



Advances in Molecular Diagnostic Approaches for Biothreat Agents

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13.1 Introduction

Bioterrorism is the purposeful and intentional delivery of bioweapons by non-state actors to harm humans, livestock and agriculture. The Biological Weapon Convention (BWC) was established in 1972, banning the development, production and stockpiling of microorganisms and toxins that can lead to mass destruction. The Center of Disease Control and Prevention classified bioterrorism agents into three major categories: Category A, B and C. The categorization depends upon the following criteria: (1) the easy dissemination (person-to-person) of biologically active infectious agents, (2) a major public health concern and causing a high case fatality rate both in humans and/or livestock, and (3) the creation of panic and terror among people [1].

Prior to the development of high-end diagnostic tools, basic microscopy techniques used to be a primary tool in the diagnosis of disease. Microscopy played a pivotal role in diagnosis of bacterial pathogens and helped to enumerate pathogens in different samples (e.g., blood, urine, sputum, stool). Different stains (e.g., gram-stain, Rhodamine, Indian ink) on histopathological specimens differentiated and classified several bacterial strains. The non-cultivable viruses, like rotaviruses, hepatitis A virus, and Norwalk virus, were also identified through microscopic techniques. Diagnosis was based on the morphological features of bacteria and virus particles, which required large amounts of samples. In the case of viruses, a high viral titer (10^5 to 10^6 virus particles/ml) was needed. In addition, sample losses were high during sample preparation (electron microscopy, EM), either due to sample dehydration or charging of biomolecules (bacteria and viruses), which used to result in reduced contrast and performance. Further, microscopic techniques

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required expertise and trained manpower. Advancement in the technology led to the development of transmission electron cryomicroscopy, for which specimens are preserved at $-150\text{ }^{\circ}\text{C}$ for maintaining the amorphous texture and for avoiding any ice-crystal damage. Nonetheless, the technique requires a high amount of sample ($\sim 1\text{ mg/ml}$ of virus particles). In addition, bacteria are far too thick for transmission electron cryomicroscopy to allow resolution of structural details [2–4]. The amalgamation of EM with cell culture practices greatly contributed in the diagnosis of bacterial and viral pathogens.

Immunochromatographic assays, polymerase chain reaction (PCR) and enzyme-linked immunosorbent assays (ELISAs) have been developed for the detection of biothreat agents. With different types of immuno-based and chip-based biosensors, biothreat agents are quickly and sensitively detected. Scientists are encouraged to use nucleic acid amplification tests (NAATs), for real-time detection of DNA and RNA using fluorescent dyes through multiplex polymerase chain reaction (PCR) assays, in which multiple pathogens can be diagnosed in a single run. PCR amplification cannot produce a large amount of high-throughput data, but next-generation sequencing (NGS) platforms provide an unbiased-in depth analysis of near-complete genomes. Molecular diagnostic techniques (Microarrays, PCR and NGS) are time saving and precise in the detection and discovery of micro-organisms in a given sample. Development of quantitative, multiplex platforms is imperative for the specific diagnosis of such pathogens, and these platforms can be easily used as point-of-care (POC) molecular diagnostic tools. With automation and miniaturization, microfluidics-based chips can be easily used as a POC platform. In addition, microfluidics integrated with various other techniques such as PCR, ELISA, and isothermal amplification enhance the sensitivity and specificity of the POC platform within financial and time constraints. These molecular diagnostic tools can be used in surveillance programs intended for biodefense preparedness.

13.2 Latest Diagnostic Techniques

13.2.1 Immunological Methods

13.2.1.1 Lateral-Flow Immunochromatographic Assays

Detection of biothreat agents by (monoclonal or polyclonal) antibodies is a standard approach in clinical diagnosis. The basic principle is the formation of an antigen-antibody complex on a solid surface followed by the visual readout. These tests are qualitative and semi-quantitative (intensity of color readout), quick (less than 20 min), cost-effective, user-friendly, and require minimal settings. Therefore, these assays are an attractive tool for POC diagnosis/prognosis in a surveillance program.

Immunochromatographic lateral flow assay are used for the detection of pathogens. Gold nanoparticles or carbon/silver/magnetic beads, upconverting phosphors and latex colored bead capture detector antibodies (a detector antibody detects the presence/absence of an analyte in a given sample) on a dry solid surface.

Once the diluted specimen is added, the detector antibodies form a complex with the cognate antigen, and the color readout indicates the test result [5–7]. A multiplexed suspension-based immuno-array can detect five biothreat agents (i.e., *Yersinia pestis*, severe acute respiratory syndrome coronavirus [SARS-CoV], staphylococcal enterotoxin B (SEB), ricin) in powder samples with a limit of detection (LOD) of 111 colony forming units (CFU)/ml, 20 CFU/ml, 110 pg, 5.4 ng, and 2 ng respectively [8].

Many rapid diagnostic kits (RDKs) are available for the detection of biothreat agents. The ENVI Assay System Gold (EnviroNics) is a lab-in-a-box device that detects various biothreat agents within 20 min [9]. New Horizon Diagnostic Inc. offers detection kits available for various biothreat agents in patient, environment, food, and water samples [10]. Response Biomedical Inc., developed RAMP® (Rapid Analyte measurement platform) for the detection of influenza A (FluA) and B (FluB) viruses, *Bacillus anthracis*, variola virus, and ricin and botulinum toxins. This platform consists of a fluorescent reader and a disposable single-use cartridge that provides specific on-field detection of pathogens [11]. Similarly, GenPrime developed a bio-detection system for the detection of US Centers for Disease Control and Prevention-listed bioterrorism agents along with others agents [12] (Table 13.1).

13.2.1.2 Enzyme-Linked-Immunosorbent-Assay

ELISA is the most widely used method detecting enzyme-linked antigen-antibody complexes on a solid phase. The technique is user-friendly, sensitive, specific, and cost-effective [14]. The method quantifies the antigen/antibody complexes in a given sample and is used as an *in-vitro* diagnostic tool in clinical laboratories.

Different types of ELISAs are in use for the pathogen detection such as: sandwich, direct, indirect, and competitive ELISAs. These different formats detect biothreat agents such as *F. tularensis*, *B. anthracis*, *Y. pestis*, *Brucella abortus*, *Burkholderia pseudomallei*, Ebola virus (EBOV), or Marburg virus [15–21].

The integrative multiplex assay and sampling system has been reported to identify eight biothreat agents (*B. anthracis*, *F. tularensis*, *Y. pestis*, *Brucella* spp., *B. mallei*, ricin toxin, botulinum toxin A/B, and SEB) within 15 min. The assay is a coded strip containing immobilized antibodies to identify these agents from surface, powder or liquid samples (<http://www.bbidetection.com/products/biothreat-detection-immass-device/>).

