Published in final edited form as:
Trends Biotechnol. 2011 May ; 29(5): 240–250. doi:10.1016/j.tibtech.2011.01.007.

Point-of-care nucleic acid testing for infectious diseases

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Abstract

Nucleic acid testing for infectious diseases at the point of care is beginning to enter clinical practice in developed and developing countries; especially for applications requiring fast turnaround times, and in settings where a centralized laboratory approach faces limitations. Current systems for clinical diagnostic applications are mainly PCR-based, can only be used in hospitals, and are still relatively complex and expensive. Integrating sample preparation with nucleic acid amplification and detection in a cost-effective, robust, and user-friendly format remains challenging. This review describes recent technical advances that might be able to address these limitations, with a focus on isothermal nucleic acid amplification methods. It briefly discusses selected applications related to the diagnosis and management of tuberculosis, HIV, and perinatal and nosocomial infections.

Introduction

Nucleic acid testing (NAT) for infectious diseases is almost exclusively performed in centralized laboratories using high-end instrumentation and skilled personnel. However, point of care (POC) testing, which is defined as near-patient testing in a hospital, doctor's office, clinic, or home, offers advantages when a rapid answer is required [1], or when suitable facilities or logistics chains are unavailable [2,3]. Compared to equivalent laboratory methods, POC testing is usually more expensive per test, although a true cost comparison needs to consider central laboratory overhead costs, better patient management and disease containment [4]. Under the United States Clinical Laboratory Improvement Amendments (CLIA), clinical diagnostic tests are categorized as high complexity, moderate complexity, or waived [5]. Most POC settings only allow the use of waived tests that are 'so simple and accurate as to render the likelihood of erroneous results by the user negligible'. Currently, there are no waived POC NAT systems. High and moderate complexity tests are distinguished based on required operator training and experience, complexity of operational steps, system troubleshooting, and equipment maintenance. High complexity tests are limited to central laboratories, whereas moderate complexity tests can be performed near patients in a hospital. The Cepheid GeneXpert, a PCR-based system that performs fully

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Disclosure statement

Keck Graduate Institute, the employer of A.N., owns intellectual property related to EXPAR/Fingerprinting. In 2009, A.N. spent a 6-month sabbatical at Roche Molecular Diagnostics. Claremont BioSolutions, the employer of T.M.F., is developing sample preparation methods described herein, and has rights to or has filed U.S. Patent Application Nos. 12/052,950, 12/732,070, and 12/823,081. D.B. is currently developing and validating RPA assays with TwistDX. PATH and Lumora are working together on a product development project.

automated ‘sample-in/answer-out’ testing is currently the only moderate complexity NAT platform approved in the USA.

POC NAT in developed countries must address patient needs that are insufficiently met by existing centralized laboratory methods, which includes rapid screening for perinatal and nosocomial infections. POC NAT in the developing world focuses on diagnosis and management of endemic infectious diseases, such as tuberculosis (TB) and HIV infection [2,3,6]. Developing countries have limited financial resources and cannot implement well-established, yet complex commercially available NAT systems or laboratory-developed tests through a network of centralized laboratories [7,8]. POC NAT can provide access to much-needed diagnostic methods in low-resource, high disease-burden areas, but appropriate devices should be affordable, robust and easy to use by minimally trained personnel, with stable, ready-to-use reagents, simple, maintenance-free instrumentation, and clear, actionable results, in addition to being suitably sensitive and specific. The World Health Organization (WHO) recently endorsed the Cepheid GeneXpert to facilitate diagnosis of multi-drug-resistant TB in high-burden, low-resource settings (http://www.who.int/mediacentre/news/releases/2010/tb_test_20101208/en/index.html). However, this system is still relatively expensive and complex. It most likely will penetrate into district hospitals and microscopy centers [2,9], but will not be appropriate for POC settings such as rural clinics with sub-optimal infrastructure [2]. POC NAT is still in the emerging stages, but technological advances are beginning to address these challenges.

Current NAT technologies

NAT involves three main steps: sample preparation, amplification, and detection. Examples of fully or partially integrated platforms that are commercially available or close to market are listed in Table 1. Integration of all three steps into a POC-compatible format has been demonstrated in the GeneXpert [10], the IQuum LIAT analyzer [11], and other real-time PCR-based benchtop systems (Table 1). Size, cost and complexity can be reduced by using isothermal amplification with real-time detection, as implemented in Lumora’s Bioluminescent Assay in Real-Time (BART) reader and in the TwistDX Twista. NAT with minimal instrumentation is facilitated by simple endpoint detection schemes, such as nucleic acid lateral flow (NALF), exemplified in the BioHelix BESt Cassette [12]. These examples also illustrate the spectrum of advances in systems integration: the GeneXpert and LIAT analyzer enable complete sample-in/answer-out testing; the BART and Twista perform amplification and detection; and the BESt Cassette performs detection only.

Sample preparation

Sample preparation is a bottleneck in NAT, especially for POC applications [13], because it involves lengthy processes that are often manually performed. In clinical laboratories, sample preparation is typically automated on large instruments. For POC NAT, sample preparation starting from the clinical specimen needs to be integrated and coupled with amplification and detection in an inexpensive, automated, miniaturized, closed system format. Microfluidic devices have been developed to automate individual steps or the entire sample preparation process [14,15], but to date, none of the systems have become commercially viable products, possibly because of the challenges related to system complexity, manufacturability, and reproducible device performance. Most infectious disease applications further require extraction and concentration of target nucleic acids from sample input volumes >500 μl to reach a suitably low limit of detection (LOD). This requirement often cannot be accommodated in micro-fluidic devices. The GeneXpert therefore employs a macro-fluidic approach to sample preparation [10] (Figure 1a).

