Short Technical Report

Creating Arrays by Centrifugation

BioTechniques 32:1322-1329 (June 2002)

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ABSTRACT

We describe a fast, low-cost, and reliable way of creating arrays from sample molecules of interest present within microformatted sample vessels (such as 1536-well microplates). The principle involves simple centrifugal transfer of molecules of interest onto a solid planar or membrane surfaces placed over the initial sample vessel. Tools and procedures are presented that validate the robustness and precision of this facile solution to an otherwise difficult problem in modern molecular genetics. The availability of transferred DNA molecules for hybridization is also demonstrated. In conclusion, this "centrifugal-array" concept should help research studies to be applied on ever-greater scales with very simple machinery.

INTRODUCTION

Genetic analysis technologies are transitioning rapidly from small numbers of large-volume reactions toward massive numbers of small-volume reactions (5). Sample processing in a format of 96 reaction wells (approximately 200 μ L each) in an 8×12 layout has come to dominate the field in recent times, and many systems for liquid handling in this format have been developed. There is now a growing need for the manipulation of even larger numbers of samples and smaller reaction volumes (7). This has prompted the development of 384- and 1536-well formats with well volumes as small as approximately 2 μ L. Systems for liquid handling in these formats are being developed, but the tight layout and the small volumes involved will tend to make the component devices sophisticated, non-robust, and expensive.

In parallel to the above developments, there has been a recent move toward solid-phase reactions (4). In typical scenarios, samples are transferred and chemically (or at least strongly) bound to a solid phase to facilitate purification and downstream processing in a high-throughput mode. The solid phase may be the inner surface of a reaction well (3), microbeads (6), a planar solid surface (e.g., glass or polymer) (1), or a membrane (2). Creating sample arrays on planar solid surfaces and membranes offers an advantage over other supports in that liquid-phase reactions (e.g., enzyme treatments or

hybridization steps) can be carried out in a highly paralleled manner by simple immersion in suitable reagent mixtures. When the desired arrays are to entail one or a few hundred samples, transfer from the starting reaction vessel to the solid surface can be mediated (albeit with at best medium throughput) via aspiration-dispensing procedures (e.g., multichannel pipets). However, creating arrays with many more samples currently requires sophisticated and thus costly transfer robotics.

To enable better use of arrays in molecular biology, we describe here a simple alternative to complex liquid-handling systems for sample transfer and creation of arrays. We demonstrate how simple centrifugation principles may be utilized to create arrays on both planar solid surfaces and membranes, starting with microplate-formatted input samples (Figure 1). We present tools and protocols that enable the rapid creation of desired arrays from any format of sample plate, ensuring the salvaging of any nontransferred excess materials and at the same time completely avoiding cross-contamination between features during centrifugal transfer.

MATERIALS AND METHODS

Samples

The "hybridization target" oligonucleotide in the membrane experiments was JTN6G-01T (5'-AGAGAAGAGAGACTATTTTCCAACAGCGTTTGT-

GTTTCTTGGATGTCATTCT-3') used at 1 μ M, 500 nM, 250 nM, 125 nM, 62 nM, 31 nM, and 16 nM concentrations, and the complementary BodipyTM TMR-labeled probe was oligonucleotide JTN6G+02P (5'-ACACAAACGCTGTTGGA-3'-Bodipy TMR) used at 1 μ M. For the full 1536-well format array, with the features laid out to spell DASH, Rox-labeled oligonucleotide HB15+10P (5'-GTTGTACGGTGGGTTGT-3'-Rox) was used at 0.5 μ M. Target sequence in the experiments with glass for solid support was the 5'-aminated oligonucleotide JPSN2+04F (C6 Aminolink-5'-CTGTCGTCAGTACACGGTCCCT-3') used at 1 μ M. The complementary probe was the Cy3-labeled oligonucleotide JPSN2+05P (Cy3-5'-AGGACCGTGTACTGACGACAG-3') used at 1 μ M. All oligonucleotides were synthesized by Thermo-Hybaidd, Interactiva Division (Ulm, Germany).

Membrane Surfaces

The membrane used for experiments in this report was Hybond[®]-N⁺ (Amersham Biosciences, Uppsala, Sweden). For array creation, the membrane was pre-wet in double-distilled water, briefly drained, and incorporated directly into the microplate, along with the

compression pad and clamp arrangement. After array creation by centrifugation, the membrane was air-dried and the samples were cross-linked to the membrane by UV treatment for 5 min on a regular UV transilluminator.

