Parallel DNA Template Preparation Using a Vacuum Filtration Sample Transfer Device

J. Dunker, U. Larsson, D. Petersson, J. Forsell, A.-L. Schiller,
A. Alderborn, and L.M. Berg
Pyrosequencing AB, Uppsala, Sweden

BioTechniques 34:862-868 (April 2003)

ABSTRACT

Solid-phase techniques have facilitated the handling of biochemical analytes. This has stimulated the development of systems by which large sample panels can be analyzed with high levels of security and quality. We describe a sample transfer device based on the principle of vacuum filtration, which enables parallel handling of 96 samples of analytes bound to Sepharose™ beads. The tool was employed for strand separation of DNA samples, by attracting the beads to filter probes while passing them between the reagent solutions. The samples were analyzed using Pyrosequencing™ technology and proved to yield genotyping results of high quality. The presented sample preparation procedure provides an important link in the development of integrated systems for rapid genetic analysis at a low cost. In addition, the same filter could be reused extensively with very low risk for detectable cross-contamination between assays and without any reduction in processing capacity, thus further reducing the cost per analyzed sample.

INTRODUCTION

The methodology of immobilization of analytes by attachment to a solid surface increases the robustness and quality of laboratory analyses and enables automation by facilitating the exchange of reagent solutions. In the case of DNA sequence analysis, solid-phase techniques have simplified the purification of single-stranded templates before sequencing reactions. The solid surface can be provided by suspended particles or by the walls of reaction containers. The former are preferred in microscale applications because they increase the area-to-volume ratio and enhance the reaction kinetics.

Immobilization of templates for DNA sequencing reactions is conveniently achieved using the strong avidin-biotin interaction (1). Pyrosequencing™ technology (Pyrosequencing AB, Uppsala, Sweden) is one example of a method that has benefited from this procedure. This is a single-tube sequencing-by-synthesis technique that relies on enzyme-mediated, real-time detection of nucleotide incorporation during primer strand elongation (2,3). Although feasible with dsDNA in liquid phase (4,5), Pyrosequencing reactions have primarily been performed with solid-phase, single-stranded templates. Biotinylation of either the upstream or the downstream PCR primer and attachment to streptavidin-coated beads (6) enable the purification of single-stranded PCR products before the Pyrosequencing reaction. This method has proven to be highly accurate for the determination of single nucleotide polymorphisms (SNPs) and short sequences and has been applied in a wide variety of contexts (7).

Template preparation for Pyrosequencing reactions has been greatly facilitated by using paramagnetic beads as the solid phase. Sample preparation devices have earlier been constructed, based on ejectable magnetic probes that enable transfer of templates in parallel between reagent solutions. As an alternative solution, a preparation protocol based on streptavidin-coated Sepharose™ beads (Amersham Biosciences AB, Uppsala, Sweden), in which denaturation and washing is performed in filter plates, has been developed by Pyrosequencing AB. Binding of the template is carried out at room temperature using this procedure, which is advantageous in many settings. The Sepharose protocol has yielded results of high quality in applications of Pyrosequencing technology (8,9). However, the efficiency of sample han-

dling is reduced with this solution, since all sample transfer must be performed by pipetting and the reagents for strand separation must be dispensed to each sample.

In this study, we developed and tested a device for the efficient handling of DNA strands bound to Sepharose beads. The instrument, referred to as the Vacuum Prep Tool, relies on vacuum filtration that captures the beads and holds them during transfer between reagent solutions. Since fluids flow freely through the filters, reagents are given full access to the bound analytes during incubation, whereas the filters are easily washed and can be reused a large number of times. The performance of the Vacuum Prep Tool was evaluated for use with Pyrosequencing technology, in the analysis of synthetic oligonucleotides and human SNP samples.

