CLONTECH Laboratories
Palo Alto, CA, USA

Inexpensive Handheld Device for the Construction of High-Density Nucleic Acid Arrays

BioTechniques 23:1087-1092 (December 1997)

ABSTRACT

We report an easy-to-use, 384-pin handheld arraying and replicating device (ARD) for constructing high-density replicas of nucleic acids and E. coli *transformants. We have modified an existing 384-pin tool to include a novel guide system to ensure vertical pin movement and enhance reproducibility. An asymmetric rectangular multiplexing frame is designed to increase the array density to 1536 dots on a standard microplate-size nylon membrane and to reduce the time and effort involved in producing array replicas. Our initial studies used the ARD to construct 1536-dot arrays of ovarian cDNA clones. We have hybridized these arrays with ^{32}P-labeled probes, which resulted in distinctive signals for either visual interpretation or semiautomatic spot detection and signal integration.*

INTRODUCTION

For the past 25 years, biologists have focused on the analysis of individual genes or proteins. With the development of new methods such as differential display (3) and mass-sequencing of entire libraries (7,12), science has come one step closer to the study of complex systems. High-density cDNA and oligonucleotide arrays permit the parallel monitoring of transcript levels in cells of different tissue types or under different physiological conditions. Original descriptions used arrays of DNA on nylon membranes (1,2,6,8) and used ^{32}P and ^{33}P for detection. Recently, glass and silicon supports have been described that greatly increase array density and allow for detection with fluorescent compounds (10,11). Arrays can consist of known genes gathered from DNA sequencing (8) or cloned genome fragments (1,11), or they can be comprised of unknown cDNAs selected at random (2,6,8). The DNA can be dotted in the form of polymerase chain reaction (PCR) products (10), as DNA from minipreparations (8) or through colonies with subsequent lysis on the membrane (1,2,6). The interrogating probes range from single sequences for genomic screening (1) to first-strand total cDNA from different tissues whose transcript patterns are thus monitored (2,6,8,10).

Dotting on glass slides at high density requires special devices for both dotting and reading (10,11). Dotting on membranes can be either performed by an automatic robotic device (1,2,6,8) or a handheld pin tool, depending on the number of clones to be arrayed and their density. Both the robotic and the manual systems use 384-well microplates for template storage and 384-pin print heads. Though robotic methods are more accurate and rapid, they are also significantly more expensive and require dedicated laboratory space.

To address these concerns, we designed and built a new handheld 384-pin replicator capable of dotting 1536 specimens on an area of a 96-well plate. The stamp-like dotting process is performed with ease and high accuracy and can produce multiple replica arrays within a few hours.

MATERIALS AND METHODS

The following is a general description of the arraying and replication device (ARD; Figure 1) that includes its dimensions and its features. Detailed instructions on how to construct the device, including the technical drawings, can be found on our World Wide Web site (**http://chroma.mbt.washington.edu/ARD**). The construction of the ARD took several weeks of repeated visits to the University of Washington Scientific Instruments Division, Seattle, where we applied the modifications described below. The final costs, including labor, amounted to approximately $3500. In a rough estimate, a second ARD could be built for $1500 to $1700. The materials the ARD were made from are commonly available, such as polycarbonate for the device itself and for the retaining frame (from

sheets of 9.5-mm thickness) and the aluminum for the multiplexing frame (from sheets of 12.7-mm thickness).

The Printing Head

The 384-pin printing head is a commercially available Multi-Blot Replicator (Model VP 384; V&P Scientific, San Diego, CA, USA) with a modified pin size. The original pin diameter was approximately 800 µm with a conical-shaped tip. We asked the manufacturer to reduce the pin diameter to 450 µm with a flat tip, which would facilitate the 1536 array density by reducing dot overlap. Each new pin delivers approximately 25 nL of solution to each dot.

