Towards integrated molecular lateral flow diagnostic tests using advanced micro- and nanotechnology

Ana Rubio-Monterde[1,2], Daniel Quesada-González[1*†], Arben Merkoçi[1,2,3,4*]

[1] Paperdrop Diagnostics S.L., MRB, Campus UAB, 08193 Bellaterra, Spain

[2] Nanobioelectronics and Biosensors Group, Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC, Barcelona, Spain

[3] The Barcelona Institute of Science and Technology (BIST), Campus UAB, 08036 Bellaterra, Barcelona, Spain

[4] ICREA, Institució Catalana de Recerca i Estudis Avançats, Pg. Lluis Companys 23, 08010, Barcelona, Spain

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INTRODUCTION

Since the COVID-19 pandemic, some of the routine methods for clinical diagnosis of infectious diseases have become closer to the general population and terms like “PCR” (polymerase chain reaction) and “antigen rapid diagnostic test (RDT)” are not restricted only to the scientific community but now also belong to the lay language. Given the emergency, the pandemic also raised awareness about supply politics and regulation of the market for diagnostic tests (e.g., Emergency Use Authorization, EUA, from the Food and Drug Administration, FDA; or the "Conformité Européenne" marking, CE marking, from the European Union, EU). PCR and RDT are two major techniques currently used in the clinical practice for diagnosis of diseases. On the one hand, PCR is a highly sensitive method, but relies on expensive equipment and qualified personnel. On the other hand, rapid diagnostic tests, despite being less sensitive, are faster, cheaper, portable, and battery/instrument-free. In this review, we will discuss the advantages and disadvantages of rapid antigen tests and different nucleic acid amplification techniques (NAAT), such as PCR, both as *in vitro* diagnostic devices. To do so, our review explains the working principle of each method, classifies the different subtypes and analyzes the impact that will have the introduction of isothermal amplification methods in the diagnostic field. In addition, this review gives an overview of the current available products in the market and the new trends generated as a result of combining the strengths of NAAT and RDT.

In vitro diagnostic medical devices

From May 26th, 2022, when the “Regulation (EU) 2017/746” came into force[1,2] replacing Directive 98/79/EC, the development and use of diagnostic tests in the EU are controlled by the new regulatory framework. According to this regulation, *in vitro* diagnostic medical devices are considered any medical device which is a reagent (or a reagent product), control material, kit, instrument, calibrator, apparatus, piece of equipment, system or even a software, being used in solitary or combined, which its intended use (according to the manufacturer) is for an *in vitro* application. The regulation also implies that the device is

intended for the examination of specimens, (which includes human tissue and blood donations), solely or mainly for providing information of relevance of at least one (or more) of the following cases: (i) a physiological or pathological process or state; (ii) congenital physical or mental impairments; (iii) the predisposition to a medical condition or a disease; (iv) the determination of the safety and compatibility with potential recipients; (v) the prediction of the treatment response or reactions; (vi) the definition or monitoring therapeutic measures.[1]

This definition includes from specimen receptacles to malaria, viral loads, or prenatal tests, and the now so well-known COVID-19 tests. Some of these tests are based on PCR or rapid immunochromatography, which are techniques that have been used in clinical practice for decades[3–6], but the COVID-19 pandemic made *vox populi*.

Lessons learnt from COVID-19 pandemic

In December 2019, an outbreak caused by "Severe acute respiratory syndrome coronavirus 2" (SARS-CoV-2) was identified in Wuhan,[7,8] soon after, the virus rapidly spread and became a global pandemic declared by the World Health Organization (WHO) on March 11th. In a short time, the need for available and reliable diagnostic tests was under an increasing demand and a flood of laboratory-developed tests (LDTs)[9,10], EUA tests[10] and CE marked tests reached hospitals and laboratories. By end of July 2020, the FDA had authorized 163 COVID-19 diagnostic tests under EUA.[10] Those diagnostic methods for detection of SARS-CoV-2 were classified in (i) molecular tests, such as the PCR, and (ii) antigen tests, also known as RDT.[11,12]

PCR of nasopharyngeal samples became the gold standard test for the diagnosis of COVID-19[11], but despite its lower limit of detection (LOD) it usually implies transport of the sample to a central laboratory to be analyzed by qualified staff, high cost and, consequently, low-frequency testing, which entails a delay of some days to obtain the result[13,14] and has de risk of being positive after the transmissible stage.[13] RDT were questioned for their sensitivity[15,16] but allowed fast detection of infected patients, at-home testing,[17] and high-frequency testing due to its low cost, which overall helps to prevent spread to others. Figure 1[14] shows a schematic of the optimal timeframe to use the different types of tests and the viral dynamics of SARS-CoV-2 infection in a symptomatic patient. Equally important to acknowledge advantages and disadvantages of every test[18], is to understand the test performance and its intended use, because no test is 100% accurate.[10]

