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Analysis of State-of-the-Art Diagnostics for Far-Forward Use

Jon M. Davis
Kristen A. Bishop
Catherine E. Scheible
Jay S. Shah

July 2022

IDA Paper P-33049

Log: H 22-000143

INSTITUTE FOR DEFENSE ANALYSES
730 E. Glebe Road
Alexandria, Virginia 22305

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About This Publication
This work was conducted by the Institute for Defense Analyses under contract HQ0034-14-D-0001, project FN-06-4947, "Medical CBRN Defense Analyses," for the U.S. Army Office of the Surgeon General (OTSG) Executive Agent. The views, opinions, and findings should not be construed as representing the official position of either the Department of Defense or the sponsoring organization.

Acknowledgements
The author wishes to thank Doug Schultz, Ted Cieslak, and Mike Finnin for their thoughtful reviews.

For More Information:
Dr. Jon M. Davis, Project Leader
jdavis@ida.org, 703-933-3570
Ms. Jessica L. Stewart, Director, SFRD
jstewart@ida.org, 703-575-4530

730 E. Glebe Road

Alexandria, Virginia 22305

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Executive Summary

The ability to accurately diagnose an illness in a timely manner is a key component of any strategy aimed at protecting people from disease that occurs naturally or otherwise. Protecting, maintaining, and recovering the health of the force is vital to ensuring ongoing military operations, and it is an especially important component in determining whether a disease is the result of a potential biological agent attack. In a previous analysis, the Army Office of the Surgeon General (OTSG) tasked the Institute for Defense Analyses (IDA) with assessing the optimal operating space for diagnostics to be able to identify an outbreak in the earliest stages, including far-forward deployment.[1]

Diagnostic modalities are most effective when deployed at the correct location so they can be used, with the correct sample specimen, at the correct point in disease progression. Deploying diagnostic technologies far-forward (for example, at Role 1 medical treatment facilities [MTFs]) may provide vital information, if it meets the other criteria of testing the correct sample drawn at the correct time. Diagnostics that are able to be deployed far-forward will likely have to meet certain criteria, such as ruggedness and portability, while maintaining their sensitivity and accuracy. Because diagnostic technology is an ever-evolving field, OTSG asked IDA to analyze the current state-of-the-art in diagnostic technology to determine if there are options that can potentially be used far-forward.

The research team performed a literature review to identify advances made in various diagnostic techniques, procedures, assays, and reagents; the scope of the literature review was limited to peer-reviewed articles published between 2019 and 2021, as the previous IDA analysis[2] looked at data published through 2019. The current literature review identified 270 peer-reviewed studies that described new diagnostic techniques; 230 of these described entire assays, 10 articles described improvements to sample preparation steps, 6 described improvements to reagent preparation steps, 40 described new materials for use in existing assays, 57 optimized current assay steps, 19 identified new assay targets, and 32 described miniaturization or increased portability of existing assays.

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1 Kristen A. Bishop, Robert L. Cubeta, Jon M. Davis, and Lucas A. LaViolet, *(U) Evaluation of Biological Agent Clinical Sampling and Analysis*, IDA Paper P-21576 (Alexandria, VA: Institute for Defense Analyses, May 2021). CONTROLLED UNCLASSIFIED INFORMATION. Only UNCLASSIFIED information is included in this paper.

2 Ibid., CONTROLLED UNCLASSIFIED INFORMATION. Only UNCLASSIFIED information is included in this paper.

The IDA team gathered data on each assay or technique, including reagent and equipment requirements, portability, personnel training requirements, and current commercialization status. Based on this information, the IDA team approximated the Technology Readiness Level (TRL) and Clinical Laboratory Improvement Amendments (CLIA) complexity score to provide guideposts for determining how mature a diagnostic was. The diagnostics identified here range from proof-of-concept in a lab setting to fully commercialized devices. A diagnostic with a high TRL and low CLIA complexity is closer to deployability.

The majority of identified diagnostics remain in the proof-of-concept stage of development. Some are brand new techniques or technologies, while others have only been tested against one pathogen or one sample type. While research and development needs remain, many are promising and could be useful in military applications. The results of this analysis can inform decision-makers looking to pursue investment or development of certain diagnostics; depending on a given priority, decision-makers could identify diagnostics that address that priority and then pursue further diagnostic development or acquisition. The following table summarizes the basic characteristics of the diagnostics identified during the literature review.

We recommend OTSG use the information developed in this analysis to:

1. Identify diagnostic technologies that will enable the placement of diagnostic assays at appropriate locations or with the most appropriate unit types to fully capitalize on the intended use of the assay (surveillance, early diagnosis to facilitate early intervention, general situational awareness, etc.).
2. Engage early and often with DOD research program managers, product developers, and Integrated Concept Team (ICT) members to highlight advances in diagnostic technology that support OTSG's intended application of the diagnostic.
3. Work closely with U.S. Army Nuclear and Countering Weapons of Mass Destruction Agency (USANCA) to ensure synchronization between the clinical needs of OTSG and overall strategic goals, policy, and direction of the Army Biological Defense Strategy Implementation.
4. Maintain ongoing situational awareness of trends in diagnostic technology. This is a fast-moving field and advances can occur rapidly, creating new opportunities that may not have been obvious before.

Summary of Diagnostic Technologies Characteristics

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Improvements to Existing Assays[c]					
Aptamer-Based Systems	Comparable or better	Specific target(s)	<2 hours	3-4	Waived to High
Resonance Energy Transfer	Comparable or better	Specific target(s)	Minutes to hours	4	Moderate to High
Lyophilized Reagents	Comparable or better	n/a	A few hours	4-7+	Moderate to High
Lyophilized Sera	Better	n/a		3	High
Atom Transfer Radical Polymer Gold Nanoparticles (ATRP-AuNP) in ELISA	Comparable or better	Specific target		4	Moderate
Gold Nanoparticles in PCR	Better	Specific target	A few hours	4	Moderate
Loop Mediated Isothermal Amplification (LAMP)	Comparable or better	Specific target	<1 hour	3-4	High
Plasmonic PCR	Comparable	Specific target	<1 hour	4	Moderate
8pG-based Microplate	Better	Specific target		4	Moderate
Graphene Nanoparticle-based ELISA	Better	Specific target	A few hours	3	High
Paper-based ELISA	Comparable or better	Specific target	A few hours	4	Waived to Moderate
Surface Plasmon Resonance (SPR)	Comparable or better	Specific target	<1–2 hours	4	Moderate to High
Fluorescent in Situ Hybridization (FiSH)	Comparable or better	Specific target	Minutes to hours	3	Moderate
CRISPR[e]	Better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-7+	Moderate to High
Recombinase Polymerase Amplification (RPA)	Comparable or better	Specific target(s) Potential screening via multiplexing	<30 minutes	3-4	Moderate to High

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Surface Enhanced Raman Scattering (SERS)[e]	Better	Specific target	<1–2 hours	3-4	Moderate
Quantum Dots[e]	Better	Specific target(s) Potential screening via multiplexing	<1–2 hours	4	Moderate to High
Whole Cell-based Biosensors	Better	Specific target	<24 hours	4	High
Electrochemical Sensors	Comparable or better	Specific target	<1 hour	3-4	Moderate to High
New Assays or Procedures[d]					
Bypassing RNA Extraction in PCR	Comparable or better	n/a	<1 hour	3-4	Moderate to High
Bypassing Nucleotide Extraction	Comparable or better	n/a	<1 hour	3-4	Moderate to High
Plasma Cell-Free DNA Metagenomic Next-Generation Sequencing (mNGS)	Comparable	Screening		7+	High
Membrane Sample Concentration	Comparable or better	n/a	<20 minutes	3	Moderate
Digital PCR	Better	Specific target		4	Moderate
Droplet Digital ELISA/SiMoA	Better	Specific target	A few hours	4-7+	Moderate to High
MicroRNA Targets (miRNA)	Comparable or better			4	High
Hybridization Chain Reaction (HCR)	Comparable or better	Specific target(s)	A few hours	4	High
CRISPR[e]	Better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-7+	Moderate to High
MALDI-TOF Mass Spectrometry	Comparable	Screening	<1 hour	3-7+	Moderate to High

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Surface Enhanced Raman Scattering (SERS)[e]	Better	Specific target	<1–2 hours	3-4	Moderate
Polymerase Spiral Reaction (PSR)	Better	Specific target	A few hours	4	Moderate
Quantum Dots[e]	Better	Specific target(s) Potential screening via multiplexing	<1–2 hours	4	Moderate to High
Microfluidics	Comparable or better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-4	Moderate to High
Lateral Flow Assays	Comparable or better	Specific target(s) Screening via multiplexing	<1 hour	4	Moderate
Shear-Horizontal Surface Acoustic Wave (SH-SAW)	Better	Specific target	Minutes	4	High

[a] The "current technology" is the technology that is currently considered the preferred method for that technique or purpose. In this case, the "current technology" is often PCR or the baseline technology for variations listed here (e.g., ELISA or mass spectroscopy).

[b] This column ("Pathogen Screening or Specific Identification") indicates whether a diagnostic technology would be useful to identifying a pathogen, without prior suspicion, from a broad spectrum of possibilities ("Pathogen Screening") or whether the diagnostic is only useful if there is some prior suspicion or knowledge of the specific pathogen for which to test ("Specific Identification").

[c] "Improvements to Existing Assays" includes: assay automation, combination of tools, change in materials or use of a new material in an existing assay, change in reagents or use of a new reagent in an existing assay, assay miniaturization, technique improvement, reference library preparation, and improvements to sample preparation.

[d] "New Assays or Procedures" includes certain combinations of tools (e.g., combinations that create a new assay or use new materials or tools), technique commercialization, combination of techniques, new techniques, and use of a new or different target.

[e] Note: CRISPR, SERS, and quantum dots fall under both categories, as there have been improvements to existing assays, procedures, and materials in addition to new techniques and assays. However, the majority of the studies fall under the "Improvements to Existing Assays" category.

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1. Introduction

A prior analysis funded by the Army Office of the Surgeon General (OTSG) examined the tactics, techniques and procedures associated with the collection and analysis of diagnostic specimens.[3] As part of that effort, the Institute for Defense Analyses (IDA) conducted research to determine what the utility may be of diagnostic specimens collected in an operational environment to enhance the situational awareness of commanders. To contain the scope of the project, diagnostic assays for biological warfare agents available as of 2019 served as a technology limit. One of the significant findings of that 2019 analysis was that it is unknown whether the technological limits of current diagnostic assays allow for low-level detection of pathogens of interest.[4] The diagnostic assays identified within that analysis served their purpose for enabling the military healthcare system to diagnose disease-causing pathogens, but the assays were not always capable of detecting sufficiently low levels of pathogens to inform commanders' operational decision-making. Sensitive, early-disease detection is an important aspect of situational awareness that provides the commander with sufficient time to implement corrective actions (e.g., post-exposure prophylaxis, restriction of movement) before a biological agent impacts operational effectiveness.

Since 2019, there has been substantial research and development in the diagnostics field, especially as a result of the coronavirus disease 2019 (COVID-19) pandemic. The purpose of this analysis is to identify and describe the current state-of-the-art for diagnostic technologies. We also identify technologies and associated concepts, such as sample preparation, that may provide earlier detection; some diagnostic technologies may provide additional benefits, such as more detailed pathogenic characterization. The literature review focused on pathogen-agnostic assays so that the technology underlying the diagnostics could be broadly examined. While many of the diagnostic assays and techniques discussed in Chapter 3 were tested on specific pathogens or biomarkers, they are included here because the underlying technology shows promise for broad use with further research and development. Not all of the diagnostics were tested with multiple pathogens.

Chapter 2 outlines the scope and methodology of the analysis, including search terms and descriptions of our assessment framework. Chapter 3 provides details on a variety of different

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3 Kristen A. Bishop, Robert L. Cubeta, Jon M. Davis, and Lucas A. LaViolet, *(U) Evaluation of Biological Agent Clinical Sampling and Analysis*, IDA Paper P-21576 (Alexandria, VA: Institute for Defense Analyses, May 2021). CONTROLLED UNCLASSIFIED INFORMATION. Only UNCLASSIFIED information is included in this paper.

4 Ibid., iii.

diagnostic categories and includes an assessment of each technology area. Chapter 4 outlines suggested next steps for the technologies.

2. Scope and Methodology

This analysis is primarily a literature review of current diagnostic technologies. We included all papers published between 2016 and 2022 that reported new or improved diagnostic technologies that had produced at least a proof-of-concept. This includes assays for all disease-causing pathogens, rather than only assays that test for biological warfare agents. Many of these assays could be adapted for a different pathogen of interest without major changes to the underlying technology. We focused our assessment on five different categories: performance, infrastructure, portability, Technology Readiness Level (TRL), and Clinical Laboratory Improvement Amendments (CLIA) complexity. While cost was not considered in the assessment process, the IDA team included cost per assay data when it was mentioned in the literature to provide a rough basis for future investments. A further explanation of each of these categories is provided in the subsequent sections in this chapter.

A. Search Parameters

We conducted our literature search using individual journals – such as *Nature*, *Biosensors & Bioelectronics*, and *PLOS One* – as well as databases, such as PubMed and Google Scholar.[5] In addition, we searched the Defense Technical Information Center (DTIC), using similar search terms, to find any non-public, unclassified Department of Defense (DOD) research that may have been conducted.

To ensure that a large number of studies could be captured, both broad and assay-specific searches were performed. Broad searches were performed using search terms created from combinations of the terms "novel," "diagnostic," "assay," "clinical," "new," "method," "identification," "disease," "infectious," "limit of detection," "sensitivity," "specificity," "diagnoses," "point-of-care," and "enhancement." Assay-specific searches were also performed using the search terms mentioned previously in combination with the name of a diagnostic assay; for example, to search for polymerase chain reaction (PCR) literature, search terms such as "novel PCR" and "PCR enhancement" were used. All searches were limited to papers published after January 1, 2016 and only included studies that were unclassified and approved for public distribution (if applicable).

[5] M. Gusenbauer, "Google Scholar to Overshadow Them All? Comparing the Sizes of 12 Academic Search Engines and Bibliographic Databases," *Scientometrics* 118**,** no. 177–214 (2019), https://doi.org/10.1007/s11192-018-2958-5.

B. Technology Readiness Level (TRL)

The IDA team used the National Institutes of Health (NIH) Centers for Accelerated Innovations (NCAI) Technology Readiness Guidelines to assess the TRL of diagnostic assays.[6] This metric describes the progression and maturation of technologies and has been adopted by several organizations, including the Department of Health and Human Services, for assessing biomedical technologies. Table 1 summarizes each TRL. Because this analysis required the existence of a proof-of-concept for each assay, the minimum TRL considered in this analysis is "3" with no upper limit.

Table 1. Technology Readiness Levels (TRLs)

TRL Level	Diagnostic (Assay/Test)
1	Active monitoring of scientific knowledge base. Identify links between disease in humans and animals
2	Activities: Scientific "paper studies" to generate research ideas, hypotheses, and experimental designs for addressing the related scientific issues. Characterize disease epidemiology. Initial intellectual property search for patentability.
3	Activities: Explore assay components via prototypes and screening; identify and evaluate critical technologies and components, and begin characterization of lead design. Survey clinical literature to characterize current care patterns and unmet need(s). Initiate user feedback Milestones: Demonstrate preliminary assay with simplified sample/artificial matrices. Demonstrate sensitivity and specificity with spike/recovery studies in the appropriate matrices.
4	Activities: Integration of critical technologies and components (including hardware and software). Select appropriate candidate reference and QC (quality control) reagents. Milestones: Assay/ test method validation in accordance with the product's intended use (Sample type, volume, assay components). Establish Draft Product Profile. Characterize current reimbursement mechanisms, economic burden of illness and treatment costs. Formulate initial regulatory and reimbursement strategies.
5	Activities: Design freeze. Develop a scalable and reproducible manufacturing process aligned with regulatory guidelines (as needed). Finalize QC criteria. Milestones: Identify supply chain and/or manufacturing partners. Demonstrate acceptable performance as necessary for regulatory filing and for impact on clinical care. Preliminary FDA meeting.
6	Milestones: Manufacture product compliant with quality protocols. Based on regulatory classification (e.g. CLIA vs IVD route), submit regulatory package

[6] NIH, "Technology Readiness Levels," accessed November 1, 2021, https://ncai.nhlbi.nih.gov/ncai/resources/techreadylevels.

TRL Level	Diagnostic (Assay/Test)
7	Milestones: Assays used to assess product quality are validated. Assays used to assess critical outcomes in clinical trials and in animal efficacy studies are validated.

Source: NIH, "Technology Readiness Levels," accessed November 1, 2021, https://ncai.nhlbi.nih.gov/ncai/resources/techreadylevels.

C. CLIA Complexity

The complexity level of each assay was estimated using the CLIA Complexity categorizations. According to these categorizations, a test may have moderate complexity, high complexity, or may be waived (i.e., technologies that are approved for home use or are sufficiently simple as to have minimal risk of error or harm[7]); the difference between moderate and high-complexity concerns the personnel requirements to perform the test. The CLIA complexity levels may be used to determine if a laboratory meets the requirements to perform a given test; laboratories performing waived tests may not be subject to inspection, while laboratories performing nonwaived tests are subject to CLIA quality system standards. While CLIA is not directly applicable to the military, the CLIA complexity can provide an estimate of the overall requirements of an assay. The DOD implements the Clinical Laboratory Improvement Program (CLIP), which regulates and enforces laboratory policies that are derived from and similar to CLIA guidelines but also consider requirements unique to the DOD.

The CLIA complexity score is determined by a summed score of seven criteria, where each criterion can be scored with a value of 1, 2, or 3. Definitions for scores of 1 and 3 are given for each criterion, while a score of 2 implies that the characteristics of a test are intermediate; these score definitions are listed in Table 2. A total score of 12 or less categorizes a test as moderately complex, while a total score greater than 12 is considered a high-complexity test. While manufacturers must request that a test be characterized as waived by the Food and Drug Administration (FDA), for the purpose of this analysis, we considered tests having the minimum total score of 7 to be "waived" tests.[8] CLIA complexities are also designed to analyze entire assays, and therefore may not be applicable in characterizing advancements that alter a single step of a complete assay.

[7] U.S. Food and Drug Administration, "CLIA Waiver by Application," accessed on May 12, 2022, updated May 2, 2022, https://www.fda.gov/medical-devices/ivd-regulatory-assistance/clia-waiver-application.

[8] The CLIA complexity estimates were created using the authors' best judgements, and may not accurately reflect the views of the FDA.

Table 2. CLIA Complexity Scores

	Knowledge	Training and Experience	Reagents and Material Preparation	Characteristics of Operational Steps	Calibration, Quality Control, Proficiency Testing Materials	Test System Troubleshooting and Equipment Maintenance	Interpretation and Judgement
1	(A) Minimal scientific and technical knowledge is required to perform the test; and (B) Knowledge required to perform the test may be obtained through on-the-job instruction	(A) Minimal training is required for pre-analytic, analytic and post-analytic phases of the testing process; and (B) Limited experience is required to perform the test.	(A) Reagents and materials are generally stable and reliable; and (B) Reagents and materials are prepackaged, or premeasured, or require no special handling, precautions or storage conditions.	Operational steps are either automatically executed (such as pipetting, temperature monitoring, or timing of steps), or are easily controlled.	(A) Calibration materials are stable and readily available; (B) Quality control materials are stable and readily available; and (C) External proficiency testing materials, when available, are stable.	(A) Test system troubleshooting is automatic or self-correcting, or clearly described or requires minimal judgment; and (B) Equipment maintenance is provided by the manufacturer, is seldom needed, or can easily be performed.	(A) Minimal interpretation and judgment are required to perform pre-analytic, analytic and post-analytic processes; and (B) Resolution of problems requires limited independent interpretation and judgment.
3	Specialized scientific and technical knowledge is essential to perform pre-analytic, analytic or post-analytic phases of the testing.	(A) Specialized training is essential to perform the pre-analytic, analytic or post-analytic testing process; or Substantial experience may be necessary for analytic test performance	(A) Reagents and materials may be labile and may require special handling to assure reliability; or (B) Reagents and materials preparation may include manual steps such as gravimetric or volumetric measurements.	Operational steps in the testing process require close monitoring or control, and may require special specimen preparation, precise temperature control or timing of procedural steps, accurate pipetting, or extensive calculations.	(A) Calibration materials, if available, may be labile; (B) Quality control materials may be labile, or not available; or (C) External proficiency testing materials, if available, may be labile.	(A) Trouble-shooting is not automatic and requires decision-making and direct intervention to resolve most problems; or (B) Maintenance requires special knowledge, skills, and abilities.	(A) Extensive independent interpretation and judgment are required to perform the pre-analytic, analytic or post-analytic processes; and (B) Resolution of problems requires extensive interpretation and judgment.

Source: FDA, “CLIA Categorizations,” accessed May 12, 2022, updated February 25, 2020, https://www.fda.gov/medical-devices/ivd-regulatory-assistance/clia-categorizations.

D. Diagnostic Performance

As part of the assessment of each assay, the performance of the diagnostic – such as sensitivity, specificity, limit of detection, and concurrence with existing assays – was considered. Many of the assays claimed to have improved performance over an existing assay; however, most tests that claimed improved performance analyzed only a single target. While performance metrics, such as limit of detection, can vary for different targets in different environments, this still demonstrates a potential for improvement. Further testing could validate these assays with multiple targets.

E. Infrastructure and Portability

Infrastructure requirements – such as refrigeration, cold chain requirements, power sources, and reagents requirements – play a major role in determining where a tool can be deployed. Assays that could be stored at room temperatures, had minimal external power requirements, had minimal reagent requirements, or did not require extensively trained personnel would be appropriate for forward deployment.

An assay's portability may depend on the infrastructure requirements, but also on whether or not the assay can be self-contained and whether it requires specific environmental conditions. Some assays designed for portability may be completely self-contained systems. Miniaturized systems can modify existing assays to have a smaller profile to increase their portability.

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3. Technology Assessment

A. Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is an assay that has been used in diagnostics and research for decades, and has since become the most common modern diagnostic method (and "gold standard") for a variety of disease-causing pathogens. Advantages of PCR include fewer laboratory safety concerns, shorter runtime (compared to bacterial or viral isolation), and increased sensitivity and specificity (over most standard antigen assays).[9] The PCR process begins with the amplification of a specific target sequence (or sequences) of bacteria or virus genetic material, followed by result detection by any applicable method, such as fluorescent spectroscopy.

Improving existing PCR techniques may include, for example, a method to increase the performance and speed of current tests. Several PCR technologies were identified that may offer improvements to existing assays or act as novel assays offering new advantages; for example, there have been multiple advances in the materials, targets, and techniques used in PCR, and a new real-time PCR assay was validated for orthopoxvirus detection with a limit of detection (LOD) of 2.5 viral copies per 5 microliter (µL).[10] A 2019 study measured host gene expression using RT-PCR (with potential markers such as calcitonin) to differentiate between viral, bacterial, and non-infectious respiratory pathogens. Their model showed 81%–95% accuracy at distinguishing between various forms of disease-causing pathogens, which was an improvement over using only a calcitonin marker.[11]

1. Plasmonic PCR

Plasmonic PCR utilizes the optical radiation of metallic nanoparticles to decrease the time required to run each PCR cycle. These nanoparticles act as photothermal heaters, or "nano heaters," and are dispersed in the reaction mixture; when a sufficient density is obtained, the light-to-heat conversion approaches 100% and the nanoparticles convectively transfer their thermal

9 Caroline Chartrand et al., "Diagnostic Accuracy of Rapid Antigen Detection Tests for Respiratory Syncytial Virus Infection: Systematic Review and Meta-Analysis," *Journal of Clinical Microbiology* 53, no. 12 (December 2015): 3738, doi:10.1128/JCM.01816-15.

10 Eric M. Mucker et al., "Validation of a Pan-Orthopox Real-Time PCR Assay for the Detection and Quantification of Viral Genomes from Nonhuman Primate Blood," *Virology Journal* 14, no. 1 (2017), https://doi.org/10.1186/s12985-017-0880-8.

11 Emily C. Lydon et al., "Validation of a Host Response Test to Distinguish Bacterial and Viral Respiratory Infection," *EBioMedicine* 48 (2019), https://doi.org/10.1016/j.ebiom.2019.09.040.

energy to the surrounding media, which decreases the reaction time. Modifications may be made to prevent the nanoparticles from inhibiting deoxyribonucleic acid (DNA) polymerase, such as adding bovine serum albumin to prevent physical contact between DNA polymerase and the nanoparticles, or PEGylating the nanoparticles (adding polyethylene glycol) with HS-PEG5000. The plasmonic PCR reaction occurs in a thin-walled glass capillary tube with a laser-based heating source.[12]

One study demonstrated the ability to run 30 cycles in 54 seconds using plasmonic PCR and the KAPA2G fast polymerase enzyme and gold nanoparticles to produce a detectable product, improving upon a similar technique from 2012. The laboratory used conventional PCR volumes to avoid microfluidics or continuous-flow systems and a 1°C chilled airflow system for rapid cooling. However, they employed a "hot start" in which the sample was preheated before PCR cycling. This technology was shown to be compatible with common fluorescent quantification techniques, though special instrumentation may be required depending on the method.[13]

A South Korean lab took this advancement a step further by combining plasmonic thermocycling and fluorescent signal detection onto a single device. This portable device was originally intended as a COVID-19 diagnostic. Standard spectrometers, magnetometers, and a dynamic light-scattering device were used to measure nanoparticle size. The prototype is enclosed in a 3D-printed structure and is powered by a 12-volt (V) lithium ion battery pack. The current "nanoPCR" prototype can measure three samples within 17 minutes. The prototype was tested on the N1, N2, and RPP30 genes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and had an LOD of 3.2 gene copies/µL, which is comparable to conventional PCR. Because the signal could only be detected after completion of the PCR reaction, the assay is limited to endpoint measurements.[14]

2. Digital PCR

In contrast to the exponential amplification of conventional PCR, digital PCR involves a linear approach of diluting and partitioning a sample into multiple parallel PCR reactions.[15] After amplification, each well is measured to produce a binary readout and the fraction of positive readouts is calculated. This partitioning means that some of the reactions may contain the target sequence, while others may not; the fraction of negative PCR reactions and a Poisson distribution

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12 Philip J. R. Roche et al., "Real Time Plasmonic QPCR: How Fast Is Ultra-Fast? 30 Cycles in 54 Seconds," *Analyst* 142, no. 10 (2017), https://doi.org/10.1039/C7AN00304H.

13 Philip J. R. Roche et al., "Real Time Plasmonic QPCR: How Fast Is Ultra-Fast? 30 Cycles in 54 Seconds"

14 Jiyong Cheong et al., "Fast Detection of SARS-CoV-2 RNA via the Integration of Plasmonic Thermocycling and Fluorescence Detection in a Portable Device," *Nature Biomedical Engineering* 4, no. 12 (2020), https://doi.org/10.1038/s41551-020-00654-0.

15 E. Pomari et al., "Digital PCR: A New Technology for Diagnosis of Parasitic Infections," *Clinical Microbiology and Infection* 25, no. 12 (2019), https://doi.org/10.1016/j.cmi.2019.06.009.

are used to estimate the target sample's initial concentration. This method is highly quantitative and does not require a reference sample. Due to the binary nature of each parallel well, digital PCR is also less prone to inhibitors that affect conventional PCR, as decreased amplification inside a single well will still result in a positive signal. Digital PCR is potentially more accurate and sensitive than conventional PCR and has been suggested as a method for SARS-CoV-2 detection.[16]

A system that combines plasmonic and digital PCR was created as a portable diagnostic tool; it has a small footprint (10 cm x 6 cm x 4 cm), can be powered by USB-C, and uses a small, low-cost, commercially available Raspberry Pi computer. This system can use either a poly(dimethylsiloxane) (PDMS) array to detect target concentrations of 100–260,000 copies/μL, or a mass-produced poly(methyl methacrylate) (PMMA) microwell array to detect 12–26,000 copies/μL.[17]

3. Gold Nanoparticles in PCR

Gold nanoparticles (AuNP) can be used to increase the sensitivity of conventional PCR. In addition to gold, other nanomaterials can be used for plasmonic/photonic PCR, such as carbon nanotubes, other metals, and titanium nitride.[18]

An AuNP-based PCR colorimetry method uses AuNPs as probes that complement the amplified DNA; an addition of a salt to the solution causes the AuNPs to accumulate and quantifiably change the solution's color. This method was approximately three times more sensitive than the traditional PCR method at detecting *Staphylococcus aureus*; the limit of detection was 8.7×10^{-9} g DNA/μL AuNP solution and the limit of quantification was 29×10^{-9} g DNA/μL AuNP solution.[19]

A colorimetric biosensor developed in 2015 for *Brucella* genomic DNA detection used AuNPs in the target solution to prevent color changes caused by the difference of optical characteristics of aggregate and dispersed gold nanoparticles, which made detection fast and relatively easy.[20] For *Staphylococcus epidermis*, multiplexed PCR with AuNP probes had a minimum LOD of 5×10^{-9} g/mL, which is appropriately sensitive and specific enough to be used

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16 Luca Falzone et al., "Sensitivity Assessment of Droplet Digital PCR for SARS-CoV-2 Detection," *International Journal of Molecular Medicine* 46, no. 3 (2020), https://doi.org/10.3892/ijmm.2020.4673.

17 Christian D. Ahrberg et al., "Plasmonic Heating-Based Portable Digital PCR System," *Lab on a Chip* 20, no. 19 (2020), https://doi.org/10.1039/D0LC00788A.

18 Minli You et al., "Ultrafast Photonic PCR Based on Photothermal Nanomaterials," *Trends in Biotechnology* 38, no. 6 (2020), https://doi.org/10.1016/j.tibtech.2019.12.006.

19 Reza Shahbazi et al., "Highly Selective and Sensitive Detection of *Staphylococcus aureus* with Gold Nanoparticle-Based Core-Shell Nano Biosensor," *Molecular and Cellular Probes* 41 (2018), https://doi.org/10.1016/j.mcp.2018.07.004.

20 N. Sattarahmady et al., "Gold Nanoparticles Biosensor of *Brucella* Spp. Genomic DNA: Visual and Spectrophotometric Detections," *Biochemical Engineering Journal* 97 (2015), https://doi.org/10.1016/j.bej.2015.01.010.

in a clinical environment. This method could potentially be used to build a commercial diagnostic kit.[21] One study documented a "universal" method, currently used for mouse-actin identification, with an LOD concentration of 4.3×10^{-15} molar (M). The results were visible to the naked eye within 30 minutes at room temperature.[22]

4. Micro RNA Targets (miRNAs)

Micro ribonucleic acid (RNA), or miRNA, are an emerging target for diagnostics that may provide a unique signature for most or all pathogens. A study showed that detection of miR5 (a miRNA target) identified 100% of histologically proven *G. lamblia* infections from biopsies and demonstrated 90% specificity and 66% sensitivity for miR6 in stool samples compared to biopsy histology. Testing for *G. lamblia* miRNA appeared to be more sensitive than testing for DNA; in addition, miRNAs are resistant to freeze-thaw cycling, RNase A digestion, and high pH solutions. The protocol uses reagents and equipment that would be available at medium-to-large-scale laboratories, including QIAamp DNA Minikit (for DNA extraction) and ABI Quant Studio6 Flex Real-Time PCR System (for qRT-PCR). These benefits – commonly used commercial reagents and equipment, high pathogen sensitivity and specificity, and target hardiness – provide multiple advantages over other pathogen-detection techniques.[23]

5. Assessment

Plasmonic PCR has allowed for an increased assay sensitivity with a significant decrease in assay time. Entire plasmonic PCR systems have been miniaturized and research teams have created portable enclosed systems powered by battery packs, with the potential of creating portable sample-to-result systems with additional development. These systems are self-contained in small form factors (e.g., measuring 15 cm x 15 cm x 19 cm and weighing 3 kg), which may allow for forward deployment. However, production of these platforms has not yet been scaled and they exist only as proof-of-concept devices. If scaled, plasmonic PCR systems could be portable enough to be used in environments where traditional PCR assays would not be possible due to their reduced infrastructure requirements. These PCR systems would have a moderate CLIA complexity, and currently are at a TRL level of 4.

Whereas plasmonic PCR can decrease assay time, digital PCR can increase the assay diagnostic accuracy. Digital PCR systems have been combined with plasmonic PCR, and similar

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21 Mahsa Osmani Bojd et al., "Thiolated AuNP Probes and Multiplex PCR for Molecular Detection of *Staphylococcus epidermidis*," *Molecular and Cellular Probes* 34 (2017), https://doi.org/10.1016/j.mcp.2017.04.006.

22 Li Zou et al., "Sensitive DNA Detection by Polymerase Chain Reaction with Gold Nanoparticles," *Analytica Chimica Acta* 1038 (2018), https://doi.org/10.1016/j.aca.2018.07.006.

23 Tal Meningher et al., "*Giardia lamblia* MiRNAs as a New Diagnostic Tool for Human Giardiasis," *PLOS Neglected Tropical Diseases* 13, no. 6 (2019), https://doi.org/10.1371/journal.pntd.0007398.

self-contained systems have been created using commercially available components, such as Raspberry Pi mini-computers and portable power banks, creating portable options for digital PCR. Commercial portable digital PCR systems already exist, and are marketed by companies such as ThermoFisher, although they are not quite as portable as assays like the one created by Ahrberg et al. An adaptation of the plasmonic digital PCR assay[24] may be suitable for forward deployment, considering the lower resource requirements. These systems would have a moderate CLIA complexity and are assessed at TRL 4.

As a new alternative reagent for an existing process, the use of AuNP probes can increase the diagnostic performance of traditional PCR assays. AuNP-based colorimetric probes can give a visual readout, reducing personnel training requirements, and can increase the sensitivity over traditional PCR; most traditional PCR equipment requirements remain the same. However, this would not affect the complexity or resource requirements of the assay, and would not help the assay be deployed further forward. Use of AuNPs in PCR would be at a TRL of 4, with a moderate CLIA complexity.

Depending on the target pathogen, miRNA targeting could be an approach to increase sensitivity and decrease susceptibility to environmental factors. While the principles and equipment requirements of PCR would remain the same, different reagents would be required to process miRNAs. This would potentially allow miRNA targeting at Role 3 facilities, but this methodology may not be feasible for lower-role facilities due to portability restrictions. This technology would have a high CLIA complexity, and would be at a TRL of 4. A summary of requirements for various PCR techniques can be found in Table 3 and Table 4.

Table 3. Assay Requirements for PCR

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Plasmonic PCR	Metallic nanoparticles Bovine serum albumin	Glass capillary tube Laser-based heat source	Maybe	Chilled airflow system (refrigeration may be required for reagents) External battery packs (e.g. 12-V lithium ion battery)	Requires training
Digital PCR	--	Raspberry Pi USB power source	Yes	PDMS array or PMMA microwell array	Requires training
AuNPs in PCR	Salts (for colorimetry)	Standard PCR equipment	No	Fridge or freezer for reagent storage	Requires training (may be reduced in future versions)

[24] Ahrberg et al., "Plasmonic Heating-Based Portable Digital PCR System."

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
MicroRNA Targets	Standard reagents (QIAmp DNA Minikit)	Standard qRT-PCR equipment	No	Standard for PCR	Requires training

Table 4. Assay Details for PCR

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Plasmonic PCR	LOD: 3.2–101 copies/µL Time: 1–50 minutes	Proof-of-concept	Faster More sensitive	Potential for miniaturization and portability
Digital PCR	100–260000 copies/µL 12–26000 copies/µL	Some commercial options	More sensitive Less prone to target inhibition	Runs parallel reactions Doesn't require a reference sample
AuNPs in PCR	5–8.7 × 10^{-9} g/µL Time: 30 minutes	Proof-of-concept	More sensitive Visual colorimetric readout	Used to supplement conventional PCR
MicroRNA Targets	LOD: 8 parasitic cells	Proof-of-concept	Unique signature for pathogens	May be useful at Role 3

B. Nucleotide Extraction

Many diagnostic assays work on the principle of identification of nucleotide sequences of the target (i.e., DNA or RNA sequences). Nucleotide sequences can be found in various clinical samples, including blood and saliva. These samples contain a multitude of other substances, which may lead to misleading results and/or block the desired reaction from occurring. Therefore, most nucleotide-identification-based assays have a step that extracts nucleotide sequences from the sample for better performance.

Nucleotide extraction is an essential sample preparation step in multiple diagnostic techniques, including PCR. Usually, nucleotide extraction uses extra reagents and is a sensitive step requiring care to prevent contamination of the sample and the person preparing the sample (especially if hazardous or highly infectious pathogens are being tested). Bypassing or improving this step could increase the usage of diagnostic technologies in austere environments and may also decrease sample-to-result time, reagent requirements, and complexity. While it is generally considered a "standard" step, some procedures are able to eliminate this step while still achieving similar or better results. For example, the Next-Generation Diagnostic System (NGDS) Increment 1 uses a simplified nucleotide extraction and purification step within the portable kit, which differs

from conventional PCR and allows users to easily go from sample-to-result within a single assay panel.[25]

1. Bypassing Nucleotide Extraction

RNA extraction is typically necessary to remove PCR inhibitors from a target solution. However, the use of dry swabs transported by a modified mild lysis buffer can avoid this RNA extraction step. RNAse inhibitors can be used to deactivate endogenous RNAses, though the amount of elution buffer required must be adjusted based on the type of clinical sample. The modified protocol detected 20 copies of RNA/reaction, which corresponds to 300–1000 RNA copies/swab. The new reverse transcription polymerase chain reaction (RT-PCR) formulation created for this method was designed to be compatible with detergent-based lysis buffers and had cryoprotectants.[26]

A laboratory study bypassed RNA extraction by using swab eluate to directly amplify samples. This simplifies the process, reduces cost, and avoids the use of toxic reagents such as guanidium thiocyanate, a common component of lysis buffers.[27] Another method for bypassing RNA extraction in PCR treats the nasopharyngeal swab directly (in this case, for SARS-CoV-2 diagnosis).[28] Another direct one-step detection method could detect SARS-CoV-2 in under 1 hour.[29]

A 2020 study in India showed that pre-treating samples with protease enzyme and dithiothreitol (DTT) before heat inactivation could allow the sample to be directly processed by PCR. This pre-treatment method was tested on SARS-CoV-2 samples and the results concurred with conventional PCR methods that included RNA extraction.[30] Similarly, another study showed that saliva pre-treatment with heat and proteinase K reduced reaction inhibitors and allowed for the RNA extraction step to be skipped in the detection of SARS-CoV-2 both by colorimetric loop

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25 Director, Operational Test and Evaluation, "Next Generation Diagnostic System (NGDS) Increment 1 Early Fielding Report," June 2017, Accessed April 12, 2022, https://apps.dtic.mil/sti/pdfs/AD1036241.pdf

26 Nuttada Panpradist et al., "Simpler and Faster Covid-19 Testing: Strategies to Streamline SARS-CoV-2 Molecular Assays," *EBioMedicine* 64 (2021), https://doi.org/10.1016/j.ebiom.2021.103236.

