

Applications of the Universal DNA Microarray in Molecular Medicine

Reyna Favis, Norman P. Gerry, Yu-Wei Cheng, and Francis Barany

Summary

Integration of molecular medicine into standard clinical practice will require the availability of diagnostics that are sensitive, rapid, and robust. The backbone technology underlying the diagnostic will likely serve double duty during clinical trials in order to first validate the biomarkers that contribute to both drug response and disease stratification. PCR/LDR/Universal DNA microarray is a promising technology to help drive the transition from the current paradigms of clinical decision making to the new era of personalized medicine. By uncoupling the mutation detection step from array hybridization, this technology becomes fully programmable. It exploits full use of the sensitivity that the ligase detection reaction can provide, while maintaining a rapid read out on a universal microarray. Thus, PCR/LDR/Universal DNA microarray is 50-fold more sensitive and 10-fold more rapid than conventional hybridization-only arrays. The intent of this article is to provide investigators with a perspective on current uses of this approach, as well as to serve as a practical guide to implementation.

Key Words: Microarray; ligase detection reaction; high throughput; multiplexing; cancer genomics; SNP; DNA methylation; mutation detection; thermostable ligase.

1. Introduction

1.1. *The Future of Molecular Medicine*

Molecular medicine holds the promise of maximizing patient benefit while minimizing risk. In a vision of the future, standard care for a patient would involve the selection of the drug most likely to be efficacious, given the particulars of an individual's indication, and an adjustment of the drug dose to accommodate the patient's drug metabolism (absorption, distribution, metabolism, and excretion [ADME]) profile. Guiding the physician in making these decisions will be information contained on the drug label, as well as results obtained from point-of-care diagnostic kits and/or the diagnostic laboratory.

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Although the present state of medical care bears little resemblance to this scenario, appropriate technologies to support this standard of care are actively being developed.

To bring molecular medicine a step closer to becoming a reality, the technology platforms of today must have the flexibility to make the transition from clinical trial to diagnostic laboratory. In the recent Food and Drug Administration draft guidance for pharmacogenomic data submission (<http://www.fda.gov/cder/guidance/5900dft.pdf>), it was recommended that the test and the drug be codeveloped and complete information on the test be submitted to the agency. Thus, a cost-effective strategy is to apply the test used during the clinical trial, very likely in fundamentally the same form, to execute the same function in a diagnostic capability after the drug is launched. The standard requirements for a marketable test include high sensitivity and specificity, reproducibility, rapid throughput, and cost effectiveness; however, clinical trials require extra considerations. Of note, tests must assess only those factors for which subjects have provided consent. A suitable technology platform must provide for the aforementioned requirements, as well as be able to accommodate molecular versatility, since there are numerous indications that would benefit from the use of biomarkers.

Molecular approaches for identifying single-base variants fall into three general strategies:

1. Target amplification, e.g., (real-time polymerase chain reaction [PCR], usually with TaqMan[®] or molecular beacon probes), whole-genome amplification (WGA), and strand displacement amplification (SDA).
2. Probe amplification, e.g., ligase chain reaction (LCR), ligase detection reaction (LDR), and rolling circle amplification (RCA).
3. Signal amplification, e.g., 3D dendrimer labeling systems, enzymatic cascade reporters, and invader assay (*see* **ref. 1** for review) (**Fig. 1**; *see* Color Plate 8 following p. 18).

These amplification strategies are often combined with identification of the product sequence by hybridization to its complement on an array. Although many of these approaches have great utility in certain contexts, various drawbacks exist that limit their use in other contexts. For example, direct hybridization schemes cannot always distinguish between closely related sequences and cannot find mutant sequence in an excess of normal sequence (**Fig. 1**, hybridization array on bottom left). Another fundamental stumbling block is that all amplification techniques have the risk of false-positive signal. Whereas PCR amplification suffers from carryover contamination and amplification of false products, LCR and RCA may amplify probe in the absence of the correct target, and signal amplification schemes may create signal arising from nonspecific binding. By combining two such techniques, i.e., PCR and LDR, we have been

able to integrate the best features of each approach, achieving high sensitivity while avoiding false positives (2–14). Furthermore, we have been able to overcome the limitations of hybridization arrays by introducing the concept of divergent zip-code sequences with similar T_m values to guide products to their correct addresses on universal arrays (Fig. 1, universal arrays on bottom right).

1.2. Development and Proliferation of Ligase-Based Detection Strategies

The ability to cycle ligation reactions efficiently was first made possible in the early 1990s, following the cloning of the *Thermus thermophilus* (*Tth*) DNA ligase-encoding gene by our laboratory (15,16). That same year, we demonstrated that LCR (the exponential amplification of DNA by ligating four adjacent primers on both DNA strands) could be used for the detection of genetic disease. LDR (linear amplification by ligating two adjacent primers on one DNA strand) was subsequently developed as a versatile method for discriminating single-base mutations or polymorphisms in a multiplexed fashion (17,18). LDR is ideal for multiplexing when combined with PCR, since several primer sets can ligate along a gene without the interference encountered in polymerase-based systems. This advance paved the way for LDR/PCR (2), which permitted amplification of the signal from the ligation detection event. The development of the Universal DNA microarray allowed different LDR products tagged with unique “zip-code” sequences to be guided to complementary addresses on a universal DNA chip (3).

The techniques and approaches described above have been shown to be versatile, robust, and accessible, having been successfully validated and extended in other laboratories (see Subheading 1.4. below). Indeed, another measure of utility and accessibility has been the conversion of ligase-based strategies into commercial products. Our industrial collaborators (Applied Biosystems [ABI], Foster City, CA) and others have subsequently adopted our universal zip-code design and/or ligation assays for distinguishing multiple signals in capillary, liquid, bead, or microchip arrays (11,19–35). For example, ABI has extended the multiplexing utility of LDR (see Subheading 1.4.3. below).

1.3. Principles of the Universal DNA Microarray

The Universal DNA microarray is a technology platform that provides an alternate strategy in microarray design. It differs from the conventional approaches to microarray technology in that mutation detection and hybridization to the array surface are completely separate events. Since the specificity for the Universal DNA microarray is determined by LDR, it avoids the false negatives and false positives associated with direct DNA hybridization arrays. For high-throughput detection of specific multiplexed LDR products, unique zip-code sequences are

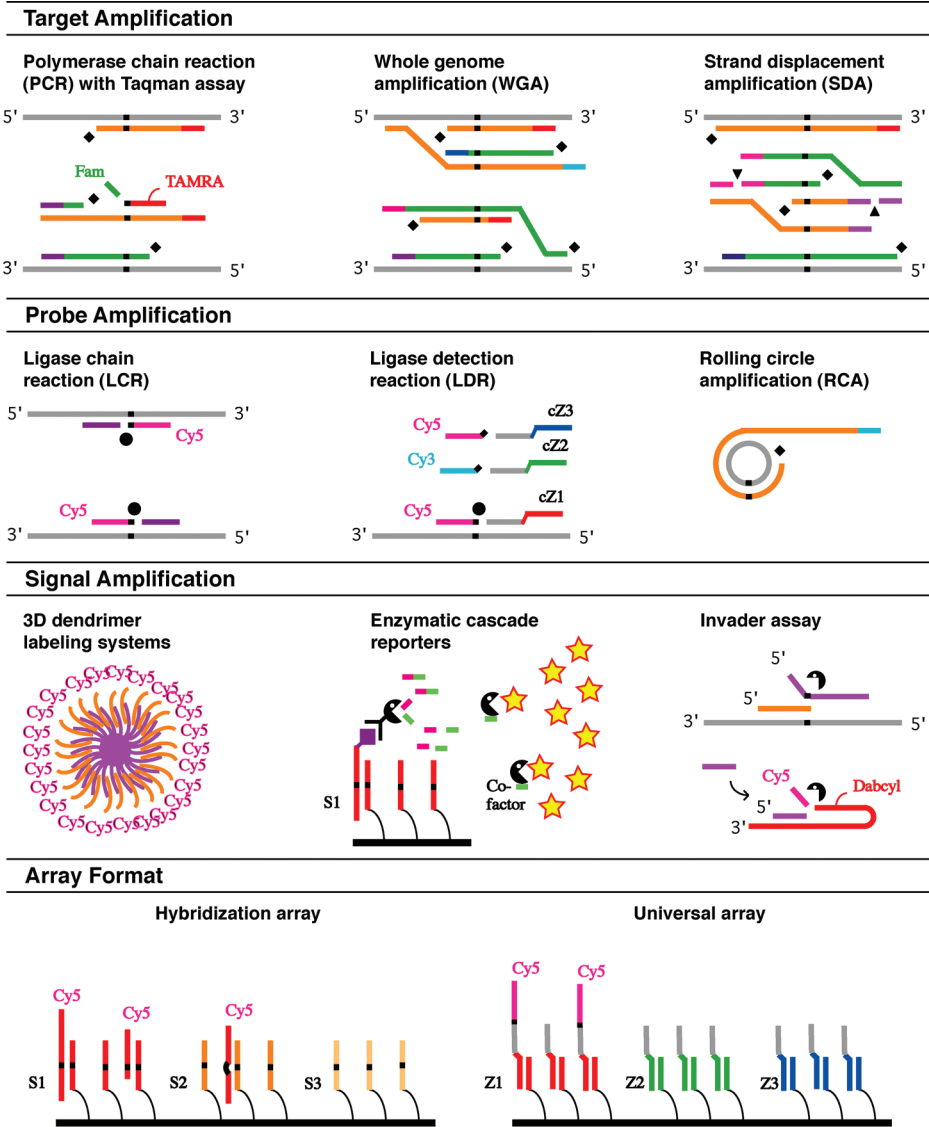


Fig. 1. (Color Plate 8 following p. 18). Overview of target amplification, probe amplification, signal amplification, and arrays used in molecular diagnostics (*see* **ref. 1** for original references). **Target amplification:** PCR/TaqMan—The 5′–3′ exonuclease activity of Taq polymerase releases a fluorescent group from its quencher during primer extension, allowing real-time monitoring of the PCR reaction. WGA—random oligonucleotide primers are used in conjunction with a processive polymerase with strand displacement

attached to each LDR product, allowing for specific capture at complementary addresses on a DNA microarray (3). The Universal DNA microarray is thus programmable and can accommodate any gene without redesigning the array. The details of the methodology provide explanations as to why this technology is sensitive, rapid, and robust.

