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Methodological improvements of pyrosequencing technology

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Abstract

Pyrosequencing technology is a rather novel DNA sequencing method based on the sequencing-by-synthesis principle. This bioluminometric, real-time DNA sequencing technique employs a cascade of four enzymatic reactions producing sequence peak signals. The method has been proven highly suitable for single nucleotide polymorphism analysis and sequencing of short stretches of DNA. Although the pyrosequencing procedure is relatively straightforward, users may face challenges due to varying parameters in PCR and sequencing primer design, sample preparation and nucleotide dispensation; such challenges are labor and cost intensive. In this study, these issues have been addressed to increase signal quality and assure sequence accuracy.

Keywords

DNA sequencing; Primer-dimers; Pyrosequencing technology; Sample preparation; Single-strand binding protein

1. Introduction

DNA sequencing is a basic and essential tool in molecular biology and applied biosciences, allowing analyses ranging from single nucleotide polymorphism identification to whole genome sequencing. Though the majority of DNA sequencing techniques are gel-based and electrophoretic, there are high-throughput techniques that are more suitable for other applications than long sequence reads. Pyrosequencing (Ronaghi et al., 1998) is a bioluminometric and real-time sequencing technique, well suited for de novo and short-read sequencing. Pyrosequencing has been demonstrated to allow sequencing of up to 100 bases (Gharizadeh et al., 2002). The method is broadly applicable for analysis of single nucleotide polymorphisms (SNPs) (Ahmadian et al., 2000a; Milan et al., 2000; Nordstrom et al., 2000) and for identification of short DNA sequences used in bacterial (Gharizadeh et al., 2003b), fungal (Gharizadeh et al., 2004) and viral typing (Gharizadeh et al., 2001; Pourmand et al., 2002). In addition, the methodological performance of the pyrosequencing technology has proven practical in the determination of difficult secondary structures (Ronaghi et al., 1999), mutation detection (Ahmadian et al., 2000b; Garcia et al., 2000), DNA methylation analysis

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(Uhlmann et al., 2002; Yang et al., 2004), tag sequencing of a selected cDNA library (Nordstrom et al., 2001) and clone checking (Nourizad et al., 2003).

Although the pyrosequencing technique is relatively simple and straightforward, users may encounter challenges due to different parameters in sample preparation (single-strand procedures) prior to sequencing, primer design, enzyme and nucleotide dispensation, primer self-looping, primer-dimers (primer-primer hybridizations) and cross-hybridizations (when more than one sequencing primer is used). Although single-stranded DNA binding protein (SSB) (Ehn et al., 2002; Ronaghi, 2000) is highly recommended for primer and template complications in pyrosequencing, it has, at times, shown limitations in resolving strong secondary structures or primer-related self/cross-hybridizations in challenging regions. This is particularly observed when there is limited flexibility allowed in primer designs.

Many of these practical concerns and troubleshooting are not discussed in the literature, and this is the first time such issues are being addressed. In this study, significant improvements have been made to assure accurate data collection and increase sequence quality of difficult templates, consequently reducing labor and cost. We introduce (i) simple and informative test templates for rapid evaluation of the system prior to each sequencing run for enzyme, substrate, nucleotides and dispensation, (ii) a primer wash step in the sample preparation procedure to remove excess primers causing false signals due to secondary structure formation i.e. primer self-looping, primer-dimers and cross-hybridization. This improves sequence results and lowers background signals from challenging templates and sequencing primers (when SSB is not effective), (iii) use of a new pyrosequencing machine (PSQ HS 96A), which though initially intended for SNP analysis, was programmed with a modified dispensation protocol for DNA sequencing. This allows a 10-fold decrease in PCR volume (from 50 to 5 μ l), which reduces the cost and effort of performing PCR reactions. Finally (iv) introduction of a new, efficient aspiration handset designed and constructed in-house, to be used with the sample preparation machine (Vacuum Prep Workstation), which remarkably improves sample preparation efficiency. In addition, suggestions for effective primer design facilitating improved sequence results are discussed.

