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Metal ion CHElate-aSSisted LIGAtion (CHESS LIGA) for SNP detection on microarrays

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ABSTRACT

We developed a metal ion chelate-assisted ligation for SNP detection by microarray. An oligonucleotide probe was separated into two 9-10-mers bearing iminodiacetic residues at the gap point. Duplex formation with the DNA target was possible only if nickel ions were added, but a nucleotide substitution opposite the gap point prevented duplex formation. Here we demonstrate the application of this approach for SNP detection (A1298C) within the 5,10-methylenetetrahydrofolate reductase gene on a microarray.

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Modified nucleic acids are widely used for inhibition of gene expression (antisense, aptamers, siRNA)[1] and as probes for DNA and RNA detection by hybridization[2] or PCR.[3] Single nucleotide polymorphism (SNP) is the most frequent mutation (more than 1.2 million in the human genome). SNPs cause a number of hereditary diseases[4] or drug resistance (i.e., for HIV-1[5]) and their list increases every year. Recently a lot of approaches for SNP detection have been suggested. Only sequencing gives a general solution for SNP detection. As a result of significant developments in next-generation sequencing,[6] it is the method of choice for personal genomics in the near future. However, today only a limited number of mutations are often identified in the area of interest, so real-time PCR[7] and microarrays[2] are more widely used. Microarrays give more opportunities, but usually suffer from low sensitivity and false-positive results. Single base extension (SBE) technique[8] and solid-phase ligation[9] are the most effective ones, but are not robust enough and are expensive. In this study we developed a metal ion chelate-assisted ligation for SNP detection on a microarray. An oligonucleotide probe was separated into two 9-10-mers bearing iminodiacetic (IDA) residues at the gap point. Duplex formation with the DNA target was possible only if Ni^{2+} ions were added. A nucleotide substitution opposite to the gap point prevented duplex formation. Here we demonstrate the application of this approach for SNP detection (A1298C) within the 5,10-methylenetetrahydrofolate reductase (NADPH) gene (rs18011331) on a home-made microarray.

Ligation of oligonucleotides on NA matrixes by unnatural chemical reactions is widely used for detection of DNAs, cellular RNAs, reaction discovery and drug release.[10] A number of papers deal with the synthesis of oligonucleotides bearing chelating groups. They have been used as artificial nucleases (RNA hydrolysis or DNA oxidative disruption)[11], for luminescent labeling with lanthanide ions,[12] for duplex or triplex stabilization,[13] fluorophor quenching in molecular beacons[14] and DNA detection.[15] Balasubramanian and co-workers[16] gave conclusive proof for enhanced cooperative complex formation by two oligonucleotides linked by metal ion chelation in the presence of a complementary DNA template.

We synthesized oligonucleotides with amino groups at 5′- or 3′-end (Table 1).[17] Two variants of amino group attachment were used: either incorporation of 5′-amino-5′-deoxythymidine (Fig. 1b), 3′-amino-3′-deoxythymidine (Fig. 1a) or 6-aminohexanol (Fig. 1c and d). Oligonucleotides with 3′-amino-3′-deoxythymidine residues were synthesized by reversed DNA synthesis. Carbodiimide-assisted acylation of amino oligonucleotides with nitrilotriacetic acid (NTA) gave desired compounds in high yields. To prevent formation of double/triple conjugates of the oligonucleotides, a large excess of NTA was used. Due to low solubility we used a NTA-based buffer for acylation.[18]

Then we studied the influence of modifications on duplex stability by UV-melting in PBS buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7) (Table 2). A gap in the duplex dramatically decreased ther-

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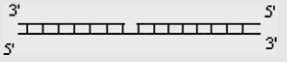
Table 1
Oligonucleotides used in this study

N	5′→3′	MALDI-TOF MS (calcd/found)
1	GTCAGCAAGGTGAG	–
1*	GTCAGCCAGGTGAG	–
2	CTCACCTTGCTGAC	–
3	pTGCTGAC-NH_2	–
4	NH_2-CTCACCT	–
5	pCTCACCT-NH-IDA	2269/2268
6	IDA-NH-TGCTGAC	2269/2268
7	CTCACCT-PO_4^--$(CH_2)_6$-NH-IDA	2369/2368
8	IDA-NH-$(CH_2)_6$-PO_4^--TGCTGAC	2449/2448
9	CTCACCT-**HEG**-TGCTGAC	–
10	CTCACCT-**TTT**-TGCTGAC	–
11	IDA-NH-$(CH_2)_6$-PO_4^--AAAGTGTCTT	3395/3402
12	IDA-NH-$(CH_2)_6$-PO_4^--CAAGTGTCTT	3371/3373
13	$NH_2(CH_2)_6$-PO_4^--T_{20}CCAGTGAAGAAAGTGTCTT	–
14	$NH_2(CH_2)_6$-PO_4^--T_{20}CCAGTGAAGCAAGTGTCTT	–
15	$NH_2(CH_2)_6$-PO_4^--T_{20}CCAGTGAAG-PO_4^--$(CH_2)_6$-NH-IDA	9364/9375
16	GAGGAGCTGACCAGTGAAG	–
17	Biotin-TGGTTCTCCCGAGAGGTAAA	–