The major components of the process are simple to perform and involve PCR followed by LDR and then hybridization of the product to the Universal DNA microarray (**Fig. 2**; see Color Plate 9 following p. 18). To amplify multiple genomic regions while minimizing primer-specific differences in efficiency,

Fig. 1. (Continued from previous page) activity, allowing isothermal, nonspecific amplification total genomic DNA with little amplification bias. SDA—two primers hybridize to each strand of the target DNA and are extended so that the upstream primer displaces the downstream primer. Two more primers anneal to the displaced product and extend in the same way, followed by restriction endonuclease nicking and extension of the double-stranded product. **Probe amplification:** LCR—two primers anneal to each strand of the target DNA region and are ligated if there is perfect complementarity at the junctions. The product molecules can act as templates for the next round of LCR, resulting in exponential amplification. LDR—a fluorescently labeled allele-specific primer and a downstream primer anneal adjacently on one strand of the target DNA. If there is perfect complementarity at the junction, a thermostable ligase joins the primers, and the resulting product is detected on an array or by electrophoresis. RCA—both sides of a long primer hybridize to the target sequence and, when ligated, produce a circular product molecule, which is then amplified by a strand-displacing DNA polymerase. **Signal amplification:** 3D dendrimer labeling systems—a dendrimer has a core that consists of a matrix of double-stranded DNA, and an outer surface with hundreds of single-stranded “arms,” which are available for hybridization to a specific sequence or to oligonucleotides that carry signal molecules. Enzymatic cascade reporters—an event, such as hybridization of the correct DNA molecule to a position on an array, stimulates an enzyme to produce a cofactor that is specific for a second enzyme. This second enzyme can, in turn, separate a fluorescent group from its quencher. Invader assay—an allele-specific oligonucleotide and a second oligonucleotide hybridize to the target so there is a single nucleotide overlap at the base in question, and cleavase exonuclease releases the “flap” at the 5′ end of the oligonucleotide only if there is perfect complementarity at the junction. This “flap” is then able to bind to a specific fluorescence resonance energy transfer (FRET) moiety, allowing cleavase to release a fluorescent signal. **Array format:** Hybridization array—biological oligonucleotides that are specific for a particular organism (e.g., cDNAs) are covalently attached to an array surface at specific locations, allowing detection of their labeled complements (e.g., RNAs). This format has difficulty distinguishing all single-base mutations. Universal array—unique oligonucleotides (zip-codes) are located at specific positions on an array surface, allowing fluorescently labeled LDR products, with a tails that are complementary to the zip-codes, to hybridize. Appropriate primer design allows a single Universal array to be used for many different assays.

PCR / PCR / LDR / Universal Array

- 1. PCR amplify all p53 exons using gene-specific/universal primers and Taq polymerase. ♦
- 2. PCR amplify all primary products using universal primers and Taq polymerase. ♦
- 3. Perform LDR using mutation-specific LDR primers, common primers containing complementary zip code sequences, and thermostable ligase. ●
- 4. Capture fluorescent products on addressable array and score for presence of mutation.

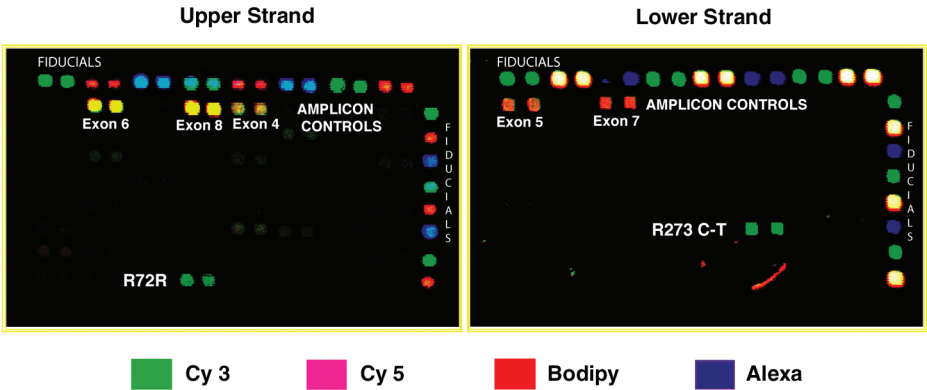
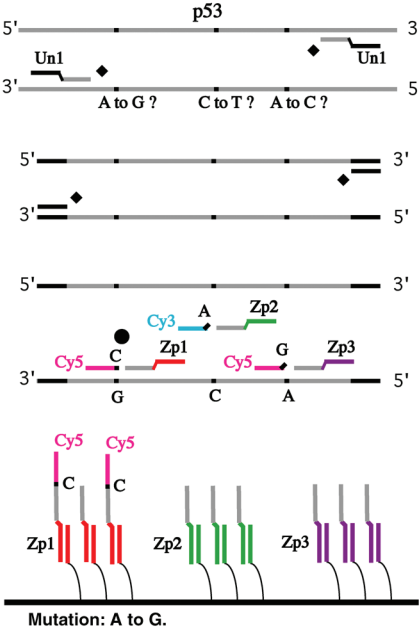


Fig. 2. (Color Plate 9 following p.18) Schematic diagram and results for the detection of multiple mutations using PCR/PCR/LDR/Universal Array. Upper panel shows schematic. p53 exons 4–8 are PCR-amplified in a multiplex format with gene-specific primers bearing 5' universal sequences, and in a second PCR, amplified simultaneously with universal PCR primers. Multiplexed LDR is performed on all PCR products with fluorescently labeled allele-specific LDR primers and common primers containing complementary zip-code sequences. LDR products are hybridized to universal array containing zip-codes. Address and color of the microarray spot scores are given for the mutation. Lower panel shows chip results. The p53 chip can detect 110 different mutations in exons

two rounds of PCR are used. PCR primers are constructed to have gene-specific portions connected to universal sequences on the 5' ends. The first round of PCR relies on a limited number of cycles using the gene-specific portions of the primers. The reaction is supplemented with a PCR cocktail containing primers complementary to the universal sequences and the majority of the amplification cycles are completed. Following this multiplex PCR/PCR amplification of the genomic regions of interest, each mutation is simultaneously detected using a thermostable ligase that joins adjacent pairs of oligonucleotides complementary to the sequences of interest. Attached to one of the paired oligonucleotides (referred to as the common oligo) are nongenic 24-base sequences that are complementary to 24-base zip-code sequences present at known locations on the microarray surface. The remaining oligonucleotide of the pair (referred to as the discriminating oligo) is fluorescently labeled. Ligation occurs only when the sequence at the junction between the paired oligos is exactly complementary to the template sequence. Thus, when the variant of interest is present, ligation joins the oligonucleotide bearing the fluorescent label to the oligonucleotide bearing the zip-code complement. Hybridizing the LDR product to the Universal DNA microarray reveals the presence of a variant. If the variant of interest is present, a fluorescent signal will be visible on the address bearing the zip-code sequence that captures the complementary zip-code on the LDR oligo. If the variant is not present, the LDR oligo with the complementary zip-code will still hybridize to the appropriate address, but no fluorescent signal will be joined to it.

The sensitivity of this system is augmented by the use of a 3D polyacrylamide surface. This surface permits hybridization times of 30–60 min and signal intensities 100-fold better than conventional microarrays (e.g., poly-L-lysine or amino/silane-coated slides) (3).

The most significant advantage of our technique is the ability to separate and therefore optimize mutation identification independently of array hybridization.

Fig. 2. (Continued from previous page) 5, 6, 7, and 8. A total of 216 LDR primers were required for detection. The mutation status of each sample and the zip-codes expected to capture signal are indicated at the bottom of each array; fiducials are along the top and right side of all arrays. Two reactions were performed for each sample containing LDR primers that were designed to hybridize to the upper strand or lower strand of *p53* sequence. The array was imaged on a Lumonics ScanArray 5000 to visualize the Cy3, Cy5, and FAM signals. The 16-bit grayscale images for each dye were captured using the MetaMorph Imaging System (Universal Imaging), rendered in color, overlaid, and merged. R72R is a polymorphism in exon 4, and R273 C→T is the mutant signal. PCR, polymerase chain reaction; LDR, ligase detection reaction.

The background signal from each step can be minimized, and consequently, the overall sensitivity and accuracy of our method can be significantly enhanced over other strategies. Direct hybridization DNA microarrays suffer from differential hybridization efficiencies owing either to sequence variation or to the amount of target present in the sample. Consequently, hybridizations are performed at low temperatures, often for several hours to overnight, and this results in increased background noise and false signals caused by mismatch hybridization and nonspecific binding, for example, on small insertions and deletions in repeat sequences (36–39). In contrast, our approach of designing divergent zip-code sequences with similar T_m s, allows for a more stringent and rapid hybridization at 65°C.

1.4. Applications of PCR/LDR and the Universal DNA Microarray

When our approaches are combined with PCR, they have been successfully applied to the simultaneous multiplex detection of numerous genetic diseases (see **Subheading 1.4.1.** below). In our own laboratory, the approach has been validated on hundreds of clinical tumor samples during detection of 19 *K-ras* and 110 *p53* gene mutations in non-microdissected tumors, as well as stool, demonstrating the ability to find mutations despite a large quantity of background normal sequence (5,10,12–14). Our approach has the sensitivity to detect 1 in 100 for a *p53* mutation in a wild-type sequence, which is impossible to achieve using standard commercial hybridization chips (3,12).