2. Materials and methods

2.1. Design and construction of aspiration handset device for sample preparation

Due to some limitations and inefficiencies occurring with use of the Vacuum Prep handset tool (Biotage, Uppsala, Sweden), a more efficient sample-preparation handset was designed and constructed at the Stanford Genome Technology Center (SGTC). The design modifications as applied to the new handset construct were intended to (i) increase liquid aspiration efficiency, (ii) prevent dilution effects caused by residual liquid volumes entering the reaction wells, and (iii) prevent uncontrolled liquid aspiration (which otherwise results in loss of reaction and template mixture). Upon disassembly, it was discovered that Biotage's handset tool is constructed with an unnecessarily large liquid chamber, divided by a wall into two chambers, and only one aspiration outlet. These flaws in the handset's design contribute to uneven and somewhat uncontrollable liquid aspiration. The new in-house tool was constructed with a thinner, non-divided liquid chamber and two aspiration outlets, one on each side of the tool.

A filter pad was designed to absorb the liquid that would run along the metal tips and into the wells of the microplate. The pad is placed around the tips at their point of insertion into the body of the handset. Fig. 1a and b exhibit both constructs. Detailed instructions for reconstruction of the handset are available at http://bioel.stanford.edu/vacuum_prep.

For evaluation of the new handset, a 1:2 dilution series of 1.4, 0.7, 0.35 and 0.17 of LS amplicon was prepared. The PCR conditions and primers (including sequencing primer) for the LS

amplicon are described in an earlier work (Gharizadeh et al., 2002). Sample preparation (single-strand separation and sequencing-primer annealing) was performed by both handsets and the efficiency of the handsets was compared by pyrosequencing (PSQ HS96A).

2.2. System and quality control template and preparation

The oligonucleotides Seq-BG (5'-GGA CTA TAA AGA TAC CAG GCG TT), BG02-short (5'-TTA ACC GGT ACG AAC GCC TGG TAT CTT TAT AGT CCA TC), and BG03-long (5'-TTC CAA GGA GTC TAC GAA CGC CTG GTA TCT TTA TAG TCC ATC) were designed for use as pyrosequencing system quality control assessments. The oligonucleotides were synthesized and HPLC purified by IDT (Coralville, IA, USA). The BG02-short and BG03-long oligonucleotides were each mixed with annealing buffer (20 mM Tris-acetate and 5 mM MgAc₂) and Seq-BG sequencing primer to obtain a final concentration of 10 pmol/μl. An annealing procedure was performed for each mix at 80 °C for 4 min and cooled to room temperature for 10 min. The hybridized oligonucleotides Seq-BG/BG02-short and Seq-BG/BG03-long were stored at -20 °C in small aliquots.

2.3. PCR products and amplification for primer wash step

The amplification oligonucleotides Biotin-pUC-5G-forward (5'-GGC TCG TAT GTT GTG TGG AA), pUC-5G-reverse (5'-CGG GCC TCT TCG CTA TTA CG) and the sequencing primer Seq-5Gb (5'-TTC GCT ATT ACG CCA GCT GGC G) were synthesized by IDT. Amplification was performed using the primer pair Biotin-pUC-5G-forward and pUC-5G-reverse for partial amplification of the vector pUC19 (Biolabs, New England, USA). PCR was carried out in 50 μl (total volume) reactions containing 2 mM MgCl₂, 50 mM KCl, 0.1% (v/v) Tween 20, 0.2 mM dNTPs, 1.5 units AmpliTaq Gold DNA polymerase (Perkin-Elmer, Norwalk, Connecticut, USA) and 10 pmol of pUC-5G-forward and Biotin-pUC-5G-reverse primers. A 2-min denaturation step at 94 °C was followed by 32 cycles of amplification with a thermocycler GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). Each cycle included a denaturation step at 94 °C for 40 s, a primer annealing step at 56 °C for 45 s, and a chain elongation step at 72 °C for 45 s. The final elongation step was prolonged by 10 min to ensure a complete extension of the amplified DNA. The length of the amplified DNA was 241 base pair.

