes of *B. anthracis* by a multiprimer extension method; two PCR-amplified fragments of *lef* (0.3 pmol) and *pag* (0.3 pmol) toxin genes were applied on a microchip containing immobilized primers, and the method was implemented at 66°C for 2 h.

FIG. 6 shows the sequence of normal alleles and mutated alleles for regions of the human  $\beta$ -globin locus.

FIG. 7 illustrates detection of  $\beta$ -thalassemia mutations in PCR-amplified DNA by both the multibase and the multiprimer set method on microchips; sense chains containing A, G, T, or C, immobilized as primers were extended at the 3'-terminus with labeled ddNTPs at 66°C for 2h. The first column of graphs shows DNA samples analyzed by a multibase assay; the second column shows DNA analyzed by the multiprimer assay; test DNAs are from a heterozygous control having two normal alleles

and from two heterozygous patients having a mutation in each  $\beta$ -globin allele (codon CD6A+T and codon CD26A+G).

### DETAILED DESCRIPTION OF THE INVENTION

5 Novel methods are developed that incorporate primer extension methods on a genetic microchip with hybridization methods to detect target nucleic acids. Biological microchips are supported structures containing large numbers of small biological molecules; genetic microchips are those containing nucleic acids. Suitable primer extension methods include single nucleotide extension using multibase primers and  
10 single nucleotide extension using multiprimer sets. An aspect of the invention is to extend by more than a single nucleotide.

An amplification product may include additional target molecules, or target-like molecules, or molecules complementary to the target molecule. If the target is a nucleic acid, an amplification product can be made enzymatically with DNA or RNA  
15 polymerases or transcriptases. Polymerases and transcriptases are enzymes which, in the presence of appropriate reaction conditions, produce complementary copies of a strand of DNA or RNA. The strand that is copied is called the template DNA or RNA.

In the present invention, gel-immobilized oligonucleotides are used as suitable substrates for nucleic acid polymerases. Gel-immobilized oligonucleotides are useful for  
20 many applications, *e.g.* for minisequencing. For example, a single base extension method was performed for the first time on a microchip on which primers are immobilized in gel elements in an array. An advantage of using an array of immobilized primers is the opportunity to identify all the variable nucleotides in a DNA target in one reaction with greater precision.

25 The term "oligonucleotide" herein means any fragment of DNA or RNA including either or both synthetic and naturally occurring molecules. Generally an oligonucleotide is between 2 and 50 nucleotides in length; the term "polynucleotide" generally refers to larger chains. However, there are no strict definitions for such terms.

"Probe" is a strand of nucleic acid having a base sequence complementary to a  
30 target base sequence. The probe may be associated with a label to identify a target base sequence to which the probe hybridizes. "Primer" is a nucleic acid molecule having a nitrogen base sequence complementary to a target base sequence, which upon

hybridization generates an amplification reaction. These reactions usually involve enzymes called polymerases and transcriptases.

“Label” is generally a molecular agent which can be detected. “Labels” can be attached to other molecules such as primers or probes.

5           Single nucleotide polymorphism (SNP) analysis by primer extension on biological (genetic) microchips containing gel-immobilized oligonucleotides is an aspect of the invention. A fluorescent signal on the immobilized primers was amplified by carrying out isothermal DNA polymerase reaction at an elevated temperature. Amplification of hybridization signals of more than 10X are aspects of the invention.  
10       Reactions on microchips with each of four fluorescently labeled ddNTPs (dideoxynucleoside triphosphates i.e., dideoxyadenosine (-cytidine, -guanosine, -uridine) triphosphates were used to identify any base in a polymorphic site using one primer.

          An alternative procedure was developed for a microchip containing four immobilized primers. The primers differ in the nucleotide at the 3'-end of the primer that  
15       matches a polymorphic site. This is an example of the multiprimer set composition.

          A target nucleic acid includes single stranded DNA, double stranded DNA, or RNA. An immobilized primer may be any synthetic or native DNA (oligonucleotides, or PCR fragments, or RNA, and so forth), which has an open 3' end (i.e., extendable by DNA polymerase). Each nucleic acid is linked by a phosphodiester bridge between the  
20       5' hydroxyl group of the sugar of one nucleotide and the 3' hydroxyl group of the sugar of an adjacent nucleotide. Each linear strand of naturally occurring DNA or RNA has one terminal end having a free 5' hydroxyl group and another terminal end having a 3' hydroxyl group. The terminal ends of polynucleotides are often referred to as being five  
25       prime (5') termini or three prime (3') termini in reference to the respective free hydroxyl group. Complementary strands of DNA and RNA form antiparallel complexes in which the 3' terminal end of one strand is oriented to the 5' terminal end of the opposing strand. ddNTPs may be conjugated with any detectable label, or any molecule which could be converted to a label, or any combination of distinguishable label. Each type of ddNTPs could be conjugated with the same label, or with different labels.

30           Gel pads, provide an environment on a microchip that is improved over surfaces such as glass slides because a gel is a 3-dimensional structure that provides a microenvironment that is more like a solution, whereas glass is essentially flat (2-D).

Oligonucleotides immobilized within polyacrylamide gel pads of a microchip are also accessible substrates for enzymes such as T4 polynucleotide kinase and T4 DNA ligase. Similarly, gel supports for primer immobilization provide a more homogeneous environment for DNA polymerases (MW~90kDa) than the solid-phase, glass-immobilized oligonucleotides suggested for primer extension by Pastinen *et al.* (1998).  
5

The three-dimensional structure of the gel allows achieving high local concentrations of immobilized primers (many copies of a primer within a single gel element) within a small volume and is characterized by significantly higher capacity for immobilization than two-dimensional glass supports. The higher concentration of the primer in each gel element results in increases in the local concentration of DNA  
10 fragments complementary to the primer during hybridization and does not affect the concentration of DNAs that do not match the primer DNAs. This phenomenon reinforces the specificity of the method and the primer extension rate dynamics. Accumulation of matching fragments within the gel is particularly important when double-stranded DNA is used in the analysis. Under the same experimental conditions, single-stranded DNA  
15 (ssDNA) produces a fluorescent signal about 5 times higher than double-stranded DNA (dsDNA). However, the use of dsDNA significantly simplifies sample preparation and may provide an additional control to increase the reliability of testing. Two primers complementary to both strands of DNA can be selected for the assay to test the same polymorphic site. That is, both sense and antisense mutations strands of DNA can be  
20 detected. In addition, not only double-stranded DNA, but fragmented DNA (chemically or enzymatically) has a nonextendable 3'-end, which rules out unwanted concurrent extension of the double stranded DNA.