The ARD Case

The case of the ARD is essentially a box made from Plexiglas® in which the print head can slide up and down. This box consists of an upper part (external dimensions [in mm; length, width and height, respectively]: $150.1 \times 101 \times 28$; internal dimensions: $134.3 \times 80.2 \times 22$) with a round hole for the handle of the print head, and a bottom part (external dimensions: $150.1 \times 101 \times 19.7$; internal dimensions: $123 \times 81.6 \times 19.7$) screwed onto the upper part with three 4.76-mm, allen-cap screws. The bottom part of the case is thus open on both ends, which, when screwed on the upper part, enables the pins of the print head to reach out of the box (Figure 2, A and B). The bottom 13 mm of the bottom part have been further drilled down to outer dimensions of 135.2-mm length and 98.5-mm width to accommodate the multiplexing frame (described below). The metal spring (45-mm-long, 30-mm-wide, 2-mm-diameter stainless steel), which encircles the handle and which is secured by a Plexiglas disk at the top of the handle, makes the print head snap back to its retracted position inside the case once it is pushed down (Figures 2B and 2A). The print head comes with two alignment pins attached to the extreme top left and right edges of it. These pins slide into two alignment grooves that were drilled in the bottom part of the case and serve as a guiding system for the print head. This ensures that the pins travel vertically and will not touch the walls of the sample storage microplate. This also ensures accurate and reproducible sample uptake and delivery to the surface of a nylon membrane or agar plate.

The bottom part of the case has the exact inner dimensions and shape to fit over a standard 384-well plate in only one orientation (Figure 2A), and has on its bottom side an asymmetric array of 7 pins that punch orientation holes into the membrane. This thus unmistakably marks the dotted area in its four corners (Figure 2B, arrow). This feature helps when cutting out small pieces of membrane as close to the dots as possible, even if the arrayed dots have dried and therefore are no longer visible. Furthermore, the case protects the fragile pins from bending.

The Retaining and the Multiplexing Frame

The retaining frame (Figure 1) is comprised of two polycarbonate plates of $215 \times 300 \times 9.5$ mm and two polycarbonate side panels of 11-mm width and height and 215-mm length, glued to the short sides of the lower plate. The nylon membrane is held between the upper and the lower plate. The upper plate is held in position by the two side panels, and it is tightly secured to the lower plate by rubber bands (17-mm diameter, 3-mm width) attached to the lower plate and sliding over eight pins (3-mm diameter × 24-mm length) extending from the sides of the upper plate (three pins on each short side and one pin halfway down the long side; Figure 1). The upper plate has four openings for the access of the ARD.

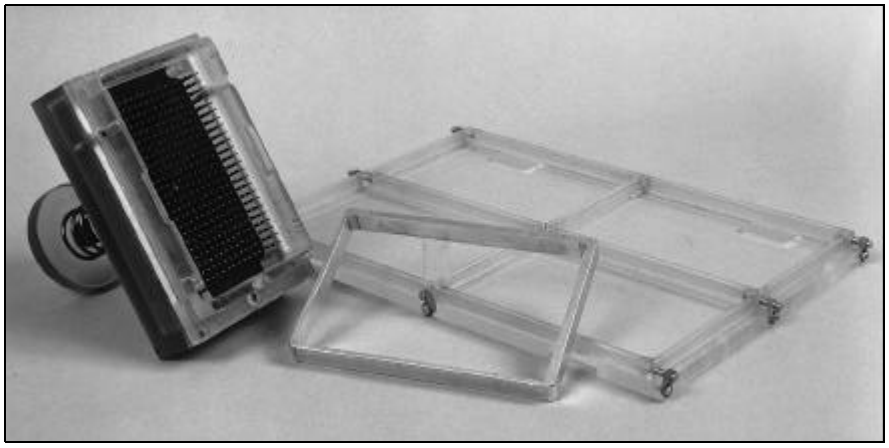

Figure 1. The ARD, the multiplexing frame and one retaining frame (from left to right). The upper part of the retaining frame is tightly secured to the lower part by eight rubber bands, three each on the short side and one each on the long side.

