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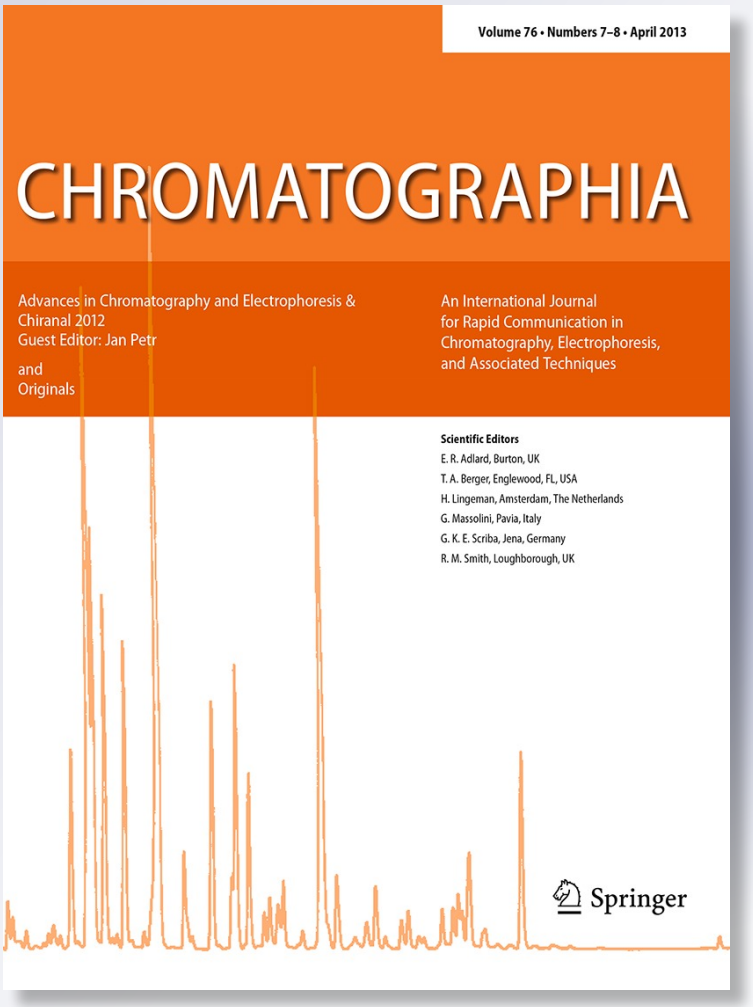

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ORIGINAL

Isolation of Xis Gen Fragment of λ Phage from Agarose Gel Using Magnetic Particles for Subsequent Enzymatic DNA Sequencing

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Abstract Gel electrophoresis is one of the most important methods used in biochemistry and molecular biology. The recovery of analytes from the gel required for subsequent analysis including amplification by polymerase chain reaction (PCR) or DNA sequencing is an issue due to the gel contamination. Among the other methods used for sample recovery, the application of nanomaterials is also being investigated. In this study, the applicability of magnetic particles (1 μm) for isolation of DNA fragment from agarose gel with subsequent DNA sequencing was investigated. Electrochemical analysis and DNA sequencing was used to investigate the recovery yield. The influence of dilution of the gel prior to the purification was investigated and the linear dependence with regression coefficient $R^2 = 0.9972$ was obtained using square wave voltammetry. Moreover, bioinformatic analysis was used for comparison of obtained sequences, and simple and easy identification of non-systematic errors caused by both fluorescence labeling reaction and electrophoretic separation. It was found that magnetic nanoparticles based isolation markedly lowered the errors occurring during sequencing of the isolated DNA fragment from 7 to 1 %.