The Universal DNA microarray allows for the detection of (1) dozens to hundreds of polymorphisms in a single-tube multiplex format, (2) small insertions and deletions in repeat sequences, (3) low-level mutations in a background of normal DNA (3,5,6,17,18), and (4) methylation status of gene promoters. In addition, it requires less manipulation of the DNA. Direct hybridization methods require (1) multiple rounds of PCR or PCR/T7 transcription and (2) processing of PCR-amplified products into fragments or rendering them single stranded. In contrast, our approach allows multiplexed PCR in a single reaction (18) but does not require an additional step to convert product into a single-stranded form.

PCR/LDR and the Universal DNA microarray have been successfully employed in studies that required the following capabilities.

1.4.1. Multiplexed Detection of Single-Nucleotide Polymorphisms and Point Mutations

PCR/LDR has been successfully applied to simultaneous multiplex detection of 61 cystic fibrosis alleles (40,41), 6 hyperkalemic periodic paralysis alleles (42), and 20 21-hydroxylase deficiency alleles (17,43). In addition to point mutations,

we demonstrated that PCR/LDR could detect instability within the transforming growth factor- β type II receptor gene and the APC1307K mononucleotide repeat allele in DNA derived from both blood and paraffin-embedded tumor samples (4,6). The cystic fibrosis test is commercialized by our corporate collaborators, ABI and Celera Diagnostics, and is used throughout the world for prenatal testing of this inherited disease.

1.4.2. Multiplexed PCR for Amplifying Many Regions of Chromosomal DNA Simultaneously

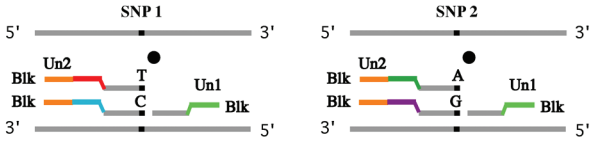
We have developed a coupled multiplex PCR/PCR/LDR assay for use in armed forces personnel. This technique was developed to mitigate the problems of false amplicons, allele dropout, and uneven amplifications, which often mar attempts to perform highly multiplexed PCR (18). A comparison of LDR profiles of several individuals demonstrated the ability of PCR/LDR to distinguish both homozygous and heterozygous genotypes at each locus (18). Others have independently validated the use of PCR/PCR in human identification to amplify 26 loci simultaneously (44) or ligase-based detection to distinguish 32 alleles, although the latter was in individual reactions (45). We have also developed a PCR/PCR/LDR assay to detect the founder Jewish BRCA1 and BRCA2 insertion and deletion mutations associated with breast cancer (8).

1.4.3. Multiplexed LDR/PCR to Determine DNA Copy Number or Score SNPs

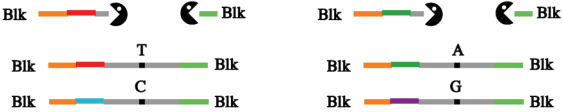
We initially developed multiplexed LDR followed by PCR to score chromosomal instability in tumors (2). Others have extended our approach to detection of deletions in the *DMD* gene, deletions in the *hMLH1* and *hMSH2* genes, and chromosomal trisomy (46–48). Our corporate collaborators at ABI have extended our LDR/PCR protocol for typing single-nucleotide polymorphisms (SNPs) directly on genomic DNA. In their protocol, one of each LDR primer pair contains a unique zip-code sequence, and locus-specific sequences are flanked by universal primer sequences. Consequently, all LDR products may be amplified in a single PCR step, and each product may be identified by its unique zip-code sequence. The products may be rendered single stranded and hybridized on a universal array (Fig. 3; see Color Plate 10 following p. 18), or alternatively used to capture premade fluorescently labeled “zip-chutes” (developed by ABI) (Fig. 4; see Color Plate 11 following p. 18), each with a unique size, for scoring by electrophoretic separation. The technique has been validated on 3000 SNPs using 96 genomic DNA samples (11).

LDR / PCR / Universal Array

1. Perform LDR using allele-specific and common LDR primers containing universal primer sequences, and thermostable ligase. ● Each LDR product contains a unique complementary zip code sequence.



2. Destroy unligated LDR primers using 5'→3' and 3'→5' exonucleases.



3. PCR amplify all LDR products using universal primers and Tag polymerase. ◆



4. Capture fluorescent products on addressable array and score each SNP.

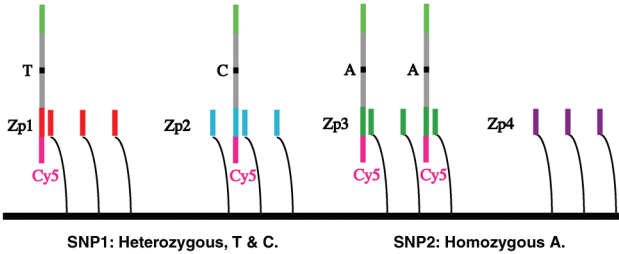


Fig. 3. (Color Plate 9 following p. 18) Schematic diagram for the detection of multiple single-nucleotide polymorphisms (SNPs) using LDR/PCR/Universal Array. Multiplexed LDR is performed on multiple SNPs using allele-specific primers containing unique zip-code sequences and a 5' universal sequence (Un2) as well as locus-specific primers containing a different universal sequence (Un1) on their 3' ends. Only if there is perfect complementarity at the junction will the ligation product form, thus distinguishing different SNPs. Unligated products are destroyed with λ exonuclease (5'→3') and exonuclease 1 (3'→5'). All remaining LDR products are coamplified simultaneously with universal PCR primers Un2 and Un1. In this illustration, Un2 is labeled with Cy5 on the 5' end, and Un1 may be phosphorylated on the 5' end, allowing for the option to convert PCR products to a single-stranded form with λ exonuclease prior to hybridization. PCR products are hybridized to universal array containing zip-codes. Fluorescent signal at a given address scores for the presence or absence of each SNP. LDR, ligase detection reaction; PCR, polymerase chain reaction.

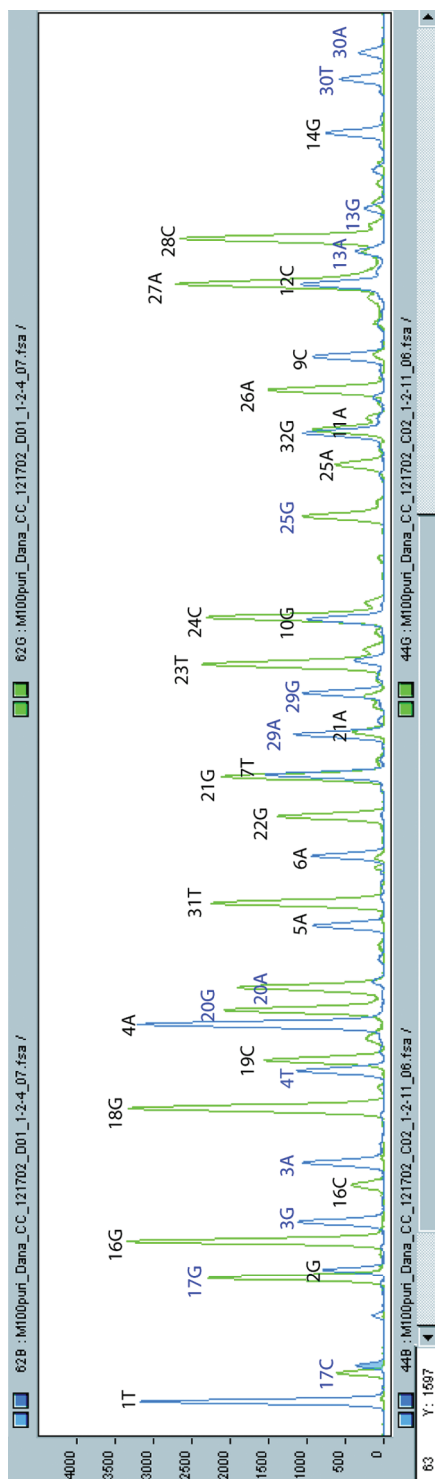


Fig. 4. (Color Plate 11 following p. 18) Electrophoretogram of 30-plex detection in genomic DNA. The data demonstrate the ability of LDR/PCR to characterize 60 alleles simultaneously in an SNP genotyping assay. The blue and green peaks are either FAM or Vic labeled mobility modified zip-chutes. The x-axis is the size of zip-chute and the y-axis is fluorescent intensity of capillary electrophoresis. The zip-chutes have the same color but different motifs for both of allele-1 and allele-2. For example, 1T (first blue peak) is homozygous, T; 17C (green) and 17G (green) are heterozygous C/G, etc.

1.4.4. Detection of K-ras, BRCA1, BRCA2, and p53 Mutations Using Multiplex PCR/LDR and Zip-Code Capture

Since the zip-code sequences remain constant and their complements can be appended to any set of LDR primers, our zip-code arrays are universal, and we and our cancer collaborators at The Rockefeller University and the Institut Curie have applied this array-based mutation detection to mutations in the *K-ras*, *BRCA1*, *BRCA2*, and *p53* genes (3,8,9,12–14). **Figure 2** shows the schematic and results for *p53* mutation analysis, where by 110 mutations could be queried simultaneously. Mutations present at 1% of the wild-type DNA level, or in pooled samples could be distinguished (8,12).

1.5. Back to the Future

The practice of molecular medicine will require technology platforms that can span the progression from clinical trial to diagnostic laboratory and rapidly deliver an answer. En route, the incipient diagnostic will be challenged with a variety of genes containing diverse assortments of genetic variation.

The Universal DNA microarray is a strong candidate to serve this need. Because the platform is programmable, it is robust enough to accommodate changes in the genes, SNPs, or mutations of interest without reengineering the array. In addition, our zip-code concept can be used for displaying mutations on a variety of platforms: universal array surfaces, gel or capillary electrophoresis, and universal encoded beads (see **Fig. 5** [Color Plate 12 following p. 18] and **Subheading 3.3.**). This programmability and display versatility are added boons in the context of clinical trials, in which the list of genes for which subjects provide consent for genotyping can vary from trial to trial, the SNP content must reflect the targeted patient/volunteer population, and the number of recruited subjects can vary by orders of magnitude.