2.4. Preparation of single-stranded DNA template

Single-stranded DNA samples were prepared semi-automatically using a Vacuum Prep Tool and Vacuum Prep Worktable (Biotage). In brief, 5–10 μl of biotinylated PCR product was immobilized onto 2.5 μl streptavidin-coated Sepharose High Performance beads (Amersham Biosciences, Piscataway, NJ, USA) under agitation at 1400 rpm using an Eppendorf Thermomixer R (Eppendorf AG, Hamburg, Germany) for at least 5 min. The immobilized DNA on Sepharose beads were treated separately with 70% EtOH, 0.2 M NaOH and TA buffer (0.1 M Tris-acetate, pH 7.6) for denaturing the double-stranded DNA according to company instructions (Biotage) for use of the Vacuum Prep Station. Single-stranded DNA immobilized on Sepharose beads was suspended in 12 μl annealing buffer (10 mM Tris-acetate pH 7.75, 5 mM Mg-acetate) and hybridized to 4 pmol sequencing-primer relevant to the specific assay at 90 °C for 2 min and incubated for 10 min at 60 °C and 5 min at room temperature (22 °C).

2.5. Preparation of single-stranded DNA template with primer wash

Template preparation was performed according to the standard protocol with an additional washing step procedure using the in-house Vacuum Prep Tool and the Vacuum Prep Worktable. In brief, 15–20 μl of biotinylated PCR product was immobilized onto 3.5 μl streptavidin-coated Sepharose High Performance beads by incubation at room temperature under agitation at 1400 rpm for at least 5 min. The immobilized DNA on Sepharose beads was treated separately with

70% EtOH, 0.2 M NaOH and TA buffer. The immobilized single-stranded DNA was then suspended in 24 μ l annealing buffer. The Vacuum Prep Tool (handset) was placed in the wells for 5 min to allow diffusion of beads. The DNA strand was hybridized to 8 pmol of the sequencing-primer relevant for the specific assay, using the GeneAmp PCR system 9700 at 90 °C for 2 min followed by incubation at 60 °C for 7 min and cooling to room temperature (22 °C). Subsequently, an additional primer wash step was performed. The DNA template immobilized on the streptavidin-coated Sepharose beads was captured by the filter tips of the in-house Vacuum Prep Tool with applied vacuum, and washed with 70% EtOH and TA buffer and then suspended in 12 μ l of annealing buffer in the microplate wells.

2.6. Pyrosequencing

The PSQTMHS96A DNA sequencing system, originally intended for SNP analysis, was used for DNA sequencing by modifying a repetitive sequencing dispensation order of n (ACGT) for de novo sequencing in the SNP Software Version 1.2 and adding a SNP in the end, such as G/C. This adjustment will allow the user to program the desired dispensation order for both de novo and resequencing. This PSQTMHS96A system uses a more sensitive CCD camera and Capillary Dispensation Tips (CDTs), permitting use of significantly lower PCR product volumes of 5–10 μ l (up to 10 times lower than the conventional pyrosequencing used previously), thus decreasing the cost and labor for both PCR and sequence reactions. Samples were pyrosequenced at 28 °C and pH 7.6 with a dispensing pressure of 625 mbar with 4 ms open time and 65 s cycle time. Sequencing was performed in a total volume of 12 μ l using the PSQ 96 SNP kit (Biotage). The detailed pyrosequencing chemistry at elevated temperature, pH and read-length limitations are described in an earlier work (Eriksson et al., 2004; Gharizadeh et al., 2002).

2.7. Minimum detection range by pyrosequencing

The LS-fragment PCR product (Gharizadeh et al., 2002) was used to determine the minimum detection limit range by the PSQTMHS96A system by two different approaches. After amplification, sample cleanup was performed using the QIAquick PCR Purification Kit (Qiagen, Valencia, USA). Thereafter the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) was used according to manufacturer's instructions to determine the quantity of the PCR amplified LS-fragment. In parallel the quantitative gel-based exACTGene 50 bp Mini DNA Ladder (Fisher Scientific, Pittsburgh, PA) was also used to measure the DNA concentration of the LS-fragment amplicon according to manufacturer's instructions. A dilution series of the quantitative PCR amplicon was thereafter sequenced by the PSQTMHS96A system. The determination of the minimum detection limit range was based on the PCR product dilution range where readable sequence information could no longer be obtained.