Lysis of most pathogens can be performed using chemical or enzymatic means. Certain microorganisms, such as *Mycobacterium tuberculosis*, however, are difficult to lyse. The GeneXpert utilizes a miniaturized sonicator for mechanical cell disruption (Figure 1b) [16], whereas Claremont BioSolutions has developed a miniaturized, battery-operated, bead beating system for mechanical pathogen lysis (Figure 1c) [17]. Following lysis, nucleic acids are typically purified through solid phase extraction (SPE), which involves capturing nucleic acids onto a solid support, followed by wash steps and elution for downstream amplification [15]. Nucleic acid purification is necessary because subsequent target amplification using polymerases can be inhibited by compounds found in clinical samples. Most automated nucleic acid sample preparation systems perform SPE on silica-coated magnetic beads, with lysis/binding buffers that contain chaotropic salts and ethanol, which also can inhibit polymerases. SPE using chitosan-coated beads [18] or other resins, such as the ChargeSwitch method (Invitrogen), do not require chaotropic salts and organic solvents [13]. SPE without wash steps has been demonstrated by moving DNA bound to magnetic beads through a layer of liquid wax (Figure 1d) [19]. Recently, PCR assays that are less-prone to inhibition have been described [20,21], which might require less rigorous sample preparation. Many isothermal technologies appear to be less affected by inhibitory compounds; especially those found in whole blood [22].

Amplification

NAT for infectious disease diagnosis often requires reaching an LOD of ≤100 copies/ml of the pathogen, which, at present, can only be accomplished through polymerase-based amplification. PCR requires thermocycling to mediate DNA melting, followed by primer annealing and extension. Fully integrated macrofluidic [10,11,23] and microfluidic [24,25] devices and many permutations of microfluidic lab-on-a-chip systems [26,27] have been developed for miniaturized PCR.

Conversely, isothermal nucleic acid amplification technologies use a single reaction temperature, which translates into less complex and less expensive instrumentation, more suited to applications in low-resource settings. Isothermal amplification can be performed in water baths, using simple resistive heaters, or via exothermic chemical heating [28]. Many isothermal amplification methods have been reported, which can be grouped based on the reaction principle (Figures 2 and 3; Table 2). Current methods include those based on RNA transcription (Figure 2a) [29–31]; DNA replication with enzymatic duplex melting and primer annealing (Figure 2b) [32–35]; strand displacement using polymerases only, with multiple linear primer sets (Figure 2c) [36–39]; and strand displacement from a circular or circularized target (Figure 2d) [40,41]. Many isothermal amplification methods use polymerase extension in conjunction with a single-strand cutting event (Figure 3). [42–50]. Transcription-mediated amplification (TMA; Gen-Probe) [29], nucleic acid sequence-based amplification (NASBA; BioMerieux) [30], and strand displacement amplification (SDA; Becton Dickinson) [42,43] are well-established and implemented in commercial laboratory-based NAT. Some methods, such as loop-mediated amplification (LAMP; Eiken) [36,37], are becoming mainstream, and many others, such as recombinase polymerase amplification (RPA, TwistDx) [34,35], are emerging.

Comparing the reaction schemes reveals a set of underlying principles to accomplish three basic goals: isothermal generation of ssDNA; exponential amplification; and intermediate target generation. To make DNA polymerase-based amplification isothermal, target ssDNA must be obtained at the same temperature that is required for annealing and extension. Different isothermal amplification methods require reaction temperatures between 30 and 65 °C (Table 2), which is mainly determined by the stability and activity of the polymerase and other enzymes in the reaction. Isothermal generation of ssDNA can be achieved through enzymatic duplex melting and primer annealing (Figure 2b), or by using a strand-displacing

polymerase to 'peel off' ssDNA through a bumper primer, through extension around a circular target, or by creating a priming site through a single-strand cutting event (Figures 2c,d and 3). Very short amplicons thermally melt off the target, in addition to strand displacement. Isothermal duplex melting can be highly efficient, as reflected by the rapid amplification achievable in RPA and other methods (Table 2).

To obtain sensitive target amplification, amplicons must feed back into the reaction. In NASBA and TMA (Figure 2a), the RNA amplicon is converted into dsDNA with a promoter region. In most DNA polymerase-based methods, the amplicon either re-primes or self-primes. In isothermal methods, exponential amplification occurs asynchronously, as opposed to PCR, in which annealing, extension and duplex melting are synchronized through thermal cycling. Asynchronous reaction progression in some methods facilitates effective isothermal amplification in ≤20 min (Table 2), which is difficult to accomplish via PCR. Other isothermal reactions require ≥1 h to achieve sufficient amplification, which is similar to standard PCR, but not ideal for POC applications. Slower, more controlled reaction progression, however, often translates into less non-specific background amplification, and is better suited for target quantification.

If polymerase-mediated exponential amplification does not directly start from the genomic target, then steps have to be incorporated to generate a suitable intermediate target. In NASBA and TMA, target viral RNA is converted into dsDNA with a promoter region. Detection of RNA through DNA-polymerase-based amplification requires a reverse transcriptase step. Other methods that require the generation of intermediate targets include LAMP, which starts from a dumbbell structure (Figure 2c), and the ramification amplification (RAM) reaction, which requires generation of a circularized probe (Figure 2d) [41]. All methods based on single strand cutting events employ often sophisticated methods to generate the intermediate targets.

Another consideration when choosing an amplification method is the ability to multiplex, or at least co-amplify the target and an internal control (Table 2). Amplification reactions used for clinical diagnostics should incorporate an internal control, co-amplified with the target in the same reaction, to eliminate false negatives due to failed amplification or failed sample preparation.