MATERIALS AND METHODS

DNA Material and Amplification

Two complementary oligonucleotides, one of which was biotinylated, were synthesized by Thermo BioSciences GmbH (Ulm, Germany). These were mixed in equal proportions and annealed for 2 min at 80°C to produce dsDNA. The base composition of the biotinylated strand was 5'-CTCC-AGCTCCTGGGCAGGTAGCGCTGCCCGTTCTAGGTC-3'. This strand was analyzed using the sequencing primer 5'-GACCTAGAACGGGCAGC-3'.

DNA sequences containing the biallelic SNP T3409C in the angiotensin-converting enzyme gene (*ACE*) were amplified and analyzed from human DNA samples as described elsewhere (10). Either the forward or the reverse PCR primer was biotinylated. PCR was performed in 50 µL 1× PCR buffer II (Perkin Elmer, Boston, MA, USA), 1.5 mM MgCl₂, 0.125 µM dNTP, 0.2 µM each primer, using 1.5 U AmpliTaq Gold® DNA polymerase (Perkin Elmer). PCR yield and quality were estimated by electrophoretic separation of products in 1.5% agarose gels followed by staining with ethidium bromide and visualization using UV-transillumination.

Pre-Sequencing Sample Preparation

The dsDNA analytes were immobilized onto streptavidin-coated 34-µm Sepharose beads (Streptavidin Sepharose High Performance). Two microliters of bead gel slurry were added to each sample, containing 0.25, 0.5, 1, 1.5, or 2 pmol DNA in 80 µL binding buffer (5 mM Tris-HCl, pH 7.6, 1 M NaCl, 0.5 mM EDTA, 0.05% Tween 20® in final concentration), in a 96-well PCR plate, and incubated at room temperature for 5 min with continuous shaking at 1400 rpm. Vacuum was applied to collect the beads with immobilized templates on the filter probes (sintered polyethylene, 2 mm thick, pores 10 µm) of either of four identical Vacuum Prep Tools, which were powered by a Millivac Vacuum pump (Millipore, Bedford, MA, USA) at a pressure of 400 mmHg. The beads were subsequently transferred between solution troughs according to the following scheme (unless noted otherwise): 70% ethanol for 5 s, 0.2 M NaOH

for 5 s, and washing buffer (10 mM Tris-acetic acid, pH 7.6) for 5 s. After denaturation and washing, the beads were released into PSQ™ HS 96 Plate wells containing 0.33 µM sequencing primer in either 12 or 14 µL (unless noted otherwise) of annealing buffer [2 mM Mg (CH₃COO)₂, 20 mM Tris-acetic acid, pH 7.6]. The release was accomplished by deactivating the vacuum source and by rubbing the filter probes against the bottom of the wells. Sequencing primers were annealed onto the immobilized single-stranded templates by incubating at 80°C for 2 min. The reactions were allowed to cool to room temperature before analysis. After preparation of one plate, the Vacuum Prep Tool was flushed with approximately 180 mL Milli-Q® water (18.2 MΩ/cm; Millipore) (i.e., one full trough). Each tool was reused without exchange of filters.

Evaluation Using Pyrosequencing Technology

The samples were analyzed using the SNP reagent kit with the PSQ HS 96A System (Pyrosequencing AB) according to standard instructions. The results were evaluated using SNP/AQ 1.2 software (Pyrosequencing AB).

RESULTS AND DISCUSSION

Transfer of DNA Strands Using Vacuum Filtration

The idea behind the Vacuum Prep Tool is to use vacuum filtration for transfer between reagent solutions of samples that are bound to Sepharose beads. Thus, instead of applying vacuum from below in a stationary filter plate and suspending solutions to each well, the underpressure over the filters is used for lifting the beads and holding them while they are passed between solutions. The tool is essentially a box, constructed from chemically resistant compounds, which is equipped with 96 protruding filter probes and connected to a vacuum station through a rubber hose. The probes were designed to enter the body of the tool to avoid any back-flow of fluids through the filters. The filters were placed at the bottom of the probes to facilitate release of beads into reaction plates through rubbing against the bottom of the wells. To be functional for strand separation of DNA sequencing templates bound to Sepharose beads, the filters should allow all liquid-phase DNA to pass through the pores while capturing the beads. Sintered polyethylene filters were chosen because of their low affinity for DNA and because their thickness makes them easy to apply to the probes. The pore diameter is 10 µm, which is sufficiently small for blocking Sepharose beads of the preferred size (34 µm).

