Combining nucleic acid amplification and detection

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Major recent advances in molecular amplification in the past year were initial validation of two new amplification technologies (rolling circle amplification and Invader), a significant increase in the number of molecular diagnostic assays, achievement of amplification directly on microarrays (by strand displacement amplification and rolling circle amplification), and description of two new read-out probes (Scorpions and nanoparticles).

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Abbreviations

bDNA	branched DNA
FDA	Food and Drug Administration
HPV	human papillomavirus
LCR	ligase chain reaction
NASBA	nucleic acid sequence-based amplification
PCR	polymerase chain reaction
RCA	rolling circle amplification
RT-PCR	reverse transcriptase PCR
SDA	strand displacement amplification
SNP	single nucleotide polymorphism
TMA	transcription-mediated amplification

Introduction

Molecular amplification methods are fundamental to laboratory research, pharmacogenomics and molecular diagnostics. They fall into two classes, enabling either target or signal amplification (Table 1). Target amplification methods include polymerase chain reaction (PCR), strand displacement amplification (SDA), ligase chain reaction (LCR), and nucleic acid sequence-based amplification (NASBA). All are very sensitive and compatible with many detection techniques, such as fluorescence, chemiluminescence, or gel electrophoresis. Signal amplification technologies include branched DNA (bDNA), hybrid capture, and cleavase, and measure nucleic acid targets by amplification of a surrogate marker. Rolling circle amplification (RCA) is a newer method that performs either target or signal amplification.

The aim of this review is to highlight advances in the use of these technologies during the past year. In addition, novel procedures for detecting the products of amplification reactions are discussed.

Polymerase chain reaction

PCR remains the most widely used DNA amplification method. Food and Drug Administration (FDA)-approved diagnostic kits (Roche) that use PCR for the detection and quantitation of HIV, *Mycobacterium tuberculosis*, and *Chlamydia trachomatis* were the subject of numerous studies during the past year that demonstrated clinical accuracies equivalent or superior to other nucleic acid detection techniques, such as bDNA and NASAB [1–4].

A significant recent advance in PCR was the development of homogenous assays for real-time fluorescence detection of PCR-amplified products in a closed-tube format. In general, homogeneous formats can confer greater sensitivity, specificity, ease of use, and multiplexing ability. An

Table 1

Properties of various nucleic acid amplification technologies.

Property	PCR	LCR	SDA	NASBA	bDNA	Invader	RCA
DNA target amplification	✓	✓	✓	✗	✗	✗	✓
RNA target amplification	✓	✗	✓	✓	✗	✗	✓
DNA signal amplification	✗	✗	✗	✗	✓	✓	✓
RNA signal amplification	✗	✗	✗	✗	✓	✓	✓
Protein signal amplification	✗	✗	✗	✗	✗	✗	✓
Multiplexing	Little	✗	Little	Little	✗	✗	✓
Mesothermal	✗	✗	✓	✓	✓	✓	✓
Amplification within cells	✓	✗	✗	✗	✗	✗	✓
Amplification on microarrays	✗	✗	✓	✗	✗	✗	✓
Sensitivity (copies)	<10	100	500	100	500	600	1
Range (logs)	5	3	4	5	3	4	7
Specificity (allele discrimination factor)	50	5000	50	50	10	3000	100,000

example is 'TaqMan', in which homogeneous detection is achieved with an oligonucleotide probe labeled with a fluorophore and fluorescence quencher that, upon binding to the PCR product, undergoes quencher removal by the 5′–3′ exonuclease activity of *Taq* DNA polymerase [5]. The past year saw significant expansion of the diagnostic applications of TaqMan assays (for examples, see [6–8]).

An alternative to TaqMan for a homogeneous assay is the molecular beacon. Beacons contain a target recognition loop flanked by a hairpin with a fluorophore and quencher on the opposing ends. Specific binding to a PCR product opens the hairpin, separating the fluorophore and quencher, thus producing fluorescence [9]. Molecular beacons appear to confer similar gains in PCR performance to TaqMan probes. Several new diagnostic applications of molecular beacons were described in 2000 [10,11].

