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DIAGNOSTICS MICROCHIPS: DETECTION OF BETA-THALASSEMIA MUTATIONS BY HYBRIDIZATION WITH AN OLIGONUCLEOTIDE ARRAY

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The development of diagnostics microchips facilitates the collection and analysis of significant information on genetic polymorphism and mutations in rather simple experiments. A microchip contains hundreds and thousands of gel elements (60×60×20 μm and larger) fixed on a glass surface and containing specific oligonucleotides (1). A low background and the chemical immobilization of the oligonucleotides through a stable chemical bond (2) allows one to carry out hybridizations many times with the same microchip. A high-output robot was constructed to manufacture microchips at rather low cost. (1) Hybridization of DNA with the entire microchip at different temperatures is monitored in real time (exposure time, 1–5 s) with a specially devised four-color fluorescence microscope equipped with a CCD camera and a data acquisition system (1). Single- and double-stranded DNA and single-stranded RNA (Fig. 1) were synthesized from the PCR-amplified DNA of a number of β-globin genomic regions. A fluorescent label was incorporated into DNA and RNA either during their synthesis of by chemical procedures after their partial fragmentation (3).

The precision in identification of a base change in DNA was significantly enhanced by several means:

1. Introducing simultaneous melting curve measurements (Fig. 2) for all duplexes (with different stabilities and A/T contents) formed on the entire microchip (4).
2. Using short immobilized oligonucleotides such as 8- and 10-mers (Fig. 3).
3. Carrying out hybridization of two samples of normal and mutated alleles simultaneously on the same microchip. These two samples were labeled with different fluorescent dyes (Table 1, exp. 6a,b) and their hybridization was measured in parallel and separately at two wavelengths (1,4).
4. Using sets of several overlapped oligonucleotides immobilized on the microchip that are complimentary to a mutated region (Table 2).
5. Fragmentation of DNA or RNA to short pieces.
6. Developing a contiguous stacking hybridization on the microchip with labeled 5-mers (1,5).

Screening of known mutations and genetic variants can be carried out with a high reliability on rather simple microchips containing partly overlapped oligonucleotides. The use, in addition of contiguous stacking hybridization with differently labeled pentamers on these hybridized microchips, can extend the procedure for identification of unknown changes in DNA (5).

A reliable identification of genomic heterozygous and homozygous β-thalassemia mutations, in a "yes" or "no" way, has been demonstrated with the microchips (1,4). The work was supported by Grant DE-FG02-93ER61538 of the U.S. Department of Energy and by Grant 558 and 562 of the Russian Human Genome Program.

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BIOGRAPHICAL SKETCH

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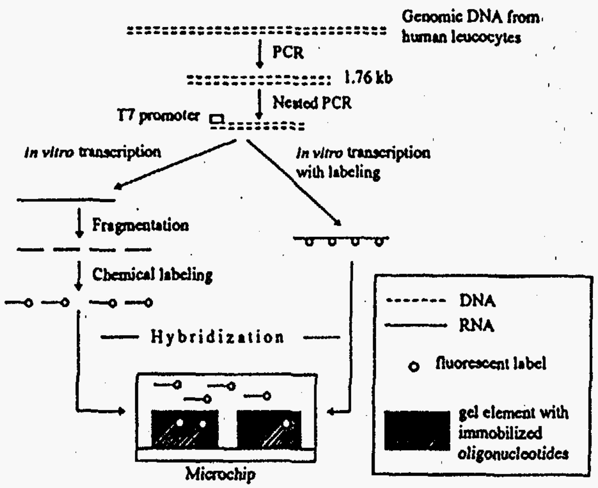
Fig. 1. Sequence analysis by hybridization of RNA transcripts with oligonucleotide microchips.