27 Panpradist et al., "Simpler and Faster Covid-19 Testing: Strategies to Streamline SARS-CoV-2 Molecular Assays."

28 Catia Mio et al., "A Streamlined Approach to Rapidly Detect SARS-CoV-2 Infection Avoiding RNA Extraction: Workflow Validation," *Disease Markers* 2020 (2020), https://doi.org/10.1155/2020/8869424.

29 Eva Kriegova, Regina Fillerova, and Petr Kvapil, "Direct-RT-QPCR Detection of SARS-CoV-2 Without RNA Extraction as Part of a COVID-19 Testing Strategy: From Sample to Result in One Hour," *Diagnostics* (Basel, Switzerland) 10, no. 8 (2020), https://doi.org/10.3390/diagnostics10080605.

30 Kalichami Alagarasu et al., "Utility of a Modified Heat Inactivation Method for Direct Detection of SARS-CoV-2 by RT-QPCR in Viral Transport Medium Bypassing RNA Extraction: A Preliminary Study," *The Indian Journal of Medical Research* 152, 1 & 2 (2020), https://doi.org/10.4103/ijmr.IJMR_3121_20.

mediated isothermal amplification (LAMP) and PCR methods.[31] A different 2020 study also showed that samples heat-inactivated at 65℃ in a process called hid-RT-PCR detected SARS-CoV-2 with similar results and accuracy to the conventional RT-PCR method.[32]

A single fluid transfer step with a heat block was combined with RT-LAMP and a colorimetric visual readout for SARS-CoV-2 detection. The sample (in this case, saliva) is directly added to a pre-filled microcentrifuge tube before being incubated at 63°C for 30 minutes. Within a single microcentrifuge tube, four analytical steps take place: heat-mediated RNA virus release, reverse transcription, LAMP, and color change. No other equipment except heat blocks and a transfer pipette are required, making this method suitable for point-of-care testing. The limit of detection was 2.7 copies/μL of saliva, and the technique had a positive percentage agreement of 96.7% and a negative percentage agreement of 97.1% compared with RT-PCR.[33]

A method for rapid Ebolavirus detection can directly perform RT-qPCR with unprocessed blood samples. Far-red dyes are used to overcome the fluorogenic inhibition effect of blood and freezing/thawing cycles were used to disturb virion structures, which releases viral genomes into the blood samples. Though the limit of detection (1.2×10^4 PFU/mL) is one order of magnitude higher than the gold standard, the test may still be clinically appropriate.[34]

2. RNA Extraction Improvements

In addition to methods that remove the need for RNA extraction, there have also been improvements in RNA extraction techniques. A technique using dendritic fibrous nanosilica (DFNS), whose large surface area may assist in RNA extraction, may be a potential basis for future diagnostic kits.[35]

Precipitation-enhanced analyte retrieval (PEARL) was developed to deal with the shortage of standard commercial RNA extraction kits. This extraction method uses a lysis solution to disrupt cell membranes and viral envelopes while providing conditions suitable for alcohol-based precipitation of RNA, DNA, and proteins; this offers comparable performance to commercial extraction kits. The PEARL lysis solution contains common laboratory reagents, such as nonionic

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31 Matthew A. Lalli et al., "Rapid and Extraction-Free Detection of SARS-CoV-2 from Saliva by Colorimetric Reverse-Transcription Loop-Mediated Isothermal Amplification," *Clinical Chemistry* 67, no. 2 (2021), https://doi.org/10.1093/clinchem/hvaa267.

32 Ioanna Smyrlaki et al., "Massive and Rapid COVID-19 Testing Is Feasible by Extraction-Free SARS-CoV-2 RT-PCR," *Nature Communications* 11, no. 1 (2020), https://doi.org/10.1038/s41467-020-18611-5.

33 Shan Wei et al., "Field-Deployable, Rapid Diagnostic Testing of Saliva for SARS-CoV-2," *Scientific Reports* 11, no. 1 (2021), https://doi.org/10.1038/s41598-021-84792-8.

34 Kavit Shah et al., "Field-Deployable, Quantitative, Rapid Identification of Active Ebola Virus Infection in Unprocessed Blood," *Chemical Science* 8, no. 11 (2017), https://doi.org/10.1039/C7SC03281A.

35 Ayan Maity et al., "Dendritic Fibrous Nanosilica (DFNS) For RNA Extraction from Cells," *Langmuir* 36, no. 42 (2020), https://doi.org/10.1021/acs.langmuir.0c02520.

detergent IGEPAL-CA-630, HEPES buffer, and TCEP reducing agent; reagents such as glycerol, sodium acetate, and linear polyacrylamide aid in precipitation. The total cost of reagents is $0.18 per reaction.[36]

3. Assessment

Nucleotide extraction is an essential sample preparation step that is common to multiple diagnostic techniques. Studies that aim to bypass conventional nucleotide extraction may instead use alternative methods for extraction, such as a different extraction solution or the application of heat, or may attempt to perform an assay directly without extraction.[37] The need for RNA extraction kits placed a bottleneck on SARS-CoV-2 tests, and alternative methods such as PEARL attempted to address the limitation by creating an alternative solution for an otherwise standard PCR procedure using common laboratory reagents that offer similar performance to commercial RNA extraction kits.[38] Methods like these could be extended to other diagnostic platforms, increasing accessibility to RNA extraction at facilities lacking the specific kits.

Instead of alternatives to traditional nucleotide extraction, some studies have managed to remove the need for a distinct extraction step.[39] This could decrease diagnostic tool complexity, and decrease resource requirements. Nucleotide extraction bypassing techniques would have TRLs ranging from 3 to 4, and would have moderate to high complexity. A summary of techniques for bypassing nucleotide extraction can be found in Table 5 and Table 6.

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36 Jose C. Ponce-Rojas et al., "A Fast and Accessible Method for the Isolation of RNA, DNA, and Protein to Facilitate the Detection of SARS-CoV-2," *Journal of Clinical Microbiology* 59, no. 4 (2021), https://doi.org/10.1128/JCM.02403-20.

37 Mio et al., "A Streamlined Approach to Rapidly Detect SARS-CoV-2 Infection Avoiding RNA Extraction: Workflow Validation"; and Kriegova, Fillerova, and Kvapil, "Direct-RT-QPCR Detection of SARS-CoV-2 Without RNA Extraction as Part of a COVID-19 Testing Strategy: From Sample to Result in One Hour.

38 Ponce-Rojas et al., "A Fast and Accessible Method for the Isolation of RNA, DNA, and Protein To Facilitate the Detection of SARS-CoV-2."

39 Xu and Zheng, "Direct RNA Detection Without Nucleic Acid Purification and PCR: Combining Sandwich Hybridization with Signal Amplification Based on Branched Hybridization Chain Reaction"; Xu and Zheng, "Hybridization Chain Reaction for Direct MRNA Detection Without Nucleic Acid Purification," in Gaspar (ed.), *RNA Detection: Methods in Molecular Biology*; and Shah et al., "Field-Deployable, Quantitative, Rapid Identification of Active Ebola Virus Infection in Unprocessed Blood."

Table 5. Assay Requirements for Bypassing or Improving Nucleotide Extraction

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Sample pre-treatment	Protease enzyme DTT Proteinase K Far-red dyes	Standard PCR equipment Standard LAMP equipment Heat source	No	Standard for PCR	Requires training (may be reduced in future versions)
PEARL	Lysis solution Glycerol Sodium acetate Linear polyacrylamide	Hand-powered centrifuge	No	Standard lab equipment	No training required

Table 6. Assay Details for Bypassing or Improving Nucleotide Extraction

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Sample pre-treatment	LOD: 2.7 copies/µL; 1.2×10^4 PFU/mL Time: <1 hour	Proof-of-concept	Low cost Eliminates need for RNA extraction	
PEARL	--	Proof-of-concept	Low cost Eliminates need for RNA extraction	$0.18/reaction

C. Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA uses antibodies to detect a given antigen or other target, such as a protein/peptide, using plates, antibodies, and reporter molecules.[40] Antigens are first attached to a plate, after which antibodies attached to a reporter molecule are added in. The binding of the antigen with the appropriate antibody causes the reporter molecule to fluoresce or otherwise produce a signal that can be detected. There may be modifications to this process, but the same basic premise applies to all ELISA assays. Most traditional ELISA assays must be performed in a laboratory.

1. Droplet Digital ELISA/Single Molecule Assay

Developed in 2010, the single molecule assay (SiMoA) is an ultrasensitive diagnostic tool that uses a bead-based sandwich immunoassay approach, with one enzyme-labeled immunocomplex per bead; the beads are sealed with oil to ensure that only one bead is present in each well. This method creates immunofluorescent images that can be paired with conventional

[40] "What is an ELISA (Enzyme-Linked Immunosorbent Assay)?" Thermo Fisher Scientific, accessed January 27, 2021, https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/overview-elisa.html.

detection technology to quantify the target molecule's concentration. It can detect as few as 10 enzyme-labeled complexes in a 100 μL sample.[41]

A potential downside to SiMoA is the challenge of isolating the beads. The commercial SiMoA HD-1 Analyzer can only isolate approximately 5% of the total beads used.[42] A study attempted to solve this problem using water-in-oil droplets to entrap beads and found that a high percentage of the beads were trapped, thereby increasing sensitivity. Droplet-generating devices are simple, making the overall process more efficient and accessible, as well as increasing the isolation efficiency, decreasing cost, and improving production speed (e.g., millions of droplets created in a few minutes). The system could detect proteins with an LOD concentration of 20×10^{-18} M; this is approximately a 25-fold improvement (corresponding to approximately 1,200 protein particles in a sample) over conventional SiMoA. One disadvantage of this method is the increased imaging time, due to the larger volume of droplets compared to the wells used in SiMoA.[43]

2. 8pG-Based Microplate

Poly-protein G-expressing cells (BALB/c 3T3 cells) that express the Fc domains of protein G can potentially increase the sensitivity of ELISA by improving the antibody-trapping ability of the microplate over commercially available polystyrene-based and G-based microplates. Protein G is a streptococcal surface protein that exhibits specific interactions with immunoglobin. Protein-G-based plates can be expensive due to the protein purification process, so a cell-based plate is advantageous and provides a large antigen-trapping area with homogenous orientation. The concentration of antibodies in the coating on 8pG-based microplates is 1.5–23 times higher than traditional microplates; this method could detect a concentration of CTLA4 of 2.5×10^{-9} M.[44]

3. Graphene Nanoparticle-Based ELISA

Graphene oxide sheets are excellent nanocarriers and can be antibody-functionalized to increase the sensitivity of ELISA. One study used graphene-based ELISA to test for parasites in

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41 David M. Rissin et al., "Single-Molecule Enzyme-Linked Immunosorbent Assay Detects Serum Proteins at Subfemtomolar Concentrations," *Nature Biotechnology* 28, no. 6 (2010), https://doi.org/10.1038/nbt.1641.

42 David H. Wilson et al., "The Simoa HD-1 Analyzer: A Novel Fully Automated Digital Immunoassay Analyzer with Single-Molecule Sensitivity and Multiplexing," *Journal of Laboratory Automation* 21, no. 4 (2016), https://doi.org/10.1177/2211068215589580.

43 Limor Cohen et al., "Single Molecule Protein Detection with Attomolar Sensitivity Using Droplet Digital Enzyme-Linked Immunosorbent Assay," *ACS Nano* 14, no. 8 (2020), https://doi.org/10.1021/acsnano.0c02378.

44 Yi-Jou Chen et al., "Development of a Highly Sensitive Enzyme-Linked Immunosorbent Assay (ELISA) Through Use of Poly-Protein G-Expressing Cell-Based Microplates," *Scientific Reports* 8, no. 1 (2018), https://doi.org/10.1038/s41598-018-36192-8.

27 filariasis patients; compared to traditional ELISA, this method had greater sensitivity.[45] The same concept was used to detect the amyloid-beta biomarker for Alzheimer's disease and showed similarly increased sensitivity, with an LOD of 50×10^{-12} M.[46] Antibody-functionalized graphene oxide has also been used to detect parvalbumin (a major allergen in fish) at a concentration of 4.29×10^{-9} g/mL, showing similar increased sensitivity and a lowered lower limit of detection compared to previous studies.[47]

4. Atom Transfer Radical Polymer – Gold Nanoparticle (ATRP-AuNP)

The use of ATRP-AuNPs as ELISA reporters may increase the sensitivity and lower the limit of detection of ELISA. An atom transfer radical polymer reaction generates in situ non-crosslinked polymer chains on particle surfaces and is relatively easy to control. The resulting polymer chains provide a large number of loci for proteins; because the polymer chains are not cross-linked, the protein substrates can migrate deep inside the chains. One study used ATRP-AuNPs to target the cancer biomarker Nogo-66 and was found to be 81 times more sensitive than conventional ELISA. This strategy could potentially be used for other immunoreactions methodologies, such as immunofluorescence. This procedure is simple, rapid, and does not require external equipment.[48]

5. Paper-Based ELISA (p-ELISA)

Paper-based ELISA was introduced in 2010 using an inexpensive cellulose fiber web that is ideal for low-cost point-of-care testing.[49] It has the advantage of being light, disposable, biodegradable, and costs can potentially reach less than $0.01.[50] Paper ELISA plates require lower sample volumes and results can be read using less expensive scanners (e.g., a $100 desktop scanner

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45 I.R. Aly et al., "Graphene Nanoparticles-Based ELISA as a Crucial New Diagnostic Tool for Diagnosis of Human Filariasis," *International Journal of Infectious Diseases* 101 (2020), https://doi.org/10.1016/j.ijid.2020.09.1101.

46 Jing Zhao et al., "Graphene Oxide-Gold Nanoparticle-Aptamer Complexed Probe for Detecting Amyloid Beta Oligomer by ELISA-Based Immunoassay," *Journal of Immunological Methods* 489 (2021), https://doi.org/10.1016/j.jim.2020.112942.

47 Yanbo Wang et al., "Graphene Oxide and Gold Nanoparticles-Based Dual Amplification Method for Immunomagnetic Beads-Derived ELISA of Parvalbumin," *Food Control* 110 (2020), https://doi.org/10.1016/j.foodcont.2019.106989.

48 Feng Chen et al., "Development of Atom Transfer Radical Polymer-Modified Gold Nanoparticle-Based Enzyme-Linked Immunosorbent Assay (ELISA)," *Analytical Chemistry* 86, no. 20 (2014), https://doi.org/10.1021/ac403872k.

49 Chao-Min Cheng et al., "Paper-Based ELISA," *Angewandte Chemie International Edition* 49, no. 28 (2010), https://doi.org/10.1002/anie.201001005.

50 Bo Pang et al., "Development of a Low-Cost Paper-Based ELISA Method for Rapid *Escherichia coli* O157:H7 Detection," *Analytical Biochemistry* 542 (2018), https://doi.org/10.1016/j.ab.2017.11.010.

vs. a $20,000 ELISA-specific plate reader).[51] A portable p-ELISA kit was designed to detect C-reactive protein (a biomarker for multiple diseases and conditions) with an LOD of 1µg/ml in blood. The kit is portable, easy to use, and costs less than $0.50 per kit; the sample (in this case, blood) and water is added onto a sliding strip, which is then moved at scheduled times before being analyzed by a scanner.[52] The potential of disposable p-ELISA kits could make it useful at Role 1 facilities.

One study found that p-ELISA has an LOD of 10^4 colony forming units (CFU)/mL for *E. coli* in under three hours. This method, which was verified to be compatible with food samples, used a smartphone for obtaining images instead of a scanner.[53] For SARS-CoV-2, the demonstrated LOD of 9.0×10^{-9} g/µL or 0.112 IU/mL is significantly lower than commercially available ELISA kits.[54]

6. Assessment

Much like digital PCR, droplet digital ELISA offers improvements, such as increased sensitivity. Commercial digital ELISA systems, such as the SiMoA Hd-1 analyzer, have entered the market; this specific system claims a 1,200-fold sensitivity improvement over the standard, has a sample-to-result time of one hour, and would be a moderately complex assay with a TRL of 7. Systems like these could offer a direct replacement for currently-used immunoassays, but are yet to be widely tested. While digital methods would increase sensitivity, they would not decrease the infrastructure/personnel requirements, nor would they assist in deploying the technology at a lower-role facility. With some assays commercially available, digital ELISA would have TRLs ranging from 4 to 7 and above, and would have moderate to high complexity.

By replacing the traditional ELISA microplates with 8pG-based microplates that contain cells expressing Fc domains of poly-protein G, an improvement to the plate's antibody-trapping ability could lead to an increase in sensitivity without affecting any other part of the assay. The TRL of 8pG-based microplates for ELISA is assessed at 4, with a moderate complexity.

Graphene oxide sheets can offer greater sensitivity when acting as an antibody carrier. While this technology has not yet been produced at scale, it has the potential to significantly impact current assays. While it would increase diagnostic performance, in the current state it would not decrease infrastructure requirements or make the assay more accessible. Graphene nanoparticle-

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51 Malik A. Anwar, "Paper Based Vs Conventional Enzyme Linked Immuno-Sorbent Assay: A Review of Literature," *International Clinical Pathology Journal* 3, no. 3 (2016), https://doi.org/10.15406/icpjl.2016.03.00079.

52 Mohit S. Verma et al., "Sliding-Strip Microfluidic Device Enables ELISA on Paper," *Biosensors & Bioelectronics* 99 (2018), https://doi.org/10.1016/j.bios.2017.07.034.

53 Anwar, "Paper Based Vs Conventional Enzyme Linked Immuno-Sorbent Assay: A Review of Literature."

54 Surasak Kasetsirikul et al., "Detection of the SARS-CoV-2 Humanized Antibody with Paper-Based ELISA," *Analyst* 145, no. 23 (2020), https://doi.org/10.1039/D0AN01609H.

based ELISA assays are still in a relatively preliminary stage of development, with a TRL of 3 and high complexity.

ATRP-AuNPs have demonstrated an ability to decrease the limit of detection for ELISA and amplify ELISA signals. This technique is simple and inexpensive, and does not require additional external equipment. With the potential to be applied to other immunoreactions, further research into the use of ATRP-AuNPs could help increase the diagnostic performance of existing assays. Currently, ATR-AuNPs would have a moderate complexity with a TRL of 4.

Paper-based ELISA assays have the advantage of being inexpensive (potentially less than $0.01/plate), require lower sample volumes, and may be used as point-of-care tests. These assays can be paired with portable scanners, such as smartphones, for readouts and could be performed by non-trained personnel. Disposable paper-based ELISA kits would likely be assessed at a TRL 4 and moderate CLIA complexity, with the potential of waived CLIA complexity and could be used at Role 1 facilities. A summary of various ELISA techniques is listed in Table 7 and Table 8.

Table 7. Assay Requirements for ELISA

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Droplet Digital ELISA/SiMoA	Bead sandwich plate	Standard immunofluorescent detectors	No	Refrigerator for reagent storage	Requires training
8pG-based Microplate	Microplate	Standard immunofluorescent detectors	No	--	Requires training
Graphene NP-based ELISA	Antibody-functionalized graphene sheets	Standard immunofluorescent detectors	No	--	Requires training
ATRP-AuNPs	--	--	No	--	Requires training
p-ELISA	Water	Smart phone or desktop scanner	Some	--	Potentially reduced or no training

Table 8. Assay Details for ELISA

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Droplet Digital ELISA/SiMoA	LOD: 60 copies/mL; 20–200 × 10^{-18} M concentrations Time: 60 minutes	Some commercial options	Increased sensitivity Faster time to result	Can be difficult to isolate the beads required for the assay

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
8pG-based Microplate	LOD: 2.5×10^{-9} M concentrations	Proof-of-concept	Increased sensitivity	
Graphene NP-based ELISA	LOD: 4.29×10^{-9} g/mL; 50×10^{-12} M concentrations	Proof-of-concept	Increased sensitivity	
ATRP-AuNPs	81x more sensitive than standard ELISA	Proof-of-concept	Increased sensitivity	
p-ELISA	LOD: 10^4 CFU/mL; 9×10^{-9} g/µL Time: 90 minutes–3 hours	Proof-of-concept	Low cost Increased sensitivity Results read with simple scanners	<$0.01/assay; $0.50/portable kit Light, disposable, and biodegradable May be useful at Role 1

D. Hybridization Chain Reaction (HCR)

HCR is a method for enzyme-free detection of specific DNA sequences based on a chain reaction of recognition and hybridization events between two sets of DNA hairpin molecules. These two DNA hairpins co-exist in a stable solution until a single-stranded DNA (ssDNA) initiator is added. This initiator opens one of the hairpins to expose a new single-stranded region, which then opens the other hairpin set to reveal a single-stranded region that is identical to the ssDNA initiator. This chain reaction forms a nicked double helix until the hairpin supply runs out. While conventional PCR causes exponential amplification, HCR causes linear amplification.[55]

In situ DNA HCR has the potential for use in detecting microbial cells, potentially including various pathogens. In situ probe hybridization followed by HCR amplification can produce bright signals with high specificity. One study showed that in situ HCR had an advantage over Fluorescent in-Situ Hybridization (FiSH) in detecting environmental microorganisms.[56] Another proposed method is based on multiplex super PCR and asymmetric tailing HCR, which can provide

[55] Daniel Evanko, "Hybridization Chain Reaction," *Nature Methods* 1, no. 3 (2004), https://doi.org/10.1038/nmeth1204-186a.

[56] Tsuyoshi Yamaguchi et al., "In Situ DNA-Hybridization Chain Reaction (HCR): A Facilitated in Situ HCR System for the Detection of Environmental Microorganisms," *Environmental Microbiology* 17, no. 7 (2015), https://doi.org/10.1111/1462-2920.12745.

a total detection time of 130 minutes for single-cell level detection. This method allows PCR to produce ssDNA products to initialize an HCR reaction.[57]

HCR signal amplification has also been used to detect *M. tuberculosis* genes, using magnetic beads for separation. Three hairpin probes were used, with one of the probes attached to magnetic beads. If the target sequence was present, HCR would be initiated and magnetic separation would result in total probe removal, resulting in a lack of fluorescence. If there were no target sequences in the sample, two of the three hairpin probes would remain after magnetic separation and result in fluorescence. The lowest detectable concentration was 10×10^{-12} M.[58] Using a flow-cytometric bead assay in conjunction with HCR, *B. cereus* was identified with a limit of detection of 7.7 CFU/mL (with an LOD in milk of 920 CFU/mL).[59]

A cyclic HCR technique was shown to detect hepatitis B virus DNA. This technique has a limit of detection of five copies per reaction and showed >97% sensitivity in clinical samples. This method uses two dumbbell-shaped primers (U1 and U2) that are each formed by two oligonucleotides (U1-1, U1-2, U2-1, U2-2); the primers are formed with two sticky ends that are complimentary, resulting in the formation of a packed, complex 3D DNA structure.[60]

HCR was combined with an aggregation-induced emission (AIE)-based fluorometric assay for sensitive nucleic acid detection and achieved an LOD of 37.2×10^{-15} M. In this wash/label/enzyme-free "seesaw" strategy, AIE luminogens (AIEgens) are used as bioprobes to interact electrostatically with DNA to turn on fluorescence signals. The AIEgen (in this case, tertiary amine-containing tetraphenylethene) also causes AuNP aggregation to change the solution tonality. An increase in DNA concentration would cause free TPE-Tas to generate a fluorescent signal, while the remaining TPE-Tas would cause a change in solution tonality. In the case of low DNA concentration, there would be weakened fluorescence but an increase in change of tonality. Two sets of signals help increase fidelity and ease of signal processing.[61]

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57 Jingjing Tian et al., "Visual Single Cell Detection of Dual-Pathogens Based on Multiplex Super PCR (MS-PCR) And Asymmetric Tailing HCR (AT-HCR)," *Sensors and Actuators B: Chemical* 260 (2018), https://doi.org/10.1016/j.snb.2018.01.017.

58 Zhao J. Chu et al., "Rapid and Sensitive Detection of the IS6110 Gene Sequences of *Mycobacterium tuberculosis* Based on Hybridization Chain Reaction and Reusable Magnetic Particles," *Sensors and Actuators B: Chemical* 282 (2019), https://doi.org/10.1016/j.snb.2018.11.146.

59 Bei Yu et al., "Hybridization Chain Reaction-Based Flow Cytometric Bead Sensor for the Detection of Emetic *Bacillus cereus* in Milk," *Sensors and Actuators B: Chemical* 256 (2018), https://doi.org/10.1016/j.snb.2017.09.199.

60 Gaolian Xu et al., "Cycling of Rational Hybridization Chain Reaction to Enable Enzyme-Free DNA-Based Clinical Diagnosis," *ACS Nano* 12, no. 7 (2018), https://doi.org/10.1021/acsnano.8b03183.

61 Jianlei Shen et al., "Dual-Mode Ultrasensitive Detection of Nucleic Acids via an Aqueous 'Seesaw' Strategy by Combining Aggregation-Induced Emission and Plasmonic Colorimetry," *ACS Applied Nano Materials* 2, no. 1 (2019), https://doi.org/10.1021/acsanm.8b01773.

1. ELISA

An ELISA-based platform combined fluorescent HCR with multicolor fluorescence concatemers (a polymer made of multiple copies of the same DNA sequence) to amplify and report signals. This system could simultaneously detect different pathogens with different fluorescent signatures, achieved PCR-level sensitivity, and did not require a nucleic acid extraction step. It demonstrated good performance in milk samples; experiments that test other sample types are still pending.[62]

To increase specificity, a novel method using a sandwich RNA capturing assay and a two-dimensional HCR bypasses RNA extraction with a capture hybridization method. A series of oligonucleotide probes, each complementary to a different region of the target and each having a specific sequence that interacts with either an adaptor or the solid support, is used. These probes are conjugated to the surface of wells in a well-plate and sandwich the RNA target, capturing the RNA onto the plate. The adaptors that are bound to the oligonucleotide probe tails initiate HCR once the hairpins are added. A 2D HCR technique includes an initial HCR reaction, triggered by the target that creates double-stranded DNA (dsDNA) with single sided branches, and triggers the second HCR reaction to create a branched double-stranded structure. This structure can be easily detected with common techniques such as DNA binding dyes, can yield a more amplified signal over conventional 1D HCR, and can be completed in less than four hours. This method used regular oligonucleotides (rather than specially prepared ones) and was performed on a standard 96-well plate, which lowers the cost and increases accessibility.[63,64] Instead of oligonucleotides, peptide nucleic acids have been shown to enable HCR. This could potentially be resistant to nucleases and proteases for *in vivo* applications.[65]

2. Multi-Branched HCR

In conjunction with gold nanoparticles, multi-branched HCR can detect vascular endothelial growth factor (VEGF) at concentrations as low as 3.7×10^{-15} M in less than 60 minutes. This system used a hairpin probe along with two dsDNA and two auxiliary ssDNAs to trigger non-

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62 Xi Lv et al., "Multicolor and Ultrasensitive Enzyme-Linked Immunosorbent Assay Based on the Fluorescence Hybrid Chain Reaction for Simultaneous Detection of Pathogens," *Journal of Agricultural and Food Chemistry* 67, no. 33 (2019), https://doi.org/10.1021/acs.jafc.9b03414.

63 Yao Xu and Zhi Zheng, "Direct RNA Detection Without Nucleic Acid Purification and PCR: Combining Sandwich Hybridization with Signal Amplification Based on Branched Hybridization Chain Reaction," *Biosensors & Bioelectronics* 79 (2016), https://doi.org/10.1016/j.bios.2015.12.057.

64 Yao Xu and Zhi Zheng, "Hybridization Chain Reaction for Direct MRNA Detection Without Nucleic Acid Purification," in I. Gaspar (ed.), *RNA Detection: Methods in Molecular Biology*, vol. 1649 (New York, NY: Humana Press, 2018), https://doi.org/10.1007/978-1-4939-7213-5_12.

65 Ki T. Kim, Simona Angerani, and Nicolas Winssinger, "A Minimal Hybridization Chain Reaction (HCR) System Using Peptide Nucleic Acids," *Chemical Science* 12, no. 23 (2021), https://doi.org/10.1039/D1SC01269J.

linear HCR.[66] Similarly, a layered-branched strategy without purification and separation steps achieved a limit of detection of 0.6×10^{-15} M for interferon gamma.[67] However, multi-branched HCR remains a complex process, and a false chain reaction initiation may occur. To improve on this, DNA strand displacement circuits have been employed, along with a hyperbranched HCR technique.[68]

One lab assembled DNA hairpins at the vertices of a quadrivalent DNA nanostructure, which accelerated the reaction kinetics of HCR due to multiple reaction orientations, increased collision probability, and enhanced local concentrations. The proof-of-concept was 70-fold faster than traditional HCR and increased probe biostability.[69]

3. Biosensors

A surface plasmon resonance (SPR) biosensing method was developed using nonlinear HCR. This method uses double-stranded DNA monomers, which dendritically assemble themselves into highly branched nanostructures upon introduction of the target sequence, to detect target DNA at concentrations of 0.85×10^{-12} M in approximately 60 minutes. This system was based on commercially available instruments, including the Biocore X analytical system, gold sending chips, BioRad ChemDoc XRS analyzer, and electrophoresis analyzers. This method remains in the proof-of-concept stage and may require some effort to bring to scale.[70]

HCR was used to create an ultrasensitive electrochemical biosensor that can detect *E. coli* at a limit of 7 CFU/mL. The target sequence was amplified by rolling circle amplification (RCA) with the use of a 3D DNA walker, and each RCA product fragment triggered HCR. The long dsDNA sequences formed, then immobilized, electrochemical indicators to create a greatly enhanced signal. When stored at 4°C for 30 days, the biosensor retained 94.3% of the initial response.[71]

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66 Chia-Chen Chang et al., "Aptamer-Based Colorimetric Detection of Proteins Using a Branched DNA Cascade Amplification Strategy and Unmodified Gold Nanoparticles," *Biosensors & Bioelectronics* 78 (2016), https://doi.org/10.1016/j.bios.2015.11.051.

67 Ting Bao et al., "Ultrasensitive Electrochemical Biosensor of Interferon-Gamma Based on Gold Nanoclusters-Graphene@zeolitic Imidazolate Framework-8 and Layered-Branched Hybridization Chain Reaction," *Sensors and Actuators B: Chemical* 296 (2019), https://doi.org/10.1016/j.snb.2019.05.083.

68 Chuyan Zhang et al., "The Recent Development of Hybridization Chain Reaction Strategies in Biosensors," *ACS Sensors* 5, no. 10 (2020), https://doi.org/10.1021/acssensors.0c01453.

69 Jing Wang et al., "Three-Dimensional DNA Nanostructures to Improve the Hyperbranched Hybridization Chain Reaction," *Chemical Science* 10, no. 42 (2019), https://doi.org/10.1039/C9SC02281C.

70 Xiaojuan Ding et al., "An Enzyme-Free Surface Plasmon Resonance Biosensing Strategy for Detection of DNA and Small Molecule Based on Nonlinear Hybridization Chain Reaction," *Biosensors & Bioelectronics* 87 (2017), https://doi.org/10.1016/j.bios.2016.08.077.

71 Yan Li et al., "A Sensitive Electrochemical Strategy via Multiple Amplification Reactions for the Detection of *E. coli* O157:H7," *Biosensors & Bioelectronics* 147 (2020), https://doi.org/10.1016/j.bios.2019.111752.

4. Assessment

As a relatively simple isothermal process, HCR may have great versatility as a diagnostic tool. This technique can be used in conjunction with techniques such as PCR, or by itself as a complete assay. HCR does not require specialized equipment and researchers have created assays with standard regents and materials, which would increase the accessibility to HCR. In its current state, HCR would be limited to higher-role facilities and would require personnel with considerable training to perform the assay, due to a lack of standardization, portability, and scaling. However, due to the simple nature of the reaction, once the technology is brought to scale, it could be less complex than procedures such as PCR and could be used in environments where instruments such as thermal cyclers are not readily available, as it is an isothermal reaction.

Combinations of HCR with technologies such as RCA, electrochemical biosensors, or fluorometric assays could provide increased sensitivity in the detection of low nucleotide concentrations. HCR could also be combined with other amplification methods with DNA for ultrasensitive detection of target molecules, such as demonstrated by Tian et al.[72] Prepared biosensors[73] could be useful for rapid and sensitive identification of pathogens. These biosensors would require a cold chain, but could be viable alternatives for standard assays such as PCR for common diagnostics, offering the advantage of reduced instrumentation and time requirements.

Improvements and modifications of the HCR process itself have been developed, such as two-dimensional (2D) HCR. This method was also performed using standard reagents such as regular oligonucleotides on a standard 96-well plate, which would be accessible at Role 3 facilities. The use of DNA nanostructures for HCR has been shown to increase reaction kinetics, and would decrease assay time significantly.[74] The CLIA complexity for HCR is currently high but could become moderately complex as production scaling increases and personnel requirements decrease. Currently, most HCR platforms would have a TRL of 4. A summary of various HCR techniques is listed in Table 9 and Table 10.

Table 9. Assay Requirements for HCR

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
ELISA-HCR	ELISA plate DNA initiator and hairpin probes DNA binding dyes	Standard immunofluorescent detectors	No	Refrigerator for reagent storage	Requires training

[72] Tian et al., "Visual Single Cell Detection of Dual-Pathogens Based on Multiplex Super PCR (MS-PCR) And Asymmetric Tailing HCR (AT-HCR)."

[73] Yan Li et al., "A Sensitive Electrochemical Strategy via Multiple Amplification Reactions for the Detection of *E. coli* O157:H7," *Biosensors & Bioelectronics* 147 (2020), https://doi.org/10.1016/j.bios.2019.111752.

[74] Wang et al., "Three-Dimensional DNA Nanostructures to Improve the Hyperbranched Hybridization Chain Reaction."

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Multi-Branched HCR	DNA initiator and hairpin probes	Standard immunofluorescent detectors	No	Refrigerator for reagent storage	Requires training
Biosensors	DNA monomers DNA walkers for amplification	Biocore X analytical system Gold sending chips BioRad ChemDoc XRS analyzer Electrophoresis analyzers	No	Refrigerator for reagent storage	Requires training

Table 10. Assay Details for HCR

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
ELISA-HCR	LOD: 5.6–98 CFU/mL Time: <4 hours	Proof-of-concept	Simultaneous detection of multiple pathogens	Bypasses nucleic acid extraction May be useful at Role 3
Multi-Branched HCR	LOD: 0.6–3.7 × 10^{-15} M concentrations Time: 60 minutes	Proof-of-concept	Increased sensitivity Faster time to result	Bypasses nucleic acid extraction May be useful at Role 3
Biosensors	LOD: 7 CFU/mL Time: 60 minutes	Proof-of-concept	Increased sensitivity Faster time to result	Uses standard commercial lab equipment May be useful at Role 3

E. Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) occurs when electrons in a metal surface layer are excited by photons at different angles of incidence, causing them to propagate parallel to the metal surface. The angle that triggers SPR is based on the refractive index of the material near the metal surface; small observable changes in the refractive index make analyte detection possible. SPR biosensors use a surface-coupled ligand to interact with the analyte when the solution is streamed over the surface, which modulates the resonance condition and can be monitored in real-time. Some commercial SPR instruments may be expensive, but labs have developed low-cost instruments that can detect pathogens such as *Salmonella*.[75]

[75] Jijo Lukose et al., "Real-Time and Rapid Detection of *Salmonella typhimurium* Using an Inexpensive Lab-Built Surface Plasmon Resonance Setup," *Laser Physics Letters* 15, no. 7 (2018), https://doi.org/10.1088/1612-202X/aabed8.

SPR has been used with plasmonic gold nanoparticles to amplify the fluorescent signal of quantum dots. This localized SPR could detect the NS1 protein of dengue virus with a limit of detection of 8 copies/mL.[76] Antimonene, a relatively new material, was used to create an SPR sensor for ultrasensitive miRNA detection. A novel process mechanically exfoliates bulk antimony through liquid-phase sonication to create antimonene nanosheets a few layers thick, which are then assembled onto gold SPR sensor chips. Gold nanorods conjugated with a probe DNA are then adsorbed onto the antimonene SPR chip. The LOD was 10×10^{-18} M.[77]

Fiber-optics based surface plasmon resonance assays can manipulate the properties of silica optical fibers to provide several advantages, including reduced cost and reduction of equipment needed. A miniature, portable, USB-powered fiber-optics based SPR instrument was created for methotrexate monitoring (a drug commonly prescribed for immunologic diseases, such as rheumatoid arthritis, which can cause major toxic side effects[78]), though the same methodology could be adapted for other clinical analytes.[79] Another portable optical fiber-based device SPR device can monitor serum antibody concentrations (e.g., infliximab); this device has many advantages over classic ELISA, including better handling, compact construction, and the ability to avoid long incubation, separation, and washing steps. The optical fibers are coated with anti-infliximab antibodies for infliximab detection, demonstrating an LOD of 73.7×10^{-9} g/mL, which is similar to conventional SPR (80×10^{-9} g/mL).[80]

A similar fiber optic method was used to detect *E. coli* in food at concentrations up to 50 CFU/mL. Antimicrobial peptides were used as the recognition elements and silver nanoparticles-reduced-graphene oxide aided signal amplification. The nanoparticles were fixed on the optical fiber and are covered in a gold film to prevent oxidation. The fixed peptides capture the bacteria and cause a shift in the wavelength peak.[81]

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76 Kenshin Takemura et al., "A Localized Surface Plasmon Resonance-Amplified Immunofluorescence Biosensor for Ultrasensitive and Rapid Detection of Nonstructural Protein 1 of Zika Virus," *PLOS ONE* 14, no. 1 (2019), https://doi.org/10.1371/journal.pone.0211517.

77 Tianyu Xue et al., "Ultrasensitive Detection of MiRNA with an Antimonene-Based Surface Plasmon Resonance Sensor," *Nature Communications* 10, no. 1 (2019), https://doi.org/10.1038/s41467-018-07947-8.

78 Kelly W. Jones and Supen R. Patel, "A Family Physician's Guide to Monitoring Methotrexate," *American Family Physician* 62, no. 7 (2000), https://www.aafp.org/afp/2000/1001/p1607.html.

79 Sandy S. Zhao et al., "Miniature Multi-Channel SPR Instrument for Methotrexate Monitoring in Clinical Samples," *Biosensors & Bioelectronics* 64 (2015), https://doi.org/10.1016/j.bios.2014.09.082.