Keywords Gel electrophoresis · Electrochemical analysis · DNA sequencing · DNA purification · Magnetic particles

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Introduction

Gel electrophoresis (GE) is one of the most important techniques used in biochemistry and molecular biology [1, 2], providing valuable information about the presence and molecular mass of proteins and/or nucleic acids in a real sample. Even though GE has several disadvantages, such as it is time consuming and laborious, however, due to their advantages such as versatility, ease of use and low costs it is not simple to replace GE. Also, the recovery of analytes following the GE separation required for subsequent analysis including amplification by polymerase chain reaction (PCR) or DNA sequencing is an issue due to gel contamination by chemical and other biomolecules. The isolation of DNA from agarose gels is a routine multipurpose procedure in molecular biology. To our knowledge, there are several methods used for purification of DNA from agarose gel. They are variably effective for DNA of different lengths in terms of yield and purity of the recovered DNA. Typically, the organic extraction method, electro-elution [3, 4], binding of DNA to glass fibers [5, 6], and ion exchange resins provide pure DNA, but a drawback of such methods is lower yields of recovered DNA. In addition, there are other methods, such as syringe squeeze [7, 8] and centrifugal filtration [9, 10] that provide higher

recovery of DNA. However, the issue with these methods involves incompatibility of purified DNA for further manipulation. It is not surprising that other assays and methods are searched for this purpose [11].

During the past decade, nanotechnology has developed to such an extent that it has become possible to design and tailor the functional properties of nanoparticles for a variety of biomedical applications. Besides using these particles for drug delivery and magnetic resonance imaging, magnetic nanoparticles (MPs) have been used for DNA purification [12–15]. Additionally, the magnetic method takes less than 15 min to extract PCR ready genomic DNA compared to the traditional phenol–chloroform extraction protocols that takes several hours. Numerous strategies and platforms for ultrafast DNA sequencing currently under development include nanopore sequencing, sequencing-by-hybridization, and sequencing-by-synthesis [16, 17]. Despite the advances in modern sequencing technologies, the development of the fluorescence-based Sanger's approach is still focusing on producing faster and cheaper sequencing reads. On the other hand, the sample pretreatment is an important step for all sequencing techniques. Especially after gel electrophoretic separation, the sample recovery prior to the sequencing analysis is crucial.

The aim of this study is to investigate the applicability of magnetic particles for isolation of DNA fragment from agarose gel prior to subsequent DNA sequencing. Electrochemical analysis and DNA sequencing was used to investigate the yield. The influence of dilution of the gel prior to the purification was investigated. Moreover, bioinformatics analysis was used to compare the obtained sequences, and to simply and easily identify the identification of non-systematic errors caused by both fluorescence labeling reaction and electrophoretic separation.

Experimental Section

Polymerase Chain Reaction

Taq PCR kit and DNA isolated from bacteriophage λ (48,502 bp) were purchased from New England BioLabs (Ipswich, MA, USA). Primers for PCR were synthesized by Sigma-Aldrich (St. Louis, MO, USA). The sequence of a forward primer was 5′-CCTGCTCTGCCGCTTCACGC-3′ and the sequence of a reverse primer was 5′-TCCGGAT AAAAACGTCGATGACATTTGC-3′. The 50 μL reaction mixture was composed of 5 μL 10× standard *Taq* reaction buffer, 1 μL of 10 mM deoxynucleotide solution mix, 1 μL of each primer (10 μM), 0.25 μL of 5 U μL^{-1} *Taq* DNA polymerase, 1 μL of 0.5 μg μL^{-1} λ DNA and 40.75 μL H_2O (sterile, ACS purity, Sigma-Aldrich). The PCR tubes with the mixture were placed into the cycler (Mastercycler ep *realplex*^{4} S, Eppendorf AG, Hamburg, Germany) and cycling conditions were as follows: initial denaturation at 95 °C for 120 s, 25 cycles of denaturation at 95 °C for 15 s, annealing at 64 °C for 15 s, extension at 72 °C for 45 s and a final extension at 72 °C for 5 min. The amplicons were analyzed by agarose gel electrophoresis.

PCR Product Agarose Gel Electrophoresis

Agarose gel (1.2 % v/v, low melt, Mercury, San Diego, CA, USA) was prepared by boiling of 1× TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid, Bio-Rad, Hercules, CA, USA) for 2 min in microwave (power 600 W). Then the gel was cooled to 60 °C and ethidium bromide was added (5 μL/100 mL of the gel). The gel was transferred into electrophoretic bath containing 1× TAE buffer. Samples prepared with 5 % (v/v) bromophenol blue and 3 % (v/v) glycerol (ACS purity, Sigma-Aldrich) were loaded into a gel in 30 μL aliquots. DNA ladder (New England BioLabs) within the size range from 0.5 to 1.5 kb was used to monitor the size of analyzed fragment. The electrophoresis (Bio-Rad) was running at 100 V and 6 °C for 45 min. The bands were visualized by UV transilluminator at 312 nm (VilberLourmat, Marne-la-Vallée Cedex, France).