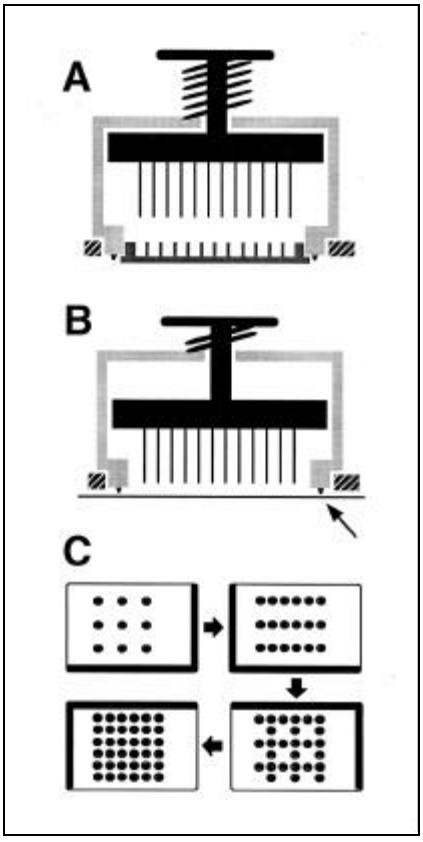

Figure 2. Schematic drawing describing the dotting procedure. (A) Cross section of the ARD with asymmetric multiplexing frame (hatched) fitting over a standard 384-well plate. (B) Cross section of the ARD pushed down on the membrane. The arrow points to a marker pin. (C) Scheme for 4×384 multiplexing (only 36 dots with 4×9 replication are shown). The relative orientations of the multiplexing frame are indicated outside the dots.

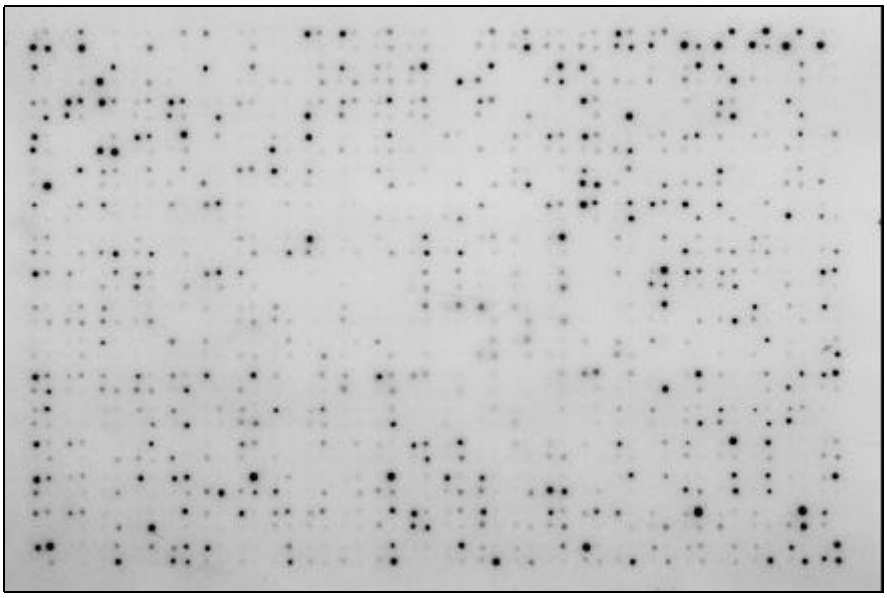

Figure 3. Autoradiogram of a membrane containing 1536 human ovarian epithelial cDNAs (1–2 fmol per dot) hybridized with ^{32}P-labeled human ovarian tumor probe. The dots are clearly separated and thus suited for automatic spotfinding.

With 90 × 144 mm each, these openings are slightly larger than the ARD's case that fits into them; the gap is filled with the asymmetric aluminum multiplexing frame (Figure 1). This rectangular frame (inner dimensions [in mm; length, width and height, respectively]: 135.9 × 90.1 × 12.7) has two adjacent sides of 3-mm width and two adjacent sides of 5-mm width each. It fits tightly around the base of the ARD (Figure 2, A and B) and less tightly into the opening of the retaining frame. Due to its asymmetry, it fits around the base of the ARD in four orientations. This layout enables the dotting from four different 384-well plates onto the same 7- × 12-cm area, each dotting being performed with another orientation of the multiplexing frame (Figure 2C).