80 Luigi Zeni et al., "A Portable Optical-Fibre-Based Surface Plasmon Resonance Biosensor for the Detection of Therapeutic Antibodies in Human Serum," *Scientific Reports* 10, no. 11154 (2020), https://doi.org/10.1038/s41598-020-68050-x.

81 Chen Zhou et al., "Fiber Optic Surface Plasmon Resonance Sensor for Detection of *E. Coli* O157:H7 Based on Antimicrobial Peptides and AgNPs-RGO," *Biosensors & Bioelectronics* 117 (2018), https://doi.org/10.1016/j.bios.2018.06.005.

A novel DNA nanotechnology-based method uses an entropy-driven strand displacement reaction (ESDR) with SPR to detect DNA. Hairpin probes are immobilized on the SPR sensor chip and bind target DNA on the terminal toehold region, which initiates toehold-mediated strand displacement reactions and causes the reuse of target DNA and the production of double-stranded complexes. These complexes hybridize with the hairpin probes and expose the double-layer DNA tetrahedon (DDT) binding sites; this causes three-decker composite formation on the SPR chip and significantly amplifies the SPR response. With its flexible sequence design, this method has excellent programmability. This enzyme-free method produces results in 60 minutes and has been tested with HIV-related DNA detection.[82]

ESDR and DNAzyme were used for an efficient DNA detection method tested on the p53 gene. ESDR systems use the entropic gain of liberated molecules and a series of ssDNA in a catalytic circuit using toehold-assisted branch migration to drive the reaction. DNAzymes are catalytic nucleic acid sequences that have hydrolytic cleavage activity on substrates in presence of specific cofactors. When a target is present, ESDR initiates toehold-mediated strand displacement to re-circulate the target DNA and release DNAzyme sequences into the solution. DNAzymes cyclically catalyze the cleavage of fluorophore/quencher-labeled DNA substrates, which amplifies the resultant fluorescent signal. This method shows excellent specificity, with a limit of detection of 220×10^{-15} M in 90 minutes, and could be easily adapted for other nucleotide sequences.[83]

1. Assessment

SPR assays have the advantage of being selective for the analyte, having fast analysis times, and potentially avoiding sample pre-processing steps, such as purification. Currently, commercially available SPR instruments are expensive to operate, though individual labs have targeted research to develop low-cost platforms.[84] The combination of fiber-optics with SPR instruments has also allowed for instrument miniaturization, with performance that matches conventional antibody assays;[85] additional research and development may be necessary to make SPR technology fully portable. While commercial SPRs exist, in-development SPR platforms (such as miniaturized versions) would still have moderate to high CLIA complexity and would be at a TRL of 4. A summary of SPR techniques is found in Table 11 and Table 12.

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82 Wei Diao et al., "Highly Sensitive Surface Plasmon Resonance Biosensor for the Detection of HIV-Related DNA Based on Dynamic and Structural DNA Nanodevices," *Biosensors & Bioelectronics* 100 (2018), https://doi.org/10.1016/j.bios.2017.08.042.

83 Yujian Li et al., "A Homogeneous Fluorescent Biosensing Strategy for Highly Sensitive Detection of DNA Based on a Programmed Entropy-Driven Strand Displacement Reaction and DNAzyme for Dual Recycling Amplification," *Analytical Methods* 11, no. 12 (2019), https://doi.org/10.1039/C9AY00061E.

84 Lukose et al., "Real-Time and Rapid Detection of *Salmonella typhimurium* Using an Inexpensive Lab-Built Surface Plasmon Resonance Setup."

85 Xue et al., "Ultrasensitive Detection of MiRNA with an Antimonene-Based Surface Plasmon Resonance Sensor."

Table 11. Assay Requirements for SPR

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Fiber Optics	--	Standard immunofluorescent detectors	Some	Refrigerator for reagent storage	Requires training
ESDR	DNA hairpin probes DNAzymes	Standard immunofluorescent detectors	No	Refrigerator for reagent storage	Requires training

Table 12. Assay Details for SPR

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Fiber Optics	LOD: 50 CFU/mL; 73.7 × 10^{-9} g/mL	Proof-of-concept	Faster time to result Simpler process	Can be expensive
ESDR	LOD: 220 × 10^{-15} M concentrations Time: 60–90 minutes	Proof-of-concept	Faster time to result Programmable process	

F. FiSH (Fluorescent in Situ Hybridization)

FiSH is a technique for identifying DNA within a genome.[86] In this technique, a fluorescent dye is attached to a purified piece of DNA. This DNA is incubated with the full set of chromosomes from the target genome. The labeled DNA hybridizes with the complementary genomic DNA if present, which can then be identified by observing fluorescence under a microscope. This technique is usually limited to laboratories.

The Accelerate Pheno system aims to automate FiSH with time-lapse imaging for rapid identification and antibiotic susceptibility testing of positive blood culture samples. A two-center study found that this system was 98.8% sensitive, 94.7% specific, and is much faster than conventional diagnostic standards, reducing diagnostic times by approximately 40 hours. While FDA-cleared for certain targets, the Accelerate Pheno system currently has a few drawbacks, including a significant number of false positives.[87]

86 "Fluorescence In Situ Hybridization," National Hyman Genome Research Institute, Accessed April 12, 2022, https://www.genome.gov/genetics-glossary/Fluorescence-In-Situ-Hybridization

87 Joseph D. Lutgring et al., "Evaluation of the Accelerate Pheno System: Results from Two Academic Medical Centers," *Journal of Clinical Microbiology* 56, no. 4 (2018), https://doi.org/10.1128/JCM.01672-17.

1. Pre-Labeled Oligomer Probes (PLOPs)

Pre-labeled oligomer probes (PLOPs) have the potential to reduce FiSH hybridization time from approximately 16 hours (when using conventional nick-translation derived rDNA probes) to approximately 5 minutes (when using PLOPs), while also significantly reducing the reagent cost. By taking advantage of conserved polymorphisms in ribosomal RNA, probes can be developed to identify various microbial species or genera.[88]

2. Fixation-Free FiSH

To detect *Salmonella* in samples such as milk and water, a fixation-free FiSH method used polyhexamethylene biguanide (PHMB), a molecule with cellular delivery properties, to achieve a turnaround time of less than five hours; the results of the fixation-free method did not differ from standard FiSH.[89]

3. Peptide Nucleic Acid (PNA)-FiSH

While peptide nucleic acid FiSH has been used extensively for research, it is rare in clinical settings. One study using a rat model used PNA-FiSH to identify multiple pathogens with swab and tissue samples from burn wounds, which reduced the need for culture-based diagnostics and decreased the diagnostic turnaround time. In addition, tissue autofluorescence did not appear to interfere with the analysis.[90] Another study used PNA-FiSH on a variety clinical samples, such as sputum and bronchoalveolar lavage, to demonstrate clinical viability, requiring only 1.5 hours to perform the procedure after sample pre-enrichment.[91]

4. Assessment

The use of pre-labeled oligomer probes (PLOPs) has the potential to decrease FiSH hybridization time by an order of magnitude, while also decreasing reagent requirements; other equipment requirements for FiSH would likely remain the same despite using PLOPs. This technique could replace current FiSH assays, though extensive studies have not yet been carried out in clinical samples. PLOPs used in FiSH would have a TRL of 3 and a moderate CLIA complexity.

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88 Nomar E. Waminal et al., "Rapid and Efficient FISH Using Pre-Labeled Oligomer Probes," *Scientific Reports* 8, no. 1 (2018), https://doi.org/10.1038/s41598-018-26667-z.

89 Oluwawemimo Adebowale and Liam Good, "Development of a Fixation-Free Fluorescence in Situ Hybridization for the Detection of *Salmonella* Species," *Biology Methods & Protocols* 5, no. 1 (2020), https://doi.org/10.1093/biomethods/bpaa024.

90 Alan J. Weaver et al., "Clinical Utility of PNA-FISH for Burn Wound Diagnostics: A Noninvasive, Culture-Independent Technique for Rapid Identification of Pathogenic Organisms in Burn Wounds," *Journal of Burn Care & Research* 40, no. 4 (2019), https://doi.org/10.1093/jbcr/irz047.

91 Laura Cerqueira et al., "Establishment of a New PNA-FISH Method for *Aspergillus fumigatus* Identification: First Insights for Future Use in Pulmonary Samples," *Microorganisms* 8, no. 12 (2020), https://doi.org/10.3390/microorganisms8121950.

Fixation-free FiSH could decrease assay time compared to traditional FiSH. However, this method has not been tested on clinical samples and further studies may be warranted before recommending clinical use; fixation-free FiSH would be assessed at a moderate CLIA complexity and a TRL of 3.

The use of peptide nucleic acids (PNAs) could also help decrease the time to perform FiSH, with labs reporting a run time of only 1.5 hours. This assay would have a TRL of 4 and moderate CLIA complexity. A summary of various FiSH techniques can be found in Table 13 and Table 14.

Table 13. Assay Requirements for FiSH

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
PLOPs	Pre-labeled oligomer probes	Standard immunofluorescent detectors	No	--	Requires training
Fixation-Free FiSH	Polyhexamethylene biguanide	Standard immunofluorescent detectors	No	Refrigerator for reagent storage	Requires training
PNA-FiSH	--	Standard immunofluorescent detectors	No	--	Requires training

Table 14. Assay Details for FiSH

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
PLOPs	Time: 5 minutes	Proof-of-concept	Faster time to result Low cost	$0.06/slide
Fixation-Free FiSH	LOD: 10^5–10^6 CFU/mL Time: <5 hours	Proof-of-concept	Faster time to result	
PNA-FiSH	LOD: 10^3 spores/mL Time: 1.5–24 hours	Proof-of-concept	Faster time to result Simpler process	

G. CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-based systems take advantage of the high specificity and sensitivity of natural CRISPR systems.[92] CRISPR is a natural defense mechanism of single-celled organisms, which identifies and cleaves specific nucleotide

[92] Xiaohong Xiang et al., "CRISPR-Cas Systems Based Molecular Diagnostic Tool for Infectious Diseases and Emerging 2019 Novel Coronavirus (COVID-19) Pneumonia," *Journal of Drug Targeting* 28, no. 7-8 (2020), https://doi.org/10.1080/1061186X.2020.1769637.

sequences foreign to the host cell. This property of nucleotide cleavage can be leveraged for identification of specific sequences with high accuracy. Cas9, Cas12, and Cas13 are the most common proteins used in conjunction with CRISPR. CRISPR/Cas systems are divided into two classes: Class 1 systems that use CRISPR RNA (crRNA) with multi-effector complexes to recognize and cleave the target sequence, and Class 2 systems that utilize a single multi-domain Cas protein with crRNA for interference. Class 2 systems are currently used for genome editing and rapid pathogen diagnosis, and include the proteins cas9, cas12, and cas13.[93] CRISPR/Cas12 and Cas13 shows non-specific trans-cleavage activity after nucleic acid recognition, giving it the potential to become a rapid and accurate diagnostic tool. CRISPR Cas13 also has the feature of being a ribonuclease, cleaving RNA instead of DNA.[94]

1. Cas9

CRISPR-dCas9 has been used with FiSH to detect methicillin-resistant *S. aureus* (MRSA). This method has an advantage over the conventional monoclonal antibody method because the protein can interact in a sequence-specific manner, yielding an LOD of 10 CFU/mL in 30 minutes. dCas9 lacks endonuclease activity, which makes it an efficient DNA probe that acts similarly to an antibody; this makes it possible to rapidly distinguish between *S. aureus* isolates based on the presence or absence of the mecA gene. dCAS9 was used with SYBR Green as a dye for staining.[95] CRISPR/Cas9 can also be used to directly detect antibiotic resistance by recognizing and cleaving sequences of the resistance genes (i.e., plasmids). Cutting circular plasmids results in a linear configuration: when Cas9 cuts plasmids at a specific location, most will be linearized at the same location and will be directly visible via microscope. These "barcodes" are created by adding netropsin and YOYO, an optical DNA mapping method.[96]

CRISDA (CRISPR-Cas9-triggered nicking endonuclease-mediated strand displacement amplification) is a method that takes advantage of CRISPR's sensitivity in recognizing target DNA to isothermally amplify and detect DNA; this method has a detection limit of 0.25×10^{-18} M (3

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93 Xiang et al., "CRISPR-Cas Systems Based Molecular Diagnostic Tool for Infectious Diseases and Emerging 2019 Novel Coronavirus (COVID-19) Pneumonia."

94 Bruno T. D. Nunes et al., "Development of RT-QPCR and Semi-Nested RT-PCR Assays for Molecular Diagnosis of Hantavirus Pulmonary Syndrome," *PLOS Neglected Tropical Diseases* 13, no. 12 (2019), https://doi.org/10.1371/journal.pntd.0007884; Shi-Yuan Li et al., "CRISPR-Cas12a Has Both Cis- and Trans-Cleavage Activities on Single-Stranded DNA," *Cell Research* 28, no. 4 (2018), https://doi.org/10.1038/s41422-018-0022-x.

95 Kyeonghye Guk et al., "A Facile, Rapid and Sensitive Detection of MRSA Using a CRISPR-Mediated DNA FISH Method, Antibody-Like DCas9/sgRNA Complex," *Biosensors & Bioelectronics* 95 (2017), https://doi.org/10.1016/j.bios.2017.04.016.

96 Vilhelm Müller et al., "Direct Identification of Antibiotic Resistance Genes on Single Plasmid Molecules Using CRISPR/Cas9 in Combination with Optical DNA Mapping," *Scientific Reports* 6, no. 1 (2016), https://doi.org/10.1038/srep37938.

copies/reaction) for the pGL3 plasmid. PNA probes are used to increase accuracy. With the addition of a reverse transcription step, this method could be adapted for RNA identification.[97]

A CRISPR/Cas9-triggered isothermal exponential amplification reaction (CAS-EXPAR) is based on nicking endonuclease (NEase) mediated nucleic acid amplification. This method does not require exogenous primers because Cas9 directs the site-specific target cleavage that generates the primers, which hybridize with the template that then allows the DNA polymerase to act. After dsDNA formation, the NEase induces a nick to result in the release of the primer due to DNA polymerase displacement activity. The limit of detection was 0.82×10^{-18} M; this method could also be used to detect DNA methylation.[98]

CRISPR/Cas9-mediated lateral flow nucleic acid assay (CASLFA) is a method that combines CRISPR with a lateral flow assay and has 100% concurrence with RT-PCR for African swine fever virus. Due to the two steps of selective gene amplification and Cas9/sgRNA recognition, this method increases specificity and sensitivity, indicating its potential as a point-of-care diagnostic tool. An AuNP probe was used to target the non-target ssDNA produced after CRISPR/Cas9 recognition in the lateral flow assay (LFA). With a portable toolkit, results with an LOD of 200 copies/reaction could be obtained in approximately 40 minutes.[99]

CRISPR-Chip is a rapid CRISPR-enhanced graphene-based field-effect transistor (gFET) method that uses graphene functionalized with dCas9 as a channel between the source and drain electrodes. The binding of the target DNA to the dCas9 complex results in electrical modulation of the gFET. This technique could detect Duchenne muscular dystrophy-associated mutations, had an LOD of 3.3 ng/μL (1.7×10^{-15} M genomic material), and could generate results in 15 minutes without DNA amplification.[100]

One lab used toehold switches combined with recombinase polymerase amplification (RPA) and a CRISPR/Cas9 system to rapidly identify and distinguish Zika genotypes. Nucleic acid sequence-based amplification (NASBA) was used to isothermally amplify RNA. Toehold switch sensors are programmable synthetic bioregulators that control gene translation by binding to and activating a ribosome when in the presence of target RNA; these sensors can detect RNA by causing translation of the lacZ enzyme to initiate a substrate color change. This system uses Cas9

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97 Wenhua Zhou et al., "A CRISPR-Cas9-Triggered Strand Displacement Amplification Method for Ultrasensitive DNA Detection," *Nature Communications* 9, no. 1 (2018), https://doi.org/10.1038/s41467-018-07324-5.

98 Mengqi Huang et al., "Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Triggered Isothermal Amplification for Site-Specific Nucleic Acid Detection," *Analytical Chemistry* 90, no. 3 (2018), https://doi.org/10.1021/acs.analchem.7b04542.

99 Xusheng Wang et al., "Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-Mediated Lateral Flow Nucleic Acid Assay," *ACS Nano* 14, no. 2 (2020), https://doi.org/10.1021/acsnano.0c00022.

100 Reza Hajian et al., "Detection of Unamplified Target Genes via CRISPR-Cas9 Immobilized on a Graphene Field-Effect Transistor," *Nature Biomedical Engineering* 3, no. 6 (2019), https://doi.org/10.1038/s41551-019-0371-x.

to cleave DNA only in the presence of the appropriate protospacer adjacent motif (PAM). If the appropriate PAM sequence and target site is present in the DNA after NASBA amplification, the Cas9 cleavage will result in an RNA product that cannot activate the toehold switch sensor. The system was able to differentiate strains at base pair resolution.[101]

The Finding Low Abundance Sequences by Hybridization (FLASH) method combines Cas9 with next-generation sequencing (NGS), which describes technologies that sequence nucleic acids more rapidly and with higher sensitivity by running in parallel.[102] By choosing optimal guide RNA targets, sequences of interest are cleaved into fragments for Illumina sequencing. FLASH can be used to identify antimicrobial resistance genes and can act as an efficient alternative to PCR.[103]

2. Cas12

Cas12a, which does not use transactivating crRNA, generates a staggered cut with a 5' overhand at DNA target sites. These staggered ends are different from the blunt ends of Cas9 and may be advantageous for integrating DNA sequences in a precise orientation.[104] Unlike Cas9 proteins, Cas12a proteins are guided by a ssRNA sequence without any transactivating CRISPR RNAs (tracrRNAs), making them simpler to design and use.[105] A system using RPA and CRISPR-Cas12a detection could sensitively quantify viral particles using a chip format incorporated into a smartphone socket.[106]

One lab used isothermal amplification to create a method called DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR). DETECTR combines the activation of non-specific single-stranded deoxyribonuclease Cas12a with isothermal amplification. The binding of the crRNA-Cas12a complex to the target causes indiscriminate cleavage of ssDNA, which is coupled with a

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101 Keith Pardee et al., "Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components," *Cell* 165, no. 5 (2016), https://doi.org/10.1016/j.cell.2016.04.059.

102 Sam Behjati and Patrick S. Tarpey, "What is Next Generation Sequencing?" *Archives of Disease in Childhood, Education and Practice Edition* 98, no. 6 (December 2013), https://doi.org/10.1136/archdischild-2013-304340; Thermo Fisher Scientific, "What is Next Generation Sequencing (NGS)?" accessed May 18, 2022, https://www.thermofisher.com/us/en/home/life-science/sequencing/sequencing-learning-center/next-generation-sequencing-information/ngs-basics/what-is-next-generation-sequencing.html.

103 Jenai Quan et al., "FLASH: A Next-Generation CRISPR Diagnostic for Multiplexed Detection of Antimicrobial Resistance Sequences," *Nucleic Acids Research* 47, no. 14 (2019), https://doi.org/10.1093/nar/gkz418.

104 Adrian Pickar-Oliver and Charles A. Gersbach, "The Next Generation of CRISPR-Cas Technologies and Applications," *Nature Reviews Molecular Cell Biology* 20, no. 8 (2019), https://doi.org/10.1038/s41580-019-0131-5.

105 Sergey Shmakov et al., "Discovery and Functional Characterization of Diverse Class 2 CRISPR-Cas Systems," *Molecular Cell* 60, no. 3 (2015), https://doi.org/10.1016/j.molcel.2015.10.008.

106 Bo Ning et al., "A Smartphone-Read Ultrasensitive and Quantitative Saliva Test for COVID-19," *Science Advances* 7, no. 2 (2021), https://doi.org/10.1126/sciadv.abe3703.

fluorescent reporter and exhibits attomolar (10^{-18} M) sensitivity.[107] A similar technique was used for point-of-care detection of African Swine Fever Virus (ASFV), with Cas12a/crRNA binding to the target DNA to form an activated complex that degrades a fluorescent ssDNA reporter. The most rapid version takes 2 hours and has a limit of detection of 1 mM, but if the sample is incubated for 24 hours, the limit of detection is 100×10^{-15} M.[108]

Similar to DETECTR, HOLMES (one-Hour Low-cost Multipurpose highly Efficient System) is a system developed after Specific High-Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) that uses Cas12a for DNA or RNA detection after pre-amplification with PCR.[109] HOLMESv2 further improves this method and was created using thermophilic CRISPR-Cas12b. It is a one-pot system using LAMP amplification to quantify the nucleic acid target and can be performed in 30 minutes. HOLMESv2 also uses the Bst 3.0 polymerase, a DNA polymerase that can work on both DNA and RNA templates and therefore avoid the need for a reverse-transcription step.[110] For detection of SARS-CoV-2, a HOLMES-based assay had a limit of detection of 1.6 copies/reaction.[111]

All-In-One Dual CRISPR (AIOD-CRISPR) is another one-pot system that was tested with SARS-CoV-2 and human immunodeficiency virus (HIV)-1. This system uses a pair of Cas12a RNA complexes to bind two sites that are in proximity to primer recognition sites in the target sequence. During incubation, the RPA amplification exposes the binding sites of the Cas12a complexes due to strand displacement. The Cas12a endonuclease then cleaves nearby ssDNA-FQ reporters to produce fluorescence. Visual detection of SARS-CoV-2 could be done with a sensitivity of 4.6 copies per pot in as little as 40 minutes.[112]

A point-of-care system for viral detection (iSCAN SARS-CoV-2 detection module) was developed using RT-LAMP and CRISPR-Cas12 in an LFA. The assay could detect 10 RNA copies

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107 Janice S. Chen et al., "CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded DNase Activity," *Science* 360, no. 6387 (2018), https://doi.org/10.1126/science.aar6245.

108 Qian He et al., "High-Throughput and All-Solution Phase African Swine Fever Virus (ASFV) Detection Using CRISPR-Cas12a and Fluorescence Based Point-of-Care System," *Biosensors & Bioelectronics* 154 (2020), https://doi.org/10.1016/j.bios.2020.112068.

109 Shi-Yuan Li et al., "CRISPR-Cas12a-Assisted Nucleic Acid Detection," *Cell Discovery* 4, no. 1 (2018), https://doi.org/10.1038/s41421-018-0028-z.

110 Linxian Li et al., "HOLMESv2: A CRISPR-Cas12b-Assisted Platform for Nucleic Acid Detection and DNA Methylation Quantitation," *ACS Synthetic Biology* 8, no. 10 (2019), https://doi.org/10.1021/acssynbio.9b00209.

111 Weiren Huang et al., "A CRISPR-Cas12a-Based Specific Enhancer for More Sensitive Detection of SARS-CoV-2 Infection," *EBioMedicine* 61 (2020), https://doi.org/10.1016/j.ebiom.2020.103036.

112 Xiong Ding et al., "All-in-One Dual CRISPR-Cas12a (AIOD-CRISPR) Assay: A Case for Rapid, Ultrasensitive and Visual Detection of Novel Coronavirus SARS-CoV-2 and HIV Virus," *bioRxiv*, 2020, https://doi.org/10.1101/2020.03.19.998724.

per reaction, takes less than one hour, requires simple equipment (i.e., is field-deployable), and uses an easy-to-read colorimetric reaction.[113]

CRISPR-Cas-only amplification network (CONAN) is a CRISPR-Cas-powered nucleic acid circuit that detects isothermally amplified genomic DNA. With one-step real-time single-base-specific DNA detection, this system uses a positive-feedback circuit to increase sensitivity to the attomolar level. It has been tested on human bladder cancer-associated single nucleotide mutations and Hepatitis B virus infections, detecting as low as 3 copies/μL of HBV genomic DNA.[114]

Microfluidics can be used to accelerate biochemical reactions, as in a Cas12-based system that uses an electric field to focus the Cas12-gRNA, reporters, and target within a chip. An electrokinetic microfluidic technique called isotachophoresis (ITP) was used, which uses a two-buffer system consisting of a high-mobility leading electrolyte and a low-mobility trailing electrolyte. When an electric field is applied, sample ions whose mobilities are between those of the two-buffer electrolytes can be focused at the interface of the electrolytes. ITP is used both to extract nucleic acids from raw biological samples and to control enzymatic activity on target recognition; this leads to a 100-fold lower consumption of reagents and an entire assay runtime of 30–40 minutes. This technique could be scaled up, but current disadvantages include requiring an off-chip amplification step.[115]

One point-of-care system uses magnetic bead-based capture and magnetofluidic transport to concentrate/purify SARS-CoV-2 with nucleic acid-binding magnetic beads; this method can also be used to transport RNA for the Cas12a-based RT-RPA assay. The assay was adapted into a plastic cartridge with a palm-size device to automate the process. The device achieved 100% concordance with RT-PCR and could produce results in 20 minutes.[116] CRISPR-Cas12a could be combined with RPA for sensitive detection of foodborne pathogens. As few as 10 copies could be detected in 45 minutes at 37°C. Only a handheld centrifuge and portable fluorescence analyzer were required for detection, making this method potentially useful in the field.[117]

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113 Zahir Ali et al., "ISCAN: An RT-LAMP-Coupled CRISPR-Cas12 Module for Rapid, Sensitive Detection of SARS-CoV-2," *Virus Research* 288 (2020), https://doi.org/10.1016/j.virusres.2020.198129.

114 Kai Shi et al., "A CRISPR-Cas Autocatalysis-Driven Feedback Amplification Network for Supersensitive DNA Diagnostics," *Science Advances* 7, no. 5 (2021), https://doi.org/10.1126/sciadv.abc7802.

115 Ashwin Ramachandran et al., "Electric Field-Driven Microfluidics for Rapid CRISPR-Based Diagnostics and Its Application to Detection of SARS-CoV-2," *Proceedings of the National Academy of Sciences* 117, no. 47 (2020), https://doi.org/10.1073/pnas.2010254117.

116 Fan-En Chen et al., "Point-of-Care CRISPR-Cas-Assisted SARS-CoV-2 Detection in an Automated and Portable Droplet Magnetofluidic Device," *Biosensors & Bioelectronics* 190 (2021), https://doi.org/10.1016/j.bios.2021.113390.

117 Hua Liu et al., "RPA-Cas12a-FS: A Frontline Nucleic Acid Rapid Detection System for Food Safety Based on CRISPR-Cas12a Combined with Recombinase Polymerase Amplification," *Food Chemistry* 334 (2021), https://doi.org/10.1016/j.foodchem.2020.127608.

CRISPR/Cas12a was also shown to have increased sensitivity over the GeneXpert MTB/RIF assay for tuberculosis, which is the current gold standard.[118] It was also shown to detect 2 copies of SARS-CoV-2 RNA, while quantitative PCR (qPCR) could not detect samples containing less than 5 copies.[119] A Cas12a variant, enAsCas12a, has been developed with an expanded targeting range that can target many more PAMs. This may be combined with other systems for efficient diagnoses.[120]

A Cas12a-dependent reporter system using functional DNA (fDNA) molecules, such as aptamers, was developed to detect non-nucleic-acid targets, such as metal ions or small organic molecules (e.g., adenosine triphosphate (ATP)). An fDNA molecule locks a DNA activator for Cas12a-crRNA, preventing ssDNA cleavage. In the presence of the fDNA targets, the DNA activator is unlocked, which activates the ssDNA cleavage that cleaves a DNA substrate with a fluorophore. ATP and sodium ions have been detected using this method at room temperature in less than 15 minutes, making it a potential point-of-care tool.[121]

A similar aptamer-based system called RADAR (Random Molecular Aptamer-Dependent CRISPR-Assisted Reporter) also detects small molecules. The binding of the aptamer to the target results in reduced binding of the aptamer to the Cas12a complex, causing a reduced fluorescent signal. The limit of detection was 10000×10^{-9} M and the assay could be performed in 25 minutes.[122]

One group used the AaCas12b enzyme, which can cleave double-stranded DNA with high specificity and sensitivity, as a detection system. In most tested cleavage sites, it had a higher sensitivity than Cas12a-based detection and could identify human papillomavirus (HPV) dsDNA at sub-attomolar concentrations. Unlike Cas13-based systems, this method does not require a T7 transcription step, which is an advantage for rapid detection.[123]

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118 Jing-Wen Ai et al., "CRISPR-Based Rapid and Ultra-Sensitive Diagnostic Test for *Mycobacterium tuberculosis*," *Emerging Microbes & Infections* 8, no. 1 (2019), https://doi.org/10.1080/22221751.2019.1664939.

119 Zhen Huang et al., "Ultra-Sensitive and High-Throughput CRISPR-Powered COVID-19 Diagnosis," *Biosensors & Bioelectronics* 164 (2020), https://doi.org/10.1016/j.bios.2020.112316.

120 Benjamin P. Kleinstiver et al., "Engineered CRISPR-Cas12a Variants with Increased Activities and Improved Targeting Ranges for Gene, Epigenetic and Base Editing," *Nature Biotechnology* 37, no. 3 (2019), https://doi.org/10.1038/s41587-018-0011-0.

121 Ying Xiong et al., "Functional DNA Regulated CRISPR-Cas12a Sensors for Point-of-Care Diagnostics of Non-Nucleic-Acid Targets," *Journal of the American Chemical Society* 142, no. 1 (2020), https://doi.org/10.1021/jacs.9b09211.

122 Chenqi Niu et al., "Aptamer Assisted CRISPR-Cas12a Strategy for Small Molecule Diagnostics," *Biosensors & Bioelectronics* 183 (2021), https://doi.org/10.1016/j.bios.2021.113196.

123 Fei Teng et al., "CDetection: CRISPR-Cas12b-Based DNA Detection with Sub-Attomolar Sensitivity and Single-Base Specificity," *Genome Biology* 20, no. 1 (2019), https://doi.org/10.1186/s13059-019-1742-z.

Lyo-CRISPR SARS-CoV-2 is a commercially available assay kit for the detection of SARS-CoV-2. This assay has almost perfect concordance with RT-PCR when tested with nasopharyngeal swabs; results are rapid (available in 1.5 hours) and specific (without cross-reactivity to other respiratory pathogens). This test could be deployed at low-resource laboratories as it only requires one additional step of RNA extraction, which can be performed using a QIAamp Viral RNA Mini Kit.[124]

To avoid relying on pre-assembled Cas-crRNA complexes, one self-powered method uses Cas12a to recruit crRNA by self-processing pre-crRNA repeats that are generated by target-responsive rolling circle transcription. In rolling circle transcription, a small circular nucleotide acts as a template for RNA polymerase to produce long, repeated product strands that are amplified copies of the circle sequence. This method suppresses non-specific background signals and does not require the presence of a PAM site in the target sequence.[125]

When Cas12a is linked to a glucose-producing reaction, the results of SARS-CoV-2 detection can be quantified by a glucometer, with a range of $10–10^4$ copies/μL. Reverse transcription recombinase-aided amplification (RT-RAA) is used to convert target viral RNA into cDNA and amplify at constant mild temperatures. Cas12a recognizes the amplified DNA and the ssDNA cleavage activity causes the release of ssDNA-conjugated invertase on magnetic beads. The samples do not require an extraction step and are treated with commercial lysis and RNA protective buffers.[126]

3. Cas13

Cas13 is an RNA-guided ribonuclease, which provides specificity through crRNA-target pairing. One lab developed a combinatorial arrayed reaction for multiplexed evaluation of nucleic acids (CARMEN)-Cas13 system. The system inputs are PCR-amplified samples and a detection mix including Cas13, a sequence-specific CRISPR RNA, and a cleavage reporter. The lab developed an assay to test a sample for 169 human-associated viruses simultaneously. When compared with 11,268 sequenced samples, 99.7% of the results were concordant.[127]

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124 Lucía A. Curti et al., "Evaluation of a Lyophilized CRISPR-Cas12 Assay for a Sensitive, Specific, and Rapid Detection of SARS-CoV-2," *Viruses* 13, no. 3 (2021), https://doi.org/10.3390/v13030420.

125 Gaoting Wang et al., "New CRISPR-Derived MicroRNA Sensing Mechanism Based on Cas12a Self-Powered and Rolling Circle Transcription-Unleashed Real-Time CrRNA Recruiting," *Analytical Chemistry* 92, no. 9 (2020), https://doi.org/10.1021/acs.analchem.0c00680.

126 Di Huang et al., "A CRISPR-Cas12a-Derived Biosensor Enabling Portable Personal Glucose Meter Readout for Quantitative Detection of SARS-CoV-2," *Biotechnology and Bioengineering* 118, no. 4 (2021), https://doi.org/10.1002/bit.27673.

127 Cheri M. Ackerman et al., "Massively Multiplexed Nucleic Acid Detection with Cas13," *Nature* 582, no. 7811 (2020), https://doi.org/10.1038/s41586-020-2279-8.

A Cas13a-based system called SHERLOCK can detect and quantify strains of Zika and Dengue virus, pathogenic bacteria, and mutations in tumor DNA. SHERLOCK combines reverse transcription recombinase polymerase amplification (RT-RPA) with Cas13a nuclease activity and uses a crRNA-Cas13a complex to bind to the target, which activates the specific and non-specific RNAse activity. This degrades the non-target RNA, which is coupled to a fluorescent reporter. This method could be used in the field with paper spotting and lyophilization; when Cas13a complexes were lyophilized and rehydrated, they were still able to detect 20×10^{-15} M of non-amplified ssRNA. SHERLOCK could also differentiate between *P. aeruginosa* strains with different resistance genes, and could detect single-base differences. A commercial SHERLOCK SARS-CoV-2 assay was authorized by the FDA for emergency use and demonstrated an LOD of 1.35 copies/μL.[128]

SHERLOCK was revised to create SHERLOCKv2, which could simultaneously detect one DNA target and three ssRNA targets in the same reaction. Cas13 works together with Csm6 (an auxiliary CRISPR type III-associated nuclease) to result in a 3.5-fold increase in signal sensitivity. Csm6 cleaves ssRNA that is complementary to the target crRNA, while collateral Cas13 activity generates Csm6-activating species to allows for amplified detection with a Csm6-specific reporter. This method can be performed in less than 90 minutes with a limit of detection of 2×10^{-18} M. By scaling the pre-amplification RPA step, a detection signal could be produced for sample concentrations as low as $8–200 \times 10^{-21}$ M. A lateral-flow readout allows for visual analysis without additional instrumentation. In addition, a closed-tube/one-pot assay was developed as an alternative.[129]

Standard SHERLOCK methodology requires an extraction step to detect viral nucleic acid. HUDSON (Heating Unextracted Diagnostic Samples to Obliviate Nucleases) pairs with SHERLOCK to provide direct instrument-free detection from patient samples in less than two hours. This combination could detect Zika virus (ZIKV) RNA in human saliva and urine at concentrations of 0.9×10^{-18} M and 20×10^{-18} M, respectively. The system was designed to be a field-deployable rapid viral diagnostic platform that requires minimal equipment, and the reagents can be lyophilized for cold chain independence.[130] This system (combined with RPA for amplification) has also been used for early diagnosis of rabies; the ultra-sensitivity allowed for detection of RNA particles in the cerebrospinal fluid of rats earlier than RT-PCR and RPA-

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128 Jonathan S. Gootenberg et al., "Nucleic Acid Detection with CRISPR-Cas13a/C2c2," *Science* 356, no. 6336 (2017), https://doi.org/10.1126/science.aam9321.

129 Jonathan S. Gootenberg et al., "Multiplexed and Portable Nucleic Acid Detection Platform with Cas13, Cas12a, and Csm6," *Science* 360, no. 6387 (2018), https://doi.org/10.1126/science.aaq0179.

130 Cameron Myhrvold et al., "Field-Deployable Viral Diagnostics Using CRISPR-Cas13," *Science* 360, no. 6387 (2018), https://doi.org/10.1126/science.aas8836.

CRISPR, and could detect the virus at three days after infection, though clinical signs started to appear at six to seven days after infection.[131]

A method called SHINE (Streamlined Highlighting of Infections to Navigate Epidemics) was created as a combination of SHERLOCK and HUDSON. SHINE improves on HUDSON to inactivate viruses in 10 minutes and optimizes SHERLOCK to allow RPA and Cas13-based detection in a single step. For detection of SARS-CoV-2, the entire assay could be performed in 50 minutes and had 90% sensitivity and 100% specificity. The limit of detection was 10 copies/μL for fluorescence reading and 100 copies/μL for LFA-based colorimetric readouts.[132] Cas13-assisted Restriction of Viral Expression and Readout (CARVER) is a platform developed for both detection and cleavage of viral DNA for treatment. It combines SHERLOCK with Cas-13's RNA cleavage ability to inhibit viral replication. However, testing was limited to cell cultures.[133]

An automated, isothermal, and fully solution-based point-of-care system was developed for Ebola RNA detection using CRISPR-Cas13a; this system has a limit of detection of 20 plaque forming units (PFU)/mL (5.45×10^7 copies/mL) of Ebola RNA, and demonstrates the proof-of-concept ability of CRISPR-Cas13a as an accurate point-of-care method. When hybridized with target Ebola RNA, the CRISPR-Cas13a complex cleaves random RNA strands to release fluorophores into the solution. The entire detection method could be completed in approximately five minutes and could potentially be applied as a finger-prick test. This system could be adapted to any viral RNA by programming the spacer sequence of the crRNA with different complementary RNA targets.[134]

CREST (Cas13-based rugged, equitable, scalable testing) was developed to address the hurdles of reagent/instrument accessibility, personnel training requirements, and upfront investment. The method used mini-thermocyclers and a 9V battery-powered cardboard fluorescence visualizer to reduce cost. For SARS-CoV-2 samples, CREST has a sensitivity of 97% and specificity of 98%.[135]

A system called COMET (Cas-CHDC-powered electrochemical RNA-sensing technology) uses an integrated, reusable electrochemical biosensor with a catalytic hairpin DNA circuit

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131 Meishen Ren et al., "Early Diagnosis of Rabies Virus Infection by RPA-CRISPR Techniques in a Rat Model," *Archives of Virology* 166, no. 4 (2021), https://doi.org/10.1007/s00705-021-04970-x.

132 Jon Arizti-Sanz et al., "Streamlined Inactivation, Amplification, and Cas13-Based Detection of SARS-CoV-2," *Nature Communications* 11, no. 1 (2020), https://doi.org/10.1038/s41467-020-19097-x.

133 Catherine A. Freije et al., "Programmable Inhibition and Detection of RNA Viruses Using Cas13," *Molecular Cell* 76, no. 5 (2019), https://doi.org/10.1016/j.molcel.2019.09.013.

134 Peiwu Qin et al., "Rapid and Fully Microfluidic Ebola Virus Detection with CRISPR-Cas13a," *ACS Sensors* 4, no. 4 (2019), https://doi.org/10.1021/acssensors.9b00239.

