Polymorphism analysis and gene detection by minisequencing on an array of gel-immobilized primers

Svetlana Dubiley[1,2], Eugene Kirillov[1,2] and Andrei Mirzabekov[1,2,*]

[1]Joint Human Genome Program, Engelhardt Institute of Molecular Biology, 32 Vavilov Str., Moscow 117984, Russia and [2]Joint Human Genome Program, Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA

Received January 22, 1999; Revised May 30, 1999; Accepted June 15, 1999

ABSTRACT

Two procedures, multibase and multiprimer, have been developed for single nucleotide extension of primers immobilized within polyacrylamide gel pads on a microchip. In the multibase assay, a primer is next to a polymorphic nucleotide; the nucleotide is identified by the specificity with which the primer incorporates fluorescently labeled dideoxyribonucleoside triphosphates. In the multiprimer assay, several primers containing different 3'-terminal nucleotides overlapping the variable nucleotide in DNA are used. The polymorphic nucleotide is identified according to the primer that is extended. The methods were compared for diagnosis of β-thalassemia mutations. Isothermal amplification of the fluorescent signal was achieved by performing both assays at elevated temperature. Anthrax toxin genes were identified in a model system using this amplification method.

INTRODUCTION

The rapidly increasing amounts of sequencing data have revealed a large number of nucleotide polymorphic sites and mutations in the human genome and in the genomes of other organisms. Polymorphism analysis is beginning to be widely applied for detection of genetic diseases and genetic variations, mapping of genes, in forensic studies and identification of pathogenic microorganisms. There is an urgent need for fast, inexpensive and reliable methods to perform such analyses on a large scale.

There are numerous approaches to identifying single nucleotide polymorphisms (SNPs) in DNA; restriction fragment length polymorphism (1) and single-strand or double-strand conformational polymorphism analysis (2,3) are two examples. Two other methods appear to be highly promising: allele-specific hybridization with oligonucleotide microarrays and single base extension. Simultaneous screening of a large number of polymorphic sites was carried out effectively by parallel hybridization of a DNA sample with many oligonucleotides immobilized on a microchip, although the accuracy of the assay needs to be enhanced (4–8).

The single base extension appears to be one of the most accurate methods for detecting nucleotide polymorphisms. In minisequencing, first described as a solid phase procedure (9), a primer is hybridized to DNA and extended with DNA polymerase by one nucleotide with the appropriate dideoxyribonucleoside triphosphate that matches the nucleotide at the variable site. Minisequencing, in solution or microtiter plate-based, is a well-established procedure for analyzing polymorphisms (10,11), diagnosing point mutations (12), determining gene copy number (13) and quantifying rare mRNA species (14). Separation of different extended primers according to their size by gel electrophoresis (11) or MALDI mass spectrometry (15) was suggested for multiplex minisequencing. Recently, the multiplex analysis was carried out on a microarray of various glass-immobilized primers (16,17).

We shall here describe two new procedures for SNP analysis by minisequencing on biological microchips containing gel-immobilized oligonucleotides. The fluorescent signal was amplified by carrying out the isothermal DNA polymerase reaction at elevated temperature. Reactions on microchips with each of four fluorescently labeled ddNTPs were used to identify any base in a polymorphic site with one primer. An alternative procedure was developed wherein just one microchip containing four immobilized primers is used. The primers differ in the nucleotide at the 3'-end that matches the polymorphic site. Both procedures were applied to identify β-thalassemia mutations and to detect anthrax toxin genes.

MATERIALS AND METHODS

Oligonucleotide synthesis, microchip manufacturing and fluorescent signal detection

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

Microchips were manufactured as described earlier (18–20). Micromatrices of $100 \times 100 \times 20$ μm^3 of gel pads were prepared by photopolymerization of a 5% polyacrylamide gel as described earlier (18). Each primer was applied to a gel pad by

*To whom correspondence should be addressed at: Joint Human Genome Program, Center for Mechanistic Biology and Biotechnology, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA. Tel: +1 630 252 3161; Fax: +1 630 252 3387; Email: amir@everest.bim.anl.gov

a pin robot (20) and immobilized through reductive coupling of the 5'-amino group of the primer with the aldehyde group of the activated gel pad (19).

The fluorescence pattern was monitored on an epifluorescent microscope equipped with a CCD camera. The image of the microchip was displayed and analyzed on a PC using specially developed software (20).

