ELSEVIER

Genetic Analysis: Biomolecular Engineering
15 (1999) 35–40

GENETIC ANALYSIS
Biomolecular Engineering

Rolling circle amplification of DNA immobilized on solid surfaces and its application to multiplex mutation detection

Anson Hatch *, Takeshi Sano, John Misasi, Cassandra L. Smith

Center for Advanced Biotechnology and Departments of Biomedical Engineering and Biology, Boston University, 36 Cummington Street, Boston, MA 02215, USA

Accepted 7 August 1998

Abstract

A new method of amplifying short DNA molecules immobilized on a solid support has been developed. This method uses a solid-phase rolling circle replication reaction, termed rolling circle amplification (RCA). The probe consists of a single-stranded DNA primer anchored at the 5′ terminus to a solid support and a single stranded DNA template hybridized to the immobilized primer. Here, DNA ligase was used to circularize the template, and DNA polymerase I was used to extend the immobilized primer in a rolling circle replication reaction. This method was used to identify a known polymorphism in BRCA1 exon 5. These results demonstrate that RCA offers considerable promise to facilitate effective mutation screening of DNA using a solid-phase format.

Keywords: Rolling circle amplification; DNA; Multiplex mutation detection

1. Introduction

The necessity of examining stretches of DNA for polymorphisms has rapidly increased with the rate of discovery of diseased genes. Due to the large number of mutations possible in genomic DNA and their diverse relationship to disease, a rapid, comprehensive, and cost-effective method of scanning these genes is essential for diagnostic and research purposes. A reduction in cost two orders of magnitude below the cost of direct sequencing may be necessary for effective genomic screening [1]. Many methods have been developed to screen DNA for mutations, which can be divided into two general categories; solid-phase methods using probes bound to a solid support, and methods requiring size fractionation by electrophoresis and other separation methods.

Examples of methods using size fractionation include single-strand conformation polymorphisms (SSCP) [2], denaturing gradient gel electrophoresis (DGGE) [3], combined chain reactions (CCR) [4], and enzyme mismatch cleavage [5,6]. Electrophoretic methods are commonly used to screen genes for mutations because of their ease of use and simplicity. An enzyme or chemical reaction is used to specifically identify a mutation followed by gel electrophoresis to analyze the results. The use of electrophoresis requires a large amount of DNA for analysis, usually obtained through PCR, and requires great effort to process large numbers of samples. These methods can be anywhere from 80 to 100% accurate in detecting a mutation, but they are less than an order of magnitude below the cost of direct sequencing. Electrophoretic separation may never reach the accuracy and cost requirements necessary for effective genomic screening.

Solid-phase scanning of genomic DNA, though complex, holds more promise in reducing cost and providing complete accuracy. It is not clear that any

* Corresponding author.

1050-3862/99/$ - see front matter
PII S1050-3862(98)00014-X

solid-phase method yet provides the cost-savings and accuracy necessary for effective genomic screening [1]. Solid-phase methods include: sequencing by hybridization (SBH) [7–11], trapped oligonucleotide nucleotide incorporation (TONI) [12], multiplex allele-specific diagnostic assay (MASDA) [13], and solid-phase chemical cleavage (SPCC) [14]. Solid-phase approaches allow the screening of large numbers of DNA sequences in parallel, which can significantly reduce the time and materials required for detecting mutations. These methods may also be automated, which could further reduce cost and increase throughput. Current solid-phase methods are limited by specificity. Conventional SBH is not highly accurate (based on physical hybridization alone). Additional refinements such as competition strategies [9,10], excessive quantities of probes and/or target [10,15], and the use of enzymes [7,10,15,16], are necessary to increase the specificity of a solid-phase method.

Another limiting factor in solid-phase screening is signal intensity. Solid-phase methods currently rely on the use of large numbers of probes isolated at different locations on a solid support as well as high concentrations of target material, which is inefficient and costly. PCR amplification can increase a small number of probes or targets by several orders of magnitude in solution, but PCR is difficult to incorporate into a solid-phase approach because the amplified DNA is not immobilized on the surface [17]. A strategy that amplifies the signal from small numbers of initial probes could significantly increase the effectiveness of solid-phase methodology.

