REVIEWS

Adv Clin Exp Med 2009, **18**, 1, 85–92
ISSN 1230-025X

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Application of Selected Methods Based on the Polymerase Chain Reaction in Medical Molecular Diagnostics

Wykorzystanie wybranych metod opartych na reakcji polimerazy do diagnostyki molekularnej chorób genetycznych

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Abstract

A breakthrough in molecular biology came with the discovery of the polymerase chain reaction. It allowed the amplification of DNA fragments *in vitro* in an efficient, rapid, and inexpensive way. Furthermore, it is so versatile that the polymerase chain reaction technique has been used for a multitude of purposes and been found to be applicable in a variety of different disciplines. The authors present the most frequently used laboratory methods based on the polymerase reaction and discuss their application in diagnosing genetic disorders (**Adv Clin Exp Med 2009, 18, 1, 85–92**).

Key words: polymerase reaction, diagnostic methods.

Streszczenie

Rozwój diagnostyki genetycznej jest ściśle związany z odkryciem łańcuchowej reakcji polimerazy (PCR). Pozwala ona na szybką, wydajną i tanią amplifikację *in vitro* fragmentów DNA. Reakcja prowadzona przez polimerazę stała się ponadto podstawą do stworzenia i rozwoju wielu różnych metod powszechnie stosowanych w laboratoriach. Autorzy przedstawiają najczęściej wykorzystywane metody biologii molekularnej oparte na reakcji polimerazy oraz opisują ich zastosowanie w diagnostyce chorób uwarunkowanych genetycznie (**Adv Clin Exp Med 2009, 18, 1, 85–92**).

Słowa kluczowe: reakcja polimerazy, metody diagnostyczne.

The synthesis of deoxyribonucleic acid catalyzed by DNA polymerase (the Klenow fragment of the enzyme) is one of the most crucial reactions in molecular biology. Its adoption in *in vitro* laboratory use has contributed to the universal and routine application of the polymerase chain reaction (PCR) in research studies on living organisms and in forensics, and medical diagnostics. This paper discusses the application of PCR in diagnosing genetic disorders.

PCR and PCR-Based Methods

The polymerase chain reaction, first described by Kary Mullis [1], gave its originator the Nobel Prize for chemistry in 1993. Since then, this simple technique has become a fundamental and standard method in molecular biology. At present, this enzymatic reaction, catalyzed *in vitro* by a thermostable enzyme, polymerase, is the only method that enables obtaining several million or more copies of a critical DNA fragment chosen from the whole genome. The enzyme which catalyses the polymerization of deoxynucleotides *in vitro* is isolated from thermophilic bacteria, originally from *Thermus aquaticus* (Taq). Like other DNA polymerases, it needs a primer to begin the reaction. Therefore, to start copying the double-stranded DNA, two oligonucleotides, a forward and a reverse, are necessary. Because of the specificity in nucleotide pairing, primers about 20 nucleotides long are enough to anneal at one target DNA site, selecting between a huge number of other sequences. PCR is a three-step reaction performed in an automatic cycler, which is able to cool and heat the reaction mixture rapidly in a small tube. A typical PCR run begins with 3–5 minutes of initial denaturation at 94–95°C, which is necessary to destroy the tertiary structure of the genomic DNA. After that, reaction in regular three-step cycles takes place: 1) denaturation at 90–94°C, necessary to melt the template double-stranded DNA to single strands, 2) annealing at the temperature of primer hybridization when the primers are matched to the complementary sequences, which initiates the polymerase chain reaction, and 3) elongation/extension at 72°C, optimal for the Taq polymerase to read the template from the 3' to 5' ends and extend a new complementary strand from the 5' to 3' ends using deoxynucleotide triphosphates. In each cycle, the number of dsDNA fragments is doubled. After about 30–35 cycles and 5 minutes of a final extension at 72°C, several million or more copies are synthesized by the polymerase from one copy of the target sequence.

This simple procedure has allowed the development of a variety of PCR-based methods both in research and in medical diagnostics, especially for diagnosing genetic disorders that result from numerical and structural chromosome aberrations, deletions or amplifications of genes or gene fragments, point mutations, or abnormal methylation.