Two types of primers, multibase and multiprimer sets, have been developed for  
25 primer extension. The method of single-nucleotide extension of primers immobilized within polyacrylamide gel pads on a microchip uses multibase primers. In this method the 3' end of the primer is positioned next to a polymorphic nucleotide in a sample of DNA to be tested. For the test sample, the position of the polymorphism is known because of the question to be answered, but not by its identity. Generally, the DNA  
30 molecule to the sample to be tested (fragment) is longer than the primers in the microchip, that is, the fragment has more nucleotides in its sequence than the primers. The nucleotide is then identified by the specificity with which the primer incorporates

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fluorescently labeled dideoxyribonucleoside triphosphates after a polymerase extends the end. In the multiprimer set method, several primers containing different 3'-terminal nucleotides and overlapping the variable nucleotide in the sample DNA are used. These primers were designed to be complementary to the target sequence, which is located  
5 downstream of the variable nucleotide. The 3' terminus nucleotide was next to the variable nucleotide (in a multibase assay) or overlapped the variable nucleotide in the multiprimer assay results shown herein (e.g., see FIGS. 2a and 2b). The polymorphic nucleotide was identified according to which primer was extended or a fluorescent label was used to determine which primer hybridized to the sample DNA. A comparison was  
10 made of the two methods for utility in the diagnosis of  $\beta$ -thalassemia mutations. For the comparison, an isothermal amplification of the fluorescent signal was achieved by performing both methods at elevated temperatures, that is, above the melting temperature for a perfectly matched nucleic acid duplex. Matched duplexes with different sequences and different lengths will have different melting temperatures. "Suitable" temperatures  
15 are above the melting temperatures ( $T_m$ ) of the primers. Small amounts of nucleic acids or low-copy genes were identified by isothermal single-base extension.

Signal amplification occurs because, at elevated temperature, the immobilized oligonucleotide-DNA or -RNA duplex will dissociate, the DNA/RNA strand will rehybridize to new unlabeled primer, with incorporation of fluorescent label at the new  
20 hybridization site.

In this way, hybridization followed by single-base extension of the immobilized oligonucleotide generates fluorescent signals that continuously amplify with time on each gel pad where hybridization has taken place.

A penultimate position is the design for the multibase primers. In FIG. 2a, the  
25 "PN" primer is an example of a multibase primer; in FIG. 2b, pCD6, pCD18, pCD26, pIVSI/1, pIVSI/2, pIVSI/5, and pVISI/6 are multibase primers. The last position in these primers (adjacent to, or 3' of the position of the mutation in the gene sequence) will match either the normal or the mutant allele. For example, if a normal allele had a sequence 5'-TTCGCGGGG-3' and the mutant allele was 5'-TTCCCGGGG-3', a primer  
30 with the sequence 3'-GCCCC-5'-gel would be a multibase primer. The normal allele would anneal and add a labeled-ddCTP; the mutant allele would anneal and add a labeled-ddGTP. The two ddNTPs would need to be labeled with different labels; for any

one nucleotide difference, both normal and mutant test DNAs would anneal to the same location on the chip, but the label for the ddCTP would be a different color from the color of the ddGTP.

An ultimate (variable) position is for the multiprimers. In FIG. 2a primers  
5 P25ntN, P20ntN, P19ntN, P18ntN, and P17ntN are examples of multiprimers; a  
multiprimer set would include four primers, each with an A, T, G, or C and the 3' end;  
primers as depicted in FIG. 2b with name designations that end in "N" are the  
multiprimer set types. FIGS. 2a and 2b illustrate that the position in the primer that is  
complementary to the mutant allele versus the complement to the normal allele is varied.

10 A primer that is complementary to the mutant allele is not complementary to the  
normal allele (and vice-versa). For example, if a normal allele had a sequence  
5'-TTCGCGGGG-3' and the mutant allele was 5'-TTC CCGGGG-3', one of the  
multiprimers in the multiprimer set would have the sequence 3'-CGCCCC-5'-gel and  
another one of the multiprimers in the multiprimer set would have the sequence  
15 3'-GGCCCC-5'-gel. The mutant allele would anneal to the 3'-GGCCCC-5'-gel primer  
and add a labeled ddGTP. The normal allele would not anneal to that primer. The  
normal allele would anneal to the 3'-CGCCCC-5'-gel and add a labeled ddGTP; but the  
mutant allele would not anneal to that primer. There would be fluorescence at a different  
location on the chip.

20 It was unexpected that primer extension at or near the  $T_m$  would be successful,  
because the single ddNTP that matched the mutated or polymorphic site was not expected  
to stay associated with the test nucleic acid molecule at this high temperature for a long  
enough period of time for an extension reaction to be completed. However, incorporation  
of labeled ddNTPs did occur, and, moreover, this transient association allowed the test  
25 DNA to hybridize with a greater number of immobilized primers, which led to a greater  
number of immobilized primers that were labeled by primer extension. In this way, the  
signal became amplified over the level of signal that was expected.

The  $T_m$  for any particular primer and gene duplex can be calculated using any of  
a variety of readily available computer applications, or the  $T_m$  can be determined  
30 experimentally. For example, experiments can be performed where the amount of  
hybridization between a primer and a fluorescently-labeled test DNA sequence can be  
measured over a temperature range, by observation of the fluorescence signal intensity.

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As indicated, there are two aspects of the method of the present invention to identify a variable base in a target DNA. The variable base is complementary to either a newly incorporated, labeled nucleotide, or to the nucleotide that is adjacent to the incorporated nucleotide in the primer. Depending on whether the variable nucleotide is included or not into the 3' end of the primer (regardless of the position of the variable nucleotide in the DNA target), after the reaction is completed, the extended primer contains the variable nucleotide, whether it is in the 3' ultimate position or in the 3' penultimate position.

In the multibase assay, the 3'-terminus of the primer is located next to the variable base N, and DNA polymerase incorporates one of the four different labeled ddNTP which is complementary to the base, whereas the mismatched ddNTPs fail to be incorporated. To identify the variable base, four ddNTPs are tested in separate reactions, or each ddNTP is associated with a distinct reporter molecule or label.

In the multiprimer set method, the 3'-terminal base of the primer corresponds to the variable base. In this aspect of the invention, four primers that each are variable at the 3'-nucleotide and are preferably used to test all four possible bases in the polymorphic site of target DNA. The polymerase recognizes only the primer that matches this base in its 3'-terminal nucleotide and incorporates the labeled ddNTP adjacent to it. The polymerase does not react with the primers that form mismatched base pairs in their 3'-terminal positions.

The relative efficiency of the various types of primers suitable for practice of the invention depends on how reliably the DNA polymerase discriminates a match against a mismatch either in the terminal position of the nucleotide primers or in the incorporated ddNTPs.

Nucleic acid hybridization assays detect the tendency of pairs of nucleic acid strands to pair with greatest stability if they contain regions of complementary sequence. Each pair of complementary nucleotides, between two strands, increases the stability of pairing between a biological binding pair formed between the two nucleic acids. DNA segments isolated from a growing organism are generally duplex DNA, a pair of perfectly complementary strands whose pairing is very stable. The term "hybridize" refers to imposing conditions which promote such pairing. The term "denature" refers to

imposing conditions which discourage such pairing. The conditions are imposed by adjusting ionic strength, pH or temperature.