Amplifluor is a new beacon variant that is a PCR primer with a hairpin structure at the 5′ end containing a fluorophore and quencher. Incorporation of an Amplifluor into an amplicon opens the hairpin producing fluorescence [12]. Amplifluors were recently used for PCR genotyping of human papillomavirus (HPV) [13] and single nucleotide polymorphism (SNP) genotyping by RCA (Faruqi *et al.*, personal communication). A disadvantage of Amplifluor in comparison with TaqMan or beacon is that specific and non-specific PCR products are indistinguishable. Scorpion is a modified Amplifluor [14] with reduced artifactual signals (Figure 1). Under fast thermal cycling conditions, Scorpion primers may outperform TaqMan or beacon probes [15••]. At least in principal, beacons, Amplifluors and Scorpions are compatible with many amplification methods and will be limited in applicability only by difficulty in synthesis.

Finally, 2000 saw the launch of several new real-time detection instruments for PCR, most notably the SmartCycler (Cepheid). A clear trend in PCR instrumentation is smaller, faster devices to increase accessibility for routine use in research and clinical applications.

Ligase chain reaction

In LCR, two contiguous oligonucleotides are joined by DNA ligase upon perfect hybridization to a DNA target. Ligated probes are amplified by thermal cycling with complementary oligonucleotides [16]. During the past year, several studies of an FDA-approved LCR diagnostic test for *C. trachomatis* infection showed comparable clinical sensitivity to PCR [17].

LCR has better allele specificity than PCR for genotyping point mutations and SNPs because of the greater discriminatory power of ligation over primer extension [18]. This benefit was recently applied to DNA microarrays; upon target binding, an allele-specific probe was ligated to a fluorescently labeled probe [19,20••]. The allele-specific probe contained a short additional sequence at the 5′ end to permit capture of the LCR product on a DNA microarray. This procedure combined the specificity of solution-phase LCR with the multiplexing benefit of a universal microarray, and may broaden the appeal of LCR to general laboratories.

Strand displacement amplification

The mechanism of amplification by SDA is shown in Figure 2. Amplified targets are detected by inclusion of a single-stranded probe containing two different fluors. Upon SDA, the probe is incorporated into a double-stranded product that is cleaved by a restriction endonuclease, eliminating fluorescence quenching. A semi-automated system (ProbeTecET, Becton-Dickinson) for carrying out SDA with real-time fluorescence detection was recently introduced, which may reinvigorate interest in this method among general researchers. Detection of *C. trachomatis* and *Neisseria gonorrhoeae* using this system was recently shown to be of equivalent sensitivity and specificity to LCR [21].

Adaptation of SDA for amplification on a microelectronic chip was recently described [22••,23•]. An electric field localized biotin-labeled primers on a microarray, templates were hybridized using another electrical field, and SDA was performed *in situ*. Although loss of potential signal-generating DNA occurred because of strand-displacement and a denaturation step (carried out after SDA to remove DNA unattached to the anchoring primer in order to permit binding of a labeled reporter probe to the anchored strand), at 10^4 molecules target sensitivity was good. Direct amplification on microarrays is necessary if the utility of multifeature microarrays is to be extended beyond mRNA expression profiling.

Nucleic acid sequence-based amplification

NASBA is commonly used for measuring HIV viral load (Organon Teknika). NASBA amplifies RNA and DNA targets as antisense, single-stranded RNA by the concurrent activity of reverse transcriptase, RNase H, and T7 RNA polymerase and two primers [24].

