# Signal amplification by rolling circle amplification on DNA microarrays

**Girish Nallur*, Chenghua Luo, Linhua Fang, Stephanie Cooley, Varshal Dave, Jeremy Lambert, Kari Kukanskis, Stephen Kingsmore, Roger Lasken and Barry Schweitzer**

Molecular Staging Inc., 300 George Street, New Haven, CT 06511, USA

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## ABSTRACT

**While microarrays hold considerable promise in large-scale biology on account of their massively parallel analytical nature, there is a need for compatible signal amplification procedures to increase sensitivity without loss of multiplexing. Rolling circle amplification (RCA) is a molecular amplification method with the unique property of product localization. This report describes the application of RCA signal amplification for multiplexed, direct detection and quantitation of nucleic acid targets on planar glass and gel-coated microarrays. As few as 150 molecules bound to the surface of microarrays can be detected using RCA. Because of the linear kinetics of RCA, nucleic acid target molecules may be measured with a dynamic range of four orders of magnitude. Consequently, RCA is a promising technology for the direct measurement of nucleic acids on microarrays without the need for a potentially biasing preamplification step.**

## INTRODUCTION

The rapidity, cost effectiveness and massively parallel nature of microarray assays make them the method of choice for translating the vast knowledge base created by genome sequencing into a set of miniaturized assays for clinical applications. The full potential of microarrays, however, will be realized only when rare targets can be detected simultaneously with more abundant ones in a single assay. Sensitivity for detection of rare targets has been a serious drawback in the utility of current microarray formats. Consequently, solution phase preamplification approaches such as PCR (1) or T7 polymerase amplification (2) are routinely employed in conjunction with microarray analysis. However, these amplification methods are of limited applicability in nucleic acid detection strategies involving a high degree of multiplexing, such as microarrays, because the products of the amplification from each assay intermingle and interfere with each other, resulting in loss of amplification efficiency and specificity (3,4). In addition, off-chip target amplification strategies greatly increase the cost of the procedures and can lead to sequence-dependent quantitation bias. In contrast, on-chip signal amplification methods have the potential to provide the necessary sensitivity and specificity, while retaining spatial multiplexing.

Rolling circle amplification (RCA) is a technology that is adaptable to an on-chip signal amplification format. In RCA, a circle of DNA, a short DNA primer (complementary to a portion of the circle) and an enzyme catalyst converts dNTPs into a single-stranded concatameric DNA molecule that is composed of thousands of tandemly repeated copies of the circle (Fig. 1). Unlike other amplification procedures, RCA produces a single amplified product that remains linked to the DNA primer. Consequently, RCA is well suited to solid phase formats such as microarrays for generating localized signals at specific microarray locations. This distinctive property of RCA should allow many assays to be performed simultaneously (multiplexing) without interference. In contrast, PCR or other solution phase amplification procedures cannot be configured for on-chip amplification due to at least one of the following: (i) lack of accumulation of amplified signal at the site of amplification, i.e. diffusion of products into the solution; (ii) deleterious effects of temperature cycling on reaction components, such as analytes, sample or microarray substrates. Being an isothermal process, RCA overcomes the need for costly and cumbersome equipment for temperature cycling. Applications of RCA signal amplification to detection of DNA targets on solid surfaces as well as for ultrasensitive detection of proteins on microarrays (5) have been previously demonstrated.

This report describes the application of RCA signal amplification to the sensitive detection of nucleic acids on microarrays. We demonstrate the flexibility of RCA as a universal detection/amplification module for integration with a variety of microarray substrates and nucleic acid detection formats. We also show the ability of RCA to provide a large increase in sensitivity when employed as a signal amplifier in combination with contemporary methods for nucleic acid detection and allele discrimination.

## MATERIALS AND METHODS

### Oligonucleotides

The oligonucleotides were either synthesized in-house or purchased from Integrated DNA Technologies (Coralville, IA) and purified by HPLC. Unless otherwise mentioned, oligonucleotides were dissolved in water and stored frozen at –20°C.

*To whom correspondence should be addressed. Tel: +1 203 772 5018; Fax; +1 203 776 5278; Email: girishn@molecularstaging.com
Present addresses:
Varshal Dave, Axon Instruments, Arlington, TX 76012, USA
Jeremy Lambert, HTS BioSystems, Hopkinton, MA 01748, USA

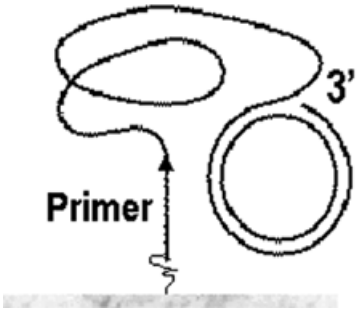



**Figure 1.** Schematic of L-RCA™ on microarrays. The RCA primer is immobilized from its 5′-end and contains a free 3′-end to which the circle hybridizes. The linear concatameric RCA product, comprising repeating units of a sequence complementary to the circle sequence, is detected by hybridization of short oligonucleotides containing fluorescent labels.

Cy5- and Cy3-labeled deoxyribonucleotides were purchased from Amersham Pharmacia Biotech (Piscataway, NJ).

### RCA in solution

Reactions contained 10 U ϕ29 DNA polymerase, 2.5 fmol 80 nt circular template (circle 1, 200 fmol as nucleotides) (5′-phos-CTCAGCTGTGTAACAACATGAAGATTGTAGGTCAGA-ACTCACCTGTTAGAAACTGTGAAGATCGCTTATTAT-GTCCTATC-3′), 50 fmol 26 nt primer 1 [5′-$(A)_{50}$-CAGCT-GAGGATAGGACATAATAAGC-3′], 1 mM each dATP, dTTP and dGTP and 1 mM [$^{32}$P]dCTP (150–300 c.p.m./pmol) in 25 µl of reaction buffer containing 50 mM Tris–HCl pH 7.9, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 0.2 mg/ml BSA. For DNA synthesis determination, aliquots were removed at individual time points and synthesis quantified as acid-precipitable DNA. For decoration with probe, RCA products were quenched with EDTA, mixed with 5′-$^{32}$P-labeled decorator probes and incubated at 37°C for 1 h before they were analyzed by electrophoresis on 1% neutral agarose gels. The $^{32}$P-labeled RCA product:decorator duplexes were quantified using a Storm PhosphorImager and ImageQuant software (Amersham Pharmacia Biotech, Piscataway, NJ).