3. Results and discussion

Pyrosequencing is a relatively straightforward and user-friendly method possessing unique methodological characteristics. The technique is currently used in multidisciplinary fields in academic, clinical and industrial settings. The disparate applications and DNA sequencing parameters of this technology may present users with difficulties, especially during sequencing of challenging DNA regions. Recent improvements to the method will assist users in assuring sequence results and increasing the quality of the signal peaks.

The test oligonucleotides BG02-short and BG03-long were designed as quality control templates to test the performance of nucleotides, enzyme and substrate dispensations in the designated wells. BG02-short can be used for short and rapid sequence results. BG03-long is designed for use with relatively longer sequences. Fig. 2a and b demonstrate the Pyrograms of

BG02-short and BG03-long, respectively. Incorrect dispensations, clogged dispensers or machine failure may be easily monitored by sequencing the BG test templates. It is highly recommended that sequencing of these templates be performed before starting a sequencing run to act as a control for the performance efficiency of the system and its instrumentation. The test templates also help to assess the quality of the enzymes, substrates and nucleotides. Additionally, the use of a test PCR amplified template as a positive sample preparation control (in the present study, the LS-fragment amplicon) is also recommended. More importantly, the LS amplicon, which can be sequenced for experiments requiring longer sequencing reads, demonstrates the performance efficiency of the sample preparation system.

The original Vacuum Prep Tool handset from the manufacturer is designed with a large liquid chamber accommodating the vacuumed liquid, with only one aspiration outlet. The overly spacious chamber is divided by a wall with a very narrow opening to allow liquid passage (Fig. 1a). This results in inefficient and unequal liquid aspiration and residual liquid remains in the chamber, causing dilution of the samples in the microplate wells. Moreover, liquid droplets bind to the outer surfaces of the metal filter tips and can contribute to the reaction volumes, thereby further diluting the sequencing reaction mixture. The standard handset also contains metal plates inside, which are prone to rusting (as observed in our laboratory's handset). To address these issues, a new aspiration handset was designed and constructed to contain a thinner vacuum chamber and two aspiration outlets, resulting in extremely efficient liquid removal (Fig. 1b). Furthermore, a liquid absorbing pad was installed at the point of tip attachment, preventing the dispersal of residual liquid volumes into the reaction wells. A 1:2 four dilution-series concentration of LS amplicon (1.4, 0.7, 3.5 and 0.17 pmol) were denatured and primer-annealed by both the standard Vacuum Prep Tool handset and the in-house constructed handset for comparison and evaluation. The samples were sequenced in parallel, and the pyrogram sequence results (Fig. 3a and b) show significantly higher signal intensities (y-axis) with the new handset, indicating remarkably decreased DNA loss. Although we have provided all the information for building the new handset, we are fully aware that not every laboratory has the facilities to construct one. This clearly indicates the need for upgrading and reconstruction of the standard handset.

The standard Sepharose beads protocol for the Vacuum Prep Station does not include a primer-washing step (which is not needed in most cases, particularly in the presence of SSB). However, there are sequencing primers or multiple sequencing primers (Gharizadeh et al., 2003a, 2005) that result in false sequence signal peaks from strong primer-dimers or secondary structures even with the inclusion of SSB. The introduced primer wash is recommended for such cases. The major challenges encountered as the result of primer removal/wash step have been (i) loss of sepharose beads containing immobilized ssDNA due to extra washes and (ii) liquid residues left in the chambers decanting back in the microtiter wells and diluting the sequencing reaction volumes. The first challenge is remedied through use of higher volumes of the PCR product (15–20 μ l) and more Sepharose beads. The second challenge was addressed through the development of a new handset. To demonstrate these improvements, a template was sequenced using a challenging sequencing primer (Seq-5Gb), which results in strong primer-dimers involving 12 nucleotide base-pairings (-29.7 kcal/mol) (Fig. 4). The Seq-5Gb primer-dimer is extended by DNA polymerase, giving rise to sequence signals that interfere with the sequence results from the target template; SSB is not capable of resolving this primer-primer interaction. Fig. 5a shows the Seq-5Gb sequencing primer in absence of template, and Fig. 5b shows the sequence of the target template 5G, sequenced with the primer Seq-5Gb. Although SSB is used in all the experiments, no interpretable sequence signal peaks are generated. By performing the primer wash step and removing the excess and non-hybridized Seq-5Gb, uniform sequence results (without interference from the sequencing primer) are produced (Fig. 5c). The primer wash allows generation of interpretable sequence results where the standard protocol fails. This is particularly important when there is no or low flexibility in