Non-covered medical needs and new biomarkers

The ongoing SARS-CoV-2 pandemic has highlighted the importance of being able to develop, produce and deploy rapidly diagnostic tests for emerging infectious diseases.[19] SARS-CoV-2 will not be the last threat, as other viruses like avian influenza (Influenza H5)[20] or monkeypox[21] have proven recently. However, not only systems for detection and surveillance of viruses are needed, the increasing prevalence of antimicrobial resistance (AMR) has become a silent pandemic which has been exacerbated by the SARS-CoV-2 pandemic.[22] AMR is a serious burden and threat to global public health,[23–25] according to the Centers for Disease Control and Prevention (CDC), only in the United States, every year more than 2.8 million antibiotic-resistant infections happen, and because of them more than 35,000 people die.[24] Particularly concerning is the spread of multi-resistant bacteria, also known as "superbugs".[25] The WHO has recognized the urgency for new *in vitro* diagnostics tests to combat AMR. Among the tests required are: (i) RDT to differentiate between bacterial and viral infections; (ii) tests to identify pathogens; and (iii) antimicrobial susceptibility tests (AST).[26]

Another gap in the market that needs to be addressed is the rapid diagnosis of sepsis. Sepsis is a life-threating condition responsible of 1 in 5 deaths worldwide,[27] defined as an organ dysfunction caused by a dysregulated host response to infection.[28,29] The lack of a reliable and fast diagnostic tests plays a part in the use of broad-spectrum

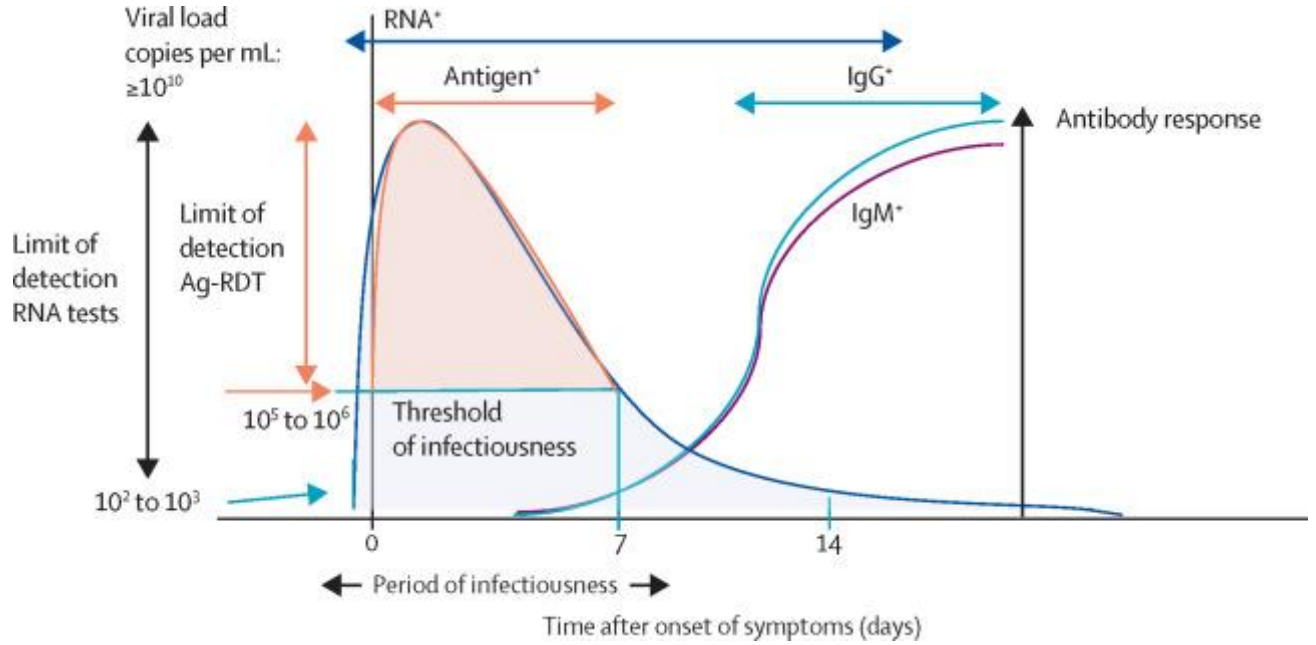

Figure 1: Timelines for optimal use of different diagnostic tests for Covid-19 detection and host response. Reprinted with permission from Peeling, R. W.; Heymann, D. L.; Teo, Y. Y.; Garcia, P. J. Diagnostics for COVID-19: Moving from Pandemic Response to Control. *Lancet* **2022**, 399 (10326), 757–768, (ref. 14). Copyright ® (2022) Elsevier.