135 Jennifer N. Rauch et al., "A Scalable, Easy-to-Deploy Protocol for Cas13-Based Detection of SARS-CoV-2 Genetic Material," *Journal of Clinical Microbiology* 59, no. 4 (2021), https://doi.org/10.1128/JCM.02402-20.

(CHDC) to detect RNA targets in human serum. The system has a sensitivity of 90% and a specificity of 95.2%, and the overall process time was 36 minutes.[136]

One system uses microfluidics to perform a Cas13 assay in picoliter-sized droplets. This system uses the same trans-cleavage property for fluorescence production as other methods, but divides the assay into picoliter-sized droplets to enable single-cell level molecular detection. This ultra-localized assay showed both a several-fold improvement over standard Cas13a assays and good linear response, with a limit of detection of 10×10^{-18} M. The entire assay is isothermal and requires a single sample loading step. This system has the potential to be a powerful tool in the future, though there are current disadvantages to multiplexed detection and droplet readout that must first be addressed.[137]

4. Cas14

Cas14 was discovered in 2018 and has unique properties that may be advantageous over other CRISPR proteins. Cas14 proteins are smaller in size (approximately 50% smaller than Cas9), target ssDNA for cleavage, and do not require the presence of a PAM for cleavage.[138] Like Cas12a, Cas14 exhibits non-specific trans-cleavage activity in addition to cis-cleavage activity. Similar to Cas12-DETECTR, DETECTR-Cas14 uses the collateral cleavage activity of Cas14 for target detection. These features make Cas14 a potential new standard for CRISPR diagnostics.[139]

CRISPR-CasX is a recently discovered RNA-guided DNA endonuclease that is similarly compact (less than 1,000 bp) that may have potential due to its double-stranded DNA cleavage ability.[140]

5. CRISPR-Related Advances

As CRISPR technologies advance, new probe technologies also advance. For example, a method to produce gold nanoparticle bioprobes more efficiently than the traditional methods has been developed, eliminating the need for a multi-step process including the use of thiol and salt-aging. This new single-step process can be completed in several minutes and can be used as a

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136 Yan Sheng et al., "A CRISPR/Cas13a-Powered Catalytic Electrochemical Biosensor for Successive and Highly Sensitive RNA Diagnostics," *Biosensors & Bioelectronics* 178 (2021), https://doi.org/10.1016/j.bios.2021.113027.

137 Tian Tian et al., "An Ultralocalized Cas13a Assay Enables Universal and Nucleic Acid Amplification-Free Single-Molecule RNA Diagnostics," *ACS Nano* 15, no. 1 (2021), https://doi.org/10.1021/acsnano.0c08165.

138 Lucas B. Harrington et al., "Programmed DNA Destruction by Miniature CRISPR-Cas14 Enzymes," *Science* 362, no. 6416 (2018), https://doi.org/10.1126/science.aav4294.

139 Guillermo Aquino-Jarquin, "CRISPR-Cas14 Is Now Part of the Artillery for Gene Editing and Molecular Diagnostic," *Nanomedicine Nanotechnology, Biology, and Medicine* 18 (2019), https://doi.org/10.1016/j.nano.2019.03.006.

140 Jun-Jie Liu et al., "CasX Enzymes Comprise a Distinct Family of RNA-Guided Genome Editors," *Nature* 566, no. 7743 (2019), https://doi.org/10.1038/s41586-019-0908-x.

sensitive probe in a CRISPR/Cas13a system that can detect RNA at concentrations as low as 8×10^{-9} g of total RNA in initial tests.[141] Anti-CRISPR proteins are antagonistic proteins encoded by mobile genetic elements, such as plasmids and phages, that interfere with CRISPR-Cas function and can provide post-translational regulation for CRISPR technologies.[142]

6. Assessment

CRISPR diagnostics have been making rapid advancements. For example, the development of the Cas13a-based diagnostic techniques of SHERLOCK was followed by the development of the HUDSON protocol and later the SHINE technique, which improved upon both of the existing techniques. These advancements helped decrease the sample processing cost and time to result and improved upon the limit of detection, demonstrating the potential for rapid improvements in current CRISPR diagnostic methods. Even with currently proposed assays, CRISPR may have increased sensitivity over the current "gold standards." For pathogens such as *Mycobacterium tuberculosis*, CRISPR-based rapid tests were shown to have better diagnostic performance than the standard GeneXpert MTB/RIF assay (Xpert).[143] Newly discovered CRISPR proteins, such as Cas14 and CasX, have certain properties that may yield advantages over existing systems.[144]

CRISPR point-of-care tools include highly sensitive and specific LFAs and "one-pot" assays, in which all steps of the assay take place in a single container. These assays would considerably reduce the cost and equipment required to detect multiple pathogens, and would allow for enhanced capabilities at lower-role facilities. The "All-In-One-Device" (AIOD-CRISPR) has been tested using a commercial disposable hand warmer as a heating source, costing $6 for the entire apparatus while demonstrating a greater sensitivity than the conventional PCR assay for the pathogen tested (SARS-CoV-2). Although it has a decreased assay time and decreased personnel training requirement, AIOD-CRISPR remains moderately complex.

LFAs have been shown to yield similar results to PCR, and could act as direct replacements as the standard diagnostic tool for various pathogens, potentially enhancing the diagnostic capability of lower-role facilities. A version of the Cas13-based system SHERLOCK has been approved by the FDA for emergency use for SARS-CoV-2, and it could be modified for different targets/sample types. SHERLOCK has been modified by different groups for LFAs, one-pot-assays, and faster (<90 minutes) assay times, any of which could be scaled for use in the field.

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141 Menglu Hu et al., "Single-Step, Salt-Aging-Free, and Thiol-Free Freezing Construction of AuNP-Based Bioprobes for Advancing CRISPR-Based Diagnostics," *Journal of the American Chemical Society* 142, no. 16 (2020), https://doi.org/10.1021/jacs.0c00217.

142 Charles Chiu, "Cutting-Edge Infectious Disease Diagnostics with CRISPR," *Cell Host & Microbe* 23, no. 6 (June 13, 2018), https://doi.org/10.1016/j.chom.2018.05.016.

143 Ai et al., "CRISPR-Based Rapid and Ultra-Sensitive Diagnostic Test for Mycobacterium Tuberculosis."

144 Aquino-Jarquin, "CRISPR-Cas14 Is Now Part of the Artillery for Gene Editing and Molecular Diagnostic."

While many exist in the proof-of-concept stage, some are beginning to be commercialized, with TRLs varying between 3 and 7.

Automated CRISPR systems[145] could act as rapid sample-to-result tools, having turnaround times of five minutes using automated microfluidics. Similarly, combining CRISPR with amplification methods, such as PCR in a portable device, would decrease resource requirements and increase accessibility, as was seen in the system developed by Rauch et al.[146]

By manipulating the natural sensitivity/specificity of CRISPR systems, CRISPR-based diagnostics offer several advantages over traditional diagnostic assays. Multiple CRISPR diagnostic systems have been identified, including Cas9, Cas12, and Cas13-based systems. The simplicity and lack of heavy instrumentation of CRISPR assays has led to it being tested as a point-of-care diagnostic tool, both as a standalone technology and in conjunction with other techniques such as lateral flow assays. For example, a CRISPR/Cas9-LFA assay can be stored in a portable toolkit, allowing for potential forward use with further research and development.[147] Depending on whether the assay is performed as an LFA, or in a lab combined with other techniques, the CLIA complexity may vary from moderate to high. Various CRISPR-based diagnostic systems have recently been given FDA approval, including for the diagnosis of COVID-19, while many concepts still exist in the lab, giving them various TRLs from 3 to 7+. A summary of various CRISPR techniques is found in Table 15 and Table 16.

Table 15. Assay Requirements for CRISPR

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Cas9	Method-dependent	Method-dependent	Maybe	Refrigerator and/or for reagent storage	Requires training (may be reduced in future versions)
Cas12	Method-dependent (e.g., standard lysis and RNA protective buffers)	Method-dependent (e.g. hand centrifuge, fluorescent analyzer)	Maybe	Freezer may be required for reagent storage	Requires training (may be reduced in future versions
Cas13	Method-dependent	Method-dependent (e.g. LFA readout, fluorescent analyzer)	Maybe	Refrigerator and/or freezer for reagent storage	Requires training (may be reduced in future versions)

[145] Qin et al., "Rapid and Fully Microfluidic Ebola Virus Detection with CRISPR-Cas13a."

[146] Rauch et al., "A Scalable, Easy-to-Deploy Protocol for Cas13-Based Detection of SARS-CoV-2 Genetic Material."

[147] Wang et al., "Clustered Regularly Interspaced Short Palindromic Repeats/Cas9-Mediated Lateral Flow Nucleic Acid Assay."

Table 16. Assay Details for CRISPR

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Cas9	LOD: 10 CFU/mL; 0.25–0.82 × 10^{-18} M concentrations; 3.3 × 10^{-9} g/µL Time: 15–40 minutes	Some commercial options	Increased sensitivity Faster time to result	Potential to distinguish between species isolates and identify antibiotic resistance Potential to be miniaturized or made portable
Cas12	LOD: 3–10 copies/µL; 10000 × 10^{-9} M concentrations Time: 15–90 minutes	Some commercial options	Increased sensitivity Faster time to result Relatively low cost	Potential to be miniaturized or made portable $0.60–$5/reaction
Cas13	LOD: 1.35–100 copies/µL; 0.9–20 × 10^{-18} M concentrations Time: <90 minutes	Some commercial options	Increased sensitivity Faster time to result	Potential to be lyophilized Could provide detection earlier in disease

H. Recombinase Polymerase Amplification (RPA)

Recombinase polymerase amplification (RPA) works on a principle similar to PCR to amplify target DNA. Like PCR, RPA uses two primers, but it also uses a recombinase protein and single-stranded DNA binding (SSB) protein. The recombinase protein inserts the primers into the complementary sites of the target dsDNA, while the SSB proteins stabilize the opening of the DNA structure; this allows the strand displacement polymerase to extend the 3' end of the primers. RPA is isothermal (i.e., works at a constant temperature of approximately 37°–42°C), and has a fast reaction time (less than 20 minutes). These advantages greatly simplify the process, compared to PCR, which has a requirement for thermal cycling. Commercially available TwistAmp RPA kits are sold in lyophilized and liquid forms, though the commercial availability is currently restricted to a single company and may be a limiting factor.[148] Some advantages of RPA include the use of a single tube, reagents that can be freeze-dried and pelletized, reduced time to a positive signal, and real-time detection using fluorescent probes.[149]

[148] Oliver W. Stringer et al., "TwistAmp® Liquid: A Versatile Amplification Method to Replace PCR," *Nature Methods* 15, no. 5 (2018), https://doi.org/10.1038/nmeth.f.407.

[149] Matthew D. Moore and Lee-Ann Jaykus, "Development of a Recombinase Polymerase Amplification Assay for Detection of Epidemic Human Noroviruses," *Scientific Reports* 7, no. 1 (2017), https://doi.org/10.1038/srep40244.

RPA amplification plus surface enhanced Raman scattering (SERS) was used for accurate detection of multiple plant pathogens and was determined to be more sensitive than PCR, as it could detect as few as two copies of *B. cinerea* DNA in a sample. Simultaneous detection of different pathogens was also possible. For out-of-laboratory use, a single-tube assay using this technique was developed and may have the potential to be used with other sample types, though it requires a portable Raman spectrometer.[150]

RPA has been coupled with CRISPR-Cas12a for sensitive colorimetry-based SARS-CoV-2 detection. Gold nanoparticles are capped with ssDNA to act as a substrate for Cas12a cleavage. In the presence of the target, the amplified dsDNA would activate Cas12a via crRNA guided binding, which causes the capped ssDNA strands to undergo degradation and cause AuNP aggregation. This aggregation changes the surface plasmon resonance, which can be measured and quantified. This combination of methods has a highly sensitive detection limit of one sequence per test, and false positives may be reduced due to the specific Cas12a process and isothermal amplification.[151]

Real-time RPA with immunomagnetic separation (RPA-IMS) is a method that achieved almost complete concordance with RT-PCR for the detection of *L. monocytogenes*. Immunomagnetic separation (IMS) uses magnetic beads and a magnetic particle concentrator to separate and concentrate the pathogens while simultaneously significantly reducing background microorganisms; DNA extraction was performed with the lysis GuSCN method.[152] Another real time-RPA assay could detect human noroviruses from stool samples in as little as six minutes and was more resistant to inhibitors than RT-PCR.[153]

One assay uses an RPA exo probe (an oligonucleotide at least 46 base pairs long with an internal structure consisting of both a fluorophore and a quencher separated by an apurinic/apyrimidinic (AP) site) to hybridize with the target, which causes exonuclease III to cleave the AP site and separate the quencher and the fluorophore. This method has been used to detect SARS-CoV-2 and could achieve results in as little as seven minutes if RNA concentrations

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150 Han Y. Lau et al., "Field Demonstration of a Multiplexed Point-of-Care Diagnostic Platform for Plant Pathogens," *Analytical Chemistry* 88, no. 16 (2016), https://doi.org/10.1021/acs.analchem.6b01551.

151 Wei S. Zhang et al., "Reverse Transcription Recombinase Polymerase Amplification Coupled with CRISPR-Cas12a for Facile and Highly Sensitive Colorimetric SARS-CoV-2 Detection," *Analytical Chemistry* 93, no. 8 (2021), https://doi.org/10.1021/acs.analchem.1c00013.

152 Alejandro Garrido-Maestu et al., "Combination of Immunomagnetic Separation and Real-Time Recombinase Polymerase Amplification (IMS-QRPA) For Specific Detection of *Listeria monocytogenes* in Smoked Salmon Samples," *Journal of Food Science* 84, no. 7 (2019), https://doi.org/10.1111/1750-3841.14662.

153 Moore and Jaykus, "Development of a Recombinase Polymerase Amplification Assay for Detection of Epidemic Human Noroviruses."

were sufficiently high; standard maximum assay runtime is 15–20 minutes. This assay has shown both 100% sensitivity and specificity when compared to RT-qPCR.[154]

In one study, RPA was combined with an LFA to detect *F. tularensis* and *Y. pestis* with an LOD of 243×10^{-15} g (121 copies) and 4×10^{-15} g (0.85 copies), respectively. A novel fishing sequence was used to remove excess primer, which prevents unwanted competition of the primer with amplicons for probe binding that can lead to lower signal intensity. A specific sequence was included in the primers to hybridize the primers for removal using methods such as magnetic beads.[155] An RPA-lateral flow assay for the detection of cutaneous leishmaniasis had a sensitivity and specificity of 87% and 86%, respectively, in a lab scenario and 75% and 89%, respectively, in a field scenario.[156] Similarly, a microfluidic-integrated LFA for SARS-CoV-2 detection mixes real time-RPA reaction components with running buffer and delivers them to LF detection strips enclosed in a microfluidic chip; this avoids both aerosol contamination and the manual steps of incubation and mixing with the running buffer. The assay has a detection limit of one copy per μL, or 30 copies per sample. The assay was completed in 30 minutes and had a sensitivity of 97% and a specificity of 100%.[157]

1. Point-of-Care RPA

In 2021, a "suitcase lab" was created to detect SARS-CoV-2 using reverse-transcriptase RPA (RT-RPA) that required approximately 15 minutes for amplification. The assay sensitivity and specificity compared to PCR was 94% and 100% for the RdRP, 65% and 77% for the envelope protein, and 83% and 94% for the nucleocapsid protein genes of SARS-CoV-2.[158] The same lab created an RPA assay to detect Ebolavirus in Guinea in 2015; that assay was a mobile laboratory "diagnostics-in-a-suitcase" device with a solar panel and power pack, and had a sensitivity and specificity of 91% and 100%, respectively, when compared with RT-PCR.[159]

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154 Ole Behrmann et al., "Rapid Detection of SARS-CoV-2 by Low Volume Real-Time Single Tube Reverse Transcription Recombinase Polymerase Amplification Using an Exo Probe with an Internally Linked Quencher (Exo-IQ)," *Clinical Chemistry* 66, no. 8 (2020), https://doi.org/10.1093/clinchem/hvaa116.

155 Miriam Jauset-Rubio et al., "Duplex Lateral Flow Assay for the Simultaneous Detection of *Yersinia pestis* and *Francisella tularensis*," *Analytical Chemistry* 90, no. 21 (2018), https://doi.org/10.1021/acs.analchem.8b03105.

156 Alexandra Cossio et al., "Diagnostic Performance of a Recombinant Polymerase Amplification Test-Lateral Flow (RPA-LF) For Cutaneous Leishmaniasis in an Endemic Setting of Colombia," *PLOS Neglected Tropical Diseases* 15, no. 4 (2021), https://doi.org/10.1371/journal.pntd.0009291.

157 Dan Liu et al., "A Microfluidic-Integrated Lateral Flow Recombinase Polymerase Amplification (MI-IF-RPA) Assay for Rapid COVID-19 Detection," *Lab on a Chip* 21, no. 10 (2021), https://doi.org/10.1039/D0LC01222J.

158 Ahmed A. El Wahed et al., "Suitcase Lab for Rapid Detection of SARS-CoV-2 Based on Recombinase Polymerase Amplification Assay," *Analytical Chemistry* 93, no. 4 (2021), https://doi.org/10.1021/acs.analchem.0c04779.

159 Oumar Faye et al., "Development and Deployment of a Rapid Recombinase Polymerase Amplification Ebola Virus Detection Assay in Guinea in 2015," *Eurosurveillance* 20, no. 44 (2015), https://doi.org/10.2807/1560-7917.ES.2015.20.44.30053.

The RT-RPA workflow was modified to create reverse transcription enzymatic recombinase amplification (RT-ERA). The reaction takes place in a single tube, which is placed in a 37°C water bath for RT before being spun and reheated to 40°C for the ERA reaction. After another spin-and-heat cycle, the results can be analyzed using fluorescence methods. This method has the potential for ultrasensitive detection, with an estimated limit of detection of 0.05–1 copy/µL (a sample with only one copy of the target in over 20 µL of sample volume was tested). Digital RNA detection would further increase the sensitivity of this method.[160]

A multiplex LFA combined with RPA and a seven-segment display output was developed to detect multiple targets; this assay uses standard molecular labeling and a generic sandwich assay for biomolecule detection. This would eliminate the need to run multiple tests for point-of-care diagnostics.[161] An RPA with a lateral flow dipstick (LFD) assay was used to detect African swine fever virus with a sensitivity of 150 copies/reaction and 100% agreement compared to RT-PCR. The reaction time was 10 minutes at 38°C. However, the assay developers noted a disadvantage for field use: the reaction tube must be opened after completion of RPA, which introduces a possible mode of contamination.[162]

A paper chip RPA had an LOD of 10^2 CFU/mL for *E. coli*, *S. aureus*, and *S. typhimurium* in milk; this paper chip had different zones to allow for multiplex detection of different pathogens. The reaction time was 20 minutes at 37°C for and the results were comparable to RPA in solution. The simple fabrication of this assay is advantageous in that it could be modified for use as a point-of-care diagnostic tool.[163]

A wearable microfluidic device intended to be worn on the wrist uses human body heat to drive the RPA reaction and uses a cellphone-based fluorescence detection system for quantification. However, DNA extraction from a blood sample must still be performed. This device could quantify HIV-1 DNA at concentrations from 10^2 to 10^5 copies/mL.[164]

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160 Simin Xia and Xi Chen, "Single-Copy Sensitive, Field-Deployable, and Simultaneous Dual-Gene Detection of SARS-CoV-2 RNA via Modified RT–RPA," *Cell Discovery* 6, no. 1 (2020), https://doi.org/10.1038/s41421-020-0175-x.

161 Jia Li, Nina M. Pollak, and Joanne Macdonald, "Multiplex Detection of Nucleic Acids Using Recombinase Polymerase Amplification and a Molecular Colorimetric 7-Segment Display," *ACS Omega* 4, no. 7 (2019), https://doi.org/10.1021/acsomega.9b01097.

162 Faming Miao et al., "Rapid and Sensitive Recombinase Polymerase Amplification Combined with Lateral Flow Strip for Detecting African Swine Fever Virus," *Frontiers in Microbiology* 10 (2019), https://doi.org/10.3389/fmicb.2019.01004.

163 Heeseop Ahn et al., "Single-Step Recombinase Polymerase Amplification Assay Based on a Paper Chip for Simultaneous Detection of Multiple Foodborne Pathogens," *Analytical Chemistry* 90, no. 17 (2018), https://doi.org/10.1021/acs.analchem.8b01309.

164 Mengqi Kong et al., "A Wearable Microfluidic Device for Rapid Detection of HIV-1 DNA Using Recombinase Polymerase Amplification," *Talanta* 205 (2019), https://doi.org/10.1016/j.talanta.2019.120155.

Competitive RPA/qRPA may overcome some challenges of field deployability while providing a semi-quantitative result. A reference molecule is competitively amplified along with the target; the amplicons are separated in an LFA for semi-quantitative endpoint detection. The ratio of the target and reference amplicon can provide a quantitative estimate to overcome the problems of non-specific product formation in normal RPA, where reactions will continue until the reagents are exhaustion, making quantification difficult.[165]

2. Assessment

Similar to HCR, RPA is an isothermal amplification technique with advantages over conventional PCR techniques. RPA works at lower, physiological temperatures (37°–42°C) and has a fast reaction time. Lyophilized RPA kits are commercially available, allowing for easy transport.

The combination of RPA with other diagnostic tools such as CRISPR, SERS, and immune-magnetic separation has demonstrated advantages over conventional PCR, such as decreased time to result, increased sensitivity/specificity, and resistance to reaction inhibitors.[166] Techniques such as immune-magnetic separation would require instruments, such as magnetic particle concentrators, which may not be appropriate for far-forward use but may offer improvements to higher-role facilities.

Due to the low reaction temperature, RPAs have the potential to be used for point-of-care tests. Multiple labs have demonstrated point-of-care RPA assays, including a completely self-contained diagnostics-in-a-suitcase powered by a solar panel and power pack.[167] RPA on paper chips for multiplexed pathogen detection is a technology that would greatly benefit far-forward diagnoses.[168] After proving the concept by detecting foodborne pathogens in food samples, the same techniques could be applied to a multiplexed system for clinical samples. Due to the physiologically relevant reaction temperature range, the assay can be powered by body heat, which reduces the equipment requirements.[169] However, further research would be required as external power would be required for other steps of the assay, such as nucleotide extraction. While the lyophilized reagents used in some RPA assays are available commercially, the assays analyzed

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165 Alejandro Garrido-Maestu et al., "Combination of Immunomagnetic Separation and Real-Time Recombinase Polymerase Amplification (IMS-QRPA) For Specific Detection of *Listeria monocytogenes* in Smoked Salmon Samples," Journal of Food Science 84, no. 7 (2019), https://doi.org/10.1111/1750-3841.14662.

166 Moore and Jaykus, "Development of a Recombinase Polymerase Amplification Assay for Detection of Epidemic Human Noroviruses"; Zhang et al., "Reverse Transcription Recombinase Polymerase Amplification Coupled with CRISPR-Cas12a for Facile and Highly Sensitive Colorimetric SARS-CoV-2 Detection."

167 Faye et al., "Development and Deployment of a Rapid Recombinase Polymerase Amplification Ebola Virus Detection Assay in Guinea in 2015."

168 Ahn et al., "Single-Step Recombinase Polymerase Amplification Assay Based on a Paper Chip for Simultaneous Detection of Multiple Foodborne Pathogens."

169 Kong et al., "A Wearable Microfluidic Device for Rapid Detection of HIV-1 DNA Using Recombinase Polymerase Amplification."

would still be at a TRL level of 3 or 4, with a moderate to high complexity. A summary of RPA techniques is listed in Table 17 and Table 18.

Table 17. Assay Requirements for RPA

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
RPA	Two primers SSB protein Recombinase protein Fluorescent probes	Fluorescent analyzer	No	Refrigerator for reagent storage	Requires training (may be reduced in future versions)
RPA-SERS	Single-tube assay	Portable Raman spectrometer	Maybe	--	Requires training (may be reduced in future versions)
RPA-CRISPR	AuNPs Cas12a	SPR analyzer	No	Refrigerator for reagent storage	Requires training
RPA-IMS	Magnetic beads	Magnetic separator	No	--	Requires training
RPA-LFA	Novel fishing sequence and primers	Magnetic separator	No	Refrigerator and/or freezer for reagent storage	Requires training
RT-RPA	--	Centrifuge Heat source Fluorescence analyzer	Maybe	Refrigerator for reagent storage	Requires training (may be reduced in future versions)

Table 18. Assay Details for RPA

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
RPA	Time: <30 minutes	Some commercial options	Faster time to result Simpler process	Reagents can be lyophilized and pelletized
RPA-SERS	LOD: 2 copies/reaction	Proof-of-concept	Increased sensitivity	May be able to be made portable
RPA-CRISPR	LOD: 1 sequence/reaction	Proof-of-concept	Increased sensitivity	May reduce false positives
RPA-IMS	LOD: 6.3 CFU/25 g	Proof-of-concept	Increased sensitivity	May reduce interference of background microorganisms
RPA-LFA	LOD: 1–121 copies/reaction Time: <1 hour	Proof-of-concept	Increased sensitivity Faster time to result Potential for multiplex	May reduce interference of background microorganisms
RT-RPA	LOD: 7.74 copies/reaction; 0.2 copies/µL Time: 6–30 minutes	Proof-of-concept	Increased sensitivity Faster time to result Miniaturization	May be more resistant to inhibitors

I. MALDI-TOF Mass Spectrometry

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometry (MS) identifies microbes by comparing intact cells or cell extracts to a library of microbial "fingerprints". The sample is mixed with an energy-absorbent matrix and is allowed to crystallize before being ionized with a laser. The protonated ions are separated based on their mass-to-charge ratio by measuring the time of flight through a flight tube.[170] Using this information, a peptide mass fingerprint (PMF) is created in a few minutes and can be compared with the PMFs of known organisms for identification. Three commonly available platforms are MALDI Biotyper (Bruker Daltonics GmbH, Bremen, Germany), VITEK MS (bioMerieux, Marcy-l'Étoile, France), and Andromas MS (Paris, France).[171] The commercial VITEK MS database includes information about different *Nocardia* species, and MALDI-TOF may be the best method for differentiating different *Nocardia* species: species could be identified with 94% accuracy. This may be of clinical interest, as different *Nocardia* species may have different resistance levels to antibiotics.[172]

[170] Neelja Singhal et al., "MALDI-TOF Mass Spectrometry: An Emerging Technology for Microbial Identification and Diagnosis," *Frontiers in Microbiology* 6 (2015), https://doi.org/10.3389/fmicb.2015.00791.

[171] Marlène Sauget et al., "Can MALDI-TOF Mass Spectrometry Reasonably Type Bacteria?," *Trends in Microbiology* 25, no. 6 (2017), https://doi.org/10.1016/j.tim.2016.12.006.

[172] Victoria Girard et al., "Routine Identification of *Nocardia* Species by MALDI-TOF Mass Spectrometry," *Diagnostic Microbiology and Infectious Disease* 87, no. 1 (2017), https://doi.org/10.1016/j.diagmicrobio.2016.09.024.

A study using blood culture and cerebrospinal fluid specimens was able to identify the correct pathogen genus 94% of the time and correct pathogen species 78% of the time.[173] Large scale studies have shown genus and species accuracies of 90–100% and 75–98%.[174] Concordance was 100% and 73% with molecular techniques when identifying non-fermenting gram-negative bacteria (such as *A. baumanii*, *B. cepacia*, and *S. maltophilia*).[175] A commercially available method called Sepsityper could correctly identify species 77% of the time; while this may not be the most clinically viable option, the Sepsityper system demonstrates an existing commercial option that may improve with further development[176] A 2018 study combining MALDI-TOF with blood culture centrifugation allowed for rapid bacterial identification with high sensitivity (93% for gram-negative bacteria and 81% for gram-positive bacteria). Furthermore, the cost per sample was low ($0.50) and the turnaround time was approximately 20 minutes.[177]

One study assessing the rapid diagnostic capability of MALDI-TOF for mycobacteria skipped the sub-culture step and directly analyzed mycobacteria growth indicator tube (MGIT) broths. While sensitivity was high in monomicrobial broths (96–100%), sensitivity was low in polymicrobial broths (41.6%) and a screening immunochromatography test was recommended before performing MALDI-TOF.[178] The use of a pure culture specimen (monomicrobial) was also emphasized in a study where MALDI-TOF identified 500 specimens of bovine mastitis bacteria. MALDI-TOF could also identify 99.5% and 97% of *Brucella* strains at the genus and species level from cultures.[179]

In addition to microorganisms, MALDI-TOF can also be used to efficiently identify toxins; one study showed that MALDI-TOF could correctly identify a variety of fungal toxins, including

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173 Manal Tadros and Astrid Petrich, "Evaluation of MALDI-TOF Mass Spectrometry and Sepsityper Kit™ for the Direct Identification of Organisms from Sterile Body Fluids in a Canadian Pediatric Hospital," *Canadian Journal of Infectious Diseases and Medical Microbiology* 24, no. 4 (2013), https://doi.org/10.1155/2013/701093.

174 Tsung-Yun Hou, Chuan Chiang-Ni, and Shih-Hua Teng, "Current Status of MALDI-TOF Mass Spectrometry in Clinical Microbiology," *Journal of Food and Drug Analysis* 27, no. 2 (2019), https://doi.org/10.1016/j.jfda.2019.01.001.

175 Vikas Gautam et al., "MALDI-TOF Mass Spectrometry: An Emerging Tool for Unequivocal Identification of Non-Fermenting Gram-Negative Bacilli," *The Indian Journal of Medical Research* 145, no. 5 (2017), https://doi.org/10.4103/ijmr.IJMR_1105_15.

176 J.D. Haigh et al., "Rapid Identification of Bacteria from BioMérieux BacT/ALERT Blood Culture Bottles by MALDI-TOF MS," *British Journal of Biomedical Science* 70, no. 4 (2013), https://doi.org/10.1080/09674845.2013.11669949.

177 Yong-Lu Huang et al., "Evaluation of an In-House MALDI-TOF MS Rapid Diagnostic Method for Direct Identification of Micro-Organisms from Blood Cultures," *Journal of Medical Microbiology* 68, no. 1 (2019), https://doi.org/10.1099/jmm.0.000866.

178 Tsi-Shu Huang et al., "Rapid Identification of Mycobacteria from Positive MGIT Broths of Primary Cultures by MALDI-TOF Mass Spectrometry," *PLOS ONE* 13, no. 2 (2018), https://doi.org/10.1371/journal.pone.0192291.

179 Bettina Nonnemann et al., "Bovine Mastitis Bacteria Resolved by MALDI-TOF Mass Spectrometry," *Journal of Dairy Science* 102, no. 3 (2019), https://doi.org/10.3168/jds.2018-15424.

aflatoxin.[180] Similarly, MALDI-TOF can be used to identify anthrax lethal factor with high sensitivity and high specificity in samples from humans, rabbits, and rhesus macaques.[181] MALDI-TOF is also being studied to detect parasites, but there is currently a limitation in the abundance and availability of reference databases. One study identified a set of MALDI-TOF signals for the identification of *Anisakis* species, indicating that parasite detection was viable using this methodology.[182] Similarly, MALDI-TOF has the potential for nematode detection (including the clinically relevant nematode *Ascaris lumbricoides*) and species differentiation, as consistent and unique species-identifying peaks were found in different species.[183]

Paper Spray Ionization Mass Spectrometry (PSI-MS) allows for mass spectrometric sample analysis with little or no sample preparation, making it a promising option for future rapid clinical assays. In PSI-MS, a solvent is applied to the substrate before subsequent electrospray ionization for direct analysis, resulting in a small sample volume and runtimes of less than two minutes. Recently, this technique was used to demonstrate strain-level bacterial differentiation from whole cell and cell lysate samples with a 30-second analytical method.[184]

1. Antibiotic Resistance

MALDI-TOF MS may also be used for direct identification of bacterial resistance by measuring antibiotic modifications due to bacterial enzymatic activity, analyzing the spectroscopic peak patterns of bacteria, or quantifying bacterial growth in the presence of an antibiotic.[185] PMFs can also be used to extract information about drug resistance. One peak was associated with methicillin resistance in *Staphylococci*, and although drug resistance identification had low sensitivity (37% and 6% for *S. aureus* and *S. epidermidis*, respectively), specificity was high (less than 98%), implying that there is value to this peak. This can help improve diagnoses with no

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180 Lukáš Hleba et al., "Detection of Mycotoxins Using MALDI-TOF Mass Spectrometry," *Journal of Microbiology, Biotechnology and Food Sciences* 7, no. 2 (2017), https://doi.org/10.15414/jmbfs.2017.7.2.181-185.

181 Maribel Gallegos-Candela et al., "Validated MALDI-TOF-MS Method for Anthrax Lethal Factor Provides Early Diagnosis and Evaluation of Therapeutics," *Analytical Biochemistry* 543 (2018), https://doi.org/10.1016/j.ab.2017.12.007.

182 Valeria Marzano et al., "Mass Spectrometry Based-Proteomic Analysis of *Anisakis* Spp.: A Preliminary Study Towards a New Diagnostic Tool," *Genes* 11, no. 6 (2020), https://doi.org/10.3390/genes11060693.

183 Sergey A. Nagorny et al., "The Application of Proteomic Methods (MALDI-Toff MS) For Studying Protein Profiles of Some Nematodes (Dirofilaria and Ascaris) For Differentiating Species," *International Journal of Infectious Diseases* 82 (2019), https://doi.org/10.1016/j.ijid.2019.02.047.

184 Casey A. Chamberlain, Vanessa Y. Rubio, and Timothy J. Garrett, "Strain-Level Differentiation of Bacteria by Paper Spray Ionization Mass Spectrometry," *Analytical Chemistry* 91, no. 8 (2019), https://doi.org/10.1021/acs.analchem.9b00330.

185 Marina Oviaño and Germán Bou, "Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for the Rapid Detection of Antimicrobial Resistance Mechanisms and Beyond," *Clinical Microbiology Reviews* 32, no. 1 (2019), https://doi.org/10.1128/CMR.00037-18.

additional cost/steps.[186] PMFs can identify the protein profile of the microorganism, which in turn may be able to provide similar information. A blaKPC signal is correlated with *K. pneumoniae* carbapenem resistance due to production of *K. pneumoniae* carbapenemase.[187]

A supervised neural network was developed that could identify MRSA strains with a positive predictive value (PPV) of 99.6%.[188] Another study demonstrated that a model could be created with up to 90% prediction accuracy in differentiating between methicillin-susceptible *Staphylococcus aureus* (MSSA) and MRSA.[189] Most studies report better performance for gram-negative bacteria.[190] This methodology may also have the potential to identify antifungal resistance.[191] Many machine-learning models have been developed to analyze MALDI-TOF results and may be widely used in the near future.[192]

2. Assessment

MALDI-TOF MS systems can offer an advantage over the standard method of blood cultures by decreasing the time to identification, which may aid in reducing mortality. One of the largest current obstacles to MALDI-TOF MS is the difficulty in distinguishing polymicrobial blood cultures, where peaks of multiple species can merge into a single mass spectrum.[193] Furthermore, MALDI-TOF may offer a rapid method to identify antibiotic resistance by identifying the PMFs of organisms with information about drug resistance. The development of machine-learning

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186 Daniel D. Rhoads et al., "The Presence of a Single MALDI-TOF Mass Spectral Peak Predicts Methicillin Resistance in Staphylococci," *Diagnostic Microbiology and Infectious Disease* 86, no. 3 (2016), https://doi.org/10.1016/j.diagmicrobio.2016.08.001.

187 Paolo Gaibani et al., "Evaluation of Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Identification of KPC-Producing *Klebsiella pneumoniae*," *Journal of Clinical Microbiology* 54, no. 10 (2016), https://doi.org/10.1128/JCM.01242-16.

188 M. Camoez et al., "Automated Categorization of Methicillin-Resistant *Staphylococcus aureus* Clinical Isolates into Different Clonal Complexes by MALDI-TOF Mass Spectrometry," *Clinical Microbiology and Infection* 22, no. 2 (2016), https://doi.org/10.1016/j.cmi.2015.10.009.

189 Wenhao Tang et al., "MALDI-TOF Mass Spectrometry on Intact Bacteria Combined with a Refined Analysis Framework Allows Accurate Classification of MSSA and MRSA," *PLOS ONE* 14, no. 6 (2019), https://doi.org/10.1371/journal.pone.0218951.

190 Menglan Zhou et al., "An Improved In-House MALDI-TOF MS Protocol for Direct Cost-Effective Identification of Pathogens from Blood Cultures," *Frontiers in Microbiology* 8 (2017), https://doi.org/10.3389/fmicb.2017.01824.

191 Antonietta Vella et al., "Potential Use of MALDI-ToF Mass Spectrometry for Rapid Detection of Antifungal Resistance in the Human Pathogen *Candida glabrata*," *Scientific Reports* 7, no. 1 (2017), https://doi.org/10.1038/s41598-017-09329-4.

192 C. V. Weis, C. R. Jutzeler, and K. Borgwardt, "Machine Learning for Microbial Identification and Antimicrobial Susceptibility Testing on MALDI-TOF Mass Spectra: A Systematic Review," *Clinical Microbiology and Infection* 26, no. 10 (2020), https://doi.org/10.1016/j.cmi.2020.03.014.

193 W. Florio, S. Cappellini, C. Giordano, A. Vecchione, E. Ghelardi, A. Lupetti, "A New Culture-Based Method for Rapid Identification of Microorganisms in Polymicrobial Blood Cultures by MALDI-TOF MS," *BMC Microbiology* 19 (2019), https://doi.org/10.1186/s12866-019-1641-1.

models to detect resistance may also increase the performance of this diagnostic tool. Other modifications to the MALDI-TOF procedure, such as the centrifugation of blood cultures, may decrease sample processing costs and lead to faster sample-to-result times.[194]

One of the major drawbacks of the current state of MALDI-TOF technology is that existing libraries may not be sufficiently large or robust, which may limit the performance of the assay. Moreover, many libraries may contain proprietary information and may not be publicly available. The identification success rate largely depends on the quality and extensiveness of the library used.[195] A 2017 study had 100% accuracy in identifying different species of *Listeria* and attributed reliable identification to the existence of a good quality reference library of MALDI-TOF MS data.[196] Possible improvements to identification may include creation of extensive libraries of MS profiles.

MALDI-TOF also has potential as a rapid clinical assay, with techniques such as paper spray ionization allowing for turnaround times of two minutes.[197] While advances in MALDI-TOF spectrometers are reducing the size, they are still relatively bulky, requiring constant power and may not yet be appropriate for "rugged" use. Commercially available miniaturized mass spectrometers may be limited in performance compared to full-size versions, including decreased compatible mass ranges.[198] At this time, MALDI-TOF would not be appropriate for far-forward or Role 1 use and would be limited to Role 3 and above, due to its power requirements and portability limitations. They also have varying TRL levels from 3 to 7+, with two commercial systems available. CLIA complexities for assays would also vary from moderate to high. A summary of mass spectrometry techniques is found in Table 19 and Table 20.