Detection

Nucleic acid amplification can be analyzed after the reaction (endpoint detection) or while the reaction is progressing (real-time detection). Endpoint detection requires less complex instrumentation and provides simpler outputs for interpretation. Real-time methods integrate amplification with detection, and are superior for quantitative analyte detection with a large dynamic range. In all cases, detection methods are preferable that can differentiate target-specific amplicons from non-specific amplification products and that minimize the risk of carry-over contamination.

Real-time fluorescence detection for PCR or isothermal amplification reactions can be mediated by intercalating dyes, by oligonucleotide probes that are cleaved during the reaction, or by using probes or primers that change conformation upon target amplification. Most current POC NAT platforms are based on PCR amplification with real-time fluorescence detection (Table 1), which requires relatively expensive and complex instrumentation. Simpler and cheaper instruments enable real-time monitoring of isothermal amplification reactions. The Loopamp Realtime Turbidimeter (LA-200; Eiken) monitors LAMP reactions: pyrophosphate ions, a byproduct of DNA synthesis, precipitate in the presence of metal cations, which leads to an increase in turbidity. BART uses a bioluminescent readout, which couples pyrophosphate generation with luciferase-mediated

light emission. Twista is a portable real-time fluorimeter that can detect twin fluorescent probes for monitoring RPA reactions.

Lateral flow (LF) devices perform endpoint detection sandwich assays using passive fluidics, and are well-established for POC diagnostics [2]. Sensitivity in NALF is provided through upstream nucleic acid amplification. Simple NALF devices without a reader can detect <10 copies of target nucleic acid per amplification reaction, which is equivalent to less than attomolar analyte concentrations. NALF has been implemented in antibody-dependent (Figure 4a) and antibody-independent (Figure 4b) configurations. Antibody-dependent NALF is similar to LF immunoassays, but antigenic labels have to be incorporated into the amplicon via the primers or labeled dNTPs, and a second labeled probe is often required. The BESt Cassette (Table 1), which detects labeled amplicons via antibody-dependent NALF, is designed to minimize the risk of carryover contamination [12]. Antibody-independent NALF has a simplified reaction scheme, but requires nucleotide immobilization on the LF strip and colored microparticles. In a device called simple amplification-based assay (SAMBA) (Table 1), isothermal nucleic acid amplification and antibody-independent NALF are integrated into a disposable cartridge in conjunction with a benchtop instrument [51]. NALF is amenable to low-level multiplexing, and has been coupled with PCR [52,53], NASBA [54], helicase dependent amplification (HDA) [12], RPA [35], LAMP [55], cross-priming amplification (CPA) [38], and other isothermal amplification methods.

Changes in turbidity owing to pyrophosphate precipitation have been utilized for visual endpoint detection of LAMP reactions [37]. By including calcein dye, turbidity is enhanced via fluorescence. Precipitates of different colors can be obtained through the addition of cationic polymers in conjunction with fluorescently labeled primers and probes [56]. Colorimetric detection can be achieved through the addition of hydroxyl napthol [57]. These approaches minimize the risk for amplicon carryover, because the reaction tube remains sealed after incubation. Many other optical and electronic biosensor technologies enable endpoint nucleic acid detection [58]. For example, on-chip isothermal amplification has been coupled with microarray-based readout for multiplexed pathogen detection [59], and electrochemical biosensors can be miniaturized and integrated into microfluidic devices [60].

A unique combination of sample preparation, amplification and detection technologies might facilitate fully integrated, low-cost POC NAT. Isothermal amplification with endpoint detection can be implemented in a non- or minimally instrumented format. Recently, several single-use, sample preparation devices have been described that interface with various isothermal amplification methods, including LAMP, RPA and HDA [34,61,62]. Ultimately, moving NAT to the POC is driven by the clinical applications, some of which are presented in the next section.

Selected applications

Tuberculosis

TB is a disease of high public health priority owing to the increase in multi-drug-resistant strains and the high incidence of complications associated with HIV/TB co-infection (http://www.who.int/tb/publications/global_report/2009/en/index.html). Rapid diagnosis and drug susceptibility testing can limit the spread of TB and facilitate effective treatment. Commercial systems for TB NAT using large analyzers with manual sample preparation steps, followed by amplification based on PCR (Roche), TMA (GenProbe) and SDA (Beckton Dickinson), are available [63]. Sample preparation is particularly challenging for TB NAT because pre-processing is required to liquefy sputum and concentrate the pathogen;

mycobacteria are lysis-resistant; and sputum contains polymerase inhibitors. Fully integrated, automated, POC-compatible TB NAT starting from raw sputum has been demonstrated in <2 h using the GeneXpert *M. tuberculosis* complex/rifampicin resistance (MTB/RIF) test [64,65]. In this method, chemically liquefied and decontaminated sputum samples are introduced into the GeneXpert cartridge, where intact mycobacteria are captured, concentrated, and purified. Following sonication lysis to liberate mycobacterial genomic material, the system performs a real-time, hemi-nested PCR assay with five molecular beacons that target the rifampicin resistance core region of the *rpoB* gene, plus one molecular beacon that detects a process/internal control. A multi-site evaluation has demonstrated that the MTB/RIF assay exhibits excellent sensitivity and specificity not only for TB diagnosis, but also for detection of rifampicin resistance as a marker for multi-drug-resistant tuberculosis (MDR-TB) [66]. The system has been endorsed by the WHO; however, the cost and complexity of the GeneXpert is a concern for broad implementation in low-resource settings. Inexpensive TB NAT in low-resource settings has been demonstrated using LAMP with visual endpoint detection [67], but the method is performed manually with separate sample preparation steps and additional instrument requirements. A need exists for simple, inexpensive, yet fully integrated POC NAT systems with performance characteristics sufficient to diagnose MDR-TB.