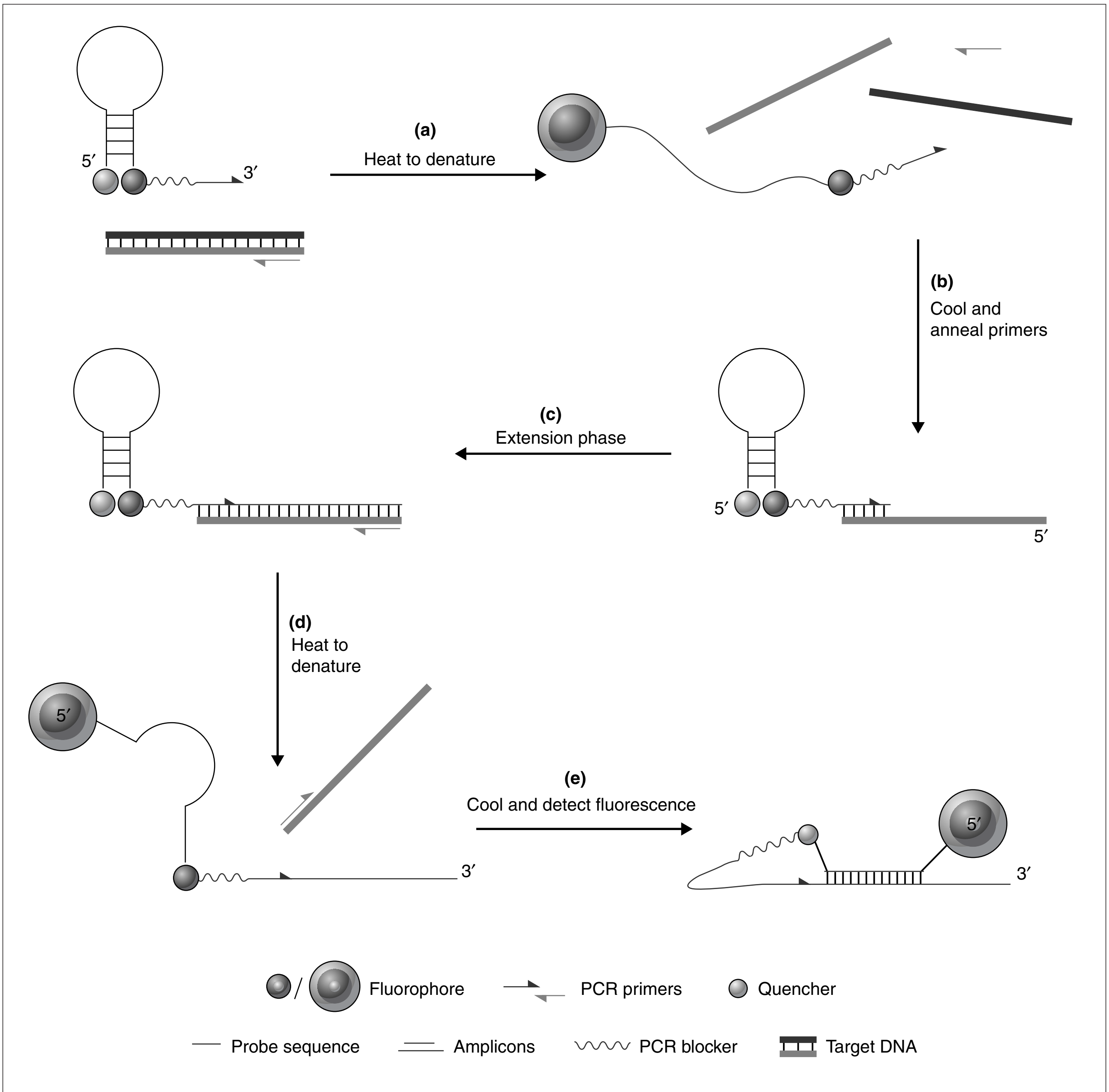
Recent comparisons of HIV-1 assays by NASBA and other methods differed in their conclusions [1,2,25,26], indicating a need for further evaluation of NASBA. This past year also saw the launch of a NASBA-based general RNA assay (Qiagen).