Here, a new method of amplifying short DNA molecules immobilized to a solid support is described, termed rolling circle amplification (RCA). RCA targets specific DNA sequences through hybridization and a DNA ligase reaction. The circular product is then used as a template in a rolling circle replication reaction.

2. Materials and methods

2.1. Oligonucleotides

Oligonucleotides (Table 1) were purchased from Operon Technologies (Alameda, CA). DNA was labeled using T4 polynucleotide kinase. A 10-pmol DNA template was labeled with ^{32}P in a 50-μl volume containing 70 mM Tris–HCl (ph 7.6)/10 mM $MgCl_2$/5 mM dithiothreitol/0.2 μM [γ-^{32}P]ATP (0.8 mCi/mmol, 1 Ci = 37 GBq; Amersham). The 5′ terminus of the DNA template was phosporylated by adding 10 U of T4 polynucleotide kinase (New England Biolabs) and incubating at 37°C for 3 h.

2.2. Immobilization of DNA probe complex

A mixture of a 5′ biotinylated priming oligonucleotide (5 pmol) and template oligonucleotide (10 pmol) containing 22 bases complementary to the 3′ terminus of the priming oligonucleotide (Figs. 1 and 2) was heated to 95°C and slowly cooled to room temperature to allow formation of a double stranded complex. The probes were immobilized on streptavidin coated paramagnetic beads (0.25 mg, Dynal, Oslo). The beads were washed three times with 50 μl of TE (10 mM Tris–HCl, pH 8/1 mM EDTA (ethylene diamine tetraacetic acid)) to remove unbound probes and finally suspended in 50 μl of TE. Binding of the probe complex using 5′ labeled template was evaluated by Cerenkov counting. Counts of the initial supernatant containing unbound template were compared to counts of washed beads containing immobilized probes to assess binding efficiency.

2.3. Ligation of immobilized probes

Two target sequences of BRCA1 from exon 5 were used. One target (N) contained the normal sequence and a second target (M) contained a G for T substitution in codon 64 (Table 1). The template sequence contained ten bases at the 5′ terminus and ten bases at the 3′ terminus complementarily juxtaposed to the normal sequence of BRCA1 exon 5 (Fig. 2). The 5′ terminus of the template DNA was complementary to the position of the point mutation in codon 64. Beads with 5 pmol immobilized probes were washed with 50 μl of 1 × T4 DNA ligase buffer containing 50 mM Tris–HCl (pH 7.5)/10 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP/25 μg/ml bovine serum albumin. Beads and immobilized probes were suspended in 50 μl of 1 × T4

Table 1
Oligonucleotides

Name	Sequence
Primer	5′-b-$(T)_{20}$GCGGCCGC$(T)_{15}$-CGCCAGGGTTTTCCCAGTCACGAC-3′
Template	5′-ATAAAGGACATT-GAATTCGTCGTGACTGGGAAAACC-CTGGGATCCTTTCATTCTTAC-3′
Target N	5′-CACAGTGTCCTTTATGTAAGAAT-GATATAAC-3′
Target M	5′-CACAGTGTCCTTTAGGTAAGAAT-GATATAAC-3′

The primer oligonucleotide has a biotinylated 5′ terminus. The template oligonucleotide contains 22 central bases (underlined) complementarily to the 3′ terminus of the primer sequence. The ten (underlined) 5′ and 3′ terminal bases of the template DNA are complementary juxtaposed to the normal sequence of BRCA1 exon 5. Target N contains the normal sequence of BRCA1 exon 5 and target M contains a G for T substitution indicated in boldface type.