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194 Huang et al., "Evaluation of an in-House MALDI-TOF MS Rapid Diagnostic Method for Direct Identification of Micro-Organisms from Blood Cultures."

195 Markus Stein et al., "Evaluation of Three MALDI-TOF Mass Spectrometry Libraries for the Identification of Filamentous Fungi in Three Clinical Microbiology Laboratories in Manitoba, Canada," *Mycoses* 61, no. 10 (2018), https://doi.org/10.1111/myc.12800.

196 Pierre Thouvenot et al., "MALDI-TOF Mass Spectrometry-Based Identification of *Listeria* Species in Surveillance: A Prospective Study," *Journal of Microbiological Methods* 144 (2018), https://doi.org/10.1016/j.mimet.2017.10.009.

197 Chamberlain, Rubio, and Garrett, "Strain-Level Differentiation of Bacteria by Paper Spray Ionization Mass Spectrometry."

198 J. Lin, M. Chu, and C. Chen, "A Portable Multiple Ionization Source Biological Mass Spectrometer," *Analyst* 145, no. 10 (2020): 3495–504, https://doi.org/10.1039/D0AN00126K.

Table 19. Assay Requirements for Mass Spectrometry

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
MALDI-TOF	Energy-absorbent matrix	Flight tube Laser source	No	Power source PMF reference library	Requires training
PSI-MS	Solvent	Electrospray ionization equipment	No	Power source	Requires training

Table 20. Assay Details for Mass Spectrometry

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
MALDI-TOF	Time: 20–70 minutes	Commercial systems available	Increased sensitivity Faster time to result Can identify bacteria, viruses, toxins, and parasites	Some variations can be low cost ($0.50/sample) May be used to detect antibiotic resistance May be useful at Role 3
PSI-MS	Time: 2–20 minutes	Some commercial options	Increased sensitivity Faster time to result	May be useful at Role 3

J. Surface Enhanced Raman Scattering (SERS)

Raman Spectroscopy identifies the "chemical fingerprint" by identifying the loss or gain of energy by a photon that is inelastically scattered by a molecular vibrational event. SERS enhances the Raman signal by amplifying the electron cloud density around metallic nanostructures. The analyte must either be adsorbed or reside very close to a dielectric surface to cause plasmons (or oscillating conduction band electrons) to increase the local electron density, improving the likelihood for inelastic scatter events. When the molecule is bound directly to the metal surface, a charge-transfer structure or electron-hole pair is generated, which mediates energy transfer from the metal to the analyte's molecular bonds. SERS makes single molecule detection possible by enhancing signal levels by a factor of 10^6 to 10^8. Colloidal plasmonic nanoparticles, both labeled and label-free, have been commonly used as substrates.[199]

SERS has been successfully used for clinical detection of hepatitis B viral RNA, with 99% accuracy.[200] It has also been used to sensitively and specifically detect oseltamivir-resistant H1N1

[199] Haley Marks et al., "Surface Enhanced Raman Spectroscopy (SERS) for In Vitro Diagnostic Testing at the Point of Care," *Nanophotonics* 6, no. 4 (2017), https://doi.org/10.1515/nanoph-2016-0180.

[200] Saira Nasir et al., "Surface Enhanced Raman Spectroscopy of RNA Samples Extracted from Blood of Hepatitis C Patients for Quantification of Viral Loads," *Photodiagnosis and Photodynamic Therapy* 33 (2021), https://doi.org/10.1016/j.pdpdt.2020.102152.

flu viruses from nasal secretions by combining oseltamivir hexylthiol (which has a 250 times greater affinity for oseltamivir-resistant strains) with gold nanoparticles, avoiding unnecessary and ineffective antiviral administration.[201] MRSA can also be detected with SERS, taking advantage of a methodology with a shorter assay time (80 minutes) and reduced resources, due to using only Raman reporter molecules as labels.[202]

A SERS-based LFA using gold nanoparticles as SERS nanotags was developed for thyroid-stimulating hormone (TSH) detection in clinical samples. TSH could be identified through a test line color change, with the SERS quantitative evaluation having an LOD of 0.025 μIU/mL. The assay takes 10 minutes and may be used on other analytes.[203] Another lab created an LFA-based system using SERS nanotags; Raman signals were greatly enhanced by the adsorption of reporter molecules on the surface of gold nanoparticles. For *Y. pestis*, *F. tularensis*, and *B. anthracis*, the LODs were 43.4 CFU/mL, 45.8 CFU/mL, and 357 CFU/mL, respectively, which is three to four times more sensitive than standard colorimetric LFA strips.[204]

A SERS-based microdroplet sensor uses a microfluidic system to segregate free and bound SERS tags by splitting droplets into two smaller parts. The target (in this case, prostate-specific antigen [PSA]) causes more tags to create an immunocomplex in one droplet than the other. PSA could be detected in serum without any washing, with a LOD of less than 0.1×10^{-9} g/mL.[205]

SERS-based immunoassays, such as the assay to detect H5N1 using digital microfluidics, can be automated. SERS tags were labeled with 4-MBA and were used in a sandwich immunoassay with antibody-coated magnetic beads. Automation greatly simplifies the procedure while reducing the risk of exposure to hazardous materials. The assay had an LOD of 74×10^{-12} g/mL for H5N1 and ran in less than one hour.[206]

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201 Gayoung Eom et al., "Diagnosis of Tamiflu-Resistant Influenza Virus in Human Nasal Fluid and Saliva Using Surface-Enhanced Raman Scattering," *ACS Sensors* 4, no. 9 (2019), https://doi.org/10.1021/acssensors.9b00697.

202 Phani R. Potluri et al., "Rapid and Specific Duplex Detection of Methicillin-Resistant *Staphylococcus aureus* Genes by Surface-Enhanced Raman Spectroscopy," *Analyst* 145, no. 7 (2020), https://doi.org/10.1039/C9AN01959F.

203 Suji Choi et al., "Quantitative Analysis of Thyroid-Stimulating Hormone (TSH) Using SERS-Based Lateral Flow Immunoassay," *Sensors and Actuators B: Chemical* 240 (2017), https://doi.org/10.1016/j.snb.2016.08.178.

204 Rui Wang et al., "Highly Sensitive Detection of High-Risk Bacterial Pathogens Using SERS-Based Lateral Flow Assay Strips," *Sensors and Actuators B: Chemical* 270 (2018), https://doi.org/10.1016/j.snb.2018.04.162.

205 Rongke Gao et al., "Wash-Free Magnetic Immunoassay of the PSA Cancer Marker Using SERS and Droplet Microfluidics," *Lab on a Chip* 16, no. 6 (2016), https://doi.org/10.1039/C5LC01249J.

206 Yang Wang et al., "Highly Sensitive and Automated Surface Enhanced Raman Scattering-Based Immunoassay for H5N1 Detection with Digital Microfluidics," *Analytical Chemistry* 90, no. 8 (2018), https://doi.org/10.1021/acs.analchem.8b00002.

SERS can also be used to test antibiotic susceptibility with bacteria-aptamer silver nanoparticles. The Raman signal intensity of *S. aureus* and *E. coli* in different antibiotic concentrations was measured in two hours, making it a rapid antibiotic susceptibility test method.[207]

SERS was also coupled with PCR for rapid and low-cycle bacterial DNA detection. Silver nanowires were used as the SERS substrate and PCR thermal cycles were limited to keep the processing time short. The LOD was 3.12×10^{-12} g/μL at 10 cycles, and the entire assay could be completed in 30 minutes (20 minutes of PCR, 10 minutes of SERS analysis).[208]

1. Portable Devices

A microfluidic device was created and patented to work in conjunction with a portable Raman spectrometer for portable and rapid pathogen detection (approximately 15 minutes). The system couples a microfluidic flow cell with a syringe pump flow system; inside the microfluidic channel, a silver spot that serves as the SERS substrate is generated by laser irradiation. This spot can be washed and reused for detecting different pathogens.[209]

A "lab in a stick" portable device was created to directly use blood lysate to detect pathogen nucleic acids without requiring nucleic acid extraction or amplification. Target sequences are tagged with ultrabright SERS-encoded nanorattles, which are metallic particles comprised of a core and shell separated by a dielectric spacer that has high electric-field enhancement inside the cavity. Tagged target sequences are concentrated into a focus spot for detection using hybridization sandwiches with magnetic microbeads. The limit of detection is 200×10^{-15} M, and *P. falciparum* RNA could be directly detected in red blood cell (RBC) lysate.[210]

Some SERS-based biosensors have used metallic nanosculptured thin films to detect bacteria (e.g., *E. coli*) at the level of a single bacterium in a 10 μL sample. T-bacteriophages are immobilized on a plasmonic nanosculptured thin film to enhance the Raman bands.[211]

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207 Shijie Fu et al., "A Sensitive and Rapid Bacterial Antibiotic Susceptibility Test Method by Surface Enhanced Raman Spectroscopy," *Brazilian Journal of Microbiology* 51, no. 3 (2020), https://doi.org/10.1007/s42770-020-00282-5.

208 Hyo G. Lee et al., "PCR-Coupled Paper-Based Surface-Enhanced Raman Scattering (SERS) Sensor for Rapid and Sensitive Detection of Respiratory Bacterial DNA," *Sensors and Actuators B: Chemical* 326 (2021), https://doi.org/10.1016/j.snb.2020.128802.

209 Nicoleta E. Dina et al., "Microfluidic Portable Device for Pathogens 'Rapid SERS Detection," *Proceedings* 60, no. 1 (2020), https://doi.org/10.3390/IECB2020-07089.

210 Hoan T. Ngo et al., "Direct Detection of Unamplified Pathogen RNA in Blood Lysate Using an Integrated Lab-in-a-Stick Device and Ultrabright SERS Nanorattles," *Scientific Reports* 8, no. 1 (2018), https://doi.org/10.1038/s41598-018-21615-3.

211 Sachin K. Srivastava et al., "Highly Sensitive and Specific Detection of *E. coli* by a SERS Nanobiosensor Chip Utilizing Metallic Nanosculptured Thin Films," *Analyst* 140, no. 9 (2015), https://doi.org/10.1039/C5AN00209E.

Miniaturized biosensors have also been created, including a sandwiched biosensor created with Fe_3O_4 nanoparticles, bacteria, and a SERS tag as part of the sandwich structure. The nanoparticle-bacteria bonding helps in magnetic separation, a boronic acid group helps binding to the cell wall, and the 4MPBA molecule can discriminate among different bacteria by modifying their "fingerprints."[212]

One LFA uses SERS and a built-in plasma separation unit to achieve an LOD of 1×10^{-9} g/mL. Plasma separation is performed by introducing salt solutions to whole blood to aggregate the red blood cells, though this method often has the secondary effect of causing the antibody-functionalized probes to also aggregate, due to the solution's ionic strength. Special nanoparticles (consisting of a gold nano-star and a thin silica shell) with a Raman reporter were created to prevent probe aggregation in high ionic strength solutions.[213] Another SERS-LFA uses modified gold nanoparticles to detect *L. monocytogenes* and *S. enterica*. RPA amplification was performed in less than 20 minutes and SERS signals were quantified on the LFA lines. An LFA of 27 CFU/mL and 19 CFU/mL was achieved for *S. enterica* and *L. monocytogenes* respectively.[214]

2. Label/Label-Free Assays

SERS methodologies may be labeled or label free. For a label-based approach, novel SERS tags were used to detect *E. coli* O157:H7 using aptamers and Raman reporter molecules that were incubated with gold nanorods before the detection process.[215] Another method uses aptamer–iron (II,III) oxide and gold (Fe_3O_4@Au) magnetic nanoparticles as the SERS substrate with vancomycin-SERS tags. The captured bacteria are magnetically separated after the SERS measurement process. The LOD for *S. aureus* was 3 cells/mL.[216]

A culture-free, label-free technique was able to detect *E. coli*-containing bioaerosols by collecting the aerosols on Klarite substrates with an eight-stage Andersen sampler before being

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212 Kaisong Yuan et al., "Antimicrobial Peptide Based Magnetic Recognition Elements and Au@Ag-GO SERS Tags with Stable Internal Standards: A Three in One Biosensor for Isolation, Discrimination and Killing of Multiple Bacteria in Whole Blood," *Chemical Science* 9, no. 47 (2018), https://doi.org/10.1039/C8SC04637A.

213 Xuefei Gao et al., "Enabling Direct Protein Detection in a Drop of Whole Blood with an "On-Strip" Plasma Separation Unit in a Paper-Based Lateral Flow Strip," *Analytical Chemistry* 93, no. 3 (2021), https://doi.org/10.1021/acs.analchem.0c02555.

214 Hai-bin Liu, Xin-jun Du, Yu-Xuan Zang, Ping Li, and Shuo Wang, "SERS-Based Lateral Flow Strip Biosensor for Simultaneous Detection of *Listeria monocytogenes* and *Salmonella enterica* Serotype Enteritidis," *Journal of Agricultural and Food Chemistry* 65, no. 47 (2017), https://doi.org/10.1021/acs.jafc.7b03957.

215 Yuzhi Li et al., "Sensitive and Simultaneous Detection of Different Pathogens by Surface-Enhanced Raman Scattering Based on Aptamer and Raman Reporter Co-Mediated Gold Tags," *Sensors and Actuators B: Chemical* 317 (2020), https://doi.org/10.1016/j.snb.2020.128182.

216 Yuanfeng Pang et al., "Dual-Recognition Surface-Enhanced Raman Scattering (SERS) Biosensor for Pathogenic Bacteria Detection by Using Vancomycin-SERS Tags and Aptamer-Fe_3O_4@Au," *Analytica Chimica Acta* 1077 (2019), https://doi.org/10.1016/j.aca.2019.05.059.

directly analyzed with SERS.[217] Another label-free method used Ag/Au nanoparticles to detect *E. coli*, *S. typhimurium*, and *B. subtilis*. The positive nanoparticle surface charge enables bacterial capture, which is followed by SERS to distinguish and classify the bacteria. Label-free SERS methods are generally less expensive and take less time than the label-mediated approaches.[218]

3. Assessment

SERS has the potential to rapidly identify targets with great accuracy, though the current state of technology may not be ready for far-forward deployment. SERS may eventually be useful as a point-of-care test and for screening for weaponizable bacteria using LFA-based systems.[219] With the use of different reagents such as AuNPs, increases in sensitivity can offer an advantage over standard LFAs. The entire process can also be automated using digital microfluidics, which would decrease the personnel training required to operate and analyze the diagnostic tool.

Multiple studies have attempted to make SERS systems portable, including the "lab in a stick" portable device.[220] The specific "lab in a stick" example did not require nucleic acid extraction or amplification, which could make it feasible for low-resource environments. Disposable SERS LFAs have the potential to be used at Role 2 facilities, given their relatively reduced resource and personnel requirements, while label-free approaches could help reduce the per-test cost. Label-free SERS assays could be used as biodetectors, as they have been tested for detecting dispersed bioaerosols.[221] The SERS assays analyzed here have TRLs varying between 3 and 4, with a moderate CLIA complexity. A summary of SERS techniques is listed in Table 21 and Table 22.

Table 21. Assay Requirements for SERS

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
SERS	Nanoparticles Nanotags Aptamers	Spectrometer Raman spectrometer	No	Refrigerator for reagent storage	Requires training

[217] Muhammad A. Tahir et al., "Klarite as a Label-Free SERS-Based Assay: A Promising Approach for Atmospheric Bioaerosol Detection," *Analyst* 145, no. 1 (2019), https://doi.org/10.1039/C9AN01715A.

[218] Om Prakash et al., "Direct Detection of Bacteria Using Positively Charged Ag/Au Bimetallic Nanoparticles: A Label-Free Surface-Enhanced Raman Scattering Study Coupled with Multivariate Analysis," *The Journal of Physical Chemistry* C 124, no. 1 (2020), https://doi.org/10.1021/acs.jpcc.9b09311.

[219] Wang et al., "Highly Sensitive Detection of High-Risk Bacterial Pathogens Using SERS-Based Lateral Flow Assay Strips."

[220] Ngo et al., "Direct Detection of Unamplified Pathogen RNA in Blood Lysate Using an Integrated Lab-in-a-Stick Device and Ultrabright SERS Nanorattles."

[221] Tahir et al., "Klarite as a Label-Free SERS-Based Assay: A Promising Approach for Atmospheric Bioaerosol Detection."

SERS-LFA	Nanotags	Raman spectrometer	Maybe	--	Requires training (may be reduced in future versions)
SERS microfluidics	Nanotags/nano-rattles	Raman spectrometer Laser	Yes	Power source	Requires training (may be reduced in future versions)
SERS immunoassays	Antibody-coated magnetic beads	--	Maybe	--	Requires training (may be reduced in future versions)
SERS-PCR	Silver nanowires	Standard PCR equipment	No	Power source	Requires training (may be reduced in future versions)
Label-Free	Klarite substrate Ag/Au nanoparticles	Andersen sampler	No	Power source	Requires training

Table 22. Assay Details for SERS

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
SERS	LOD: 1 PFU/sample; 10^4 copies/reaction; 3 cells/mL Time: 15 minutes–5 hours	Proof-of-concept	Increased sensitivity Faster time to result	Can be performed with or without labels Potentially lower cost
SERS-LFA	LOD: 0.025 µIU/mL; 19–357 CFU/mL Time: 10–20 minutes	Proof-of-concept	Increased sensitivity Faster time to result	May be useful at Role 2
SERS microfluidics	LOD: 0.1×10^{-9} g/mL; 200×10^{-15} M concentrations Time: <1 hour	Proof-of-concept	Increased sensitivity Faster time to result	
SERS immunoassays	LOD: 74×10^{-12} g/mL Time: <1 hour	Proof-of-concept	Increased sensitivity Faster time to result	
SERS-PCR	LOD: 3.12×10^{-12} g/µL Time: 30 minutes	Proof-of-concept	Increased sensitivity Faster time to result	
Label-Free	LOD: 8 CFU/mL; 3 cells/mL Time: <1 hour	Proof-of-concept	Lower cost Faster time to result	

K. Loop Mediated Isothermal Amplification (LAMP)

Loop mediated isothermal amplification (LAMP) is a single-tube technique with the potential to be a low-cost alternative in the detection of certain pathogens. Unlike PCR, the target is amplified at a constant temperature (usually 60°–65°C) with two to three sets of primers and a DNA polymerase capable of strand displacement; the variety of primers increases specificity by amplifying distinct regions of the target DNA sequence. This results in a turbid amplified product that can be detected with dyes, photometric approaches, or other biochemical methods.

Gold nanoparticles (AuNPs) with single-stranded DNA probes allow for the turbid LAMP amplified product to be visually measured by a color change. By adding magnesium salt to the mixture and incubating for 20 minutes at 65°C, the solution developed a high ionic strength environment that caused AuNP probe aggregation. This method demonstrated higher sensitivity and ease of visualization compared to LAMP turbidity, with a limit of detection of 10^2 and 10^1 copies for HPV16 and HPV18, respectively.[222] This sensitivity was approximately ten times greater than the modified LAMP turbidity assay.[223] Furthermore, the probes remained stable for more than one year, extending the shelf life of the assay.[224]

1. LAMP-HCR

LAMP can be combined with HCR to create an ultrasensitive diagnostic tool. Compared to Loop Mediated Isothermal Nucleic Acid Amplification – One Strand Displacement (LAMP-OSD), LAMP-HCR has a significantly enhanced signal-to-background ratio and can detect as few as 30 copies of the NoV gene in 2% fecal samples.[225]

2. RT-LAMP

In one lab, RT-LAMP was used instead of RT-PCR when combined with CRISPR-Cas12 for the detection of SARS-CoV-2. The assay took 30–40 minutes and results were visualized using a lateral flow strip with a FAM-biotin receptor.[226] A microfluidic RT-LAMP assay was developed

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222 Ratchanida Kumvongpin et al., "High Sensitivity, Loop-Mediated Isothermal Amplification Combined with Colorimetric Gold-Nanoparticle Probes for Visual Detection of High Risk Human Papillomavirus Genotypes 16 and 18," *Journal of Virological Methods* 234 (2016), https://doi.org/10.1016/j.jviromet.2016.04.008.

223 Chitladda Saetiew et al., "Rapid Detection of the Most Common High-Risk Human Papillomaviruses by Loop-Mediated Isothermal Amplification," *Journal of Virological Methods* 178, 1-2 (2011), https://doi.org/10.1016/j.jviromet.2011.08.007.

224 Ratchanida Kumvongpin et al., "High Sensitivity, Loop-Mediated Isothermal Amplification Combined with Colorimetric Gold-Nanoparticle Probes for Visual Detection of High Risk Human Papillomavirus Genotypes 16 and 18," *Journal of Virological Methods* 234 (2016), https://doi.org/10.1016/j.jviromet.2016.04.008.

225 Qing Dong et al., "A Signal-Flexible Gene Diagnostic Strategy Coupling Loop-Mediated Isothermal Amplification with Hybridization Chain Reaction," *Analytica Chimica Acta* 1079 (2019), https://doi.org/10.1016/j.aca.2019.06.048.

226 James P. Broughton et al., "CRISPR-Cas12-Based Detection of SARS-CoV-2," *Nature Biotechnology* 38, no. 7 (2020), https://doi.org/10.1038/s41587-020-0513-4.

for rapid H1N1 detection. The assay could be performed in 40 minutes and had an LOD of 3×10^{-4} hemagglutinating units per reaction. The entire process could be automated without requiring human intervention, and the estimated cost per assay is $3.[227]

3. Assessment

LAMP is an alternative amplification technique with the advantage of being isothermal, and therefore does not require thermal cycling equipment. With lyophilized LAMP (L-LAMP), the simpler technique and lower equipment requirements could also be advantageous over the high price of standard LAMP kits for point-of-care use.[228] However, some LAMP devices have been created to decrease resource requirements, including a fully automated microfluidic device that would cost an estimated $3/assay.[229] Current techniques such as the use of AuNPs may increase reagent requirements, but as AuNPs become more widely available, they may influence a decrease in infrastructure requirements due to the visual readout ability of AuNPs. They have not been commercialized, and the assays analyzed would have TRLs of 3 or 4. Currently, most LAMP assays would have a high CLIA complexity, but this may decrease with the use of automated devices. A summary of LAMP techniques can be found in Table 23 and Table 24.

Table 23. Assay Requirements for LAMP

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
LAMP	Primers DNA polymerase AuNPs	Colorimetric or other photovisual readouts	Maybe	Refrigerator for reagent storage	Requires training
LAMP-HCR	Primers DNA polymerase HCR reagents	Colorimetric or other photovisual readouts	No	Freezer for reagent storage	Requires training
RT-LAMP	Lateral flow strip with a FAM-biotin receptor	Colorimetric or other photovisual readouts	No	--	Requires training

[227] Yu-Dong Ma, Yi-Sin Chen, and Gwo-Bin Lee, "An Integrated Self-Driven Microfluidic Device for Rapid Detection of the Influenza A (H1N1) Virus by Reverse Transcription Loop-Mediated Isothermal Amplification," *Sensors and Actuators B: Chemical* 296 (2019), https://doi.org/10.1016/j.snb.2019.126647.

[228] Kumar et al., "Advanced Lyophilised Loop Mediated Isothermal Amplification (L-LAMP) Based Point of Care Technique for the Detection of Dengue Virus."

[229] Ma, Chen, and Lee, "An Integrated Self-Driven Microfluidic Device for Rapid Detection of the Influenza A (H1N1) Virus by Reverse Transcription Loop-Mediated Isothermal Amplification."

Table 24. Assay Details for LAMP

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
LAMP	LOD: 10–10^2 copies/reaction Time: 25–40 minutes	Proof-of-concept	Lower cost Increased sensitivity	Compatible with multiple result visualization methods
LAMP-HCR	LOD: 30 copies/2% fecal sample	Proof-of-concept	Increased sensitivity Low cost	Cost is <$1/test
RT-LAMP	LOD: 3×10^{-4} units/reaction; 10 copies/µL Time: 30–40 minutes	Proof-of-concept	Low cost	Cost is <$3/test

L. Polymerase Spiral Reaction (PSR)

Polymerase spiral reaction (PSR) is an isothermal nucleic acid amplification method that requires only one pair of primers and one polymerase enzyme, using the principles of both PCR and LAMP. The single primer/enzyme simplifies the optimization process and reduces non-specific amplification, compared to techniques such as LAMP. A Bst polymerase is used as the single enzyme because it is more resilient to Taq DNA polymerase inhibitors, a common inhibitor for PCR reactions. The PSR primers have a 3' end that is complimentary to a portion of the target sequence and a 5' end that is the reverse of the other primer's 5' end. This allows the single strand to curl onto itself and form a spiral structure after extension. The use of Betaine, a chemical that destabilizes the DNA helix structure, allows the strands of DNA to unlock at 61°–65°C.[230]

For the detection of *S. aureus* in meat, PSR showed 10 times and 100 times more analytical sensitivity than conventional PCR and real-time PCR, respectively. The detection limit was 19.9×10^3 CFU after 2 hours and 19.9 CFU after 8 hours.[231] The analytical sensitivity (100×10^{-15} g) was tenfold greater than qPCR when detecting *Salmonella*, with a PPV and NPV of 100%.[232] In PSR, reverse transcription and amplification can also be conducted in a single tube,

[230] Wei Liu et al., "Polymerase Spiral Reaction (PSR): A Novel Isothermal Nucleic Acid Amplification Method," *Scientific Reports* 5, no. 1 (2015), https://doi.org/10.1038/srep12723.

[231] A. A. P. Milton et al., "Development of a Novel Polymerase Spiral Reaction (PSR) Assay for Rapid and Visual Detection of *Staphylococcus aureus* in Meat," *LWT* 139 (2021), https://doi.org/10.1016/j.lwt.2020.110507.

[232] Kasanchi M. Momin et al., "Development of a Novel and Rapid Polymerase Spiral Reaction (PSR) Assay to Detect *Salmonella* in Pork and Pork Products," *Molecular and Cellular Probes* 50 (2020), https://doi.org/10.1016/j.mcp.2020.101510.

as demonstrated by the detection of Coxsackievirus A16.[233] When identifying the envelope gene to detect West Nile Virus, the LOD was found to be 100 times better than conventional PCR (one RNA copy/reaction).[234]

A designed pair of accelerating primers can be taken from between the primer and target sequence to accelerate the extension process, so the reaction could be completed in 40 minutes with an increased sensitivity 100 times greater than conventional PCR.[235] Visual detection of PSR results can be achieved without specialized instrumentation by using dyes, as demonstrated with the detection of porcine epidemic diarrhea virus; this study also reported a tenfold lower LOD compared to conventional PCR.[236]

PSR reagents can be lyophilized for ease of transport and stability, as demonstrated by a study that conducted the first test for detection of a human RNA virus (chikungunya virus) from samples using RT-PSR. Reagents were stable for at least six months at 4°C and for at least one week at room temperature with no change in sensitivity.[237] One study combined PSR with a nanoparticle LFA that produced results in 60 minutes (including blood sample processing, reaction time, and visualization). This method has an LOD of 5.4 copies/mL of genomic hepatitis B viral DNA and the true positive rate is 100%.[238]

1. Assessment

As a recent technique developed in 2015, PSR is not yet sold as a commercial kit/platform. It is an isothermal amplification process that aims to increase specificity to the amplicon. It may be advantageous compared to conventional PCR techniques, as demonstrated by the 100-fold

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233 Shiyu He et al., "A Reverse Transcription-Polymerase Spiral Reaction (RT-PSR)-Based Rapid Coxsackievirus A16 Detection Method and Its Application in the Clinical Diagnosis of Hand, Foot, and Mouth Disease," *Frontiers in Microbiology* 11 (2020), https://doi.org/10.3389/fmicb.2020.00734.

234 Priyanka S. Tomar et al., "Polymerase Spiral Reaction Assay for Rapid and Real Time Detection of West Nile Virus from Clinical Samples," *Frontiers in Cellular and Infection Microbiology* 10 (2020), https://doi.org/10.3389/fcimb.2020.00426.

235 Shiyu He et al., "Rapid Visualized Isothermal Nucleic Acid Testing of *Vibrio parahaemolyticus* by Polymerase Spiral Reaction," *Analytical and Bioanalytical Chemistry* 412, no. 1 (2020), https://doi.org/10.1007/s00216-019-02209-y.

236 Xueyu Wang et al., "Visual Detection of Porcine Epidemic Diarrhea Virus Using a Novel Reverse Transcription Polymerase Spiral Reaction Method," *BMC Veterinary Research* 15, no. 1 (2019), https://doi.org/10.1186/s12917-019-1851-7.

237 Shashi Sharma et al., "Development of Magnetic Bead Based Sample Extraction Coupled Polymerase Spiral Reaction for Rapid On-Site Detection of Chikungunya Virus," *Scientific Reports* 10, no. 1 (2020), https://doi.org/10.1038/s41598-020-68469-2.

238 Lin Lin et al., "Rapid Detection of Hepatitis B Virus in Blood Samples Using a Combination of Polymerase Spiral Reaction with Nanoparticles Lateral-Flow Biosensor," *Frontiers in Molecular Biosciences* 7 (2020), https://doi.org/10.3389/fmolb.2020.578892.

lower detection limit for PSR compared to conventional PCR for West Nile Virus detection.[239] Researchers have claimed that RT-PSR has the potential to be easily scaled up for a high-throughput reaction, and can be used for rapid screening assays. Published research has already outlined the potential to speed the PSR reaction rate, lyophilize reagents, and remove instrumentation requirements for readouts by providing a visual result, highlighting the future potential to use PCR as a point-of-care test.[240] PSR has a similar complexity to PCR but is not yet commercially produced, so it would be assessed as moderate CLIA complexity with a TRL of 4. A summary of PSR techniques is listed in Table 25 and Table 26.

Table 25. Assay Requirements for PSR

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
PSR	Two primers Polymerase	Dyes for visualization	Maybe	--	Requires training
RT-PSR	Lyophilized reagents	--	No	--	Requires training
PSR-LFA	Nanoparticles	--	No	Refrigerator for reagent storage	Requires training

Table 26. Assay Details for PSR

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
PSR	LOD: 19.9–19.9 × 10^3 CFU/reaction; 1 RNA copy/reaction; 2.4 CFU/mL Time: 1–8 hours	Proof-of-concept	Increased sensitivity Simpler process No special equipment for result visualization	May be more resistant to inhibitors
RT-PSR	LOD: 24–240 copies/µL; 10 copies/reaction Time: 40 minutes	Proof-of-concept	Increased sensitivity Simpler process	May be useful for high throughput or screening
PSR-LFA	LOD: 5.4 copies/mL Time: 60 minutes	Proof-of-concept	Increased sensitivity Simpler process	

[239] Tomar et al., "Polymerase Spiral Reaction Assay for Rapid and Real Time Detection of West Nile Virus from Clinical Samples."

[240] Momin et al., "Development of a Novel and Rapid Polymerase Spiral Reaction (PSR) Assay to Detect Salmonella in Pork and Pork Products."

M. Plasma Cell-Free DNA Metagenomic Next-Generation Sequencing (mNGS)

mNGS is emerging as a potentially universal one-test approach for the diagnosis of a wide variety of pathogens. mNGS involves sequencing the entirety of nucleic acids in a clinical sample, followed by a computational analysis to identify targets of interest, either in the form of species identification or identification of sequences within a genome. mNGS can be performed using plasma cell-free DNA or DNA/RNA from cerebrospinal fluid or respiratory secretions.[241] The most common commercial mNGS is the Karius Test, which can detect and quantify pathogen cell-free DNA from a selection of 1,250 bacteria, viruses, fungi, and eukaryotic parasites. A meta-analysis of Karius tests showed that test results led to a change in the treatment course in 11% of patients, due to the test providing information not available otherwise. It is yet to be determined if this test can be used as a primary diagnostic, as it may not be able to distinguish colonization from infection. In another study, mNGS demonstrated good utility in immunocompromised pediatric patients, but had low PPV and NPV.[242] mNGS sequencing would have a TRL of 7+, with commercial platforms available, and a high CLIA complexity. A summary of mNGS is listed in Table 27 and Table 28.

Table 27. Assay Requirements for Plasma Cell-Free DNA mNGS

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Karius Test	--	--	No	--	Requires training

Table 28. Assay Details for Plasma Cell-Free DNA mNGS

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Karius Test	--	Commercial options exist	Simpler process Increased accuracy	Can distinguish among over 1,000 different viruses, bacteria, fungi, and parasites

241 Catherine A. Hogan et al., "Clinical Impact of Metagenomic Next-Generation Sequencing of Plasma Cell-Free DNA for the Diagnosis of Infectious Diseases: A Multicenter Retrospective Cohort Study," *Clinical Infectious Diseases* 72, no. 2 (2021), https://doi.org/10.1093/cid/ciaa035.

242 Role A. Lee et al., "Assessment of the Clinical Utility of Plasma Metagenomic Next-Generation Sequencing in a Pediatric Hospital Population," *Journal of Clinical Microbiology* 58 no.7 (2020), https://doi.org/10.1128/JCM.00419-20.

N. Quantum Dots

Quantum dots (QDs) are inorganic semiconductor nanocrystals, with diameters from $2–10 \times 10^{-9}$ m, that display a range of unique optoelectronic properties. They have broad excitation spectra and narrow emission spectra, and the emission wavelength can be tuned by changing the size of the nanoparticle; this creates great potential for fluorescence sensing applications. QDs are brighter and more stable against photobleaching than conventional organic dyes.[243]

Quantum dots can be used to improve the detection limit of immunochromatography. A QD-based immunochromatographic test strip (ICTS) could detect PSA in a 40 μL sample within 15 minutes with high specificity and sensitivity. The limit of detection was several times better than traditional ICTS platforms.[244] Quantum dots can be used in "barcode" systems to simultaneously detect multiple markers. One lab created a smartphone-based miniature device that could detect 1,000 viral copies/mL for HIV and hepatitis B in less than one hour.[245] A QD-based fluorescent immunochromatography method showed 93.75% sensitivity and 100% specificity for influenza A.[246] Another barcode assay achieved a lower limit of detection of 10^2 CFU/mL for *S. aureus*, MRSA, and *K. pneumoniae*. This method requires standard laboratory equipment and a range of automated instruments, but has the potential to be miniaturized into a portable device.[247]

A fluorescent biosensor uses quantum dots and magnetic separation to achieve a lower limit of detection for *E. coli* of 14 CFU/mL in two hours. The bacteria were captured first by magnetic nanoparticles (MNPs) and then by quantum dots to form MNP-bacteria-QD complexes, which can be detected using a portable optical system.[248]

Magnetic Bead-Quantum Dots (MB-Qdot) have been used with CRISPR for viral DNA detection; a colorimetric assay makes this method promising for a rapid, simple, visual diagnostic

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243 María Díaz-González et al., "Quantum Dot Bioconjugates for Diagnostic Applications," *Topics in Current Chemistry (Cham)* 378, no. 2 (2020), https://doi.org/10.1007/s41061-020-0296-6.

244 Xue Li et al., "Rapid and Quantitative Detection of Prostate Specific Antigen with a Quantum Dot Nanobeads-Based Immunochromatography Test Strip," *ACS Applied Materials & Interfaces* 6, no. 9 (2014), https://doi.org/10.1021/am5012782.

245 Kevin Ming et al., "Integrated Quantum Dot Barcode Smartphone Optical Device for Wireless Multiplexed Diagnosis of Infected Patients," *ACS Nano* 9, no. 3 (2015), https://doi.org/10.1021/nn5072792.

246 Anh V. T. Nguyen et al., "Sensitive Detection of Influenza A Virus Based on a CdSe/CdS/ZnS Quantum Dot-Linked Rapid Fluorescent Immunochromatographic Test," *Biosensors & Bioelectronics* 155 (2020), https://doi.org/10.1016/j.bios.2020.112090.

247 Kristyna Cihalova et al., "Antibody-Free Detection of Infectious Bacteria Using Quantum Dots-Based Barcode Assay," *Journal of Pharmaceutical and Biomedical Analysis* 134 (2017), https://doi.org/10.1016/j.jpba.2016.10.025.

248 Li Xue et al., "An Ultrasensitive Fluorescent Biosensor Using High Gradient Magnetic Separation and Quantum Dots for Fast Detection of Foodborne Pathogenic Bacteria," *Sensors and Actuators B: Chemical* 265 (2018), https://doi.org/10.1016/j.snb.2018.03.014.

tool that does not require sample processing or lab instruments. This assay demonstrated a detection limit of 0.5×10^{-9} M and 1.25×10^{-9} M in buffer and porcine plasma.[249]

A single-step platform using graphene oxide microplates and photoluminescent probes (consisting of quantum dot-antibody complexes) has been used to detect pathogenic bacterial food contamination. The microplates deactivate the probes' luminescence if not conjugated with the target, while probes that interact with the target maintain their photoluminescence. The limit of detection was 2 CFU/mL after 30 minutes, demonstrating an improvement over culture-based methods.[250]

A portable smartphone-based convective PCR (cPCR) device combines a lateral flow assay with quantum dot-labeled reporter probes. cPCR relies on buoyancy-driven natural convection for amplification, which can be performed in less than 30 minutes; when the bottom of a sample tube is heated, the temperature gradient is equivalent to the three phases of PCR (denaturation, annealing, extension). A lateral flow assay with quantum dot labels was used for clinical sample detection and could be completed in 10–15 minutes. The limit of detection was 4.7×10^3 DNA copies for clinical samples of MRSA. This device was created with off-the-shelf components, can amplify nucleic acids without the need for electric power, and can capture the fluorescent signal using a smartphone camera.[251]

A quantum dot based system has been used to detect multiple viruses coinfecting a single cell with a single staining cycle. This system uses multicolor, self-assembled QD-probes consisting of *S. aureus* protein A, conjugated QDs, and virus-specific antibodies. Multicolor probes for H1N1, H3N2, H9N2 were tested using this rapid self-assembly process and did not require purification.[252]

A photographic paper-based assay that uses gold nanoparticles grafted with cysteamine A to immobilize ssDNA with silver-graphene quantum dots (Ag/GQDs) was able to identify *L. pneumophila*. The Ag/GQDs increased the probe immobilization as well as the sensitivity. This

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249 Mengdi Bao et al., "Magnetic Bead-Quantum Dot (MB-Qdot) Clustered Regularly Interspaced Short Palindromic Repeat Assay for Simple Viral DNA Detection," *ACS Applied Materials & Interfaces* 12, no. 39 (2020), https://doi.org/10.1021/acsami.0c12482.