HEG—hexaethylene glycol.

Table 2
Duplex stability measured by UV-melting

Oligos in duplex with 1 or 1*	Schematic structure of the duplex	T_m (ΔT_m)		
		0 mM Ni^{2+}	1 mM Ni^{2+}	
		Oligo 1	Oligo 1	Oligo 1*
2		60	nd	50 (−10)
3+4		22 (−38)	nd	nd
5+6		20 (−40)	35 (−25)	–
7+8		19 (−41)	45 (−15)	–
9		43 (−17)	44 (−16)	39 (−21)
10		43 (−17)	44 (−16)	38 (−22)

Nd—not determined.
$\Delta T_m = [T_m(n) - T_m(\mathbf{1}+\mathbf{2})]$.

mal stability (T_m changed from 60 to 22 °C). A slightly negative influence of IDA residues on duplex stability was observed due to charge repulsion (Table 2). First we studied use of magnesium ions for complex formation, but observed a two-phase melting curve that suggested an equilibrium in IDA-Mg^{2+} interactions. Then we used Ni^{2+}, which forms more stable complexes with EDTA (log K = 20.4 vs 8.8 for Mg^{2+}).[19] We expected the same increase in complex stability for two IDA residues that approach each other. Addition of nickel ions significantly increased duplex stability in the case of IDA-oligonucleotides (Table 2). The use of a long linker gave better results in comparison to a short linker (Table 2). The observed duplex stability was close to that obtained for a bulged 17-mer (bulge was formed either by three dT residues or by a hexaethylene glycol insertion). Lower stability in the case of the short linker could be a result of the duplex distortion during chelate formation. In both cases such ligation is reversible and addition of EDTA destroys the complex.

As the aim of this study was to analyze PCR mixtures containing magnesium ions, we made a model experiment. Three duplex-forming oligos were dissolved in PCR buffer (10 mM Tris–HCl, 50 mM KCl, 2.5 mM Mg^{2+}, pH 8.3) and then 2.5 mM EDTA was added to capture magnesium ions followed by 1 mM nickel ions over 1 h. EDTA complexes are kinetically stable under basic conditions, but we observed rather modest stabilization. Only slight duplex stabilization was observed when we changed the buffer to PBS with 2.5 mM Mg^{2+}, 2.5 mM EDTA followed by 3 mM nickel ions or the same without EDTA. The mixture was stored at rt overnight or more to achieve equilibrium, but again only partial duplex

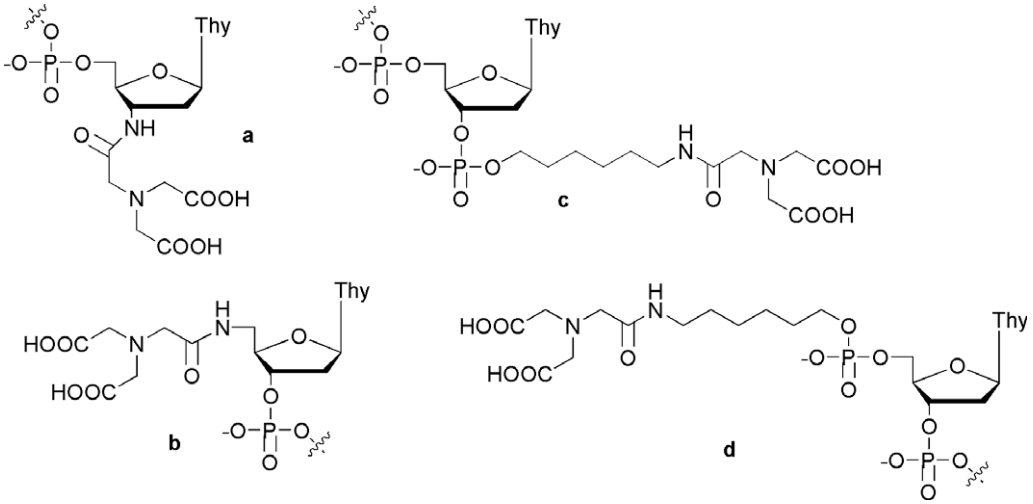


Figure 1. IDA-modified oligonucleotides. a,b—short linkers, c,d—long linkers.

stabilization was observed. So the only way to analyze the PCR mixtures was to remove magnesium ions from the solution and to work in PBS buffer.

