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RESEARCH

Short Oligonucleotide Tandem Ligation Assay for Genotyping of Single-Nucleotide Polymorphisms in Y Chromosome

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Abstract We propose a novel universal methodology, Short Oligonucleotide Tandem Ligation Assay (SOTLA), for SNP genotyping. SOTLA is based on using a tandem of short oligonucleotide (TSO) probes consisting of three fragments: the core oligonucleotide and two flanking oligomers, one of which is immobilized onto a solid support and another one contains the biotin label. TSO is self-associated on a complementary DNA template, forms the complex containing two nicks, which are efficiently ligated with DNA ligase giving biotinylated oligonucleotide covalently bound to polymer beads. No ligation of TSO on an imperfect DNA template bearing the base substitution in the core binding site is occurred. We used SOTLA for the highly selective SNP analysis in different DNA fragments of human Y chromosome. Comparison of SOTLA results with those of PCR-RFLP and allele-specific PCR techniques demonstrates that SOTLA ensures the univocal reliable SNP analysis in different PCR fragments varying in length and base composition. The fundamental difference between SOTLA and well known OLA approaches while using T4 DNA ligase is that the accuracy of SNP analysis in OLA is ensured only by the specificity of ligase while that in SOTLA is provided by the specificity of both ligation and hybridization of TSO probes.

Keywords Single-nucleotide polymorphism · Point mutation · Y chromosome · DNA diagnostics

Abbreviations

SOTLA	Short oligonucleotide tandem ligation assay
Bio	Biotin
BCIP	5-bromo-4-chloro-3-indolylphosphate
NBT	Nitro blue tetrazolium
Stv–AP	Streptavidin–alkaline phosphatase conjugate

Oligodeoxyribonucleotides are abbreviated without prefix "d".

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Introduction

The discerning of single-nucleotide polymorphisms (SNPs) in DNA is of great fundamental importance for investigations of human genome, pharmacogenetics, and genetic diseases. The revealing of DNA polymorphisms is of great importance in human population and evolutionary genetics [1–4]. DNA-based reconstruction of human population history, started initially with maternally inherited mitochondrial DNA, now exploits the wide range of DNA sequence variation, including SNPs, micro- and minisatellites, and mobile genetic elements located both on autosomes and sex chromosomes. Y chromosome used for reconstruction of male lineages has similarity with mtDNA due to its behavior in the uniparental mode of inheritance and to the lack of recombination in the non-recombining part. Until recently, population studies of Y chromosome

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were hampered by the limited number of the known DNA sequence variations in non-recombining region of the Y chromosome (NRY) [5]. Recent developments have now revealed a variety of available polymorphic markers, which can be powerful molecular tools for the detailed study of the Y chromosome diversity [6–10].

Two types of DNA markers are currently used for Y chromosome haplotyping: biallelic, or binary, loci such as base substitutions and some insertions–deletions [3, 5, 6, 11, 12] and multi-allelic systems such as microsatellites [13–16]. For the massive genotyping and screening of new markers, powerful methods such as denaturing high-performance liquid chromatography (DHPLC) and sequence analysis [3, 6–8] are still unsurpassed. They are, however, too laborious for population screening of any known SNPs markers. Some known Y-linked SNPs markers can be analyzed by the PCR-RFLP technique [11, 17–19] but the application of this technique is confined within the range of some restriction site sequences.

Recent analyses of a large set of binary polymorphisms associated with NRY, most of which were discovered by DHPLC, revealed the global pattern of Y chromosome lineages evolution in human populations and put some insight into the demographic and population history of modern humans [6, 7, 12, 20, 21]. Some of the known Y chromosome biallelic polymorphisms are geographically or ethnically specific. Investigation of polymorphisms restricted to modern Eurasian populations is of special interest for reconstruction of population history in North Eurasia [18, 19, 22–24]. For this purpose it is necessary to develop effective and reliable methods of testing SNPs.

