Rapid purification of fluorescent dye-labeled products in a 96-well format for high-throughput automated DNA sequencing

Karen Krakowski, Julie Bunville, Jason Seto¹, Dale Baskin¹ and Donald Seto*

Division of Applied Biotechnology, Experimental Station, The DuPont Merck Pharmaceutical Company, Wilmington, DE 19880-400, USA and ¹Department of Molecular Biotechnology, School of Medicine, University of Washington, Seattle, WA 98195, USA

Received September 12, 1995; Revised and Accepted October 31, 1995

Large-scale DNA sequencing projects can be limited by various technical bottlenecks. As more DNA templates are generated and successfully processed, the mechanisms and technologies continue to be improved and optimized. Fluorescence-based automated sequencing (1) has replaced radioisotope-based manual DNA sequencing (2) as the primary approach in large-scale genome projects. For this approach, the preferred DNA sequencing chemistry had been fluorescence dye-primer based reactions. Reiterations of this automated protocol generated large amounts of DNA sequence data with its corresponding biological information. This, in turn, spurred the continued improvements and modifications that allow the rapid production of data that is generated routinely today using thermostable DNA polymerases and single-stranded or double-stranded DNA templates.

Dye terminator chemistries are more flexible. This approach, coupled with a thermostable DNA polymerase and the need for less template (0.1-1 µg) as well as the availability of single-tube reactions per sample, allows for greater sample throughput. Its utility in primer walking for difficult templates and for gap closures has led to the widespread use of Taq polymerase-mediated dye-terminator chemistries in genome sequencing laboratories. This is in spite of the additional manipulations required. For example, due to the inefficiency of Taq DNA polymerase to incorporate the dye-labeled terminators, there is a need to remove the unincorporated molecules from the DNA sequencing ladder, as these unincorporated dyes interfere with the resolution of bands near the priming site and with base calling. This step is tedious and not readily amenable to automation. We describe a method to rapidly, efficiently and inexpensively remove these contaminating unincorporated dye-terminators from the DNA sequencing ladder prior to electrophoresis on a 373A or 377 ABI Sequencer (Perkin Elmer/Applied Biosystems Division; Foster City, CA).

There are three main steps involved in this protocol: the preparation of the resin, the preparation of the multi-well minicolumns and the purification of the DNA sequencing ladder.

Step 1. Preparation of resin. Sephadex G-75 (40-120 micron beads) is purchased from Sigma Chemical Company (St Louis, MO). The resin is prepared according to the manufacturer’s instructions. Subsequently, the suspended resin solution is gently inverted or swirled a few times to give a homogenous slurry, and allowed to settle briefly. It is preferable to store the prepared resin at room temperature, as the cold resin produces air bubbles in the bed unless it has been pre-equilibrated at room temperature prior to use.

Step 2. Preparation of multi-well minicolumns. Multi-well minicolumn blocks (Fig. 1), in the 96-well microtiter plate format, with a glass fiber filter frit may be purchased as ‘Deepwell filter plates, UNIfilter DW GF/C from Polyfiltronics (RockJand, MA). Alternatively, pre-poured units, ‘96-well gel filtration kits’, are now available from Advanced Genetic Technologies Corp. (Gaithersburg, MD). This array of minicolumns is placed on top of a 96-well microtiter plate, which acts as a physical support as well as a collection device. An aliquot (~800 µl) of the settled slurry is pipetted into each of the wells. This resin is packed under centrifugal force in a Jouan CR422 centrifuge, adapted with microtiter plate carriers (Jouan, Inc.; Winchester, VA). Centrifugation is carried out at 2100 r.p.m. (~750 g) for 2 min. The resultant bed volume is approximately one-half the height of the well, and is ~400 µl in volume; a second and a third aliquot of this slurry produces a 600 µl and an 800 µl settled bed, respectively. The 600 µl bed is optimal for separating the unincorporated dye-terminators from the sequencing ladder (Fig. 2). N.B., if the packed bed is cracked vertically from top to bottom, the most common cause is too much packing force. That particular well should be repacked.