13.2.1.3 Time-Resolved Fluorescence Immunoassay and Immunomagnetic Separation Electrochemiluminescence Assay

Time-resolved fluorescence immunoassay (TRF) is a version of immunoassay with extended fluorescence decay time. The long fluorescence signal helps to measure the signal after the background noise has subsided. TRF assays can detect botulinum toxin in patient samples at low concentrations (0.01 pM) [22]. The commercialized platform, dissociation-enhanced lanthanide fluorescence immunoassay, was developed by Perkin-Elmer for the detection of various pathogens. The test is similar to ELISA: a 96-well plate is coated with streptavidin and Europium- (Eu^{3+} , lanthanide

Table 13.1 Different platforms for detection of pathogens and toxins

	Product	Company	Pathogens/Toxins detected	Total run time
1.	NIDS [®] handheld biothreat assay and handheld reader	ANP technologies	<i>Bacillus anthracis</i> , vaccinia virus, Brucellae, Venezuelan equine encephalitis virus, <i>Listeria</i> , SEB, <i>Francisella tularensis</i> , botulinum toxin A, <i>Vibrio cholerae</i> , <i>Escherichia coli</i> 0157, ricin, <i>Coxiella burnetii</i> , <i>Yersinia pestis</i> , <i>Salmonella</i> sp.	15 min
2.	PRO STRIPS 5 Agent biowarfare threat detection kit	ADVNT Biotechnology	<i>B. anthracis</i> , ricin, botulinum toxin A and B, <i>Y. pestis</i> and SEB	Not specified
3.	Zephyr	PathSensors, Inc.	<i>B. anthracis</i> , <i>Y. pestis</i> , ricin, <i>F. tularensis</i> , orthopoxviruses, <i>Salmonella</i> sp.	15 min
4.	PrimeAlert	GenPrime, Inc.	Ricin, botulinum toxin, SEB, <i>F. tularensis</i> , <i>Y. pestis</i>	15 min
5.	ENVI Assay System Gold	Environics Oy	Ricin, botulinum toxin, SEB, orthopoxviruses, <i>B. anthracis</i> , <i>Y. pestis</i> and <i>F. tularensis</i>	<20 min
6.	Aegis 1000	BioDetection Instruments (BDI)	Foodborne pathogens, toxins, infectious agents, protein biomarkers, waterborne pathogens	<30 min
7.	RAMP 200 Biowarfare Detection System	Response Biomedical Corporation	<i>B. anthracis</i> , ricin, botulinum toxin, variola virus	>30 min

Adapted from <http://www.cbrmetechindex.com/Biological-Detection/Technology-BD/Immunological-BD-T/Lateral-Flow-Hand-Held-Immunoassay-BD-I> [13]

series) tagged detector antibody. The immobilized Eu-tagged antibody produces a fluorescent signal when it interacts with the antigen and releases Europium. The limit of detection of the system is 4–20 pg/ml [23].

In the IMS-ECL assay, the immunomagnetic separations are coupled with electrochemiluminescence assay (ECL) for the rapid detection of biothreat agents, *B. anthracis*, SEB toxins, and *Clostridium botulinum* in clinical specimens within 1 h with the LOD ranging from 1 pg/ml to 100 pg/ml [24].

13.2.2 Biosensors

A biosensor is an analytical device integrated with biologically active components, bioreceptors, and transducers for detection of analytes in a given sample. Bioreceptors may be enzymes, antibodies, single-stranded DNAs (ssDNA), aptamer proteins, or cells. Methods for detection of pathogen/toxins using biosensors are rapid, sensitive, and cost-effective. Immunoassays-based biosensors detect specific antigens in patient sample(s) or identify biomarkers for studying the host-immune

response during infection [25]. Scientists using biosensor(s) may opt for either label-free assays or labeled assays for detection of an analyte. A label-free assay detects the presence of an analyte directly through a transducer that can be an optical, electrical or mechanical [26]. In contrast, labeled assays use a second detector coupled with an enzyme, fluorophore or radioisotope to detect an analyte [14, 27–29].

The integrated multiplexed microarray biosensor CombiMatrix ElectraSense microarray detects *Y. pestis*, *B. anthracis*, and SEB. The LOD for SEB and *Y. pestis* is 5 pg/ml and 10^6 CFU/ml, respectively [30]. The electrochemical multiplexed immunosensor with the indirect labeled assay can diagnose *F. tularensis* infection with a LOD of 1000 CFU/ml within 25 min [31]. Furthermore, a piezoelectric immunosensor identifies *F. tularensis* with an LOD of 10^5 CFU/ml within 5 min [32].

Biosensor-based detection of EBOV glycoprotein (GP_{1,2}) is based on surface plasmon resonance and a quartz crystal microbalance sensor [33]. The on-paper gene circuit and visualization platform detects small molecules and RNA and strain-specific EBOV molecular patterns. This platform is cost-effective, quick and sensitive and can be used in industry, research, and biodefense programs [34].

A cell-based biosensor named CANARY (Cellular Analysis and Notification of Antigen Risks and Yields) developed at Massachusetts Institute of Technology detects emerging disease pathogens or biothreat agents relevant in sectors such as biodefense, agriculture, and food safety. Within the CANARY biosensor, engineered B lymphocyte cells express a calcium-dependent bioluminescent protein, aequorin, coupled with an antigen-specific membrane-bound antibody. The antigen-antibody binding activates an intracellular calcium ion channel, and aequorin will emit light [35, 36]. The CANARY technology was commercialized by PathSensors Company for developing the BioFlash-E Biological Identifier and by Zephyr for screening liquid and powder samples for biological threat agents and toxins. The biosensor is a stand-alone, state-of-the-art device that can be used for both indoor and outdoor applications with an LOD of less than 100 CFU/ml within 5 min and 2–15 min, respectively [35]. The various types of advanced biosensors and their applications in the detection of biowarfare agents and infectious pathogens were extensively reviewed elsewhere [37]. The various types of biosensors used for the detection of biothreat agents are listed in Table 13.2.

13.2.3 Nucleic Acid-Amplification-Based Techniques

The development of PCR by Kary B. Mullis in 1987 revolutionized molecular diagnostics in clinical laboratories. PCR rapidly detects both biothreat agents and other pathogens in small patient samples in minimal time without compromising sensitivity or specificity. The basic principle of PCR is the isolation of nucleic acid (DNA, RNA) from a given sample and amplification by using a set of primers and thermostable polymerases. Later, the amplified products can be analyzed on gel electrophoresis or by using fluorescence-based detection systems.

Table 13.2 Biosensors for detection of biothreat agents

S. No.	Transducer	Biothreat agents	Limit of detection	Readout time	Samples tested	References
1	Electrochemical biosensor with gold-nanoparticle	Botulinum neurotoxin type E	10 pg/ml to 10 ng/ml	65 min	Orange juice and milk	[38]
2	Impedometric biosensor with gold nanoparticle	<i>Brucella melitensis</i>	4×10^5 CFU/ml	1.5 h	Milk	[39]
3	Electrochemical biosensor with bimetallic gold and palladium nanoparticles on boron-nitride nanosheets	<i>B. anthracis</i> surface array proteins	5 pg/ml to 100 ng/ml	1 h	Cell culture	[40]
4	Surface plasmon resonance and electrochemical impedance spectroscopy	<i>Brucella abortus</i>	0.05 pM	10 min	Cell culture	[41]
5	Surface plasmon resonance with antibody against F1 antigen	<i>Y. pestis</i>	10^6 CFU/ml	1 h	Environmental samples	[42]
6	Surface plasmon resonance coupled with electrochemical impedance spectroscopy	Botulinum neurotoxin A	0.045 fM		Cell culture	[43]
7	Piezoelectric Immunosensor coupled with quartz crystal microbalance (QCM) and covalently immobilized <i>F. tularensis</i> antigen	<i>F. tularensis</i>	5×10^6 cells/ml	35 min	Cell culture	[44]
8	Quartz crystal microbalance	staphylococcal enterotoxin A (SEA)	0.02 mg/L	25 min	Milk	[45]

Originally developed PCR was used for virus detection in early 1990s [46]. However, post-amplification contamination and false positives were major drawbacks. New and improved versions of PCR have been introduced, called Nucleic Acid Amplification Tests (NAAT). Following the detection of SARS-CoV in 2003 and swine influenza A (H1N1) virus in 2009, NAAT has become an integrative part for clinical diagnostics and detection of bio threat agents [47, 48]. Real-time PCR, quantitative-PCR (q-PCR), and reverse transcription-PCR (RT-PCR) are widely used for the detection and quantification of pathogens [49]. The q-PCR platform quantifies the amount of amplified product in “real-time” by using fluorescent dyes such as SYBR green and light emission upon extension primers, or using probe-based detection systems such as TaqMan™, scorpion probes, and molecular beacons. In RT-PCR, the complementary-DNA (cDNA) copy is synthesized by using reverse transcriptase, which can be amplified later by using gene-specific primers and polymerase enzyme. The human and avian influenza A viruses can be detected in patient samples by using RT-PCR [50, 51].