The bands of interest were cut out with a sterile blade and transferred to a microcentrifuge tube. After melting agarose gel at 65 °C for 15 min in thermoblock (Thermomixer 5355, Eppendorf AG, Hamburg, Germany) the liquid was divided into three aliquots and TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, ACS purity, Sigma-Aldrich) was added (dilution gel:TE buffer 1:0.5, 1:1 and 1:2).

Isolation by MPs

For isolation of DNA from gel, a ChargeSwitch PCR Clean-Up Kit (Invitrogen, Grand Island, NY, USA) was used. The experiments with magnetic particles (1 μm diameter) were performed in RNA/DNA UV cleaner box UVT-S-AR (Biosan, Riga, Latvia).

DNA Immobilization to MPs

To 50 μL of melted gel with λ DNA fragment in microcentrifuge tube, 50 μL of purification buffer and 25 μL of ChargeSwitch magnetic beads were added. The suspension was mixed with the pipette and incubated for 1 min at 21 °C. In this step the DNA binds to the MPs surface. After incubation the microcentrifuge tube with the mixture was transferred to a magnetic stand (Dynal, Oslo, Norway) for 1 min. Due to the magnetic force, the MPs coupled with DNA were attracted to the microcentrifuge tube wall and were separated from contaminants. The solution with contaminants was pipetted out.

Washing DNA

The microcentrifuge tube was removed from the magnetic stand and 150 µL of washing buffer (heated to 50 °C) was added to the pellet of MPs. After mixing with pipette the microcentrifuge tube was placed on a magnetic stand for 1 min and then the washing buffer was pipetted out. The washing step was repeated twice.

DNA Elution

The microcentrifuge tube was removed from magnetic stand and 25 µL of elution buffer (10 mM Tris–HCl, pH 8.5) was added to MPs. After mixing with pipette the microcentrifuge tube was placed on a magnetic stand for 1 min. In this step the DNA was eluted from MPs to elution buffer. After adhesion of MPs to the microcentrifuge tube wall by magnetic force, purified DNA was removed to clean microcentrifuge tube.

Electrochemical Analysis

Quantitative analysis of purified DNA was carried out by AUTOLAB PGS30 Analyzer (EcoChemie, Utrecht, The Netherlands) connected to VA-Stand 663 (Metrohm, Zofingen, Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3 M KCl electrode served as the reference electrode. Pt wire was used as the auxiliary electrode.

Adsorptive transfer technique was used for the electrochemical determination of DNA. The adsorptive transfer technique is based on the sample accumulation (120 s) onto the working electrode surface and consequently on the electrode washing and square wave voltammetric (SWV) measurement. All experiments were carried out at room temperature (22 °C). SWV measurements were carried out in the presence of acetate buffer pH 5.0. SWV parameters: start potential 0 V, end potential −1.8 V, potential step 5 mV, frequency 280 Hz, and amplitude 25.05 mV. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie (Utrecht, The Netherlands) was employed [18–20].