We and others have also shown that our assay can detect a greater range of human genetic variation compared with other systems. In addition to SNPs and low-level mutations (10), insertion/deletion mutations (8) and length polymorphisms in mononucleotide (6) and dinucleotide repeats (7) can also be reliably detected—two blind spots for direct hybridization arrays (49–51). Even though this latter type of variation is invisible to most mutation detection technologies, such variations are known to have pharmacological relevance owing to their prevalence in ADME genes. For example, CYP2D6 has numerous insertion/deletions that impact enzyme activity (52); UGT1A1 contains a promoter polymorphism consisting of variable numbers of TA repeats that influences enzyme concentration (53).

This technology is also amenable to the rapid provision of results in a clinical setting. Given that LDR can be performed in 5–10 min (54), a microfabricated

LDR / PCR / Universal Display

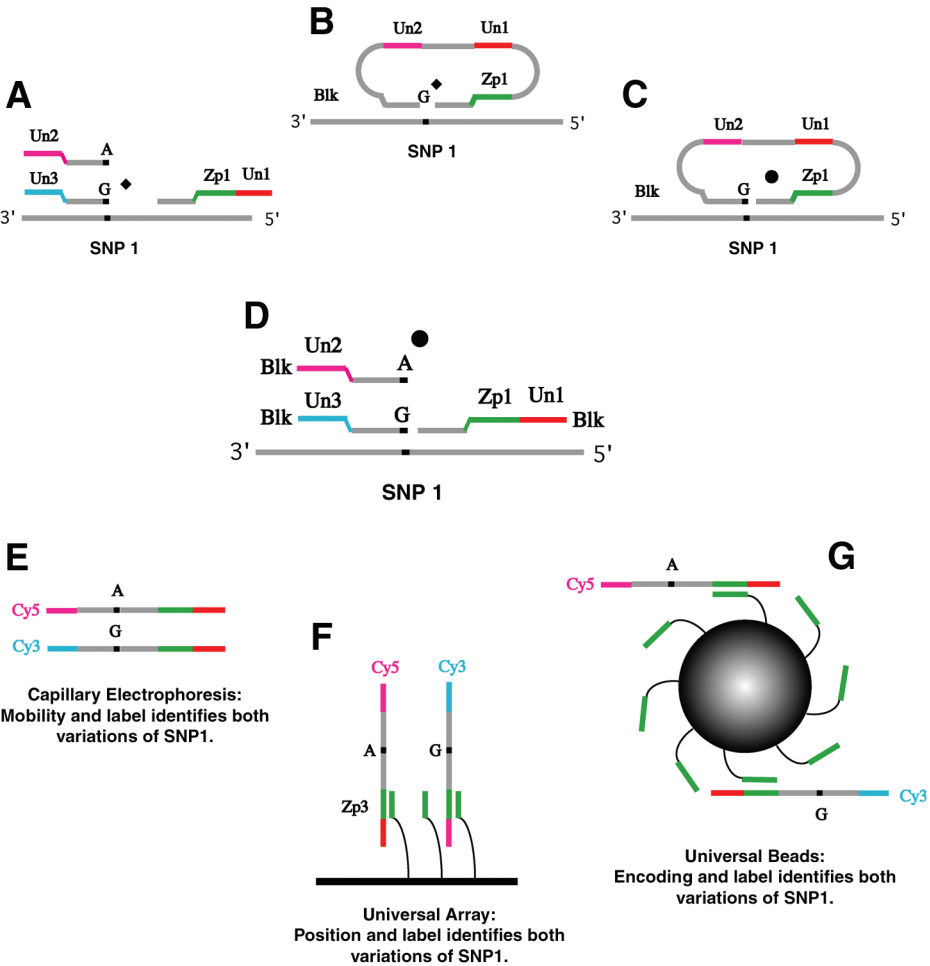


Fig. 5. (Color Plate 12 following p. 18) Ligase-based multiplexed single-nucleotide polymorphism (SNP) assays combined with zip-code universal display. The original LDR/PCR procedure combines ligation of multiple oligonucleotide probes on multiple target sequences followed by PCR coamplification of ligation products using universal primer sequences (2). The generic version for SNP detection is illustrated in the middle of the figure (D). Allele-specific oligonucleotides may hybridize on target DNA adjacent to a downstream locus-specific sequence containing a specific zip-code sequence and will ligate if there is perfect complementarity at the junction. Use of blocked ends allows for exonuclease to degrade unligated probes but not the ligation products. Universal primers Un1, Un2, and Un3 are used to coamplify all the products. A variation of this scheme uses only two universal primers but distinguishes alleles by appending different zip-code sequences to the allele-specific oligonucleotides (as shown in Fig. 3). In

device that integrates sample prep with multiplexed genetic variation identification (PCR/LDR) and molecular profiling (LDR/PCR) would be anticipated to provide results in less than 30 min. A reason for optimism lies in the significant progress recently made in developing microfluidic and microchip-based platforms for point-of-care analysis. Although they are beyond the scope of this chapter, platforms that integrate sample preparation (55–57), purification and concentration of the DNA/RNA component (58,59), component delivery (60–63), thermocycling (58,64,65), and capillary or channel-based separation and signal detection (54,66–80) will be considered for enriching our assay development.

2. Materials

1. Oligonucleotides (*see Note 1*).
2. dNTPs (PE Biosystems, cat. no. 4303441).
3. AmpliTaq Gold (PE Biosystems, cat. no. 4311820).
4. Taq DNA ligase (NEB, cat. no. M0208L).
5. Proteinase K (Qiagen, cat. no. 19131).
6. Three 1-in microscope slides with etched circles (VWR, cat. no. 48349-057 or Erie, cat. no. 2960; *see Note 2*).
7. 24 × 50-mm Cover slips (VWR, cat. no. 48393-081; *see Note 2*).
8. Corning crystallizing dishes, 170-mm diameter × 90-mm height (VWR, cat. no. 25411-140 or Corning, cat. no. 3140-170; *see Note 3*).
9. 20-Slide glass slide racks (VWR, cat. no. 25463-009 or Wheaton, cat. no. 900204).
10. Glass slide rack handle (VWR, cat. no. 25464-001 or Wheaton, cat. no. 900205).
11. 50-Slide rack and staining dish (VWR, cat. no. 25461-024 or Wheaton, cat. no. 900400).
12. Acrylamide (Boehringer Mannheim, cat. no. 1871757).
13. Bis-acrylamide (Boehringer Mannheim, cat. no. 1685830).
14. Acrylic acid (Aldrich, cat. no. 14,723-0).

Fig. 5. (*Continued from previous page*) another variation of this scheme, allele-specific oligonucleotide primers are extended with a polymerase prior to ligation to the common oligonucleotide (**A** [23]). Alternatively, the nonligating ends of the upstream and downstream probes may also be blocked by synthesizing a single long probe, which is subsequently linearized after exonuclease digestion in preparation for the PCR step (**B** [21]) In one version of this variation, an extra polymerase step is used to add a single base prior to ligation (**B**), although the approach works as well by using straight ligation (**C** [19]). In the examples illustrated here, two different fluorescent labels are used for the UN2 and Un3 PCR primers, although schemes using a single label or more may be used. The PCR products may be displayed using gel or capillary electrophoresis (**E** [2,11]), universal array surfaces (**F** [3,8–10,12–14,19,21]), or universal encoded beads (**G** [22–24]).

15. Ammonium persulfate (APS; VWR, cat. no. JT0762-11).
16. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (Aldrich, cat. no. 16,146-2).
17. *N*-hydroxysuccinimide (NHS; Aldrich, cat. no. 13,067-2).
18. 3-(Trimethoxysilyl)propyl methacrylate (Aldrich, cat. no. 44,015-9).
19. Concentrated NH_4OH (VWR, cat. no. JT9721-4).
20. 30% Hydrogen peroxide (Aldrich, cat. no. 21,676-3).
21. Concentrated HCl (VWR, cat. no. JT9535-4).
22. High-performance liquid chromatography (HPLC) grade methanol (VWR, cat. no. JT9093-3).
23. HPLC grade acetone (VWR, cat. no. JT9002-3).
24. HPLC grade chloroform (Fisher, cat. no. C606-4).
25. Triethylamine (Aldrich, cat. no. 47,128-3).
26. 0.4 M $\text{K}_2\text{HPO}_4 / \text{KH}_2\text{PO}_4$, pH 5.5.
27. 1 M 2-Morpholinoethanesulfonic acid (MES), pH 6.0 (light sensitive; filter-sterilize and store at room temperature; stable for 3–4 mo; do not use if the solution appears yellow).
28. 2X Hybridization buffer: 0.6 M MES, pH 6.0, 20 mM MgCl_2 , 0.2% sodium dodecyl sulfate (SDS; light sensitive; store at room temperature; stable for 3–4 mo).
29. 1X Wash buffer: 0.3 M bicine, pH 8.0, filter-sterilized, 0.1% SDS (store at room temperature).
30. Drierite (VWR, cat. no. WLC3712T).
31. Cover wells, 9-mm diameter \times 1-mm height (Grace, Sunriver, OR).
32. Razor blade.
33. Plastic slide box for hybridization (SPI Supplies, cat. no. 01253A-CF).
34. Dessicator.
35. Shaker.
36. Heat blocks.
37. Hot plates.
38. Thermocycler.
39. Rotating hybridization oven.
40. Cartesian Pixsys 5500 array spotting robot with a quill-type spotter in a controlled atmosphere chamber or similar instrument.
41. Perkin-Elmer ScanArray 5000 or similar instrument.

3. Methods

3.1. Array Fabrication

Below is a description of the process used to produce universal arrays. Commercially available arrays with 3D surfaces are an alternative to in-house production.