TaqMan probes have been developed by combining gene specific primers with fluorescently labeled probes. TaqMan chemistry employs the 5'-3' exonuclease activity of Taq polymerase, which cleaves the probe-containing reporter and quencher [52]. TaqMan-based qPCR panels detect and quantify filoviruses, arenaviruses, and hantaviruses with an LOD of 0.001–10 PFU/PCR [53, 54].

Although probe-based or non-probe-based methods are commercialized for pathogen detection the detection of RNA viruses is still a challenging task. The error-prone replication of RNA viruses results in changes in target gene sequences, making standard primer/probes ineffective over time. Consensus PCR assays based on primers and probes with wobble codes (degenerate primers) can detect various biothreat agents such as paramyxoviruses in bats and rodents [55, 56].

In nested PCRs, two sets of primers (hemi-nested and fully-nested) are used for amplification. This method detected influenza A virus in 2001 [57, 58]. Several laboratories do not use nested-PCR due to increased work load with double amplification process and higher chances of contamination [57, 59].

Multiplex RT-PCR has been increasingly used by diagnostic laboratories for the detection of multiple pathogens in a single run. The technique is efficient and less time-consuming than singleplex PCR, even in detecting co-infections in patient samples. This technique minimizes false positives, is highly sensitive, and aids in better diagnosis and prognosis than singleplex PCR. The multiplex RT-PCR platform detects and differentiates between the influenza A, B and C viruses [60].

During the Operation Iraqi Freedom 2003, US Marines troops were infected with multiple *Shigella* sp. and Norwalk virus, resulting in an outbreak of gastroenteritis. Multiplex-RT-PCR, enzyme immunoassay and sequencing technologies confirmed the presence of these pathogens [61]. The novel set of primers and probes are used with an LOD of >95% in *in-vitro* transcribed RNA technique [62]. The multiplex qRT-PCR platform detects EBOV and Marburg virus by using conserved regions of the genomes of these viruses with the sensitivity of 28.6 copies/μl and 30.5 copies/μl, respectively [63]. The outbreak of swine influenza in 2009 led to the development of multiplexed PCR-microfluidics and a silicon nanowire module for sequencing.

These POC devices have a sensitivity of 20–30 µg/µl to detect H1N1 influenza A virus in samples [25]. In addition, the World Health Organization (WHO) licensed RT-PCR for detection of Middle East respiratory syndrome coronavirus in 2012 [64]. Nguyen et al. [65] developed a multiplex PCR assay for the detection of *E. coli*, *Salmonella* and *Listeria monocytogenes* in patient samples and food samples with the sensitivity of 10 CFU/ml [65].

Many multiplex PCR kits have been developed for the quick detection of various biothreat agents. Altogether, the sensitivity and specificity of multiplex PCR assays are very good, and the assays can be used in routine diagnosis [66]. The Food and Drug Administration (FDA) licensed the use of the xTAG[®] respiratory virus panel (developed by Luminex Corporation, USA), which is a multiplex molecular method for the detection of respiratory viral infections. Pillet et al. 2013 reviewed elsewhere the six commercially available kits for the diagnosis of acute respiratory infections [67]. Human respiratory syncytial virus (type A and B) and FluA and FluB nucleic acids can be detected in one-step multiplex RT-PCR assay named, ProFlu-1 (developed by Prodesse, Waukesha, WI) [68].

The GeneXpert technology was developed by Cepheid, USA, and is based on the use of microfluidics and multiplexed RT-PCR for the detection and diagnosis of various pathogens. In 2010, WHO endorsed the GeneXpert *Mycobacterium tuberculosis* and rifampicin resistance assay for the diagnosis of multi-drug resistant and extensively-drug resistant tuberculosis. This platform is rapid, user-friendly, and has a clinical sensitivity of 98–100% and a specificity of 99% [69–73]. The GeneXpert platform detects and analyzes *B. anthracis* in clinical isolates within 90 min and has an LOD of 10 CFU/ml and 100% specificity [69]. Methicillin-resistant *Staphylococcus aureus* infections are also rapidly diagnosed by using the GeneXpert platform from clinical specimens [74].

After the Ebola virus disease outbreak in 2013, the GeneXpert platform was endorsed by both WHO and FDA under emergency use authorization. The assay used for the diagnosis of EBOV performed better in the field compared to other RT-PCR assays and culture methods [54, 75–77]. Real-time PCR or immunofluorescence assays are used to diagnose acute Q fever using clinical specimens (blood or formalin-fixed tissues) [78, 79]. The Primerdesign Company commercialized the Genesig Easy kit for the detection of *Coxiella burnetii* with a specificity of 66.6% and a sensitivity of 100% [80]. LightCycler real time PCR coupled with RET probes (Roche Applied Science) can be used to diagnose orthopoxvirus infection with an LOD of 5–10 copies of virus DNA in 45 min [81].

A broad-spectrum PCR coupled with electrospray ionization mass spectroscopy run on an automated platform developed by Abbott-PLEX-ID [82] is able to detect 10 bacterial and 4 viral biothreat agents listed by the National Institute of Allergy and Infectious Diseases and the U.S. Department of Agriculture, and U.S. Department of Health and Human Services [83]. The IRIDICA is a combination of PCR and electrospray ionization mass spectroscopy techniques for quick and specific detection of pathogens in patient samples. Bloodstream infections, including both bacterial and yeast, may be analyzed by the IRIDICA BAC BSI assay [66].

13.2.4 Next Generation Sequencing

Sanger and Gilbert introduced DNA sequencing in 1977. Using their method, a DNA sequence can be deciphered by adding a terminal di-deoxy nucleotide phosphates (ddNTPs) labeled with fluorescent-dye by DNA polymerase in a reaction mixture which terminates the reaction. Later, the terminated nucleic acid stretches can be detected by capillary electrophoresis, and the laser excitation values are captured on charge-coupled device (CCD) camera. The disadvantages of the system include inaccurate read out, formation of DNA secondary structures, and limitation to the short length of DNA sequences. Next-generation sequencing (NGS) opened new horizons for molecular diagnosis but still has limited use in clinical diagnosis due to the requirement of trained personnel, long duration, and sophisticated setup. This technique can be used for the detection of homopolymer or repeat sequences.

NGS is useful in the characterization of pathogen genomes, genetic mutations or drug resistance patterns, and novel pathogen discovery [84–90]. NGS application includes an unbiased in-depth analysis of complete genomes [91], whole transcriptome shotgun sequencing (WTSS) [92], whole exome sequencing and methylation sequencing, or candidate gene sequencing. Another major application of NGS includes metagenomic sequencing, which detects multiple microorganisms in homogenous or heterogeneous samples simultaneously even if microorganisms are present in low abundance. Further, NGS can be applied to non-cultivable microorganisms. The future of NGS promises great potential for the development of precision medicine. Second generation NGS includes the construction of cDNA libraries for amplification and sequencing genomes. The libraries are synthesized by fragmenting DNA strands and ligating them with adaptor molecules. Once the libraries are constructed, the adaptor-ligated sequences are amplified through emulsion PCR which uses immobilized adaptor-ligated sequences on microbeads or bridge PCR which uses solid-surface to form colonies. NGS is efficiently used for the detection of biothreat agents [93].