Transcription-mediated amplification

Transcription-mediated amplification (TMA) is an RNA transcription amplification method that uses RNA polymerase and reverse transcriptase in an isothermal reaction to amplify either DNA or RNA targets. In the TMA assay (Gen-Probe Inc., San Diego, CA, USA), amplicons are detected homogenously by chemiluminescence.

TMA is FDA-approved for detection of *C. trachomatis* and *M. tuberculosis*. A TMA assay for quantification of HIV-1 RNA was recently shown to be more sensitive than reverse transcriptase PCR (RT-PCR) or bDNA [27].

Figure 1

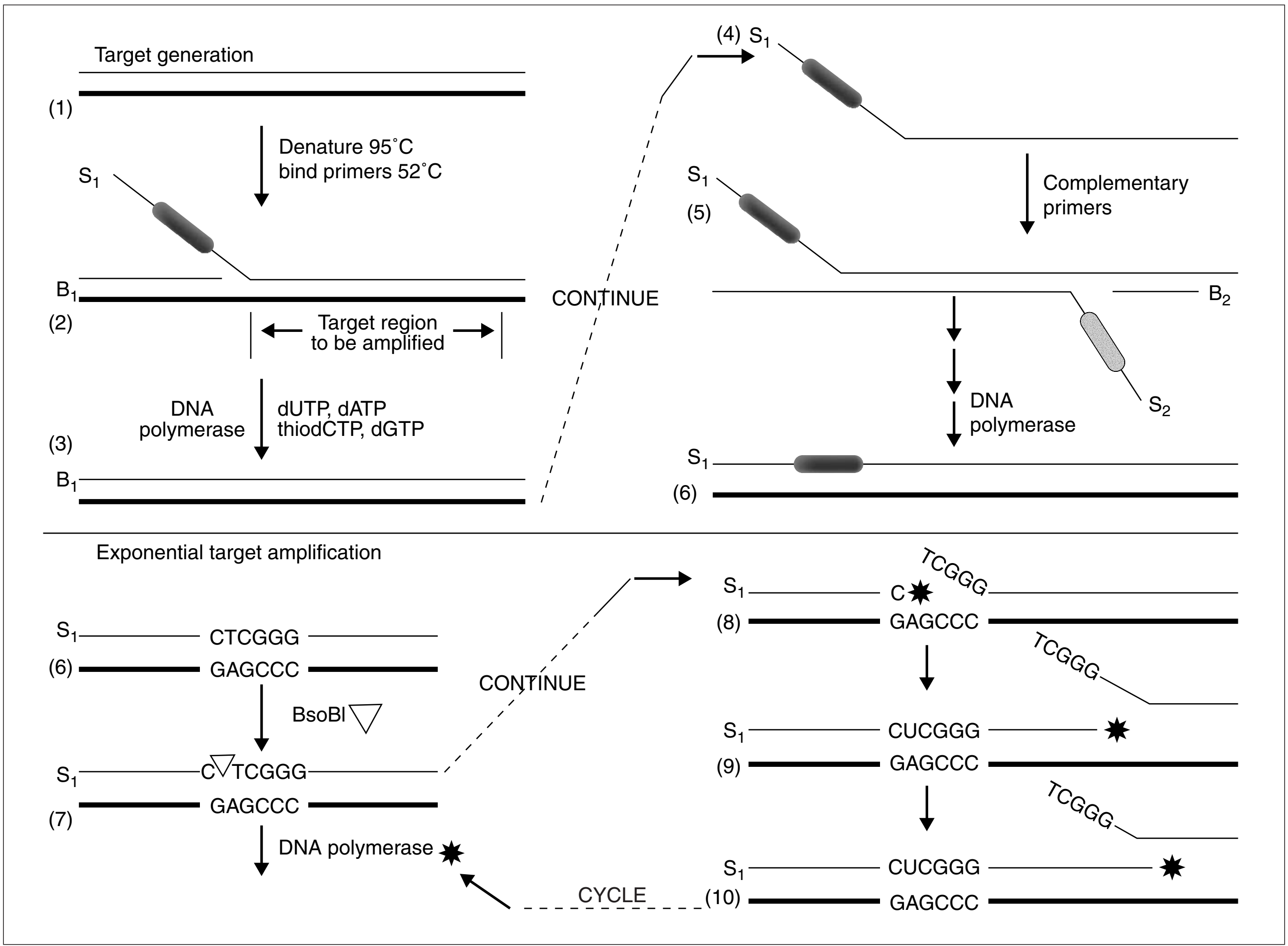


Scorpion primers. **(a)** Initial denaturation of target and Scorpion stem sequence. **(b)** Annealing of Scorpion primer to target. **(c)** Extension of Scorpion primer produces double-stranded DNA. **(d)** Denaturation of double-stranded DNA produced in (c). This gives a single-stranded target molecule with the Scorpion primer attached. **(e)** Upon cooling, the Scorpion probe sequence binds to its target in an intramolecular manner. Figure reproduced from [15••] with permission.

Branched DNA

bDNA achieves signal amplification by attaching many alkaline phosphatase molecules to a DNA dendrimer. Several tree-like structures are built in each molecular recognition event. The Quantiplex bDNA assay (Chiron) uses a dioxetane substrate for alkaline phosphatase to produce chemiluminescence. Although RT-PCR is the only FDA-approved method for quantitation of HIV-1 RNA in plasma, bDNA is frequently used in clinical laboratories. An improved bDNA test was recently shown to be linear from 100–500,000 HIV-1 RNA copies/ml, which is superior to RT-PCR [28]. Recent studies have also shown that the improved bDNA test has equivalent sensitivity and dynamic range for multiple hepatitis C virus (HCV) genotypes [29–31].

Figure 2