The enzymatic ligation of oligonucleotide probes on a DNA template (Oligonucleotide Ligation Assay: OLA) is a promising and convenient approach to the single-nucleotide polymorphism genotyping and point mutations testing. Accuracy of SNPs analysis is usually provided by the specificity of DNA ligase. Ligation is known to be much less efficient if a non-complementary pair in a probe-template complex is close to the ligation site [25–28]. It is obvious that lengthy oligonucleotide probes can form stable both perfect and imperfect complexes with DNA. If the formed complex has only one nucleotide discrepancy, its melting temperature (T_m) and other physicochemical properties are close to those of the perfect complex. Sometimes the ligation also takes place in imperfect complexes. It is known that the specificity of DNA ligase is not high enough to completely distinguish base pair mismatches [25, 27, 29]. The efficiency of ligation with T4 DNA ligase of two lengthy oligonucleotide probes bearing a wrong base at the end of an oligonucleotide depends on the combination of bases forming the mismatch [29]. Short oligonucleotides are more sensitive to SNP but they have many binding sites and form unstable complexes.

We have earlier investigated properties of a tandem of short oligonucleotides (TSO) in order to evaluate a possibility of their application as oligonucleotide probes [30, 31]. The study was fulfilled using a model oligonucleotide template. In this case TSO (for example, 8+4+8 mers) were self-associated on complementary DNA. Duplex formed by the central tetramer and DNA was stabilized by adjacent oligonucleotides. If one or both adjacent oligonucleotides were absent, the core short oligonucleotide could not form the stable complex. Complementary DNA analyte and TSO formed the perfect complex containing two nicks, which were ligated. Using the model oligonucleotide systems we have shown that single-base substitutions in DNA (20-mer) can be identified with a high accuracy by ligation of TSO with T4 DNA ligase. The formation of the 20-nt ligated product was shown to occur only when the tetramer was completely complementary to its binding site on the DNA analyte. Both high selectivity of hybridization of short oligonucleotides and high specificity of ligation of three-component tandems provide the efficient discrimination of single-base substitutions in the tetramer-binding site on a DNA template [31, 32].

Here we propose a novel SNP genotyping platform designated as of Short Oligonucleotide Tandem Ligation Assay (SOTLA). This method is based on the use of the tandem of short oligonucleotides (TSO) as oligonucleotide probes intended for ligation on the complementary DNA template (the double stranded PCR fragment). The genotyping of a number of known Y-linked SNPs in human Y chromosome was carried out by SOTLA, and the results were compared with those of most common techniques such as PCR-RFLP and allele-specific PCR.

Materials and Methods

The general approach to the choice of the oligonucleotide tandems was identical to that described previously [33] with the exception that the oligonucleotide sequences were SNP locus-specific. All reagents and conditions were the same, if not specified.

Oligonucleotides were synthesized by the common phosphoramidite protocol on an ASM-700 synthesizer (Biosset, Novosibirsk, Russia) using the solid support and phosphoramidite synthons from Glen Research (Sterling, Verginia, USA). The synthesized oligomers were deblocked and removed from the CPG support by the treatment with 25% ammonia at 56°C for 8 h. Deprotected oligonucleotides were isolated by successive HPLC on ion-exchange (Polysil SA-500, Theoretical Practice, Novosibirsk, Russia) and reverse phase (LiChrosorb RP-18, Darmstadt, Merck, Germany) columns. All oligonucleotide samples were at least 98% pure according to electrophoretic analysis in 20%

denaturing PAG. Oligonucleotide spots on electropherograms were stained with the Stains-all reagent (Sigma–Aldrich Inc., USA). Concentrations of oligonucleotides and their derivatives were measured spectrophotometrically using the summarized molar absorption coefficient (ε_{260}) of mono- and di-nucleotides at 260 nm [34]. Immobilization of oligonucleotides onto polymethacrylic beads (DMEG-7, 30–60 μm) was carried out in accordance with the published procedure [35].