Hybridization of DNA with an immobilized primer and primer extension were carried out in one step at an elevated temperature in the presence of thermostable DNA  
5 polymerase and fluorescently labeled dideoxynucleotide triphosphates. By terminating chain elongation with such triphosphates, the incorporation of only one base was ensured and, therefore, the appearance of a fluorescent signal was from only such gel pads wherein the correct hybridization had taken place.

Isothermal amplification was carried out at a temperature above the melting  
10 temperature of a matched nucleic acid duplex. Melting temperature is defined as that temperature at which quantity of duplexes is 50% of maximal possible quantity of duplexes at given conditions (salt concentration and concentration of both strands.) The temperature is determined empirically or theoretically with known matches. A DNA strand to be sequenced will temporarily anneal and provide a template for single base  
15 extension of the complementary primers. As the process of annealing and denaturing recurs, a plurality of primers in a gel element -- that is, containing multiple copies of the same primer -- has its signal enhanced.

Initial amplification was performed with ThermoSequenase<sup>®</sup> at temperatures above the melting points ( $T_m$ ) of the duplexes of all primers to be used. To achieve  
20 amplification of the fluorescent signal, the methods of the present invention were carried out at a temperature slightly above the melting temperature of a perfectly matched duplex formed by DNA and the immobilized primer. Under these conditions, the target DNA undergoes a rapid turnover between the duplex and its dissociated state. The same DNA molecule successively interacts with different primer molecules, producing duplexes  
25 many times. The lifetimes of at least some of these duplexes are sufficiently long for the enzyme to extend the primer. Thus, in theory, a single DNA molecule would suffice for extending all available primers.

The rate of amplification proceeded linearly in time, and the rate was higher for longer and more stable oligonucleotides (FIG. 3). The power of discrimination between  
30 perfect matched versus mismatched oligonucleotides also increased with time. A direct correlation between the fluorescent signal dependence on the target DNA concentration was observed (FIG. 4). With this method it is possible to carry out comparative

quantitative DNA analysis on the microchip within a wide range of target concentrations. Table 1 shows the fidelity of ThermoSequenase® in a multibase primer extension method tested for 16 combinations of four DNAs (D50ntA, D50ntG, D50ntT, D50ntC) with four fluorescently labeled ddNTPs (ddATP, ddGTP, ddUTP, ddCTP) and immobilized  
5 (P25nt) primers (the sequence is represented in FIG. 2a). Discrimination of a mismatched base was at least 50 fold, and the method demonstrated high specificity when single-stranded DNA was used as compared to double stranded DNA. Specificity as used in this context means the probability of mistakes due to, *e.g.*, misincorporation of a nucleotide(s).

10 The term "melting temperature" is helpful to explain different experimental phenomena. Despite the "common sense" that in described experiments the optimal temperature for primer extension should be lower than the melting temperature of primers (because of the higher concentration of duplexes), for "short" primers (15-30 nt long) the optimal temperature was higher than melting temperature of said primers. For longer  
15 primers (*e.g.* 100 nt long) the optimal temperature would be lower than the melting temperature of the primers.

When a universal base (a base such as inosine that will base pair with any of the four naturally occurring bases) or a plurality of naturally occurring bases is present in an internal position of primers, the number of primers could be lower if the polymorphic  
20 sites are located close to each other. Mismatches near the 5' end do not considerably change the yield of the extension.

Multibase primers and multiprimer sets designed to have internal mismatches are compositions of the present invention. These primers with internal mismatches would add an additional accuracy parameter to distinguish an exact match from non-matching  
25 oligonucleotides to serve as an on-chip internal control (or could be used to detect both 3' variation and an internal variation). An example of a primer with internal mismatch sequence would be a P17ntN sequence (see FIG. 2a) with a sequence of Ngacttttcgtgactt 5'-gel, rather than Ngacttttccgtgactt 5'-gel.

30 Methods of the present invention are useful for detection of small amounts of the target (as low as  $10^6$  molecules per microliter or less, see FIG. 4). Methods are also useful for detection and measurement of relative amounts of target nucleic acid in a mixture of highly homologous nucleic acids (*e.g.*, that differ in one nucleotide)

represented in the mixture in different proportions from equal ratios (for heterozygous samples) to 1/10 and lower ratios. For example, in an analysis, which aims to detect *B. anthracis* in a sample from the soil, a confounding factor is that *B. thuringiensis* is a widespread microorganism in the soil. There are only few nucleotide differences in the 16S RNA of these microorganisms, that is, their 16S RNA genes are highly homologous. The most probable situation is that *B. thuringiensis* 16S RNA would be in large excess over *B. anthracis* 16S.

The methods of the present invention are also useful where a large set of different types of molecules of any nature is obtained and each type of molecule is conjugated separately with a specific DNA fragment. By identification of the specific DNA fragment the type of molecule conjugated to it is identified. In addition, the dNTP is a suitable alternative to ddNTP in a "multiprimer assay". In this case, it would not be single base extension, but rather a "multiple" base extension.

15

## EXAMPLES

The following examples illustrate embodiments of the invention:

### Example 1: Determination of Preferred Temperature for Signal Enhancement

Optimization experiments were carried out with synthetic 50-nt-long DNA fragments of the *B. anthracis* *lef* toxin gene in which a polymorphic (variable) site (N) had been introduced. A set of primers was derived from a 17-nt-long oligonucleotide by elongating its 5'-end; all primers except P25nt contained one of the four possible nucleotides (A, T, C or G) at the 3'-terminal position. (FIG. 2a).

Different amounts (from 0.2 to 1 pmol) of primers were immobilized per 100 x 100 x 20  $\mu\text{m}^3$  gel pad to determine the optimum primer concentration. No significant rise in the fluorescent signal was observed for immobilized primer concentrations above 0.6 pmol per pad. The best discrimination of 3' mismatches was attained for this amount of primers within a wide range of target DNA concentrations, and therefore was used in subsequent experiments.

The melting temperature ( $T_m$ ) of primers calculated by using different formulas (Mitsubishi 1996 a, b; Fotin *et al.* 1998) did not correlate well with the experimental values. In addition, as shown earlier,  $T_m$  was different for duplexes formed on a

30

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microchip versus duplexes formed in solution. Microchip values are not predictable from other empirical temperatures.  $T_m$  values, which are determined for perfectly matched duplexes of target DNA (1  $\mu$ M D50ntN) hybridized with microchip immobilized primers of different lengths  $T_m$  for duplexes formed on a microchip was determined according to

5 Fotin *et al.* 1998, were within the range of 48-53°C. Adding Perfect Match PCR Enhancer (Stratagene) did not considerably affect the melting temperature for perfect duplexes.