Strand displacement amplification. In the target generation phase (top panel) of SDA, a double-stranded DNA target (1) is denatured and hybridized with two primers (2). One primer (B_1) is designated as a 'bumper' primer, and the other primer (S_1) contains a BsoBI restriction enzyme sequence 5′ to the target binding region. The B_1 (3) and S_1 (4) primers are simultaneously extended by the thermostable enzyme *Bst* DNA polymerase in the presence of thiolated dCTP. Extension from the bumper primer displaces the S_1 extended product, which can then hybridize to the opposite strand primers, B_2 and S_2 (5). Extension of both of these primers produces species 6, which is utilized in the exponential target amplification phase of the reaction (lower panel). The strand that has been extended from the S_1 primer is nicked by BsoBI (7), but the complementary strand is refractory to cleavage owing to the presence of a thiolated dCTP within the restriction site. DNA polymerase binds to the nick and begins synthesis of a new strand while displacing the downstream strand (8–10). This recreates the double-stranded species 7, and the process repeats. The displaced strands bind to opposite strand primers, thus producing exponential amplification. Figure reproduced from [21] with permission.

A limitation of bDNA has been sensitivity. A variant of bDNA was recently described in which signal amplification was achieved through nucleotide extension and excision on the DNA platform itself. In this application, multiple oligonucleotide cassettes that can serve as templates for T7 DNA polymerase are attached to DNA dendrimers [32•]. Reaction of these dendrimers with polymerase generates inorganic pyrophosphate that is enzymatically converted to bioluminescence. The sensitivity of this variant was 5 zeptomoles, which approaches that of PCR.

Hybrid capture

In Hybrid Capture (Digene), target DNA molecules hybridize to specific RNA probes. RNA–DNA hybrids are captured on a solid phase coated with specific antibodies and detected with a chemiluminescent substrate using antibodies conjugated with alkaline phosphatase.

The system has been approved by the FDA for the detection of *N. gonorrhoeae*, *C. trachomatis*, and cytomegalovirus. Hybrid Capture is the only FDA-approved DNA-based assay for the detection of human papillomavirus (HPV) [33••]; this year, the assay was approved for screening women for HPV strains associated with a high risk of cancer.