The following algorithm for image analysis was used to obtain fluorescent signals. The background originating from the absorbed dye-labeled ddNTPs was assumed to be uniform between 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 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, i.e. $J = (C - B)/B$. Under the above-mentioned assumptions, the registered hybridization signal (*J*) differs from the 'real' one (*D*): $D = J_s - J_g$, where J_s 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 the β-globin gene was prepared by PCR amplification from the genomic DNA of patients with different β-thalassemia mutations (20).

Aliquots of 100 µl of reaction mixture contained 50 ng of genomic DNA, 50 pmol of each corresponding primer (5'-tgcc-agaagagccaaggacaggta-3' and 5'-taagggtgggaaaatagaccaata-3'), 200 µM of each dNTP (Pharmacia), 20 µM dUTP (Pharmacia) and 5 U *Taq* Plus Precision enzyme mixture (Stratagene) in 1× *Taq* Plus Precision buffer (Stratagene). Amplification was carried out for 30 cycles of 95°C for 30 s, 57°C for 20 s and 72°C for 30 s. Upon completion of the reaction, DNA was purified from the unincorporated dNTPs and the primers with a QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocol. DNA was eluted from the column with 50 µl of 1× ThermoSequenase buffer for enzymatic cleavage or with 20 µl of water for chemical fragmentation. Enzymatic fragmentation was carried out by adding 3 U of uracil DNA glycosylase (Boehringer Mannheim) to 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 with 80 µl of formic acid, incubated at room temperature for 20 min and precipitated with 1 ml of 0.2 M $LiClO_4$ in acetone at –20°C for 20 min. The pellet was dissolved in 100 µl of 10% piperidine and incubated at 95°C for 1 h. Piperidine was twice extracted with 0.5 ml of chloroform. The fragmented DNA was precipitated with 1 ml of 0.2 M $LiClO_4$ in acetone, washed with 70% ethanol and acetone and then dried and dissolved in water.

Isothermal single base primer extension assay

Identification of single base polymorphism with multiprimer assay. From 0.2 to 1 pmol of primers were loaded per 100 × 100 × 20 μm^3 gel pad of the microchip. Each 50 µl of reaction mixture contained 0.06–6 nM DNA, 10 µM each fluorescein-conjugated dideoxynucleotide triphosphate (ddNTP-FL; NEL400–NEL403; NEN DuPont), 5 U of Perfect Match PCR Enhancer (Stratagene) and 30 U of ThermoSequenase (Amersham) in 1× ThermoSequenase reaction buffer (Amersham). Double-stranded (ds)DNA was denatured at 95°C for 5 min before applying to the microchip. The reaction mixture was placed on the microchip at 75°C, covered with a chamber for *in situ* PCR (AmpliCover Disc and AmpliCover Clips; Perkin Elmer Co.) and incubated for 10–360 min at a constant temperature ranging from 58 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 0.5× TBE buffer to remove fluorescently labeled ddNTPs. Then the microchip was rinsed with water and dried. The fluorescence pattern was analyzed as described above.

Identification of polymorphism with primers located next to polymorphic sites (multibase assay). Aliquots of 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 reaction (A, C, G or T reaction). The reaction conditions were as described above, except using the following concentrations of one of the four fluorescein-labeled ddNTPs: 4 µM ddATP-Fl, 7 µM ddCTP-Fl, 5 µM ddGTP-Fl or 14 µM ddUTP-Fl; each labeled ddNTP was completed with a mixture of the three other unlabeled ddNTPs, each at 10 µM concentration.

RESULTS

Amplified multibase and multiprimer single-strand extension

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

To achieve amplification of the fluorescent signal, the assay is carried out at a temperature above the melting temperature of the 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. Because the system is in dynamic equilibrium, the same DNA molecule successively interacts with different primer molecules, producing many duplexes. 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 to extend all available primers.

There are two variants of the assay to identify a mutation or a polymorphic base in the target DNA, depending on the position of the variable base, N, which is recognized by DNA polymerase. The variable base is complementary to either the newly incorporated nucleotide or the nucleotide that is adjacent to the incorporated one in the primer.