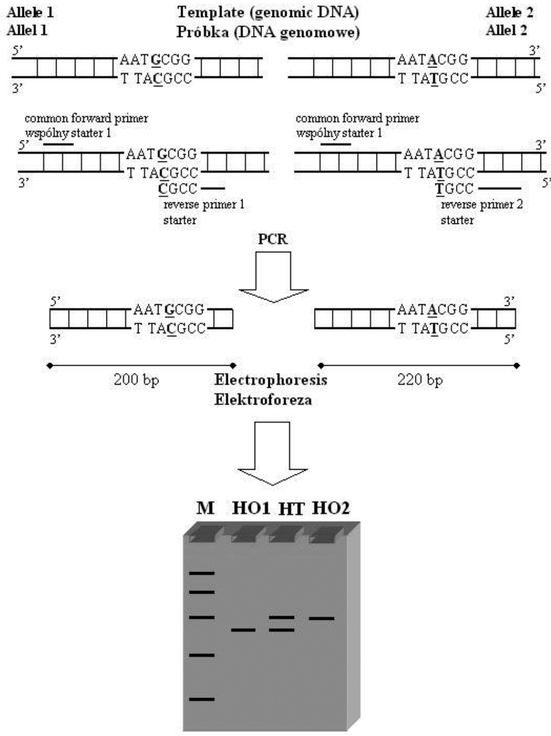

Fig. 1. ASA-PCR. M – mass marker, HO1 – homozygote 1, HT – heterozygote, HO2 – homozygote 2. Description in the text

Ryc. 1. ASA-PCR. M – marker masy, HO1 – homozygota 1, HT – heterozygota, HO2 – homozygota 2. Opis w tekście

ASA-PCR

One of the simplest and least expensive methods of detecting DNA point mutations is allele-specific amplification PCR (ASA-PCR) [2]. In this technique, three primers are used to amplify a fragment of a critical gene and simultaneously identify a change in the DNA sequence. One of the reverse primers is complementary to the wild-type allele and a second to the mutated allele. The forward primer is the same for both alleles [3]. The difference between the reverse primers is at the 3' end, in which the critical single-nucleotide polymorphism (SNP) or mutation is located. Due to the unique conditions of the reaction, amplification of the wild-type and mutated alleles is only possible between their specific reverse and common forward primers. In order to reach such a high affinity of both reverse primers, the reaction conditions must be precisely determined and restrictively adhered to.

Because one of the reverse primers is complementary to the DNA sequence about 20 nucleotides upstream and produces a longer product, the difference in the lengths of the PCR products, visible on an agarose gel, allows distinguishing between the mutated and wild-type alleles. One band for homozygotes and two bands for heterozygotes are present [3].

Examples of Clinical Application

ASA-PCR is used to detect point mutations in the *BRCA1* (breast cancer 1) gene. The *BRCA1* mutations predispose their carriers to the development of breast and/or ovarian cancer. Identification of this DNA defect enables the introduction of preventive surveillance programs for people at high risk of cancer development. In recent years, many mutations in *BRCA1* gene have been identified in different populations. Four founder mutations in *BRCA1* in the Polish population have been reported: 5382insC, C61G (300 T/A) and 4153delA [4], and 185delAG [5]. The most frequently used diagnostic test in Poland based on ASA-PCR and PCR-RFLP was described by Lubinski et al. [6]. There is also another multiplex diagnostic test for the mutations 5382insC in exon 20 and 185delAG in exon 2 of *BRCA1* and 6147delT in exon 11 of *BRCA2* (breast cancer 2) with three sets of primers described by Chan et al. [7].

PCR-RFLP and ACRS-PCR-RFLP

In the PCR-RFLP (PCR-restriction fragment length polymorphism) method, PCR amplification products are cleaved into shorter fragments by a specific endonuclease that recognizes a specific DNA sequence. The restriction enzyme usually cleaves the DNA sequence at a symmetric fragment called the palindrome sequence, for example 5'-GAT↓C-3'. Any alteration in this sequence makes it unrecognizable for a given enzyme but, on the other hand, an alteration in a non-palindromic sequence may result in the creation of a digestion site. Cleavage of the PCR product produces two or more shorter fragments that can be visualized on an agarose gel. In the commonly used agarose electrophoresis, restricted and unrestricted PCR fragments differing in no less than 20–25 nucleotides are necessary for detection. The variety of commercially available restriction enzymes and the low cost and equipment requirements make PCR-RFLP a widely applicable method.

