High-Density Small-Volume Gel Loading Directly from Capillary Tubes

ABSTRACT

A technique has been developed for high lane density loading of small-volume DNA samples in a horizontal agarose gel. This technique has been investigated with a simple hand-held tool that is made to couple to sample output from a new capillary-based sample automation system. The approach consists of piercing the gel with pressurized sample capillaries and relieving the pressure shortly before withdrawal. The pressurization prevents the capillary from aspirating the gel buffer and keeps the sample at the tip of the capillary, so that it may be sucked into the gel during withdrawal. This method is shown to be adequate for a wide range of DNA ladders and PCR-based screening. In addition to allowing smaller lanes and a higher lane density than is achievable with traditional well-forming techniques, it relaxes the need for well formation and the alignment of the sample loader with those wells, providing an easy, efficient means of loading agarose gels.

INTRODUCTION

Using agarose gel electrophoresis to separate and view DNA reaction products typically involves loading samples into a gel individually, which is a tedious and time-consuming process. This is eased somewhat by parallelization through the use of multichannel hand pipettors and by fully automated systems that use robot arms with single-or multichannel pipettors (3). While these techniques reduce the time required to load a gel, they do little to address a more fundamental bottleneck that exists in large-scale genomic operations: the time-and manpower-consuming process of reliably loading and forming the gel as compared to the amount of information extracted from it. Standard agarose gel loading can also be difficult to automate because a loading dye must be added to the reaction mixture before processing or between the processing and gel loading steps.

One way to ease the bottleneck of gel formation and loading is to increase the information obtained per gel forming operation by increasing the number of lanes per gel. Typical gels are loaded at a maximum density of one well every 4 mm and <100 wells per gel. Limiting factors include the difficulty of forming and loading narrow wells, the time required to load large numbers of samples and the time available for gel loading, which is limited by sample diffusion. Some have loaded as many as 200 lanes simultaneously by transferring samples to a membrane that is then placed onto the gel (2), but this requires the use of additional disposables, and the technique has not yet been perfected commercially.

The use of narrow lanes also facilitates the use of ever-smaller reaction volumes, allowing conservation of expensive reagents. Using narrow lanes concentrates the fluorescing dye-DNA samples spatially, preserving the “brightness” of the DNA bands compared to traditional wells.

The process of forming and loading gels becomes more of a bottleneck when automated high-throughput sample handling is used to increase the “upstream” sample processing rate. In particular, this paper is concerned with a technique compatible with Acapella, a capillary-based reaction automation device (4). In this device, small glass capillaries are used as reaction chambers to reduce reaction volumes and thermal cycling times (5) in biological and chemical procedures. Acapella uses 55 mm long, 5 µL capacity capillaries (OD = 0.864 mm, ID = 0.340 mm; Drummond Scientific, Broomall, PA, USA) for 1 or 2 µL reaction volumes. Acapella processes more than 1000 samples in 8 h, and a system to process 5000 samples in 8 h is being built.

After processing, the samples can be loaded from the capillary into a standard gel well. However, the care required to safely eject a 1–2 µL volume from a hydrophilic capillary makes it difficult to match the required gel loading throughput to the upstream sample handling throughput: injecting air bubbles while loading the well leads to significant sample loss. Additionally, in Acapella, the starting position of the fluid may be hard to control, and in optimization experiments, the reaction volumes may vary from capillary to capillary. These factors preclude simple parallel loading into traditional wells.

Therefore, to best couple gel loading to the needs of Acapella, both the sample loading rate and Acapella’s output format (capillaries) must match. This has been achieved by loading the samples directly from capillary tubes by gel puncture. In this procedure, the capillary creates its own sample well while piercing the gel, and the sample is sucked into this well by smoothly withdrawing the capillary. Using small capillaries provides a means of loading sub-microliter samples into a standard gel while also providing a means to increase the lane density in the gel. As an added benefit, it has been found that a loading dye is not required to keep the samples contained in these nonstandard wells.

MATERIALS AND METHODS

Principle

After thermal processing, capillaries containing the sample are stabbed perpendicularly into an agarose gel that is immersed in a buffer solution (see Figure 1a). The sample is at or near the end of the capillary that pierces the gel. The capillaries must not pass completely through the gel, or else a plug of agarose may become lodged in the capillary, trapping the fluid in the capillary. The stabbing action forces agarose away from the capillary as it burrows into the gel, as illustrated in Figure 1b. The end of the capillary that is away from the gel must be such that buffer cannot enter the capillary by capillary action, and the sample inside cannot be forced away from the gel during insertion. This is accomplished by sealing the back end of the capillary; the pressure of the trapped volume of air behind the sample serves to offset any forces encountered during insertion.