Then we designed the microarray assay for SNP detection (Fig. 2). Probe 1 (oligonucleotide **15**) was immobilized on a glass slide, and then we added labeled ssDNA to be studied, probe 2 (oligonucleotides **11** or **12**) and Ni^{2+}. Since hybridization occurs only in case of a perfectly matched duplex, we expected to carry out SNP discrimination. In a control experiment a full length 19-mer oligonucleotide (oligonucleotides **13** or **14**) was immobilized on an aldehyde slide.

We synthesized probes that could be used in the microarray experiments (Table 1). A 5′-MMTr-protected diamino oligonucleotide was 3′-acylated followed by 5′-amino group deprotection (Scheme 1). Probe 1 was immobilized on the aldehyde glass microscope slide.[20]

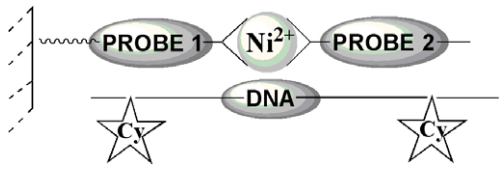
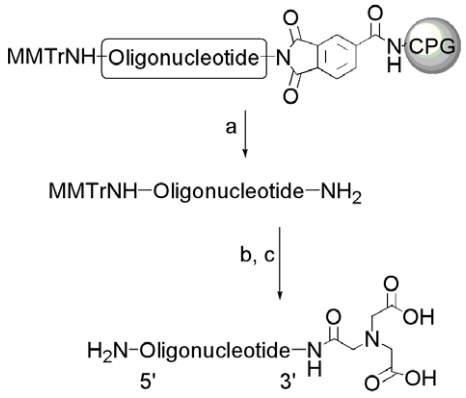


Figure 2. Principle of SNP detection on microarrays based on IDA-modified oligonucleotides.



Scheme 1. Synthesis of probe 1 (Fig. 2A and B). (a) aq ammonia, 55 °C, overnight; (b) NTA, water-soluble carbodiimide; (c) 80% AcOH, 1 h.

The DNA target was labeled during PCR by use of Cy3- or Cy5-dCTP (GE Healthcare). Fluorescently labeled samples for hybridization were generated by nested PCR with primers **16**, **17** (see Table 1). dsDNA (one chain with biotin) was immobilized on streptavidin-coated magnetic beads and one chain was eluted.[21] Then ssDNA and probe 2 (oligonucleotide **11** or **12**) were dissolved in PBS buffer with nickel ions, mixed with hybridization buffer and applied to the microarray area. Hybridization was performed overnight at rt.[22]

If standard 19-mer oligonucleotide **13** or **14** was used for hybridization, only 1.42 and 1.27 discrimination factors were observed respectively (Fig. 3). In the case of the metal chelation assisted ligation, the discrimination factor increased up to 16.7 and 6, respectively. In spite of a slight decrease of fluorescence due to lower duplex stability, the resolution between the two genotypes dramatically increased. We hope that this approach would be widely used for SNP detection.

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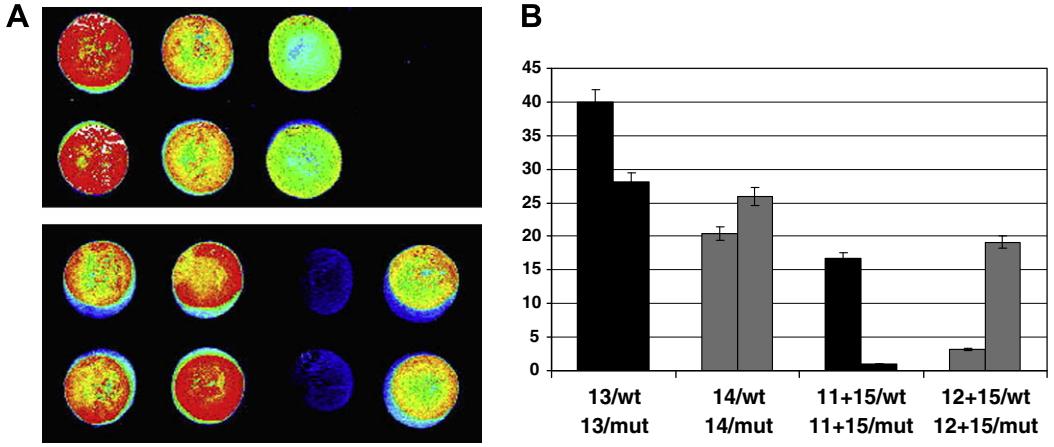