In the multibase assay, the 3'-terminus of the primer is located next to the variable base N and the polymerase incorporates one of the four different labeled ddNTPs which is

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target DNA
5′-attatgtagaaaatNctgaaaaggcactgaacgtttattatgaaataggt  D50ntN
Primers:          gacttttccgtgacttgcaaataat 5′-gel      PN
                 Ngacttttccgtgacttgcaaataa 5′-gel      P25ntN
                 Ngacttttccgtgacttgca 5′-gel           P20ntN
                 Ngacttttccgtgacttgc 5′-gel            P19ntN
                 Ngacttttccgtgacttg 5′-gel             P18ntN
                 Ngacttttccgtgactt 5′-gel              P17ntN
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Figure 1. DNA sequences and primers used in model experiments. Synthetic DNA (D) and microchip-immobilized primers (P) contain one of the four bases A, G, T or C in the polymorphic site, N. The length of sequences (nt) is incorporated into their names.

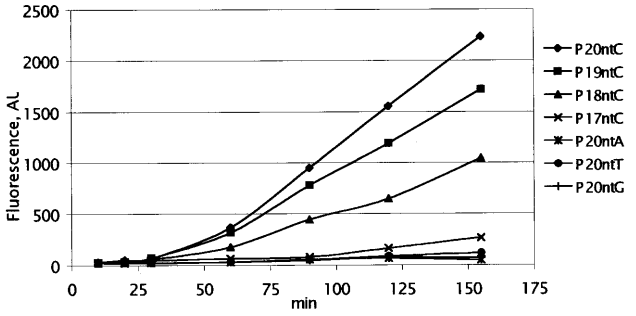

Figure 2. Time dependence of fluorescence signal amplification in the microchip multiprimer assay. The assay was carried out at 66°C with D50ntG DNA (2 nM), matched primers P20ntC, P19ntC, P18ntC and P17ntC and terminally mismatched primers P20ntA, P20ntG and P20ntT.

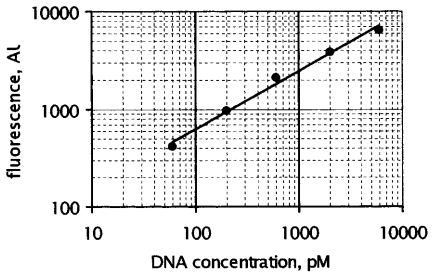

Figure 3. Effect of DNA concentration on microchip multiprimer extension assay. The assay was carried out with D50ntA as a target and primer P25ntT labeled with ddATP at 66°C for 6 h.

complementary to this base, whereas the mismatched ddNTPs fail to be incorporated. To identify the variable base, four ddNTPs should be tested in separate reactions or each should carry a distinct reporter molecule.

In the multiprimer assay, the variable base corresponds to the 3'-terminal base of the primer. In this case, four primers variable at the 3' nucleotide should be 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 next to it. The polymerase does not react with the primers that form mismatched base pairs in their 3'-terminal positions.

The relative efficiency of these two assays depends on how reliably the DNA polymerase discriminates a match against a mismatch either in the terminal position of the primers or in incorporated ddNTP.

Optimization of the base extension assay

Optimization experiments were carried out with synthetic 50 nt long DNA fragments of the *Bacillus anthracis lef* toxin gene into which a polymorphic 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 PN contained one of the four nucleotides at the 3'-terminal position (Fig. 1).

Different amounts (0.2–1 pmol) of primers were immobilized per 100 × 100 × 20 μm^3 gel pad to determine the optimum primer concentration. No significant rise in the fluorescent signal was observed for immobilized primer concentrations >0.6 pmol/pad. The best discrimination of 3' mismatches was attained for this amount of primers within a wide range of target DNA concentrations (data not shown) and, therefore, we used it in all subsequent experiments.

The melting temperature (T_m) of primers calculated using different formulae (21,22) did not correlate well with the experimental values. In addition, as shown earlier, T_m was different for duplexes formed on a microchip and in solution (23). T_m values, which were determined for perfect duplexes of target DNA (1 μM D50ntN) hybridized with microchip-immobilized primers of different length (for procedure see 24), were within the range 48–53°C (data not shown). Adding Perfect Match PCR Enhancer did not considerably affect the melting temperature for perfect duplexes.

The highest yield of the extension reaction on the microchip was found at 66 ± 2°C for all tested primers (data not shown). This range is above the T_m but below the optimum temperature for ThermoSequenase (74°C).

Figure 2 shows the rise in fluorescent signal with time during the extension reaction with 2 nM D50ntG DNA and terminally matched primers of different lengths (P20ntC, P19ntC, P18ntC and P17ntC) and mismatched primers (P20ntA, P20ntG and P20ntT). It was found that after a 60 min lag, the fluorescent signal became linear for all primers and higher for longer ones. The 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 by, for example, up to 22- to 25-fold in 120 min for the 20mer primer. The discrimination was about the same for all tested mismatches.