After insertion, briefly holding the capillaries at their lowest insertion depth (typically a few millimeters) for
<1 s allows the agarose to seal around the capillary. The seal at the back end of the capillary is then removed, and the capillary is smoothly withdrawn in approximately 1 s, as illustrated in Figure 1c. Because the back end of the capillary is open to the atmosphere, the fluid is able to flow into the vacuum that results from the removal of the capillary. At this point, the sample is loaded and ready for electrophoretic separation. The volume of sample loaded is roughly equal to the volume of the crevice created by the capillary. With our capillaries and a 2-mm insertion depth, roughly 1 µL of sample is loaded. Smaller loading volumes may result from imperfect sealing of the gel around the capillary.

Device and Operation

The viability of this technique was investigated by using a prototype handheld device. The device, designed to easily accept capillaries from an Acapella output cassette, has been used to load thousands of gel lanes and is illustrated in Figure 2. The loader consists of two metal pieces and a gasket. The capillaries are inserted through small holes drilled into the facing plate, and their ends then pass through a 1/8″ thick silicone rubber gasket (not shown) into a small backing chamber in the second metal piece. The gasket is compressed by fastening the facing plate to the second piece and provides a tight air seal. The backing chamber is connected to the atmosphere through a relief hole that is designed to couple to a syringe. If not under pressure, it is important for the air volume of the backing chamber to be small—on the order of 10 µL per capillary—to prevent capillary action from drawing gel buffer during insertion. The capillaries are held in the loader on 2 mm centers to match the spacing of the Acapella output cassette. An additional plastic, grooved support piece is clamped around the capillaries in this design to ensure that the capillaries are parallel and evenly spaced.

After sealing the capillaries in the gasket, their fluids are moved to the open ends of the capillaries in preparation for loading. This is done passively by increasing the pressure in the backing chamber and using surface tension to keep the fluid volumes within their capillaries. The fluid volumes are thus moved to the ends of their capillaries, insensitively to their initial positions. This simple method could be used in an open-loop fashion by maintaining the backing chamber at a fixed elevated pressure that moves the fluid without ejecting it, thereby avoiding the use of sensors, detectors and feedback control.

Once the fluid volumes are in their desired positions, the backing chamber may either be vented and resealed or held at its slightly elevated pressure. The capillaries are then stabbed into the gel. Two limit posts that flank and are 2 mm longer than the sample capillaries pass through the gel and rest upon the gel.

Figure 1. Puncture loading of an agarose gel with a capillary. (a) Capillary (sealed) is inserted into a gel immersed in buffer solution. (b) Capillary is briefly held at lowermost insertion depth to allow agarose to seal around the capillary. (c) Capillary seal is removed, and the capillary is withdrawn from the gel leaving the sample behind.
gel bed, providing a uniform piercing depth and ensuring that the capillaries will not pass through the gel. To load the samples, the backing chamber is vented to the atmosphere and the handheld unit is withdrawn. Alternatively, the slightly elevated backing pressure may be retained during withdrawal to inject more of the sample more deeply into the gel.

**Biological Protocols**

The capillary gel loading technique and device were investigated on 0.7%-2.0% agarose gels. The samples used included a 100-bp and 1-kb DNA ladder (Promega, Madison, WI, USA) and a large marker used for multiple-complete-digest (MCD) restriction fragment mapping (largest fragment: 39.4 kb), kindly provided by the University of Washington Genome Center (6). In addition, polymerase chain reaction (PCR) product from the amplification of a length polymorphism that is 5' to the human antithrombin III (AT III) gene was loaded (1). Three different human genomic templates from the CEPH/Utah pedigree (Repository Nos. NA07057, NA06990B and NA10848; Coriell Cell Repositories, Camden NJ, USA) that are known to be heterozygous and homozygous in both alleles were screened with genomic templates from the CEPH/Utah pedigree (Repository Nos. NA07057, NA06990B and NA10848; Coriell Cell Repositories, Camden NJ, USA) yielding 143 and 218 bp PCR products. The reactions were performed on Acapella.