13.2.4.1 454 Pyrosequencing

The GS20 was the first second-generation sequencing method commercialized by 454 Life Sciences in 2005 (later acquired by Roche in 2007). This technology employs pyrosequencing chemistry in which three different enzymes, adenosine 5' phosphosulfate (APS), luciferase, and apyrase are used. The pyrophosphates generated during the addition of nucleotides by DNA polymerases act as substrates for APS enzyme to produce adenosine triphosphate. Adenosine triphosphate is used for the conversion of luciferin to oxyluciferin by luciferase, and this conversion leads to the emission of light, which is captured by highly sensitive CCD cameras. Therefore, the amount of light produced is proportional to the number of nucleotides added to the growing chain. Apyrase degrades any unincorporated nucleotide.

The pyrosequencing system uses microscopic beads embedded with DNA sequences of 100–150 bp. The system amplifies the sequences by using emulsion PCR and provides 2,00,000 reads per run [94]. The upgraded versions of the GS20 genome sequencer, the 454 GS FLX Titanium platform, was introduced in 2007 and

provides 4–6 million reads of ≥ 400 bp-long sequences within 24 h [95]. The major drawbacks are the expensive reagents and inability of the system to distinguish between homopolymers (AAA or CCC), and hence the product was withdrawn from the market

13.2.4.2 Illumina Sequencing

Solexa introduced the HiSeq and MiSeq platforms for sequencing of shorter DNA templates in 2007. These techniques rely on the sequence-by-synthesis method using modified deoxyribonucleotide triphosphates labeled with fluorescent dyes for chain termination (similar to the Sanger method) and detection by a CCD camera. The 100–300 bp-long DNA ligated with adaptors is amplified on a solid surface through “flowcells” for bridge PCR amplification. The clonal amplification results in approximately more than 1 million copies of each 100–300 bp-long template. The laser excitation captures the emitted light and records the first base, the reaction is called “reversible terminator reaction”. Thereafter, the cycle continues, and multiple sequence reads are recorded, aligned and compared to the reference template. Unlike 454 Pyrosequencing, which produces 1 million reads, the Hi-Seq platforms produces 120–1500 GB reads in 3–10 days, whereas the MiSeq platforms can produce up to 0.3–15 GB reads within 1–2 days for clinical testing or laboratory purposes [96]. The Illumina can detect *B. anthracis* both from the soil and aerosol samples, with a LOD of 10 genomic copies of DNA [97].

13.2.4.3 Sequencing by Oligonucleotide Ligation and Detection

Sequencing by oligonucleotide ligation and detection (SOLiD) was commercialized by Life technologies and released by Applied Biosystems in 2008. The technique is mostly similar to Roche or Illumina sequencing but differs by using DNA ligases during sequencing. After the library preparation and colony formation, sequences are detected by a modified probe named “interrogation probe.” The probe is an octamer consisting of degenerate sequences covalently attached to a fluorescent dye. The first two bases are specific to the DNA template and provide 16 different combinations for annealing (e.g., AT, AG, AA, AC). The interrogation probe provides a free 5′ phosphate group instead of providing a free 3′ hydroxyl group for ligation during each step of reaction by thermostable DNA ligases. The fluorescent signals are recorded by four different channels before the last 3 bp are cleaved for the next cycle. The newly synthesized sequence is removed, and a new complementary primer binds to the $n - 1$ region of DNA sequence and continues the annealing and ligation cycles. Following this approach, every DNA sequence is sequenced twice, thereby providing accuracy of 99.94% to the system. The read length of this technique ranges from 25 to 35 bp-long sequences [98]. The longer duration time (7–14 days) and requirement of skilled personnel are drawbacks of this system [99, 100]. Strain-specific polymorphisms of *B. anthracis* and *Y. pestis* were studied by using SOLiD high-throughput-sequencing [101] with high genome coverage and low error rates (>99.99 accuracy).

13.2.4.4 Ion Torrent

The ion torrent platform was released in 2010 by DNA Technologies. The method uses semiconductor chips incorporated with an ion-sensitive field-effect transistor (ISFET) sensor. The ISFET is an electrical biosensor that records the changes in concentrations of H^+ or OH^- ions. ISFET sensors are used to measure the changes in the H^+ concentration after every addition of a nucleotide by DNA polymerases during a sequencing step. The biosensors convert the chemical energy to electronic signals. These ion sensors are situated right beneath microwells containing microbeads covered with amplified target molecules. The microwell chip is successively flooded with only one type of nucleotide at a time. When the nucleotide is complementary to the target template molecule at the leading position, the nucleotide will be incorporated into a growing nucleic acid. The earlier versions (2011) of ion torrents could read a 50 bp-long sequence with 99.99% accuracy and produced 100 MB of data per run. The upgraded version, the ion personal genome machine (PGM™) reads up to 400 bp with >99.1% accuracy in 7.3 h [102]. The Ion proton system, the successor to the Ion PDM, can read up to 200 bp within 2–4 h with 10 GB of data per read and can be used for genome sequencing, *de-novo* sequencing, chromatin immuno-precipitation (ChIP), transcriptome, exome, methylation patterns, gene expression by sequencing, and small RNAs [96].

NGS platforms have been integrated with several molecular diagnostic techniques which amplify even small amounts of nucleic acid randomly. This platform can detect both known and unknown pathogens in the clinical samples using the integrated digital transcriptome subtraction technique [103, 104]. The detection of a highly divergent rhabdovirus, Bas-Congo virus, through NGS highlights the potential of sequencing for the detection of novel viruses during major disease outbreaks [105]. The Ion Proton platform (BGISEQ-100) and Roche 454 v4.9 were used for sequencing and phylogenetic and phylogeographic analysis of EBOV genomes during the major outbreak in 2013–2016 [106].

13.2.5 Microarrays

Microarray technology was established in 1995, when Ron Davis and Pat Brown used cDNA as a probe to quantitate the gene expression pattern in *Arabidopsis* [107]. Microarrays are miniature lab-on-chip devices made from glass or silicon, containing 25–70 mer-long complementary oligonucleotide probes spotted on the slide through mechanical deposition [107], inkjet printing [108] or through photolithography [109]. Every single spot on a microchip contains several folds (10 nM to 100pM DNA) of oligonucleotide copies. Depending upon the need, a microarray may have multiple signature probes for different microorganisms or a complete genome from a single microorganism. The commercialized microchips from Affymetrix and Illumina have been used for the detection of more than 20,000 to several million genes. Different types of microarrays are available (protein, peptide, carbohydrate, lipid,, tissue, reverse phase, or antibody microarrays).

Steps in microarray protocols include sample preparation, labelling of probes with fluorescent dyes, hybridization of the sample on the chip, washing and image acquisition followed by data normalization, analysis and interpretation.

A highly broad-spectrum multiplexed-microarray, resequencing pathogen microarray of tropical emerging infections (TessArray[®] RPM-TEI 1.0, TessArae LLC, Potomac Falls, VA) detects and differentiates between 84 pathogens and 13 toxins, including Category A, B and C Pathogens with an LOD of 10^4 per test. The tests are very sensitive and can further differentiate between EBOV, Machupo virus, and Lassa virus [110].

A pan-viral array named “ViroChip” was developed for the detection of viruses. The 70-mer oligonucleotide probes recognize the conserved regions (1600 probes) from 140 viral genomes, and can be used to detect viruses, such as human herpesvirus 8, human respiratory syncytial virus, parainfluenza virus type 3, adenoviruses, and multiple rhinovirus serotypes [111]. During the 2003 SARS outbreak, a DNA microchip was used for identification and sequencing the then-uncharacterized coronavirus isolated from SARS patients [112]. An improved and upgraded version of ViroChip detects viruses of 53 families and 214 genera using full-length viral genomes [113]. In addition, ViroChip was used for the diagnosis of acute respiratory tract infection in children. Similarly, the pan-microbial array named GreeneChipPm was developed for the quick and unbiased detection of bacteria in different samples. The platform includes densely spotted oligonucleotides (29,495) from the GreeneChipVr v1.0 database. For bacterial and fungal/protozoan detection, 11,479 16s rRNAs and 18s rRNA sequences were used for pathogen surveillance and detection [114, 115].