HIV viral load monitoring

Improved access to antiretroviral therapy in low-resource settings has decreased AIDS-related morbidity and mortality, but the emergence of drug-resistant HIV strains, coupled with the increasing rates of treatment failure, might undermine long-term therapeutic success [68–70]. Monitoring HIV viral load is routinely performed in developed countries to identify virological treatment failure, which prompts enhanced adherence counseling and possibly a change to second-line therapy. The WHO recognizes that increased access to viral-load monitoring in low-resource settings can help prevent emergence of drug-resistant HIV strains and patient health decline; however, the WHO does not recommend routine monitoring owing to the expense, complexity, and required infrastructure to implement current technologies [71].

NAT remains the preferred method for HIV viral-load monitoring in developed and developing countries [72], and commercial systems are available for high-volume centralized laboratory testing based on real-time quantitative PCR (e.g. Roche and Abbott), NASBA (BioMerieux), or a branched sandwich assay called bDNA (Bayer/Siemens) [68]. Several African countries are utilizing viral-load monitoring from dried blood spots sent to centralized laboratories, which poses logistical challenges and cannot reach all patients [7,68]. Technologies are in development that can move HIV viral-load monitoring out of the central laboratory. IQuum recently has demonstrated POC-compatible sample-in/answer-out HIV viral-load monitoring in 1.5 h using the LIAT analyzer [11], with analytical performance comparable to, and quantitative results concordant with, US Food and Drug Administration (FDA)-approved central laboratory systems.

Infant HIV diagnosis

Early diagnosis and timely intervention with antiretroviral drugs are essential to extend the life expectancy of HIV-1-infected infants [73]. Infant HIV diagnosis using standard serological assays is compromised owing to the presence of maternal HIV antibodies [74]. The WHO and the United Nations Children's Fund (www.unicef.org) therefore recommend virological testing for infant HIV diagnosis, which can be accomplished through HIV NAT that targets viral RNA or proviral DNA using commercially available assays or laboratory-developed tests [75,76]. Central laboratories enable large-volume NAT [77], but transporting specimens and reporting results can result in turnaround times of 1–3 months

[78]. Several POC-compatible platforms are under development. The SAMBA system (Table 1) currently performs HIV diagnosis through an isothermal nucleic acid amplification method similar to NASBA (Figure 2a), coupled with LF detection in an integrated cartridge combined with a benchtop instrument [51]. Inclusion of sample preparation is planned for a future version of this device. BioHelix has developed an HDA-based assay for HIV diagnosis with NALF detection using the BESt Cassette [78,79]; however, sample preparation, isothermal amplification, and NALF detection are performed as separate steps.

Group B streptococcus

Maternal carriage of group B streptococcus (GBS) in the vagino-cervical tract can cause severe infections in the neonate. Maternal carriage might be asymptomatic, therefore, rapid diagnosis of GBS during labor (intrapartum) is needed to minimize transmission risk at birth [1]. Screening for GBS colonization at 35–37 weeks via culture is insufficient because not all cases of GBS are detected [80]. The GeneXpert GBS enables testing directly from a vaginal swab during labor in 75 min, with sensitivity and specificity similar to GBS culture methods [80,81].

Methicillin-resistant Staphylococcus aureus surveillance and diagnosis

The incidence of hospital-acquired *Staphylococcus aureus* infections is increasing, particularly in the USA. *S. aureus* is a common hospital-acquired infection and is the leading cause of ventilator-associated pneumonia and surgical site infections [82]. Methicillin-resistant *S. aureus* (MRSA) accounts for 64% of all hospital-acquired *S.aureus*infections [83]; is resistant to vancomycin as well as most β-lactam antibiotics; and can lead to severe complications once the infection spreads within the body, particularly in immuno-compromised individuals. NAT has been recommended recently by the Clinical and Laboratory Standards Institute (www.CLSI.org) for active MRSA surveillance of patients admitted to hospitals and other healthcare facilities [84]. Roche, Becton Dickinson and Cepheid market FDA-approved PCR-based MRSA NATs. The Roche and Becton Dickinson tests are conducted in central laboratories, but the GeneXpert MRSA test enables POC MRSA surveillance in hospital wards and outpatient clinics [85]. GeneXpert MRSA tests performed in hospital wards have been shown to provide results >10 h faster than GeneXpert MRSA tests performed in a central laboratory [86]. Isothermal MRSA NAT using HDA [12] and RPA [35] have also been reported. RPA integrated into a ‘lab on a foil’ system has enabled detection of the MRSA *mecA* gene at low copy numbers in under 20 min [34]. This system consists of a microfluidic centrifugal disk that contains all reagents on-board and a benchtop analyzer for disk rotation, incubation, and real-time fluorescence detection. The system can perform up to 30 amplification/detection reactions in parallel, but does not currently include sample preparation.

Conclusions

The progress in developing POC-compatible NAT technologies has increased dramatically in the past 5 years; driven by applications that require rapid turnaround in developed countries, and by expanding markets related to endemic diseases in the developing world. Cepheid has demonstrated that POC NAT is possible: the GeneXpert, originally developed to detect biothreat agents, has successfully crossed over to clinical diagnostic applications. After receiving FDA clearance for the GBS assay as the first moderate complexity NAT, Cepheid has rapidly expanded the GeneXpert test menu to a range of nosocomial infections, including MRSA, and has demonstrated, through the MTB/RIF test, that fully integrated sample-in/answer-out NAT can meet crucial needs that are related to infectious disease diagnosis in the developing world. Other integrated platforms are in the late stages of development (Table 1).