DNA cleavage-based signal amplification

Two signal amplification methods based on DNA cleavage have been described. In cycling probe technology (CPT;

ID Biomedical), a chimeric DNA–RNA probe is cleaved by RNAse H upon target binding. Probe cleavage is detected by either immunoassay on a lateral flow device or colorimetry. Two recent studies reported the use of CPT for diagnosis of methicillin-resistant *Staphylococcus aureus* [34] and vancomycin-resistant enterococci [35].

Invader (Third Wave) is the second cleavage-based assay. Invader uses two partially overlapping probes that are cleaved by an endonuclease upon target DNA binding. By use of a thermostable endonuclease and elevated temperature, 3000 cleavage events can be evoked per target molecule [36]. A homogeneous Invader assay with greater sensitivity was recently described in which the cleaved product binds to a second probe containing a fluorophore and quencher [37••]. This second probe is also cleaved by endonuclease, generating up to 10^7 fluorescence events for each target molecule, which is sufficient for detection of <1000 targets.

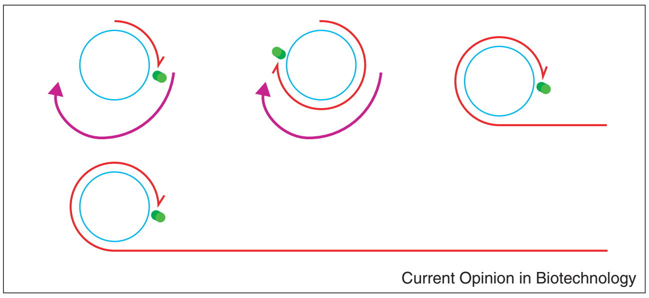
Invader can score SNPs close to the cleavage site because mispairing prevents cleavage. Good concordance between Invader and allele-specific PCR was recently obtained in a genotyping study of the Factor V Leiden mutation [38•]. These advances have significantly expanded the usefulness of Invader.

Rolling circle amplification

RCA is a new amplification method that is useful both for signal and target amplification [39]. Linear and exponential forms of RCA have been described. In linear RCA, a DNA circle is amplified by polymerase extension of a complementary primer. Up to 10^5 tandemly repeated, concatemerized copies of the DNA circle are generated by each primer (Figure 3). Target amplification by linear RCA is limited to circular nucleic acids, such as circular viruses, plasmids, and circular chromosomes. Using random primers and highly processive polymerases, this method allows genome sequencing from plaques or colonies without additional culturing (Dean *et al.*, personal communication). This method has considerable potential for *in vitro* library generation.

Linear RCA is the only method to generate a single, amplified product linked to an initiating primer. When used as a signal amplification method, this property enables high multiplexing without interference, direct amplification on a solid phase, and precise product localization [39]. Several recent studies demonstrated the practical utility of linear RCA signal amplification on microarrays. On two- and three-dimensional DNA arrays, RCA can give up to a 10,000-fold increase in signal over hybridization of a fluorescently labeled probe (Nallur *et al.*, personal communication). Schweitzer *et al.* [40••] showed that attachment of the 5′ end of an RCA primer to an antibody resulted in RCA products attached to the antibody. Protein analytes captured on microarrayed antibodies were measured with a detector antibody attached to an RCA primer. RCA products were labeled with fluorescent probes and detected on a microarray scanner, with a sensitivity of 0.1 pg protein/mL and dynamic range of 5 logs. Clinical performance of an RCA

Figure 3



Linear rolling circle amplification. A DNA primer (red) hybridizes to a DNA circle (blue) and is copied multiple times by DNA polymerase (green), making one single-stranded, concatemerized product.

protein chip for measurement of IgE to multiple allergens in serum samples was recently shown to be superior to conventional immunoassays [41•]. Another study demonstrated RCA signal amplification of targets in cells and formalin-fixed tissues (Gusev *et al.*, personal communication).