3.1.1. Clean Slides to Remove Oxidized Surfaces

1. Assemble slides in the glass slide racks. Put slides in back to back so there are two slides per slot (*see Note 4*).

2. In a hood using hot plates, boil 600 mL of ultrapure water in two glass crystallizing dishes partially covered with a glass plate to prevent evaporation. Fill a third dish with water, and heat to 60–70°C.
3. When the water boils, add 120 mL concentrated NH_4OH and 120 mL 30% peroxide to the first dish (5:1:1 water/ NH_4OH /peroxide; the solution will bubble vigorously). Place racked slides in solution, and boil for 10 min (*see Note 5*).
4. Rinse slides in 60–70°C dish of water for 2–3 min. Drain excess water from slide racks.
5. Add 120 mL concentrated HCl and 120 mL 30% peroxide to second dish of boiling water (5:1:1 water/ HCl /peroxide; the solution will bubble vigorously). Add slide racks, and boil for 10 min.
6. Rinse slides in water followed by HPLC-grade methanol and finally HPLC-grade acetone. Air-dry (*see Note 6*).

3.1.2. Silanize Slides

1. Place cleaned slides in 50-slide rack and immerse in 400 mL CHCl_3 solution containing 2% γ -(trimethoxysilyl)propyl methacrylate and 0.2% triethylamine. Agitate gently at room temperature on a shaker table for 30 min (*see Note 7*).
2. Wash slides in 400 mL CHCl_3 for 15 min on a shaker table. Repeat wash with fresh CHCl_3 (*see Note 8*).
3. Drain excess CHCl_3 , blot racked slides on paper towel, and let air-dry in a hood. Store slides in slide box until ready to prepare polymer surface. Do not touch surface of slides; handle slides by frosted end only (*see Note 9*).

3.1.3. Preparation of Polymer Surface

1. Heat heating blocks to 70°C, and then invert blocks so the solid bottom surface is up.
2. Make up monomer solution. The individual monomer solutions should be made fresh (no more than 1 or 2 d in advance). The solution is as follows: 200 μL 40% w/v acrylamide, 50 μL 40% w/v acrylic acid, 20 μL 1% w/v bis-acrylamide, 80 μL 10% APS, and 650 μL water.
3. Vortex to mix, and allow mixture to sit at room temperature for 1 h before making slides (*see Note 10*).
4. Spot 20 μL of monomer solution onto the center of a silanized slide from above.
 - a. Cover with a cover slip by lowering the cover slip parallel to the surface of the slide until it makes contact with the top of the monomer solution droplet.
 - b. Release the cover slip, and allow the monomer solution to spread.
 - c. The solution should spread to the edges of the cover slip on its own.
 - d. If numerous or large bubbles are caught under the cover slip, it can be slid off, the surface gently wiped with a Kimwipe, and the procedure repeated using a fresh cover slip (*see Note 11*).
5. Place slide on heated blocks for 4.5 min with cover slip side up (*see Note 12*).
6. Following polymerization, place the slide into a slide rack immersed in water for 5 min.

7. Use a razor blade to wedge up one of the short sides of the cover slip, and then slowly peel the cover slip from the polymer.
8. Place slides that have new polymer surfaces in racks. (We use test tube racks.)
9. Rinse the slides under running ultrapure water, use a forced air line to gently blow off excess liquid, and then allow slides to air-dry. Store at room temperature in slide boxes until ready to activate surfaces for spotting (*see* **Note 13**).

3.1.4. Activation of Polymer Surfaces

1. Place polymer-coated slides in a 50-slide rack, and immerse in 400 mL 0.1 M potassium phosphate, pH 6.0, containing 0.1 M (EDC) 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and 20 mM NHS. Agitate gently at room temperature on a shaker table for 30 min.
2. Dunk racked slides in receptacle of ultrapure water approx 20 times to rinse. Replace water, and immerse approx 10 times.
3. Tap racked slides on paper towel to blot excess water, and blow off water using forced air.
4. Heat slides at 65°C in an oven (cracked open slightly for good circulation) until completely dry (30 min to 1 h).
5. Store slides desiccated at room temperature in slide boxes (*see* **Note 14**).

3.1.5. Array Spotting

1. Prepare spotting plates by mixing 5 μ L 1000 μ M zip-code oligonucleotide solutions with 5 μ L 0.4 M K_2HPO_4/KH_2PO_4 , pH 8.5, in 384 conical well spotting plates (*see* **Note 15**).
2. Place slides in spotter, and set relative humidity to 60–70%. Allow the slides to incubate for 15–20 min (*see* **Note 16**).
3. Spot slides in desired layout (*see* **Note 17**).
4. Following spotting, removed uncoupled oligonucleotides from the polymer surfaces by soaking the slides in 300 mM bicine, pH 8.0/300 mM NaCl/0.1% SDS for 30 min at 65°C (*see* **Note 18**).
5. Rinse the slides with ultrapure water, and dry as described above in **Subheading 3.1.4**.
6. Store slides desiccated at room temperature in slide boxes (*see* **Note 19**).

3.1.6. Array Quality Control

Spotting failures are detected by staining two newly minted arrays from the beginning and end of a spotting run with SYBR Green II via the method of Battaglia et al. (81) (**Fig. 6A**; *see* Color Plate 13 following p. 18) This dye clearly shows whether addresses failed to spot during the array printing process; if critical addresses used by all assays are affected, defective arrays can be discarded. If addresses deeper into the array are affected, these arrays can be set aside for use in small assays that will be unaffected by the missing addresses (e.g., *K-ras* mutation detection only requires the first four addresses).

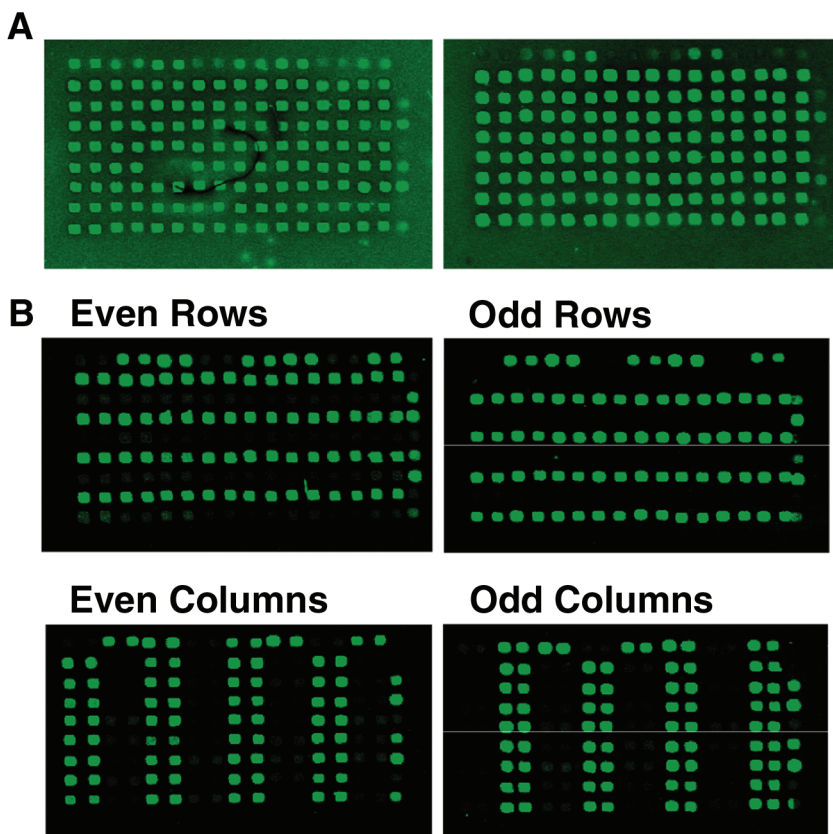


Fig. 6. (Color Plate 13 following p. 18) Validation of arrays following spotting. **(A)** Arrays are stained with a solution of SYBR Green II to determine whether all the zip-codes have been spotted successfully. The left panel shows an array, which has had a spotting failure, and the right panel shows the complete array. **(B)** Arrays are hybridized with mixtures of fluorescein-labeled zip-code complements to look for cross-talk resulting from either well-plate cross-contamination or poor washing of the pins between spotting cycles.

Four random arrays are next chosen from array sets that pass this first round of inspection and are subjected to hybridization with mixtures of fluorescein-labeled zip-code complements (*see Subheading 3.2.* for conditions). Mixtures are prepared for the even and odd rows, and the even and odd columns. This test confirms that there is no crosstalk between addresses caused by either well-plate crosscontamination or poor washing of the pins between spotting cycles. **Figure 6B** shows an array that has passed this level of inspection: specific hybridization to odd or even rows and odd or even columns are visible with no extraneous signals.

3.2. PCR/LDR/Array Hybridization

3.2.1. PCR

1. Multiplex PCR is performed with 50–100 ng of genomic DNA in a 25 μ L reaction using AmpliTaq Gold and 2 pmol of each gene-specific primer bearing the universal primer on the 5' ends.
 - a. Overlay the reaction with mineral oil and incubate for 10 min at 95°C.
 - b. Amplify for 15 cycles using conditions optimum for the genes of interest.
 - c. Add a second 25 μ L aliquot of the reaction mixture through the mineral oil containing 25 pmol universal primer.
 - d. Continue cycling for 25 cycles at a higher annealing temperature than that used for the gene-specific primers.
2. Digest the reaction with the addition of 1 μ L proteinase K (18 mg/mL) and incubation at 70°C for 10 min. Inactivate the proteinase K by incubating at 95°C for 15 min.
3. Analyze a 1–2 μ L aliquot by agarose gel electrophoresis to verify the presence of amplification product of the expected size.

3.2.2. LDR

1. LDR reactions are carried out under oil. The following reactants are combined in a PCR tube for each sample: 1 μ L 10X ligase buffer, 1 μ L 100 mM dithiothreitol (DTT), 1 μ L 10 mM NAD⁺, 500 fmol of each primer, and 2 μ L of PCR product from **Subheading 3.2.1., step 3** in a total volume of 10 μ L.
2. Dilute the 40,000 U/mL (0.3 pmol/ μ L) *Tth* DNA ligase to 2.5 fmol/ μ L in 1X ligase buffer. The volume of the diluted ligase should be sufficient to add 10 μ L to each reaction.
3. Heat-denature the 10 μ L reaction at 94°C for 2 min, and add 10 μ L (25 fmol) of diluted *Tth* DNA ligase.
4. Cycle the reaction for 20 rounds of 94°C for 30 s and 65°C for 4 min.