Technical obstacles remain that are related to achieving appropriate integration of multiple crucial components in a robust, user-friendly format, while minimizing complexity and cost. Combination of sample preparation with amplification and detection in one integrated system is the greatest challenge. New sample preparation technologies are needed with simplified workflow and high nucleic acid recovery, which are capable of processing a large sample input volume and purifying nucleic acids from a variety of sample matrices, without introducing additional inhibitory compounds. Isothermal amplification methods often require less-stringent sample preparation, and can facilitate rapid, sensitive, and specific target amplification via single-temperature incubation, which reduces system complexity and cost compared to PCR-based methods. Established and emerging isothermal amplification methods vary in terms of complexity (e.g. use of multiple enzymes and/or complex primer design), reaction speed, attainable sensitivity and specificity, and ability to multiplex. To date, no method is clearly superior, and different methods will probably find their way to market. Real-time detection offers superior performance, especially for analyte quantification. However, system cost and complexity can be reduced significantly through simple visual endpoint detection methods, such as NALF. Through a combination of such new technologies, POC NAT might be able to advance towards CLIA waived status and beyond testing near patients in hospital. Systems developed for non-clinical applications related to biothreat detection, food-safety, agricultural and veterinary diagnostics require the same inherent characteristics, but have an easier path to market, and serve as a stepping stone towards clinical diagnostic POC NAT.

Sustained implementation of POC NAT requires that new technologies truly address the needs of the targeted settings, which is especially true in developing countries [87]. Once a suitable method has been developed, stakeholders within the countries need to be convinced through rigorous evaluation, demonstration, and assessment studies that the new diagnostic method leads to better patient outcomes and has a positive impact on society overall, while being cost-effective and implementable in the existing healthcare system.

Acknowledgments

The authors acknowledge NIH NIAID funding through awards R01AI090831 and R01AI076247, which supports ongoing collaborative efforts to develop integrated POC NAT systems based on isothermal amplification.

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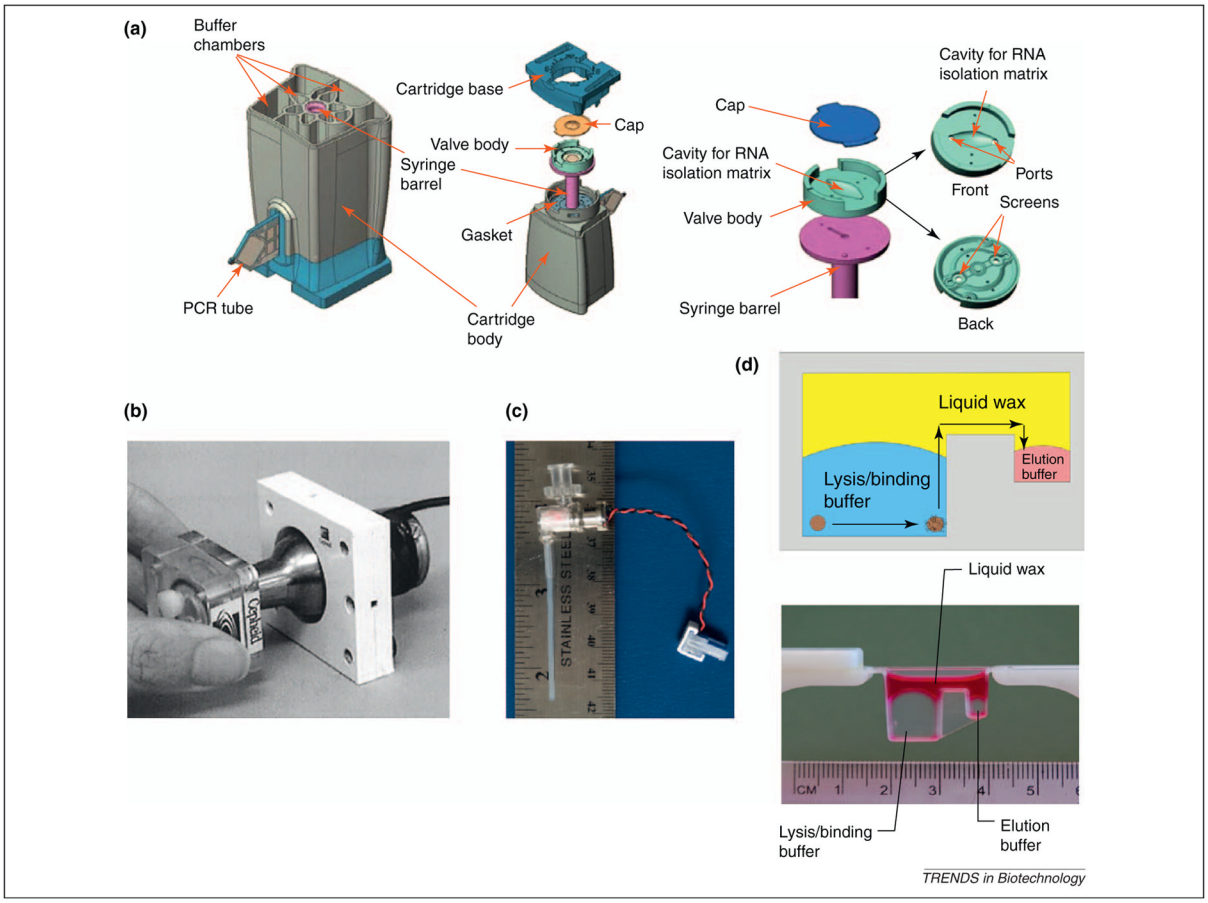

Figure 1.