250 Mariana D. Avila Huerta et al., "Real-Time Pathogen Determination by Optical Biosensing Based on Graphene Oxide," *Proceedings* 60, no. 1 (2020), https://doi.org/10.3390/IECB2020-07016.

251 Vinoth K. Rajendran et al., "Smartphone Detection of Antibiotic Resistance Using Convective PCR and a Lateral Flow Assay," *Sensors and Actuators B: Chemical* 298 (2019), https://doi.org/10.1016/j.snb.2019.126849.

252 Thaer K. Fayyadh et al., "Simultaneous Detection of Multiple Viruses in Their Co-Infected Cells Using Multicolour Imaging with Self-Assembled Quantum Dot Probes," *Microchimica Acta* 184, no. 8 (2017), https://doi.org/10.1007/s00604-017-2300-6.

inexpensive genosensor can provide rapid detection with a bacterial detection limit of 1×10^{-21} M, which is better than comparable sensors.[253]

Quantum dots can also be used to provide sensitive detection using Fluorescence Resonance Energy Transfer (FRET)-based biosensors. FRET occurs when the electronic excitation energy of a donor molecule is transferred to an acceptor molecule. Cyanine 5-labeled oligonucleotide probes are attached to the quantum dots and added to the DNA target solution to form a sandwich hybrid. This sandwiching brings the Cy5 fluorophore (acceptor) close enough to the quantum dot (donor) so that FRET can occur. The detection limit was 0.2×10^{-9} M.[254]

Antibody-attached quantum dots have been used in QLISA (Quantum Linked Immunosorbent Assay), an ELISA-like assay in which half-antibodies are immobilized by thiol groups on the quantum dots. The lower limit of IL-6 detection was 50×10^{-12} g/mL.[255]

1. Assessment

The various characteristics of quantum dots can be leveraged to enhance existing diagnostic assays, especially for fluorescence detection. QDs could be implemented in multiple assays, including CRISPR, PCR, and LFAs. Due to the broad excitation spectra, narrow emission spectra, and brightness compared to conventional organic dyes, quantum dot-based substances may help decrease the limit of detection of existing assays. Depending on the assay with which they are paired, QD-based assays may have the potential to be portable with more research and development. Most QD-based assays would be assessed at a TRL level of 4, due to lack of production scaling and testing beyond the laboratory. The CLIA complexity of QD-based assays would be moderate to high complexity, but would decrease as QDs become more mainstream to use in assays. A summary of quantum dot techniques can be found in Table 29 and Table 30.

Table 29. Assay Requirements for Quantum Dots

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Quantum Dots	Semi-conductor nanoparticles	Fluorescence analyzer Standard lab equipment	Maybe	Standard lab equipment Refrigerator for reagent storage	Requires training

[253] Ahmad Mobed et al., "Immobilization of SsDNA on the Surface of Silver Nanoparticles-Graphene Quantum Dots Modified by Gold Nanoparticles Towards Biosensing of Microorganism," *Microchemical Journal* 152 (2020), https://doi.org/10.1016/j.microc.2019.104286.

[254] Mojtaba Shamsipur et al., "A Highly Sensitive Quantum Dots-DNA Nanobiosensor Based on Fluorescence Resonance Energy Transfer for Rapid Detection of Nanomolar Amounts of Human Papillomavirus 18," *Journal of Pharmaceutical and Biomedical Analysis* 136 (2017), https://doi.org/10.1016/j.jpba.2017.01.002.

[255] Miho Suzuki, Hikari Udaka, and Takeshi Fukuda, "Quantum Dot-Linked Immunosorbent Assay (QLISA) Using Orientation-Directed Antibodies," *Journal of Pharmaceutical and Biomedical Analysis* 143 (2017), https://doi.org/10.1016/j.jpba.2017.05.014.

QD-PCR	LFA with quantum dot-labeled reporter probes	Smartphone COTS components	Yes	No need for external power	Requires training
QD-FRET	Cy5-labeled oligonucleotide probes	FRET equipment	No	--	Requires training
QLISA	Antibody-labeled QDs	Fluorescence analyzer	No	Refrigerator for reagent storage	Requires training

Table 30. Assay Details for Quantum Dots

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Quantum Dots	LOD: 1,000 copies/mL; 2–10^2 CFU/mL; 0.5–1.25 × 10^{-9} M concentrations Time: 15 minutes–2 hours	Proof-of-concept	Increase the sensitivity of other quantification methods Semi-customizable Brighter than standard dyes	
QD-PCR	LOD: 4.7 × 10^3 DNA copies/reaction	Proof-of-concept	Increased sensitivity	
QD-FRET	LOD: 0.2 × 10^{-9} M concentrations	Proof-of-concept	Increased sensitivity	
QLISA	LOD: 50 × 10^{-12} g/mL	Proof-of-concept	Increased sensitivity	

O. Aptamer-Based Systems

Aptamers are nucleic acid probes that can bind to a range of nucleic acid targets (such as proteins, antibodies, toxins, and enzymes) with high affinity and specificity.[256] Advantages of aptamers include the ability to attach to targets with high affinity, high surface density, and less spatial blocking, and the ability to fold after target binding. Aptamers also maintain their structure over a wide range of temperature and storage conditions, and can be chemically synthesized under non-physiological conditions.[257]

Aptamers are selected using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process of binding, partition, elution, amplification, and conditioning. Sequences are selected from a DNA library containing random sequences by identifying those that bind to the target, which are then amplified by PCR. This process is repeated multiple times to obtain the probe with highest affinity and specificity; additional steps, such as structure processing and

[256] Edith Torres-Chavolla and Evangelyn C. Alocilja, "Aptasensors for Detection of Microbial and Viral Pathogens," *Biosensors & Bioelectronics* 24, no. 11 (2009), https://doi.org/10.1016/j.bios.2008.11.010.

[257] Naser Alizadeh et al., "Aptamer-Assisted Novel Technologies for Detecting Bacterial Pathogens," *Biomedicine & Pharmacotherapy* 93 (2017), https://doi.org/10.1016/j.biopha.2017.07.011.

affinity determination, may also be performed. However, SELEX is a time-consuming process that can take multiple months. Non-SELEX methods do exist, but they typically require capillary electrophoresis and are usually suitable only for macromolecular substances.[258]

A colorimetric aptasensor using G-quadruplex DNAzyme with peroxidase activity could detect *V. parahemolyticus* in food at levels as low as 10 CFU/mL, which is consistent with the standard plate counting method. In this method, the aptamer and its complementary sequence are fixed onto magnetic beads. In the presence of the target organism, both the complementary sequence and the embedded G-quadruplex sequences dissociate from the beads to create a colorimetric signal after the addition of 3,3′-5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide.[259] A lateral-flow assay aptasensor could detect *V. parahaemolyticus* at a similar level without a DNA extraction step requirement, and could be completed in less than 60 minutes.[260]

A label-free electrochemical aptasensor was able to detect *S. typhimurium* at concentrations as low as 3 CFU/mL.[261] Aptamers were used with SERS, which measures the Raman spectral signature to identify and quantify a target, to detect thrombin. Aptamers recognize the target and are then quantified with SERS tags. The limit of detection was 1.6×10^{-11} M.[262] When used in tandem with aptamers, a self-powered photoelectrochemical electrode with a zinc oxide nanowire array modified with gold nanoparticles was able to recognize and quantify *E. coli* at concentrations as low as 1.125 CFU/mL.[263]

RNA aptamers can bind to fluorogens to enhance their fluorescence by many orders of magnitude.[264] An RNA aptamer method in which there are no steps of separation, purification, or enrichment was developed and tested on foodborne *S. aureus* as a proof of principle. The target-aptamer binding facilitates the subsequent binding of a "binder" sequence with a "blocker"

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258 Wei Wu et al., "Research Advances of DNA Aptasensors for Foodborne Pathogen Detection," *Critical Reviews in Food Science and Nutrition* 60, no. 14 (2020), https://doi.org/10.1080/10408398.2019.1636763.

259 Yuhan Sun et al., "Colorimetric Aptasensor Based on Truncated Aptamer and Trivalent DNAzyme for *Vibrio parahemolyticus* Determination," *Journal of Agricultural and Food Chemistry* 67, no. 8 (2019), https://doi.org/10.1021/acs.jafc.8b06893.

260 Wei Wu et al., "A Sensitive Aptasensor for the Detection of *Vibrio parahaemolyticus*," *Sensors and Actuators B: Chemical* 272 (2018), https://doi.org/10.1016/j.snb.2018.05.171.

261 E. Sheikhzadeh et al., "Label-Free Impedimetric Biosensor for *Salmonella typhimurium* Detection Based on Poly Pyrrole-Co-3-Carboxyl-Pyrrole Copolymer Supported Aptamer," *Biosensors & Bioelectronics* 80 (2016), https://doi.org/10.1016/j.bios.2016.01.057.

262 Liyuan Yang et al., "Aptamer-Based Surface-Enhanced Raman Scattering (SERS) Sensor for Thrombin Based on Supramolecular Recognition, Oriented Assembly, and Local Field Coupling," *Analytical and Bioanalytical Chemistry* 409, no. 1 (2017), https://doi.org/10.1007/s00216-016-9992-z.

263 Xiuxiu Dong et al., "CdS Quantum Dots/Au Nanoparticles/ZnO Nanowire Array for Self-Powered Photoelectrochemical Detection of *Escherichia coli* O157:H7," *Biosensors & Bioelectronics* 149 (2020), https://doi.org/10.1016/j.bios.2019.111843.

264 Jeremy S. Paige, Karen Y. Wu, and Samie R. Jaffrey, "RNA Mimics of Green Fluorescent Protein," *Science* 333, no. 6042 (2011), https://doi.org/10.1126/science.1207339.

sequence, which is hybridized with the aptamer in absence of the target. The quantification was similar to the conventional plate counting method. These RNA aptamers can be easily obtained and amplified through in vitro transcription, which is simple and efficient.[265]

Another fluorescence aptasensor technique uses carboxyfluorescein-labeled complimentary DNA (FAM-cDNA). When the target (*P. aeruginosa*) is present, it displaces FAM-cDNA from the aptamer and releases it from magnetic nanoparticles. The result is quantified by measuring the fluorescence after magnetic separation. The LOD was 1 CFU/mL and the quantification range was 10–10^8 CFU/mL, which is more sensitive than most conventional methods over a similarly wide range. The entire detection process can be completed in 1.5 hours, making it an efficient technique that could provide additional options for existing laboratories; however, specialized equipment is required to perform some steps, such as magnetic separation and detection of fluorescence.[266] In another study, the aptamers were conjugated with photoluminescent carbon dots and graphene oxide was used as an anchor and quencher to reduce background signals. This method had a lower LOD of 9 CFU/mL.[267]

A novel aptamer-capped nanoporous anodic alumina (NAA) is able to provide quick, cheap, and ultrasensitive (5 CFU/mL in blood) detection of *S. aureus*. The NAA scaffold is loaded with a fluorescent indicator and pore entrances are capped by an aptamer that targets *S. aureus*. In the presence of the target, the pore entrances are uncapped due to aptamer displacement, releasing a dye into the medium. This method could be suitable for a point-of-care detection system and could be useful for detecting other pathogens.[268]

A piezoelectric quartz crystal-based system also achieved a lower detection limit of 9 CFU/mL for *P. aeruginosa* in buffer and 52 CFU/ml in blood. A sandwich complex of magnetic bead, aptamer, and poly-adenylated DNA with a gold electrode was attached to the quartz crystal. When the target was present, the poly-adenylated DNA would be replaced with the target on the aptamer; the newly-released poly-adenylated DNA would then adsorb onto the gold electrode,

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265 Lele Sheng et al., "A Transcription Aptasensor: Amplified, Label-Free and Culture-Independent Detection of Foodborne Pathogens via Light-up RNA Aptamers," *Chemical Communications* 55, no. 68 (2019), https://doi.org/10.1039/C9CC05036A.

266 Zitao Zhong et al., "Selective Capture and Sensitive Fluorometric Determination of *Pseudomonas aeruginosa* by Using Aptamer Modified Magnetic Nanoparticles," *Microchimica Acta* 185, no. 8 (2018), https://doi.org/10.1007/s00604-018-2914-3.

267 Hongying Wang et al., "Development of a Fluorescence Assay for Highly Sensitive Detection of *Pseudomonas aeruginosa* Based on an Aptamer-Carbon Dots/Graphene Oxide System," *RSC Advances* 8, no. 57 (2018), https://doi.org/10.1039/C8RA04819C.

268 Luis Pla et al., "Aptamer-Capped Nanoporous Anodic Alumina for *Staphylococcus aureus* Detection," *Sensors and Actuators B: Chemical* 320 (2020), https://doi.org/10.1016/j.snb.2020.128281.

resulting in a frequency shift in the quartz-based sensor response. This assay was selective and fast and may be used clinically with future developments.[269]

A novel method used aptamers to release two types of "walking" strands upon binding to the target. Using Exo III, these strands walk on an AuNP-based 3D track and can bind to other strands to induce a cycle of cleavage and hybridization. This results in destabilized aggregation of probes, which induces an observable color change. A "dual walker" system such as this improves the reaction kinetics, allowing analysis to be performed in approximately 15 minutes with an LOD of 1 CFU/mL.[270] This method improved upon a 2019 study in which a single stochastic DNA walker was used for bacterial detection.[271]

A "SpinChip" combines aptamer-specific recognition and nanoparticle-catalyzed pressure amplification for multiplexed point-of-care testing. A sample reacts with immobilized aptasensors, which are magnetically retained in a microwell, to form binding complexes that are then catalytically amplified. Catalytic amplification requires mixing the sample solution with a hydrogen peroxide (H_2O_2) solution to generate oxygen (O_2) (catalyzed by platinum nanoparticles) to increase the internal pressure that drives the movement of dyes into channels. A higher concentration of pathogens will increase the pressure that transduces the dyes to create a visual bar chart-like signal.[272]

One study demonstrated the use of a stem-looped oligonucleotide aptamer probes instead of a standard probe. The study's goal was to detect amyloid-beta oligomers for the early detection of Alzheimer's. The stem-looped oligonucleotide aptamer probes showed multiple advantages over standard probes, such as a strong conformational change upon target binding that increased background currents, and 80% reagent efficacy after storage for two weeks. The amyloid-beta oligomers could be detected at picomolar (10^{-12} M) concentrations.[273]

Aptamer-based systems usually use peroxidase to label targets. Peroxidase is costly and relatively unstable, prompting the development of labeling alternatives. A stable nanocomposite consisting of cerium oxide nanoparticles on Zeolite Y had superior performance and was used to

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269 Xiaohong Shi, Jialin Zhang, and Fengjiao He, "A New Aptamer/Polyadenylated DNA Interdigitated Gold Electrode Piezoelectric Sensor for Rapid Detection of *Pseudomonas aeruginosa*," *Biosensors & Bioelectronics* 132 (2019), https://doi.org/10.1016/j.bios.2019.02.053.

270 Haihong Yang et al., "Stochastic DNA Dual-Walkers for Ultrafast Colorimetric Bacteria Detection," *Analytical Chemistry* 92, no. 7 (2020), https://doi.org/10.1021/acs.analchem.9b05149.

271 Mingshu Xiao et al., "Stochastic DNA Walkers in Droplets for Super-Multiplexed Bacterial Phenotype Detection," *Angewandte Chemie* 131, no. 43 (2019), https://doi.org/10.1002/ange.201906438.

272 Xiaofeng Wei et al., "Multiplexed Instrument-Free Bar-Chart SpinChip Integrated with Nanoparticle-Mediated Magnetic Aptasensors for Visual Quantitative Detection of Multiple Pathogens," *Analytical Chemistry* 90, no. 16 (2018), https://doi.org/10.1021/acs.analchem.8b02055.

273 Yuting Zhang et al., "Amperometric Aptasensor for Amyloid-B Oligomer Detection by Optimized Stem-Loop Structures with an Adjustable Detection Range," *ACS Sensors* 4, no. 11 (2019), https://doi.org/10.1021/acssensors.9b01630.

detect serum glucose levels.[274] The use of a $ZnFe_2O_4$-reduced-graphene oxide nanostructure showed a peroxidase-mimetic effect and was used to detect *Salmonella* detection with a lower limit of detection of 11 CFU/mL.[275]

1. Assessment

Aptamer probes offer multiple advantages over traditional probes, including having a higher affinity for their targets. Traditionally, the selection of an appropriate aptamer using the SELEX process has been a time-consuming bottleneck, but newer methods utilizing in-silico analyses may considerably reduce the time required to a few days or hours.[276] Aptamers may also be combined with novel technologies, such as the aptamer-capped NAA, which has the potential for quick, ultrasensitive diagnoses of bloodborne pathogens.[277] Various novel categories of functional aptamers are being created, such as stem-looped oligonucleotides aptamers.[278] While stem-looped aptamers were discovered to have decreased storage requirements and increased sensitivity, further research into aptamer-based assays could lead to other improvements.

Aptamers are also stable over a wide range of temperatures and storage conditions, which are essential properties for use in forward facilities. This would allow them to potentially have a waived complexity with devices such as the SpinChip,[279] with CLIA complexities for the aptamer assays analyzed here varying from waived to high. The TRLs for these assays would range from 3 to 4. A summary of aptamer-based techniques is listed in Table 31 and Table 32.

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274 Xiaowei Cheng et al., "Rational Design of a Stable Peroxidase Mimic for Colorimetric Detection of H_2O_2 and Glucose: A Synergistic CeO_2/Zeolite Y Nanocomposite," *Journal of Colloid and Interface Science* 535 (2019), https://doi.org/10.1016/j.jcis.2018.09.101.

275 Shijia Wu et al., "Colorimetric Aptasensor for the Detection of *Salmonella enterica* Serovar Typhimurium Using $ZnFe_2O_4$-Reduced Graphene Oxide Nanostructures as an Effective Peroxidase Mimetics," *International Journal of Food Microbiology* 261 (2017), https://doi.org/10.1016/j.ijfoodmicro.2017.09.002.

276 Tao Wang, Changying Chen, Leon M. Larcher, Roberto A. Barrero, Rakesh N. Veedu, "Three Decades of Nucleic Acid Aptamer Technologies: Lessons Learned, Progress and Opportunities on Aptamer Development," *Biotechnology Advances* 37, no. 1 (2019): 28–50, https://doi.org/10.1016/j.biotechadv.2018.11.001.

277 Pla et al., "Aptamer-Capped Nanoporous Anodic Alumina for *Staphylococcus aureus* Detection."

278 Yuting Zhang et al., "Amperometric Aptasensor for Amyloid-B Oligomer Detection by Optimized Stem-Loop Structures with an Adjustable Detection Range."

279 Wei et al., "Multiplexed Instrument-Free Bar-Chart SpinChip Integrated with Nanoparticle-Mediated Magnetic Aptasensors for Visual Quantitative Detection of Multiple Pathogens."

Table 31. Assay Requirements for Aptamer-Based Systems

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Colorimetric aptasensor	DNAzyme Peroxidase Magnetic beads TMB H_2O_2	Standard lab equipment	No	Refrigerator and/or freezer required for reagent storage	Requires straining
Electrochemical aptasensors	SERS tags Zinc oxide nanowire array AuNPs NAA scaffold Piezoelectric quartz	Raman spectrometer	No	Refrigerator and/or freezer required for reagent storage	Requires straining
Fluorescence aptasensors	Fluorogens FAM-cDNA Photo-luminescent carbon dots	Plate counter Magnetic separator	No	Refrigerator and/or freezer required for reagent storage	Requires straining

Table 32. Assay Details for Aptamer-Based Systems

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Colorimetric aptasensor	LOD: 1–10 CFU/mL Time: 15–60 minutes	Proof-of-concept	Simpler process Faster time to result	
Electrochemical aptasensors	LOD: 1.125–52 CFU/mL; 0.1×10^{-9} M concentrations Time: 45–60 minutes	Proof-of-concept	Increased sensitivity Simpler process Faster time to result	
Fluorescence aptasensors	LOD: 1 CFU/mL Time: 1.5 hours	Proof-of-concept	Increased sensitivity Faster time to result	Requires some specialized equipment

P. Sample Preparation and Analysis Technologies

1. Lyophilized Reagents

Lyophilizing, or freeze-drying, reagents and samples may allow techniques such as PCR to become more accessible to non-trained personnel. [280] Lyophilized reagents can be reconstituted when needed, which can allow for reduced storage requirements and increased portability. Multiple studies have shown that this method is useful in diagnosing diseases caused by a variety

[280] Nuttada Panpradist et al., "Simpler and Faster Covid-19 Testing: Strategies to Streamline SARS-CoV-2 Molecular Assays," *EBioMedicine* 64 (2021), https://doi.org/10.1016/j.ebiom.2021.103236.

of agents and may increase sensitivity and transportability, as described below. This principle may be used for multiple different assay types, which may warrant future studies to identify the feasibility of lyophilization for different reagents.

One study used lyophilized PCR reagents and a high-throughput automated DNA extraction instrument to detect African swine fever virus in less than two hours, which is quicker than conventional PCR. This method had both high sensitivity (100%) and specificity (>96%).[281] Lyophilised reagents were used to detect the foot-and-mouth disease virus in clinical specimens with comparable accuracy to conventional real-time reverse-transcriptase PCR (rRT-PCR) assays. This allowed for assay/reagent portability and a decrease in time required for results (<1.5 hours).[282]

According to one study, lyophilized PCR reagents may be stored for a minimum of three months at 37°C and remain active.[283] A Chinese lab developed a similar PCR method for SARS-CoV-2 detection using lyophilized reagents, but estimated that reagent activity may only last for 10 days at 37°C.[284] An assay for foodborne *Salmonella*, *Staphylococcus*, and *Bacillus cereus* used lyophilized reagents that remained stable at 25°C for one month and could detect these pathogens with a sensitivity of 10 CFU/mL.[285] A French lab standardized a process for preparing and stabilizing oligonucleotide primers and hydrolysis probes in a single test tube using lyophilization. The resulting reagents were stable for four days at 37°C and two weeks at 4°C, and the rehydrated reagents were stable for at least 14 days at 4°C; this indicates suitability for transport, both with and without a cold chain. These reagents showed increased sensitivity over conventional PCR for detecting Chikungunya virus and Rift Valley Fever virus.[286]

Lyophilization of reagents has also been shown to benefit LAMP assays. Lyophilized LAMP (L-LAMP) showed comparable or better sensitivity and specificity (100% and 92%, respectively)

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281 Aiping Wang et al., "Development of a Novel Quantitative Real-Time PCR Assay with Lyophilized Powder Reagent to Detect African Swine Fever Virus in Blood Samples of Domestic Pigs in China," *Transboundary and Emerging Diseases* 67, no. 1 (2020), https://doi.org/10.1111/tbed.13350.

282 E. L. A. Howson et al., "Direct Detection and Characterization of Foot-and-Mouth Disease Virus in East Africa Using a Field-Ready Real-Time PCR Platform," *Transboundary and Emerging Diseases* 65, no. 1 (2018), https://doi.org/10.1111/tbed.12684.

283 Nur A. Khazani et al., "A Thermostabilized, One-Step PCR Assay for Simultaneous Detection of *Klebsiella pneumoniae* and *Haemophilus influenzae*," *Journal of Tropical Medicine* 2017 (2017), https://doi.org/10.1155/2017/7210849.

284 Jiasu Xu et al., "Room-Temperature-Storable PCR Mixes for SARS-CoV-2 Detection," *Clinical Biochemistry* 84 (2020), https://doi.org/10.1016/j.clinbiochem.2020.06.013.

285 Narong Arunrut, Wansika Kiatpathomchai, and Chiraporn Ananchaipattana, "Multiplex PCR Assay and Lyophilization for Detection of *Salmonella* Spp., *Staphylococcus aureus* and *Bacillus cereus* in Pork Products," *Food Science and Biotechnology* 27, no. 3 (2018), https://doi.org/10.1007/s10068-017-0286-9.

286 Laurence Thirion et al., "Lyophilized Matrix Containing Ready-to-Use Primers and Probe Solution for Standardization of Real-Time PCR and RT-QPCR Diagnostics in Virology," *Viruses* 12, no. 2 (2020), https://doi.org/10.3390/v12020159.

than RT-PCR (100% and 88%, respectively) in detecting dengue virus. Reagent lyophilization allows for easier access to LAMP, especially in regions where it is difficult to store reagents at temperatures of -20°C.[287]

2. Lyophilized Sera

In addition to reagents, lyophilization also demonstrates potential benefit when applied to serum, plasma, blood cells, and tissue samples. In one study, sera lyophilization appeared have no negative effect on assay accuracy and may increase sensitivity. The LOD decreased 100-fold, compared to non-lyophilized sera, which may be affected by the bacterial DNA concentration in the sample. This method was tested clinically using the sera of patients with *Coxiella burnetii* infections.[288] A study using mouse tissue found that lyophilization did not alter protein activities and there was no significant difference in RNA amplified by RT-PCR.[289]

Lyophilized serum has been shown to improve sensitivity for qPCR-based bacterial DNA detection. The use of lyophilized sera produced a limit of detection for *C. burnetti* of 1 bacterium/mL, while the use of conventional sera had a limit of detection of 100 bacteria/mL.[290] An older study found that sample lyophilization for viral nucleic acid detection was viable, but clinical studies have not recently been performed.[291]

In one study, a commercially available tool (QIAmp Tissue Kit in the QIAGEN Biorobot EZ1 Workstation) was used for DNA extraction before performing lyophilization using a Lyovac GT2 instrument in 1.5 mL tube. This technique to detect PSA lyophilizes patient sera to increase the target concentration of PSA four-fold, which resulted in the detection of recurring cancers 300 days before traditional PSA assays; the increased sensitivity could be attributed to the increased target concentration following rehydration. For Q fever, this lyophilization technique could result

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287 Sandeep Kumar et al., "Advanced Lyophilised Loop Mediated Isothermal Amplification (L-LAMP) Based Point of Care Technique for the Detection of Dengue Virus," *Journal of Virological Methods* 293 (2021), https://doi.org/10.1016/j.jviromet.2021.114168.

288 Sophie Edouard and Didier Raoult, "Lyophilization to Improve the Sensitivity of QPCR for Bacterial DNA Detection in Serum: The Q Fever Paradigm," *Journal of Medical Microbiology* 65, no. 6 (2016), https://doi.org/10.1099/jmm.0.000253.

289 Yonghong Wu et al., "Lyophilization is Suitable for Storage and Shipment of Fresh Tissue Samples Without Altering RNA and Protein Levels Stored at Room Temperature," *Amino Acids* 43, no. 3 (2012), https://doi.org/10.1007/s00726-011-1212-8.

290 Edouard and Raoult, "Lyophilization to Improve the Sensitivity of qPCR for Bacterial DNA Detection in Serum: The Q Fever Paradigm."

291 Helen Vaughan et al., "Stability of Lyophilised Specimens for the Molecular Detection of Viral DNA/RNA," *Journal of Clinical Virology* 35, no. 2 (2006), https://doi.org/10.1016/j.jcv.2005.06.001.

in an earlier diagnosis during the acute stage of disease. However, this study did not assess the effects of long-term storage on the lyophilized sera.[292]

A 2020 study found that lyophilized platelets can be used for anti-platelet antibody detection in solid phase red cell adherence tests. Storage at 2°–8°C was possible for up to 14 months and the reconstituted sample was stable for 48 hours.[293] Another study found that lyophilized blood samples remained stable for at least seven days at 45°C, and HIV RNA quantification did not differ between the lyophilized samples and samples tested directly after extraction. This sample preparation process eliminates cold-chain requirements for sample transport and short-term storage.[294]

A study found that lyophilizing plasma with trehalose reduced freeze drying-induced protein aggregation and reduced the accumulation of reactive oxygen species and protein oxidation products. Inhibiting protein aggregation helps preserve the protein/antibody and nucleotide recognition sites that are used in detection. The plasma's immunoglobulin-G (IgG) contents were not affected by freeze-drying when measured with ELISA.[295]

3. Membrane Sample Concentration

Membrane-driven pressure preconcentration devices may help increase the analyte concentration in biofluids, which would increase the sensitivity of any subsequent assay performed on the sample. Using a 5 kDa pore polyethersulfone membrane filter, the devices can create an influenza A nucleoprotein preconcentration up to 33 times the original concentration; when these devices are used in conjunction with an LFA, results could be obtained in a few minutes. However, each analyte must be tested for analyte loss before usage and the devices may not be relevant or useful for large analytes.[296]

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[292] Alexander Haese et al., "Ultrasensitive Detection of Prostate Specific Antigen in the Followup of 422 Patients after Radical Prostatectomy," *Journal of Urology* 161, no. 4 (1999), https://doi.org/10.1016/S0022-5347(01)61635-5.

[293] Shengbao Duan et al., "Application of Lyophilised Human Platelets for Antibody Detection in Solid Phase Red Cell Adherence Assay," *Journal of Immunological Methods* 487 (2020), https://doi.org/10.1016/j.jim.2020.112868.

[294] Jürgen Weidner et al., "Storage and Transportation of "HIV" RNA in Plasma Samples up to 45°C in a Lyophilized Stabilizer," *Clinical Laboratory* 65, no. 8 (2019), https://doi.org/10.7754/Clin.Lab.2019.190126.

[295] Raffaele Brogna et al., "Increasing Storage Stability of Freeze-Dried Plasma Using Trehalose," *PLOS ONE* 15, no. 6 (2020), https://doi.org/10.1371/journal.pone.0234502.

[296] A. Drexelius et al., "Analysis of Pressure-Driven Membrane Preconcentration for Point-of-Care Assays," *Biomicrofluidics* 14, no. 5 (2020), https://doi.org/10.1063/5.0013987.

4. Assessment

Using lyophilized reagents may increase the shelf life of reagents and allow assay techniques to become more accessible by non-trained personnel.[297] Lyophilized reagents, which can be reconstituted as needed, have allowed for increased portability of PCR assays and can allow for far-forward deployment by removing the need for bulky storage equipment and a cold chain. While lyophilization of reagents has only been tested on a limited selection of assays, the process could theoretically be used with a larger set of techniques, including many of the novel assays in this document. Similarly, L-LAMP has been proposed as a way to make LAMP more accessible in regions where storage at -20°C is not possible.[298] While lyophilized LAMP kits are not yet commercially available, lyophilized reagents are. Overall, assays using lyophilization techniques have TRLs ranging from 4 to 7+, with moderate or high CLIA complexities.

Lyophilization may be useful for serum sample transport, or as a sera-processing step before the actual assay. Some studies have shown an increase in sensitivity, due to increases in concentration after lyophilization.[299] These potential advantages would allow increased diagnostic capabilities further forward (e.g., a Role 1 facility could lyophilize a sample and send it to a higher role for processing). This would decrease logistical requirements, such as the presence of a cold chain. However, this technology has not been extensively tested and would still be at a TRL of 3 with a high CLIA complexity, which may be reduced with further research and development.

Pressure-driven membrane preconcentration devices may increase the sensitivity of subsequent assays performed on a sample. A proof-of-concept device was able to create a 33-fold concentration increase, which corresponded to a decrease in the limit of detection in a subsequent LFA.[300] Such devices could help increase the performance of existing assays with minimal increase in equipment or personnel. The current TRL of this technology is 3 with a moderate CLIA complexity.

A summary of sample preparation and analysis technologies is listed in Table 33 and Table 34.

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297 Panpradist et al., "Simpler and Faster Covid-19 Testing: Strategies to Streamline SARS-CoV-2 Molecular Assays."

298 Kumar et al., "Advanced Lyophilised Loop Mediated Isothermal Amplification (L-LAMP) Based Point of Care Technique for the Detection of Dengue Virus."

299 Edouard and Raoult, "Lyophilization to Improve the Sensitivity of qPCR for Bacterial DNA Detection in Serum: The Q Fever Paradigm."

300 Drexelius et al., "Analysis of Pressure-Driven Membrane Preconcentration for Point-of-Care Assays"

Table 33. Assay Requirements for Sample Preparation and Analysis Technologies

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Lyophilized Reagents	--	DNA extraction equipment	Maybe	Battery-powered Refrigerator optional	Requires training
Lyophilized Sera	--	DNA extraction equipment Lyophilizing equipment	Maybe	Freezer required for preparation	Requires training
Membrane Sample Concentration	Polyethersulfone membrane filter	Pressure-driven preconcentration device	No	Power source	Requires training (may be reduced in future versions)

Table 34. Assay Details for Sample Preparation and Analysis Technologies

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Lyophilized Reagents	LOD: 10–1,000 CFU/mL; 0.5–100 copies/µL Time: 1.5–3 hours	Some commercial options	Simpler process Increased sensitivity Faster time to result Longer shelf life	Could be useful at Role 1
Lyophilized Sera	LOD: 1 bacterium/mL	Proof-of-concept	Increased sensitivity Faster time to result Longer shelf life	Could be useful at Role 1
Membrane Sample Concentration	Increased target concentration 33-fold Time: <20 minutes	Proof-of-concept	Increased sensitivity	Used in conjunction with other methods to increase sensitivity

Q. Whole Cell-Based Biosensors

Whole cell-based biosensors (WCBB) are living cells that act as biosensors. In a typical WCBB, the target interacts with a regulator protein in the cell to cause the transcription of a reporter gene, leading to the production of a reporter protein. These sensors are not limited to bacteria and viruses and can be used to detect a range of analytes, including environmental contaminants. With recent advantages in synthetic biology, these sensors may emerge as a new diagnostic platform.

A whole-cell biosensor was created to detect *P. aeruginosa* and *B. pseudomallei* in water using the QscR quorum-sensing system in an *E. coli* host cell. Quorum sensing is a cell-to-cell communication method that allows bacteria to detect and respond to cell population density by regulating certain genes. This *E. coli* quorum-sensing system-based biosensor was modified to express enhanced green fluorescent proteins and has a sensitivity of 5.9×10^{-9} M. A paper-based

assay based on this biosensor was developed to produce a red spot upon successful detection.[301] A similar biosensor had a detection limit for human fibrinogen of 10×10^{-12} M in diluted human plasma. However, the analysis time was 18 hours; the use of centrifugation or filtration could theoretically reduce the time to result visualization.[302]

E. coli-based and *B. subtilis*-based WCBBs were able to detect the presence and activity of elastase, an enzyme released by *S. mansoni* cercarial larvae. The biosensors were lyophilized and could be reconstituted before detection. The WCBBs are labeled but proteolytic cleavage by elastase prevents labeling, resulting in an observable loss of color in the presence of *S. mansoni*.[303]

To detect antibiotic resistance to gentamicin, an integrated platform called DropFAST traps bacteria in 20 pL droplets to detect fluorescent signals from a fluorescent growth assay; this assay produces results after one hour of incubation, or approximately two to three replications. Similarly, change in fluorescent intensity emitted from resazurin reduction, which correlates with bacterial growth, could be measured.[304] This method was tested for *E. coli*, *K. pneumoniae*, and *E. faecalis* and could produce a result in one to three hours, depending on the species.[305]

A simplified modular yeast biosensor uses fungal mating G-protein coupled receptors to detect pathogen-specific peptides and uses red lycopene as a visual readout. The system has been reconfigured as a dipstick assay that can detect micromolar peptide concentrations in samples such as blood, soil, urine, and serum. This biosensor dipstick assay could be stored for 38 weeks at room temperature and has been reproducibly tested on *P. brasiliensis* and *C. albicans* peptides.[306]

1. Assessment

WCBBs have the potential to be a standard tool for medical diagnostics and environmental monitoring, with microelectronics aiding the development of real-time portable devices.[307] There

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301 Ying Wu et al., "A Whole-Cell Biosensor for Point-of-Care Detection of Waterborne Bacterial Pathogens," *ACS Synthetic Biology* 10, no. 2 (2021), https://doi.org/10.1021/acssynbio.0c00491.

302 Nicolas Kylilis et al., "Whole-Cell Biosensor with Tunable Limit of Detection Enables Low-Cost Agglutination Assays for Medical Diagnostic Applications," *ACS Sensors* 4, no. 2 (2019), https://doi.org/10.1021/acssensors.8b01163.

303 A.J. Webb et al., "A Protease-Based Biosensor for the Detection of Schistosome Cercariae," *Scientific Reports* 6, no. 1 (2016), https://doi.org/10.1038/srep24725.

304 Aniruddha M. Kaushik et al., "Accelerating Bacterial Growth Detection and Antimicrobial Susceptibility Assessment in Integrated Picoliter Droplet Platform," *Biosensors & Bioelectronics* 97 (2017), https://doi.org/10.1016/j.bios.2017.06.006.

305 Morteza Azizi et al., "Nanoliter-Sized Microchamber/Microarray Microfluidic Platform for Antibiotic Susceptibility Testing," *Analytical Chemistry* 90, no. 24 (2018), https://doi.org/10.1021/acs.analchem.8b03817.

306 Nili Ostrov et al., "A Modular Yeast Biosensor for Low-Cost Point-of-Care Pathogen Detection," *Science Advances* 3, no. 6 (2017), https://doi.org/10.1126/sciadv.1603221.

307 Qingyuan Gui et al., "The Application of Whole Cell-Based Biosensors for Use in Environmental Analysis and in Medical Diagnostics," *Sensors* 17, no. 7 (2017), https://doi.org/10.3390/s17071623.

are multiple potential advantage of WCBBs; being capable of genetic modification, they can operate over a wide range of conditions, such as temperatures and pH values. They can also be lyophilized for ease of storage and transportation.[308] While WCBBs have the potential to reduce personnel requirements through the development of all-in-one chips, they currently would have a high CLIA complexity, due to the knowledge required to create and test sensors. WCBBs are assessed at a TRL of 4, with production not yet scalable. A summary of whole cell-based biosensors can be found in Table 35 and Table 36.