SNP Genotyping with PCR-RFLP

Y-chromosomal regions containing SNP were amplified using primers and PCR conditions described previously [6, 17, 33]. M 46 (Tat), M130, and SRY 1532 PCR products were digested with appropriate restriction enzyme, separated on agarose gel and visualized with ethidium bromide. Tat PCR products were digested with *Hsp92II* restriction endonuclease (Promega, USA). The **C** allele product was resistant to *Hsp92II* cleavage, while the **T** allele product was cleaved by the enzyme, yielding the 81- and 29-bp fragments. The M130 PCR products were digested with *Bsc4I* restriction endonuclease (SybEnzyme, Novosibirsk, Russia). **T** allele (the absence of the restriction site) corresponds to the 205-bp fragment, and **C** allele (the presence of the *Bsc4I* site) corresponds to the 159- and 46-bp fragments. SRY 1532 PCR products were treated with *AdeI* (MBI Fermentas, Vilnius, Lithuania) producing the 167-bp fragment (allele **A**), or the 122- and 55-bp fragments (allele **G**).

M89 SNP was genotyped by allele-specific PCR using the forward primer described in [6] and the modified reverse primers specific to either **C** or **T** alleles [18].

SNP Genotyping by the Tandem of Short Oligonucleotide Ligation Assay (SOTLA)

PCR products were precipitated overnight from the PCR mixtures in the presence of 2 M ammonium acetate by addition of 3.2 volumes of ethanol at –20°C and centrifuged then at 14000 rpm for 15–20 min. The precipitates were washed by 400 μl of an ethanol–water (4:1, v/v) mixture, dried, and redissolved in double distilled water in one-two fifth of the initial volume of the PCR sample. Just before adding to the SOTLA reaction mixture, the DNA solution was heated at 95–100°C for about 5 min.

A standard SOTLA sample consisted of polymethacrylic beads (30–60 μm, 0.5 mg) bearing immobilized 5′-flanking oligonucleotide (1 μmol/g) and solution (15 μl) containing tetranucleotide (10^{-4} or 10^{-5} M), biotinylated 3′-flanking oligonucleotide (10^{-5} M), T4 DNA ligase (30–50 U), and DNA template (PCR product) in ligase buffer (10 mM $MgCl_2$, 0.1 M NaCl, 10 mM DTT, 1 mM ATP in 20 mM Tris–HCl, pH 7.5) [32]. The preheated DNA sample (equivalent to 5–10 μl of the standard PCR mixture) was added to the reaction mixture at the last moment. The ligation was carried out at 30–37°C for 30 min in thermostat (Eppendorf, Germany) with the occasional stirring of the beads. The reaction was stopped by the addition of 1 ml EDTA-Na_2 (20 mM, pH 7.5–8.0) followed by the heating of the sample at 100°C for 5 min.

The polymer beads containing the immobilized biotinylated ligation products were washed, incubated with the Stv ~ AP conjugate (Sigma–Aldrich, USA) and, after additional washing, treated with BCIP/NBT chromogenic substrates (Molecular Probes, Eugene, Oregon, USA) in the manner described earlier [32].

Microtiter panel with the SOTLA beads after revealing the immobilized ligation product was scanned with a "PowerLook 1000" (UMAX, Taipei, Taiwan).

Results

The sequences of the oligonucleotide components are listed in Table 1. The oligonucleotide tandems were designed so that the melting temperatures of complementary duplexes formed by core tetranucleotides and DNA were in the range of 25–37°C, which was optimal for T4 DNA ligase

Table 1 Oligonucleotide sequences of tandems for the SOTLA typing of the single-nucleotide substitutions in human Y chromosome

SNP test system	5′-flanking oligonucleotide of tandem (5′-immobilized)	Central tetranucleotide of tandem (variable)	3′-flanking oligonucleotide of tandem (3′-biotinylated)	Analyzed amplicon (length/SNP position and type)
SRY 1532	pCTTTTTCACAC	pAGT**A**	pTAACATTTTCp**Bio**	167 bp/115 **G** → **A**
		pAGT**G**		
M46 (Tat)	pTTGTGAAT	pTCA**T**	pGTTGTTTTp**Bio**	112 bp/28 **T** → **C**
		pTCA**C**		
M130	pCCTTGGAT	pTTC**T**	pCTGCCCAGp**Bio**	205 bp/41 **C** → **T**
		pTTC**C**		
M89	pTGTACAAAAA	pTCT**T**	pATCTCTCACp**Bio**	527 bp/347 **C** → **T**
		pTCT**C**		