### Microarray production

Microarrays were printed on streptavidin-coated glass microscope slides (Xenopore Corp., Hawthorne, NJ) using the OmniGrid microarraying robot (GeneMachines, San Carlos, CA). For printing, oligonucleotides were dissolved at the appropriate concentrations in 1× phosphate-buffered saline (PBS) containing 0.05 mg/ml BSA. Their sequences were: primer 1, 5′-biotin-$(A)_{50}$-ACACAGCTGAGGATAGGACATAATAAGC-3′; primer 2, 5′-biotin-$(A)_{50}$-TGTCCGTGCTAGAAGGAAACAGTTACA-3′. After printing, the slides were blocked by incubation with biotinylated BSA (1 mg/ml in 1× PBS; Pierce, Rockford, IL) for 1 h at 37°C and washed twice for 2 min in 1× PBS, 0.05% Tween-20. Microarrays on 3D-Link slides (SurModics, Eden Prairie, MN) were produced by depositing oligonucleotides with a terminal amino group dissolved in 300 mM sodium phosphate buffer, pH 8.5. Microarrays were produced as described for the streptavidin slides, allowed to incubate on the arrayer platform at 80% humidity for 16 h and blocked as recommended by the manufacturer.

Gel-coated microarrays were prepared as described (4) with several modifications. The gel mix consisted of 3% acrylamide, 0.15% bisacrylamide, 0.75% polyethyleneglycol (200) diacrylate (PEG-200), 0.0002% methylene blue, 40% glycerol and 1% *N*,*N*,*N*′,*N*′-tetramethylethylenediamine in PBS, pH 7.2. Aliquots of 5 µl of the gel mix were placed on the surface of glass slides (Fisher Scientific) coated with (3-acryloxypropyl)trimethoxysilane and the drop was covered with a glass coverslip (13 mm diameter). Slides were placed on a UV transilluminator, coverslip side up, and irradiated for 30 min at 8000 µW/cm$^2$. After polymerization, the coverslip was removed, the gel-coated arrays were washed with several changes of distilled water to remove unpolymerized acrylamide monomers and dried by placing on a hot plate at 55°C. Prior to depositing oligonucleotides, gel-coated arrays were activated by immersion in 25% glutaraldehyde (in distilled $H_2O$) for 12 h at 37°C. Samples of 0.2 µl of a solution (in distilled $H_2O$) of oligonucleotides containing a terminal $NH_2$ group were hand spotted onto the gel surfaces. Immobilization was performed by incubation at 37°C for 2 h in a humid chamber. Gel-coated arrays were deactivated in 0.1 M $NaBH_3CN$ (Sigma, St Louis, MO) for 2 h at 37°C to reduce free aldehydes, then washed twice in water for 2 min and dried on a 55°C hotplate. Microarrays were stored at room temperature under vacuum prior to use.

### RCA on microarrays

Twenty microliters of a solution containing 200 nM circle 1 or circle 2 (TAGCACGGACATATATGATGGTACCGCAGT-ATGAGTATCTCCTATCACTACTAAGTGGAAGAAATG-TAACTGTTTCCTTC) in 1× PBS, 0.05% Tween-20 was annealed to the arrays at 37°C for 30 min and excess circle removed by washing as described. RCA was performed at 37°C in a volume of 20 µl/array containing 10 U ϕ29 DNA polymerase (generously provided by Amersham Pharmacia Biotech), 1 mM each dNTP, 50 mM Tris–HCl, pH 7.9, 10 mM $MgCl_2$, 20 mM $(NH_4)SO_4$ and 0.2 mg/ml BSA. Slides were washed twice for 2 min in 2× SSC, 0.05% Tween-20. An aliquot of 20 µl of 0.5 µM decorator probe (Pr1 decorator, 5′-Cy3-GCT-TATTATGTCCTATCCTCAGCTG-3′; Pr2 decorator, 5′-Cy3-TGTAACTGTTTCCTTCTAGCACGGACA-3′) in 2× SSC, 0.05% Tween-20 was hybridized to the RCA products on the slides for 30 min at 37°C. Slides were washed twice for 2 min in 2× SSC, 0.05% Tween-20, rinsed for 30 s in 1× SSC, dried by centrifugation and analyzed by scanning in a laser scanner. The RCA reaction mix for T7 DNA polymerase consisted of 0.5× sequenase reaction buffer, 1 mM each dATP, dCTP, dGTP and dTTP, 0.0125 U/µl T7 native polymerase (US Biochemical Corp., Cleveland, OH), 30 µg/ml *Escherichia coli* single-strand-binding protein (SSB) (Promega, Madison, WI), 50 nM each decorator oligo and 8% DMSO. For gel-coated microarray experiments, RCA template circle hybridization was performed for 2 h at 37°C. Slides were washed in 20 mM Tris–HCl, 25 mM NaCl, 10 mM $MgCl_2$, pH 8.0, for 5 min at room temperature and dried by centrifugation. Gel-coated arrays were incubated with 100 µl of RCA reaction mixture for 4 h at 37°C under a coverslip. Following RCA, slides were washed for 5 min in 2× SSC. Decorator probes were hybridized as described, except that 0.1 mg/ml herring sperm DNA was included in the hybridization reaction and the incubation at 37°C was for 2 h. The slides were then washed with 2× SSC,

0.05% Tween-20 twice for 2 min, rinsed in 2× SSC for 2 min and dried at 55°C before scanning.