$T_m$  was measured in real time on an automatic experimental setup consisting of a two-wave-length fluorescent microscope, CCD-camera, Peltier thermostable,

10 temperature controller and a computer equipped with a data acquisition board. Special software was designed for experimental control and data processing, which used a LabVIEW virtual instrument interface (National Instruments, Austin, Texas). Equilibrium melting curves were registered at increasing temperature at a rate from 1°C/h at low temperatures (+4°C) to 1°C/2 min. at high temperatures (+60°C).

15 The highest yield of the extension reaction on the microchip was found at  $66 \pm 2^\circ\text{C}$  for all tested primers. This range is above the  $T_m$  but below the optimum temperature for ThermoSequenase® (which is about 74°C).

### Example 2: Effect of Extension Time on Intensity of Signal

20 FIG. 3 shows the rise of the fluorescent signal with time during the extension reaction with 2 nM D50ntG DNA and terminally matched (P20ntC, P19ntC, P18ntC, P17ntC) primers of different lengths and mismatched (P20ntA, P20ntG, and P20ntT) primers. After a 60 min lag, the slope of the fluorescent signal became linear for all primers. The velocity of signal accumulation was higher for longer primers. The

25 amplification curves for the mismatched primers were lower, changing little with time, and were similar for the G-A, G-T, and G-G terminal mismatches. Therefore, the discrimination of the matched duplex from the mismatched ones - the ratio of their fluorescent signals, increased with, for example, up to 22 to 25-fold in 120 min. for the 20-mer primer. The discrimination was about the same for all tested mismatches.

30

**Example 3: Effect of Concentration of DNA Targets on Signals**

FIG. 4 shows the direct correlation between fluorescent signal and concentration of target was observed in a wide range of target concentration (three orders of magnitude).

5 Inorganic pyrophosphatase in the reaction mixture prevents removal of extended 3' terminal dideoxymononucleotides. Misincorporation of ddNTPs with ThermoSequenase<sup>®</sup> in a microchip assay was in most cases much less than 2% in the model experiments with ssDNA (Table 1) and less than 10-20% of the correct extension in the detection of  $\beta$ -thalassemia mutations (FIG. 7).

10

**Example 4: Toxin Gene Detection**

Oligonucleotide microchips were used previously for bacterial identification by means of oligonucleotide hybridization with variable regions of 16S ribosomal RNA. However, some closely related microorganisms, such as human and insect pathogens *B.*  
15 *anthracis* and *B. thuringiensis*, respectively, differ from one another by only a few bases in their 16S ribosomal RNA, and may be, therefore, difficult to discriminate by this technique. Primer extension amplification of toxin- or antibiotic-resistant genes can be used as an alternative procedure for identifying pathogenic microorganisms and for accomplishing what is currently difficult or impossible.

20 Double-stranded PCR fragments of *B. anthracis* plasmid pUX1-borne protective antigen, *pag* (positions 1933-2179, GeneBank accession number M22589) and lethal factor, *lef* (positions 1153-1256, GeneBank accession number M30210) were used in a multiprimer extension assay. The microchip contained *lef* gene-specific primers (fully matched P20ntT and terminally mismatched P20ntC as a control, see FIG. 1) and a *pag*  
25 gene-specific primer, PAG22nt, (5' - AGAACTAGGAATAGATAAATCCCCT-3'). 0.3 pmol of the 246-bp-long DNA of *pag* gene were chemically fragmented. 0.3 pmol of the 103-bp-long DNA of the *lef* gene was used without fragmentation. FIG. 4 shows the results of the microchip extension assay carried out for these two DNAs. Positive fluorescence signals demonstrated that DNA in such amounts can be specifically  
30 identified by the assay.

In a control experiment, 0.3 pmol of a synthetic fragment of the *lef* gene DNA were conjugated with a Texas Red fluorescent label and hybridized with the microchip.

Only an insignificant fluorescence signal was detected in the P20ntT gel pad. At least a 10-fold amplification of the hybridization signal was achieved by the extension assay.

### 5 **Example 5: Use of Multibase and Multiprimer Primer Extension on Diagnosis of $\beta$ -Thalassemia**

Comparative studies of  $\beta$ -thalassemia mutations have revealed similar specificities of the multibase and multiprimer set single nucleotide extension methods.

The comparative usefulness of multibase and multiprimer set single nucleotide extension methods was tested for diagnosis of seven commonly occurring  $\beta$ -thalassemia  
10 mutations within the  $\beta$ -globin gene (FIG. 6). The mutations were codon substitutions in the first exon: CD6 A/T (A/T, substitution of A for T); CD18 T/C; CD26 G/A, T; and splice-site mutations in various positions of the first intron (IVS I) IVS I/1 G/A,T; IVS I/2 T/A, C,G; IVS I/5 G/A,T,C; and IVS I/6 T/C. FIG. 7 shows the results of genotyping of two patients.

15 For diagnosis of these mutations using a multibase method, microchips containing 7 site-specific primers were prepared. The primers (pCD6, pCD18, pCD26, pIVSI/1, pIVSI/2, pIVSI/5, pIVSI/6, see FIG. 6) were complementary to the sense chain of the  $\beta$ -globin gene and adjacent to the polymorphic sites. Four fluorescein-labeled ddNTPs were used on the four microchips in four extension assays.

20 For diagnosis of mutations in a multiprimer set method, the seven sets of primers (FIG. 2b) were immobilized on a microchip. Each set consisted of four site-specific primers differing in their 3'-end nucleotides, which overlapped the position of a possible mutation (pCD6N, pCD18N, pCD26N, pIVSI/1N, pIVSI/2N, pIVSI/5N, or pIVSI/6N, where N was A, G, C, or T, see FIG. 6). A mixture of all four fluoroscein-conjugated  
25 ddNTPs was used for the extension reaction. The results shown in FIG. 7 enable one to identify the mutations as homozygous at CD 6 A, CD 18 T, IVSI/2 T, IVS I/5 G, and IVS I/6 T; and heterozygous at CD 26 G+A and IVS I/1 G+A.

All identified mutations were confirmed by standard sequencing of the tested DNAs. (Sanger *et al.*, 1977) The effect of penultimate mismatches on the yield of  
30 primer extension was determined by a multiprimer assay. Detection of beta thalassemia mutations was performed by a multiprimer assay. Columns were sets of primers (for sequences see FIG. 6 and FIG. 2b); rows were the terminal nucleotides of primers. The

fluorescent pattern of normal DNA was determined and for DNA with a mutation in IVSI/2 (the fluorescent signal shifted from pIVS12A in normal DNA to pIVSI/2T). This mutation is homozygous (the only gel pad in column pIVSI/2 that fluoresces). The signal from pIVSI/1 primer set disappeared completely, since the duplexes that form this primer set with target DNA contain a penultimate mismatch (the sequence of target DNA complementary to the pIVSI/1 primer set is changed from 5'gTtggtatcaaggttacaagacaggt3' to 5'gAtggtacaaggttacaagacaggt3'). Therefore penultimate mismatches could be used equally with ultimate mismatches for determining the variable nucleotide. So, to analyze the possible mutation in IVSI/1 two primer sets are designed, one for DNA normal in position IVSI/2 (3'NAaccatagttccaatggttctgtcc5'), and another one – for DNA with mutation in position IVSI/2 (3'NTaccatagttccaatggttctgtcc5'). Introducing a universal base (*I*) or a mixture of natural bases (*M*) in considered position allows us to design the only primer set (3'NIaccatagttccaatggttctgtcc5') or (3'NMaccatagttccaatggttctgtcc5').