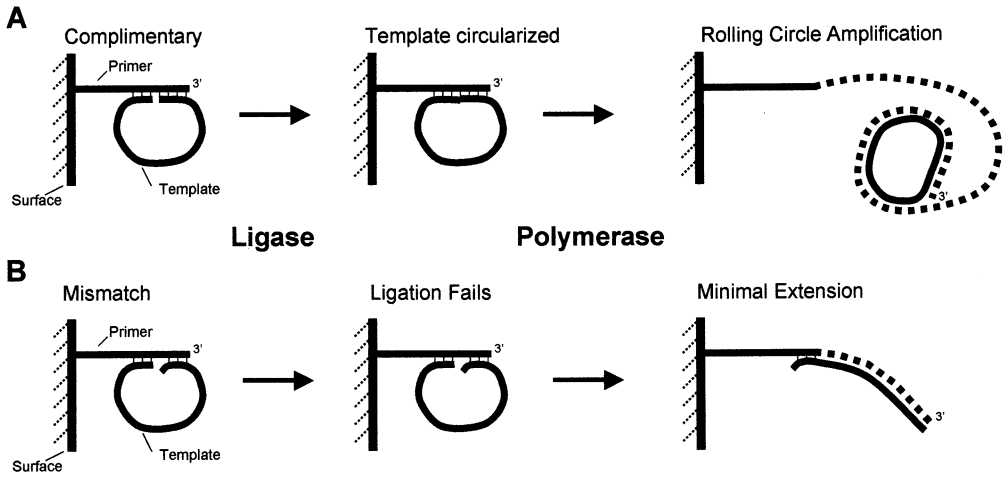

Fig. 1. Solid-phase rolling circle amplification. (A) Template is perfectly complementary at the 3′ and 5′ termini to the primer sequence. Ligase circularizes the template, and polymerase enzyme extends the primer in a rolling circle replication reaction. (B) The template strand is not perfectly complementary, at the 3′ and 5′ termini, to the DNA primer sequence. Ligation fails, and polymerase extends the primer to complement the linear template.

DNA ligase buffer containing 10 pmol target DNA. Mixtures were incubated at room temperature for 15 min with 40 U of T4 DNA ligase (New England Biolabs). Ligation reactions were terminated by adding 1 μl of 0.5 M EDTA. The beads were washed three times with 50 μl of TE and finally suspended in 50 μl of TE. Samples were incubated with 1000-fold excess biotin to binding capacity and heated to 75°C for 30 min to displace the biotinylated product from the beads. The displaced product was fractionated using denaturing polyacrylamide gel electrophoresis and the results quantified using the Storm 860 phosphoimager (Molecular Dynamics).

2.4. Rolling circle amplification of immobilized probes

Beads containing product of the ligation reaction were washed once with 50 μl of 1 × extension buffer (10 mM Tris–HCl (pH 7.5)/5 mM $MgCl_2$/7.5 mM dithiothreitol) and suspended in a final volume of 50 μl