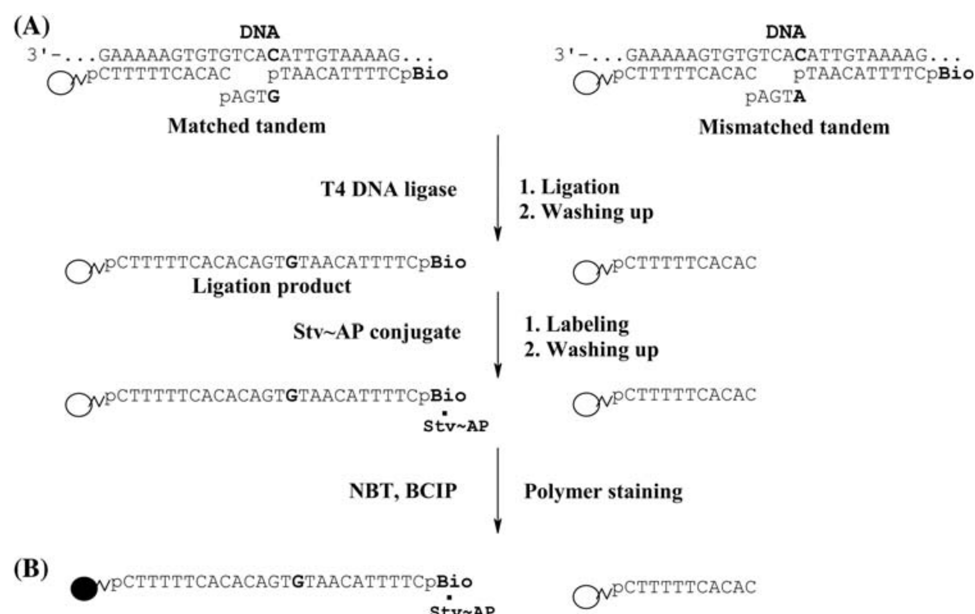

Fig. 1 Scheme of the SOTLA testing protocol. **a** Typical scheme of revealing SRY 1532 G allele. **b** Scanned image of the panel after SRY 1532-SOTLA testing of DNA samples [1–10]; **T25**, the synthetic oligonucleotide template identical to SRY 1532 **G** allele

[27, 36, 37]. In this case the enhanced reliability of the DNA testing by SOTLA is achieved due to the high selectivity of hybridization between short probes and DNA. The structures of oligonucleotide tandems for typing the single-nucleotide substitutions in human Y chromosome and characteristics of the corresponding DNA analytes are presented in Table 1.

The SNP analysis of DNA by the SOTLA technique is presented in scheme (Fig. 1a) by the example of testing SNP (A → G transition) in SRY 1532 marker within the promoter region of SRY gene. The non-radioactive SOTLA test-system contains three short oligodeoxyribonucleotide components: 5′-flanking oligomer immobilized on the solid support, tetranucleotide (the selective probe), and 3′-flanking oligomer containing the biotin residue at the 3′-end. In the presence of T4 DNA ligase and the complementary DNA template corresponding to SRY 1532 **G** allele in this case, these short oligonucleotides are self-associated on the template, form the complementary complex, and are efficiently ligated at 37°C giving the 25-mer biotinylated oligonucleotide immobilized covalently on the polymer beads. The biotin residues on the bead surface can be revealed by the standard procedure (streptavidin–alkaline phosphatase conjugate plus chromogenic substrates) resulting in staining beads. In the presence of the imperfect DNA template, such as SRY 1532 **A** allele in this instance, containing the base substitution in the tetranucleotide binding site, both instability of the tandem complex and a mismatch in the ligation site inhibit ligation of the oligonucleotides and prevent attachment of the biotin residues to the beads. Thus, in accordance with the structure of tetranucleotide pAGT**G** or pAGT**A** used in the analysis, the stained samples reveal the SRY 1532 **G** or SRY 1532 **A** genotype, respectively.