15 **Example 6: Use of Primer Extension on a Microchip to Detect a Specific Antibody**

An embodiment of the invention is to have an antigen (e.g. protein) and a library of many different antibodies. A procedure to elucidate which antibody from the library interacts with the said antigen is to immobilize all antibodies in separate vials and add the antigen to all of the vials. However, this approach needs a large quantity of the antigen and of the antibodies. Instead, using the present invention, the method is as follows:

1. coupling each antibody from the library with a distinguishable nucleic acid tag;
2. putting all antibodies and the said antigen into the one vial to allow antigen-antibody interactions;
3. removing all antibodies that do not interact with antigen;
4. preparing a microchip with immobilized primers that are complementary to the tags; and
5. determining which antibodies were associated with the antigen by detecting their nucleic acid tags by primer extension on a microchip.

## MATERIALS AND METHODS

### Oligonucleotide synthesis, microchip manufacturing, and fluorescent signal detection.

Oligonucleotides and short DNA fragments were synthesized on a 394  
5 DNA/RNA synthesizer (Applied Biosystems) by standard phosphoramidite chemistry. Primers to be immobilized on microchips contained 5'-amino group and were synthesized with C<sub>18</sub> linker (Glen Research) and Unilink aminomodifier (Clontech). The oligonucleotides containing trityl were purified by reverse-phase HPLC (Dinamax, Rainin Instrument Co. Inc.).

10 Microchips were manufactured as described by Guschin *et al.* (1997) and Timofeev *et al.* (1995). A polyacrylamide gel micromatrix was prepared by photopolymerization of 4% acrylamide (acrylamide:bisacrylamide, 19:1) for oligonucleotide and DNA immobilization and 3% acrylamide (acrylamide:diallyltartardiamide, 3:1) for protein immobilization, in 40% glycerol,  
15 0.002% methylene blue, 0.012% TEMED, and 0.1M sodium phosphate buffer, pH 7.0. The mixture was applied to an assembled polymerization chamber, which consisted on a quartz mask (100 x 100 x 1.4 mm) pretreated with Repel-Silane (LKB) and then with 0.01% Tween 20; two 20- $\mu$ m-thick 2.5 x 25-mm Teflon spacers; and a 75 x 25 x 1-mm glass microscope slide (Corning Micro Slides) pretreated with Bind-Silane (LKB) placed  
20 on the top of the spacers. The resulting glass chamber was fastened by two metal clamps. The internal side of the quartz mask has a nontransparent 1- $\mu$ m-thick chromium film prepared by photolithography. The assembled chamber, filled with acrylamide solution by capillary forces, was exposed to UV transilluminator (FisherScientific) or a 254-nm UV Stratalinker 1800 (Stratagene) from a distance of 1 in.

25 After polymerization, the chamber was disassembled. The micromatrix was washed with water to remove nonpolymerized acrylamide, dried, and kept at room temperature. Micromatrices having gel elements 25 x 25 x 20  $\mu$ m and larger were produced by this method.

An oligonucleotide solution was transferred to each gel element using a pin robot.  
30 The thermostabilized, gold-plated, glass fiber optic pin, 240  $\mu$ m in diameter, has a hydrophobic side surface and a hydrophilic upper surface. The pin temperature is kept close to dew point by a Peltier thermostated plate to avoid evaporation of the

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oligonucleotide solution. A minute volume of oligonucleotide solution. A minute volume of oligonucleotide solution is loaded on the pin, and then the solution from the pin is transferred by one or several loadings (about 1 nl per loading) to the gel pad.

Micromatrices of  $100 \times 100 \times 20 \mu^3$  of gel pads were prepared by  
5 photopolymerization of 5% polyacrylamide gel. Each primer was applied on a gel pad by a pin robot and immobilized through reductive coupling of the 5' amino group of the primer with the aldehyde group of the activated gel pad.

The fluorescent pattern was monitored on an epifluorescent microscope equipped with a CCD camera. The image of the microchip was displayed and analyzed on a PC  
10 using HybResP software (HybResP, Tool Kit for Hybridization Results Analysis, Argonne National Laboratories). However, analysis may be performed using any conventional image analysis software.

The following algorithm for image analysis was used to obtain fluorescent signals. The background originating from the absorbed dye-labeled ddNTPs was  
15 assumed to be uniform between the gel pads throughout the chip, and absorption of dye-labeled ddNTPs on the gel was assumed to be nearly uniform. Each gel pad is surrounded by two concentric square frames: an inner frame that totally covers the gel pad and an outer frame that is larger than the inner one, but does not overlap any surrounding gel pads. The fluorescent signal was averaged inside the inner frame (C) and  
20 in the space between the inner and outer frames (B, background). The fluorescent signal (J) from the gel-pad was obtained by subtracting the background from the inner frame signal and dividing the difference by the background to compensate for variations in intensity of the exciting light, that is,  $J=(C-B)/B$ . Under the above mentioned assumptions, the registered hybridization signal (J) differs from the "real" one (D):  
25 wherein  $D=J_S-J_G$  is the fluorescent signal obtained from the gel pad with immobilized primer, and  $J_G$  is the fluorescent signal from an empty gel pad.

#### **DNA preparation.**

A 421 bp long fragment of  $\beta$ -globin gene was prepared by PCR amplification  
30 from the genomic DNA of patients with different  $\beta$ -thalassemia mutations.

100  $\mu$ l of reaction mixture contained 50 ng of genomic DNA, 50 pmol of each corresponding primer (5'-TGCCAGAAGAGCCAAGGACAGGTA-3', and 5'-

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'T'AAGGGTG GGAAAATAGACCAATA-3'), 200  $\mu$ M of each dNTP (Pharmacia), 20  $\mu$ M dUTP (Pharmacia), and 5 U Taq Plus Precision enzyme mixture (Stratagene) in 1x Taq Plus Precision buffer (Stratagene). Amplification was carried out in 30 cycles: 95°C for 30 s, 57°C for 20 s, and 72°C for 30 s. Upon completion of the reaction, DNA  
5 was purified from the unincorporated dNTPs and the primers with a QIAquick PCR purification kit (QIAGEN, Inc.) according to the manufacturer's protocol. DNA was eluted from the column with 50  $\mu$ l of 1x ThermoSequenase® buffer for enzymatic cleavage or with 20  $\mu$ l of water for chemical fragmentation. Enzymatic fragmentation was carried out by adding 3 U of Uracil DNA Glycosylase (Boehringer Mannheim) to  
10 eluted DNA at 37°C for 1 h to create cleavable sites, then heated to 95°C for 15 min to split the DNA. Alternatively, chemical fragmentation was carried out as follows: the DNA solution was diluted 80  $\mu$ l of formic acid, incubated at room temperature for 20 min, and precipitated with 1 ml of 0.2 M LiClO<sub>4</sub> in acetone at -20°C for 20 min. The pellet was dissolved in 100  $\mu$ l of 10% piperidine and incubated at 95°C for 1 h.  
15 Piperidine was twice extracted with 0.5 ml of chloroform. The fragmented DNA was precipitated with 1 ml of 0.2 M LiClO<sub>4</sub> in acetone, washed with 70% ethanol and acetone, then dried and dissolved in water.