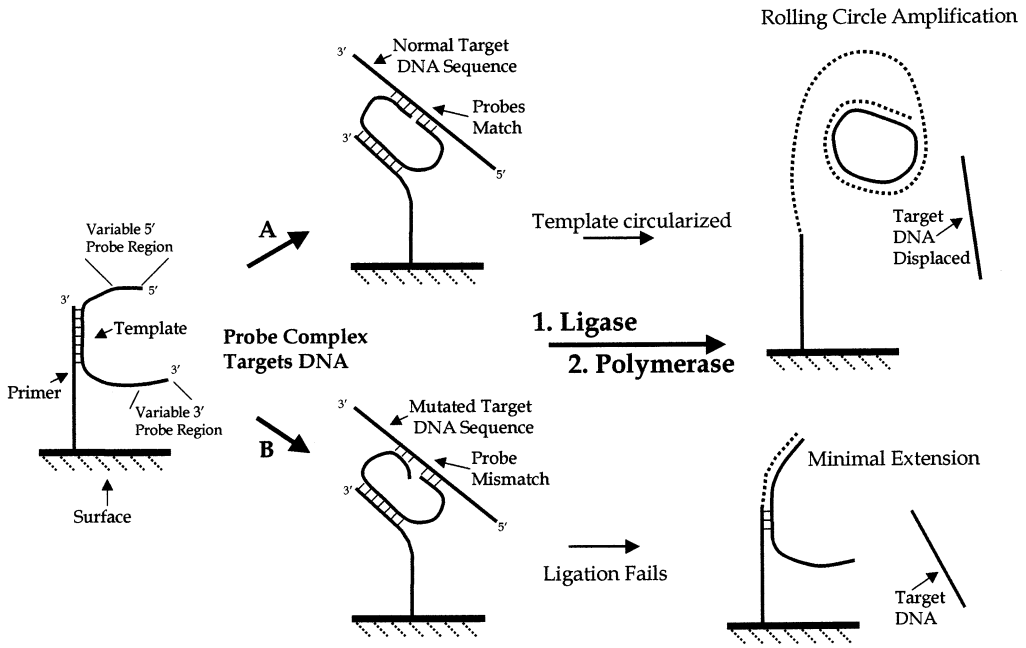

Fig. 2. Applying RCA to probe for polymorphisms. (A) Template is perfectly complementary at the 3′ and 5′ termini to the normal target DNA sequence. Ligase circularizes the template, and polymerase enzyme extends the primer in a rolling circle replication reaction. (B) The template strand is not perfectly complementary at the 3′ and 5′ termini to the mutated target DNA sequence. Ligation fails, and polymerase extends the primer complementary to the linear template.

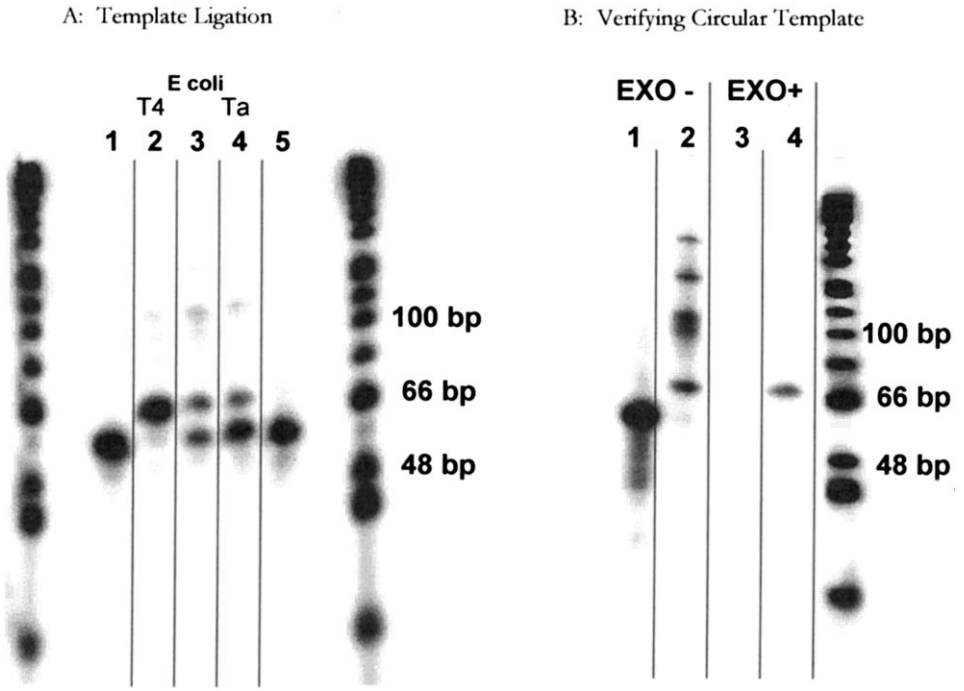

Fig. 3. Ligation of template DNA. (A) Linear 57-base DNA template (1,5) is circularized by ligase (2, T4; 3, *E. coli*, 4, Taq). Circular template is observed as a band shifted upward closer to the 66-base size standard. (B) Template circularization is verified by treating linear template (1) and ligated product under conditions generating several bands of product (2) with exonuclease (3, treated template; 4, treated ligation product).