For each DNA sample we performed SRY 1532-SOTLA tests using either pAGT**G** or pAGT**A** tetranucleotides. The additional tests either without the DNA template or in the presence of the synthetic oligonucleotide template were performed as negative or positive controls, respectively.

Ten SRY 1532 PCR samples (five **A** and five **G**) were chosen randomly from the set of amplicons characterized earlier by RFLP techniques and were tested with SRY 1532-SOTLA at the tetranucleotide concentration 10^{-5} M at 37°C. The SRY 1532-SOTLA genotyping of 10 DNA samples (Fig. 1b) allows one to reveal easily the corresponding allele due to the absolute difference between the signals “yes” or “no”. In all cases the SOTLA results are similar to the data of RFLP tests (data not shown).

Another example of SNPs in Y chromosome is M46 (Tat) mutation. Tat is the T → C transition within the RBF5 locus in proximal Yq [38] and lies within the recognition site for *Hsp92II* and *MaeII* endonucleases. [33], who identified this mutation, developed the PCR-RFLP protocol for its genotyping. The analyzed DNA samples included subsets from Altai and Kirghiz populations described elsewhere [22, 23]. PCR amplification of 112-bp fragments containing the CA**T**GT (Tat **T** allele) or CA**C**GT (Tat **C** allele) segments was carried out using Tat1 and Tat3 primers as described by [33]. Using the PCR-RFLP protocol, genotypes of 96 males from Altai and Kirghiz populations were determined. Ten samples (five Tat C and five Tat T) were chosen randomly for further experiments. For each of 10 chosen samples, the 112-bp PCR product was isolated and analyzed by SOTLA at the

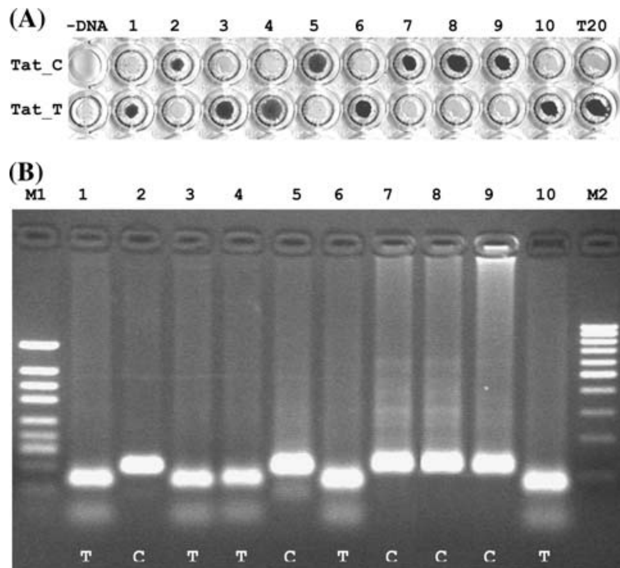

Fig. 2 Analysis of M46(Tat) DNA fragment of human Y chromosome. **a** Scanned image of the panel after M46-SOTLA testing of ten DNA samples. **T20**, the synthetic oligonucleotide template identical to M46 **T** allele. **b** Tat PCR-RFLP testing of ten DNA samples. M1, pUC19/MspI marker DNA; M2, 100-bp marker DNA

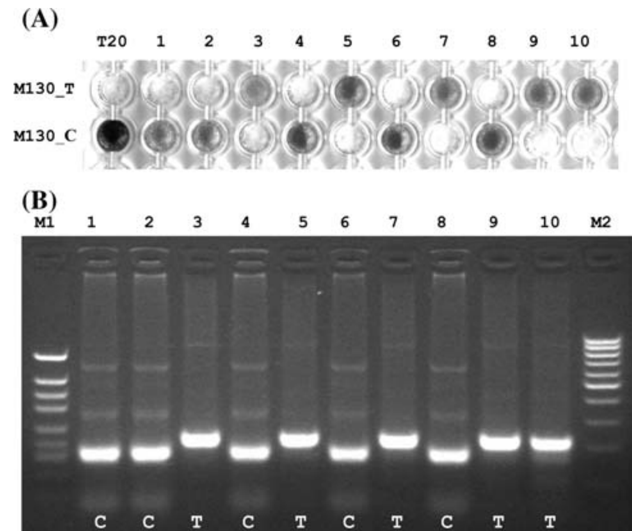

Fig. 3 Analysis of M130 DNA fragment of human Y chromosome. **a** Scanned image of the panel after M130-SOTLA testing of ten DNA samples. **T20**, the synthetic oligonucleotide template identical to M130 **C** allele. **b** M130 PCR-RFLP testing of ten DNA samples. M1, pUC19/MspI marker DNA; M2, 100-bp marker DNA