Handling of samples with the Vacuum Prep Tool was routinely initiated within 3 min after immobilization and shaking, since the beads could more easily be attracted to the filter probes while they remained in suspension. DNA (0.25–2 pmol) was transferred using this procedure. Transfer of larger amounts was not attempted but is likely to be feasible. Preparation of single-stranded templates from a full 96-well plate took approximately 30 s.

Reproducibility

Several analyses based on Pyrosequencing technology were performed using well-characterized DNA samples to optimize the performance and to evaluate the well-to-well and assay-to-assay robustness of sample preparation with the Vacuum Prep Tool. In Pyrosequencing reactions, the one-to-one relationship between the nucleotide incorporation events and the eventual release of photons, triggered by the enzyme system, enables an assessment of the amount of template DNA of a given base composition (11). Hence, the strength of the nucleotide incorporation signal at a given template position, expressed as the maximum height of detected light peaks, was used to estimate the amount of template that was prepared and transferred. In a first study using human SNP samples (see below), slight variations in the strand separation procedure were evaluated to establish the robustness of the scheme. The samples were analyzed in triplicates within a single assay, and conditions varied between assays as follows. The duration of the denaturation and washing treatments varied between 1 and 10 s, and the final reaction volume varied between 10 and 20 μ L. Of these alterations, treatment duration had no detectable effect on the resulting sequencing signal, whereas the dilution of the reaction led to a 49% average decrease of peak heights and a 31% increase in variation between sample wells. This is most likely a consequence of dilution of the enzyme system (3) and bears little relevance to the performance of the Vacuum Prep Tool.

As a next step, assays with 96 samples of identical synthetic oligonucleotides, present in three different concentrations (0.5, 1, and 1.5 pmol/12 μ L), were run in triplicates using standardized procedures. In these analyses, the incorporation signal from a single G base was measured. The average peak height increased with increasing amount of template processed, in an approximately linear fashion (not shown). The mean coefficient of variation of peak heights was slightly smaller between wells than between corresponding wells in different replicate assays (Table 1). In turn, the variation between results from the same well position when handled by different Vacuum Prep Tool specimens was similar to or smaller than the corresponding variation between assays prepared with the same specimen.

These figures most likely overestimate the variability of sample handling with the Vacuum Prep Tool, as the variation also has external causes. First, the sequencing signal in applications of Pyrosequencing technology is expected to vary slightly between reaction wells,

Table 1. Reproducibility of Vacuum Prep Tool

	Amount of DNA Template (pmol)		
	0.5	1	1.5
Between wells	17.1	17.7	18.3
Between assays	24.0	22.6	24.1
Between specimens	20.0	22.1	21.0

Table shows mean cv (%) in peak heights obtained upon incorporation of dGTP at position 8 in the analyzed sequence 5'-GCTACCTGCCCAGGAGCTGGAG-3', in replicate assays prepared using Vacuum Prep Tools and analyzed with Pyrosequencing technology.

since the location of the light detection system relative to that of the wells varies over the reaction plate. Second, minor irregularities during handling of samples, such as evaporation from reaction wells before analysis, may cause variation in

Figure 1. Vacuum Prep Tool filter lifetime. Output of Pyrosequencing reactions prepared using the same Vacuum Prep Tool specimen for analysis of a human DNA sample with regard to the T3409C SNP of the *ACE* gene. The analyzed sequence 5'-TGGCCGGGTC-3' was correctly determined in the initial assay (a) as well as in the 117th consecutively prepared replicate (b) without exchange of filters, and the strength of the sequencing signal was approximately the same in both runs.

Product Application Focus

the results between assays. Therefore, it is not surprising that the variation in signal strength between assays was slightly larger than the one between wells within assays.