1 × extension buffer containing 50 μM dATP/50 μM dTTP/50 μM dGTP/50 μM dCTP/2 μM [α-^{32}P]dCTP (0.8 mCi/mmol, 1 Ci = 37 GBq; Amersham). The 3′ terminus of the priming strand was extended by adding 2.5 U of DNA polymerase I *Escherichia coli* (Boehringer Mannheim) to the 50-μl reaction and incubating at 37°C for 3 h while mixing. Reactions were terminated by adding 1 μl of 0.5 M EDTA. The beads with bound product were washed once with 50 μl of TE and then transferred to another Eppendorf tube. The beads were then washed four times with 50 μl of TE to remove any unincorporated [α-^{32}P]dCTP. The incorporated radioactivity was measured by Cerenkov counting. Product was displaced from the beads by incubation with 1000-fold excess biotin to binding capacity and heating to 75°C for 30 min. The displaced product was analyzed as described above.

3. Results

3.1. Principles of RCA

Solid-phase RCA was developed to provide an effective method of detecting constituents of a solution. A simple scheme outlining the use of rolling circle amplification is shown in Fig. 1. A recognition step is used to generate a complex consisting of a DNA primer duplexed with a circular template bound to a surface. A polymerase enzyme is then used to amplify the bound complex. RCA uses small DNA probes that are amplified to provide an intense signal.

These principles were used to design a detection system to recognize polymorphisms in DNA (Fig. 2). The probes consist of a DNA primer anchored at the 5′ terminus to a solid support and a DNA template hybridized to the primer (Fig. 2A). The 3′ and 5′ ends of the template DNA anneals juxtaposed to the target DNA (Fig. 2B). This provides discrimination of target DNA with and without mismatches. DNA ligase is then used to circularize a matched DNA template perfectly duplexed with the target DNA. The ligation provides enhanced discrimination of perfectly matched target DNAs from those containing mismatches, because DNA ligase is sensitive to single-base mismatches near the ligation site [18–20].

DNA polymerase can then extend the anchored primer in a rolling circle replication type reaction (Fig. 2C). Extension of DNA in a rolling circle replication process has previously been achieved in solution using a short DNA primer and a small circular DNA template [21,22]. This type of DNA amplification can significantly extend the primer, generating multiple tandem repeat copies of the circular DNA template. However, if the target DNA contains a mutation in the probed region, ligation fails, and minimal extension of the primer would occur.

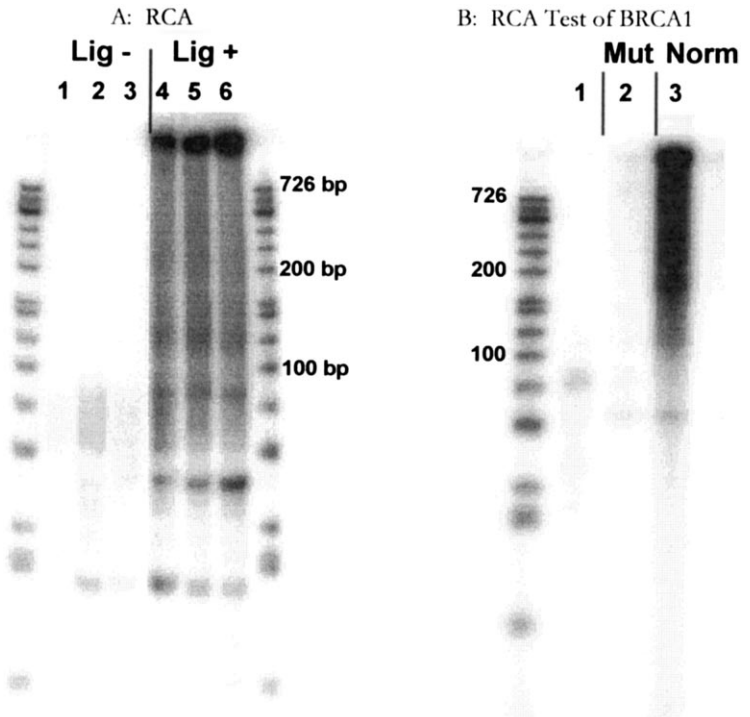

Fig. 4. Rolling circle amplification product. (A) RCA of DNA template directly ligated to a primer immobilized to streptavidin coated beads. Ligated template (4–6) allowed rolling circle replication generating long strands seen by incorporation of ^{32}P-labeled dNTPs. (B) RCA test for a targeted mutation in the sequence of BRCA1 (codon 64/G for T substitution). Conditions containing the normal target sequence (3) were significantly amplified. Conditions containing the mutated target sequence (2) were minimally amplified. Control containing normal target not exposed to ligase enzyme (1) was also minimally amplified.