antimicrobials, which contributes to increase the prevalence of AMR and this at the same time complicates the sepsis treatment.[29,30] Blood culture remains as the gold standard for sepsis diagnostic, however it is time-consuming (which can delay critical therapeutic decisions) and has poor sensitivity. Culture-independent techniques (e.g. molecular methods and mass spectrometry) may overcome some of the these limitations,[30,31] but they are not techniques suitable for point-of-care testing (POCT) or low-resource settings. The WHO wish list for the properties of the sepsis diagnostic test needed includes: (i) rapid identification of pathogen type (viruses, bacteria, fungi, parasites); (ii) high sensitivity and specificity to guide antimicrobial treatment and limit antimicrobial overuse; (iii) no need to culture (iv) detection of multiple pathogens at the same time; (v) detection of AMR (vi) easy to use; and (vii) low price.[29]

Apart from the lack of diagnostic tests, the difficulty to diagnose sepsis relies in the absence of a validated biomarker.[28,31] There is special interest in biomarkers that could be detected in the emergency room (ER) or critical care POCT to provide rapid results, and therefore rapid and adequate treatment.[28] Non-coding RNAs, particularly microRNAs (miRNAs), and extracellular vesicles (EVs) are examples of potential biomarkers that are being investigated to aid in sepsis diagnostic.[28,31,32] Outside the market of infectious diseases, miRNAs and EVs are also emerging as a disease biomarkers, standing out in cancer diagnose and liquid biopsy.[33,34] EVs are lipid-bilayer membrane structures secreted by cells which have an important role in cell-to-cell communication via the transfer of nucleic acids (NAs), proteins and lipids that can affect physiological processes.[34] Among the NAs transferred there are miRNAs, which are small non-coding RNAs, 21-25 nucleotides long, that have an important role in gene expression regulation. miRNAs are emerging as non-invasive biomarkers for a variety of diseases such as cancer, endometriosis or cardiovascular and infectious diseases.[33–38] The current methods for analyzing miRNAs are reverse transcription qPCR (RT-qPCR), Norther blot-based systems, microarrays, in situ hybridization and next-generation sequencing (NGS). Each of these techniques have their advantages and disadvantages but all of them are far from being POCT platforms. This unmet need could be covered by isothermal amplification assays and RDT based on Lateral Flow (LF), which hold potential as POCT systems for miRNA detection.[38–40]

POCT is defined as testing performed at or near the site of patient care with rapid turnaround time of test results to the patient[41]. POCT are developed to fulfill the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid & robust, Equipment-free, and results Delivered to the end-users) guidelines of the WHO.[42] As proven during the SARS-CoV-2 pandemic, POCT can improve healthcare in different aspects, from enabling earlier disease detection and monitoring to reaching low-resources settings.[43,44] From cardiac markers to infectious disease testing, the menu of POCT is wide and growing, by 2025 the global POCT market is expected to exceed US $44.6 billion with a 9% of compound annual growth rate.[44] RDT and molecular POCT systems are candidates to fulfill this demand in the near future.[45]

LATERAL FLOW

LF biosensors are RDT based on paper. These tests are low-cost, portable, easy-to-use, and equipment/battery-free, characteristics which make them highly suitable for POCT, low-resource settings and emergency use.

From immunochromatography to rapid diagnostic tests

Paper chromatography, invented by Martin and Synge in 1943, can be considered the first paper-based sensor.[46] Later, in 1970s, the first LF test was commercialized: the pregnancy test, which is one of the most used POCT.[47,48] Following this milestone, the first infectious disease LF test was commercialized in the late 1980s for the detection of Group A *Streptococcus pyogenes* from throat swabs samples.[49] From there, LF biosensors extended widely to other fields including veterinary, environmental control, food safety, diagnosis and others.[50]