Table 35. Assay Requirements for Whole Cell-Based Biosensors

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
WCBBs	--	Fluorescence analyzer	No	--	Requires training

Table 36. Assay Details for Whole Cell-Based Biosensors

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
WCBBs	LOD: 10^{-12} – 5.9×10^{-9} M concentrations Time: 1–18 hours	Proof-of-concept	Increased sensitivity Simpler process	Uses modifiable, inherent cellular processes to detect analytes Can be lyophilized

R. Resonance Energy Transfer (RET)

Resonance energy transfer is the phenomenon in which energy is transferred between two light-sensitive molecules (i.e., chromophores). Bioluminescence resonance energy transfer (BRET) uses the energy transfer between luciferase and fluorescent molecules to produce a ratiometric response to a target, which can correct for uncontrolled disturbances in the result.[309] This response can be measured to identify a target of interest. This principle can be used for portable and field-ready devices, such as a paper-based system that combines RCA and BRET. The paper-based system uses a smartphone to record and analyze the ratiometric signals and is

[308] P. Riangrungroj, C.S. Bever, B.D. Hammock et al. "A Label-Free Optical Whole-Cell *Escherichia coli* Biosensor for the Detection of Pyrethroid Insecticide Exposure," *Scientific Reports* 9 (2019): 12466. https://doi.org/10.1038/s41598-019-48907-6

[309] Kristiina Takkinen and Aurelija Žvirblienė, "Recent Advances in Homogenous Immunoassays Based on Resonance Energy Transfer," *Current Opinion in Biotechnology* 55 (2019), https://doi.org/10.1016/j.copbio.2018.07.003.

stable at room temperature, eliminating the need for a cold chain or specialized equipment for deployment.[310]

A Forster resonance energy transfer (FRET) between carbon dots and molybdenum sulfide (MoS_2) nano-couples has been tested in the detection of cardiac troponin T in serum. The MoS_2 nanosheets hold the antibody-labeled carbon dot and, in the presence of the target, the energy transfer process is hindered by the antigen/antibody interaction, which restores up-conversion intensity and can be measured by fluorescence with an LOD of 0.12×10^{-9} g/mL.[311]

1. Assessment

Resonance energy transfer-based assays have the advantage of potentially removing the requirement for specialized analytical equipment and extensive reagent storage requirements, replacing them with a simple assay process.[312] Resonance energy transfer assays would currently have moderate to high CLIA complexity, which could be reduced with further development of assays such as the paper-based BRET. These assays have not been scaled for manufacturing and have an estimated TRL of 4. A summary of RET technologies can be found in Table 37 and Table 38.

Table 37. Assay Requirements for Resonance Energy Transfer

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
BRET	Luciferase Fluorescence molecules	Smartphone	Maybe	No special equipment	Requires training (may be reduced in future versions)
FRET	Antibody-labeled carbon dots MoS_2 nanocouples	--	No	Refrigerator may be required for reagent storage	Requires training

Table 38. Assay Details for Resonance Energy Transfer

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
BRET	LOD: 2.8–19.3 × 10^{-9} M concentrations	Proof-of-concept	Faster time to result Potential for portability	

[310] Yong Li et al., "Portable and Field-Ready Detection of Circulating MicroRNAs with Paper-Based Bioluminescent Sensing and Isothermal Amplification," *Analytical Chemistry* 91, no. 23 (2019), https://doi.org/10.1021/acs.analchem.9b04422.

[311] Satyabrat Gogoi and Raju Khan, "Fluorescence Immunosensor for Cardiac Troponin T Based on Förster Resonance Energy Transfer (FRET) Between Carbon Dot and MoS_2 Nano-Couple," *Physical Chemistry Chemical Physics* 20, no. 24 (2018), https://doi.org/10.1039/C8CP02433B.

[312] Li et al., "Portable and Field-Ready Detection of Circulating MicroRNAs with Paper-Based Bioluminescent Sensing and Isothermal Amplification."

	Time: 20 minutes–3 hours		
FRET	LOD: 0.12×10^{-9} g/mL; 0.2×10^{-9} M concentrations	Proof-of-concept	Faster time to result Increased sensitivity

S. Microfluidics

Paper microfluidic analytical devices are recently emerged technologies that contain multiple patterned hydrophobic layers that guide a sample through the device while processing the sample (e.g., sample division, filtering, adding reagent, etc.), which allows for point-of-care diagnoses. One such device was able to differentiate and characterize neuraminidase activity due to influenza virus, as opposed to potentially assay-interfering upper respiratory infections such as *S. pneumoniae* and parainfluenza virus.[313] A paper-based device used BRET switches for target recognition and signal generation. Antibody binding and signal generation is combined into a single protein switch, referred to as LUMABS, and eliminates sample washing steps. Antibody binding changes the emitted bioluminescence color and without background fluorescence, direct and simultaneous antibody detection of multiple pathogens (e.g., anti-HIV1, anti-hemagglutinin [HA], and anti-Dengue [DEN]-1 antibodies) was possible with a mobile phone camera. BRET-based switches can also be used to detect low-weight molecular compounds and nucleic acids.[314]

A paper-based device used wax-patterned MF1 paper to allow plasma to separate from the RBCs by capillary force; this device could effectively detect dengue and Zika non-structural NSA1 viral protein in blood and plasma at a concentration of 10×10^{-9} g/mL within eight minutes. This device required minimal special equipment and results could be read with a smartphone, while traditional LFA requires the assembly of several pads and different materials.[315]

A microfluidic platform, prepared with a fast single-step method, with a monolith column was able to identify the *K. pneumoniae* carbapenemase gene, demonstrating the potential to simultaneously identify both pathogens and antibiotic susceptibility.[316] A small microfluidic device, measuring 13 cm x 10 cm x 12 cm and weighing 600 g, provides automated multiplex point-of-care RNA testing for infectious pathogens. The assay could be completed in 42 minutes, has a lower limit of detection of 10^2 copies/reaction, and has 100% concordance with lab-based

313 Richard C. Murdock et al., "Development of a Point-of-Care Diagnostic for Influenza Detection with Antiviral Treatment Effectiveness Indication," *Lab on a Chip* 17, no. 2 (2017), https://doi.org/10.1039/C6LC01074A.

314 Keisuke Tenda et al., "Paper-Based Antibody Detection Devices Using Bioluminescent BRET-Switching Sensor Proteins," *Angewandte Chemie International Edition* 57, no. 47 (2018), https://doi.org/10.1002/anie.201808070.

315 Frederic Bedin et al., "Paper-Based Point-of-Care Testing for Cost-Effective Diagnosis of Acute Flavivirus Infections," *Journal of Medical Virology* 89, no. 9 (2017), https://doi.org/10.1002/jmv.24806.

316 Radim Knob et al., "Sequence-Specific Sepsis-Related DNA Capture and Fluorescent Labeling in Monoliths Prepared by Single-Step Photopolymerization in Microfluidic Devices," *Journal of Chromatography A* 1562 (2018), https://doi.org/10.1016/j.chroma.2018.05.042.

molecular testing. The device uses 3D printing, commercial off-the-shelf components, and a wide-field camera for fluorescence imaging.[317]

A self-powered microfluidic chip can detect and directly quantify nucleic acid from human blood samples in approximately 30 minutes. The devices could be vacuum-stored for years, though the commercially available enzyme kits used in the assay typically expire in about one year.[318] A similar device, a microfluidic sample preparation multiplexer (SPM), has been developed for Ebola virus detection with a sample preparation time of one hour. The device uses magnetic beads with a high concentration of capture probes, which improves the efficiency and demonstrates a sensitivity of 0.021 PFU/mL.[319]

A mobile analytical platform (MAP) uses pneumatics, microfluidics, and a PCR system with readers to analyze samples in the field. This system has approximately 97% agreement with standard clinical tests against influenza A, influenza B, respiratory syncytial virus (RSV), and Middle East respiratory syndrome (MERS). The entire process is automated, from swab-loading to results analysis.[320]

One lab used free-flow electrophoresis to concentrate viral samples before performing thermal lysis and gel electrophoretic nucleic acid extraction. The process was miniaturized on a microfluidic chip with a compact power supply device, chip holder, and peristaltic pump, which could have great potential in point-of-care diagnostics. The detection limit on a test bacteriophage (PhiX174) was approximately 1 PFU/mL or 0.02 viral copies/μL. The processing time is currently 2.5 hours, which the lab is working to shorten.[321]

A microfluidic device, which uses MoS_2 nanosheets functionalized with cetyltrimethylammonium bromide (CTAB) on an indium-tin-oxide microelectrode, reached an LOD of 1.56 CFU/mL after 30 minutes for *S. typhimurium*. The device was specific even in the

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317 Bowen Shu et al., "A Pocket-Sized Device Automates Multiplexed Point-of-Care RNA Testing for Rapid Screening of Infectious Pathogens," *Biosensors & Bioelectronics* 181 (2021), https://doi.org/10.1016/j.bios.2021.113145.

318 Erh-Chia Yeh et al., "Self-Powered Integrated Microfluidic Point-of-Care Low-Cost Enabling (SIMPLE) Chip," *Science Advances* 3, no. 3 (2017), https://doi.org/10.1126/sciadv.1501645.

319 K. Du et al., "Multiplexed Efficient on-Chip Sample Preparation and Sensitive Amplification-Free Detection of Ebola Virus," *Biosensors & Bioelectronics* 91 (2017), https://doi.org/10.1016/j.bios.2016.12.071.

320 Justin Hardick et al., "Initial Performance Evaluation of a Spotted Array Mobile Analysis Platform (MAP) For the Detection of Influenza A/B, RSV, and MERS Coronavirus," *Diagnostic Microbiology and Infectious Disease* 91, no. 3 (2018), https://doi.org/10.1016/j.diagmicrobio.2018.02.011.

321 Matthias Hügle et al., "A Lab-on-a-Chip for Free-Flow Electrophoretic Preconcentration of Viruses and Gel Electrophoretic DNA Extraction," *Analyst* 145, no. 7 (2020), https://doi.org/10.1039/C9AN02333J.

presence of interferents such as *E. coli*. The chip demonstrated total stability when stored for three to four weeks at 4°C, with a 14.2% decrease in signal at the end of seven weeks.[322]

A method designed to improve upon immunochromatography uses electroosmotic force to increase the driving force and up-conversion of nanoparticles to increase sensitivity. Electroosmotic flow uses an electric field to manipulate liquid flow while in contact with a solid surface. This method increased signal intensity by 64% and reduced assay time to five minutes, with a limit of detection of 1.2×10^4 CFU/mL in soil samples.[323]

A novel method using hydrogel formation on nanofluidic pores was devised for rapid SARS-CoV-2 detection. On a microfluidic pore-containing mesh, immobilized probes hybridize with target DNA to form a DNA hydrogel by rolling circle amplification, which consequently blocks the mesh pores; this blockage is observable with a limit of detection for SARS-CoV-2 of 3×10^{-18} M at 15 minutes and 30×10^{-18} M in five minutes. This method does not require any sophisticated instrumentation, as a colored dye in a sample tube is sufficient to provide visual indication of pore blockage. This method has the potential to become a rapid and simple point-of-care test in the future.[324]

1. Assessment

Microfluidic devices can simplify the diagnostic process by enabling multiple sample processing steps in a single device and minimizing external input and personnel requirements. These devices can be self-powered and generally have short sample processing times. Platforms such as paper microfluidics can act as disposable point-of-care diagnostic tools and may provide advantages, such as simple, accessible result readability (e.g., a visually read result or through a smartphone).[325] The microfluidic devices assessed in this paper would have TRLs ranging from 3–4. Their CLIA complexity would be moderate to high, though the potential to develop self-contained portable microfluidic systems may make them useful at far-forward locations.

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322 Chandan Singh et al., "Functionalized MoS_2 Nanosheets Assembled Microfluidic Immunosensor for Highly Sensitive Detection of Food Pathogen," *Sensors and Actuators B: Chemical* 259 (2018), https://doi.org/10.1016/j.snb.2017.12.094.

323 Yong Zhao et al., "A Novel Electro-Driven Immunochromatography Assay Based on Upconversion Nanoparticles for Rapid Pathogen Detection," *Biosensors & Bioelectronics* 152 (2020), https://doi.org/10.1016/j.bios.2020.112037.

324 Hwang-soo Kim, Naseem Abbas, and Sehyun Shin, "A Rapid Diagnosis of SARS-CoV-2 Using DNA Hydrogel Formation on Microfluidic Pores," *Biosensors & Bioelectronics* 177 (2021), https://doi.org/10.1016/j.bios.2021.113005.

325 Bedin et al., "Paper-Based Point-of-Care Testing for Cost-Effective Diagnosis of Acute Flavivirus Infections."

T. Lateral Flow Assays (LFAs)

Lateral flow immunoassays (LFIAs) are based on the movement of a sample through a strip consisting of a nitrocellulose membrane and functional pad. The target molecules bind to surface antibodies on the nitrocellulose membrane to produce a visible line, which indicates a positive result. Multiplexing uses several detection antibodies to detect a variety of antigens and produce results with multiple distinct lines.[326] Multiplex lateral flow assays have potential as point-of-care tests for rapid and accurate diagnoses. Multiplex LFAs can be categorized into those that detect several analytes on one strip, those that detect several analytes with multiple strips, or those that integrate lateral flow and micro-assay technologies.

A smartphone-based LFIA was developed to miniaturize the tools required for rapid point-of-care diagnosis of Zika virus using the NS1 protein. The LOD was 0.045×10^{-9} g/mL and 0.15×10^{-9} g/mL in buffer and serum, respectively. The test could be carried out in approximately 20 minutes.[327] The same team developed an LFA and signal detection device for integrated multiplexed point-of-care diagnostics, which could detect HIV antibody, *Treponema pallidum* antibody, hepatitis C antibody, and hepatitis B surface antigen. The integrated platform is low cost ($200), rapid (20 minutes), and performs sample distribution, signal acquisition, data analysis with cartridge processing, strip reader loading, and optical signal scanning. The limits of detection were four to ten times lower than commercially available colloidal gold test strips (0.11 China National Clinical Unit (NCU)/mL for HIV antibody, 0.62 IU/L for TP antibody, 0.14 NCU/mL for HCV antibody, and 0.22 IU/mL for HBV antigen).[328]

An LFIA rapid test was developed to detect subclinical malaria from 2–5 mL of saliva. The study identified one protein (out of 35 detected) that was most abundant in subclinical saliva samples; the LFIA assay was developed with two high-affinity IgG monoclonal antibodies for capture and detection. The result is analyzed via fluorescence detection and the LOD is 50×10^{-12} g/mL (approximately 1–16 gametocyes/μL of blood); the assay has a sensitivity of 100% (gametocytes), 92% (trophozoites), 92% (pfs25), and 91% (18S rRNA) when compared to microscopy for the first two and molecular detection for the latter two. The overall estimated

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326 Hanbi Kim, Doo-Ryeon Chung, and Minhee Kang, "A New Point-of-Care Test for the Diagnosis of Infectious Diseases Based on Multiplex Lateral Flow Immunoassays," *Analyst* 144, no. 8 (2019), https://doi.org/10.1039/C8AN02295J.

327 Zhen Rong et al., "Smartphone-Based Fluorescent Lateral Flow Immunoassay Platform for Highly Sensitive Point-of-Care Detection of Zika Virus Nonstructural Protein 1," *Analytica Chimica Acta* 1055 (2019), https://doi.org/10.1016/j.aca.2018.12.043.

328 Zhen Rong et al., "Integrated Fluorescent Lateral Flow Assay Platform for Point-of-Care Diagnosis of Infectious Diseases by Using a Multichannel Test Cartridge," *Sensors and Actuators B: Chemical* 329 (2021), https://doi.org/10.1016/j.snb.2020.129193.

sensitivity for symptomatic cases is 75% compared to microscopy and 83% compared to PCR. The assay can be run in three to five minutes, with a maximum time of 30 minutes.[329]

Silver nanoparticles (AgNPs) have been used in a lateral flow chromatography technique to distinguish among dengue, yellow fever, and Ebola virus. The limit of detection was shown to be 150×10^{-9} g/mL in human serum.[330] One study used antimicrobial peptides (AMPs) labeled with colloidal gold and a target-specific membrane-bound antibody to detect Shiga toxin-producing *E. coli*. The limit of detection was 10^4 CFU/mL, which is a similar LOD to methods using antibodies instead of AMPs. *E. coli* could also be detected at very low levels in beef after an 18-hour enrichment process. The broad binding capacities of AMPs may confer advantages to this LFA sub-type.[331]

Proteinticles are nano-scaled protein particles formed by the self-assembly activities of protein monomers/constituents in cells. Adding antigen genetics to the protein sequence allows them to be presented in a homogenous orientation and a native conformation, which can enhance sensitivity over peptides by acting as a 3D-probe. One experiment used a modified human ferritin heavy chain with viral antigens linked to its C-terminus and self-assembled intracellularly in *E. coli*. Proteinticle probes have higher sensitivity and specificity and have been examined as replacements for peptide probes in testing for HIV, hepatitis B (HBV), and hepatitis C (HCV), though this method could be applied to other infectious diseases antigens or biomarkers. In addition to increased accuracy, detection signals also increased compared to traditional protein probes.[332]

An up-conversion nanoparticle-based LFA (UCNP-LFA) was developed to detect five types of target analyte in a miniaturized device (weighing 0.9 kg and measuring 24 cm x 10 cm x 6 cm). UCNPs are fluorescent nanoparticles with properties of long-term photostability and negligible background signal. The smartphone-based analyzer provides real-time quantitative analysis and was tested on heavy metal ions, bacteria, nucleic acids, and proteins. Correlation coefficients compared with the gold standard method are greater than 0.992.[333]

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329 Dingyin Tao et al., "A Saliva-Based Rapid Test to Quantify the Infectious Subclinical Malaria Parasite Reservoir," *Science Translational Medicine* 11, no. 473 (2019), https://doi.org/10.1126/scitranslmed.aan4479.

330 Chun-Wan Yen et al., "Multicolored Silver Nanoparticles for Multiplexed Disease Diagnostics: Distinguishing Dengue, Yellow Fever, and Ebola Viruses," *Lab on a Chip* 15, no. 7 (2015), https://doi.org/10.1039/C5LC00055F.

331 Taro Yonekita et al., "Development of a Novel Multiplex Lateral Flow Assay Using an Antimicrobial Peptide for the Detection of Shiga Toxin-Producing *Escherichia coli*," *Journal of Microbiological Methods* 93, no. 3 (2013), https://doi.org/10.1016/j.mimet.2013.03.006.

332 Jong-Hwan Lee et al., "Multiplex Diagnosis of Viral Infectious Diseases (AIDS, Hepatitis C, and Hepatitis a) Based on Point of Care Lateral Flow Assay Using Engineered Proteinticles," *Biosensors & Bioelectronics* 69 (2015), https://doi.org/10.1016/j.bios.2015.02.033.

333 Yan Gong et al., "A Portable and Universal Upconversion Nanoparticle-Based Lateral Flow Assay Platform for Point-of-Care Testing," *Talanta* 201 (2019), https://doi.org/10.1016/j.talanta.2019.03.105.

1. Assessment

Lateral flow assays are commonly used as a point-of-care diagnostic tool. Various studies are aimed at improving currently-used LFAs by increasing sensitivity or adding characteristics, such as the ability to distinguish among different pathogens. Miniaturization and smartphone-based readouts can reduce the need for trained personnel and increase portability. Automated systems demonstrating multiple improvements have decreased result time and increased sensitivity. [334]

Combinations of LFAs with other novel tools, such as RPA, can also help increase sensitivity and specificity, as demonstrated by Jauset-Rubio et al.[335] By enclosing the entire assay in a microfluidic chip, assays can also require fewer manual preparation steps and avoid contamination.[336] LFAs have the potential for waived CLIA complexities; however, most of the assays analyzed currently have a moderate CLIA complexity. While LFAs are widely available on the commercial market, the LFAs analyzed in this paper have a TRL of 4.

U. Electrochemical Sensors

Multiple assays using electrochemical sensors to detect a target molecule have recently been developed. These assays leverage the properties of materials such as graphene and gold for sensitive identification. Many of these assays involve the binding of the target antigen to a sheet of a conductive material, such as graphene, which causes a change in conductance. This change in conductance can be measured to characterize the bound substance.

Graphene is increasingly being used to enhance the accuracy of diagnostic tests. One lab created a paper-based electrochemical sensor to detect a cancer antigen using reduced graphene oxide/thionine/gold nanoparticles; the immune complex formed by the antibody-antigen reaction can reduce the electrical current response of thionine proportional to the antigen concentration. The assay shows acceptable agreement with traditional ELISA results, and the relative error was less than 8%. This method can be applied to different antigens, is low cost, has a short detection time, and may be used as a point-of-care method. Furthermore, this method does not require extensive equipment (measurements are taken by electrochemical workstation CHI 660E), the reagents/immunosensor can be stored at 4°C, and the reaction occurs at room temperature.[337]

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334 Rong et al., "Smartphone-Based Fluorescent Lateral Flow Immunoassay Platform for Highly Sensitive Point-of-Care Detection of Zika Virus Nonstructural Protein 1."

335 Jauset-Rubio et al., "Duplex Lateral Flow Assay for the Simultaneous Detection of *Yersinia pestis* and *Francisella tularensis*."

336 Cossio et al., "Diagnostic Performance of a Recombinant Polymerase Amplification Test-Lateral Flow (RPA-LF) For Cutaneous Leishmaniasis in an Endemic Setting of Colombia."

337 Yan Fan et al., "A Paper-Based Electrochemical Immunosensor with Reduced Graphene Oxide/Thionine/Gold Nanoparticles Nanocomposites Modification for the Detection of Cancer Antigen 125," *Biosensors & Bioelectronics* 135 (2019), https://doi.org/10.1016/j.bios.2019.03.063.

A graphene-wrapped copper (II) assisted cysteine hierarchical structure fabricated on a gold electrode, made with a novel technique, provides the basis of a label-free ultrasensitive sensor. Antibodies are immobilized on the electrodes and an autolab potentiostat is used for electrochemical analysis. The device can quantify up to 10^8 CFU/mL and has a lower LOD of 3.8 CFU/mL for *E. coli* O157: H7.[338]

A disadvantage of many electrochemical biosensors is nonspecific binding, which can affect an instrument's capacitance and resistance properties and potentially lead to inaccurate results. Gold nanowires or carbon nanotubes can overcome nonspecific binding, though they are currently prohibitively expensive. Graphene oxide nanoflakes offer a viable alternative to reduce fouling, as demonstrated by a multiplex biomarker system developed in 2020. Nanocomposite coatings of bovine serum albumin and graphene oxide nanoflakes were crosslinked with glutaraldehyde to increase sensitivity and significantly reduce cost compared to AuNPs. This assay was integrated into a microfluidic chip, has an analysis time of less than 10 minutes, and can detect sepsis biomarkers, such as procalcitonin, C-reactive protein, and pathogen-associated molecular patterns from whole blood. While still in the proof-of-concept stage, manufacturing these microfluidic chips could provide easy point-of-care diagnostics that require relatively minimal equipment.[339]

Similarly, a graphene field-effect biosensor was developed for ultrasensitive biomolecule detection and can be tailored for point-of-care diagnosis. This sensor takes advantage of the strong covalent interaction between avidin and biotin: an avidin-immobilized graphene channel monitors the current change when a biotin-containing solution is added. Biotin can conjugate with a variety of biomolecules including proteins and nucleotides, making this system tailorable based on the target molecule. The sensor has a sensitivity limit of 0.37×10^{-12} M (90×10^{-15} g/mL), has high specificity, and offers real-time detection. A Keysight 4155B semiconductor analyzer is used to measure current and would be the only required equipment, besides reagents, if this technology were to be scaled.[340]

A porous, nucleic acid aptamer-modified, reduced-graphene oxide/MoS_2-based electrode system was developed for HPV detection. This assay demonstrated a limit of detection of

338 Chandra MouliPandey et al., "Highly Sensitive Electrochemical Immunosensor Based on Graphene-Wrapped Copper Oxide-Cysteine Hierarchical Structure for Detection of Pathogenic Bacteria," *Sensors and Actuators B: Chemical* 238 (January 2017), https://doi.org/10.1016/j.snb.2016.07.121.

339 Uroš Zupančič et al., "Graphene Enabled Low-Noise Surface Chemistry for Multiplexed Sepsis Biomarker Detection in Whole Blood," *Advanced Functional Materials* 31, no. 16 (2021), https://doi.org/10.1002/adfm.202010638.

340 Shiyu Wang et al., "Graphene Field-Effect Transistor Biosensor for Detection of Biotin with Ultrahigh Sensitivity and Specificity," *Biosensors & Bioelectronics* 165 (2020), https://doi.org/10.1016/j.bios.2020.112363.

0.1×10^{-9} g/mL in saliva and spiked serum.[341] A similar MoS_2-based platform was coupled with a smartphone as a point-of-care diagnostic test; this assay has an LOD of 0.1×10^{-9} g/mL for the detection of prostate-specific antigen in serum.[342] Poly-xanthurenic acid (PXA) film functionalized with MoS_2 nanosheets could detect DNA at 1.8×10^{-17} mol/L; the PXA/MoS_2 nanocomposite served as a substrate for DNA immobilization and reflected electrochemical transduction, due to the label-less immobilization.[343] Another MoS_2-based system uses Ag/MoS_2/rGO nanocomposites for carcinoembryonic antigen detection with an LOD of 1.6×10^{-15} g/mL.[344]

Cellular prion protein bioreceptors with gold nanoparticles are used in a highly sensitive electrochemical impedance sensor that can be modified to identify different proteins. The lower detection limit is at the sub-femtomolar (10^{-15} M) level; it could sense amyloid-beta oligomer, indicating a potential for early diagnosis of Alzheimer's disease.[345] Another electrochemical sensor was developed to detect cryptosporidium using antibodies immobilized on gold electrodes as capture probes, with an LOD of 40 cells/mm^2. The formation of the target-antibody complex changes the electrode's capacitive properties, which can be measured using a potentiostat. This device still exists at the proof-of-concept stage, but could be adapted to detect other biomarkers.[346]

A novel electrochemical biosensor has the potential to identify antibiotic resistance. A deposited conductive polymer functionalized with lectin allows the sensor to capture intact bacterial cells. After glucose is added to capture the signal, adding antibiotics alters the signal, which can be used to measure the spectrum of antibiotic sensitivity; this study used *E. coli* and tetracycline, erythromycin, and kanamycin. While the LOD is 7.1×10^3 CFU/mL, concentrations

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341 Fereshteh Chekin et al., "Nucleic Aptamer Modified Porous Reduced Graphene Oxide/MoS2 Based Electrodes for Viral Detection: Application to Human Papillomavirus (HPV)," *Sensors and Actuators B: Chemical* 262 (2018), https://doi.org/10.1016/j.snb.2018.02.065.

342 Memoon Sajid et al., "All-Printed Highly Sensitive 2D MoS_2 Based Multi-Reagent Immunosensor for Smartphone Based Point-of-Care Diagnosis," *Scientific Reports* 7, no. 1 (2017), https://doi.org/10.1038/s41598-017-06265-1.

343 Wei Zhang et al., "High-Performance Electrochemical Sensing of Circulating Tumor DNA in Peripheral Blood Based on Poly-Xanthurenic Acid Functionalized MoS_2 Nanosheets," *Biosensors & Bioelectronics* 105 (2018), https://doi.org/10.1016/j.bios.2018.01.038.

344 Yaoguang Wang et al., "Label-Free Electrochemical Immunosensor Based on Flower-Like Ag/MoS_2/rGO Nanocomposites for Ultrasensitive Detection of Carcinoembryonic Antigen," *Sensors and Actuators B: Chemical* 255 (2018), https://doi.org/10.1016/j.snb.2017.07.129.

345 Jieling Qin, Misuk Cho, and Youngkwan Lee, "Ultrasensitive Detection of Amyloid-B Using Cellular Prion Protein on the Highly Conductive Au Nanoparticles-Poly(3,4-Ethylene Dioxythiophene)-Poly(Thiophene-3-Acetic Acid) Composite Electrode," *Analytical Chemistry* 91, no. 17 (2019), https://doi.org/10.1021/acs.analchem.9b02266.

346 George Luka et al., "Label-Free Capacitive Biosensor for Detection of *Cryptosporidium*," *Sensors* 19, no. 2 (2019), https://doi.org/10.3390/s19020258.

above 10^7 CFU/mL are recommended for antibiotic screening. The devices are low cost, easy to use, and potentially scalable.[347]

A bioassay developed with gold nano-architecture could identify *L. pneumophila* at a concentration of 1×10^{-21} M. A chitosan composite film immobilizes pDNA to act as a sensing platform before performing electrochemical analysis.[348] A peptide nucleic acid (PNA) biosensor based on functionalized graphene with cadmium sulfide (CdS) quantum dots could detect *M. tuberculosis* at 8.9×10^{-13} M. PNA was used because it can easily attach to the transducer surface, and by the charge of the peptide backbone it can eliminate the electrochemical repulsion between the two hybridized strands.[349]

Using electrospun Mn_2O_3 nanofibers for DNA hybridization detection produced ultrasensitive results for the dengue consensus primer sequence, with an LOD of 120×10^{-21} M. The ultrasensitivity may be derived from synthesizing a low bandgap electrospun nanomaterial that corresponds to a specific oxidation state of manganese; having a low bandgap enhances electron transfer at the electrolyte-electrode surface, thereby enhancing the signal. In this study, the method was not performed on clinical samples, so an RNA extraction step was not performed.[350] A chemiresistive platform uses metal electrodes topped with electrospun nanofibers to target Dengue virus and *S. aureus* genes; this platform successfully avoids inter-device variability that often plagues electrospun nanofiber technologies. Target interaction (such as with negatively-charged DNA) changes the surface charge of the electrodes, which influences the mobility of charge carriers in the nanofibers to ultimately cause a conductivity change. Dengue virus could be detected via a DNA sequence called Dengue virus-specific consensus primer at an LOD of 1.9×10^{-15} M.[351]

A method of using electrorheological materials was demonstrated for detection of *Y. pestis*. Electrorheological fluids are electrically insulating fluids that contain a suspension of electrically polarizable (i.e., non-conducting) particles. Electrorheological displays were constructed to

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347 Nuvia M. Saucedo, Sira Srinives, and Ashok Mulchandani, "Electrochemical Biosensor for Rapid Detection of Viable Bacteria and Antibiotic Screening," *Journal of Analysis and Testing* 3, no. 1 (2019), https://doi.org/10.1007/s41664-019-00091-2.

348 Ahmad Mobed et al., "DNA-Based Bioassay of *Legionella* Pneumonia Pathogen Using Gold Nanostructure: A New Platform for Diagnosis of Legionellosis," *International Journal of Biological Macromolecules* 128 (2019), https://doi.org/10.1016/j.ijbiomac.2019.01.125.

349 Mohd H. Mat Zaid et al., "PNA Biosensor Based on Reduced Graphene Oxide/water Soluble Quantum Dots for the Detection of *Mycobacterium tuberculosis*," *Sensors and Actuators B: Chemical* 241 (2017), https://doi.org/10.1016/j.snb.2016.10.045.

350 Suryasnata Tripathy et al., "Electrospun Manganese (III) Oxide Nanofiber Based Electrochemical DNA-Nanobiosensor for Zeptomolar Detection of Dengue Consensus Primer," *Biosensors & Bioelectronics* 90 (2017), https://doi.org/10.1016/j.bios.2016.12.008.

351 Suryasnata Tripathy et al., "Chemiresistive DNA Hybridization Sensor with Electrospun Nanofibers: A Method to Minimize Inter-Device Variability," *Biosensors & Bioelectronics* 133 (2019), https://doi.org/10.1016/j.bios.2019.03.031.

contain the microspheres, which adsorb magnetic nanoparticles and *Y. pestis* antibodies, and to observe changes in transmittance. Antigen-antibody coupling could be detected by measuring the maximum transmittance frequency. The limit of detection was 30×10^{-9} g/μL within 30 seconds. This method could be used for rapid label-free detection that can be quantified by the naked eye.[352]

An electrochemical assay could identify influenza viruses with high sensitivity using a standard glucometer and handheld potentiostat. The influenza surface glycoprotein neuraminidase cleaves substrates to release galactose, which is detected amperometrically with the potentiostat and dehydrogenase-bearing glucose strip. Depending on which substrate was cleaved, the assay could also detect a bacterial coinfection; in this case, the 4,7di-OMe N-acetylneuraminic acid attached to galactose was cleaved by only viral neuraminidase. The entire assay could be performed in 15 minutes.[353]

An electrolyte-gated graphene field-effect transistor (GFET) demonstrated an LOD of 25×10^{-18} M; at this concentration, the assay could distinguish between single nucleotide polymorphism (SNPs) in the target DNA sequence. The GFET requires 40 minutes of interaction time between the target and chip before a wash step and measurements.[354] A similar GFET used ultrathin Al_2O_3, which could detect *E. coli* in river water within 50 seconds with an LOD of 1 CFU/μL.[355]

1. Assessment

The electrochemical-sensor based assays analyzed in this paper have multiple advantages, including rapid sample-to-result times, low limits of detection, simple assay protocols, reduced equipment requirements, and reduced cost. As materials such as graphene become more accessible, assays manipulating the properties of these materials can be scaled for wider use The TRL for electrochemical-sensor based assays analyzed here range from 3–4, with estimated CLIA complexities of moderate to high.

V. Shear-Horizontal Surface Acoustic Wave (SH-SAW)

In SH-SAW assays, an acoustic wave travels along the surface between interdigital transducers, which are electrically excited by a radiofrequency signal. The substrate has an electric field that extends several micrometers into and interacts with the adjacent liquid on the chip, which

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352 Pai-Chien Chou et al., "Electrorheological Sensor Encapsulating Microsphere Media for Plague Diagnosis with Rapid Visualization," *ACS Sensors* 5, no. 3 (2020), https://doi.org/10.1021/acssensors.9b01529.

353 Xikai Cui et al., "Highly Specific and Rapid Glycan Based Amperometric Detection of Influenza Viruses," *Chemical Science* 8, no. 5 (2017), https://doi.org/10.1039/C6SC03720H.

354 Rui Campos et al., "Attomolar Label-Free Detection of DNA Hybridization with Electrolyte-Gated Graphene Field-Effect Transistors," *ACS Sensors* 4, no. 2 (2019), https://doi.org/10.1021/acssensors.8b00344.

355 Bhawana Thakur et al., "Rapid Detection of Single *E. coli* Bacteria Using a Graphene-Based Field-Effect Transistor Device," *Biosensors & Bioelectronics* 110 (2018), https://doi.org/10.1016/j.bios.2018.03.014.

affects the SH-SAW velocity. Measurements of this wave can characterize the presence of the target in the sample. A SH-SAW biosensor was developed as a digital detection device for HIV in clinical samples. The biochip has a sensing area with waterproof electrodes and the antibody detection channels are coated with anti-analyte antibodies. The biosensor is low cost ($1.50 per disposable biochip) and highly sensitive and specific (100% in five minutes in patient samples with viral loads greater than 5,000 copies/mL). Inkjet printing automates assay preparation and is part of efficient and cost-effective manufacturing. Unfunctionalized chips can be stored at room temperature, while functionalized chips can be stored at 4°C for one to two weeks. The chip can be connected to a laptop or smartphone for readout.[356]

Another lab created an aptamer-based SH-SAW sensor for endotoxin detection with an LOD of 3.53×10^{-9} g/mL. It uses a single layer, chemical vapor-deposited graphene film to immobilize the aptamer; the assay was verified with *E. coli*, *P. aeruginosa*, and aflatoxin.[357] An SH-SAW biosensor developed for *E. coli* detection in food demonstrated an LOD of 1.8×10^{-15} M and could distinguish between single-mismatched sequences.[358]

1. Assessment

SH-SAW biosensors are novel devices that are not currently used for clinical diagnoses. These biosensors could potentially act as point-of-care devices, due to their minimal storage requirements, potentially inexpensive cost ($1.50/chip in a pilot study), and readouts requiring minimal instrumentation and personnel training.[359] Currently, they would be at a TRL level of 4, having been tested on clinical samples. The CLIA complexity would be high.

W. Other Novel and Point-of-Care Assays

Multiple other novel assays have been developed, which may not fit in any of the technology categories listed above. These may include self-contained point-of-care assays, the use of different materials/reagents, or a novel type of assay.

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356 Eleanor R. Gray et al., "Ultra-Rapid, Sensitive and Specific Digital Diagnosis of HIV with a Dual-Channel SAW Biosensor in a Pilot Clinical Study," *npj Digital Medicine* 1, no. 1 (2018), https://doi.org/10.1038/s41746-018-0041-5.

357 Junwang Ji et al., "An Aptamer-Based Shear Horizontal Surface Acoustic Wave Biosensor with a CVD-Grown Single-Layered Graphene Film for High-Sensitivity Detection of a Label-Free Endotoxin," *Microsystems & Nanoengineering* 6, no. 1 (2020), https://doi.org/10.1038/s41378-019-0118-6.

358 S. T. Ten et al., "Highly Sensitive *Escherichia coli* Shear Horizontal Surface Acoustic Wave Biosensor with Silicon Dioxide Nanostructures," *Biosensors and Bioelectronics* 93 (2017), https://doi.org/10.1016/j.bios.2016.09.035.

359 Gray et al., "Ultra-Rapid, Sensitive and Specific Digital Diagnosis of HIV with a Dual-Channel SAW Biosensor in a Pilot Clinical Study."

A novel discovery and maturation strategy led to the development of a thermally stable peptide-based capture receptor platform.[360] This platform was based on an in situ "click" chemistry screen against a library of peptide macrocycles, with a novel screening strategy against folded protein structures to allow for accurate interactions with three-dimensional protein structures, which increases receptor performance. Multivalent cooperate constructs were produced with the guidance of in-silico experiments, demonstrated by detection of the E2 protein of the chikungunya virus, with a 200-fold improvement in affinity performance compared to mono-valent macrocycles. The top-matched construct was thermostable and did not experience significant affinity loss, even after being heated for one hour at 90°C. Peptide-based bioreceptors are more thermally stable and need fewer modifications than antibody methods, and they can be used in sandwich or label-free assays.

Magnetic nanomaterials can also be used for high-sensitivity pathogen detection. One system, which combines the nanomaterial's magnetic properties for enrichment and separation and the photothermal effects for detection and inactivation, was able to detect *S. typhimurium* in 1.5 hours with a limit of detection of 300 CFU/mL.[361] As an alternative to traditional cultures, a fully integrated bacterial detection system with immunomagnetic concentration allows for an assay to be performed in a few hours. This system has a sensitivity of 92.9%, specificity of 100%, and a detection limit of 10 CFU/10 mL. Although this device was designed for raw food analysis, the method may be useful in other domains.[362] Magnetic nanotrap particles have been used to improve the stability of Venezuelan Equine Encephalitis Virus (VEEV) in whole blood samples, potentially leading to longer sample storage at high temperatures; viral particles were still detectable after an incubation period of 72 hours at 40°C.[363]

A continuous-flow PCR reactor using a paper-based nucleic acid detection chip was studied for rapid and efficient detection of bacteria without laboratory equipment. This device had a detection limit of 10^4 CFU/mL for *L. monocytogenes*.[364] A paper-based device allows for nucleic

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360 Matthew B. Coppock et al., "A Novel Discovery, Maturation, and Assay Integration Approach for the Development of Ruggedized Multi-Valent Capture Receptors Exemplified Against the Chikungunya Virus E2 Protein," *Sensing and Bio-Sensing Research* 22 (2019), https://doi.org/10.1016/j.sbsr.2018.100248.

361 Zhen Zhang et al., "Rapid and Sensitive Detection of *Salmonella typhimurium* Based on the Photothermal Effect of Magnetic Nanomaterials," *Sensors and Actuators B: Chemical* 268 (2018), https://doi.org/10.1016/j.snb.2018.04.043.