Figure 3 shows that the extension signal grows approximately linearly on double logarithmic coordinates in the range 0.06–6 nM DNA concentration.

Table 1 shows the fidelity of ThermoSequenase in a multibase primer extension assay tested for 16 combinations of four DNAs (D50ntA, D50ntG, D50ntT and D50ntC) with four fluorescently labeled ddNTPs (ddATP, ddGTP, ddUTP and ddCTP) and immobilized PN primer (Fig. 1). It appears that discrimination of a mismatched base was at least 50-fold and the assay demonstrated high fidelity when single-stranded (ss)DNA was used.

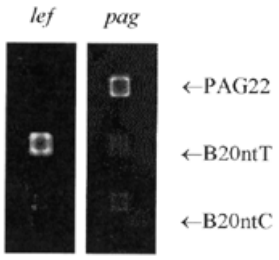

Figure 4. Detection of DNA of toxin genes of *B.anthracis* by a multiprimer extension assay. Two PCR-amplified fragments of *lef* (0.3 pmol) and *pag* (0.3 pmol) toxin genes were applied to a microchip containing immobilized primers, and the assay was carried out at 66°C for 2 h.

Table 1. Specificity of multibase primer extension assay

	Flourescence (AU) 'N' DNA50ntN			
	A	G	T	C
ddATP	2.2	1.7	828.2	6.0
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

The immobilized primer PN was extended with one of four target DNAs (D50ntN with A, G, T or C in the N position) in the presence of one of fluorescently labeled ddATP, ddGTP, ddTTP or ddCTP at 66ºC for 2 h.

Toxin gene detection

Double-stranded PCR fragments of *B.anthracis* plasmid pOX1-borne protective antigen, *pag* (positions 1933–2179, GenBank accession no. M22589), and lethal factor, *lef* (positions 1153–1256, GenBank accession no. 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* gene-specific primer, PAG22nt (5'-agaactaggaatagataaatcccct-3'). Aliquots of 0.3 pmol of the 246 bp long DNA of the *pag* gene were chemically fragmented as described above. Aliquots of 0.3 pmol of the 103 bp long DNA of the *lef* gene were used without fragmentation. Figure 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 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 (data not shown). It appears that at least a 10-fold amplification of the hybridization signal was achieved by the extension assay.

β-Thalassemia diagnostics

The comparative usefulness of multibase and multiprimer assays was tested for diagnostics of seven commonly occurring

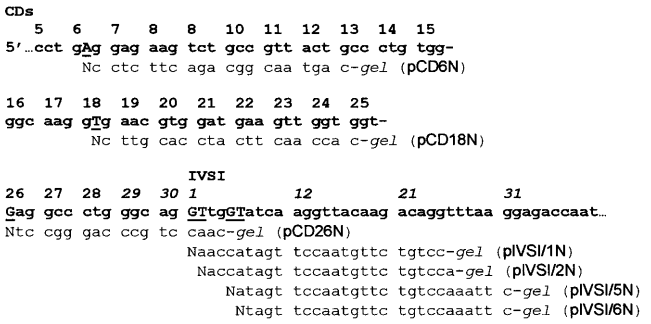

Figure 5. Sequences of a PCR-amplified fragment of β-globin gene and microchip-immobilized primers. β-Thalassemia mutations are indicated in capital letters. Marked are the codon positions in the first exon and the nucleotides in the first intron (IVS I). Each primer sequence represents a set of five primers differing in their 3'-terminal nucleotide, N. Primers for the multibase assay did not contain the N nucleotide and were used to identify polymorphism with four fluorescently labeled ddNTPs. The four primers for multiprimer assay each contained one of four nucleotides in position N.

β-thalassemia mutations within the β-globin gene (Fig. 5). The mutations were codon substitutions in the first exon [CD6 A/T (A/T, substitution of A for T), CD18 T/C and 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). DNA from eight different patients was tested. Figure 6 shows the results of the genotyping.

For diagnostics of these mutations in a multibase assay, microchips containing seven site-specific primers were prepared. The primers (pCD6, pCD18, pCD26, pIVSI/1, pIVSI/2, pIVSI/5 and pIVSI/6; see Fig. 5) were complementary to the sense chain of the β-globin gene and adjoined the polymorphic sites. Four fluorescein-labeled ddNTPs were used on the four microchips in four extension assays. The data shown in Figure 6A enable us to identify the mutations as heterozygous CD6 A+T, homozygous CD18 T, heterozygous CD26 G+A and homozygous IVS I/1 G, IVSI/2 T, IVS I/5 G and IVS I/6 T.