Selected sample preparation technologies for POC NAT. **(a)** Detailed schematic of Cepheid's GeneXpert cartridge reproduced with permission from [10], most of which is devoted to sample preparation and reaction setup. A plunger from the instrument engages with the syringe barrel to draw sample and lysis/binding, wash, and elution buffers through the rotating valve body at the bottom of the cartridge into the cavity that holds beads for NA SPE. Eluted, purified nucleic acids are combined with lyophilized mastermix reagents and transferred into the PCR chamber at the side of the cartridge for amplification with real-time fluorescence-based detection. **(b)** Miniaturized ultrasonic horn incorporated into the GeneXpert system reproduced with permission from [16], which engages with the bottom of the cartridge to facilitate pathogen lysis. **(c)** Miniaturized bead blender developed by Claremont BioSolutions [17] to mechanically disrupt lysis-resistant pathogens. **(d)** "No wash" sample preparation, based on moving nucleic acids bound to magnetic beads from the lysis/binding buffer on the left through a layer of liquid wax into the elution buffer chamber on the right, reproduced with permission from [19].

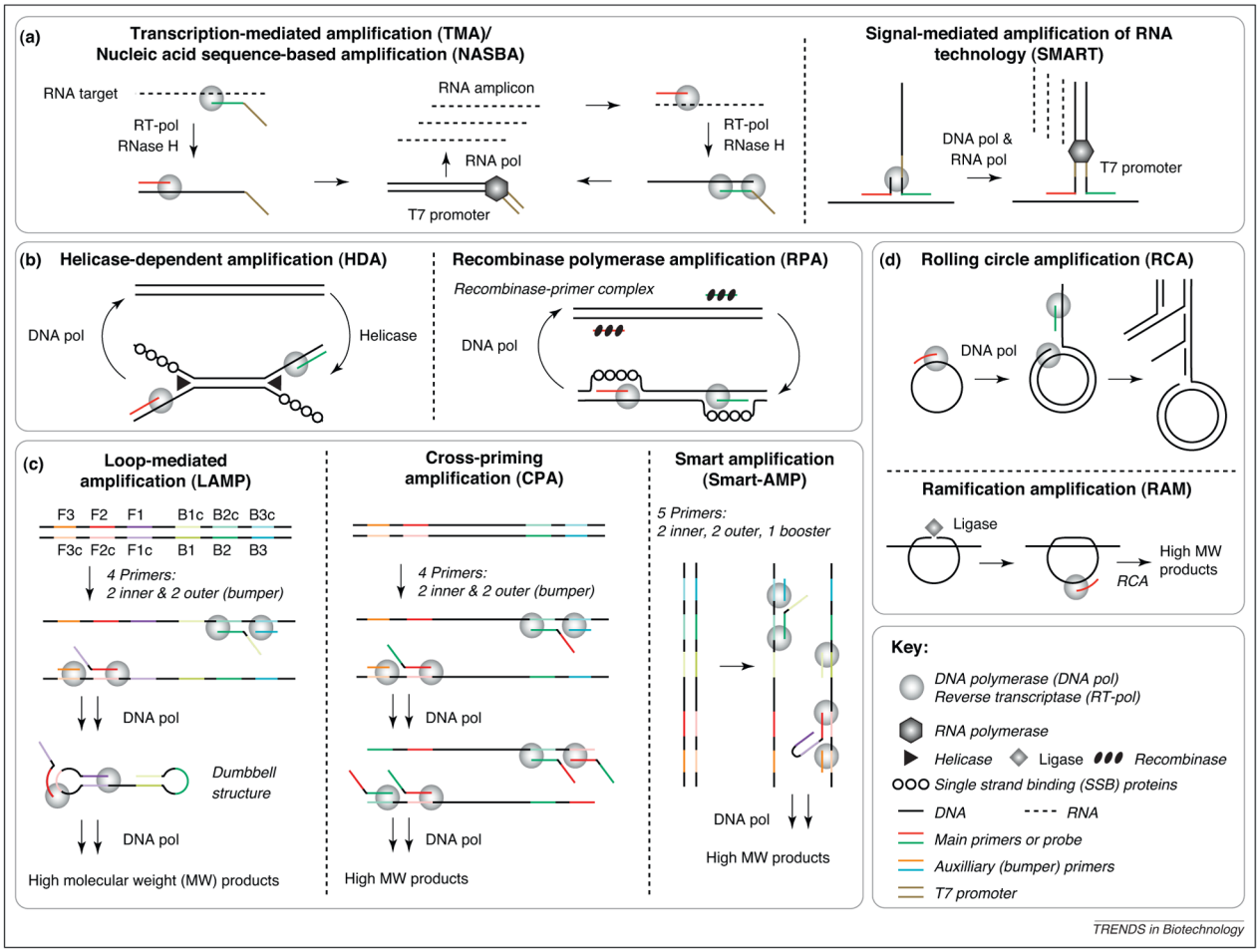

Figure 2.