However, point mutations are usually distributed randomly over the genome, which means that a critical point mutation predisposing to or causing a genetic disorder rarely appears within a palindrome sequence. Therefore the PCR-RFLP method has been modified to enable the detection of point mutations that not only destroy/create a palindrome sequence, but which are also present in other sequences. In ACRS-PCR (amplification-created restriction site or artificially created/constructed restriction site PCR), one of the primers is designed in such a way that it creates a new "mutation" in the PCR product [8]. This alteration may create or abolish a palindromic sequence itself or together with the critical mutation. After that, RFLP may be applied. It is possible to distinguish between wild-type and mutated alleles using polyacrylamide or agarose gel electrophoresis provided that the examined point mutation or SNP is present about 20–25 nucleotides from the 5' or 3' ends of the PCR product.

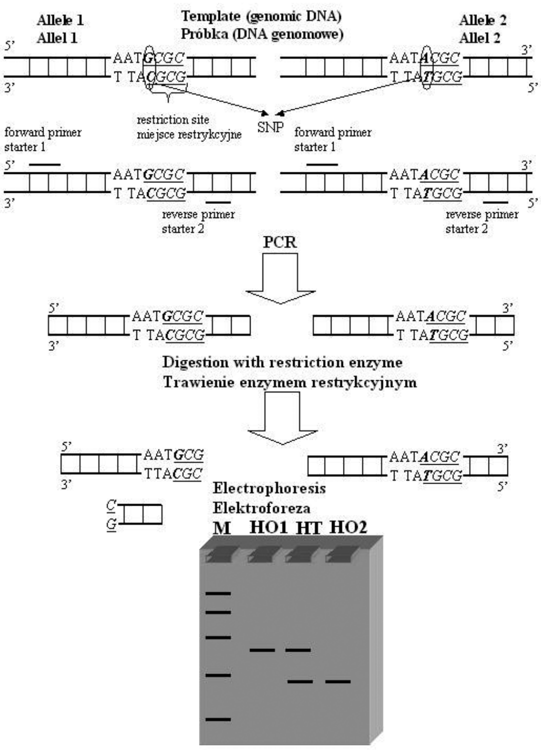

Fig. 2. PCR-RFLP. M – mass marker, HO1 – homozygote 1, HT – heterozygote, HO2 – homozygote 2. Description in the text

Ryc. 2. PCR-RFLP. M – marker masy, HO1 – homozygota 1, HT – heterozygota, HO2 – homozygota 2. Opis w tekście

Examples of Clinical Application

PCR-RFLP has been adopted to diagnose a variety of monogenic disorders, such as Rett syndrome [9, 10], or as an alternative method to ASA--PCR in the detection of *BRCA1* mutations (C61G, 5382insC, 185delAG) [4, 11]. Rett syndrome is a cause of mental retardation in girls and is characterized by regression of skills after a period of apparently normal development until 6–18 months of age. Verbal communication, gait, and purposeful hand usage are severely impaired and stereotypical hand movements such as washing, wringing, or clapping appear. In the majority of cases this disorder is caused by a point mutation in the *MeCP2* gene, usually a missense mutation [10].

MS-PCR

MS-PCR (methylation-specific PCR) is a method which allows evaluating the methylation pattern in CpG islands. Methylation is an essential process for epigenetic gene expression regulation. Hypermethylation causes down-regulation of genes and hypomethylation up-regulation. Examples of this phenomenon include X chromosome inactivation and tumor suppressor or mismatch-repair gene silencing in cancer or regulation of expression of imprinted genes [12]. This method is based on the conversion of all unmethylated cytosines to uracils, while methylated cytosines remain unmodified during sodium bisulfide treatment of DNA. Two sets of primers that distinguish between methylated and unmethylated (unmodified and modified, respectively) fragments are used. The result is indicated by the presence or absence of the PCR products.

Examples of Clinical Application

MS-PCR is used for the detection of promoter region hypermethylation of the tumor suppressor genes p16/CDKN2, 14-3-3 sigma DAP kinase, and p15 [12–14]. It can also be applied for the diagnosis of Prader-Willi and Angelman syndromes, which may be caused by a lack of a paternal or maternal pattern of CpG island methylation in the region of 15q11-13, respectively [15, 16].