### L²RCA on microarrays

The reaction mix for L²RCA contained, in 20 µl, 0.5× Sequenase buffer (US Biochemical), 1 mM each dNTP, 1 µM Cy5-labeled P2 primer, 100 µM Cy5-labeled dUTP, 0.2 mg/ml SSB and 0.5 U/µl native T7 DNA polymerase. Microarrays hybridized with RCA circles as mentioned before were incubated in the reaction mix for 2 h at 37°C. The slides were then washed with 2× SSC, 0.05% Tween-20 twice for 2 min, rinsed in 2× SSC for 2 min and dried at 55°C before scanning. Quantitation was performed using the GenePix quantitation software.

### Allele discrimination using on-chip ligation

Microarrays containing immobilized P1 ligation oligonucleotides were incubated with 8 µl of a solution containing 200 nM P2 (one each for the wild-type and mutant alleles), 2× SSC, 0.1% Triton X-100 and target at 55°C for 2 h in a humidified chamber. The slides were washed in 2× SSC, 0.05% Tween-20 at room temperature for 2 min, then in 2× TE for 1 min at room temperature and dried. Hybridized microarrays were incubated with 8 µl of ligation mix [1× Ampligase reaction buffer and 1.6 µl Ampligase enzyme (5 U/µl; Epicentre Technologies Corp., Madison, WI)] at 55°C for 30 min, washed twice with agitation in 0.1× SSC, 0.1% Tween-20 at 80°C for 3 min and twice in water at 80°C for 3 min each. After drying, the slides were used for RCA amplification as described above. The sequences of the oligonucleotides used were: G542X P1, 5′-phos-GAGAAGGTGGAATCACACTGAGTGGACGTCAACGA-GCAAAAAAAAAAAAAAAAA-(carbon$_7$)-NH$_2$-3′; G542X wild-type P2, 3′-GTTCTTGATATAACAGAAAGTAAA-(carbon$_{12}$)-AAACATGTTGTTACACAGCTGAGGATAGGA-3′; G542X mutant P2, 3′-TTTCTTGATATAACAGAAAG-TAAA-(carbon$_{12}$)-AAATGTCCGTGCTAGAAGGAAACA-GTTACA-3′; G542X wild-type target, 5′-TTGCTCGTTGAC-GTCCACTCAGTGTGATTCCACCTTCTCCATGAACTAT-ATTGTCTTTCA-3′. The mutant target contained an A nucleotide instead of C at the underlined position.

### Laser scanning of microarrays and image analysis

Microarrays were imaged using a ScanArray 5000 laser scanner (GSI Lumonics, Billerica, MA). Except where mentioned, the instrument was set at 60% laser and 60% photomultiplier tube (PMT). Images were analyzed with the QuantArray Image Analysis software package (GSI Lumonics) using the fixed circle method. Gel-coated microarray images were quantitated using the GenePix Analysis software (Axon Instruments, Union City, CA). Data normalization was performed in the following manner. The RCA signal intensity value for Pr1 (100 nM) spots in a single subarray was arbitrarily set as a reference standard. A normalization factor was derived for each subarray by dividing the intensity of Pr1 RCA spots on the subarray by the intensity of Pr1 spots on the reference subarray. Normalized fluorescence intensities for the spots of interest were then derived by multiplying the fluorescence intensity with the normalization factor for that subarray. For ligation experiments, normalized intensities from 'no target' controls were subtracted prior to normalization.

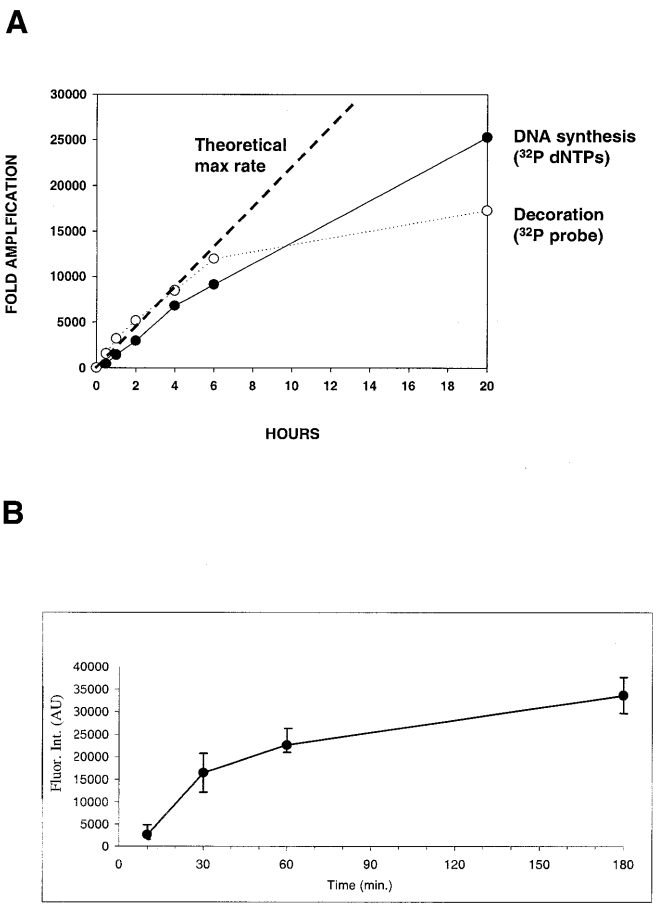



**Figure 2.** Amplification rate for ϕ29 DNA polymerase. (**A**) RCA reaction performed in solution with primer 1:circle 1 duplexes. Total DNA synthesis (filled circles) was measured as acid-precipitable counts. Decoration of RCA product with $^{32}$P-labeled probe (open circles). The dashed line indicates the estimated intensity values assuming a maximal rate of extension by the polymerase. Fold amplification was calculated by dividing either the number of tandem repeats (calculated from total DNA synthesis) or the amount of decorator annealed to RCA products by the input template circle. (**B**) An RCA reaction performed as described in (A) except that the reactions were assembled on glass microarrays printed with primer 1. The RCA product was detected by hybridization with decorator probes and scanning in a laser scanner. Error bars show the averages of three replicate experiments.