The Dotting Procedure

For the generation of multiple repli-

cas of cDNA arrays, a positively charged nylon membrane (Nytran® Plus; Schleicher & Schuell, Keene, NH, USA) cut to 210 × 300 mm is placed on 3MM paper (Whatman, Clifton, NJ, USA) between the upper and the lower part of the polycarbonate retaining frame, and the rubber bands are secured in place. The ARD with the multiplexing frame attached to its bottom is fitted over a 384-well plate that holds the specimens (Nalge Nunc International, Rochester, NY, USA), and the handle is pushed down (Figure 2A). Upon release, a tiny drop of the samples (ca. 25 nL) is held on the pin tips by surface tension. The ARD is then placed into one of the four openings of the retaining frame and pushed down to transfer the 384 droplets onto the membrane (Figure 2B). These steps can be repeated three additional times with the other three openings in the frame using the same sample plate, thus generating four identical replica filters. After printing a set of 384 samples, the pins are rinsed with water and ethanol and air-dried for 1 min. Dotting into the same opening from three additional 384-well plates with the four distinct orientations of the multiplexing frame allows the replication of 4 × 384 specimens (Figure 2C). With the use of several retaining frames, many identical replicas can be generated simultaneously.

The ARD combines the ease of one-hand stamping with the rapid and precise (replica-) dotting of the samples. The arraying pins are precisely positioned over the 384-well plate and on the membrane. The template selection options for arraying are extensive and include purified, cloned DNA (8,11), PCR products (10) and *E. coli* transformants (1,2,6). We arrayed 20 000 cDNAs derived from normal and neoplastic ovarian tissues prepared using a large-scale minipreparation method (5) on nylon membranes at 3–5 ng (1–2 fmol) of DNA per dot. Utilizing the ARD with three retaining frames for arraying the cDNAs, we dotted 144 membranes (12 replicates of 12 membranes of 1536 dots each) at a speed of approximately 12 replicas in 20 min.

Hybridization

The immobilized DNA was denatured and neutralized on the membrane using a Southern blot protocol (9). Each set of membranes was hybridized with a complex probe consisting of ^{32}P-labeled, first-strand cDNA [30 µg of total RNA were reverse-transcribed using SUPERSCRIPT™ II Reverse Transcriptase (Life Technologies, Gaithersburg, MD, USA) and oligo(dT_{12}) primers with 30 µCi of a ^{32}P[dCTP] (3000 Ci/mmol) and unlabeled dATP, dGTP and dGTP at 1 mM each; after 20 min, unlabeled dCTP was added to a concentration of 1 mM, and the reaction was continued for another 40 min; this unpurified probe was used for 12 membranes] and washed at conditions described previously (13). To assess the amount of DNA that was actually delivered to each dot, a 30-mer oligonucleotide [5′-GGTGGCCTAACTCCG-GCTACACTAGAAGG-3′ located at the ColE1 replication origin region of the cDNA cloning vector pBK-CMV (Stratagene, La Jolla, CA, USA)] was labeled by phosphorylation and hybridized with the membranes (6). The membranes were exposed to X-ray film [X-Omat; Scientific Imaging Systems