Primer extension is based on the high precision of ddNTP incorporation using methods of the present invention. ThermoSequenase® is an exonuclease-free polymerase  
20 with a phenylalanine-to-tyrosine mutation, which strongly reduces the discrimination against ddNTPs (Reeve and Fuller, 1995; Tabor and Richardson, 1995), thereby facilitating the ddNTP incorporation and reducing the uncertainty in base calling caused by the uneven rate of ddNTP incorporation.

ThermoSequenase® is not error prone. DNA polymerases are known to be highly  
25 selective in extending the 3' end of perfectly matched nucleotides over 3' mismatched ones. The lack of 3'-5' exonuclease activity in ThermoSequenase® allows the use of standard phosphoramidite chemistry for primer synthesis, whereas enzymes that bear proofreading activity would require introduction of a thiophosphate bond between the 3'-end nucleotides. Presence of mismatches within the 6-nt-long region from the 3' primer  
30 end significantly affects the extension reaction yield. Penultimate mismatches in the second position from the 3' terminus of the primer decrease the extension rate to the background level.

**Isothermal single-base primer extension method***A. Identification of single base polymorphism with multiprimer sets.*

From 0.2 to 1 pmol of primers were loaded per 100 x 100 x 20  $\mu\text{m}^3$  gel pad of a microchip. 50  $\mu\text{l}$  of reaction mixture contained 0.06 - 6 nM DNA, 10  $\mu\text{M}$  each  
5 fluorescein-conjugated dideoxynucleotide triphosphate (ddNTP-FL, NEL400-NEL403, NEN DuPont), 5 U of Perfect Match PCR Enhancer (Stratagene), and 30 U of ThermoSequenase<sup>®</sup> (Amersham) in 1x ThermoSequenase<sup>®</sup> reaction buffer (Amersham). Double-stranded DNA was denatured at 95° for 5 min before applying it to the microchip. The reaction mixture was placed on the microchip at 75°C, covered with a  
10 chamber for *in situ* PCR (AmpliCover<sup>™</sup> Disc and AmpliCover<sup>™</sup> Clips, PerkinElmer Co.) and incubated for 10-360 min at a constant temperature ranging from 58°C to 74°C. Upon completion of the reaction, the microchip was placed in a submarine electrophoretic chamber. Electrophoresis was carried out at 9 V/cm for 5 min. in 0.5x TBE buffer to remove fluorescently labeled ddNP, Then the microchip was rinsed with  
15 water and dried. The fluorescence pattern was analyzed as described herein.

*B. Identification of polymorphism with primers located next to polymorphic sites (multibase primer method).*

0.6 pmol of primers were immobilized within a gel pad of the microchip. Each type of fluorescein-conjugated dideoxynucleotide triphosphate was used in a separate  
20 reaction (A-, C-, G-, or T-reaction). The reaction conditions were as described herein, except using the following concentrations of one of four fluorescein-labeled ddNTPs: 4  $\mu\text{M}$  ddATP-FI, 7  $\mu\text{M}$  ddCTP-FI, 5  $\mu\text{M}$  ddGTP-FI, or 14  $\mu\text{M}$  ddUTP-FI; each labeled ddNTP was completed with the mixture of three other unlabeled ddNTPs, each in 10  $\mu\text{M}$  concentration.

Table 1. SPECIFICITY OF MULTIBASE PRIMER EXTENSION ASSAY

		Fluorescence (AU) «N» DNA50ntN			
		A	G	T	C
	ddATP	2.2	1.7	828.2	6.0
5	ddGTP	1.9	1.9	4.8	813.2
	ddUTP	439.8	3.2	5.9	6.1
	ddCTP	1.8	317.8	2.7	17.2

10 The immobilized primer P25nt was extended with one of four fluorescently labeled DNAs (D50ntN with A, G, T, or C in N position) in the presence of one of four target DNA's labeled ddATP, ddGTP, ddTTP, or ddCTP at 66°C for 2 h.

## DOCUMENTS CITED

- Fotin, A.V., Drobyshev, A.L., Proudnikov, D.Y., Perov, A.N. and Mirzabekov, A.D. (1998) *Nucleic Acids Research*, 26, 1515-1521.
- 5 Guschin, D., Mobarry, B.K., Proudnikov, D., Stahl, D.A., Rittmann, B.E., and Mirzabekov, A.D. (1997) *Appl. Environ. Microbiol.*, 63, 2397-2402.
- Mitsubishi (1996a) *J. Clin Lab. Anal.* 10, 277-254.
- Mitsubishi (1996b) *J. Clin Lab. Anal.* 10, 285-293.
- Pastinen, T., Perola, M., Niini, P., Terwilliger, J., Salomaa, V., Vainen, E., Peltonen,  
10 L., and Syvanen, A. (1998) *Hum. Mol. Genet.*, 7, 1453-1462.
- Reeve, M.A. and Fuller, C.W. (1995) *Nature*, 376, 796-797.
- Sanger, F., Nicklen S. and Coulson A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74, 5463-5467.
- Syvanen, A.C., Aalto, K., Harju, L., Kontula, K., and Soderlund, H. (1990) *Genomics*,  
15 8, 684-692.
- Tabor, S. and Richardson, C.C. (1995) *Proc. Natl. Acad. Sci. USA*, 92, 6339-6343,
- Timofeev, F., Kochetkova, S., Mirzahekov, A, and Florentiev, V. (1996) *Nucleic Acids Res.*, 24, 3142-3148.

**WE CLAIM:**

1. A method for determining the identity of a nitrogen base in a position of a sequence of a nucleic acid molecule from a sample, said method comprising;
  - 5 (a) obtaining a microchip comprising primers immobilized in gel pads, said primers complementary to the nitrogen base sequence in the nucleic acid molecule that is adjacent to the nitrogen base whose identity is to be determined;
  - 10 (b) having labeled dideoxynucleoside triphosphates available for a reaction between the primers in the gel pads and the nucleic acids in the sample;
  - (c) reacting the microchip with the sample at a temperature sufficient to extend the primers and effect hybridization of the primer with the target nucleic acid molecule; and
  - 15 (d) identifying the nitrogen base by determining the identity of the labeled dideoxynucleoside triphosphate that is incorporated into the primers by primer extension.
2. The method of claim 1, wherein the dideoxynucleotide triphosphates conjugated with the label are a plurality of dd NTP's selected from A, T, C, G  
20 triphosphates and universal substitutes.
3. The method of claim 2, wherein the label is different for each triphosphate.
4. The method of claim 1, wherein the reacting is carried out at an elevated temperature.
- 25 5. The method of claim 4 wherein the temperature is approximately the  $T_m$ .
6. The method of claim 1, wherein the reacting is carried out first at a lower temperature to allow the target nucleic acid to hybridize with a primer and enzyme to extend the immobilized primer then the temperature is raised to achieve denaturing conditions, and then the temperature is lowered to allow another round of hybridization  
30 to occur.