A low-density oligonucleotide microarray can detect neurotropic viruses (meningitis and encephalitis) from cerebrospinal fluid and non-cerebrospinal fluid samples. Multiplex PCR-amplified virus sequences are hybridized to a panviral central nervous system array slide for the detection of echoviruses, human herpes virus (HHV)-2, -4, -5, -6BA, -6B, and -7, vesicular stomatitis Indiana virus, and polyomavirus JC1 [116]. These microarrays integrated with microfluidic scans detect influenza A virus [117], *Y. enterocolitica* [118], and *Bacillus* sp. in milk and various other samples. The success of microarray depends upon the sensitivity of the system to detect pathogens in a single sample even if the pathogens belong to same genus.

13.2.6 Isothermal Amplification

Isothermal amplification is a robust technique for exponential amplification of nucleic acids at a single temperature. This technique is a sequence-specific amplification without using a thermocycler, thereby cutting down the cost of instruments and making the technique easily available for POC platforms. The processing steps in isothermal amplification can be divided into three categories: (1) sequence-specific amplification, (2) enzymatic duplex melting and primer annealing, and (3) strand displacement using multiple PCR primers or strand displacement from a circular target and PCR extension with single-strand cutting.

13.2.6.1 Sequence-Specific Amplification

Sequence-specific amplification is derived from a transcription-based amplification system, which includes transcription-mediated amplification (TMA) [119], nucleic acid sequence-based amplification (NASBA) [120], self-sustained sequence replication (3SR) [121], and signal-mediated amplification of RNA technology (SMART) [122]. Unlike PCR, the primer annealing and extension occurs at a constant temperature of 37 °C, thereby reducing time and cost of equipment. This method can easily be automated and multiplexed.

These methods depend upon RNA polymerase activity for amplifying the nucleic acids (ssDNA or RNA) at an isothermal temperature, which may range from 30 °C to 70 °C depending upon the reaction. The hot-start temperature of 95 °C breaks the double-stranded DNA into ssDNA; followed by the amplification at 41 °C (or for transcription-mediated amplification at 60 °C). Amplification occurs in two phases: during the linear phase, the promoter-primer containing T7 promoter region binds at the 5' end of the target sequence and synthesizes cDNA with the help of avian myeloblastosis virus reverse transcriptase. Subsequently, the RNA-cDNA hybrid formed is degraded by RNase H with the release of cDNA. A second forward primer binds to the cDNA containing the T7 promoter region and extends via reverse transcriptase. The newly formed strand enters the amplification phase, in which T7 polymerase binds to the DNA and synthesizes complementary RNA, again followed by the linear phase. Thus, billion-fold copies of RNA or DNA are generated within 90 min (Fig. 13.1) [123].

Signal Mediated Amplification of RNA Technology

SMART is another type of isothermal amplification. It is based on the identification and amplification of the target sequence in the sample with the help of two single stranded oligonucleotide probes (extension probe and template probe). The template probe contains a T7 polymerase promoter sequence and a transcription template. These probes hybridize together with the target RNA or DNA and form a three-way junction or “T-like” structure. The Bst DNA polymerase, which lacks 5'-3' exonuclease activity is obtained from *Bacillus stearothermophilus*, which can amplify the nucleic acid at 70 °C. Once amplified, the target RNA or DNA is used for synthesizing the complementary strand using the extension probe. Thereafter, T7 RNA polymerase synthesizes multiple copies of RNA from the formed DNA duplex (Fig. 13.2). An enzyme-linked oligosorbent assay quantifies the SMART-generated product. Amplicons are captured and detected by biotinylated probes and separated by microfluidics or through molecular beacons [125, 126]. SMART detects genomic DNA (10 ng) and total RNA (0.1 ng) from *E. coli* in a few hours [127].

13.2.6.2 Enzymatic Duplex Melting and Primer Annealing Method

This method includes three techniques that circumvent the initial heating step for denaturing the DNA or RNA duplexes. Under *in vivo* conditions, DNA amplification is performed at an isothermal temperature, at which the DNA duplexes are opened by helicases and topoisomerases. The recombinase polymerase enzyme for amplification (RPA), helicase-dependent amplification (HDA) and rolling circle

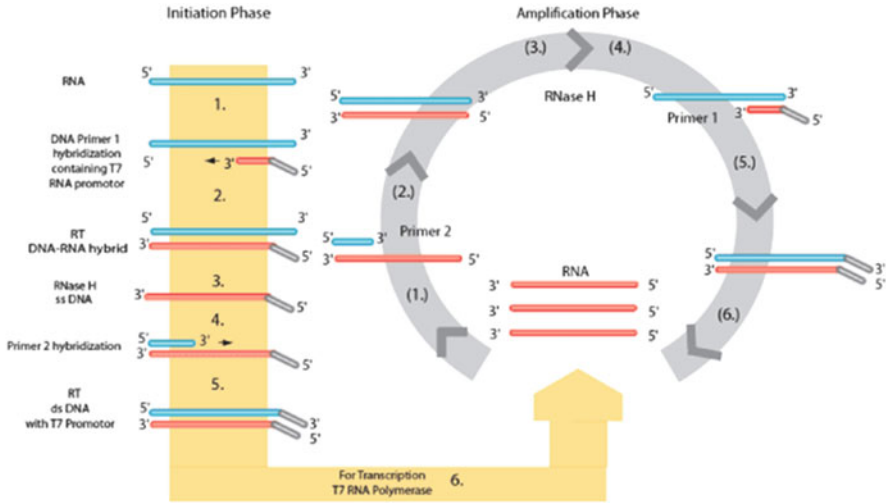


Fig. 13.1 Nucleic-acid-sequence-based Amplification (NASBA). Initial phase: 1. Annealing of DNA primer to RNA template; 2. RT for primer extension; 3. Removal of RNA by RNase H; 4. Annealing of second set of primer to the newly synthesized DNA template; 5. Double strands of DNA are synthesized by RT; 6. For transcription, T7 RNA polymerase produces several RNA copies. The amplification phase is similar to initial phase but more copies of RNA are produced steps 1–6. (Troger V and Niemann K 2015)

amplification (RCA) enzymes amplify the nucleic acids. This technique is less time consuming, than other Isothermal amplification based diagnostic techniques, and does not require any sophisticated instruments; therefore, this method can be adopted for POC diagnostic platforms.

Recombinase Polymerase Amplification

This technique utilizes three enzymes: recombinase, single strand binding proteins (SSBs), and strand displacement DNA polymerases. Initially, the recombinase enzyme pairs with the primer and binds to the target sequence, the SSBs then bind the displaced DNA strand and form a “D” loop like structure to stabilize the DNA. This displaced DNA provides a free 3'-hydroxyl site for DNA polymerase to bind and amplify the target sequence. Therefore, both the strands are amplified exponentially within 20 min. The amplified product can be visualized either by fluorescent probes or non-fluorescent probes. RPA can be easily multiplexed by using multiple primer set and can detect more than one disease simultaneously within min (Fig. 13.3). Crimean-Congo hemorrhagic fever virus (CCHFV-AY277672 Europe 1 strain) in patient samples was detected within 35 min using isothermal amplification [128]. A commercialized RPA kit (TwistAmp) was launched by TwistDx Inc.; Cambridge, UK, for the rapid detection of pathogens, like *Y. pestis*, within 1 h with a limit of detection for ssDNA and dsDNA up to $4.04 \times 10(-13)$ and $3.14 \times 10(-16)$