Overview of isothermal nucleic acid amplification reactions. **(a)** Methods based on RNA transcription. In TMA (Gen-Probe) [29] and NASBA (BioMerieux) [30], an RNA target is converted to ds cDNA with a promoter region through reverse transcription, followed by RNase H degradation of the original strand and DNA polymerization initiated by a second primer. RNA polymerase (pol) amplification creates products that feed back into the original reaction. TMA and NASBA involve the same reaction scheme, but NASBA requires three enzymes (RT-DNA pol, RNase H, and RNA pol); TMA requires only two enzymes, because the RT-DNA pol has intrinsic RNase H activity. SMART (Cytocell) [31] utilizes a three-way junction with target and extension probes to initiate linear RNA polymerization based amplification (no exponential feedback). **(b)** Methods based on DNA replication with enzymatic duplex melting/primer annealing. In HDA (Biohelix) [32,33], a helicase enzymatically “melts” dsDNA. In RPA (TwistDx) [34,35], a recombinase–primer complex scans dsDNA for the target site and facilitates primer binding. In both HDA and RPA, single strand binding proteins stabilize the separated strands; the rest of the reaction sequence is analogous to PCR. **(c)** Methods based on strand displacement using polymerases only from a linear target, through use of sacrificial outer bumper primers. LAMP (Eiken) [36,37] involves six recognition sites on the target DNA; the 5′ overhangs of the inner primers recognize sequences in the amplicon, which leads to the generation of a dumbbell structure. CPA (Ustar) [38] involves four recognition sites on the target DNA, and the inner primers lead to cross-priming after the first round. SMART-AMP (Riken Institute) [39] includes five

recognition sites on the target DNA as well as a fifth booster primer; single nucleotide polymorphism discrimination is facilitated through use of the MutS protein. **(d)** Methods based on strand-displacing polymerization from inherently circular targets in RCA (Molecular Staging) [40], or from padlock probes that are circularized through action of a ligase in RAM (Thorne Diagnostics) [41]. In both cases, branched amplification can be initiated through a second primer.

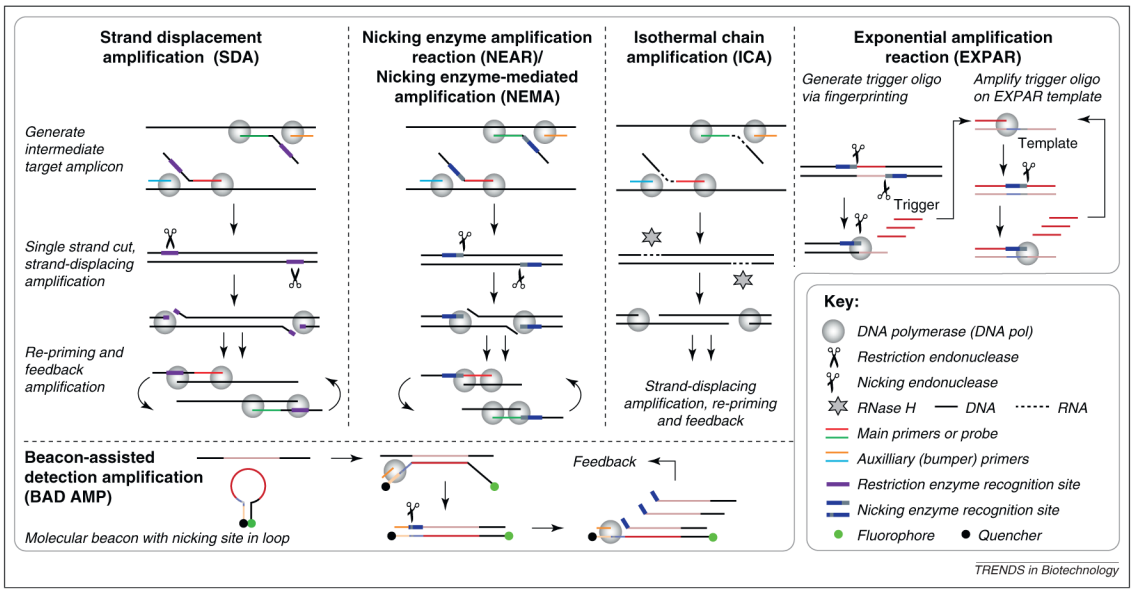

Figure 3.

Isothermal nucleic acid amplification methods that are based on polymerase extension plus a single-strand cutting event. In SDA (Becton Dickinson) [42,43], NEMA (Ustar) [45] and ICA (RapleGene) [46], an intermediate target is generated using strand-displacing amplification via sacrificial outer bumper primers. NEAR (Ionian Technologies) [44] does not use bumper primers and involves shorter amplicons than the other methods. In SDA, phosphothioates are incorporated into the amplicon during polymerization so that a restriction endonuclease only cuts one strand. NEAR and NEMA both use nicking endonucleases that are inherently single strand cutting. In ICA, a single strand cut is facilitated through RNase H and DNA–RNA–DNA chimeric primers. After the single-strand cutting event, amplicons are generated through strand-displacing amplification, for short amplicons further facilitated by thermal denaturation. These amplicons re-prime, and lead to exponential feedback amplification. EXPAR [47] amplifies short trigger oligonucleotides, which can be generated via the so-called “fingerprinting” reaction [48] from adjacent nicking enzyme recognition sites in genomic DNA. This is followed by rapid exponential amplification, mediated by a template sequence that contains two copies of the trigger complement that are separated by the nicking enzyme recognition site complement. BAD-AMP [49] uses a molecular beacon for signal generation and as a template for single-strand nicking and re-priming.