3.2.3. Array Hybridization

1. Dilute LDR reactions with an equal volume of 2X hybridization buffer to produce a final buffer concentration of 300 mM MES, pH 6.0, 10 mM MgCl₂, 0.1% SDS (see **Note 20**).
2. Denature the mixture at 94°C for 3 min and chill on ice.
3. Preincubate arrays for 15 min at 25°C in 1X hybridization buffer. Remove the arrays from the buffer and blow the surface dry of excess liquid.
4. Attach cover wells to the arrays and fill with 35 μ L of the diluted LDR products (see **Note 21**). Use adhesive plastic to seal the cover well openings to prevent drying.
5. Place the arrays in plastic slide holders and humidify using a moistened sponge. Secure the arrays to the rotisserie of the rotating hybridization oven, and incubate for 1 h at 65°C and 20 rpm (see **Note 22**).
6. After hybridization, wash the arrays in 300 mM bicine, pH 8.0/0.1% SDS for 10 min at 25°C. Rinse the arrays briefly in water, dry, and scan.

3.3. Variations on a Theme

In addition to the standard PCR/LDR technique outlined in **Subheading 3.2.** above, there are several variations of LDR as an assay tool for mutation identification, SNP detection and DNA methylation analysis.

3.3.1. LDR/PCR

To genotype hundreds of thousands SNPs accurately in multiple samples in a high-throughput format, one variation is to perform LDR on the DNA samples followed by PCR amplification of the ligation products. By performing LDR directly on the DNA samples using primers bearing universal sequences on the 5' end (*see Fig. 3*), ligation products can be subsequently simultaneously amplified using universal primers. There are several advantages to performing LDR prior to PCR amplification: (1) it eliminates the time-consuming steps of design and optimization of multiplex gene-specific PCR primers; (2) it reduces the cost of synthesizing hundreds of fluorescently labeled allele-specific LDR primers, since product labeling can be accomplished using fluorescently labeled universal PCR primers; (3) it reduces the complexity of the assay system to ensure an accurate and efficient DNA analysis by avoiding the common pitfalls associated with multiplex PCR amplification (e.g., formation of primer dimers and other nonspecific amplicons that may interfere with downstream applications); (4) it reduces the time required for LDR primer design and reaction optimization; and (5) it provides an initial linear amplification of the targeted genomic information that is nonbiased, it promotes allelic balance, and it may minimize the need for later PCR cycles that may detract from this balance.

3.3.1.1. LDR/PCR/UNIVERSAL MICROARRAY

The use of multiplex LDR followed by PCR was initially developed to score chromosomal instability in tumors (*see Subheading 1.4.* above). A schematic diagram of multiplex LDR/PCR/Universal Array to determine DNA copy number or score SNPs is shown in **Fig. 3**. In this approach, the universal primer sequences are added to the 5' end of discriminating LDR primers and to the 3' end of common LDR primers. After ligase detection reaction, the excess unligated LDR primers and DNA templates can be digested using 5'→3' and 3'→5' exonucleases. The ligation products are protected from digestion, since blocking groups are added at both their 5' and 3' ends. This exonuclease digestion step reduces the potential of nonspecific hybridization and false-positive results on the universal array readout. The ligation products are simultaneously amplified with universal PCR primers. Only one of the universal PCR primers is fluorescently labeled to serve as the detection signal when these amplicons are captured on a universal array.

3.3.1.2. LDR/PCR/UNIVERSAL DISPLAY

ABI recently extended the LDR/PCR concept with the development of an ultra-high-throughput genotyping method, SNPLEX. This technology utilizes multiplexed oligonucleotide ligation assay on genomic DNA. Each LDR primer pair was synthesized with universal primer sequences flanking the locus-specific sequences. A unique zip-code sequence is designed within the LDR primers to uniquely identify each LDR product. The excess LDR primers and genomic DNA are eliminated through enzymatic digestion. Consequently, all LDR products may be amplified in a single PCR step with two universal PCR primers, one of which is biotinylated. Biotinylated amplicons may be rendered single stranded and captured on streptavidin-coated plates. Each single-stranded PCR product may be identified by its unique zip-code sequence through interrogation with a set of universal ZipChute probes. These probes have fluorescence labels, unique complementary zip-code sequences, and ABI mobility modifiers. ZipChute probes can be eluted and electrophoretically separated on an ABI 3730xl DNA analyzer. **Figure 4** shows an example of a 60-plex reaction using this approach. This technique has been validated on 3,000 SNPs using 96 genomic DNA samples. Compared with other genotyping platforms, the SNPLEX system demonstrates 98.7 and 99.2% concordance with dideoxy sequencing and TaqMan assays, respectively. This variation is an alternative approach to existing genotyping methodologies and has the advantage of a robust detection strategy and low DNA consumption.

3.3.2. Bisulfite/PCR-PCR/LDR/Universal Microarray

One application of LDR/PCR/Universal Array is to study DNA methylation. In particular, this variation focuses on the detection of aberrant promoter methylation occurring at the 5-position of cytosine within the CpG dinucleotide. Sodium bisulfite conversion of cytosines to uracils is one of the most commonly used methods to study DNA methylation. 5-Methylcytosines are resistant to conversion, and deamination only occurs on unmethylated cytosines. The modified DNA sequences can then either be amplified and sequenced, or one can perform methylation-specific PCR (MSP) to determine cytosine methylation status. Our bisulfite/PCR-PCR/LDR/Universal Array approach provides a sensitive and accurate high-throughput format that can detect methylation status in virtually any gene sequence of interest.

A multiplex PCR-PCR/LDR assay is shown in **Fig. 7**; (see Color Plate 14 following p. 18) to illustrate this approach. When possible, the gene-specific PCR primers used for multiplexing are designed to avoid CpG sites present in the promoter sequences. As a further improvement to accommodate situations in which bisulfite-modified bases cannot be avoided, pyrimidine and purine nucleotide

Bisulfite/PCR-PCR/LDR/Universal Array

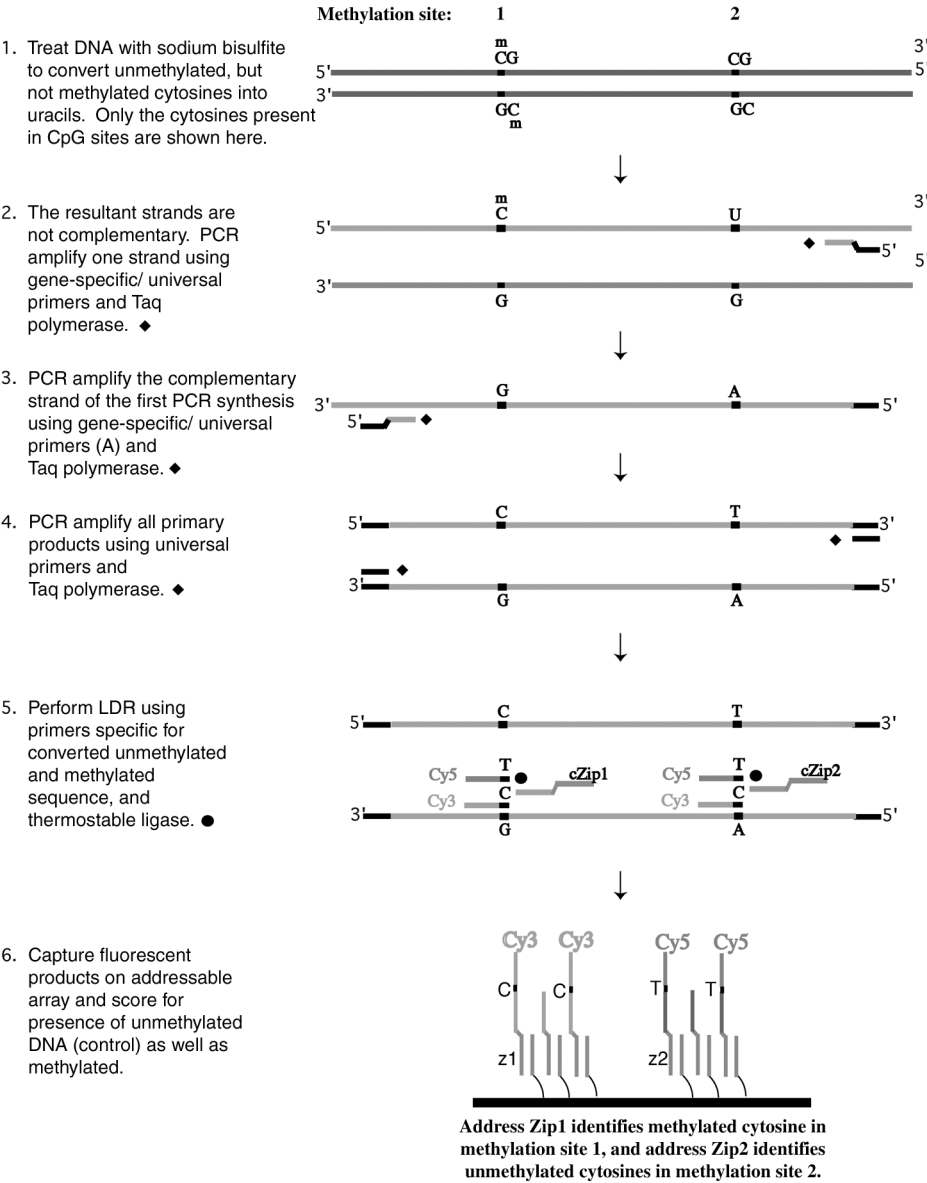


Fig. 7. (Color Plate 14 following p. 18) Schematic diagram, illustrating the procedure for high-throughput detection of promoter methylation status with the combination of bisulfite treatment, multiplex PCR, multiplex LDR, and universal array approaches. The different fluorescently labeled (Cy3 and Cy5) LDR products are captured on the same addressable array. PCR, polymerase chain reaction; LDR, ligase detection reaction.

analogs are incorporated within the PCR primers. These modified bases, designated P and K, show considerable promise as degenerate bases. The pyrimidine derivative P base pairs with either A or G, whereas the purine derivative K base pairs with either C or T; thus the target DNA can be amplified regardless of its methylation status. Multiple promoter regions are amplified in a two-stage nested PCR reaction simultaneously. The first-stage multiplex amplification uses pairs of gene-specific PCR primers with universal sequences attached to their 5' ends. The second-stage amplification uses universal PCR primers to amplify the first-stage PCR products. The final PCR products are usually verified on an agarose gel prior to LDR analysis.