MLPA

Multiplex ligation-dependent probe amplification (MLPA) was developed by Microbiology Research Center Holland in 2002 and is now commonly applied for the analysis of large exon deletions and amplifications. This method enables 1) the detection of large deletions/amplifications in one allele (which is impossible using PCR) [17, 18], 2) the identification of small changes such as SNPs or point mutations [19], 3) finding the breakpoint sites in deleted DNA fragments [20], and 4) methylation pattern analysis [21]. MLPA also allows mRNA analysis [22]. Furthermore, it can be employed as a routine technique for large groups of patients and up to 45 sequences can be examined in one reaction.

In this method, pairs of specific probes that hybridize to critical DNA fragments are used. Only precise hybridization of both probes of a pair to the complementary sequence allows them to be joined by a ligase in the following step. The product of the probe ligation then serves as a target sequence for PCR amplification. PCR products fluorescently labeled by one of the common primers are separated according to their size in a sequence-type electrophoresis device, for example a sequencer. Analysis of the peaks representing the intensities of the products' fluorescence obtained after separation and fluorescence detection allows evaluating the amount of alteration in the examined DNA fragment compared to the control probe [23].

Examples of Clinical Application

Commercially available MLPA kits have been applied in many laboratories for the diagnosis of selected critical mutations that may be found in hereditary cancer syndromes, such as hereditary breast/ovarian cancer (*BRCA1*), hereditary non-polyposis colorectal cancer (*MLH1*, *MSH2*, *MSH6*, *PMS2*), neurofibromatosis (*NF2*), and retinoblastoma (*RB1*), for pre- and postnatal analyses such as aneuploidy detection and subtelomeric deletions, and for the diagnosis of a variety of clinical syndromes, such as Rett syndrome (*MeCP2*), cystic fibrosis (*CFTR*), and Hirschsprung's disease (*RET*, *ZFHX1B*, *EDN3*, *GDNF*). So far, MLPA may be applied only in research studies as it has not yet been certified by the responsible committees and its results must be confirmed by another, alternative method.

Real-Time PCR

Real-time PCR (or quantitative real-time PCR – qRT-PCR) is not only used for the detection, but most of all for the quantification of a small amount of specific DNA or (after a reverse transcription reaction) RNA sequences. Therefore it is used in gene expression analysis as well as viral/bacterial copy number quantification, SNP or methylation pattern estimation, and the detection of mosaicism. Real-time PCR, as its name implies, allows observing the progress of PCR in real time and measures the amount of the reaction products in its exponential phase, when the copy number of PCR products correlates with the starting amount of the DNA target sequence [24]. This method is based on the measurement of a reporter molecule's fluorescence level, which increases during the time of the reaction [25, 26]. Several different fluorescent probes (reporters) are used in real-time PCR: double-stranded DNA binding dyes (SYBR-green I and II), hydrolysis probes (5' nuclease probes: TaqMan Probes), and hybridization probes (molecular beacons, sunrise primers, and scorpion primers) [27].

The most popular reporter is SYBR-green I or II because of its low price and simplicity of application. SYBR-green fluorescence is very low in solution but strong when intercalating dsDNA [28]. SYBR-green-based real-time PCR requires very rigorous optimization of the reaction conditions because it is impossible to distinguish between specific PCR products and nonspecific amplifications or a primer-dimer complex [29].

One of the specific reporters for real-time PCR is the TaqMan probe, about 10 nucleotides longer and with a Tm (melting temperature) about 10°C higher than the primers used. Usually a fluorescent dye is tied at the 5' end of the probe and a quencher at the 3' end (e.g. TAMRA). If the fluorescent and quenching molecules are connected with the oligonucleotide and are close together, the energy is transferred from the first to the second, which prevents fluorescence emission (FRET –

fluorescence resonance energy transfer) [30, 31]. TaqMan probes are complementary to the middle part of the PCR product. When DNA polymerase is replicating using the PCR product as a template, it cleaves, as a 5' exonuclease, the 5' end of the reporter and releases the fluorescent dye, which is far from the quencher and can emit fluorescence. The accumulation of the free fluorescent reporter dye correlates with the increase in the number of the PCR products. The important thing is that the probe attaches only to its complementary sequence of the PCR product, so fluorescence is present only if specific amplification occurs. Another variant of reporter-quencher probes, including molecular beacons, are sunrise and scorpion primers, which are designed in such a way that enable keeping the reporter and quencher together. When the reaction is initiated, the quencher and reporter are disconnected, which generates fluorescence during amplification.

Examples of Clinical Application

Real-time PCR may be used for the detection and quantification of the BCR/abl fusion transcripts (present in the Philadelphia chromosome) in chronic myeloid leukemia (CML), which has implications for therapy and prediction of response to treatment, drug resistance, and disease monitoring in Ph-positive CML [32].