For mutation diagnostics in a multiprimer assay, seven sets of primers 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. 5). A mixture of all four fluorescein-conjugated ddNTPs was used for the extension reaction. The results shown in Figure 6B enable one to identify the mutations as homozygous at CD6 A, CD18 T, IVSI/2 T, IVS I/5 G and IVS I/6 T and heterozygous at CD26 G+A and IVS I/1 G+A.

All identified mutations were confirmed by standard sequencing of the tested DNAs.

Comparison of the multibase and multiprimer assays performed with the same DNA demonstrated their similar efficiencies at discriminating mutations.

DISCUSSION

It has been demonstrated that oligonucleotides immobilized within polyacrylamide gel pads of a microchip are accessible

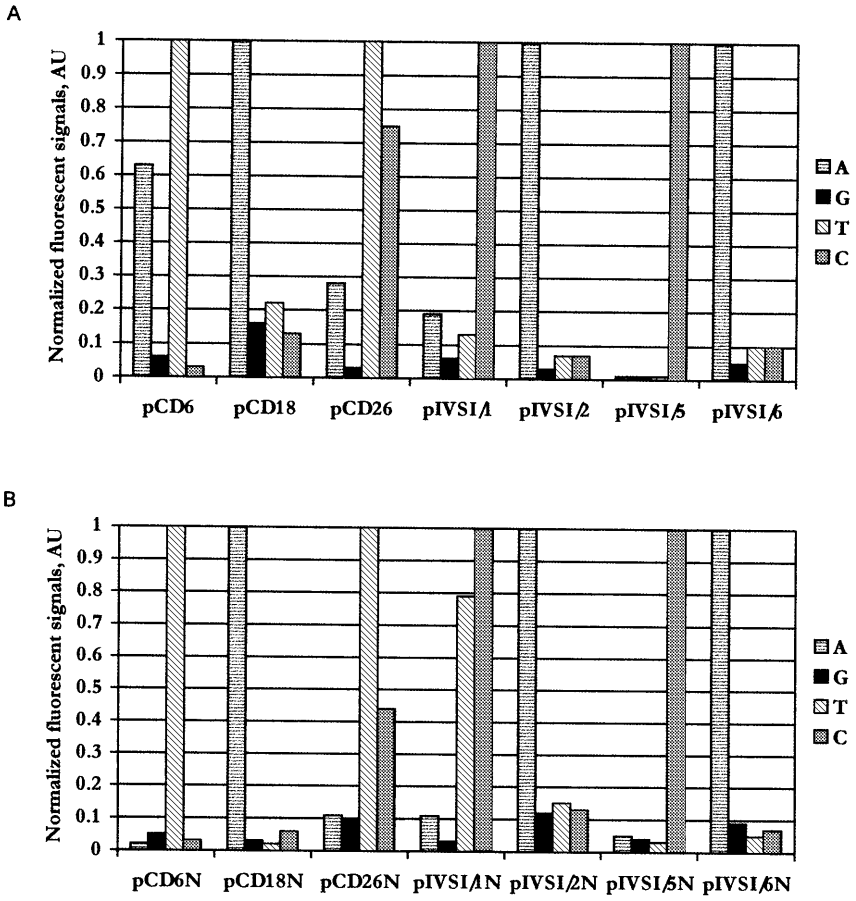

Figure 6. Detection of β-thalassemia mutations in PCR-amplified DNA by multibase and multiprimer extension assays on microchips. (**A**) Multibase assay. The primer for the polymorphic nucleotide was extended with one of four fluorescently labeled ddNTPs at 66°C for 2 h. A comparatively high false positive signal for CD26 T (corresponding to a 25–40% admixture of CD26 T) was observed for all tested target DNAs; we cannot explain this yet. Different results in Figure 5A and B are due to analyzing different patients. (**B**) Multiprimer assay. Immobilized primers containing A, G, T or C at the 3'-terminus were extended with labeled ddNTPs at 66°C for 2 h.

substrates for some enzymes, such as T4 polynucleotide kinase and T4 DNA ligase (25). Gel supports for primer immobilization provide a more homogeneous environment for DNA polymerases (mol. wt ~90 kDa) than the solid phase, glass-immobilized oligonucleotides as suggested for minisequencing (17).