The details of LDR primer design have been described in **Subheading 3.** and in **Note 1.** Briefly, three LDR primers are designed for each CpG dinucleotide site. Two discriminating primers are labeled at the 5' end with either Cy3 or Cy5 and at the 3' end with a G or A, respectively. The single common primer for the reaction consists of a 5' phosphate and terminates at the 3' end with a zip-code complement sequence. Degeneracy is also accommodated in the LDR primers by using pyrimidine and purine nucleotide analogs. After the LDR products are captured on a universal array, the methylated cytosine residues are detected by the presence of Cy3 signals; the presence of unmethylated cytosines is revealed by the presence of Cy5 signals.

Typically, 1–2 μg of genomic DNA in a volume of 40 μL is incubated with 0.2 *N* NaOH at 37°C for 10 min. Then 30 μL freshly made 10 *mM* hydroquinone and 520 μL of freshly made 3 *M* sodium bisulfite, pH 5.0 (Sigma, ACS grade) is added. This mixture is next incubated for 16 h in a DNA thermocycler using alternating cycles of 50°C for 20 min followed by a denaturing step of 85°C for 15 s. The bisulfite-treated DNAs can be desalted using MICROCON centrifugal filter devices (Millipore, Bedford, MA) or, alternatively, cleaned with a Wizard DNA clean-up kit (Promega, Madison, WI). The eluted DNAs are incubated with 1/10 volume of 3 *N* NaOH at room temperature for 5 min prior to ethanol precipitation. The DNA pellet is then resuspended in 20 μL deionized H_2O and stored at 4°C. Bisulfite-modified DNA is stable at 4°C for at least 1 mo.

The current assay is designed to detect the extent of DNA methylation within the promoters of the tumor suppressor genes *p15^{INK4b}*, *p16^{INK4a}*, *p19^{ARF}*, *p21^{CIP}*, *p27^{KIP}*, *p53*, and *BRCA1*, as well as the imprinted gene small nuclear ribonucleoprotein N (*SNRPN*). Using the same design parameters, the promoter regions of seven additional genes were chosen to investigate their promoter methylation status in human tumors. These include O6 methyl guanine DNA methyl transferase (*MGMT*), adenomatous polyposis coli (*APC*), retinoic acid receptor (*RAR β*), tissue inhibitor metalloproteinase (*TIMP-3*), death-associated protein kinase (*DAPK*), E-cadherin (*ECAD*), glutathione S-transferase (*GSTP1*),

and Ras association domain family 1 (*RASSF1*). The hemimethylated SNRPN is used as a positive internal control.

As seen in **Fig. 8** (see Color Plate 15 following p. 178), to demonstrate that LDR primers are working properly, genomic DNAs of normal lymphocytes with and without in vitro methylation are included in experiments as controls. DNA extracted from colorectal cancer cell lines SW1116 and DLD1 is also used to validate this strategy. All experiments were performed minimally in duplicate to avoid ambiguity. For each promoter region, three CpG sites were chosen to analyze their methylation status. The presence of Cy5 signals indicates efficient amplification during multiplexing PCR steps. The promoter regions will be considered to be hypermethylated only when at least two CpG sites can be detected by Cy3 labeling. In most cases, the universal arrays provide very high capture specificity. The methylated promoters identified in this method may be reconfirmed by either bisulfite sequencing or uniplex PCR/PCR/LDR under more stringent hybridization conditions on a fresh array in a separate experiment.

In contrast to MSP-based methods, the bisulfite/PCR/LDR approach circumvents the issues of incomplete bisulfite conversion (C to U modification is not 100% efficient) and the potential primer extension of unmethylated DNA by extension of a G:U mismatch. The requirement of scoring methylation at three CpG sites per promoter using LDR should help the assay retain its exquisite specificity.

4. Notes

1. We recommend the software program Oligo for LDR primer design. This program is also useful in designing PCR and multiplex PCR primers. This program calculates T_m using the nearest neighbor method. Gene-specific PCR and LDR primers are generally designed with T_m s around 70°C. To perform multiplex PCR/PCR, gene-specific oligonucleotide primers with universal primer sequence attached to the 5' ends are required. The sequence of the universal primer is 5'-ggagcagctatc-cggttagac-3'. LDR discriminating primers are labeled at the 5' end with fluorophores that can be detected by the array-scanning instrument and that have sufficient spectral separation to avoid confounding owing to overlapping signals. The final base on the discriminating primer is the query base. The LDR common primer is modified with a 5' phosphate and the 24-base zip-code complement is appended to the 3' end (see **Table 1** for sequences). When synthesized on a 1- μ mol scale or larger, zip-code oligonucleotides used for spotting arrays should be gel purified. When synthesized on a smaller scale, a reversed-phase, solid-phase extraction column produces satisfactory results. The zip-code oligonucleotides are synthesized on a 3' amino modifier C3 column (Glen Research) with a spacer C18 (Glen Research) inserted before the first base.

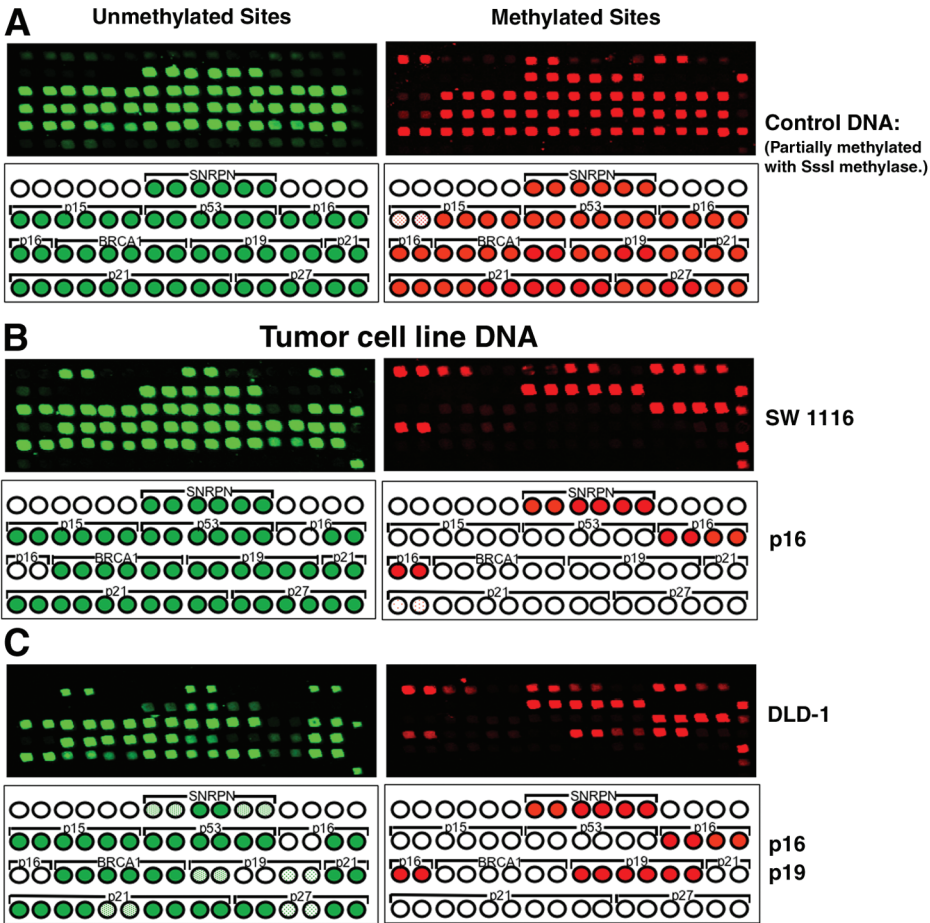


Fig. 8. (Color Plate 15 following p.178) Universal array images of methylation profiles of selected promoter regions (SNRPN, p15, p16, p19, p21, p27, p53, and BRCA1) in normal and colorectal tumor cell line genomic DNAs. False color green represents the status of unmethylated promoter regions detected by Cy5-labeled LDR primers. False color red represents the status of methylated promoter regions detected by Cy3-labeled LDR primers. **(A)** LDR results of normal human lymphocyte genomic DNAs in the presence (right panel) and absence (left panel) of in vitro methylation using SssI methylase. **(B,C)** The methylation profiles of two colorectal cancer cell line genomic DNAs were analyzed. Among the eight genes that were analyzed in cell line SW1116, Cy3 labeled LDR products only present on the p16 promoter region. This indicates that only the p16 promoter was hypermethylated. The presence of Cy3 signal on both p16 and p19 promoters in cell line DLD-1 indicates that both of these promoters are hypermethylated.