362 Won-Il Lee et al., "A Fully Integrated Bacterial Pathogen Detection System Based on Count-on-a-Cartridge Platform for Rapid, Ultrasensitive, Highly Accurate and Culture-Free Assay," *Biosensors & Bioelectronics* 152 (2020), https://doi.org/10.1016/j.bios.2020.112007.

363 Ivan Akhrymuk et al., "Magnetic Nanotrap Particles Preserve the Stability of Venezuelan Equine Encephalitis Virus in Blood for Laboratory Detection," *Frontiers in Veterinary Science* 6 (2019), https://doi.org/10.3389/fvets.2019.00509.

364 Yu Fu, Xiaoming Zhou, and Da Xing, "Integrated Paper-Based Detection Chip with Nucleic Acid Extraction and Amplification for Automatic and Sensitive Pathogen Detection," *Sensors and Actuators B: Chemical* 261 (2018), https://doi.org/10.1016/j.snb.2018.01.165.

acid extraction, amplification, and detection to be performed in a single device. The device is comprised of an ultrathin heater with a positive temperature coefficient, on-chip dried enzyme storage, an integrated battery, and a sponge-based reservoir with a paper-based valve for nucleic acid extraction. For *S. typhimurium*, the LOD was 10^2–10^3 CFU/mL, depending on the sample medium (spiked wastewater, milk, juice, and egg).[365] While the device was tested on non-clinical samples, such as milk, juice, eggs, and wastewater, the authors have stated that work is ongoing to focus on nucleic acid detection in different biological samples.

Fluorescence imaging can aid in rapid diagnosis of infections, especially in wounds. The MolecuLight i:X imaging device uses the endogenous autofluorescence of pathogens for detection, thereby avoiding the need for contrast reagents or prolonged patient contact. This device has a PPV of 92.9% for *P. aeruginosa*.[366] Some bacteria, such as *S. aureus*, emit a red-colored fluorescence signal due to porphyrin production, while other bacteria, such as *P. aeruginosa*, emit a cyan signal due to pyoverdine production.[367] In a study of hand wound infections, this device correlated with clinical signs and swab results in 97.1% of cases.[368] In an outpatient plastic surgery wound care clinic, the device had a sensitivity of 100% and specificity of 78%.[369] Another research group developed a prototypic set of goggles to be used in conjunction with a fluorescent-linked immunosorbent assay (FLISA) for portable fluorescence imaging. The goggles could detect the AF647 fluorescent reporter at a resolution of 99 μm, and the assay could reliably detect MERS-CoV spike protein at a concentration of 25×10^{-9} g/mL.[370]

MinION, a portable sequencing device by Oxford Nanotechnologies, was tested in a deployable field laboratory during a North Atlantic Treaty Organization (NATO) live agent exercise in a hot climate. MinION can be controlled by a laptop and could be used for biomedical reconnaissance. This device can identify and differentiate upper respiratory disease organisms with 87%–98% alignment against reference genome databases, an improvement over existing

365 Ruihua Tang et al., "A Fully Disposable and Integrated Paper-Based Device for Nucleic Acid Extraction, Amplification and Detection," *Lab on a Chip* 17, no. 7 (2017), https://doi.org/10.1039/c6lc01586g.

366 Rose Raizman, William Little, and Allie C. Smith, "Rapid Diagnosis of *Pseudomonas aeruginosa* in Wounds with Point-of-Care Fluorescence Imaging," *Diagnostics (Basel, Switzerland)* 11, no. 2 (2021), https://doi.org/10.3390/diagnostics11020280.

367 Ciaran M. Hurley et al., "Efficacy of a Bacterial Fluorescence Imaging Device in an Outpatient Wound Care Clinic: A Pilot Study," *Journal of Wound Care* 28, no. 7 (2019), https://doi.org/10.12968/jowc.2019.28.7.438.

368 Bryan J. W. Chew et al., "The Use of MolecuLight I:X Device in Acute Hand Trauma," *Journal of Plastic, Reconstructive & Aesthetic Surgery* 73, no. 7 (2020), https://doi.org/10.1016/j.bjps.2020.03.004.

369 Hurley et al., "Efficacy of a Bacterial Fluorescence Imaging Device in an Outpatient Wound Care Clinic: A Pilot Study."

370 Manuel Y. Caballero, Tho Hua, and Yang Liu, *Development of a Highly Sensitive Goggle for Fluorescence-Based Detection of Middle East Respiratory Syndrome Coronavirus (MERS-CoV) And Other Pathogens*, (San Antonio, TX: U.S. Air Force 59th Medical Wing, August 2020), https://apps.dtic.mil/sti/citations/AD1128433.

technologies; the 100-base error rate averaged 1.2^{-1}. With the development of a cell phone interface (SmidgION), future technologies can enable rapid RNA virus identification.[371]

A device called the Antibody-free Dual-Biomarker Rapid Enrichment Workflow (ANDREW) was developed to improve the sensitivity of malaria rapid diagnostic tests, but may be ported to other workflows. Most rapid tests have a small input volume, which limits the delivered biomarker amount. ANDREW purifies the target by capturing the biomarker with an aptamer-conjugated magnetic bead before elution. ANDREW was tested on PLDH and HRP2 and demonstrated a potential 9- to 11-fold increase in detection sensitivity.[372]

A method of nucleic acid extraction uses dipsticks and multiple solutions, which can improve the efficiency of detection in low-resource settings; the dipsticks may also be produced using an easy and low-resource method. This method takes advantage of the release kinetics of cellulose matrices, which can rapidly bind nucleic acids from complex biological samples, retain them during a washing step, and then release them into the DNA amplification reaction. The nucleic acid capture, purification, and release can be completed in less than 30 seconds by sequentially dipping a dipstick into the sample, wash, and DNA amplification solutions. This method has been tested in conjunction with PCR, LAMP, and RPA.[373]

A portable plasmonic diagnostic device based on nanohole arrays can detect ultrathin protein layers and was tested on H1N1 influenza samples. After the target analytes attach onto the plasmonic substrate, the nanohole arrays undergo a spectral shift within the extraordinary light transmission (EOT) response. A complementary metal oxide semiconductor (CMOS) camera records the nanohole arrays' diffraction field intensities under a light emitting diode (LED) light tuned to the plasmonic mode of interest.[374]

The U.S. Army Medical Research and Development Command (USAMRDC) is currently developing a tuberculosis (TB) triage test that uses a finger-stick blood assay to distinguish active TB from prior infection. The cartridge-based test being developed aims at reducing the required 9-gene signature; an mRNA signature has been identified. The platform is intended to be compatible with liquid PCR reagents or lyophilized reagent beads. The study aims for greater than

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371 Wanda J. Lyon et al., "Evaluating an Upper Respiratory Disease Panel on the Portable MinION Sequencer," *bioRxiv*, 2018, https://doi.org/10.1101/436600.

372 Andrew G. Kantor et al., "An Antibody-Free Dual-Biomarker Rapid Enrichment Workflow (AnDREW) Improves the Sensitivity of Malaria Rapid Diagnostic Tests," *Analytical Biochemistry* 612 (2021), https://doi.org/10.1016/j.ab.2020.114020.

373 Michael G. Mason and José R. Botella, "Rapid (30-Second), Equipment-Free Purification of Nucleic Acids Using Easy-to-Make Dipsticks," *Nature Protocols* 15, no. 11 (2020), https://doi.org/10.1038/s41596-020-0392-7.

374 Arif E. Cetin et al., "Handheld Plasmonic Biosensor for Virus Detection in Field-Settings," *Sensors and Actuators B: Chemical* 344 (2021), https://doi.org/10.1016/j.snb.2021.130301.

90% sensitivity and 70% specificity against reference standards.[375] Another assay developed for the U.S. Army Medical Research and Materiel Command is the D4 (Dispense, Dissolve, Diffuse, Detect) assay. Developed to be a point-of-care platform, the D4 assay uses one drop of the target solution and does not require external reagents, power, or other equipment except a smartphone for detection. This system uses a coated glass chip imprinted with microspots that contain capture antibodies and fluorophore-labeled detection antibodies in concentric strings. The methodology has four steps: blood dispensation, detection antibody dissolution, detection antibody diffusion, and capture antibody binding. This creates an optic signal that can be quantified with a handheld detector. This method is inexpensive, stable at room temperature for months, and can be read with a smartphone. This platform demonstrated an LOD of less than 1×10^{-9} g/mL for Ebolavirus antibodies.[376]

1. Assessment

The novel method for development of multi-valent capture receptors acts as a proof-of-concept for the development of receptors for existing assays. The current personnel requirements to replicate this methodology would be very high, though the development of a streamlined process could allow for rapid production of assay antibody replacements for better performance. Having been validated in an artificial solution, the TRL of this technique would be 3, with a high CLIA complexity.

The use of magnetic nanomaterials can allow for reduced runtimes for existing assays, with the potential to also enhance performance of the assay, as demonstrated in the assay developed by Zhang et al.[377] The assays analyzed would have a TRL of 4 and a moderate CLIA complexity.

The paper-based nucleic acid detection methods discussed in this paper have the advantage of reducing infrastructure requirements and cost. The assays were confined to a single device, offering the advantage of portability; similar assays may be useful for far-forward deployment. These assays would be at TRLs ranging from 3–4, with a moderate CLIA complexity.

Advances in wound imaging, such as the commercially available MolecuLight imaging device, would aid in diagnosis without requiring contrast reagents and lengthy diagnostic assays. While this technique may not provide a definitive diagnosis, it could be helpful in suggesting a treatment plan in far-forward applications. The devices described here would have a moderate

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375 Antonino Catanzaro, Timothy Rodwell, Naomi Hillery, and Laura Myhovich, *A Rapid Blood Test to Differentiate Latent Tuberculosis from Active Disease* (La Jolla, CA: University of California, San Diego, October 2020).

376 Ashutosh Chilkoti and Michael Gunn, *Smartphone Enabled Point-of-Care Diagnostics for Operationally Significant Pathogens* (Durham, NC: Duke University, October 2017), https://apps.dtic.mil/sti/citations/AD1045963.

377 Zhang et al., "Rapid and Sensitive Detection of *Salmonella typhimurium* Based on the Photothermal Effect of Magnetic Nanomaterials."

CLIA complexity and, as the Moleculight device is commercially available, would have a TRL of 4–7+.

The MinION sequencer has already been tested in a NATO live agent exercise, and has been shown to allow for rapid and accurate diagnoses in a far-forward environments. This currently available technology has a TRL of 7+ and has a moderate CLIA complexity, as it is similar to other similar sequencing platforms with the advantage of portability.

Assays to increase sensitivity of existing methods, such as the ANDREW device, can act to improve existing assays and platforms, while assays such as the portable plasmonic diagnostic device developed by Cetin et al. represent novel platforms that may be viable replacement options with further development.[378] Both assays offer similar advantages of increased sensitivity, low-resource requirements, short runtimes, and portability. These assays would have a TRL of 4, with moderate CLIA complexity.

Other point-of-care tests, such as the those developed with USAMRDC, have been developed with the explicit goal of use in a far-forward facility. The identified assays are portable and require minimal external preparation, and are designed to be used without extensive personnel training. They are still being developed, but currently are a TRL of 4 with moderate, potentially waived, CLIA complexity.

A summary of various point-of-care technologies is listed in Table 39 and Table 40.

[378] Kantor et al., "An Antibody-Free Dual-Biomarker Rapid Enrichment Workflow (AnDREW) Improves the Sensitivity of Malaria Rapid Diagnostic Tests"; Cetin et al., "Handheld Plasmonic Biosensor for Virus Detection in Field-Settings."

Table 39. Assay Requirements for Point-of-Care Diagnostics

Assay or Technique	Reagents	Instrumentation	Portability	Infrastructure	Personnel
Microfluidics	Commercially available enzyme/reagent kits	Fluorescence analyzer Smartphone Peristaltic pump	Maybe	Power source (for some)	Requires some training (may be reduced in future versions)
LFA	AuNPs AMPs	Smartphone Fluorescence analyzer	Maybe	No special equipment	Requires some training (may be reduced in future versions)
Electrochemical Sensors	Metallic NPs and/or nanowires	Autolab potentiostat Semiconductor analyzer	Maybe	Some special equipment Refrigerator may be required for reagent storage	Requires some training
SH-SAW	--	Laptop or smartphone	Maybe	No special equipment	Requires some training (may be reduced in future versions)
In situ Click Chemistry Screen	Labels		No	Refrigerator and/or freezer may be required for reagent storage	Requires training
MinION	--	Smartphone	Yes	--	Requires training
Nucleic Acid Extraction Dipsticks	Various solutions	--	Yes		Little to no training required
D4	None	Smartphone	Yes	None	Little to no training required

Table 40. Assay Details for Point-of-Care Diagnostics

Assay or Technique	Performance	Commercialization	Primary Benefit	Notes
Microfluidics	LOD: 10×10^{-9} g/mL; 10^2 copies/reaction; 0.021–1 PFU/mL Time: <1–2.5 hours	Some commercial options	Simpler process and small size Faster time to result Increased sensitivity	Potential for multiplexing and point-of-care diagnosis May be useful for Role 1
LFA	LOD: 0.045–0.15 × 10^{-9} g/mL; 50×10^{-12} g/mL; 10^4 CFU/mL Time: 5–30 minutes	Commercial options exist	Simpler process Faster time to result Increased sensitivity	Potential for multiplexing
Electrochemical Sensors	LOD: 3.8– 10^7 CFU/mL; 1.6–90 × 10^{-15} g/mL; 0.1×10^{-9} g/mL; 1.9×10^{-15} M concentrations Time: <15–40 minutes	Proof-of-concept	Low cost Faster time to result Increased sensitivity	Generally low equipment requirements
SH-SAW	LOD: 3.53–25.5 × 10^{-9} g/mL; 1.8×10^{-15} M concentrations Time: <5 minutes	Proof-of-concept	Low cost Simpler process Faster time to result	$1.50/disposable all-in-one biochip Could be useful at Role 1 or Role 2
In situ Click Chemistry Screen	Time: hours	Proof-of-concept	Increased sensitivity Multiplex/screening	Can screen for multiple epitopes on a protein
MinION	87–98% alignment with reference database	Some commercial options	Portability Faster time to result	Used in NATO live exercise in deployable field lab
Nucleic Acid Extraction Dipsticks	Time: 30 seconds	Proof-of-concept	Low cost Faster time to result	Cost is <$0.02/dipstick May be useful in low-resource settings Can be combined with other techniques
D4	LOD: $<1 \times 10^{-9}$ g/mL	Proof-of-concept	Increased sensitivity Portability Simpler process Low cost	

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4. Technology Next Steps and Recommendations

A variety of technological and process advancements have led to improvements and new options for diagnostics. As a result of the literature review, the IDA team identified 270 peer-reviewed studies from 2019 to 2021 that described new diagnostic techniques, 230 of which described entire assays; 10 articles described improvements to sample preparation steps, 6 described improvements to reagent preparation steps, 40 described new materials for use in existing assays, 57 optimized current assay steps, 19 identified new assay targets, and 32 described miniaturization or increased portability of existing assays. However, many of these advancements are currently in the early stages of the development process and additional research is required to make them viable for military or civilian clinical use. Table 41 summarizes the TRL and CLIA complexity IDA assessed for each technology type, as detailed in Chapter 3.

Table 41. IDA-Assessed TRL and CLIA Complexity for Each Technology

Technology	Assessed TRL	Assessed CLIA Complexity
Plasmonic PCR	4	Moderate
Digital PCR	4	Moderate
Gold Nanoparticles in PCR	4	Moderate
MicroRNA Targets (miRNA)	4	High
Bypassing Nucleotide Extraction	3-4	Moderate to High
Droplet Digital ELISA/SiMoA	4-7+	Moderate to High
8pG-based Microplate	4	Moderate
Graphene Nanoparticle-based ELISA	3	High
Atom Transfer Radical Polymer Gold Nanoparticles (ATRP-AuNP) in ELISA	4	Moderate
Paper-based ELISA	4	Waived to Moderate
Hybridization Chain Reaction (HCR)	4	High
Surface Plasmon Resonance (SPR)	4	Moderate to High
Fluorescent in Situ Hybridization (FiSH)	3	Moderate
CRISPR	3-7+	Moderate to High
Recombinase Polymerase Amplification (RPA)	3-4	Moderate to High
MALDI-TOF Mass Spectrometry	3-7+	Moderate to High
Surface Enhanced Raman Scattering (SERS)	3-4	Moderate

Technology	Assessed TRL	Assessed CLIA Complexity
Loop Mediated Isothermal Amplification (LAMP)	3-4	High
Polymerase Spiral Reaction (PSR)	4	Moderate
Plasma Cell-Free DNA Metagenomic Next-Generation Sequencing (mNGS)	7+	High
Quantum Dots	4	Moderate to High
Aptamer-Based Systems	3-4	Waived to High
Lyophilized Reagents	4-7+	Moderate to High
Lyophilized Sera	3	High
Membrane Sample Concentration	3	Moderate
Whole Cell-based Biosensors	4	High
Resonance Energy Transfer	4	Moderate to High
Microfluidics	3-4	Moderate to High
Lateral Flow Assays	4	Moderate
Electrochemical Sensors	3-4	Moderate to High
Shear-Horizontal Surface Acoustic Wave (SH-SAW)	4	High

Note: see Table 1 for a description of each TRL.

An ideal diagnostic assay for use in far-forward environments would have many characteristics: high diagnostic performance, low reagent requirements, low instrumentation requirements, portability, ruggedization, minimal infrastructure requirements, and low personnel training requirements. To be field deployable in far-forward settings, such as a Role 1 MTF, diagnostic technologies would likely need to reach at least a TRL 6 or 7; at this level of maturation, product manufacture would be underway with regulatory approval packages submitted for consideration. As shown in Table 41, most of the novel diagnostic assays and techniques are not mature enough to be used in far-forward clinical settings; furthermore, many have not yet been clinically validated. However, most of the technologies are currently rated with a moderate CLIA complexity, which is an indicator that the diagnostics may currently be (or have the future potential to be) simple and integrated enough to be used by non-specialized personnel in austere or far-forward environments.

A. Observations and Recommendations

The purpose of this analysis is not to provide recommendations on specific diagnostic technologies to be used by or invested in by OTSG, but rather to provide OTSG with situational awareness on the current state of the diagnostics field so decision-makers can determine what may be most useful in a given situation. The IDA team did not identify or prioritize any technologies from this research regarding which technologies should receive immediate governmental partnership; additional operational guidance is needed to identify specific diagnostic goals and

ensure that the most appropriate technology is selected to support that goal. Once specific operational goals are known, this document could be useful in identifying technologies that could have the greatest impact on achieving those particular goals. Diagnostic technology is an ever-changing field, and this analysis aims to provide a basis of knowledge to be updated in the future as technology continues to advance. A high-level summary of the literature review findings can be found in Table 43.

The variety and constant advancements in diagnostic technology provides many options and opportunities for far-forward use. There are technologies and techniques that minimize equipment and reagent requirements (e.g., p-ELISA, microfluidics, SH-SAW), making it easier to deploy in austere environments for use by non-specialized personnel. There are also options that provide rapid and sensitive results (e.g., droplet digital ELISA/SiMoA, PLOPs, RPA), which may be useful for earlier detection. If far-forward detection at Role 1 is not feasible, techniques such as lyophilization may make it more feasible to quickly transport samples taken at a Role 1 to a higher role of care for more robust testing. Depending on a desired priority (such as low cost, increased sensitivity, or equipment requirements), there are likely a number of diagnostics in development or production that can address that priority. Table 42 bins the diagnostics based on potential use cases.

Table 42. Potential Uses for Diagnostic Technologies

Potential Use	Desired Characteristics	Diagnostics	Diagnostics with TRL≥4 and CLIA Complexity ≤ Moderate
Early detection/ quarantine decisions	Faster time to result Increased sensitivity	Plasmonic PCR AuNPs in PCR Sample pre-treatment Droplet digital ELISA/SiMoA p-ELISA Multi-branched HCR Biosensors ESDR PLOPs CRISPR-Cas13 CRISPR-Cas12 RPA RPA-LFA RT-RPA PSI-MS SERS SERS-LFA SERS microfluidics SERS immunoassays SERS-PCR Label-free SERS LAMP	p-ELISA Plasmonic PCR AuNPs in PCR ESDR CRISPR-Cas13 CRISPR-Cas12 RPA RPA-LFA RT-RPA SERS SERS-LFA SERS microfluidics SERS immunoassays SERS-PCR Label-free SERS RT-PSR PSR-LFA QDs Colorimetric aptasensors Electrochemical aptasensors Fluorescence aptasensors Lyophilized reagents

Potential Use	Desired Characteristics	Diagnostics	Diagnostics with TRL≥4 and CLIA Complexity ≤ Moderate
		RT-LAMP	BRET
		RT-PSR	FRET
		PSR-LFA	LFA
		QDs	Droplet digital ELISA/SiMoA
		Colorimetric aptasensors	MinION
		Electrochemical aptasensors	
		Fluorescence aptasensors	
		Lyophilized reagents	
		Lyophilized sera	
		BRET	
		FRET	
		Microfluidics	
		LFA	
		Electrochemical sensors	
		SH-SAW	
		Nucleic acid extraction dipsticks	
		MinION	
Treatment decisions	Increased sensitivity	MicroRNA targets in PCR	CRISPR-Cas9
		CRISPR-Cas9	MALDI-TOF
	Pathogen distinction (bacteria vs. virus)	MALDI-TOF	
		Plasma Cell-Free DNA mNGS (Karius test)	
Throughput capacity or multiplexing	Capability for multiplexing	Digital PCR	Digital PCR
		PEARL	Fiber optics SPR
	Increased sensitivity	ELISA-HCR	RPA-LFA
		Fiber optics SPR	RT-PSR
		RPA-LFA	LFA
		RT-PSR	
		LFA	
		In situ click chemistry screen	
Confirmation	Increased sensitivity	8pG-based microplate	8pG-based microplate
		Graphene NP-based ELISA	ATRP-AuNPs
		ATRP-AuNPs	PNA-FiSH
		Fixation-free FiSH	RPA-SERS
		PNA-FiSH	RPA-CRISPR
		RPA-SERS	RPA-IMS
		RPA-CRISPR	PSR
		RPA-IMS	QD-PCR
		LAMP	QD-FRET
		LAMP-HCR	QLISA
		PSR	D4
		QD-PCR	
		QD-FRET	
		QLISA	
		Membrane sample concentration	

Potential Use	Desired Characteristics	Diagnostics	Diagnostics with TRL≥4 and CLIA Complexity ≤ Moderate
		WCBBs D4	

OTSG may find it useful to identify diagnostic technologies or procedures they wish to use in the future. If that technology is not mature enough for use in the near future, it may be beneficial to encourage DOD product developers, such as the Joint Program Executive Office for Chemical, Biological, Radiological and Nuclear Defense (JPEO-CBRND), to partner with the commercial owner of the technology. The earlier in the development cycle a partnership can be developed, the easier the selected technology can be customized for a specific use with the lowest cost. This may provide a more efficient and streamlined approach to acquiring new diagnostic technology for the military: rather than contracting a lab or company to develop a new, specific diagnostic from scratch, OTSG can use this information and any new information developed to identify technology and work with the DOD developers to advance an already-promising technology. Additional research would still likely be required, and such research may include conducting required validation studies to determine whether the technology can be applied to the pathogens of interest to OTSG. If a diagnostic will be used to screen for multiple pathogens, additional testing will need to be done to ensure that the assay is effective for the desired pathogens or targets. Similarly, the clinical sample used by the diagnostic must be considered; a diagnostic will only be effective if it is performed on the correct clinical sample (i.e., blood, urine, sputum) at the correct time in the disease progression.[379]

We recommend OTSG use the information in this paper to:

1. Identify diagnostic technologies that will enable the placement of diagnostic assays at appropriate locations or with the most appropriate unit types to fully capitalize on the intended use of the assay (surveillance, early diagnosis to facilitate early intervention, general situational awareness, etc.).
2. Engage early and often with DOD research program managers, product developers, and Integrated Concept Team (ICT) members to highlight advances in diagnostic technology that support OTSG's intended application of the diagnostic.
3. Work closely with U.S. Army Nuclear and Countering Weapons of Mass Destruction Agency (USANCA) to ensure synchronization between the clinical needs of OTSG and overall strategic goals, policy, and direction of the Army Biological Defense Strategy Implementation.

[379] Kristen A. Bishop, Robert L. Cubeta, Jon M. Davis, and Lucas A. LaViolet, *(U) Evaluation of Biological Agent Clinical Sampling and Analysis*, IDA Paper P-21576 (Alexandria, VA: Institute for Defense Analyses, May 2021). CONTROLLED UNCLASSIFIED INFORMATION. Only UNCLASSIFIED information is included in this paper.

4. Maintain ongoing situational awareness of trends in diagnostic technology. This is a fast-moving field and advances can occur rapidly, creating new opportunities that may not have been obvious before.

Table 43. Summary of Diagnostic Technologies' Characteristics

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Improvements to Existing Assays[c]					
Aptamer-Based Systems	Comparable or better	Specific target(s)	<2 hours	3-4	Waived to High
Resonance Energy Transfer	Comparable or better	Specific target(s)	Minutes to hours	4	Moderate to High
Lyophilized Reagents	Comparable or better	n/a	A few hours	4-7+	Moderate to High
Lyophilized Sera	Better	n/a		3	High
Atom Transfer Radical Polymer Gold Nanoparticles (ATRP-AuNP) in ELISA	Comparable or better	Specific target		4	Moderate
Gold Nanoparticles in PCR	Better	Specific target	A few hours	4	Moderate
Loop Mediated Isothermal Amplification (LAMP)	Comparable or better	Specific target	<1 hour	3-4	High
Plasmonic PCR	Comparable	Specific target	<1 hour	4	Moderate
8pG-based Microplate	Better	Specific target		4	Moderate
Graphene Nanoparticle-based ELISA	Better	Specific target	A few hours	3	High
Paper-based ELISA	Comparable or better	Specific target	A few hours	4	Waived to Moderate
Surface Plasmon Resonance (SPR)	Comparable or better	Specific target	<1–2 hours	4	Moderate to High

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Fluorescent in Situ Hybridization (FiSH)	Comparable or better	Specific target	Minutes to hours	3	Moderate
CRISPR[e]	Better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-7+	Moderate to High
Recombinase Polymerase Amplification (RPA)	Comparable or better	Specific target(s) Potential screening via multiplexing	<30 minutes	3-4	Moderate to High
Surface Enhanced Raman Scattering (SERS)[e]	Better	Specific target	<1–2 hours	3-4	Moderate
Quantum Dots[e]	Better	Specific target(s) Potential screening via multiplexing	<1–2 hours	4	Moderate to High
Whole Cell-based Biosensors	Better	Specific target	<24 hours	4	High
Electrochemical Sensors	Comparable or better	Specific target	<1 hour	3-4	Moderate to High
New Assays or Procedures[d]					
Bypassing RNA Extraction in PCR	Comparable or better	n/a	<1 hour	3-4	Moderate to High
Bypassing Nucleotide Extraction	Comparable or better	n/a	<1 hour	3-4	Moderate to High

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Plasma Cell-Free DNA Metagenomic Next-Generation Sequencing (mNGS)	Comparable	Screening		7+	High
Membrane Sample Concentration	Comparable or better	n/a	<20 minutes	3	Moderate
Digital PCR	Better	Specific target		4	Moderate
Droplet Digital ELISA/SiMoA	Better	Specific target	A few hours	4-7+	Moderate to High
MicroRNA Targets (miRNA)	Comparable or better			4	High
Hybridization Chain Reaction (HCR)	Comparable or better	Specific target(s)	A few hours	4	High
CRISPR[e]	Better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-7+	Moderate to High
MALDI-TOF Mass Spectrometry	Comparable	Screening	<1 hour	3-7+	Moderate to High
Surface Enhanced Raman Scattering (SERS)[e]	Better	Specific target	<1–2 hours	3-4	Moderate
Polymerase Spiral Reaction (PSR)	Better	Specific target	A few hours	4	Moderate
Quantum Dots[e]	Better	Specific target(s) Potential screening via multiplexing	<1–2 hours	4	Moderate to High

Technology	Sensitivity Compared to Current Technology[a]	Pathogen Screening or Specific Identification[b]	Time to Result	Assessed TRL	Assessed CLIA Complexity
Microfluidics	Comparable or better	Specific target(s) Potential screening via multiplexing	Minutes to hours	3-4	Moderate to High
Lateral Flow Assays	Comparable or better	Specific target(s) Screening via multiplexing	<1 hour	4	Moderate
Shear-Horizontal Surface Acoustic Wave (SH-SAW)	Better	Specific target	Minutes	4	High

[a] The "current technology" is the technology that is currently considered the preferred method for that technique or purpose. In this case, the "current technology" is often PCR or the baseline technology for variations listed here (e.g., ELISA or mass spectroscopy).

[b] This column ("Pathogen Screening or Specific Identification") indicates whether a diagnostic technology would be useful to identifying a pathogen, without prior suspicion, from a broad spectrum of possibilities ("Pathogen Screening") or whether the diagnostic is only useful if there is some prior suspicion or knowledge of the specific pathogen for which to test ("Specific Identification").

[c] "Improvements to Existing Assays" includes: assay automation, combination of tools, change in materials or use of a new material in an existing assay, change in reagents or use of a new reagent in an existing assay, assay miniaturization, technique improvement, reference library preparation, and improvements to sample preparation.

[d] "New Assays or Procedures" includes certain combinations of tools (e.g., combinations that create a new assay or use new materials or tools), technique commercialization, combination of techniques, new techniques, and use of a new or different target.

[e] Note: CRISPR, SERS, and quantum dots fall under both categories, as there have been both improvements to existing assays, procedures, and materials in addition to new techniques and assays. However, the majority of the studies fall under the "Improvements to Existing Assays" category.

Appendix A. References

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Appendix C. Abbreviations

AIE	aggregation-induced emission
AIEgens	AIE luminogens
AIOD-CRISPR	All-In-One Dual CRISPR
AMP	antimicrobial peptides
ANDREW	Antibody-free Dual-Biomarker Rapid Enrichment Workflow
AP	apurinic/apyrimidinic
ASFV	African Swine Fever Virus
ATP	adenosine triphosphate
ATRP	atom transfer radical polymer
Au	gold
AuNP	gold nanoparticles
BRET	bioluminescence resonance energy transfer
CARMEN	combinatorial arrayed reactions for multiplexed evaluation of nucleic acids
CARVER	Cas13-assisted Restriction of Viral Expression and Readout
CAS-EXPAR	CRISPR/Cas9-triggered isothermal exponential amplification reaction
CASLFA	CRISPR/Cas9-mediated lateral flow nucleic acid assay
CdS	cadmium sulfide
CFU	colony forming units
CHDC	catalytic hairpin DNA circuit
CLIA	Clinical Laboratory Improvement Amendments
CLIP	Clinical Laboratory Improvement Program
cm	centimeter
CMOS	complementary metal oxide semiconductor
COMET	Cas-CHDC-powered electrochemical RNA-sensing technology
CONAN	CRISPR-Cas-only amplification network
COVID-19	coronavirus disease 2019
cPCR	convective polymerase chain reaction
CREST	Cas13-based rugged, equitable, scalable testing

CRISDA	CRISPR-Cas9-triggered nicking endonuclease-mediated Strand Displacement Amplification
CRISPR	clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
CTAB	cetyltrimethylammonium bromide
DDT	DNA tetrahedron
DENV	Dengue virus
DETECTR	DNA Endonuclease Targeted CRISPR Trans Reporter
DFNS	dendritic fibrous nanosilica
DNA	deoxyribonucleic acid
DOD	Department of Defense
dsDNA	double-stranded DNA
DTIC	Defense Technical Information Center
DTT	dithiothreitol
ELISA	enzyme-linked immunosorbent assay
EOT	extraordinary light transmission
ESDR	entropy-driven strand displacement reaction
FAM-cDNA	carboxyfluorescein-labeled complimentary DNA
FDA	Food and Drug Administration
fDNA	functional DNA
Fe_3O_4@Au NP	iron (II,III) oxide gold nanoparticles
FiSH	fluorescent in situ hybridization
FLASH	Finding Low Abundance Sequences by Hybridization
FLISA	fluorescent-linked immunosorbent assay
FRET	fluorescence resonance energy transfer
FRET	Forster resonance energy transfer
GFET	graphene field-effect transistor
H_2O_2	hydrogen peroxide
HA	hemagglutinin
HBV	hepatitis B virus
HCR	hybridization chain reaction
HCV	hepatitis C virus
HOLMES	one-hour low-cost multipurpose highly efficient system
HPV	human papillomavirus
HUDSON	Heating Unextracted Diagnostic Samples to Obliviate Nucleases
ICT	Integrated Concept Team

ICTS	immunochromatographic test strip
IDA	Institute for Defense Analyses
IgG	immunoglobulin-G
IMS	immunomagnetic separation
ITP	isotachophoresis
JPEO-CBRND	Joint Program Office for Chemical, Biological, Radiological, and Nuclear Defense
kDa	kilodalton
kg	kilogram
LAMP	loop mediated isothermal amplification
LED	light emitting diode
LFA	lateral flow assay
LFD	lateral flow dipstick
LFIA	lateral flow immunoassay
L-LAMP	lyophilized loop mediated isothermal amplification
LOD	limit of detection
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight
MAP	mobile analytical platform
MB-Qdot	magnetic bead-quantum dots
MERS	Middle East respiratory syndrome
MGIT	mycobacteria growth indicator tube
miRNA	micro RNA
mL	milliliter
mNGS	metagenomic next-generation sequencing
MNP	magnetic nanoparticles
MoS_2	molybdenum sulfide
MRSA	methicillin-resistant *S. aureus*
MS	mass spectrometry
MSSA	methicillin-susceptible Staphylococcus aureus
NAA	nanoporous anodic alumina
NASBA	nucleic acid sequence-based amplification
NATO	North Atlantic Treaty Organization
NCAI	NIH Centers for Accelerated Innovations
NEase	nicking endonuclease
NGS	next-generation sequencing
NIH	National Institutes of Health

NP	nanoparticles
NPV	negative predictive value
OSD	one strand displacement
OTSG	Office of the Surgeon General
PAM	protospacer adjacent motif
PCR	polymerase chain reaction
PDMS	poly(dimethylsiloxane)
PEARL	precipitation-enhanced analyte retrieval
PEG	polyethylene glycol
p-ELISA	paper-based ELISA
PFU	plaque forming unit
PHMB	polyhexamethylene biguanide
PLOP	pre-labeled oligomer probe
PMF	peptide mass fingerprint
PMMA	poly(methyl methacrylate)
PNA	peptide nucleic acid
PPV	positive predictive value
PSA	prostate-specific antigen
PSI	paper spray ionization
PSR	polymerase spiral reaction
PXA	poly-xanthurenic acid
QD	quantum dots
QLISA	Quantum Linked Immunosorbent Assay
qPCR	quantitative PCR
RADAR	Random Molecular Aptamer-Dependent CRISPR-Assisted Reporter
RBC	red blood cell
RCA	rolling circle amplification
RNA	ribonucleic acid
RPA	recombinase polymerase amplification
rRT-PCR	real-time reverse-transcriptase PCR
RSV	respiratory syncytial virus
RT-ERA	reverse transcription enzymatic recombinase amplification
RT-PCR	reverse transcription polymerase chain reaction
RT-RAA	reverse transcription recombinase-aided amplification
RT-RPA	reverse transcription recombinase polymerase amplification
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2

SELEX	Systematic Evolution of Ligands by Exponential Enrichment
SERS	surface enhanced Raman scattering
SHERLOCK	Specific High-Sensitivity Enzymatic Reporter UnLOCKing
SHINE	Streamlined Highlighting of Infections to Navigate Epidemics
SH-SAW	Shear-Horizontal Surface Acoustic Wave
SiMoA	single molecule assay
SNP	single nucleotide polymorphism
SPM	sample preparation multiplexer
SPR	surface plasmon resonance
SSB	single-stranded DNA binding
ssDNA	single-stranded DNA
TB	tuberculosis
TMB	3,3′-5,5′-Tetramethyl benzidine
tracrRNA	transactivating CRISPR RNA
TRL	Technology Readiness Level
TSH	thyroid-stimulating hormone
µL	microliter
UNCP-LFA	upconversion nanoparticle-based lateral flow assay
USAMRDC	U.S. Army Medical Research and Development Command
USANCA	U.S. Army Nuclear and Countering Weapons of Mass Destruction Agency
VEEV	Venezuelan equine encephalitis virus
VEGF	vascular endothelial growth factor
WCBB	whole cell-based biosensors
ZIKV	Zika virus

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REPORT DOCUMENTATION PAGE	Form Approved OMB No. 0704-0188

1. REPORT DATE (DD-MM-YY)	2. REPORT TYPE	3. DATES COVERED (From – To)
XX-07-2022	Final	

4. TITLE AND SUBTITLE

Analysis of State-of-the-Art Diagnostics for Far-Forward Use

5a. CONTRACT NO.

HQ0034-14-D-0001

5b. GRANT NO.

5c. PROGRAM ELEMENT NO(S).

6. AUTHOR(S)

Jon M. Davis
Kristen A. Bishop
Catherine E. Scheible
Jay S. Shah

5d. PROJECT NO.

5e. TASK NO.

FN-06-4947

5f. WORK UNIT NO.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Institute for Defense Analyses
4850 Mark Center Drive
Alexandria, VA 22311-1882

8. PERFORMING ORGANIZATION REPORT NO.

IDA Paper P-33049
Log: H 22-000143

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

OTSG
7700 Arlington Blvd,
Falls Church, VA 22042

10. SPONSOR'S / MONITOR'S ACRONYM(S)

OTSG

11. SPONSOR'S / MONITOR'S REPORT NO(S).

12. DISTRIBUTION / AVAILABILITY STATEMENT

Distribution State A. Approved for public release; distribution unlimited.

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The Office of the Army Surgeon General (OTSG) maintains awareness of technologies important for protecting U.S. Army personnel from infectious diseases and continually seeks ways to improve existing technologies, especially those that support diagnostic assays. OTSG determined that there was a need to more fully understand the current state-of-the-art in diagnostic technology, particularly following the COVID-19 pandemic. The Institute for Defense Analyses was tasked to perform an assessment of current technologies that could be utilized to improve existing infectious disease diagnostic assays and make them more useful for a deployed force.

15. SUBJECT TERMS

diagnostic; molecular; advanced development; rapid; point of care; CLIA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NO. OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			LTC Mark Williams
U	U	U	U	166	19b. TELEPHONE NUMBER (Include Area Code) 571-278-4216

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