3.2. Immobilization of RCA probes

Close to 100% of the DNA probes can be captured to the magnetic beads in the form of primer/template probe complex, determined by Cerenkov counts of the immobilized product. The template strand was in a 2:1 ratio to the primer and 49% of the labeled template was bound to the washed magnetic beads. This information is useful for interpreting the amplification system in later steps.

3.3. Ligation of template DNA

A known mutation in the sequence of *BRCA1* gene (exon 5/codon 64/G for T substitution) [23] was targeted as a test of RCA. In these model experiments streptavidin coated magnetic beads were used as the solid support because the beads can be easily manipulated and retain the biotinylated probe complex even under harsh conditions. The template DNA was radiolabeled at the 5′ terminus and hybridized to the target DNA. T4 DNA ligase was found to efficiently ($>70\%$) circularize a 57-base DNA template bound to a 67-base anchored DNA primer, when a 40-base target N (normal BRCA1 sequence) was introduced. T4 DNA ligase was not efficient ($<2\%$) in ligating probes which were exposed to target M, containing a point mutation. Ligation of the template was observed as a shift in the electrophoresis pattern of radio-labeled template following ligation (Figs. 3 and 4A). The shifted band was verified to contain circular DNA by exposing the product to exonuclease, which degraded linear template and not the shifted band (Fig. 4B).

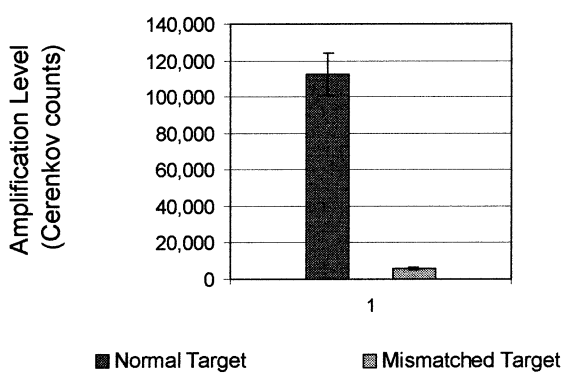

Fig. 5. Test of a BRCA1 exon 5 mutation using RCA. Amplification level of RCA to detect the presence of a mutation in BRCA1. Conditions with the normal target DNA strand were amplified 19-fold greater than conditions with target containing a single base substitution. Measurements were based on Cerenkov counts of [α-^{32}P]dCTP incorporated during the RCA extension reaction.

3.4. Extension of immobilized DNA primer

The amount of ^{32}P-labeled nucleotides incorporated into the extension product was evaluated to quantify the level of amplification. DNA polymerase I amplified probe complexes containing ligated and non-ligated template. However, the bound extension products of DNA probes exposed to the target N, the perfectly matched target, were amplified 19 times greater than probes exposed to target M, containing a base mismatch (Fig. 5). Gel analysis of displaced extension product demonstrated that rolling circle replication generates strands of DNA much longer than the original primer anchored to the solid support (Fig. 3).

4. Discussion

RCA amplifies the solid-phase signal using small initial DNA probes immobilized to the solid support. Since the probes are fixed to a solid support, different groups of probes can be fixed at known locations on a surface to generate a testing array capable of screening many unique sequences in parallel. This technology could be highly modular if unique primers are bound to each array location, allowing the addition of different sets of complimentary template probes.

RCA technology is versatile enough to make use of many different methods of DNA recognition to detect the amplification level of the immobilized DNA probes. Although incorporation of radio-labeled nucleotides was used to detect signal amplification in initial testing, other approaches could be used, including direct incorporation of fluorescent-labeled nucleotides in the amplification process, or hybridization of probes to the amplified product.

RCA combines the specificity of hybridization and ligation with the amplification possible through rolling circle replication to achieve an effective solid-phase method of screening target DNA. RCA can offer several advantages for mutation detection. It provides highly specific DNA sequence recognition and intense signal amplification from small starting probes. Using RCA, highly accurate parallel screening of DNA for many mutations may be possible.