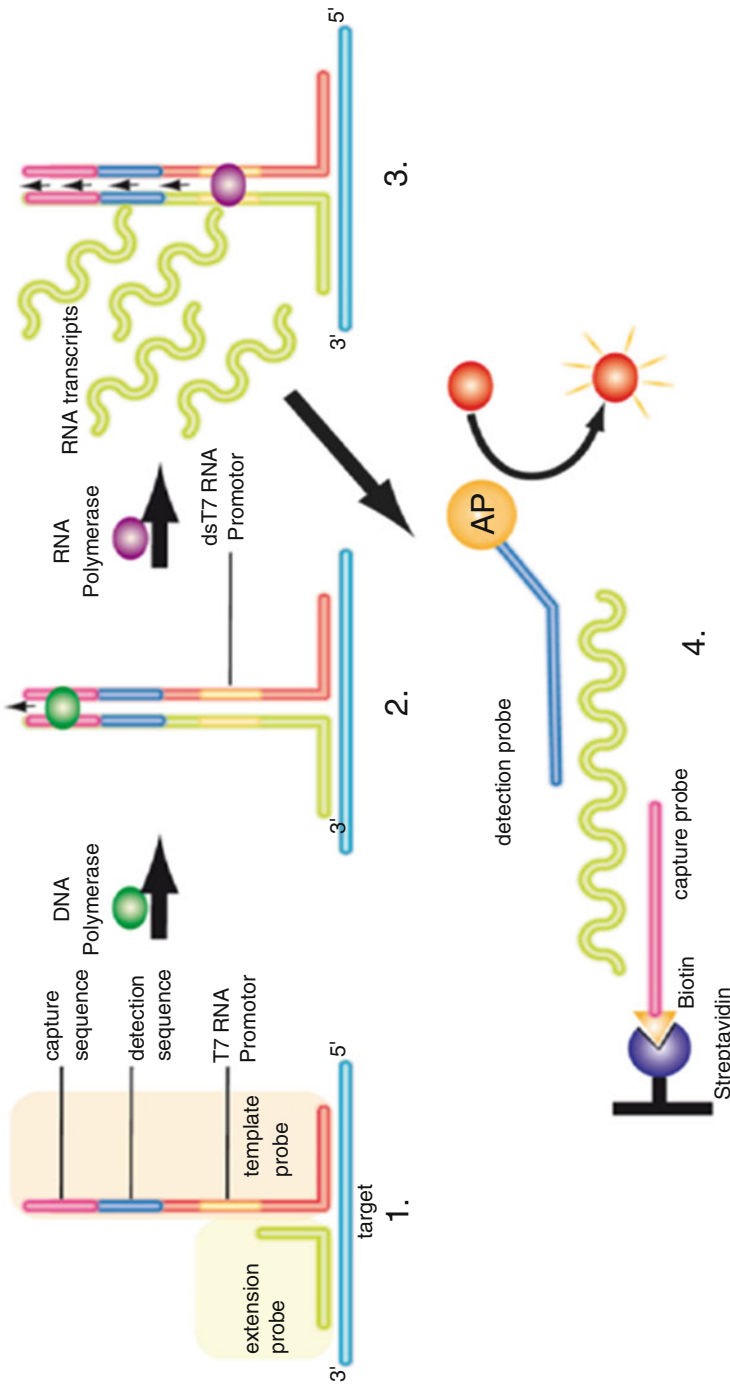


Fig. 13.2 Signal-mediated amplification of RNA technology (SMART). 1. The annealing of “extension” and “template” probes to the DNA template to form a three-way junction. 2. The extension probe is elongated by DNA polymerase, forming a double stranded T7 RNA promoter region. 3. The binding of RNA polymerase to the double strand T7 promoter produces several copies of RNA transcripts, serving as signal. 4. The signal is captured by an RNA-binding biotinylated probe, which is attached to immobilized streptavidin, and signal is detected by an alkaline phosphatase-linked probe following alkaline phosphatase substrate conversion [124]

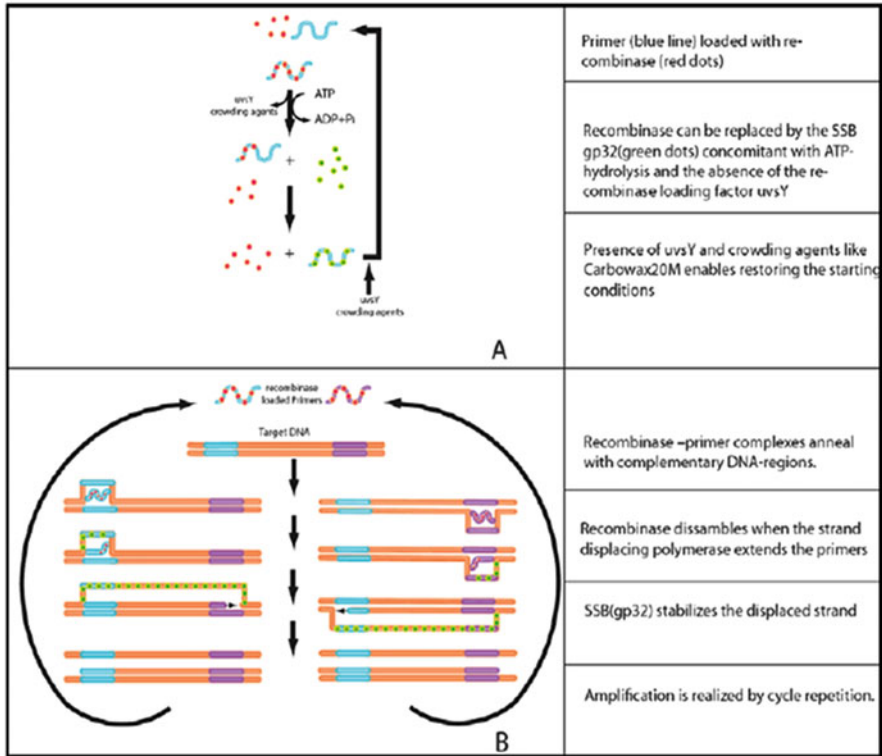


Fig. 13.3 Recombinase polymerase amplification (RPA). (a) The combination of the primer-recombinase complex and single-stranded-binding proteins (SSBs). (b) RPA cycle, in which the RPA complex anneals to the DNA template for initiation. Later, the strand displacement enzymes disassemble the recombinase and stabilize the strands by SSBs protein for further extension. Thereby, multiple copies are generated [124]

M [129]. *C. burnetii* was detected in formalin-fixed tissues and blood plasma of patients with 90% clinical sensitivity using RPA-isothermal amplification [130].

A RPA and RT-RPA fluorescent based POC platform for the detection of potentially hazardous bioterrorism pathogens (*Y. pestis*, *F. tularensis* and *B. anthracis*), gram positive and negative bacteria (*Salmonella enterica*), DNA (vaccinia and variola viruses) and RNA (EBOV, Sudan virus, and Marburg virus) was developed, with a LODs ranging from 16–21 molecules within 10 min [131].