*To whom correspondence should be addressed
Step 3. Purification and recovery of DNA sequencing ladder. A 20 μl sample is placed onto the top of each bed. Recovery of the sample is accomplished by centrifugation under the above conditions for 4 min. The recovered eluate can be transferred to microcentrifuge tubes and subsequently prepared as suggested by ABI for electrophoretic resolution on the 373A or 377 Sequencers. Alternatively, polycarbonate ‘V-bottom’ 96-well microtiter plates (USA Scientific; Ocala, FL) may be used for elution, the advantage being that these samples are dried by placing into an oven (~96°C) and stored directly in this disposable microtiter plate (Cecile Boysen, California Institute of Technology).

Since there is a high background of unincorporated dideoxy-nucleotides (ddNTPs) due to the inefficiency of DNA polymerases to incorporate them, the fluorescently tagged ddNTPs must be removed prior to electrophoresis. Currently, unincorporated dye-terminators are purified from the DNA sequencing ladder by gel-filtration through ‘Centri-Sep’ spin columns (Princeton Separations; Princeton, NJ) (3). Alternatives include phenol/chloroform extraction and precipitation with cetyl-trimethyl ammonium bromide (CTAB) (3). Due to the lack of more efficient alternatives, the Centri-Sep spin column has been the method of choice. These manipulations are not readily amenable to automation, and the cost of the spin columns is relatively high, running approximately $2.40 per sample. Multi-minicolumns cost approximately $0.23 per sample, excluding the resin which may be purchased in bulk. The resin is economical, swelling to 12–15 ml of bed volume per gram of dry resin. Typically, 600 μl of packed bed volume is required, at the minimum, per sample, at an average cost of $0.06 per bed. As mentioned earlier, pre-poured 96-well block arrays are now commercially available (‘96 well gel filtration kits’). Their cost, with a proprietary resin, is approximately $120 per two plates (or $0.63 per sample).

Screening a panel of Sephadex resins (G50-80, G50-150, G50-300 and G75-120) has identified G75-120 as the optimal medium for these multiwell columns (data not shown). Figure 2 shows an ABI 373A DNA Sequencer gel profile of the sequence ladders with samples purified with either Centri-Sep or Sephadex G75-120 resin in the microtiter plate format. Shorter well formats, such as with 0.3 ml wells, do not work well because the smaller volume of resin contained is not enough to separate the unincorporated dyes.

Although not as convenient, a Sorvall RT6000 refrigerated centrifuge may be used. The parameters for this instrument are washing/packing at 500 r.p.m./15 s and eluting at 500 r.p.m./90 s. The main drawback to this centrifuge is that the microtiter plate carriers are not constructed for these non-standard sized plates. For example, the center bar must be removed each time the multi-well minicolumns are accessed. Eluate volumes of ~100 μl are recovered.

It should be noted that another version of the method described above has been used successfully in a genome sequencing laboratory (4). This method involves mounting 12 or 24 empty 1.0 ml columns onto a collection rack, and filling with Sephadex G-50 superfine resin. The throughput appears limited as the elution volumes are large and the separation is based on gravity flow. It seems to be not as convenient as the commercially available 96-well block described in this work. Finally, changes in the reaction conditions, e.g. novel enzymes, may have an impact on post-reaction processing. Perkin Elmer/Applied Biosystems Division and Amersham Life Science Inc. (Arlington Heights, IL) have recently introduced modified Taq polymerases for fluorescence-based DNA sequencing. Our experiences with Taq FS reactions suggest that it is still worthwhile to continue using gel filtration purification, in some form, to remove the residual free unincorporated dyes.

ACKNOWLEDGEMENTS

We thank G. Hollis, R. Horlick, H. George, E. Anton and J. Doetkott for comments on the manuscript. We also thank Jon Lipsky (Polyfiltronics) and also Hal Noer and Bill Miller (Advanced Genetic Technologies Corp.) for providing samples and information on their products.

REFERENCES