DNA Sequencing

For sequencing reaction the DTCS Quick Start Kit (Beckman Coulter, Brea, CA, USA) was used. To 20 µL sequencing reaction mixture, 10 µL of isolated λ DNA fragment (every dilution analysis was performed in duplicate), 1 µL of 10 µM forward primer, 8 µL of DTCS Quick Start Master Mix and 1 µL of H_2O (sterile, ACS purity, Sigma-Aldrich) were added and the mixture was transferred to the cycler (Eppendorf AG, Hamburg, Germany). The conditions of 30 cycle-reaction were as follows: 96 °C for 20 s, 50 °C for 20 s and 60 °C for 4 min. For purification of sequencing product Clean-SEQ kit (Beckman Coulter) was used. 20 µL of sequencing product was transferred in microcentrifuge tube and 10 µL of Clean-SEQ MPs and 62 µL of 85 % ethanol (v/v, with water) were added to the product. The suspension was mixed with the pipette and the fluorescent-marked DNA binds to the MPs surface. Microcentrifuge tube with the mixture was placed on a magnetic stand (Dynal) for 5 min. The solution was pipetted out and MPs was washed twice with 100 µL of 85 % ethanol (v/v, with water). After removing ethanol the microcentrifuge tube was transferred from magnetic stand and 40 µL of sample loading solution was added to the MPs. After mixing, MPs were placed in magnetic stand for 5 min. Then purified samples was transferred to the plate containing sample loading solution and DNA sequencing was performed using Genetic Analysis System CEQ 8000 (Beckman Coulter). After denaturation at 90 °C for 2 min, a fluorescence-marked DNA fragments were separated in 33 cm long capillary with 75 µm i.d. (Beckman Coulter), which was filled with linear polyacrylamide denaturing gel (Beckman Coulter). The separation was run at capillary temperature of 50 °C and voltage of 4.2 kV for 85 min.

Results and Discussion

Phage λ is a viral particle containing double-stranded linear DNA as its genetic material. The phage particle recognizes and binds to its host, *E. coli*, causing injection of DNA into the cytoplasm of the bacterial cell through the bacterial tail [21, 22]. The genome of this phage is very well known and therefore its DNA fragments are excellent models for biochemical experiments. Phage λ genome is shown in Fig. 1 and the position of DNA fragment used in this study is highlighted. The phage λ genome includes gene xis [23], which encodes excisionase (Xis) protein. For excision the phage λ genome out of its host cell chromosome, Xis protein is required [24], which is the major factor that regulates the recombination direction [25].

λ DNA fragment was amplified by PCR and reached the required amount of DNA. The obtained PCR mixture was subsequently separated by GE to purify the fragment from other components of the mixture, such as primers, nucleotides and polymerase. GE analysis confirmed the purity of the sample excluding the presence of unspecific products and verifying the DNA fragment size. Due to large sample volume GE analysis had to be performed in 14 wells. As expected, the obtained product size was 498 bp and no unspecific contaminations were determined. All 14 GE

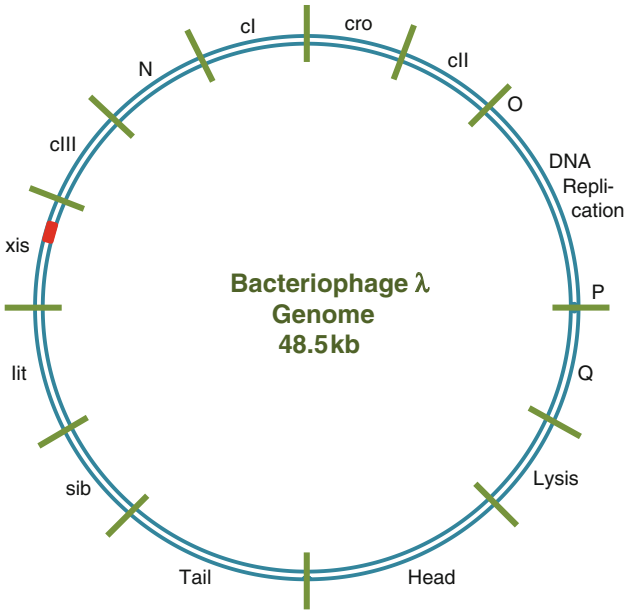

Fig. 1 λ phage genome. The *red part* in gene *xis* is amplified section. This gene encodes Xis (excisionase) protein

bands were cut out, transferred into the tube and liquidized by heating at 65 °C for 15 min. The obtained liquid was divided into three aliquots and diluted by TE buffer ("gel dilution") into three volume ratios (1:0.5, 1:1 and 1:2). These three aliquots underwent the isolation procedure by magnetic particles to extract the DNA fragment. Magnetic particles are able to bind and release nucleic acid due to the switchable surface charge, dependent on the surrounding buffer pH. Under low pH, the beads are positively charged and bind the negatively charged nucleic acid backbone. To elute nucleic acids, the charge on the surface is neutralized by raising the pH to 8.5 using a low salt elution buffer. The isolation procedure consists of an immobilization step, several washing steps and an elution step. The main advantage of this procedure is its speed, because extraction of a sample takes 10 min. Isolated DNA fragment was subsequently analyzed by three independent methods (1) GE, (2) square wave voltammetry and (3) DNA sequencing based on capillary electrophoresis.