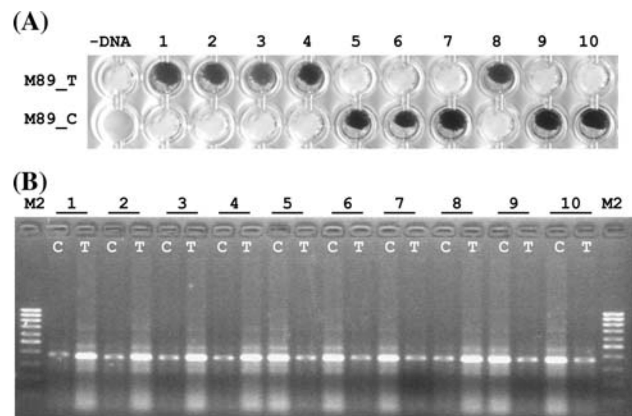

Fig. 4 Analysis of M89 DNA fragment of human Y chromosome. **a** Scanned image of the panel after M89-SOTLA testing of ten DNA samples. **b** M89 allele-specific PCR testing of ten DNA samples: **M2**, 100-bp DNA marker

tetranucleotide concentration 10^{-4} M at 30°C. The data of Tat-SOTLA and the Tat PCR-RFLP assay for the corresponding samples (Fig. 2a, b, respectively) demonstrate that the results of genotyping obtained by these two techniques are identical.

The M130 marker is the T → C transition within the RPS4Y locus is the additional SNP, which was genotyped by the PCR-RFLP approach in the same subsets from Altai and Kirghiz populations. PCR amplification of the 205-bp M130 fragment containing **C/T** SNP at position 41 was carried out using primers and reaction conditions described by [6]. Ten randomly chosen M130 PCR samples (five **C** and five **T**) were tested by M130-SOTLA at the tetranucleotide concentration 10^{-4} M as described above. The comparative results of M130 genotyping both by SOTLA and PCR-RFLP approaches (Fig. 3) show the absolute coincidence between these genotyping techniques.

The comparison between the SOTLA technique and allele-specific PCR was performed with the use of M89 marker, which is characterized by **T** → **C** transition in the non-coding part of Y chromosome. We used the allele-specific PCR genotyping of M89 described in [18]. PCR amplification of 527-bp M89 fragment containing **C/T** SNP at 347 position was carried out using the forward primer described in [6] and the allele-specific reverse primers as described by [18]. Ten randomly chosen M89 PCR samples (five **C** and five **T**) were tested with M89-SOTLA as described above for Tat with the exception that tetranucleotide concentration was 10^{-5} M for M89. The results of M89-SOTLA genotyping of 10 DNA samples are shown in Fig. 4. In this case, the results of genotyping based on the difference in the amount of the amplicons in the electrophoretic bands obtained with the allele-specific primers are transformed to reliable ("yes" or "no") colorimetric signals of M89-SOTLA tests.

Discussion

We propose a new simple and reliable methodology for the detection of SNPs. It is based on ligation of a cooperative tandem of short oligonucleotides complementary to the

binding (normal or mutant) site of an analyzed DNA template. To provide the proper interaction with absolutely unique sites in nucleic acids, 20–30-mer oligonucleotides are usually used. Extended oligonucleotides having high hybridization ability form, however, not only perfect, i.e., completely complementary complexes but also stable enough complexes containing mismatches. Therefore, the accuracy in the revealing of the SNPs in the case of the extended probes is ensured only by the ligase specificity. The ligation efficiency in imperfect complexes is lower than that in perfect complexes and depends on the combination of bases forming mismatch. For example, the probe forming the G–T mismatch with DNA is ligated more efficient than in the case of G–G or G–A base pairs by factors of 15–20 [29].