Figure 3. SNP detection on miroarrays based on IDA-modified oligonucleotides. (A) Image of a slide—Cy 3 (upper image) and Cy5 (lower image). (B) Histogramm of fluorescence intensivity.

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17. Oligonucleotides were synthesized by automatic phosphoramidite chemistry (ABI394, Applied Biosystems) and double purified by PAGE and RP-HPLC (Akta Purifier, HPLC column Jupiter 300, C18, 5 μm, 0–50% MeCN gradient in 0.1 M triethylammonium acetate during 50 min). Structures of IDA-modified oligonucleotides were confirmed by MALDI-MS.
18. Amino oligonucleotide (1 OU_{260}) was dissolved in a buffer (40 mM NTA, pH 4.5), 1 mg 1*H*-imidazole and 5 mg of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride were added. After 3 h at rt the oligonucleotide was precipitated by 4% $LiClO_4$ in acetone, dissolved in water and purified by RP-HPLC.
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20. (a) Chizhikov, V.; Wagner, M.; Ivshina, A.; Hoshino, Y.; Kapikian, A. Z.; Chumakov, K. *J. Clin. Microbiol.* **2002**, *40*, 2398; (b) Probes were printed on aldehyde-coated glass slides (Cell Associate, Inc.) using a ChipMaker microspotting device with a single CMP-7 pin delivering approximately 2–3 nl of a spotting mixture per spot (TeleChem International, Inc.). Reaction between amino oligonucleotide (50 μM) and an aldehyde slide was carried out in 0.1 M borate buffer, 50% DMSO (pH 8.0) at 75% humidity and followed by sodium borohydride reduction (0.25% water solution for 5 min). Printed slides were washed with a 0.2% SDS, twice with water and dried.
21. PCR (35 cycles, 30 s at 94 °C, 30 s at 50 °C, and 60 s at 72 °C) was done in 25 μl of a reaction mixture (1× Taq PCR buffer with 2.5 mM $MgCl_2$, 300 nM each primer, 20 μM Cy5-dCTP, 20 μM dCTP, 100 μM each dATP, dGTP, and dTTP, 1U Taq polymerase (Interlabservis), and 0.1 μl of pET-15b plasmid with a variant of 5,10-methylenetetrahydrofolate reductase gene). The resulting dsDNA with one biotinylated chain was immobilized on streptavidin-coated GenoPrep magnetic beads (GenoVision Inc.) according to the manufacturer protocol. The beads were washed twice with 1× TE buffer (Tris–EDTA), and the single-stranded fluorescent probe was eluted by 50 μl of 0.1 M NaOH at rt. The fluorescent probe was desalted by spin centrifugation through CentriSep columns (Princeton Separations) and concentrated by ethanol precipitation. Finally, the fluorescent probe was resuspended in a small volume (5 μl) of water and the probe concentration was measured with a UV spectrophotometer (Nanodrop).
22. Before hybridization, labeled ssDNA and a probe 2 (oligonucleotide **11** or **12**) were dissolved in PBS buffer with 4 mM Ni^{2+}, mixed with an equal volume of 2X hybridization buffer (0.9 M NaCl, 10 mM NaH_2PO_4, pH 7.0 plus 10X Denhardt's solution and 0.2% Tween 20) and denatured for 1 min at 95 °C, followed by chilling on ice for 1 min. Aliquots (2–3 μl) from each sample were applied to the microarray area and covered with a plastic coverslip (4 by 7 mm) to prevent evaporation of the sample during hybridization in the incubation chamber (ArrayIt). Hybridization was performed overnight at rt. Slides were then washed once with 1× PBS containing 0.01% Tween 20, once with 1× PBS and dried. Microchip fluorescent images were obtained at 570 nm (Cy3) and 694 nm (Cy5) by MArS scanner (Ditabis) and analyzed using Quant-Array software (Packard BioScience).