Analysis of the Isolated DNA

Primarily, GE of all three "gel dilution" aliquots, obtained by isolation using magnetic particles, was performed. The isolated DNA samples provided sharp bands with size of 498 bp (Fig. 2a). Moreover, the visual inspection suggested that the fluorescence intensity of each band corresponded to the gel dilution (dilution gel:TE buffer 1:0.5, 1:1 and 1:2). To obtain the quantitative information about DNA fragment concentration, as well as recovery of the magnetic particle-based isolation, square wave voltammetry was used. Voltammetry is very sensitive method for DNA detection, suitable for miniaturization as well as monitoring of DNA interactions [26–28]. The electrochemical signals of extracted DNA are shown in Fig. 2b illustrating that the extraction process is linearly dependent on DNA concentration present in the gel. The linearity of this dependence is expressed by correlation coefficient $R^2 = 0.9972$.

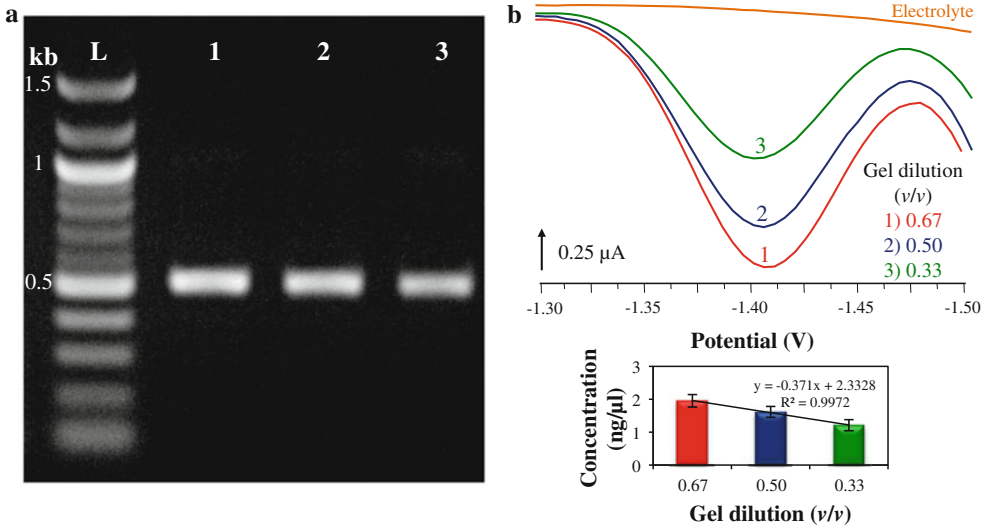

Fig. 2 **a** Gel electrophoresis of PCR product in 1.2 % agarose gel, the experimental parameters: 45 min, 100 V. *L* ladder, *1–3* PCR product (498 bp) isolated from agarose gel using MPs, dilution of gel before isolation: *1* gel:TE buffer 1:0.5 (gel dilution v/v 0.66), *2* gel:TE buffer 1:1 (gel dilution v/v 0.5), *3* gel:TE buffer 1:2 (gel dilution v/v 0.33). **b** Electrochemical determination of isolated DNA. Typical SW voltammograms of several gel dilutions. SWV parameters were as follows: time of accumulation 120 s, start potential 0 V, end potential −1.8 V, potential step 5 mV, frequency 280 Hz, amplitude 25.05 mV. *Inset*: Dependence of DNA concentration on gel dilution (v/v)