In the last decade, the inclusion of nanomaterials lead to better performance with lower LOD and other benefits.[51–54] Nanomaterials (those of 100 nm size or smaller) have properties that diverge from those of the same materials at the macroscopic scale, presenting phenomena such as electromagnetic field enhancement, quantum confinement, and plasmonic resonance, which can be exploited for signal amplification.[53] There are recent reviews devoted to nanomaterials and their promising applications in LF, which are too wide to be included in this review. Refer to the reviews of Nguyen et al.,[55] Sena-Torralba et al.,[52] Vahid Shirshahi and Guozhen Liu,[56] and Mirica et al.[57] for more information about novel nanomaterials employed in LF tests. It must be highlighted that gold nanoparticles (AuNPs; a.k.a. colloidal gold) are the most used nanomaterial on LF tests due its high biocompatibility (easy to conjugate with bioreceptors), easiness and reproducibility in synthesis, tuneability (size, shape and color) and strong colorimetric response. Other nanomaterials as carbon nanoparticles and upconverting nanoparticles (e.g. Eu-based nanoparticles),[39] also presenting fluorescence capabilities, are becoming popular in research papers but not in the same way (yet) in commercial systems, as later shown in Table 1. Large scale production for novel nanomaterials and their use in commercial test could still be challenging.

Recently, the global outbreak of SARS-CoV-2 has been a boost for the use of RDT based on LF, which has improved the trust in these tests in the diagnostic field.[50] The LF tests success is also reflected in the commercial market, considering that the global market for LF tests was valued at USD 8.2 billion in 2020 and is projected to reach USD 11.7 billion by 2028, growing at a CAGR (compound annual growth rate) of 4.6% from 2021 to 2028.[58]

Working principle

Briefly, the physical principle of the LF assays is based on capillarity action. When the extracted specimen is added to the sample pad, the flow goes through the paper reaching the conjugate pad, where the nanoparticles (NPs) conjugated[57] with the bioreceptor are stored, ready to react to the target analyte (if present). Once the flow reaches the detection pad (membrane), if the specimen contains the target, the test line (TL) will appear indicating a positive result due to the presence of a specific bioreceptor that recognizes the target. Therefore, if there is no target on the sample, the TL will not appear visible. The sample continues flowing

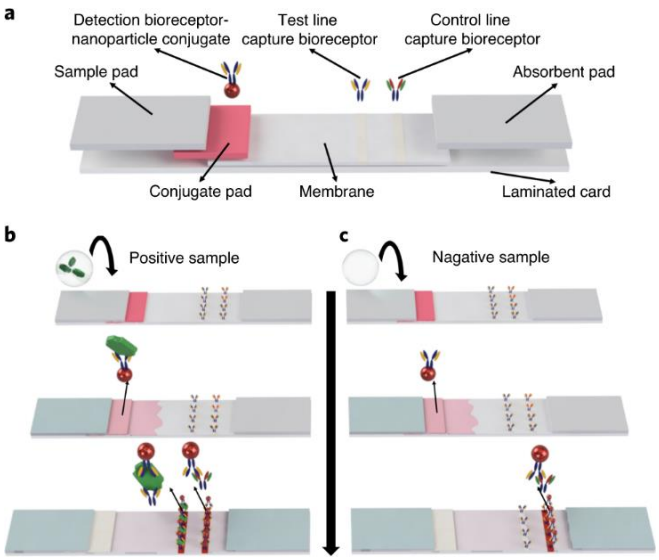

Figure 2: Components and working principal of a LF test. a) The main parts of a LF strip are the sample pad, the conjugate pad, the membrane (where the bioreceptors are printed) and the absorbent pad, all of which are assembled on a laminated card. b) and c) working principle of a classical LF immunoassay b) The presence of the target in the sample leads to the appearance of two red lines due to the accumulation of nanoparticles on TL and CL. c) In the absence of the target, only the CL appears. Reprinted with permission from Parolo, C.; Sena-Torralba, A.; Bergua, J. F.; Calucho, E.; Fuentes-Chust, C.; Hu, L.; Rivas, L.; Álvarez-Diduk, R.; Nguyen, E. P.; Cinti, S.; Quesada-González, D.; Merkoçi, A. Tutorial: Design and Fabrication of Nanoparticle-Based Lateral-Flow Immunoassays. *Nat. Protoc.* 2020, *15* (12), 3788–3816 (ref. 59). Copyright © (2020) The Authors (Quesada-González, D. and Merkoçi, A.), under exclusive license to Springer Nature Limited.

through the detection pad and a control line (CL), containing a secondary bioreceptor, should appear for all valid tests. Finally, an absorbent pad is located at the end of the strip for absorbing the fluid excess (Figure 2).[59]