Sequencing

Sequencing, i.e. determining the DNA sequence, is the ultimate and most precise technique for DNA analysis. It may be applied as a method for the detection of small DNA alterations, but also as a confirmation technique for PCR-RFLP or ASA-PCR because of its accuracy. DNA sequencing has enabled determining the whole sequence of the human genome, and has contributed to finding the genetic principles of susceptibility to human diseases (the complete human DNA sequence as well as those of an increasing number of other organisms are currently known) [33, 34]. The automation of DNA sequence analysis using a sequencer and the application of fluorescent labeling enabled reductions in the costs and time of analysis. It is possible to analyze up to 96 templates per run using a 96-capillary sequencer.

Sequencing by Sanger's method

The Sanger method of DNA sequencing is undoubtedly the most frequently used and, since 1977, many various refinements of the protocol have been developed, rather than inventing new technologies [34, 35]. The Sanger technique is based on DNA synthesis by DNA polymerase with the incorporation of either dNTP or ddNTP (dideoxynucleotide) triphosphate analogues which, because of their chemical structure, are the last nucleotides in the newly formatted oligomer. The population of specifically terminated products is afterwards separated using high-resolution electrophoresis [34]. Its application in terminating the polymerase reaction of fluorescently labeled dideoxynucleotides has allowed enclosing the reaction in one tube and makes the method non-radioactive and safe.

Minisequencing

Minisequencing is used for the detection of single-nucleotide polymorphisms or point mutations. The first step is the amplification of about 200 bp of target DNA using PCR. Afterwards, PCR product purification to remove primers and unincorporated dNTPs is necessary. There are three possible methods: 1) shrimp alkaline phosphatase (SAP) and exonuclease I (ExoI) treatment, 2) PCR product cleaning on a minicolumn, 3) separation of the PCR product by agarose gel electrophoresis and elution from the gel using a minicolumn.

The minisequencing method is based on the incorporation of a single fluorescently labeled dideoxynucleotide at the 3' end of a special oligonuclotide which is complementary to the sequence located one nucleotide before the examined polymorphic site [36]. The incorporation of one of four ddNTPs labeled by differently colored dyes depends on the genotype. Furthermore, the products of the reaction are separated by capillary electrophoresis. The color of the one (for homozygote) or two (for heterozygote) signals obtained allows identifying the incorporated ddNTP and, furthermore, the complementary deoxynucleotide in the DNA sequence. If multi-length oligonucleotides are used, several different polymorphisms/mutations in one multiplex reaction can be detected [36].

Minisequencig is an adequate method for detecting multimutational genetic diseases (e.g. cystic fibrosis, muscular dystrophy) in one reaction and for pre-implantation genetic diagnosis (PGD) of monogenic disorders from a single fetal cell [37].

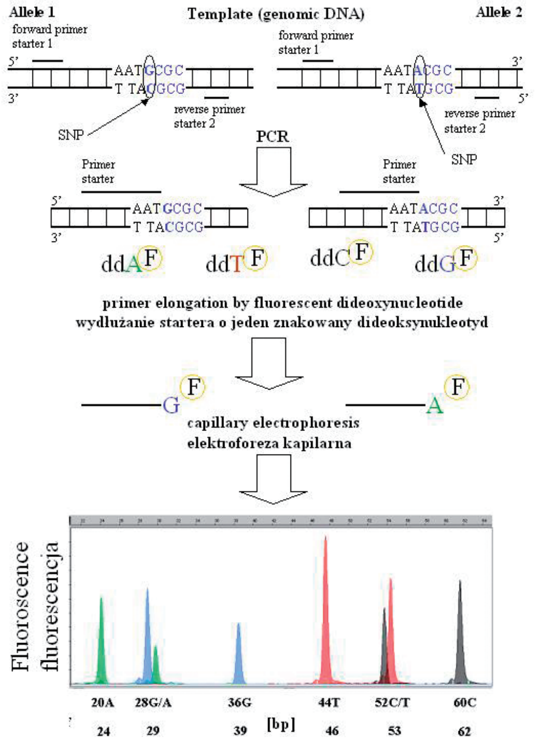

Fig. 3. SNaPshot. ddA[F] – fluorescent labeled dideoxyadenosine triphosphate, ddT[F] – fluorescent labeled dideoxythymidine triphosphate, ddC[F] – fluorescent labeled dideoxycytidine triphosphate, ddG[F] – fluorescent labeled dideoxyguanosine triphosphate. Description in the text