Although similar to linear RCA, exponential RCA uses a second DNA primer of identical sequence to the DNA circle. A two-primer RCA system achieves isothermal, exponential amplification [39]. Exponential RCA has been applied to non-circular DNA target amplification through use of a linear DNA probe that binds at both of its ends to contiguous regions of a target DNA, followed by circularization by DNA ligase. The advantage of this approach is exquisite specificity. Thomas *et al.* [42••] demonstrated sensitivity of 10 target molecules and 10^7-fold amplification in 1 hour in a homogenous closed-tube format using open circle probes, exponential RCA, and Amplifluor detection probes (see above). These results were duplicated by Faruqi *et al.* (personal communication), and extended to genotyping of SNPs directly from genomic DNA with specificity (allele discrimination) of over 100,000:1. This method, which also is quantitative when performed in a real-time detection instrument, appears promising for research and pharmacogenomic use.

Nanoparticles

Many DNA sequence detection applications utilize hybridization of labeled oligonucleotides. A novel detection scheme based on nanoparticles was recently described that uses oligonucleotides covalently bound to gold particles [43••].

One benefit of using nanoparticle labels was alteration of the melting profile of the labeled probe to improve discrimination of single base mismatches by hybridization. In addition, sensitivity was increased 100-fold compared with fluorescence by signal amplification based on nanoparticle-promoted reduction of silver (I).

Conclusions

The past year has seen substantial development in new technologies for DNA amplification and detection as well as increased diversification in the ways various amplification methods are applied. PCR remains the most widely used method for DNA amplification for both research as well as diagnostic applications. With regard to the latter, PCR still faces competition from other technologies such as LCR, SDA, NASBA, TMA, bDNA, and hybrid capture, which, for the most part, show comparable clinical utility. Few new molecular assays have received FDA approval for clinical use in the past year, the notable exception being a hybrid capture assay for detection of cancer-causing strains of HPV.

The trend to make molecular assays faster, less expensive, and easier to use is evidenced by the introduction of instrumentation for high-speed PCR and Scorpion probes that are compatible with homogeneous detection using this instrumentation. This trend is likely to continue until these assays can be carried out in hand-held devices in a matter of minutes. Another trend this year has been the harnessing of the enormous signal boosting potential of DNA amplification techniques for use in novel formats such as microarrays. A rapid expansion of work in this area should be anticipated as microarrays continue to increase in importance for applications such as gene expression analysis and pharmacogenomics. Additional expansion of amplification utilization is also likely to occur in the areas of bead-based arrays and biosensors.

The past year has also seen the establishment of several newcomers to the area of DNA amplification and detection. An improved version of the DNA cleavage-based Invader assay was validated as a useful technology for genotyping patients directly from genomic DNA. RCA was introduced as a versatile new technology for DNA target amplification, genotyping, and signal amplification on microarrays. RCA is unique among the DNA amplification technologies in its ability to be applied to protein detection on microarrays and in cells. As the field of proteomics grows, this technology, as well as other DNA detection technologies such as nanoparticles, which could be applied to proteins, will probably gain in importance.

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• The original report on immunoRCA is extended to include detection of IgE from patient sera on microarrays of multiple allergens. Specificity and sensitivity is shown to be comparable to clinically established assays; thus, combining the rolling circle amplification based signal amplification technology with microarrays has potential for transforming the manner in which allergy testing is carried out in the clinic.

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•• The combination of padlock probes, exponential rolling circle amplification, and energy-transfer probes is first described. This work has recently been extended to include real-time single nucleotide polymorphism detection directly from genomic DNA, thus providing a new platform for accurate, low-cost, and high-throughput genotyping.

43. Taton T, Mirkin C, Letsinger R: **Scanometric DNA array detection with nanoparticle probes.** *Science* 2000, **289**:1757-1760.
•• Oligonucleotides covalently attached to gold nanoparticles provide both improved hybridization discrimination as well as increased sensitivity on microarrays. Detection can be carried out on a conventional flatbed scanner; if developed further, this technology could substantially alter the way microarray analysis is carried out in the future.