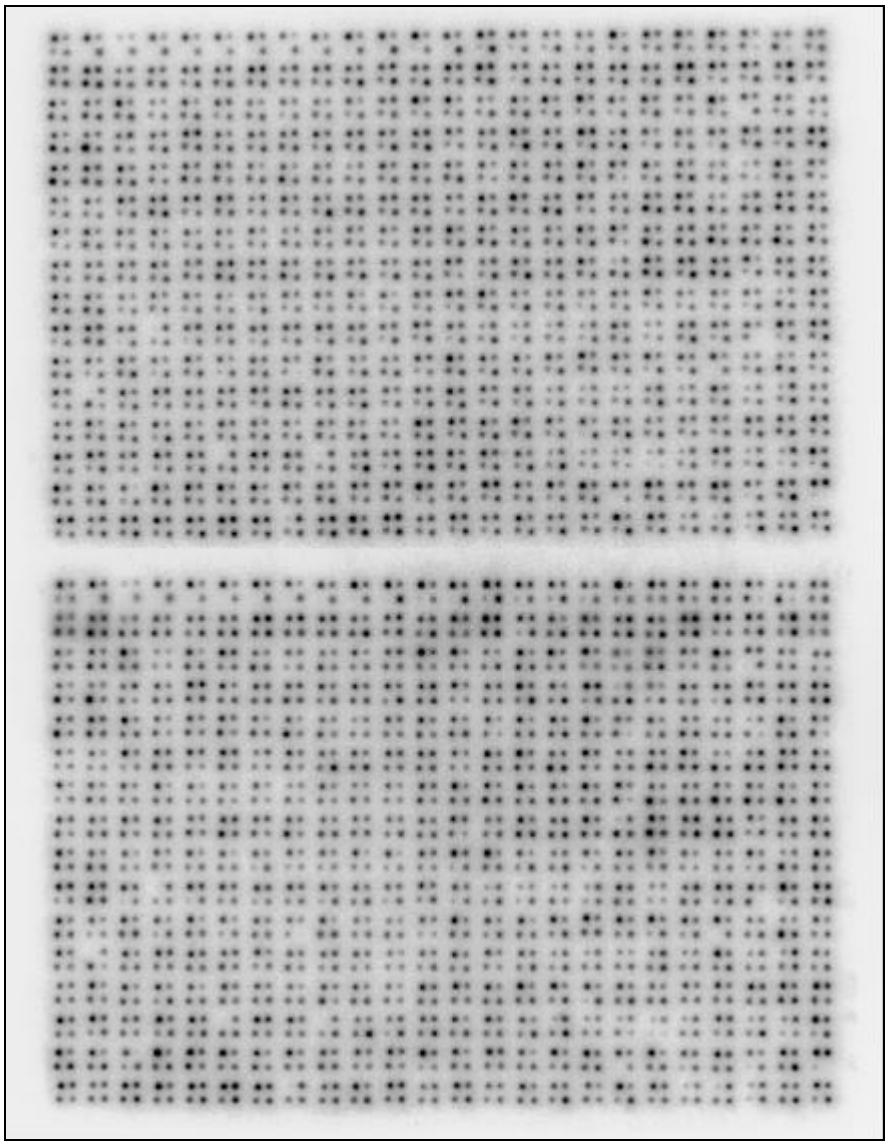

Figure 4. Autoradiogram of two identical replica membranes containing 1536 human ovarian epithelial cDNAs. Membranes were hybridized with a ^{32}P-labeled, vector-specific oligonucleotide. Variations in intensities are due to variable yields of minipreparations. At first glance, both membranes look like copies of one another, but upon closer inspection, some minor differences can be observed. Hence, the low average value of the ratios of the intensities of two corresponding dots, which is 1.02 ± 0.2.

(Eastman Kodak), New Haven, CT, USA] for 2 days at -80°C without an intensifying screen or to a phosphor capture screen for 16 h, which was read by a PhosphorImager® 420 (Molecular Dynamics, Sunnyvale, CA, USA). The hybridization results indicated that the dots were clearly separated and evenly spaced (Figure 3). Replica filters hybridized with the same vector oligonucleotide probe resulted in identical patterns (Figure 4). The visual comparison of hybridization patterns was easily performed, as was the automated detection and extraction of individual spot intensities using a custom software program (R.E. Bumgarner, personal communication).

DISCUSSION

The recently developed methods for high-throughput differential gene transcript-level analysis rely heavily on custom fabricated technology or use expensive robotic tools. The handheld array and replication device described here approaches the speed and accuracy of robotic devices at a small fraction of the cost of initial acquisition and upkeep. The ARD is capable of delivering 1536 dots onto a membrane with high precision and high speed. It delivers a volume to the membrane that is low enough to clearly distinguish the dots at high density, and to eliminate false assignment of cDNAs, it fits over a standard 384-well plate in only one orientation. Each gridded area is marked in a characteristic way for easy location and orientation on a large membrane. Membranes are held in a fixed orientation, allowing for a fourfold increase in array density (4 × 384) using a foolproof asymmetric retaining frame.