The quality of recovered DNA samples separated by GE is crucial for further analytical analysis. DNA sequencing can be used to identify co-migrating DNA fragments occurring during gel electrophoresis. Therefore, qualitative characterization of the extraction process by DNA sequencing was preformed. The sequences of DNA isolated from agarose gel by MPs and DNA without isolation were compared (Fig. 3). The electrophoretic peaks of isolated DNA fragment were well identified by the sequencing software (Fig. 3a) and the comparison of analyzed sequences with the sequence of phage λ (GenBank: J02459.1) showed 99 % identity for 1:0.5 and 1:1 gel dilution and 98 % for 1:2 gel dilution. The 1–2 % error is probably caused by incomplete labeling reaction caused by polymerase. Moreover, sporadic errors can be caused by the electrophoretic separation and the peak identification by the software. On the other hand, the DNA fragment without magnetic particle-based isolation provided very low signal, which caused 7 % errors in the sequence reading (Fig. 3b). This is probably caused due to the inhibition of sequencing labeling reaction by the presence of agarose gel.

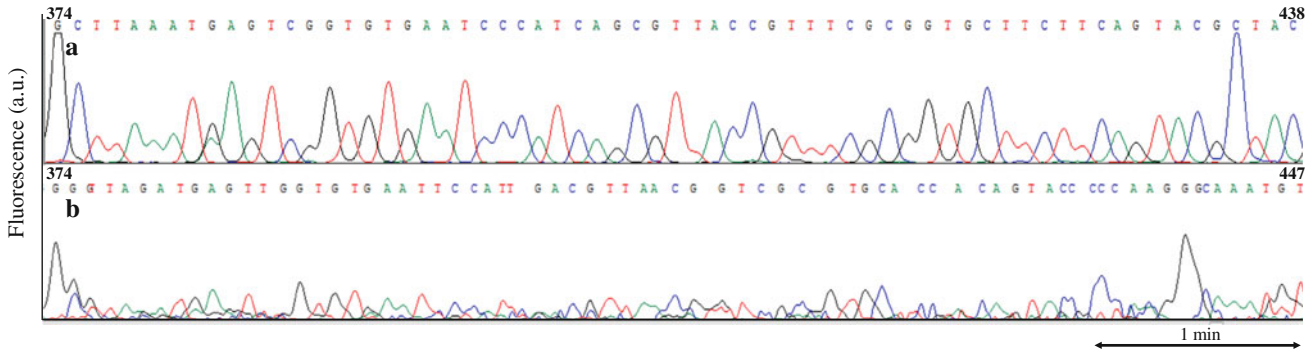

Fig. 3 *a* Part of λ DNA fragment sequence isolated from agarose gel using MPs. Dilution of sample before isolation: gel:TE buffer 1:0.5. *b* Part of λ DNA fragment sequence after agarose gel electrophoresis and excision from gel but without isolation