## RESULTS

### Comparison of metrics of RCA in solution and on glass microarrays

*RCA in solution.* Extension of an oligonucleotide hybridized to an 80mer synthetic DNA circle by ϕ29 DNA polymerase in the presence of excess dNTPs in solution occurs with linear kinetics over 20 h, achieving 25 000-fold amplification (Fig. 2). ϕ29 DNA polymerase, phage T7 DNA polymerase and Sequenase are all DNA polymerases that are well suited for such RCA (3). Favored properties include moderate temperature optima (30–37°C) and good processivity. However, ϕ29 DNA polymerase out-performs the others by virtue of exceptional processivity and good strand displacement synthesis. The replication rate of ϕ29 polymerase in this system is 50 nt/s, generating ~1000 copies of the circle in 30 min (data not shown). Phage T7 DNA polymerase and

Sequenase have a higher initial rate of synthesis (300 nt/s) (6), however, the rate for native T7 DNA polymerase and Sequenase decreases with time and ultimately the yield is ~10-fold less than for ϕ29 (data not shown).

In solution, the efficiency with which short oligonucleotide probes hybridize to the RCA product was determined by running an RCA reaction in the presence of a $^{32}$P-labeled probe and quantifying the amount of probe associated with high molecular weight DNA. In parallel experiments, the total DNA synthesized during RCA in solution was measured by incorporation of $^{32}$P-labeled dNTP. Comparison of total DNA synthesis and moles of decorator probe demonstrated near 100% efficiency in probe annealing to each tandem copy synthesized (Fig. 2A). Synthetic circles used in RCA reactions for signal amplification on microarrays are designed with one to three probe-binding sites. Typical 'decorator' probes for these sites are oligonucleotides with one or two fluorescent tags.

*RCA on glass microarrays.* On a glass microarray, in contrast, using a tethered oligonucleotide, the same synthetic circle DNA, ϕ29 DNA polymerase and dNTPs, RCA occurs with linear kinetics for 3 h, achieving 8125-fold amplification, and then plateaus (Figs 1, 2B and 3C). For practical microarray experiments, the signal generation efficiency of RCA using a tethered oligonucleotide is a function of the amount of primer present per spot, the efficiency of circle primer duplex formation and polymerase initiation frequency. Each of these steps was studied individually.

The accessibility of tethered RCA primers was determined by annealing to an excess of a fluorophore-labeled complementary probe and measuring the fluorescence intensities of hybridization spots by scanning the slide in a ScanArray 5000 laser scanner with the following settings: laser 70; PMT 60 (Fig. 3A). The limit of sensitivity of detection of immobilized primers by hybridization under these conditions was 7.8 nM at a signal-to-noise ratio of 2 (Fig. 3B).

The efficiency of hybridization of immobilized primers to 80mer DNA circles was measured by comparing signal from hybridization of a linear probe to the immobilized primer with the signal from hybridization of a linear probe to the annealed circle (Fig. 3A). By this measure, circle hybridization efficiency was at least 75% of the linear probe hybridization efficiency for all concentrations of immobilized primer (Fig. 3B).

The priming efficiency of a circle hybridized to an immobilized primer was examined by comparing the hybridization signal with the signal from single base extension by Sequenase (Fig. 3A). Sequenase was used because of its ability to incorporate dideoxy nucleotides as well as a lack of 3′-exonuclease activity. These experiments demonstrated that oligonucleotides immobilized on planar microarrays were accessible to hybridization with linear or circular probes and that ~90% of such hybridized circles are replicated by the polymerase (Fig. 3B).

The following are optimal structural features of an RCA primer: a 5′-terminal surface attachment group (e.g. $NH_2^-$ or biotin), a linker arm of 6 or 12 carbon residues, a spacer of 50 adenosine nucleotides and a 23–28 nt 3′-terminal sequence complementary to the RCA circle. Interestingly, the same primers with spacers of 15 thymidine nucleotides performed poorly in RCA. The limit of sensitivity for such surface-immobilized primers by RCA signal amplification on microarrays was determined by serial dilution studies. The RCA product was detected by hybridizing 'decorator' oligonucleotides complementary to the RCA product and determining the fluorescence intensity at each spot. The minimum concentration of spotted primer that could be detected at a signal-to-noise of 2 was 480 fM, representing detection of 150 molecules (Fig. 3C). The RCA signal was specific to the primer–circle pair, since control primers (of unrelated sequence) located on the same microarrays showed little or no RCA signal (not shown). The sensitivity achieved by hybridization of a fluorescently labeled probe to surface-bound primers in the absence of RCA was only 3.9 nM, indicating ~8125-fold signal amplification by RCA (Fig. 3C). Reproducibility of fluorescence signal intensities, an indicator of precision, was at least 90%.