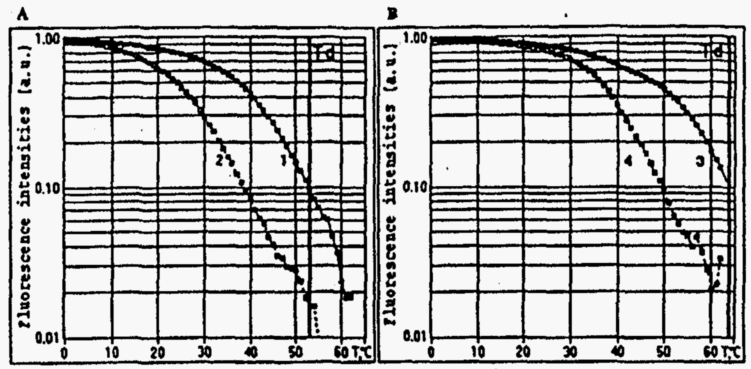


Fig. 2. Non-equilibrium melting curves of duplexes of RNA with microchip oligonucleotides.

75-nt long RNA enzymatically labeled with fluorescein was synthesized from the genomic DNA of a homozygote IVS I-2 T/A and CD26 (N) α-thalassemia patient (Table 2, probe 2a). The RNA was hybridized with the following microchip-immobilized 10-mers: 1, IVS I/2 T/A; 2, IVS(N); 3, CD26(N); 4, CD26 G/A to produce perfect, 1 and 3, and mismatched duplexes, 2 and 4. The oligonucleotide microchip is an array of 12 polyacrylamide gel pads (100×100×20 μm) fixed on a hydrophobic glass surface and spaced by 200 μm (Yershov et al., 1996). Each gel pad contains chemically bonded 10-mers (see Table 1). The melting curves were monitored simultaneously for all microchip elements by gradually increasing the temperature (1°C/min). The hybridization signals were measured at 1°C intervals for 1–5 s each time in parallel for all microchip elements using a fluorescence microscope equipped with a CCD camera and two sets of filters for fluorescein and TMR (Yershov et al., 1996). Td = discrimination temperature, a.u. - arbitrary units.

Table 1. Identification of β-thalassemia mutations by hybridization with the microchip.

Hybridized Probe				Immobilized 10-mer oligonucleotide											
				IVS (N)	I/1 G/A	I/1 G/T	I/2 T/A	I/2 T/C	I/2 T/G	I/5 G/A	I/5 G/G	I/5 G/T	I/6 T/C	CD26 (N)	CD26 G/A
#		Allele	Size (nt)	R at Td=											
				42°C	39°C	38.5°C	42°C	48°C	43.5°C	37°C	44.5°C	40°C	50°C	54.5°C	49°C
1	a	IVS (N)	75	1.00	0.04	0	0.20	0.05	0.07	0	0	0	0.04	1.00	–
	b	IVS (N)	19^a	1.00	0.09	0.07	0.02	0.03	0.01	0.03	0.03	0.07	ND	0	0
2	a	IVS I/2 T/A	Fl 75	0.15	0	0	1.00	0.12	0.08	0	0	0	0	1.00	–
	b	IVS I/2 T/A	Fl 133	0.03	0	0	1.00	0	0.30	0	0	0	0	1.00	0.19
	c	IVS I/2 T/A	19^b	0.01	0	0	1.00	0.07	0.03	0	0	0	0	0.01	0
3	a	IVS I/1 G/A	133fr	0.03	1.00	0	0.01	0	0.02	0	0	0	0	1.00	–
	b	IVS I/1 G/A	19^c	0.01	1.00	0.01	0.01	0	0	0.01	0	0	0	0	0
4	a	IVS I/1 G/A & IVS I/6 T/C	133fr	0.2	0.25	0	0.2	0	0.05	0	0	0	1.00	1.00	–
	b	IVS I/1 G/A	19^c	0.01	1.00	0.01	0.01	0	0	0.01	0	0	0	0	0
	c	IVS I/6 T/C	19^d	0.1	0	0	0	0	0	0	0	0	1.00	0	0
5	a	IVS I/5 G/T	19^e	0	0	0	0	0	0	0.03	0.02	1.00	0	0	0
	b	CD26 (N)	19^f	0	0	0	0	0	0	0	0	0	0	1.00	0.03
	c	CD26 G/A	19^g	0.03	0	0	0	0	0	0	0	0	0	0.04	1.00
6	a	IVS (N)	75	1.00	0.04	0	0.20	0.05	0.07	0	0	0	0.04	1.00	–
	b	IVS I/2 T/A	Fl 133	0.03	0	0	1.00	0	0.30	0	0	0	0	1.00	–