The three-dimensional structure of the gel allows achievement of high local concentrations of immobilized primers within a small volume and has a significantly higher capacity for immobilization than two-dimensional glass supports. A higher concentration of the primer increases the local concentration of DNA fragments complementary to the primer and does not affect the concentration of non-specific DNAs. This phenomenon reinforces the specificity of the assay and the primer extension rate. Accumulation of specific fragments within the gel is particularly important when dsDNA is used in the analysis.

Under the same conditions, ssDNA produces a fluorescent signal about five times higher than 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 can be selected for the assay to test the same polymorphic site. In addition, fragmented DNA has a non-extendable 3'-end, which rules out unwanted concurrent reaction of DNA extension in cases of double-stranded targets.

Minisequencing is based on the high precision of ddNTP incorporation by ThermoSequenase. ThermoSequenase is an exonuclease-free polymerase with a Phe→Tyr mutation, which strongly reduces the discrimination against ddNTPs (26,27), thereby facilitating ddNTP incorporation and reducing the uncertainty in base calling caused by the uneven rate of ddNTP incorporation. Inorganic pyrophosphatase in the reaction mixture prevents removal of extended 3'-terminal dideoxymononucleotides (26). Misincorporation of ddNTPs with ThermoSequenase in a microchip assay was in most cases much less than 2% in the model experiments with ssDNA (Table 1) and <10–20% of the correct extension in the detection of β-thalassemia mutations (Fig. 6).

DNA polymerases are known to be highly 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 end significantly affects the extension reaction yield. Penultimate mismatches in the 3'-terminus decrease the extension rate to the background level (data not shown). Therefore, if two polymorphic sites are located closer than 6 nt from each other, two primers for the first site have to be designed to attain reliable results. If a universal base or mixture of bases is present in an internal position of primers, the number of primers could be lower if the polymorphic sites are located close to each other. Mismatches near the 5'-end do not change the extension yield considerably.

Comparative studies of β-thalassemia mutations have revealed comparable specificity of the multibase and multiprimer nucleotide extension methods. We plan to further compare these two methods in a large-scale analysis of genomic polymorphic sites.

Isothermal amplification was performed with ThermoSequenase at temperatures above the melting points (T_m) of the duplexes. The amplification rose linearly with time and its rate was higher for longer and more stable oligonucleotides (Fig. 2). The power of discrimination between perfect and mismatched nucleotide variants also increased with time. A direct correlation between the fluorescence intensity and the amount of tested DNA was observed (Fig. 3). The fluorescent signal dependence on the target DNA concentration was linear, with the coefficient values lower than 1 on double logarithmic coordinates over the whole tested range of concentrations (three orders of magnitude). This makes it possible to carry out comparative quantitative DNA analysis on the microchip within a wide range of target concentrations.

Small amounts of nucleic acids or low copy genes can be identified by isothermal single base extension. Estimates suggest that at least 10-fold amplification of the hybridization signal could be achieved by the extension assay for anthrax toxin gene regions. Oligonucleotide microchips were applied earlier for bacterial identification by means of oligonucleotide hybridization with variable regions of 16S rRNA (28). However, some closely related microorganisms, such as the human and insect pathogens *B.anthracis* and *Bacillus thuringiensis*, respectively, differ from one another by only a few bases in this RNA and are, therefore, difficult to discriminate by this technique (Bavykin *et al*., unpublished results). Minisequencing amplification of toxin or antibiotic resistance genes can be used as an alternative procedure to identify pathogenic microorganisms.

ACKNOWLEDGEMENTS

We thank Dr Joany Jackman of USAMRIID for helpful discussion and for providing us with PCR fragments of *B.anthracis* plasmid pOX1 lethal factor and protective antigen and Dr T. Molchanova for donating the samples of genomic DNA from patients with β-thalassemia. We gratefully acknowledge contributions to this work by Dr A. Rubinov, G. Yershov, A. Belgovsky, A. Gemmell, Dr I. Taran, E. Kreindlin and V. Chupeeva. Thanks are also due to Drs V. Shick and A. Drobyshev for their useful advice and to Dr M. Bhattacharyya, Dr Eu. Boulygina and E. Novikova for critical reading of this manuscript, helpful discussions and editorial assistance. This work was supported by contract 943352403 with the Argonne National Laboratory, 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.

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