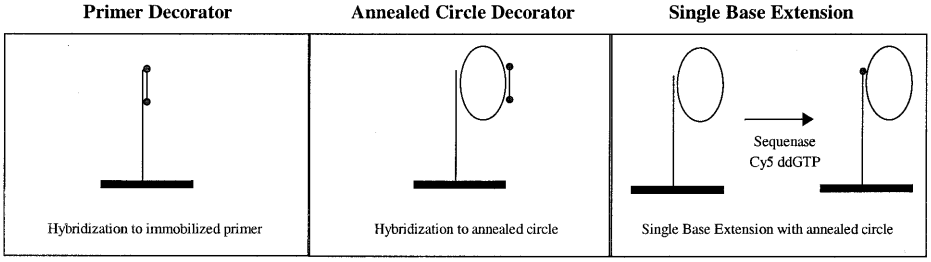
## RCA on porous substrates

Although planar glass is currently the most widely used substrate for microarrays, planar surfaces coated with porous polyacrylamide (gel-coated arrays) have been shown to immobilize larger amounts of oligonucleotides in applications such as sequencing by hybridization (7). Gel-coated arrays are compatible with several enzyme reactions and immunoassays (8). Of several acrylamide gel-coated array formulations that we examined, those polymerized with PEG-200 were found to provide superior oligonucleotide primer immobilization; in addition, these gel-coated arrays provided optimal accessibility for circle hybridization to immobilized primers (data not shown). The limit of detection sensitivity for primers immobilized in the gel-coated arrays following RCA signal amplification was determined as described previously for the planar glass microarrays. ϕ29 DNA polymerase gave a robust RCA reaction on PEG-200 gel-coated arrays, producing signal intensities that were up to 1000-fold greater than with hybridization at the same primer spots (Fig. 4). Similarly, the increase in detection sensitivity by RCA with ϕ29 was 1000-fold greater than with hybridization. In contrast, RCA reactions using *Bst* DNA polymerase, T7 native DNA polymerase and Sequenase were only 5–10-fold greater than hybridization alone. Equilibration of gel-coated arrays with these polymerases prior to RCA failed to produce increased signal (data not shown). Furthermore, gel-coated arrays with lower porosity, such as those polymerized using bisacrylamide as a crosslinker, were poor substrates for carrying out RCA using all of the enzymes, including ϕ29 (not shown). Thus, achievement of a strong RCA signal on porous array substrates is dependent upon an appropriate combination of gel pad chemistry and enzyme.

## L$^2$RCA on microarrays

Although the linear form of RCA enables signal amplification of 3–4 log on microarrays, at least another 2 log amplification could be required to enable direct detection of single copy sequences from unamplified genomic DNA. While the exponential form of RCA amplifies DNA targets by 9 or 10 log (3), most of the amplified product is released into the reaction solution and, consequently, it lacks the product localization and multiplexing advantages of on-chip amplification by linear RCA. In this report, a third RCA format, termed L$^2$RCA, is described. In L$^2$RCA, additional rounds of signal amplification occur on a linear RCA single-stranded DNA product, using a P2 primer that is complementary to the linear RCA product (Fig. 5A).

**A**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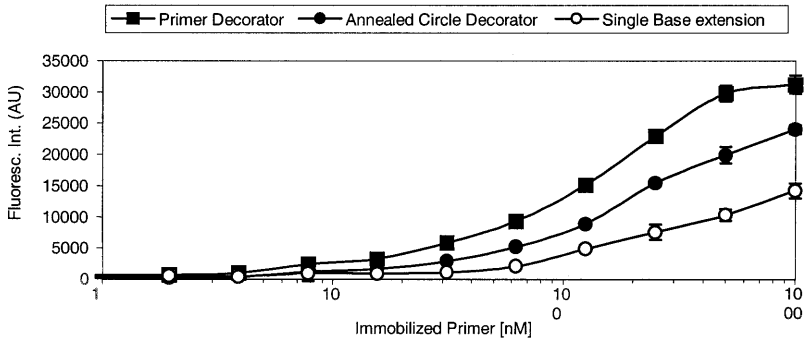
**B**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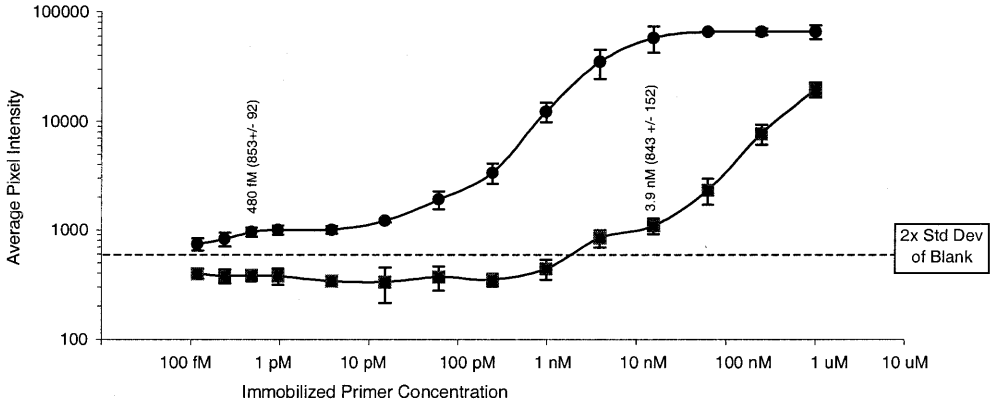
**C**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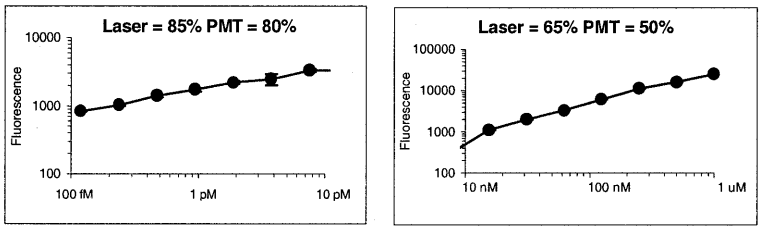
10 uM
Immobilized Primer Concentration

**D**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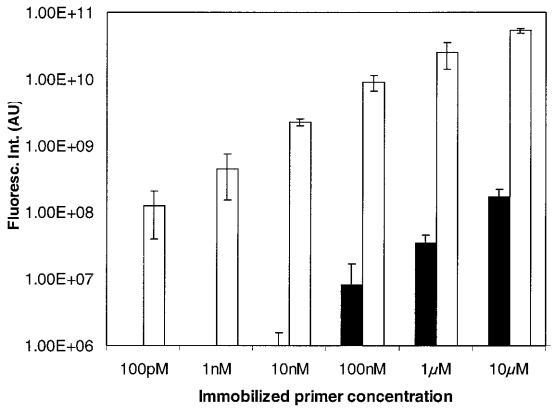


**Figure 4.** Comparison of RCA amplification and hybridization sensitivities on gel-coated arrays. RCA primers were immobilized on gel-coated arrays in a 10-fold dilution series in the concentration range 10 μM–100 pM, in 0.2 μl spots. RCA was performed as described and images were quantitated with GenePix 4.0 image quantitation software. The total fluorescence intensity values for RCA (open bars) and hybridization (filled bars) are plotted against the primer concentration in the spot. Average background was subtracted from each data point. Error bars indicate the standard deviations from the means of three spots.