Repeated Use of Filters

To keep the cost per analysis at a minimum, it is of interest to consider the possibility of repeated use of sample preparation equipment. The feasibility of repeated use of

probe filters was evaluated using human DNA samples, which were analyzed repeatedly with regard to genotype in the T3409C SNP of the *ACE* gene, in a long series of consecutive assays that were prepared using a single set of filters. A sample homozygous for the T allele was analyzed in three template concentrations (0.5, 1, and 2 pmol template/14 μ L) and in triplicate for each concentration. To save time and expense, every tenth assay preparation was performed using template, while the nine preparations in between involved

beads without attached template. The performance of the sample preparation tool was unchanged after 117 repeated assay preparations, as judged by the overall quality of the resulting pyrograms (Figure 1). After these repeated preparations, the experiment was terminated. From this, it is clear that the filters can be reused several times without any noticeable decrease in capacity.

Processed template that remains on the filters after washing may cause cross-contamination between corresponding wells in consecutively prepared reaction plates. This phenomenon was studied in experiments performed using homozygous T/T and C/C samples, in template concentrations as above and in one replicate each. First, normal SNP-scoring assays were alternated with assays in which a number of wells were free of template, to evaluate the effect of contamination in negative controls. A triple-G position in the studied sequence was analyzed, since this generates a stronger signal and therefore improves the ability to measure cross-contamination. The sequencing signal in empty wells with risk for cross-contamination was on average 1.2% (SD 0.8%) of the corresponding peaks in the same assay, obtained from wells to which template of the same genotype had been added. These cross-contamination ratios, which undoubtedly were to some extent inflated by background noise in the light detection system, were more pronounced in cases of low template concentrations (Figure 2a). One outlier with a comparably high contamination ratio can be explained by an overall poor performance of

Figure 2. Repeated use of filters and cross-contamination between assays. Cross-contamination between consecutive assays in a series of repeated Pyrosequencing reactions performed with the T3409C SNP of the *ACE* gene, using the same Vacuum Prep Tool specimen for template preparation without exchange of filters, quantified (a) as relative peak height in wells without addition of bound template compared to wells with added template and (b) as the ratio of estimated frequency of contaminating material to background noise in a regime of alternation between addition of samples containing opposite homozygous genotypes. $\square$: C/C homozygote and $\diamond$: T/T homozygote cross-transferred. Each datapoint corresponds to a single reaction. Markers of small, medium, and large size denote the amount of template added in each repeated assay, 0.5, 1, and 2, respectively.

Product Application Focus

the assay in question, which reduced the signal from the control genotype.

Second, the effect of cross-contamination between samples was studied by alternating between handling of homozygous C/C and T/T templates. The relative magnitude of peaks caused by cross-contaminating and currently processed samples was expressed in terms of the relative frequencies of the respective templates, as calculated by the Allele Quantification software for allele frequency estimation in pooled samples (Pyrosequencing AB) (12,13). To estimate cross-contamination without disturbance from background noise, the estimated frequencies of contaminating material were compared with the corresponding allele frequencies in wells where the same genotype was added in each sequential assay and where there was no risk of cross-contamination. The ratio of cross-contamination allele frequency to the relative magnitude of background was in general close to unity ($\times 1.28$, SD 0.48; Figure 2b). Also, the variance in relative peak height among assays was similar for wells with and without risk for cross-transfer, indicating a small contaminating effect.

The signal from contaminating template tended to be stronger in well positions where high template concentrations were routinely being processed. This indicates that the amount of cross-transferred template may increase with the template concentration. This in turn means that the risk of noticeable contamination increases when the template concentration varies widely between assays. At the same time, keeping DNA content high in all assays reduces the proportionate magnitude of background noise in the signal detection systems of Pyrosequencing technology, which makes any false signals easy to detect. When using the PSQ HS 96A System it is recommendable to keep DNA content in the range 0.5–1.5 pmol and to make efforts to ensure an equal amount across assays, to maximize the number of assays that can securely be prepared using a particular set of Vacuum Prep Tool filters. On the other hand, if precise estimates of allele frequencies in pooled DNA samples are requested (12), it may be necessary to exchange filters in the probes between assays or, alternatively, use a more efficient washing procedure (e.g., involving hypochlorite or ultrasonic bath).