Glass Surfaces

We employed a silylated glass surface (CSS-25; Cel Associates, Sunnyvale, CA, USA) for experiments described in this report. For array creation, this was incorporated directly into the microplate, along with the compression pad, sealing gasket, and clamp arrangement. The sealing gasket was created by drilling 1 mm diameter holes in a silicon rubber sheet (RIMA, Göteborg, Sweden), using as a drill a hollow steel pipe sharpened at one end to form a circular knife edge. Punching holes in the rubber sheet with this steel pipe is also possible. After array creation by centrifugation, the glass plate was briefly rinsed in HEN buffer (0.1 M HEPES, 50 mM NaCl, 10 mM EDTA) before signal visualization.

Pressure Clamps

The clamps were made out of 6-mm Plexiglas[®] measuring 144 $\times$ 105 mm. Eight holes for screws were drilled

three on each long side, 45 mm apart, and one centered on each short side. All holes were drilled 6 mm from the edge of the Plexiglas plate. One clamp consists of two such plates with nylon screws and nuts holding them together (Figure 2A).

Centrifugal Transfer

Polypropylene 1536 sample plates (Greiner bio-one, Frickenhausen, Germany) contained 2 or 5 μ L liquid sample/well. In the case of arraying onto a membrane, the membrane was placed directly on top of the sample plate. In the case of arraying onto a solid surface (e.g., glass), a sealing gasket with round holes aligned to the square sample plate wells was placed between the solid surface and the sample plate. These assembled components were inserted into the pressure clamp device, with compression pads (HB-TD-SFOAM; Thermo-Hybaidd, Ashford, Middlesex, UK) positioned immediately internal to each Plexiglas plate of the clamp. The clamp was tightened by hand, and the assembly placed in an ordinary microplate centrifuge (model B4i; Jouan, St. Herblain, France). Centrifugation was conducted at 1500 rpm (250 $\times$ *g*) for 30 s. For the membrane studies reported here, surface binding occurs very rapidly. Therefore, as soon as the centrifuge had slowed to a stop, binding was considered to be complete, and the clamp assembly was turned around so that excess reagents and liquid could be centrifuged back into the microplate wells (same speed and duration). When binding to glass, the reagents were left to bind for 1 h before centrifuging back excess reagents. Once the centrifuge had again come to a standstill, the clamps were disassembled and the glass plate or membrane removed for analysis of the created array.

Hybridization

Hybridization of Bodipy TMR-labeled oligonucleotide probe JTN6G+02P to membrane-bound oligonucleotide JTN6G-01T was accomplished by rehydrating the membrane in 2 $\times$ SSC and then immersing it in APH solution consisting of 5 $\times$ SSC, BLOTTO (5% fat-free powder milk and 0.02% sodi-

Figure 1. Experimental overview. (A) The array surface is applied on the sample plate (B), which is then inverted and centrifuged. (C) After sample immobilization, the array is processed (i.e., rinsing, blocking, and probe hybridization). (D) The array is analyzed.

um azide), and 0.5% SDS for 30 min at 60°C. The probe was diluted to 1 μM in APH and hybridized by placing the membrane on a glass plate, pipetting an even layer of probe solution over the membrane (approximately 4 $\mu\text{L}/\text{cm}^2$), and covering with another glass plate. The sandwiched membrane was then placed on a heating block at 85°C for 5 min and air-cooled to room temperature. Unhybridized probe was removed by three room-temperature washes of 5 min each in 2 $\times$ SSC and 0.1% SDS before signal visualization.

Hybridization of JPSN2+04F and JPSN2+05P before attaching the molecules to the glass slide was accomplished by mixing the oligonucleotides at a final concentration of 1 μM in HEN buffer (0.1 M HEPES, 50 mM NaCl, and 10 mM EDTA) at room temperature for 1 h. Hybridization to glass-bound target was done by pipetting 200 μL probe solution, 1 μM in HEN, between two glass plates and heating to 85°C. After cooling to room temperature, the glass slide was washed three times in HEN buffer.