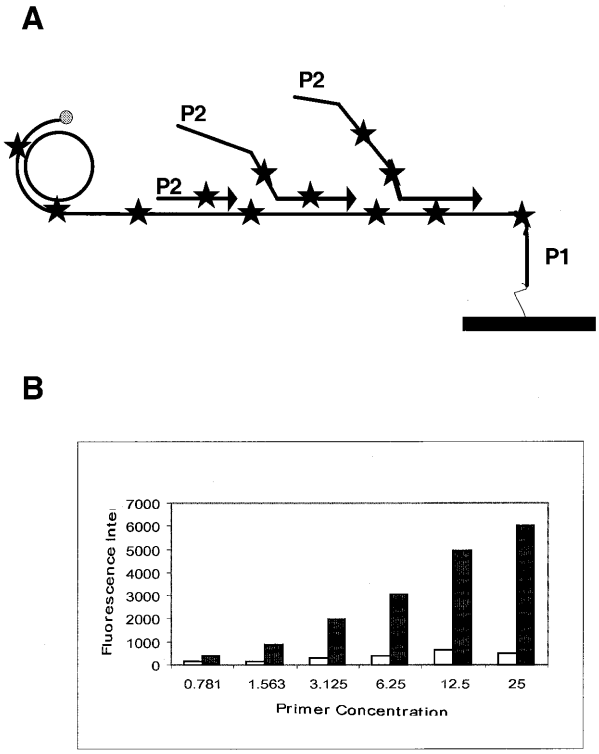


**Figure 5.** L²RCA on microarrays. (**A**) Schematic of L²RCA. In L²RCA, a primary round of RCA amplification occurs from polymerase initiation of a primed circle, just as in RCA. Simultaneously, additional rounds of signal amplification also occur using a second primer, P2 (solid arrows), which is present in the reaction mix and which hybridizes to the linear RCA product. The P2 primers contain a fluorophore tag (filled ovals). Fluorophore-labeled dNTPs are incorporated by the polymerase (asterisks). (**B**) Comparison of RCA and L²RCA. The chart shows the relative normalized intensities for RCA (filled bars) and L²RCA (open bars) in the primer concentration range 0.78–25 nM showing a linear response for RCA signals at the scanner settings employed.

Circle-dependent amplification from the immobilized P1 primer occurs simultaneously, with circle-independent polymerase extension of the P2 primer, which is present in the reaction mix. Therefore, when a strand-displacing polymerase is used, the products of L²RCA are likely to be branched structures, wherein secondary amplified products remain hybridized to the primary amplification product. As shown in Figure 5A and B, the sensitivity of detection of surface-bound primers by L²RCA was 20- to 50-fold higher than linear RCA on primers spotted at 150 pM to 1 nM. Thus, the signal generation efficiency of L²RCA is significantly higher than RCA, yet L²RCA retains the desirable property of product localization for microarray-based signal amplification assays. Comparable gains in sensitivity with L²RCA were obtained with 3D-Link slides and with planar glass microarrays (not shown).

## Allele discrimination using on-chip ligation

The practical utility of RCA signal amplification was examined in a genotyping application. Ligation was chosen as a model system for these studies in view of the specificity for allele discrimination offered by ligases (9). Synthetic oligonucleotide targets were used to determine the limit of sensitivity with allele discrimination on microarrays in a previously described two-color ligation/RCA method (3). Briefly, an immobilized oligonucleotide probe (P1), complementary to the target sequence and containing a phosphate group at the 5′-terminus, underwent allele-specific ligation to one of a pair of oligonucleotide probes (P2mu and P2wt) in the presence of oligonucleotide target. The P2 probes possess two 3′-ends, by virtue of reversed backbone synthesis; one end that is complementary to a 20 base sequence of the target contiguous with P1 and the other end having a sequence that does not hybridize with the target, but

**Figure 3.** (Previous page) Metrics of RCA on glass arrays. (**A**) Schematic representation of RCA metrics. The accessibility of surface-immobilized primers was measured by hybridizing a Cy5-labeled decorator to primer 1 immobilized in a dilution series on microarrays. Hybridization of RCA circles to the immobilized primers was measured using a Cy5-labeled oligonucleotide probe complementary to circle 1 (which hybridizes to a different sequence of circle from the primer 1 annealing site). Accessibility of primed circles for initiation by a polymerase was measured by T7 Sequenase incorporation of a single Cy5-ddGTP nucleotide using circle 1 as the template. (**B**) Primer accessibility on planar microarrays. Fluorescence intensities of hybridization of the decorator probe (filled squares) and the RCA circle (filled circles) are shown. The efficiency of polymerase initiation was measured by single base extension (open circles). Fluorescence signal intensities are plotted against each concentration of primer deposited. Average background was subtracted from each data point. Error bars indicate the standard deviations from the means of two spots from three different arrays. (**C**) Comparison of RCA amplification and hybridization sensitivities on glass arrays. Microarrays containing a dilution series of immobilized primer 1 were hybridized with circle 1 and RCA amplification was performed using native T7 DNA polymerase. After RCA signal amplification, slides were washed as described and scanned using a ScanArray 5000 laser scanner and analyzed with the QuantArray software package (GSI Lumonics). The figure shows a plot of the average pixel intensity at each spot by RCA (filled circles) and hybridization (filled squares) after subtraction of the average background from each point. Error bars indicate the standard deviations from the means of 10 arrays and two spots per array. (**D**) Dynamic range of RCA signal amplification. The scanner settings were optimized to determine signal intensities at the lower (left) or higher (right) primer concentration ranges shown in (C).