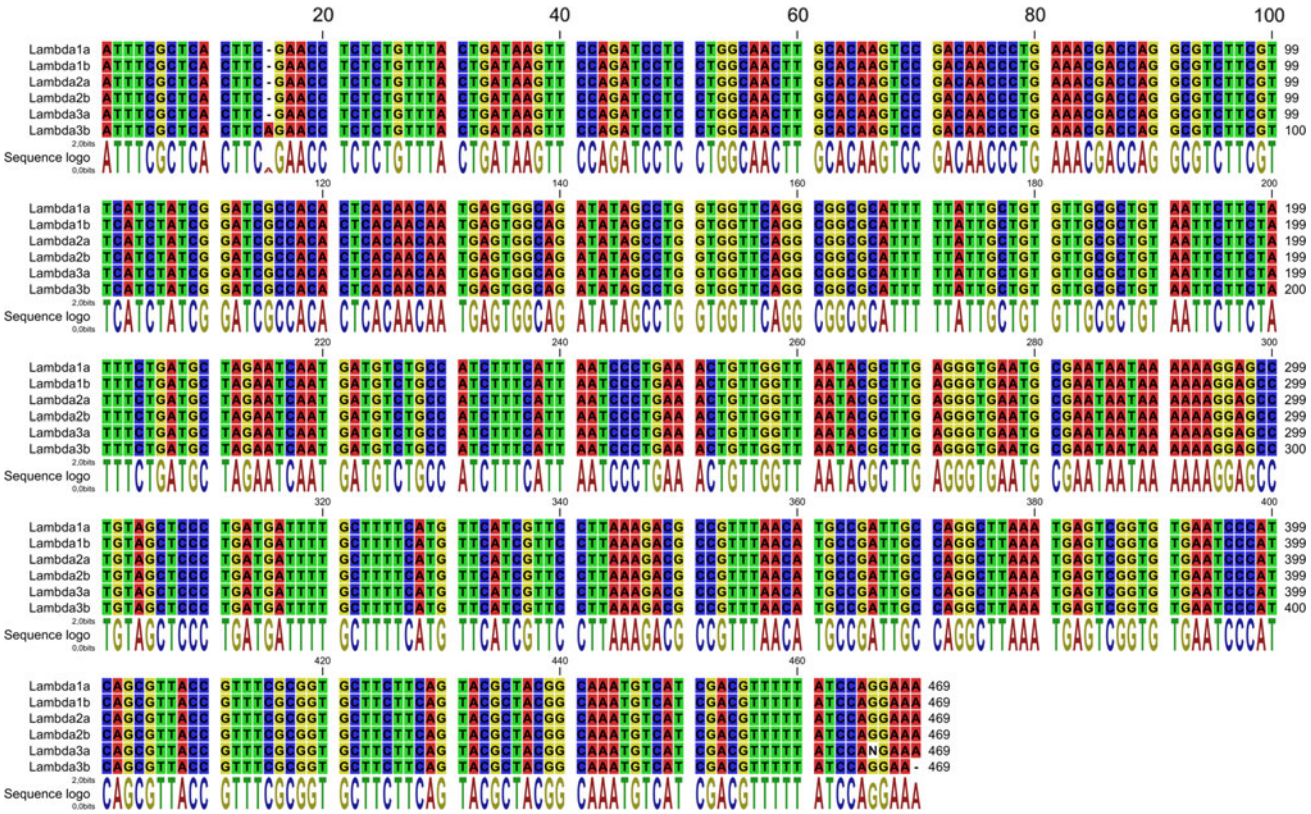

Fig. 4 Alignment of sequences of λ DNA fragment isolated from agarose gel using MPs. Dilution of gel before isolation: *1a,1b* gel:TE buffer 1:0.5; *2a, 2b* gel:TE buffer 1:1; *3a, 3b* gel:TE buffer 1:2

Bioinformatic Analysis

The obtained sequences were processed by bioinformatic tools. All sequences (two sequences for each gel dilution) were aligned to highlight the differences between each other. It was found that the errors were at the beginning and at the end of the fragments. The alignment of all six sequences was in the 99.3 % agreement. Interestingly, the average number of errors was linearly dependent on the dilution of the gel prior to the isolation by MPs. At higher dilution the number of errors observed was higher ($R^2 = 0.9908$). After bioinformatic analysis, the discrepancies in sequences were revised in electropherograms and it was found that most of the errors were caused by incorrect sequencing analysis. These discrepancies were visually revised, repaired and then another comparison followed (Fig. 4). The alignment of all six sequences was in the 99.9 % agreement. Finally, the "Sequence Logo" (Fig. 4) was created graphically showing the statistical difference between the aligned sequences. The higher the size of the letter of each basis caused, the higher the probability of occurrence of the particular base at the particular position.

Conclusions

Even though several methods for isolation of DNA from the agarose gel by magnetic particles have been presented [12, 29], in this work the applicability of such type of extraction was tested paying special attention to subsequent DNA sequencing and investigating the compatibility of these methods. It was shown that the isolation by MPs is a modern, effective and time saving method for recovery of DNA from agarose gel improving significantly the efficiency of DNA sequencing. The performance of the MPs isolation was monitored by three analytical methods confirming its efficiency and applicability. Bioinformatic tool were employed for rapid interpretation of obtained results and it is easy to use. To our best knowledge, previously presented works did not focus on combination of the extraction procedure with PCR amplification, which is a key step of DNA sequencing ensuring the labeling of the DNA by fluorescent nucleotides. We believe that possible contamination of extracted DNA with remains of the agarose gel might preclude the fluorescent labeling a therefore intercept the sequencing.

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