Helicase Dependent Amplification (HDA)

Vincent et al. used DNA helicases to unwind duplex DNA or RNA and hybridize primers onto the target sequence. The enzymes separate the strands at an isothermal temperature of 37 °C, excluding the heating step [132]. The MutI protein is a mismatch DNA repair protein found in *E. coli* that activates the DNA helicases and separates the strands. The SSBs proteins stabilize the unwinded duplex. The

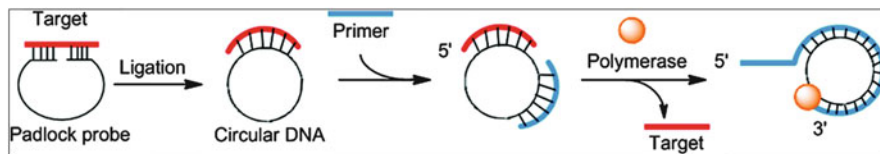


Fig. 13.4 Rolling circle amplification (RCA). The padlock probe anneals at a specific genomic location (ssDNA) forming a circular DNA and disassembles. The primers specific to the padlock probe and DNA polymerase anneal to the circular-DNA and amplify the template. These steps are performed at an isothermal temperature [124]

primers anneal to the target sequence, and DNA polymerases amplify the strands, thereby synthesizing million-fold copies of DNA within 120 min (Fig. 13.4). To further improve the sensitivity of the system, heat-stable DNA helicases derived from *Thermoanaerobacter tengcongensis* were used at 45 °C [133, 134]. The end-point results are quantified by using fluorescently-labeled probes, electrochemical detectors [135, 136], a droplet microfluidics system [137] or chip-based hybridization [138]. The HDA-TaqMan probe can detect *V. cholerae* and *B. anthracis* [139]. Motre et al. 2011 customized a HDA probe by reducing the detection time from 60 to 30 min [140].

Rolling Circle Amplification

RCA was first introduced in 1990s for amplifying the small circular DNA by using strand displacement DNA polymerases (e.g., phage phi-29 DNA polymerase of *E. coli*) [141]. RCA synthesizes ssDNA complementary to target DNA (10^5 copies from 1 DNA copy) [142, 143] at an isothermal temperature (30 °C). The method is sensitive, simple and easy to perform; therefore, this amplification method is considered as an attractive tool for POC diagnosis in clinical settings. RCA detects bacterial and viral DNA/RNA [144–146]. The amplified product can be detected by fluorescent dyes, biosensors, gel electrophoresis, electric signals, luminescence or colorimetric assays.

Padlock probes have been introduced with RCA for linear DNA amplification. The probes have two target sequences of the linear DNA at the 3' and 5' ends. These probes hybridize, circularize, and ligate on the target DNA and serve as a template for strand displacement DNA polymerases, simultaneously elongating and displacing the amplified product. The technique can be coupled with the set of primers that hybridize with the amplified product and synthesize hyper-branched structures (Fig. 13.5).

A liquid and solid phase hyper-branched RCA for the rapid detection of SARS-CoV RNA from clinical samples can detect a single-copy of SARS-CoV RNA in the patient sample and can be used in diagnostic setting for quick detection [147]. A colorimetric assay using hydroxy naphthol blue (HNB) coupled to hyper-branched RCA rapidly detected H5N1 influenza A virus with an LOD of 28 fM in clinical isolates. An upgrade version detects H5N1 influenza A virus using a fluorometric real time platform. The LOD of the system was 9 fM in clinical samples [148, 149].

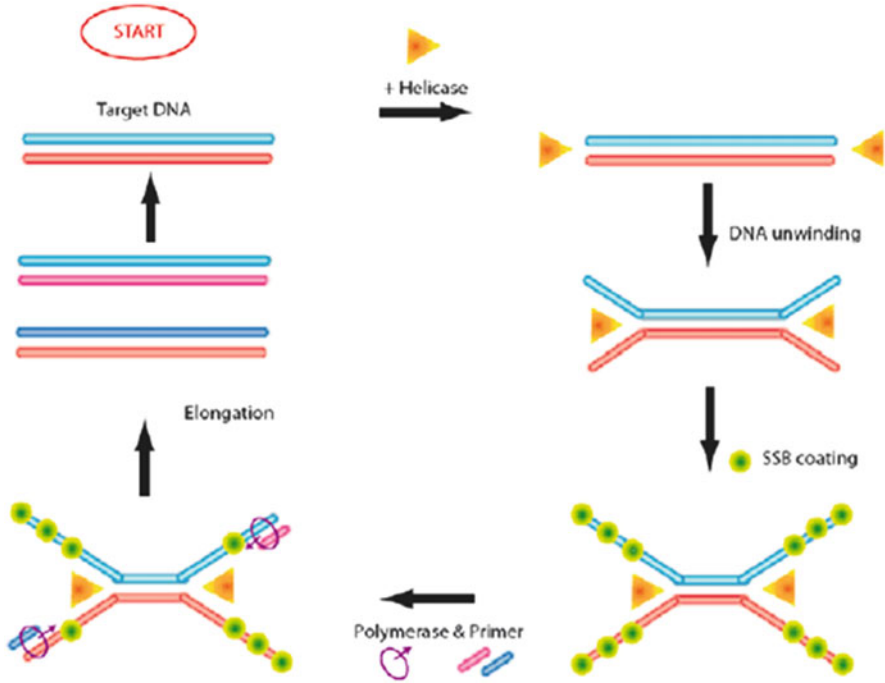


Fig. 13.5 Helicase-dependent amplification (HDA). The helicases bind to the DNA template and unwind the DNA template. The SBs stabilize the DNA strand. The primers and DNA polymerases bind to the DNA template and amplify the template [124]

Circle-to-circle amplification, another variant of RCA, detects circular DNA. Mahmoudian et al. 2008 integrated circle-to-circle amplification with a microchip electrophoresis system for the detection of *V. cholerae* with an LOD of 25 ng of bacteria within 65 min [150, 151]. An exponential linear RCA (with colorimetric detection for pathogenic bacteria, such as *E. coli*, *Salmonella* Typhimurium, and *Clostridium difficile*), was developed with LODs ranging from 10 fM to 100 fM of DNA. In this method, the amplified RCA products are cleaved by nicking endonucleases (Nb.BsrDI). The DNA fragments fold into G-quadruplex structures that form a complex with hemin and become DNazymes. The DNazymes undergo oxidation reactions that lead to a colorimetric detection [152].

Loop-Mediated Isothermal Amplification

Notomi et al., developed *loop-mediated isothermal amplification (LAMP)* in 2000 [153]. In this technique, the low copy number of DNA can be amplified up to 10^9 copies of target DNA within 1 h at an isothermal temperature. The basic principle is based on highly processive strand displacement DNA polymerases (*Bst* DNA polymerases) with four primer pairs (recognizing six different regions of target DNA), which amplifies the DNA and ensures the selectivity of target identification.

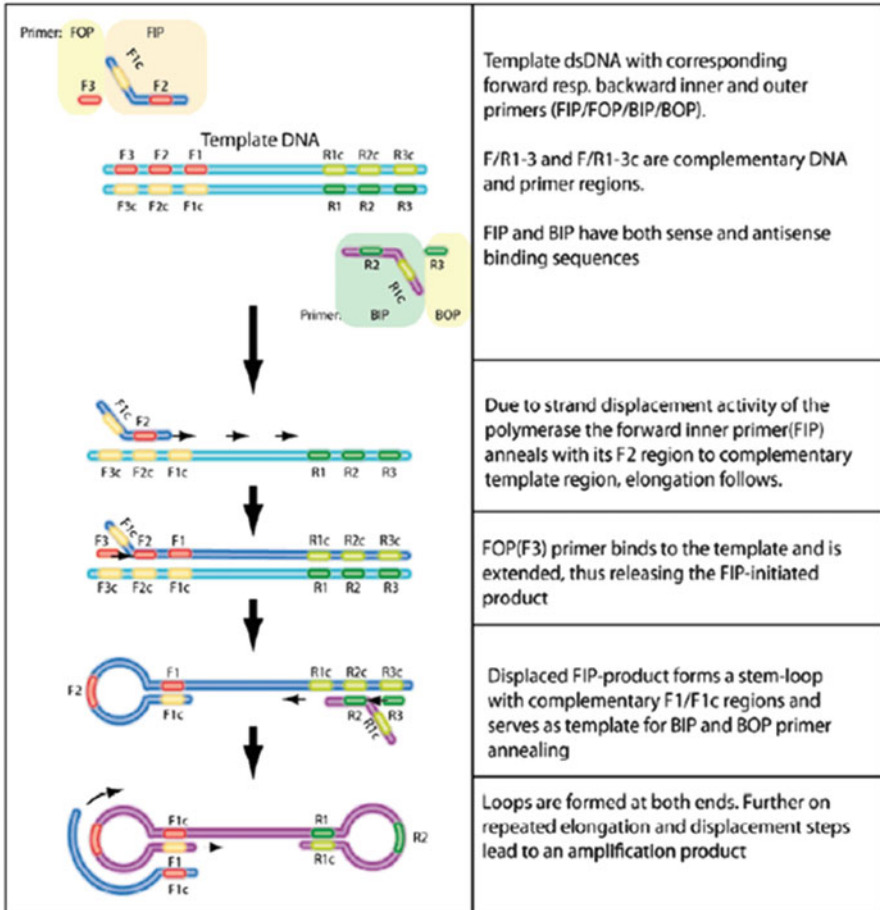
During the initial amplification, the four pairs of primers along with DNA polymerase amplify the DNA, forming a stem loop-like structure. The stem-loop structure becomes the template for cyclic amplification with two additional primer pairs and progresses to elongation and recycling. These additional primer pairs ensure the specificity of subsequent amplification, thereby providing a threefold amplification at every half cycle. The final amplified product consists of cocktail of stem-loop DNAs with different stem lengths and multiple loop structures (Fig. 13.6).