hybridizes to an RCA circle. At the gene-specific end, the two P2 probes differ in sequence by a single 3′-terminal nucleotide. After hybridization of the target to the immobilized P1 probes, a thermostable DNA ligase joins the cognate P2 to the P1 probe, thereby immobilizing P2 on the microarray. Following a stringent wash, two circles containing sequences complementary to P2wt or P2mut, respectively, are hybridized with the array. RCA is performed and the amplified product from the wild-type primer and circle is decorated by hybridization with an oligonucleotide labeled with Cy5, while the RCA product amplified from the mutant primer and circle is decorated by hybridization with an oligonucleotide labeled with Cy3. Then the microarrays are scanned in both the Cy3 and Cy5 fluorescence channels to identify the genotype of the original target oligonucleotide.

Ligation/RCA signals were robust at 100 nM target concentrations over a 20-fold range of P1 concentrations (Fig. 6A). As the concentration of target decreased, signals were seen only at higher concentrations of P1. The limit of detection of target with an allele discrimination ratio of 2.5 by ligation/RCA was 10 pM. Further, the fluorescence intensities of ligation/RCA spots were proportional to the amount of target present in the ligation step (Fig. 6B), consistent with the quantitative nature of the RCA assay. The RCA signals were absent when ligase was omitted from the reaction or in the presence of target with a mismatched nucleotide at the corresponding position (data not shown). In contrast, the limit of detection of ligated P2 molecules by hybridization to a Cy3-labeled probe under the same conditions was 10 nM target DNA (not shown). Thus, RCA signal amplification improves sensitivity by three orders of magnitude in a genotyping application, consistent with the expected signal amplification of RCA.

By utilization of a two-color RCA readout method, we generated distinct RCA signals for matched and mismatched alleles in the target. The RCA amplified ligation signals correlated quantitatively and qualitatively with the amount of target present during the ligation step and the genotype, respectively (Fig. 6B). Replicate experiments demonstrated that coefficients of variation (CVs) for ligation/RCA signal intensities were the same as for direct hybridization of ligated P2 probes (6–10%) (not shown).

Allele discrimination, which is the ratio of signal intensities for the matched and mismatched P2 probes (the Cy5 and Cy3 channels, respectively), was at least 25:1 at 100 nM target. However, in contrast to direct detection of ligation signals by hybridization, even under conditions of limiting target concentration (10 pM), the allele discrimination factor of RCA amplified ligation was sufficient to permit genotyping (2.5:1) (Fig. 6B). Ligation/RCA signal intensities and allele discrimination factors were unchanged in the presence of a $10^5$-fold (w/w) excess of non-specific DNA (not shown). Similar results were obtained with a second, different pair of targets (data not shown).

## DISCUSSION

Many microarray-based assays have been developed that use high density oligonucleotide or cDNA capture probes for sequence-specific analysis of RNA or DNA targets (reviewed in 10). These assays, however, have limitations, such as a requirement for high concentrations of input target for efficient hybridization and signal generation. Several approaches have been taken to improve sensitivity, including amplification of the target nucleic acid within the starting sample (11,12), increasing the efficiency of target capture on the array (13), amplification of the signal generated by the target capture event (14) or a combination of these strategies (reviewed in 15). To fully realize the power of microarrays, assays must be sensitive, quantitative over a broad dynamic range, capable of considerable multiplexing and compatible with a wide range of microarray formats and strategies. In this report, we have demonstrated that RCA is a sensitive method for multiplexed measurement of nucleic acid signals on microarrays. For DNA targets, the observed detection limit of RCA is ~150 molecules/200 μm spot, representing at least an 8000-fold increase in detection sensitivity over hybridization under the same conditions. This level of amplification on microarrays by RCA was similar to that achieved in solution, indicating that RCA operates at near maximal efficiency in a microarray format.

For microarray applications such as gene expression analysis, the dynamic range of the method should be at least as large as the anticipated biological response. For other applications, such as single nucleotide polymorphism (SNP) scoring, the dynamic range of the signal response provided by RCA is less important since only a qualitative determination is required. As shown in Figure 3C, RCA signal intensities varied linearly with primer concentrations ranging from 30.8 pM to 3.94 nM, corresponding to the linear dynamic range of the scanner at the settings employed. By optimizing scanner settings, the concentration ranges above and below these values were also found to be linear, however, it is not clear if the linear ranges at the scanner settings shown in Figure 3D were co-linear with that shown in Figure 3C. If so, the overall dynamic range of RCA using a tethered oligonucleotide could be four orders of magnitude. This property of RCA could be utilized in developing robust applications, including gene expression analysis and protein detection from complex biological samples, where a large dynamic range and quantitation are both necessary.

Routinely used methods for nucleic acid target detection on microarrays requires micromolar or nanomolar concentrations of target (16). It would be highly desirable, however, to be able to carry out target recognition and allele discrimination directly on genomic DNA using microarrays. Such a capability would greatly increase the throughput and reduce the cost of large-scale genotyping projects. The high fidelity of RCA amplification of hybridization/ligation signals enables mismatch detection with a high degree of confidence. We have demonstrated picomolar sensitivity for oligonucleotide targets using an approach that combines an on-chip allele-specific ligation reaction with an RCA signal amplification step. Detection of the ligated product by RCA was three orders of magnitude more sensitive than hybridization, consistent with the signal enhancement potential of RCA on microarrays. It should be noted that at 10 pM target, the signal-to-noise ratio for detection using RCA was at least 10, suggesting that further improvements in detection sensitivity are possible. Moreover, RCA signals were shown to be proportional to the amount of target present in the ligation step over a 4 log range. Investigations using modified forms of RCA, such as $L^2$RCA, are currently underway that may further improve the sensitivity of RCA on microarrays. We have also recently demonstrated that RCA provides a 1000-fold amplification in signal on microarrays