Table 1
Sequences of Zip-Code-Related Oligonucleotides

Zip-code	Zip-code complement
TTGAAATCCAGCGCAAAATCTGCG	CGCAGATTTTGCCTGGATTTCAA
TTGAAAAGCCTACACGACGGCGAA	TTCCCGTCGTGTAGGCTTTTCAA
TTGATCTGCCATACGGGCTTACGG	CCGTAAGCCCGTATGGCAGATCAA
TTGACTTGTCCTCCAGCACGGCCAT	ATGGCCGTGCTGGGGACAAGTCAA
TTGACGTTGACCAGCCCGTTGCAA	TTGCAACGGGCTGGTCAACGTCAA
TTGACGAAGCTTTCCCCCATGATG	CATCATGGGGGAAAGCTTCGTCAA
TTGAGCAAGGACGACCGCAAACGG	CCGTTTGCCTGCTCCTTGCTCAA
TTGAGATGACGGACGGTGCGGCAA	TTGCCGCACCGTCCGTCATCTCAA
TTGATCCCATCGAAAGGGACGATG	CATCGTCCCTTTTCGATGGGATCAA
TTGATGCGTCTGGGACGTGCCTTG	CAAGGCACGTCCCAGACGCATCAA
TTGACACGTGCTCAGCTCCCGTGC	GCACGGGAGCTGACGACGTGTCAA
TTGACAGCCTGTTGCGGTGCGTCT	AGACGCACCGCAACAGGCTGTCAA
TTGAGTGCGGTACTTGCAGCGATG	CATCGCTGCAAGTACCGCACTCAA
TTGAACGGTCTGCACGTCCCAGCC	GGCTGGGACGTGCAGACCGTTCAA
TGATTCTGGTGCGTGCCAGCCAGC	GCTGGCTGGCAGCACCAGAATCA
TGATTGTCGCTTTCTGACGGAGCC	GGCTCCGTGAGAAAGCGACAATCA
TGATCGTTTGCGGGTATCCCTCGT	ACGAGGGATACCCGCAAACGATCA
TGATCGAAAGGACAGCAGCCTCCC	GGGAGGCTGCTGTCCTTTTCGATCA
TGATGCAAGCAACGAACACGCTGT	ACAGCGTGTTTCGTTGCTTGATCA
TGATTGCGAGTGGAACATCGCCAT	ATGGCGATGGTCCACTCGCAATCA
TGATCACGCTTGCCATGGACGGAC	GTCCGTCCATGGCAAGCGTGATCA
TGATGTGCCTCAACGGGTGCAGCC	GGCTGCACCCGTTGAGGCACATCA
TGATGGACCGTTAGCCGATGTTGA	TCAACATCGGCTAACGGTCCATCA
TGATACGGAGGAGGACTGCGTGCG	CGCACGCAGTCCCTCCTCCGTATCA
TTAGGATGAGCCAGCCTGCGAGCC	GGCTCGCAGGCTGGCTCATCTTAA
AATCTCGTCGTTTCCCCTCATGCG	CGCATGAGGGGAAACGACGAGATT
AATCGCAACTGTCGTTACGGTGC	GCACCGTGAACGACAGTTGCGATT
AATCAGGACACGCAGCGACCTGCG	CGCAGGTGCTGCGTGTCTGATT
AATCGACCCTGTGTCTGCTTTGCG	CGCAAAGCAGACACAGGGTCGATT
AATCAGCCAAAGCGAAGTGCGATG	CATCGCACTTCGCTTTGGCTGATT
ATACGACCTCGTGAGTTCCCGCAA	TTGCGGGAACACAGGTCGTAT
AAAGCTTGACCTATCGAGCCGTGC	GCACGGCTCGATAGGTCAAGCTTT
AAAGAGCCGCTTGAGTCGAAATCG	CGATTTGACTCAAGCGGCTCTTT
TCTGCTTGCTCACCTACCATTGCG	CGCAATGGTAGGTGAGCAAGCAGA
TCTGATCGCTAGGTAACGGGGAC	GTCCCCGTTACCTAGGCGATCAGA
TCTGCAGCGGTACTGTGGACCCAT	ATGGGTCCACAGTACCGCTGCAGA
TCTGAGCCACCTAATCTCCACGG	CCGTGGGAGATTAGGTGGCTCAGA
TGTCTCGTTCCACCTCCATTCCC	GGGAATGGAGGTGGGAACGAGACA
TGTCATCGCAGCGAGTCAGCCACG	CGTGGCTGACTCGCTGCGATGACA

(Continued)

Table 1
(Continued)

Zip-code	Zip-code complement
TGTCCCTAACCTGATGGTGC GCAA	TTGCGCACCATCAGGTTAGGGACA
TGTCTGCGGTCTCCATATCGGTGC	GCACCGATATGGAGACCGCAGACA
TGTCCACGCGTTACCTTGTCGATG	CATCGACAAGGTAACGCGTGGACA
TGTCGTGCTCTGACCTTGCGCTCA	TGAGCGCAAGGTCAGAGCACGACA
TGTCACGGAATCGTGCTGCGGCTT	AAGCCGCAGCACGATTCCGTGACA
TCGTTCTGGCTTGACGCTTCTCA	TGAGAAGCGTCCAAGCCAGAACGA
TCGTTGCGTGTCGGACCTTG GATG	CATCCAAGGTCCGACACGCAACGA
CTTGCGTTGATGCGAATCGTCGAA	TTCGACGATTTCGCATCAACGCAAG
CTGTACGCTCAACCTTCCCCGTT	AACGGGGAAGGTTGAGCGTGACAG
CTGTGTGCCGTTTCGTGTGCAGTG	CACTGCACACGAAACGGCACACAG
CCATGCAATCCCAGGATGTCGGTA	TACCGACATCCTGGGATTGCATGG
CCATCAGCTCTGGCAATGCGGAGT	ACTCCGCATTGCCAGAGCTGATGG
CGAATCTGGGTAAGGAAGCCATCG	CGATGGCTTCCTTACCCAGATTTCG
CGAATGTCCTGTCCATCGAATGCG	CGCATTCGATGGACAGGACATTTCG
CGAACGTTTACATGCGTCGTAGCC	GGCTACGACGCATGTAAACGTTTCG
CGAAAGTGAGCCGCAACTTGGGAC	GTCCCAAGTTGCGGCTCACTTTCG
CGAAGGACAGTGAGTGTGCGCACG	CGTGCGCACACTCACTGTCCTTCG
GCAAAGCCATACCTTGGCTTGCTT	AAGCAAGCCAAGGTATGGCTTTGC
ACCTCTTGCCCTACGAACAGCCGAA	TTCGGCTGTTTCGTAGGCAAGAGGT
ACCTGCAAGTGCCCATGTGCCCTA	TAGGGCACATGGGCACCTTGCAGGT
AGGATCTGGACCGGACTCCCCGAA	TTCGGGGAGTCCGGTCCAGATCCT
AGGAACGGCAGCTACACACGAGCC	GGCTCGTGTGTAGCTGCCGTTCTT
GATGGACCACCTCAGCGCTTGACC	GGTCAAGCGCTGAGGTGGTCCATC
TCCCTTAGGACCCAGCGTCTGTGC	GCACAGACGCTGGGTCTTAAGGGA
TGCGCCATAAAGGACCTTAGCCAT	ATGGCTAAGGTCCTTTATGGCGCA
TGCGAGGACCATGGTAGGTAAGCC	GGCTTACCTACCATGGTCCCTCGCA

2. Use only sealed boxes of slides. Opened boxes do not clean well, probably because of to either heavy oxidation or reaction with chemicals present in the lab.
3. Each dish holds two glass slide racks.
4. We use slides with two etched circles when making arrays. A single array is spotted within each circle.
5. This process can be repeated for a second set of slides with the existing solutions before the reagents boil out and too much liquid evaporates. Use fresh solutions for the next rounds.
6. Silanize as soon as possible or leave in water overnight (rinse #1, **step 7**) if you cannot silanize immediately.
7. Do not touch the surface of slides after cleaning; handle slides by frosted end only.

8. If you are preparing a large number of slides, the second wash of one batch can be used as the first wash for the next batch of slides.
9. The silanized slides are stable for several weeks prior to coating with the polymer. For long-term storage, place in a desiccator.
10. Allowing the solution to sit produces more uniform surfaces.
11. If slides were cleaned and silanized properly, the solution should form a relatively small, well-defined droplet on the surface. Do not worry about small voids or bubbles at the edges of the cover slip; you will not be using that area of the polymer.
12. Using two to four blocks, you can heat 8–16 slides simultaneously if you stagger them by 20 s. The two outer slides on the blocks will hang off the edges slightly, but this does not effect the polymerization.
13. The slides are stable for at least 6 mo prior to activation.
14. The activated slides are stable for a minimum of 6 mo if stored properly desiccated.
15. Plates can be sealed and stored refrigerated for spotting, on consecutive days. Prior to spotting, spin the plates to collect all the liquid in the bottom of the wells. For long-term storage, dry the spotting plate, seal, and store at -20°C . The day before spotting, redissolve the wells in the appropriate amount of water (assume $\sim 0.5\ \mu\text{L}$ loss in volume/spotting run), vortex the plate several times, and store refrigerated. The day of spotting, vortex several times, and then spin the plate to collect all the liquid in the bottom of the wells.
16. The slides are very hygroscopic when dry. If not allowed to partially rehydrate prior to spotting, the first few slides will suck all the spotting solution out of the pin.
17. We normally spot each zip-code in duplicate. Additionally, we spot a set of fiducials along the top and one side of the array (*see* bottom panel of **Fig. 2**) for alignment purposes. The fiducials are made on a DNA synthesizer and have the following structure: amino-group-T-T-fluorescent dye. The fiducials should be printed after the zip-code oligonucleotides because the dyes are sticky, and carryover contamination can be a problem. The pins should be sonicated thoroughly after spotting of the fiducials to prevent contaminating the next spotting run with fluorescent dye.
18. This can be done immediately following spotting or any time just prior to hybridization.
19. The spotted slides are stable for a minimum of 6 mo.
20. The LDR can remain in the original reaction tube/plate, and the 2X buffer can be added through the oil. Following denaturation, the tubes/plate should not need to be spun down, and the hybridization mix can be drawn out from under the oil.
21. There are cover wells available in a variety of sizes for different size arrays. The important thing is not to fill the chamber completely so there is an air bubble present to facilitate mixing.
22. Avoid excessive liquid in the sponge, since this may create unnecessary moisture and result in a capillary stream between the adjacent hybridization chambers under cover well gaskets that have not been securely sealed against the array surface. This cross-contamination issue can easily be solved by just slightly dampening the sponge, that is, make the sponge damp but without the ability to squeeze out any liquid.

Acknowledgments

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