Apart from DNA, RNA can be amplified by LAMP using RT and *Bst* DNA polymerase [153]. Viruses, such as West Nile virus and SARS-CoV, can be detected by RT-LAMP assay [154, 155]. The amplified product is visualized by using a turbidimeter [155, 156], gel electrophoresis, fluorescent detection reagents, or by observing the production of white insoluble precipitate of pyrophosphate ions with Mg^{2+} ions [157]. The fluorescent detection reagent, calcein, and the DNA binding dye, SYBR green, used in RT-LAMP assay detect the amplified product [158, 159]. A low molecular weight cationic polymer, polyethylenimine, was used for genetic testing of West Nile virus by using *Bst* DNA polymerase at 63 °C [155].

LAMP is an excellent technique for POC applications. With advancement in microfluidic systems, LAMP has been successfully integrated for the specific and rapid detection of pathogens. A POC platform for the quick detection of pseudorabies virus used microLAMP (μ LAMP), for on-site application with an LOD of 10 fg/ μ l in less than 1 h at 63 °C [160]. A similar microfluidic platform with the facility of multiplexing was developed for the detection of bacteria, with an LOD of 270 copies/ μ l [160]. Wang et al. 2011 designed a device that combines isothermal amplification and magnetic-beads on microfluidics chip for the detection of methicillin-resistant *S. aureus* with an LOD of 10 fg/ μ l within 60 min [161]. *E. coli* and *S. aureus* were detected within 30 min with LODs of 20 and 30 copies/ μ l, respectively, by using an electrochemical sensor made from Ruthenium hexa-amine redox molecules to detect LAMP-amplified product [162].

13.2.6.3 Strand Displacement Amplification

The strand displacement amplification (SDA) method amplifies DNA by using a multifunctional probe that has a restriction endonuclease site at the 5' end and a strand-displacement DNA polymerase at the 3' end. This probe extends and displaces the nicked strand. Thereafter, the displaced strand acts as a template for the second probe, leading to exponential amplification. The amplified product can be detected by fluorescently-labeled probes, molecular beacons, or intercalating dyes. SDA has been integrated with various other detection methods like gold nanoparticle-based detection on lateral flow chip or a microelectric chip-array for the detection of several mutations [163, 164]. Westin et al. 2000 developed a multiplex SDA with multiple primers immobilized on a microarray chip [165], whereas Yang et al. 2002 developed a stacked micro-laboratory platform that uses electric field for immunoassays, DNA hybridization, and amplification by SDA. These systems efficiently analyze and handle different kinds of bacteria and toxins in the sample mixture [164].



Template dsDNA with corresponding forward resp. backward inner and outer primers (FIP/FOP/BIP/BOP).
 F/R1-3 and F/R1-3c are complementary DNA and primer regions.
 FIP and BIP have both sense and antisense binding sequences

Due to strand displacement activity of the polymerase the forward inner primer(FIP) anneals with its F2 region to complementary template region, elongation follows.

FOP(F3) primer binds to the template and is extended, thus releasing the FIP-initiated product

Displaced FIP-product forms a stem-loop with complementary F1/F1c regions and serves as template for BIP and BOP primer annealing

Loops are formed at both ends. Further on repeated elongation and displacement steps lead to an amplification product

Fig. 13.6 Loop-Mediated Isothermal Amplification (LAMP). Four different primer pairs bind to different locations of the DNA template along with DNA polymerases. The Bst (highly processive strand displacement polymerase) polymerase then displaces and extends the DNA template along with the primer pair to form multiple copies of the DNA template [124]

13.2.7 Microfluidics

Microfluidics technology uses the close dimensions, preferably a channel that holds fluids with a capacity of nl to few hundred μ l, for both qualitative and quantitative analysis of a given sample. The basic principle behind microfluidics technology is to create a laminar flow between the channels. The flow of fluid can be regulated by pressure-driven pumps, such as syringe pumps or electrokinetic pumps. Electrokinetic pumps apply electro-osmosis through the walls to generate pressure and flow of fluid. Microfluidics offer multiple applications in the field of molecular biology, enzyme kinetics, capillary electrophoresis, immunoassays, flowcytometry, cell manipulation, PCR amplification, DNA analysis, and clinical diagnosis [166]. The BV M-series

platform (BioVeris Corp., Gaithersburg, MD) detects antigen through electrochemiluminescence and sandwich ELISA. The antibodies, tagged with BV-TAG containing ruthenium, are immobilized on paramagnetic beads, and are passed through flow cells. The bead-antigen-TAG complexes are magnetically captured, and a voltage is applied to excite the TAG to emit multiple photons. The device is capable of detecting *E. coli* (O157), *Yersinia* sp., *S. Typhimurium*, and toxins [167, 168].

Microfluidic chips are designed by using glass, silicon or poly dimethylsiloxan, which hold small quantities of both sample and reagent in its channels for easy separation, detection and data analysis. Due to the miniaturization of the system, it is portable, cuts down the cost, and does not require any skilled labor. Therefore, microfluidics can provide a gamut of laboratory requirements on a single chip and that can be used as POC device in clinical settings.

The microfluidics platform integrated with methods such as PCR, isothermal amplification, and microarrays rapidly detects pathogens [117, 126, 137, 138, 161, 169, 170]. A lab-on-centrifugal-disk has been introduced in the field of POC devices; here, isothermal amplification (reverse transcriptase-LAMP) and optical detectors are fabricated on a centrifugal microfluidic system for the detection of influenza A virus strains. This POC device has an LOD of 10 viral copies and produces results in 47 min [171]. These lab-on-centrifugal-disk devices are more portable and robust compared to traditional assays for viral and bacterial disease diagnosis [172]. To further move in the “sample-to-result” analysis, 3M and Focus diagnostics developed a direct amplification disk that can amplify and detect pathogen in ~1 h.

13.3 Conclusion

Molecular techniques have revolutionized the diagnosis of biothreat agents and led to the development of POC devices. A number of FDA- approved and commercialized kits and devices have been launched for the rapid and quick detection of biothreat agents to strengthen the biodefense preparedness program. Although techniques like microarrays and NGS have potential to revolutionize the detection of biothreat agents, they are time-consuming and generate huge amounts of data that require skilled personnel for interpretation. Therefore, devices or platforms should be designed that are simple and user friendly and low in cost and maintenance for use in various biothreat detection surveillance programs in the field.

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