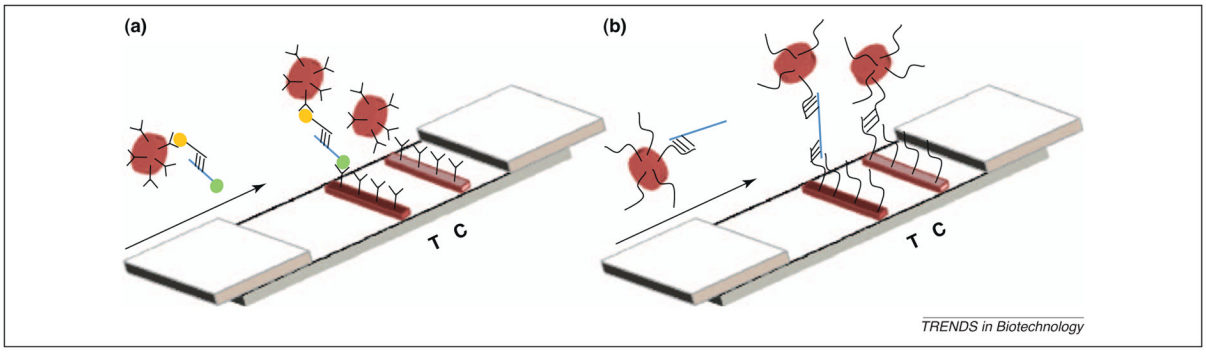

Figure 4.

(a) Antibody-dependent NALF, which uses antibody-conjugated colored particles and LF strips, and also requires an antigen-functionalized capture oligonucleotide and a target amplicon (blue) that contains an antigen. **(b)** Antibody-independent NALF involves direct hybridization of unlabeled target amplicon (blue) with oligonucleotide-functionalized colored particles and with oligonucleotides deposited on the LF strip.

Table 1

Example POC NAT platforms that are commercially available or close to market

Platform	Manufacturer	Sample prep included?	Amplification	Detection	Time to result (min)[a]	Website
GeneXpert	Cepheid	Y	PCR	RTF	<120	www.cepheid.com
Liat Analyzer	IQuum	Y	PCR	RTF	<60	www.iquum.com
MDx	Biocartis	Y	PCR	RTF	Unknown	www.biocartis.com
FL/ML	Enigma	Y	PCR	RTF	<45	www.enigmadiagnostics.com
FilmArray	Idaho technologies	Y	PCR	RTF	60	www.idahotech.com
Razor	Idaho technologies	N	PCR	RTF	<60	www.idahotech.com
R.A.P.I.D.	Idaho technologies	N	PCR	RTF	<30	www.idahotech.com
LA-200	Eiken	N	Isothermal (LAMP)	RTT	< 60	www.eiken.co.jp
Twista	TwistDX	N	Isothermal (RPA)	RTF	< 20	www.twistdx.co.uk
BART	Lumora	N	Isothermal (LAMP)	RTB	< 60	lumora.co.uk/
Genie II	Optigene	N	Isothermal (LAMP)	RTF	< 20	www.optigene.co.uk
SAMBA	Diagnostics for the Real World	N	Isothermal (similar to NASBA)	NALF	> 60	Not available
BESt Cassette[b]	BioHelix/ Ustar Biotech	N	Not included, but typically isothermal	NALF	N/A	www.biohelix.com; www.bioustar.com

[a]Time to result depends upon the particular assay. Longer times may be required for assays with a reverse transcriptase step.

[b]Device sold by BioHelix in the USA; manufactured and sold by Ustar Biotech in China.Abbreviations: RTB real-time bioluminescence; RTF real-time fluorescence; RTT real-time turbidimetry.

Table 2

Current isothermal nucleic acid amplification methods

Assay	Reaction temperature (°C)[a]	Reaction duration (min)[a]	Multiplex[b]	Rapid detection formats[c]	Target	Amplification product	Refs.
Methods based on RNA transcription							
NASBA	41[d]	105	Y	RTF, NALF	RNA (DNA)	RNA, DNA	[30]
TMA	60[d]	140	Y	RTF	RNA (DNA)	RNA, DNA	[29]
SMART	41[d]	180	N/A	RTF	RNA, DNA	RNA	[31]
Methods based on DNA replication with enzymatic duplex melting/primer annealing							
HDA	65	75–90	Y	RTF, NALF	DNA[e]	DNA	[32,33]
RPA	30–42	20	Y	RTF, NALF	DNA[e]	DNA	[34,35]
Methods based on DNA-polymerase-mediated strand displacement from linear or circular targets							
LAMP	60–65[d]	60–90	N/A	RTF, NALF, RTT, TE	DNA[e]	DNA	[36,37]
CPA	65	60	N/A	RTF, NALF	DNA	DNA	[38]
SMART-AMP	60	45	N/A	RTF	DNA[e]	DNA	[39]
RCA	65	60	N/A	RTF	DNA[e]	DNA	[40]
RAM	63[d]	120–180	N/A	RTF	DNA[e]	DNA	[41]
Methods based on polymerase extension/strand displacement, plus a single strand cutting event							
SDA	37	120	Y	RTF, NALF	DNA[e]	DNA	[42,43]
NEAR	55	10	Y	RTF, NALF	DNA[e]	DNA	[44]
NEMA	65	30	N/A	NALF	DNA	DNA	[45]
ICA	60	60	N/A	RTF	DNA	DNA	[46]
EXPAR	55	10–20	Y	RTF, NALF	DNA	DNA	[47,48]
BAD AMP	40	40	N/A	RTF	DNA	DNA	[49]
PG-RCA	60	60–120	N/A	RTF	DNA	DNA	[50]

[a] Typical incubation temperature and reaction time, variability might exist.

[b] Capability to multiplex, defined as the ability to amplify simultaneously at least two different targets (or one target and an internal control): yes (Y) or data not available (N/A).

[c] Most commonly reported formats only. Other formats might exist, but are not included here.

[d] Initial incubation at a higher temperature is sometimes recommended.

[e] RNA can be the target if a reverse transcriptase step to generate cDNA is first included.

Abbreviations: RTF, real-time fluorescence; RTT, real-time turbidity; TE, turbidity-related endpoint, including fluorescence and colorimetric enhancement.