Short oligonucleotides are known to be more sensitive to mismatches in their binding sites. On the other hand, short oligonucleotides can form unstable duplexes with many DNA sites. Disadvantages of either extended or short oligonucleotides are virtually overcome with the use of tandems consisted of derivatives of short oligonucleotides because all of the tandem components are assembled only on that DNA region where there is a full site of their binding. The shortest core tetranucleotide is very sensitive to point mutation in its binding site. Its complex with DNA is stabilized by the flanking oligomers, one of which is immobilized on the solid support and another one contains the biotin residue. In the presence of T4 DNA ligase short oligonucleotides are self-associated on the DNA template, form the complementary complex having two nicks, which is efficiently ligated at 30–37°C giving a full-length signal product. In the case of the imperfect DNA template containing any base substitution in the tetranucleotide binding site, both instability of the DNA-tetranucleotide complex and the mismatch in the ligation site completely inhibit ligation of the tandem of short oligonucleotides. The fundamental difference between SOTLA and OLA approaches while using ordinary T4 DNA ligase is that the accuracy of SNPs analysis in the OLA system is ensured only by the specificity of DNA ligase while that in the SOTLA system is provided by the specificity of both ligation of three-component tandems and hybridization of short oligonucleotides within specific region of a DNA template [31, 32].

The discrimination factor, which is the ratio of the ligation products in the perfect and mismatched complexes, can be increased if the reaction is carried out at the temperature equal to the melting temperature for the perfect duplexes. This expedient was used in the Tobe's work [39]. The rather high ligation specificity in this case is achieved due to the use of thermostabile Ampligase DNA Ligase, which allows the reaction to be carried out at the high temperature (58–93°C). The accuracy of the analysis in this case [39], as in our SOTLA system, is ensured not only by the ligase specificity but also by the selectivity of hybridization of probes in the complex under ligation. The observed values of the discrimination factors are varied in the range from 60 to 260 [39] and achieve the values of more than 300 in case of ligation of TSO probes [31]. These results show that the only and the universal variant to provide the high accuracy in the SNP analysis when utilizing mesophilic DNA ligases (e.g., available T4 DNA ligase) is the use of three-component probes. Only in this case the melting temperature of the duplex formed by the mismatch-sensitive probe (the central tetramer) can be close to the temperature of the ligation reaction. Only in the case of ligation of TSO, biotinylated oligonucleotide is bound covalently to the polymer beads. Covalent attachment of the biotin residue to the support allows thorough washing without the loss of signals intensity. The biotin residues on the bead surface can be revealed by the standard procedure resulting in staining beads (Figs. 1, 2a, 3a, 4a). The comparative data (Figs. 2, 3, 4) showed the excellent agreement between the new SOTLA approach and the known PCR RFLP and allele-specific PCR tests. At the same time, the proposed systems are universal, simple, more rapid, and less expensive as compared to the conventional hybridization techniques.

The SOTLA test systems enable identification of SNP in any nucleotide position in a tetramer hybridization site on the target DNA [30]. This assay allows univocal and reliable genotyping of SNPs. The presented data demonstrate that SOTLA can be applied for the reliable analysis of SNPs within different PCR fragments varying in lengths and base composition around the polymorphic site. One of the important features of SOTLA is that, unlike the majority of the other SNP-genotyping methods, it is sequence-independent. This method can be useful for evolutionary and biomedical studies and for massive genotyping of any SNPs in human population. SOTLA can be used as a basis for the elaboration of any other SNP revealing tests taking into account that the sequences of tandem components, length of flanking oligonucleotides, tetranucleotide concentration, and ligation temperature should be optimized for each particular case. This technique can be, moreover, applied not only to haploid systems such as Y-linked polymorphisms, but also for diploid, i.e., autosomal markers. SOTLA can be used for the analysis of any prokaryotic or eukaryotic genome.

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