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7. The method of claim 1 wherein the nucleic acid molecule is double stranded.
8. The method of claim 1, wherein the nucleic acid molecule is RNA.
9. The method of claim 1, wherein the primers are about 10 to about 50  
5 nucleotides in length.
10. The method of claim 1, wherein the concentration of primers within a gel element is from 100 pM to 1000 pM.
11. The method of claim 1, wherein the nucleic acid molecule is selected from a group consisting of RNA fragments, total RNA, fractionated mRNA, ribosomal RNA,  
10 low molecular weight RNA, as well as RNA transcripts of DNA.
12. A method for determining the identity of a nitrogen base in a position of the sequence of a nucleic molecule from a sample said method comprising:
- (a) obtaining a microchip comprising a plurality of primers containing different 3' terminal nucleotides, immobilized in gel  
15 pads, said primers overlapping the nitrogen base when the primers are hybridized to the molecule;
  - (b) reacting the microchip with the sample to extend the primers; and
  - (c) identifying the nitrogen base by determining the identity of the primer that is extended.
- 20 13. A method for detection of a small quantity of a nucleic acid target by single base primer extension on a gel microchip, said method comprising:
- (a) obtaining a microchip containing immobilized primers for the nucleic acid target;
  - (b) introducing a label into the 3' terminus of those primers which are  
25 complementary to the nucleic acid target by incubation of said microchip comprising a plurality of target nucleic acid fragments, a DNA- or RNA-dependent polymerase and a plurality of dideoxynucleotide triphosphates conjugated with any detectable label; and
  - (c) detecting the label coupled to the immobilized primers from which  
30 presence of the target is inferred.

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14. The method of claim 12, wherein the nucleic acid target is a plurality of nucleic acids that differ in one nucleotide from each other and are represented in different proportions from each other.

5 15. A method for detection of a nucleic acid target by a polymerase reaction on a gel microchip comprising:

- (a) preparing a microchip of independently immobilized 5' primers;
- (b) extending the primers which are complementary to target nucleic acids by exposing the microchip to a mixture of target nucleic acid fragments, enzymes and deoxynucleotide triphosphates; and
- 10 (c) detecting the extended primers from which presence of the target is inferred.

16. The method of claim 14, wherein the deoxynucleotide triphosphates are conjugated with a detectable label.

17. The method of claim 14, where the primer is a synthetic nucleic acid.

15 18. The method of claim 17 wherein the molecule is a specific antibody.

19. A method for detecting a molecule wherein (a) specific target nucleic acid is coupled to said molecule, and (b) the nucleic acid is identified in accord with claim 1.

20. Gel elements of a microchip comprising nucleic acid primers immobilized in the gel pads.

20 21. The gel element of claim 18, wherein the primers have sequences with at least one internal mismatch.

22. The gel elements of claim 18, wherein the primers are multibase primers.

23. The gel elements of claim 18, wherein the primers are a multiprimer set.

24. The gel elements of claim 18, wherein the primers are about 10 to about  
25 50 nucleotides in length.

25. A method to detect a specific antigen using a microchip comprising:

- (a) coupling a plurality of antibodies with a distinguishable nucleic acid tag;
- (b) putting the antibodies and the antigen into a container to allow antigen-antibody interactions;
- 30 (c) removing the antibodies that do not bind to the antigen;

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- (d) preparing a microchip with immobilized primers that are complementary to the tags on the antibodies; and
- (e) determining which antibodies were associated with the antigen by detecting their nucleic acid tags by primer extension on the microchip.

5

Fig. 1

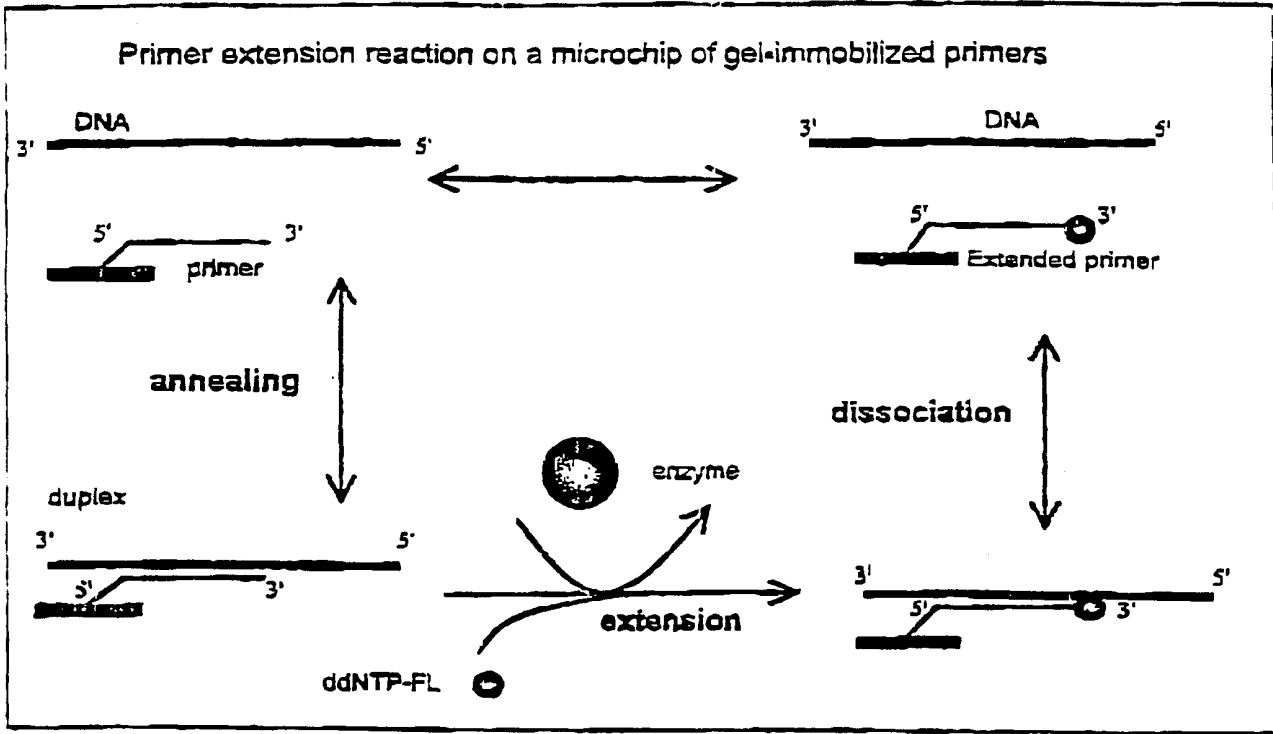


Fig. 2<sup>a</sup>

target DNA

5'-attatgtagaaaatNctgaaaaggcactgaacgtttattatgaaataggt

Primers:

- gacttttccgtgacttgcaaataat 5'-gel
- Ngacttttccgtgacttgcaaataa 5'-gel
- Ngacttttccgtgacttgca 5'-gel
- Ngacttttccgtgacttgc 5'-gel
- Ngacttttccgtgacttg 5'-gel
- Ngacttttccgtgactt 5'-gel

- D50ntN
- PN
- P25ntN
- P20ntN
- P19ntN
- P18ntN
- P17ntN

Figure

2b

```

5   6   7   8   9   10  11  12  13  14  15
5'...cot gAg gag aag tct gcc gtt act gcc ctg tgg-
      Nc ctc ttc aga cgg caa tga c-gel (pCD6N)
      c ctc ttc aga cgg caa tga c-gel (pCD6)
16  17  18  19  20  21  22  23  24  25
ggc aag gIg aac gtg gat gaa gtt ggt ggt-
      Nc ttg cac cta ctt caa cca c-gel (pCD18N)
      c ttg cac cta ctt caa cca c-gel (pCD18)
      IVSI
26  27  28  29  30  1   12   21   31.
Gag gcc ctg ggc ag GTtgGTatca aggttacaag acaggtttaa ggagaccaat..
Ntc cgg gac ccg tc caac-gel (pCD26N)
tc cgg gac ccg tc caac-gel (pCD26)
Naaccatagt tccaatgttc tgtcc-gel (pIVSI/1N)
aaccatagt tccaatgttc tgtcc-gel (pIVSI/1)
Naccatagt tccaatgttc tgtcca-gel (pIVSI/2N)
accatagt tccaatgttc tgtcca-gel (pIVSI/2)
Natagt tccaatgttc tgtccaaatt c-gel (pIVSI/5N)
atagt tccaatgttc tgtccaaatt c-gel (pIVSI/5)
Ntagt tccaatgttc tgtccaaatt c-gel (pIVSI/6N)
tagt tccaatgttc tgtccaaatt c-gel (pIVSI/6)

```

Fig. 3

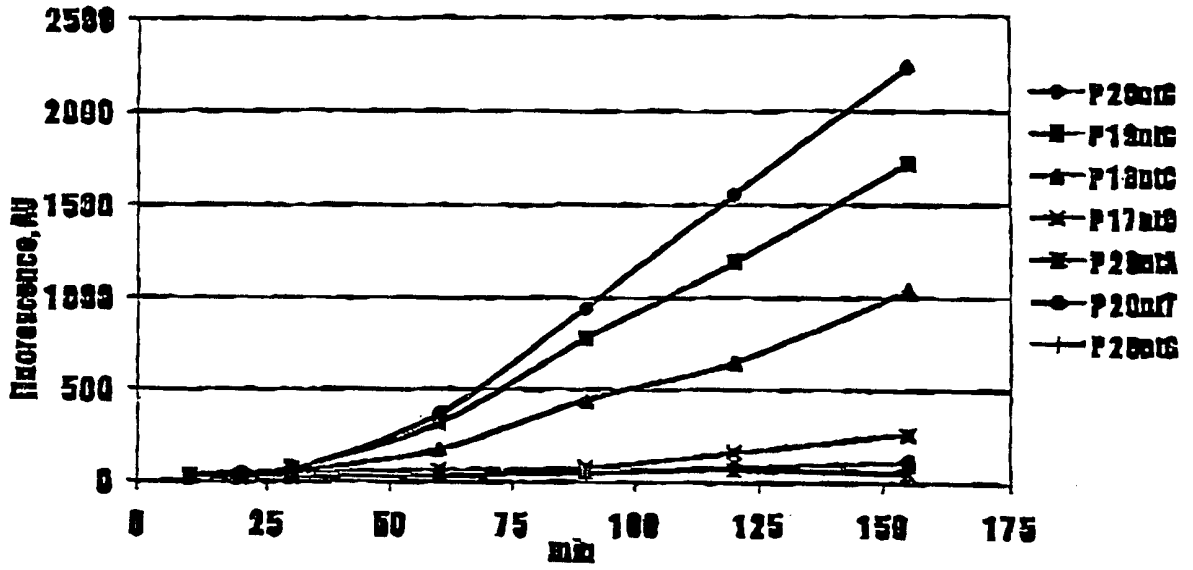


Fig. 4

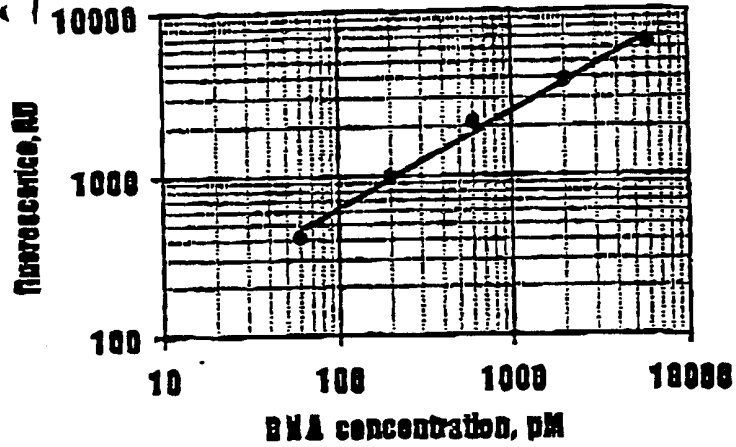
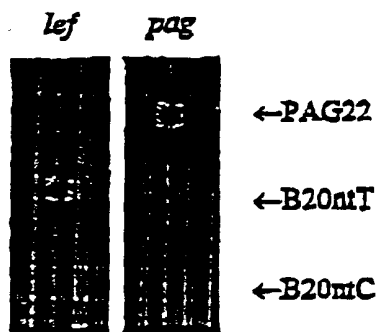


Fig. 5



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Fig. 6

Position	Normal genotype	Mutated genotype
CD6	GAG	GTC
	GAG	GCG
CD18	GTG	GGG
CD26	GAG	AAG
	GAG	TAG
IVS1/1	AGGTTGGT	AGATTGGT
	AGGTTGGT	AGTTTGGT
IVS1/2	AGGTTGGT	AGGATGGT
	AGGTTGGT	AGGCTGGT
	AGGTTGGT	AGGGTGGT
IVS1/5	AGGTTGGT	AGGTTGAT
	AGGTTGGT	AGGTTGCT
	AGGTTGGT	AGGTTGTT
IVS1/6	AGGTTGGT	AGGTTGGC

Fig. 7