All gels were run in a noncirculating gel box (Shelton Scientific, Shelton, CT, USA) and were 12.5 cm wide by 20 cm long and approximately 4-5 mm thick. Gels were formed with 0.1 µg/mL ethidium bromide and photographed while on a UV transilluminator. Capillary thermal cycling was done using a Rapid Cycler™ (Idaho Technology, Idaho Falls, ID, USA) with the custom-built Acapella output cassettes.

**RESULTS AND DISCUSSION**

The hand-held device described above has been used to successfully load hundreds of gel lanes with a wide range of samples. Figures 3 and 4 present two representative gels. Figure 3 shows a 100-bp DNA ladder (Promega) loaded onto a 2% agarose gel. The bands are clearly visible despite each lane having 1 µL or less (<100 ng) of the ladder. Figure 4 shows AT III PCR product prepared by Acapella and loaded directly from the sample capillaries following thermal cycling. The genotypes are easily distinguished despite the small volume per lane of roughly 1 µL.

The capillary gel loading technique and device have been demonstrated for 0.7%-2.0% agarose gels and, in principle, can be applied to other gel concentrations. Capillaries with outer diameters of 1 mm or less have given the best results with this technique. Using larger capillaries will increase the amount of sample loaded per lane; however, this can also yield visibly distorted sample bands and risks taking on an agarose plug during insertion.

This technique could be expanded to uses outside of Acapella to take advantage of its ease of loading high-density gels. For example, reactions could be prepared in standard 96-well trays and then drawn into capillaries by surface tension forces. By using capillaries that would be filled by the sample, the surface tension at the ends of the capillary would provide enough force to prevent the sample from being displaced by gel buffer on insertion into the gel. This implementation would thus not require sealing or pressurization of the capillaries, greatly simplifying the design. One could envision this technique being used in an automated system with a cleaning station to quickly load samples row by row from a 96- or 384-well microplate format using small, reusable capillary tubes. If desired, the lane density could be increased from the well density of the tray by moving the capillaries more closely together before loading.

Despite the attractiveness of this technique for use in horizontal gels, it can have limited resolution with large products and is not readily applicable to vertical gels, including polyacrylamide sequencing gels. These limitations arise from the fact that the sample is loaded not into a traditional well but into what is essentially a crevice that collapses around the sample once the capillary is withdrawn. Although this eliminates the burden of aligning samples with preformed wells, the sample is prevented from settling to the bottom of the “well” even when loading dye is used, resulting in a trapped, vertical distribution of the sample. In horizontal gels,
this has been found to lead to band smearing and limited resolution due to variation in the DNA mobility across the thickness of the gel. The cause of this irreproducible mobility gradient has not been determined. It is believed to be an artifact of the gel formation process, not the electric field: it is hoped that an improved gel formation protocol will eliminate the gradient, allowing the amount of sample per lane to be increased by simply using thicker gels. In vertical gels, however, the spatial distribution of the sample over a few millimeters in the direction of migration leads to very smeared bands, making this technique impractical.

In practice, the technique has other limitations. Figures 3 and 4 show that considerable lane-to-lane variation in intensity can occur. In a typical operation of the prototype device, it is estimated that approximately 1 µL is loaded for each sample, with a SD of roughly 160 nL or 16%. In 1466 capillaries, 2.4% of the samples failed to load with the handheld prototype. This is largely due to errors in handling the device, which are being addressed in future designs. Fundamentally, though, variation results from the passive nature of sample loading and capillary-to-capillary variations in the seal between the gel and capillary. This can be alleviated somewhat by pressurizing the air chamber behind the samples during loading, but ultimately this gel loading method is better suited for qualitative PCR than for quantitative PCR. This approach thus meets the requirements of high-throughput screening, but is not a practical substitute for all types of experiments.

The pneumatic positioning of samples before injection has been very reliable; there have been no instances of a sample being ejected during positioning, but in 1.8% of cases, the sample did not move. This is thought to be due to a bubble trapped in the fluid sample, deposits on the capillary wall from sample drying during thermal cycling or human handling error. The pneumatic positioning method is robust enough to work well simultaneously for different fluid samples, such as deionized water, 50% glycerol or 0.2% nonionic detergent (Nonidet® P-40).