primer design or when using multiplex sequencing primers for microbial and viral detection (Gharizadeh et al., 2003a,b, 2005). Although SSB has shown to be useful in eliminating such complications in most cases, there are challenging sequencing primers and templates that it cannot resolve. Therefore, an extra wash procedure in template preparation is recommended as to remove unbound and excess primers in such cases. Furthermore, use of SSB is encouraged in all sequencing reactions to improve sequence reads. Recently, there is a new commercial sequencing kit available (Pyro Gold from Biotage) with SSB integrated as a reagent.

Two different methods were used for determining the minimum detection range of the PSQTMHS96A System; the results of both approaches are in agreement. The minimum detection range for DNA sequencing was measured to be 0.1 pmol by the quantitative gel-based exACTGene and 0.15 pmol with NanoDrop spectrophotometer. This indicates that considerably low quantities of DNA can be sequenced, i.e. amplification products showing a weak band on agarose stained gel. As a general rule, amplicons generate the best sequencing signal heights when a clear and specific product band is detected on ethidium bromide-stained agarose gels after PCR amplification. Moreover, we recommend that a nested sequencing primer instead of a PCR (general) primer be used for sequencing to maximize specificity and accuracy. Nested PCR is advised for direct amplification of low copy numbers from cell lysates or in cases of nonspecific amplification from DNA extracts.

We also suggest the sequencing primers be designed before PCR primers in order to biotin-label the most suitable and practical DNA strand for sequencing. When designing sequencing primers for pyrosequencing, the possibility of strong hairpin loops or self-primer-primer hybridizations should be considered, as they give rise to sequence signal peaks due to the enzymatic activity of polymerase and subsequent extension during the course of DNA sequencing. There is commercial software available (Assay Design Software from Biotage) that practically covers such issues. When primer design is limited for a specific region, the additional primer wash is a useful approach, but does require more PCR product.

In conclusion, pyrosequencing technology is a fairly novel DNA sequencing technique with room for improvements in its chemistry and instrumentation. The purpose of this work has primarily been to discuss the challenges that users may face and to introduce solutions and improvements. These will result in labor and cost savings in cases of challenging templates while assuring data attainment and providing more refined DNA sequence information.

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Fig. 1. Schematic illustrations of sample-preparation handsets for the Vacuum Prep Workstation. (a) The manufacturer's standard and (b) the in-house designed. See text for details.

Fig. 2. Pyrograms of the two oligonucleotide templates (a) BG02-short and (b) BG03-long for general quality control of the pyrosequencing DNA sequencing system.

Fig. 3.

Comparison of standard- and in-house-constructed handsets for sample preparation of four 1:2 dilution series of the LS amplicon (1.4, 0.7, 0.35 and 0.17) for DNA sequencing. (a) and (b) demonstrate four pyrograms, respectively, with concentration noted above each trace.

Fig. 4.

Schematic illustration of (a) strong primer–dimer formation and (b) hairpin loop formation by the Seq-5Gb primer that cannot be removed by SSB. This self-dimer formation results in sequence signal peaks.

Fig. 5.

(a) The sequencing primer Seq-5Gb gives rise to false sequence signal peaks in absence of template in pyrosequencing reaction (b) false sequence signals are generated by the seq-5Gb sequencing primer when used for sequencing of the 5G template without the primer wash, which results in non-interpretable sequence signals, and (c) after removing the excess Seq-5Gb from the pyrosequencing reaction mixture by the primer wash step, specific sequence signals peaks are obtained. SSB has been used in all these sequencing reactions.