Microchip I (see Fig. 2) was successively hybridized with RNA 75 and 133 nt long without fragmentation or after fragmentation (133fr, probes 3a and 4a) and with 6 synthetic 19-mer oligodeoxyribonucleotides corresponding to β-thalassemia mutations. The RNA and 19-mers were labeled with TMR except for RNA probes 2a, 2b, and 6b, which were labeled with fluorescein (Fl). The melting curves (Fig. 2) were measured simultaneously for all microchip oligonucleotides at each hybridization. These curves provided values of hybridization intensities at the discrimination temperature, Td. R is the ratio of the hybridization signal of a mismatched duplex (Im) to the signal of the perfect duplex (Ip) estimated at Td in parallel for all microchip oligonucleotides. R = Im/Ip. d_{19}, synthetic 19-deoxymers were complementary to allele-specific 10-mers immobilized on the microchips.

Position of allelic base	19-mer			RNA		
	$T_{0.1}$ of perfect duplex IVS(N)	$T_{0.1}$ of (G-A) mismatched duplex IVS I/2 T/G	$\Delta T_{0.1}$	$T_{0.1}$ of perfect duplex IVS(N)	$T_{0.1}$ of (G-A) mismatched duplex IVS I/2 T/G	$\Delta T_{0.1}$
9	40	32	8	35	37	-2
8	47	32	15	49	38	11
7	42	30	12	44	41	3
6	47	28	19	49	41	8
5	52	38	14	50	42	8
4	54	39	15	54	44	10
3	55	46	9	59	54	5
2	52	46	6	58	53	5

Table 2. The effect of the position of the allelic base within 10-mers on mutation detection.

Microchip II contains two sets of 10-mers corresponding to the normal and IVS I/2 T/G alleles. The microchip was hybridized with the TMR-labeled normal allele 19-mer and to an RNA 75 nt long. $T_{0.1}$ *is the temperature at which the hybridization signals for a microchip duplex drops to 1/10 of its initial value at 0°C.* $\Delta T_{0.1} = T_{0.1}$ (a perfect duplex) $T_{0.1}$ (the corresponding mismatched duplex).

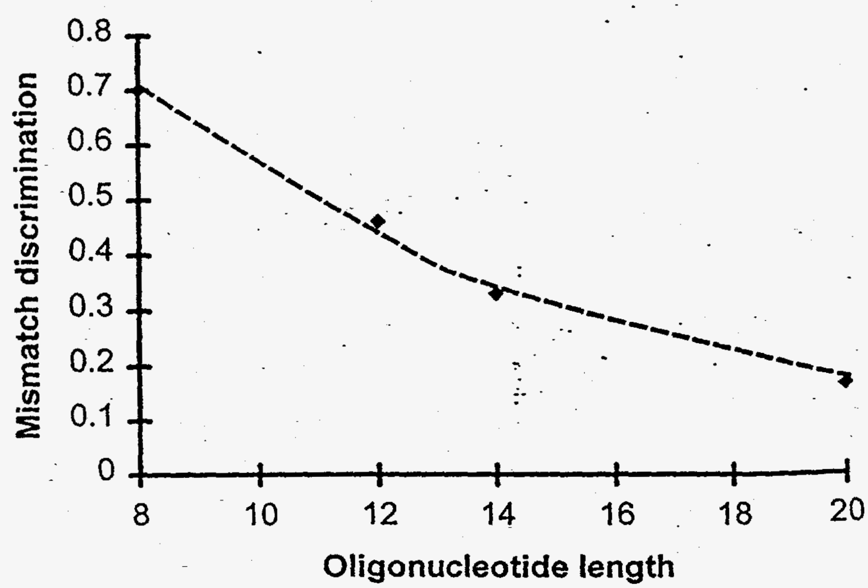


Figure 3. The effect of the length of the immobilized oligonucleotide on the hybridization discrimination of perfect and mismatched duplexes

A 135-nucleotide-long RNA fragment (+45 to +177) was hybridized with a microchip containing oligonucleotides of different length to form perfect duplexes (oligonucleotides "A" in Fig. 4) or t-T mismatched duplexes (oligonucleotides "B"). The mismatch discrimination is calculated as (P-M)/P, where P and M are the hybridization signals of perfect and mismatched duplexes, respectively.