Analysis

The full microplate-sized membrane was examined by placing in an Illumatool LT-9500 instrument (Lighttool Research, Encinitas, CA, USA) inside a GelDoc2000 gel imager (Bio-Rad Laboratories, Stockholm, Sweden). The membrane was epi-illuminated on the Illumatool board with 540 nm light and imaged through a 590-nm viewing glass. The smaller membrane was placed on a glass slide and analyzed in a GMS 418 Array Scanner (Genetic Microsystems; Affymetrix, Santa Clara, CA, USA) using the 532-nm laser for excitation, 30% power, and 30% gain. The silylated glass slide was analyzed in a ScanArray 4000XL (Packard BioChip Technologies, Meriden, CT, USA) using the 543-nm laser for excitation, 80% power, and 80% gain.

RESULTS

The goal of our study was to establish a practical system for rapidly creating solid surface arrays from samples in microplates or similar, with no need for

liquid-handling devices or robotics. The principle followed (Figure 1) was to employ centrifugal forces to move liquid-phase molecules of interest from a multiple-well-formatted plate to bring them into contact with a solid surface positioned over that plate, thereby precisely maintaining the 2-D sample arrangement present with the initial plate. Once in contact with the solid surface, a suitable reaction captures the

sample molecules as an array, and any excess molecules plus the complete liquid volume may then be transferred back into the sample plate (again by centrifugal transfer).

Several problems had to be overcome to make this system work. Principal among these were the optimal centrifugation conditions for quick but efficient transfer and establishing a means to ensure that no cross-contami-

Figure 2. Clamp arrangement. (A) The clamp consists of two Plexiglas plates and eight nylon bolts. Panel B shows the arrangement when samples are centrifuged on to a membrane and (C) onto a solid surface (e.g., glass).

nation of samples occurred. Below we present a procedure that achieves the stated goals, and we demonstrate its use on two types of solid support. Specifically, from microliter-scale samples in a dense initial layout (1536-well microplate starting vessel), we show how to create high-precision derivative arrays rapidly on a filter membrane and on a glass plate, with no need for any liquid-handling tools or robotics.

Centrifugation

The centrifugal transfer step needs to be brief (for high-throughput application), sufficiently forceful (to overcome surface tension effects that tend to retain small-volume samples in wells), but not so severe that reagents cross-contaminate each other by escaping from the junction between the upper rim of the starting vessel and the destination solid surface. In fact, we found that surface tension effects made it necessary to employ rather high relative centrifugal forces (g) compared to those typically used for activities such as simply aggregating liquid volumes in Eppendorf® tubes ($<100\times g$). We employed a regular bench-top microplate centrifuge to conduct our studies and found that g forces of between 200 and 400 were suitable for our objectives (regardless of the type of solid surface). We now routinely run the centrifuge at 1500 rpm, thereby imposing approximately $270\times g$. The duration of the transfer is less critical. Once the full g force is reached, sample transfer is complete, and so the centrifuge may be stopped immediately, making our system very fast to execute. However, keeping the centrifuge running beyond this does no harm as far as we are aware from our various trials.

Pressure Clamps and Sealing

Upon centrifugation, the samples form an even coating of the destination surface covering the sample vessel. The junction between the rim of the well and the surface makes a natural barrier, but this must be made liquid-tight to prevent sample leakage between array features. To achieve this, pressure clamps were designed to press the solid support onto the sample plate during the centrifuga-

tion step. The clamps we use are comprised of two Plexiglas slabs with eight nylon screws distributed around the edges (Figure 2A). Nylon nuts are used to tighten the clamps just finger tight.

When centrifuging samples onto a membrane, we placed rubber compression pads above the membrane and below the plate. This distributes the applied pressure evenly and slightly distorts the membrane around the well rims to ensure reliable sealing (Figure 2B).

When spinning samples onto a solid planar surface (e.g., glass), the clamps and outer pressure pads alone were not sufficient to seal effectively between the sample plate and the transfer surface. We found it necessary to add some additional means for sealing the surface to the well-rim junction. A 1-mm-thick silicon gasket with holes positioned to line up with the well positions was fabricated and placed between the solid surface and the sample plate. To achieve additional sealing, the silicon gasket was treated with a thin layer of mineral oil, which acts as a hydrophobic barrier. This solution is illustrated in Figure 2C.