This capillary-based loading technique allows high-density loading of small-volume samples and thus suits the needs of a high-throughput, small-volume system such as Acapella. Further, it has been found that adequate sample volumes are injected when the capillaries are inserted into the gel so closely that the capillaries touch, allowing further increases in the lane density. This limit is difficult to approach in practice because a means of sealing and supporting the capillaries is required. However, the lane density may also be increased by using smaller capillaries, so long as the resulting amount of loaded sample remains detectable.

Loading one 32-capillary Acapella cassette with the prototype device takes 5 min. This is comparable to the time needed to load 32 capillaries by hand. However, the device was designed only for a proof-of-concept, and the actual gel loading time of 5 s to prepare, insert and withdraw the capillaries is dominated by the time needed to load and unload the capillaries from the device. An improved design currently in development will increase the throughput rate, and using more capillaries per insertion would further increase it. However, even at its present stage of development, the device and technique provide higher lane density and reliability than traditional loading from capillaries has achieved.

In conclusion, a new method of high-density loading of small sample volumes has been presented. The proof-of-concept design described here was used in conjunction with Acapella, a high-throughput, capillary-based reaction automation system. It is appropriate for loading samples from PCR-
Isolation of Pure Human Mucosal Epithelium for RNA Analysis


ABSTRACT

Techniques for isolating the desired cell populations from complex tissues are essential for characterizing cells through mRNA analysis. We established a procedure for isolating pure mucosal epithelium from the human alimentary tract. To do this, we made rotating hooks that hold mucosal strips and detach the epithelial sheets from the irregular mucosal surface in medium containing EDTA. An additional step using a cell strainer was required to reduce contamination by lymphoid cells. Sheets of epithelial cells were detached successfully from mucosal samples derived from five different parts of the human alimentary tract. Contamination by lymphoid cells or fibroblasts was monitored by competitive RT-PCR and was no more than 0.5% of the total cells. Total RNA yields were 12.5–17 μg for each separation, and the integrity of the RNA was as good as that of RNAs extracted from mucosa immediately after resection. In conclusion, our method permits isolation of RNAs from a pure population of epithelial cells that can be used for mRNA-based gene expression analyses.

INTRODUCTION

Recent advances in high-throughput sequencing have permitted the collection of the majority of active gene sequences from any tissue (1,11). Microarray technology has allowed simultaneous quantification of the expression levels for thousands of genes by single hybridization experiment (7,10,12,15). The results of such experiments provide an explanation of the physiological and pathological status of cells in terms of global gene expression. For such purposes, all we need is to have the right quantity of mRNAs extracted from the cells of interest. Accordingly, finding methods for isolating particular types of cells from complex tissues becomes increasingly important.

The alimentary tract is composed of layers of different cell types. Between the epithelium and the proper muscle layer, there is a layer of loose connective tissue called the lamina propria that is infiltrated by numerous lymphoid cells. To examine development of pathological changes in the epithelium or to diagnose these changes through mRNA analyses, epithelial cells must be collected selectively before lysing the tissue for RNA preparation.

Bjerknes and Cheng (2) established a method to isolate intestinal epithelium by mechanical disruption of mucosa with the aid of a chylating agent. This method, with various modifications at the disruption step, has been applied successfully to the isolation of intact glands from the stomach and colon (3,5,13,14). In all of these studies, however, the means to disrupt the tissue were not easily controlled or reproduced. Sometimes, isolated glands were collected manually under microscopy. Neither of these steps is suitable for preparation of thousands of glands for the extraction of microgram quantities of RNA for cDNA library construction or hybridization probe preparation unless an amplification step (which may bias the gene expression information) is included. Consequently, isolated glands have rarely been used as an RNA source for molecular biology applications (3,4). We describe a method to isolate more than 10⁶ epithelial cells at one time from any portion of the alimentary tract, using competitive RT-PCR to evaluate the purity of the isolated cells.

MATERIALS AND METHODS

Mucosa Samples

Tissue samples of the human esophagus, proximal corpus of the stomach, antrum of the stomach, ileum end, ascending colon and sigmoid colon were obtained from the margins of surgically resected tissues. (Informed consent was obtained from all patients.) Mucosal layers, detached by injecting cold saline into the submucosal layer, were placed in cold Hank’s balanced salt solution (HBSS). Mucosal samples were cut into 3 × 10 mm strips, after being

References


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