Acknowledgements

This work was supported by grant DAMD17-97-I-7310 from the DOA to C.L.S.

References

[1] Cotton RGH. Detection of unknown mutations in DNA: a Catch-22. Am J Hum Genet 1996;59:289–91.
[2] Chen X, Baumstark T, Steger G, Riesner D. High resolution SSCP by optimization of the temperature by transverse TGGE. Nucleic Acids Res 1995;23:4524–5.
[3] Nissen H, et al. Diagnostic strategy, genetic diagnosis and identification of new mutations in intermittent porphyria by denaturing gradient gel electrophoresis. Hum Mutat 1997;9(2):122–30.
[4] Bi W, Stambrook PJ. CCR: a rapid and simple approach for mutation detection. Nucleic Acids Res 1997;25(14):2949–51.
[5] Lu A, Hsu I. Detection of single DNA base mutations with mismatch repair enzymes. Genomics 1992;14:249–55.
[6] Youil R, Kemper BW, Cotton RGH. Screening for mutations by enzyme mismatch cleavage with T4 endonuclease VII. Proc Natl Acad Sci USA 1995;92:87–91.
[7] Broude NE, Sano T, Smith CL, Cantor CR. Enhanced DNA sequencing by hybridization. Proc Natl Acad Sci USA 1994;91:3072–6.
[8] Cronin MT, et al. Cystic fibrosis mutation detection by hybridization to light-generated DNA probe arrays. Hum Mutat 1996;7:244–55.
[9] Hacia JG, Brody LC, Chee MS, Fodor SPA, Collins FS. Detection of heterozygous mutations in BRCA1 using high density oligonucleotide arrays and two-colour fluorescence analysis. Nat Genet 1996;14:441–7.
[10] Kozal MJ, et al. Extensive polymorphisms observed in HIV-1 clade B protease gene using high-density oligonucleotide arrays. Nat Med 1996;2(7):753–9.
[11] Lockhart DJ, et al. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 1996;14:1675–80.
[12] Prezant TR, Fischel-Ghodsian N. Trapped-oligonucleotide nucleotide incorporation (TONI) assay, a simple method for screening point mutations. Hum Mutat 1992;1:159–64.
[13] Shuber AP, et al. High throughput parallel analysis of hundreds of patient samples for more than 100 mutations in multiple disease genes. Hum Mol Genet 1997;6(3):337–47.
[14] Hansen LL, Justesen J, Kruse TA. Sensitive and fast mutation detection by solid-phase chemical cleavage. Hum Mutat 1996;7:256–63.
[15] Syvanen A, Landegren U. Detection of point mutations by solid-phase methods. Hum Mutat 1994;3:172–9.
[16] Shumaker JM, Metspalu S, Caskey CT. Mutation detection by solid-phase primer extension. Hum Mutat 1996;7:346–54.
[17] Khudyakov YE, Gaur L, Singh J, Patel P, Fields HA. Primer specific solid-phase detection of PCR products. Nucleic Acids Res 1994;22(7):1320–1.
[18] Landegren U, Kaiser R, Sanders J, Hood L. A ligase-mediated gene detection technique. Science 1988;241:1077–80.
[19] Barany F. Genetic disease detection and DNA amplification using cloned thermostable ligase. Proc Nat Acad Sci USA 1991;88:189–93.
[20] Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U. Padlock probes: circularizing oligonucleotides for localized DNA detection. Science 1994;265:2085–8.
[21] Fire A, Xu S. Rolling replication of short DNA circles. Proc Natl Acad Sci USA 1995;92:4641–5.
[22] Liu D, Daubendiek SL, Zillman MA, Ryan K, Kool ET. Rolling circle DNA synthesis: small circular oligonucleotides as efficient templates for DNA polymerases. J Am Chem Soc 1996;118:1587–94.
[23] Castilla LH, et al. Mutations in the *BRCA1* gene in families with early-onset breast and ovarian cancer. Nat Genet 1994;8(4):387–91.