Demonstration Transfer to a Membrane

To demonstrate array creation on a membrane, we transferred 2- μ L aliquots of a solution of unlabeled oligonucleotide molecules (JTN6G-01T) from a 1536-well microplate. We then hybridized the resulting array with Bodipy TMR-labeled oligonucleotide JTN6G+02P to show the availability of the transferred molecules for subsequent reactions. The sample layout involved six identical features per row. The first (top) row contains sample molecules as described above. The second row is a copy of the first row, with features 3 and 4 omitted to provide a duplicate negative control for the transfer. Subsequent pairs of rows were arranged the same way as for the first two rows, but employing sequential 2-fold dilutions of JTN6G-01T, concentrations ranging from 1 μ M down to 16 nM. The resulting 6×14 feature array of target molecules was hybridized to JTN6G+02P, and then the membrane was visualized to detect any bound fluorescent molecules. As can be seen in

Figure 3A, the hybridization signals on the created array features are faithful representations of the sample layout in the initial microplate, and there is no evidence of any cross-contamination between the wells. Evenly shaped features are produced by sample concentrations above 125 nM.

To demonstrate the transfer strategy from a full-size 1536-well microplate, 5 μ L Rox-labeled 17-mer oligonucleotide (HB15+10P at 0.5 μ M) were pipetted into the wells of a 1536-well plate in a mirror-image layout of the word "DASH" with a surrounding frame (Figure 3B).

Demonstration Transfer to Glass

To demonstrate array creation on glass, we transferred 2- μ L aliquots of a solution of 5'-aminated oligonucleotide JPSN2+04F that we had prehybridized to an equimolar amount of Cy3-labeled oligonucleotide JPSN2+05P (1 μ M each). The starting plasticware was again a 1536-well microplate with square wells, and the glass surface was silylated, allowing covalent binding of JPSN2+04F.

A 7×10 array of samples was transferred. Wells in a diagonal line through the array were left empty. A sealing gasket with round holes, coated with a thin layer of mineral oil on the side facing the glass, was used to ensure no leakage between adjacent wells. After creating the array by centrifugation, the glass surface was rinsed briefly in HEN buffer and analyzed in a microscope-slide laser scanner. To ensure that the bound oligonucleotides were available for hybridization, the probes were denatured away by immersing the slide in 95°C water. The array was then rescanned to ensure that the labeled probes had been fully removed (data not shown). New labeled probes were then hybridized to the bound targets, and the array was again scanned (Figure 3C). The created array features are valid representations of the sample layout in the initial microplate, the target DNAs were thus fully available for hybridization, and there is no evidence of any cross-contamination between the wells. The slight misalignment of the features is due to imprecision in the shape of the homemade sealing gasket.

Figure 3. Results of the centrifugation sample transfer strategy. Panel A shows duplicated rows of samples transferred by centrifugation from wells in a 1536-well microplate. The two central features in the second row of every row pair were omitted to demonstrate that no leakage was occurring between wells. The row pairs are representing a stepwise dilution series, sequentially halving the concentration at each step. Unlabeled target DNA was transferred and subsequently hybridized to fluorescence labeled oligonucleotide probes. Panel B is a demonstration of sample transfer by centrifugation from a full-size 1536-well microplate onto a membrane. Fluorescently labeled DNA was transferred. Panel C shows an array created on a glass slide by centrifugation of aminated target molecules subsequently hybridized to fluorescently labeled probes.

DISCUSSION

We have demonstrated facile, low-cost, and very fast creation of arrays on two types of solid supports, from a 1536-well microplate by simple centrifugation. The sample transfer principle entailed no complex liquid-handling manipulations or expensive robotic devices. Importantly, the same basic concept would be applicable to any format of starting vessel and destination surface, of almost any scale or density, and based on a whole range of binding chemistries. In the membrane experiments reported here, only a fraction of the full binding capacity of the membrane was utilized (Hybond-N⁺ can bind several micrograms of DNA/mn², while we used at most 13 ng). Therefore, the signal strengths of the system should be able to support highly multiplexed assays employing many different target molecules per feature. By contrast, the binding capacity of modified glass is typically low, in the range of fmol/cm² (8). This would motivate the creation of many replicate arrays from the same starting material, even with limited sample concentrations.

Employing microplates of greater than 384-well densities would probably

require the initial use of a multichannel pipetting device for plate loading. This could alternatively be done via robotics, but such devices are expensive and might raise total costs for array creation to an unaffordable level for small laboratories. Nevertheless, in most situations, the "centrifugal-array" concept would offer significant cost-savings, suggesting that this simple innovation has great potential to help research studies to be applied on ever-greater scales with simple and affordable machinery.

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Received 10 January 2002; accepted 26 March 2002.

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