We have demonstrated a procedure for parallel transfer of multiple samples between reagent solutions that provides an important link in the sample handling procedure at a low cost and that allows high levels of throughput. Whether automated or not, parallel sample transfer reduces processing time and labor demands in many biochemical applications. The present study has established the functionality of the Vacuum Prep Tool for the rapid preparation of ssDNA templates for Pyrosequencing reactions. These results suggest that the Vacuum Prep Tool provides a highly cost-efficient solution for the handling of macromolecules held on beads. It is likely to prove itself useful in a wide range of applications that require sample transfer or exchange of reagents.

ACKNOWLEDGMENTS

J.D. and U.L. contributed equally to this report.

REFERENCES

1. Ståhl, S., T. Hultman, A. Olsson, T. Moks, and M. Uhlén. 1988. Solid phase DNA sequencing using the biotin-avidin system. *Nucleic Acids Res.* 16:3025-3038.
2. Ronaghi, M., M. Uhlén, and P. Nyrén. 1998. A sequencing method based on real-time pyrophosphate. *Science* 281:363-365.
3. Ronaghi, M., S. Karamohamed, B. Pettersson, M. Uhlén, and P. Nyrén. 1996. Real-time DNA sequencing using detection of pyrophosphate release. *Anal. Biochem.* 242:84-89.
4. Nordström, T., K. Nourizad, M. Ronaghi, and P. Nyrén. 2000. Method enabling Pyrosequencing on double-stranded DNA. *Anal. Biochem.* 282:186-193.
5. Nordström, T., M. Ronaghi, L. Forsberg, U. De Faire, R. Morgenstern, and P. Nyrén. 2000. Direct analysis of single-nucleotide polymorphism on double-stranded DNA by pyrosequencing. *Biotech. Appl. Biochem.* 31:107-112.
6. Hultman, T., S. Ståhl, E. Hornes, and M. Uhlén. 1989. Direct solid phase sequencing of genomic and plasmid DNA using magnetic beads as solid support. *Nucleic Acids Res.* 17:4937-4946.
7. Berg, L.M., R. Sanders, and A. Alderborn. 2002. Pyrosequencing technology and the need for versatile solutions in molecular clinical research. *Exp. Rev. Mol. Diagn.* 2:361-369.
8. Andréasson, H., A. Asp, A. Alderborn, U. Gyllenstein, and M. Allen. 2002. Mitochondrial sequence analysis for forensic identification using Pyrosequencing technology. *BioTechniques* 32:124-133.
9. Ringquist, S., A.M. Alexander, W.A. Rudert, A. Styche, and M. Trucco. 2002. Pyrosequence-based typing of alleles of the HLA-DBQ1 gene. *BioTechniques* 33:166-175.
10. Alderborn, A., A. Kristofferson, and U. Hammerling. 2000. Determination of single nucleotide polymorphisms by real-time pyrophosphate DNA sequencing. *Genome Res.* 10:1249-1258.
11. Nyrén, P., B. Pettersson, and M. Uhlén. 1993. Solid phase DNA minisequencing by an enzymatic luminometric inorganic pyrophosphate detection assay. *Anal. Biochem.* 208:171-175.
12. Gruber, J.D., P.B. Colligan, and J.K. Wolford. 2002. Estimation of single nucleotide polymorphism allele frequency in DNA pools using Pyrosequencing. *Hum. Gen.* 110:395-401.
13. Wasson, J., G. Skolnick, L. Love-Gregory, and M.A. Permutt. 2002. Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by Pyrosequencing technology. *BioTechniques* 32:1144-1150.

Address correspondence to Jenny Dunker, Pyrosequencing AB, Vallongatan 1, SE-752 28 Uppsala, Sweden. e-mail: jenny.dunker@pyrosequencing.com