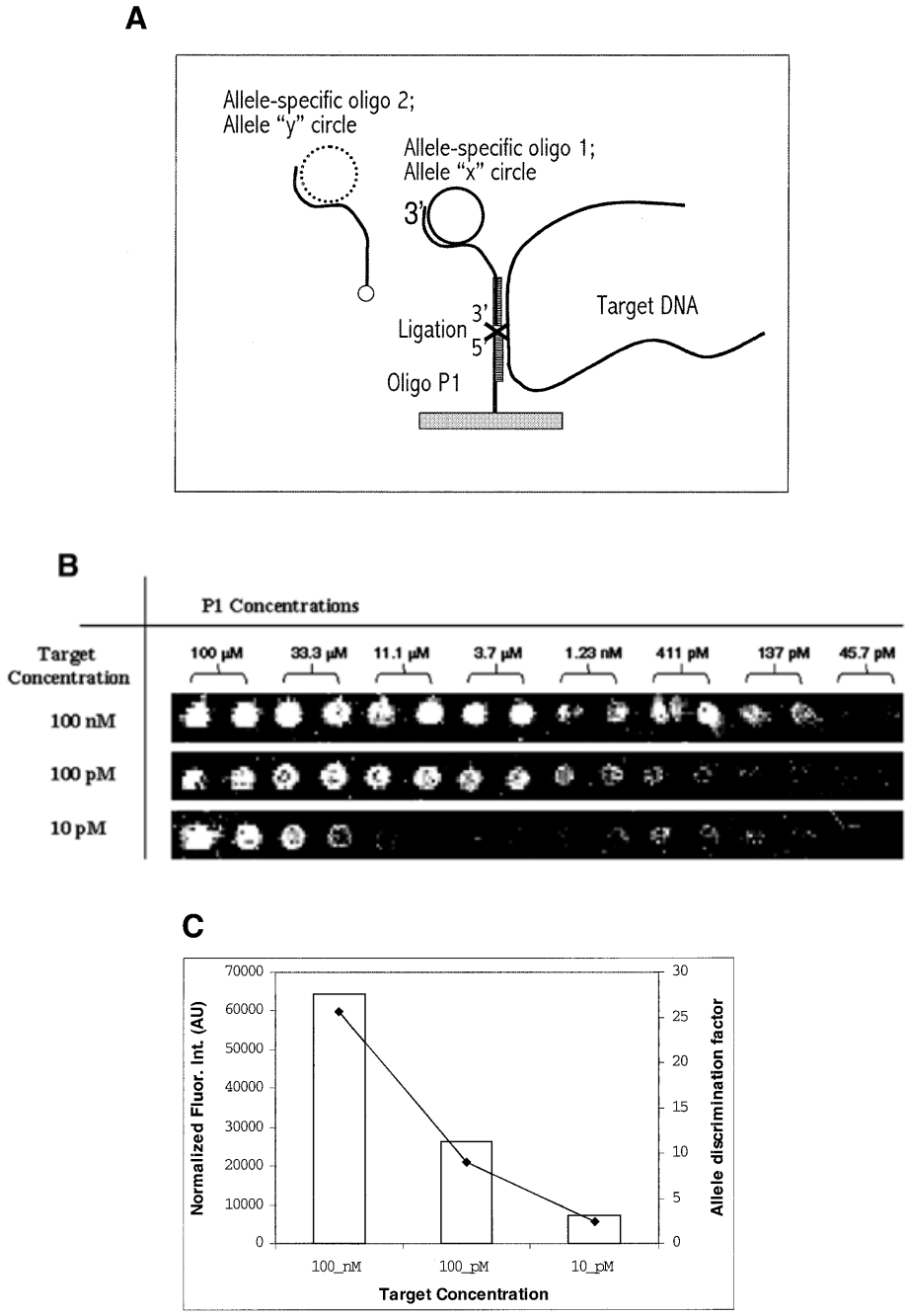


**Figure 6.** Target recognition by ligation on microarrays. Microarrays containing a concentration range of immobilized P1 oligonucleotides were ligated in the presence of target and a mixture of wild-type (wt) and mutant (mu) P2 oligonucleotides. The microarrays were washed and ligation signals were detected by RCA amplification. (**A**) Schematic representation of two-color ligation and RCA signal amplification on microarrays. (**B**) An image showing Cy5 ligation/RCA signals using synthetic wild-type G542X target. The deposited concentrations of P1 oligonucleotides for microarray preparation are denoted above the figure. The final concentrations of matched targets used for the ligation step are shown on the left. (**C**) Ligation and RCA signal intensity and allele discrimination factors at different target concentrations. The concentration of P1 oligonucleotide on the microarray spot in this experiment was 33 µM.

when used in conjunction with a single base extension approach for genotyping PCR products (Nallur *et al*., in preparation). These studies demonstrate the utility of RCA for detection of signals derived from contemporary methods for target identification on microarrays.

RCA signal amplification on microarrays involves a universal amplification circle, regardless of the nature and number of targets being assayed. This method minimizes bias during amplification. The CV of RCA signal amplification following ligation over a range of target concentrations was

similar to the variability observed with direct detection of the ligation signals by hybridization, indicating that on-chip RCA amplification did not result in significant additional variability.

In contrast to target preamplification methods, such as PCR, universal RCA signal amplification may be expected to introduce significantly less sequence-dependent bias. In PCR, the relative amplification efficiencies of targets in a complex sample will vary due to differences in primary and secondary structure of the target. Multiplexed ligation studies, using a 4–5 log range of target concentrations, demonstrated no evidence of target sequence-dependent bias at the RCA amplification step. In summary, the sensitivity, precision and compatibility of RCA signal amplification with a variety of nucleic acid sequence detection paradigms suggest it to be a useful tool for several microarray applications, including SNP genotyping, gene expression analysis and infectious disease monitoring.

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