We have shown that a substantial portion of a cDNA library can easily be arrayed in one day using the ARD. With 1536 dots on an area of a standard microplate, the arraying density is the same as reported for robotic dotting on nylon membranes for interrogation by differential hybridization (2,6,8). Arraying 100 000 cDNAs, a significant portion of a higher eukaryote's expressed genes, can thus be performed within a few days. For a simple organism such as yeast, even full coverage of mRNAs can be achieved.

Rescreening of genes found using representational difference analysis (RDA) (4), differential display (3) or RNA fingerprinting (14) can be performed efficiently at low cost and tested with probes from cells at different physiological states. A few hundred expressed sequence tags (ESTs) can be arrayed and hybridized with first-strand cDNA probe from a few dozen tissues or cell lines at various conditions, which will identify the genes associated with a certain behavior or pathway.

REFERENCES

1. **Francis, F., G. Zehetner, M. Höglund and H. Lehrach.** 1994. Construction and preliminary analysis of ICRF human P1 library. GATA *11*:148-157.
2. **Gress, T.M., J.D. Hoheisel, G.G. Lennon, G. Zehetner and H. Lehrach.** 1992. Hybridization fingerprinting of high-density cDNA-library arrays with cDNA pools derived from whole tissues. Mamm. Genome *3*:609-619.
3. **Liang, P. and A.B. Pardee.** 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science *257*:967-971.
4. **Lisitsyn, N., N. Lisitsyn and M. Wigler.** 1993. Cloning the differences between two complex genomes. Science *259*:946-951.
5. **Ng, W.-L., M. Schummer, F. Cirisano, R.L. Baldwin, B.Y. Karlan and L. Hood.** 1996. High-throughput plasmid minipreparations facilitated by micro-mixing. Nucleic Acids Res. *24*:5045-5047.
6. **Nguyen, C., D. Rocha, S. Granjeaud, M. Baldit, K. Bernard, P. Naquet and B.R. Jordan.** 1995. Differential gene expression in the murine thymus assayed by quantitative hybridization on arrayed cDNA clones. Genomics *29*:207-216.
7. **Orr, S.L., T.P. Hughes, C.L. Sawyers, R.M. Kato, S.G. Quan, S.P. Williams, O.N. Witte and L. Hood.** 1994. Isolation of unknown genes from human bone marrow by differential screening and single-pass cDNA sequence determination. Proc. Natl. Acad. Sci. USA *91*:11869-11873.
8. **Piétu, G., O. Alibert, V. Guichard, B. Lamy, F. Bois, E. Leroy, R. Mariage-Samson, R. Houlgatte, P. Soularue and C. Auffray.** 1996. Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridization of a high density cDNA array. Genome Res. *6*:429-503.
9. **Sambrook, J., E.F. Fritsch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, 2nd ed., CSH Laboratory Press. Cold Spring Harbor, NY.
10. **Schena, M., D. Shalon, R.W. Davis and P.O. Brown.** 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microassay. Science *270*:467-470.
11. **Shalon, D., S.J. Smith and P.O. Brown.** 1996. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res. *6*:639-645.
12. **Velculescu, V.E., L. Zhang, B. Vogelstein and K.W. Kinzler.** 1995. Serial analysis of gene expression. Science *270*:384-387.
13. **Wang, K., L. Gan, I. Lee and L. Hood.** 1995. Isolation and characterization of the chicken trypsinogen family. Biochem. J. *307*:471-479.
14. **Welsh, J., K. Chada, S.S. Dala, R. Cheng, D. Ralph and M. McClelland.** 1992. Arbitrary primed PCR fingerprinting of RNA. Nucleic Acids Res. *20*:4965-4970.

This work was partly supported by grants from the Deutsche Forschungsgemeinschaft and the National Science Foundation (BIR9214821). *Address correspondence to Michèl Schummer, Dept. of Molecular Biotechnology, University of Washington, Box 357730, Seattle, WA 98195, USA. Internet: kikjou@u.washington.edu*

Received 18 March 1997; accepted 24 June 1997.

M. Schummer, W.-l. Ng, P.S. Nelson, R.E. Bumgarner and L. Hood
University of Washington
Seattle, WA, USA