Ryc. 3. SNaPshot. ddA[F] – znakowany fluorescencyjnie trifosforan dideoksyadenozyny, ddT[F] – znakowany fluorescencyjnie trifosforan dideoksytymidyny, ddC[F] – znakowany fluorescencyjnie trifosforan dideoksycytydyny, ddG[F] – znakowany fluorescencyjnie trifosforan dideoksyguanozyny. Opis w tekście

Pyrosequencing

Pyrosequencing is a technique which uses four different enzymes for reading short DNA sequences (at least 20 nucleotides) in real time. Six reactions with four enzymes take place in one tube: 1) the Klenow fragment of the DNA polymerase catalyses the polymerization of the added nucleotide (one of four at a time) with the DNA template if it is complementary, 2) ATP sulfurylase uses as a substrate APS (adenosine 5'–phosphosulfate) and an inorganic pyrophosphate (PPi) released by the polymerase, producing ATP, 3) and 4) luciferase uses ATP, D-luciferin, and an oxygen molecule (O_2) to produce light in two reactions, and 5) and 6) between the addition of different nucleotides, apyrase is added to remove unincorporated dNTPs and ATPs and therefore prevent light production in the next cycle, which starts without any additional dNTPs except for the one provided by the sequencer:

1) $(DNA)_n + dNTP \rightarrow (DNA)_{n+1} + PPi$
2) $PPi + APS \rightarrow ATP + SO_4^{2-}$
3) luciferase + D-luciferin + ATP $\rightarrow$ luciferase-luciferin-AMP + PPi
4) luciferase-luciferin-AMP + O_2 $\rightarrow$ luciferase + oxyluciferin + AMP + CO_2 + LIGHT
5) ATP $\rightarrow$ AMP + 2Pi
6) dNTP $\rightarrow$ dNMP + 2Pi

In this way, light is produced only when adding a certain nucleotide and incorporation occurs (Fig. 4) [34, 38].

Pyrosequencing is applied for the detection of known and unknown mutations and SNPs with a definite position as well as taq sequencing and microorganism typing. An unquestionable advantage of pyrosequencing is the possibility of automation, economical reagent use, and multiple probe sequencing (96 to 384) in one process [34]. It is particularly important in studies with a large number of DNA alterations and/or a large number of tested samples.

Since the second half of the 20th century, a variety of applications of the *in vitro* polymerase chain reaction have been discovered. The specificity of this technique makes it one of the most important and frequently used in biomedical investigations and other biological analyses. There are many other techniques based on the polymerase chain reaction that can be applied in laboratory practice apart from those described in this paper. The authors presented those methods that are the most interesting in their opinion and/or commonly used.

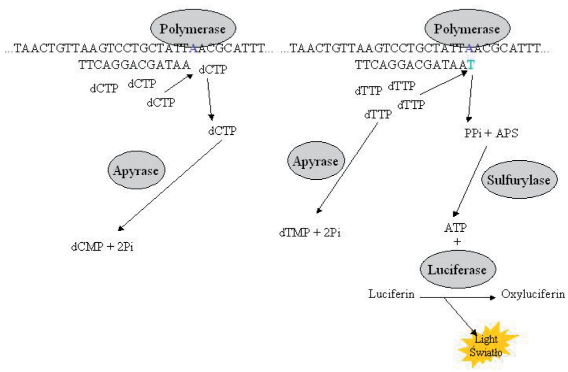

Fig. 4. Pyrosequencing. dCTP, dTTP, dATP – deoxynucleotide triphosphates, PPi – pyrophosphate, Pi – phosphate, APS – adenosine-5'-phosphosulfate, ATP – Adenosine triphosphate

Ryc. 4. Pirosekwencjonowanie. dCTP, dTTP, dATP – trójfosforany deoksynukleotydów, PPi – pirofosforan, Pi – reszta fosforanowa, APS – adenozyno-5'-fosfosiarczan, ATP – adenozynotrójfosforan

Acknowledgements

The authors would like thank Prof. Maria M. Sąsiadek for the help with the manuscript.

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Conflict of interest: None declared

Received: 26.09.2008
Revised: 6.01.2009
Accepted: 18.02.2009