The same principle applies in the multiplexed LF assays, which allow simultaneous detection of more than one target. The most straightforward way to develop a multiplexed LF is just by printing multiple TL with the corresponding bioreceptor in the detection pad. However, the number of targets to be multiplexed by this mechanism is limited by the length of the detection pad and/or the different labels available. Recent advances and novel strategies have been developed lately to address these challenges (e.g. microarrays, fluidic devices or the use of different NPs in the same assay).[52]

Types of LF tests according to the bioreceptors used

The bioreceptors are biological compounds used for the capture of the target and, in the case of LF tests, for its recognition by means of a sandwich format (Figure 2). There is a minimum of three bioreceptors needed for this design of a LF test: (i) one labelled with the NPs; (ii) another one printed in the detection pad as a TL; and (iii) the last one that is able to capture the first bioreceptor or other molecules at the surface of the NPs and is printed as a CL.[59]

The classic bioreceptors used in LF tests are antibodies (Ab), but other bioreceptors such DNA probes[40,60–62] or aptamers[63–65] can be integrated depending on the target to be detected.

In LF immunoassays (LFIA), monoclonal and polyclonal antibodies are the more common bioreceptors applied. Nevertheless, recently nanobodies and antibody fragments are standing out due to some advantages such less unspecific binging and low batch-to-batch variability in comparison with monoclonal and polyclonal Ab.[52,59] The usual target of LFIA are of protein nature[66] (e.g., hormones, antigen virus or bacteria, toxins), although other targets such as drugs of abuse[67], cofactor molecules[68] or even heavy metals[69] can be also detected.

However, despite its high applicability in LF, Ab present some limitations including high batch-to-batch variability, long development time, need for ethical approval and refrigerated storage.[63,70] In addition, there are some non-immunogenic targets like small molecules (e.g. antibiotics, bacterial spores, pesticides) for which is difficult to obtain high-affinity Ab. Aptamers, also called "chemical Ab", have been investigated as bioreceptors for LF to address these restrictions from the Ab.[70]

Aptamers are single-stranded DNA (ssDNA) or single-stranded RNA (ssRNA) molecules that fold in a complex 2D or 3D structure and bind to a target with high affinity and selectivity.[71,72] They have several clear advantages over Ab, including high stability, *in vitro* generation, ease in functionalization, and capacity of large scale synthesis.[63,73] Aptamers are developed by an *in vitro* iterative process called "systematic evolution of ligands by exponential enrichment" (SELEX),[73] which has been modified depending on the target of interest[74,75,76]. By SELEX or its variants, aptamers have been generated for hormones, drugs, heavy metals, proteins, toxins, enzymes, cancer cells, and bacterial, viral and fungal pathogens.[73] Aptamer-based LF tests use aptamers as bioreceptors, yet they do not solve the lack of sensitivity of the LF[70] and their binding activity is highly dependent on the ionic strength of the buffer and the presence of certain cations.[59]

Non structured nucleic acids (i.e., DNA probes) are also used as bioreceptors. There are Nucleic Acid Lateral Flow (NALF) tests, also called DNA-based LF tests[62], and Nucleic Acid Lateral Flow Immunoassay (NALFIA).[49,77] In NALF, different DNA probes are conjugated to the NPs and printed in TL and CL. These DNA probes are designed to be complementary according to Watson-Crick base pairing to the target (e.g., a miRNA or genetic amplicon, which is the product of a molecular amplification, with primers extended with a ssDNA tail). In the case of NALFIA, dual labeled targets (proteins and genetic amplicons) typically with biotin and another antigen with high Ab affinity, i.e., digoxigenin or fluorescein, are captured in a sandwich complex between biotin and streptavidin in one side, and antigen and antibody immunoreactions in the other.[78]

Test in the market

Apart from the huge distribution of LFIA (antigen rapid tests) for SARS-CoV-2[79] detection (709 CE mark by October 2022),[80] LF tests play a role in the diagnosis of other pathologies and as POCT or in the ER. The following table 1 summarizes the portfolio of some of the most relevant companies with *in vitro* diagnostic LFIA products available nowadays.

In the table it can be observed that colloidal gold, AuNPs, are the most common nanomaterial in LF tests, as we previously said, together with latex beads (often above the 100 nm, thus not being considered nanomaterials). Latex beads need to be dyed in order to produce color and their surface must be functionalized for their conjugation (often with carboxylic groups), while AuNPs can directly interact with thiolated bioreceptors, among others. It must not go unnoticed that those kits using fluorescent nano- or microparticles (it includes Eu beads) will always require the use of an additional equipment for reading the results, while using other reporters the response, in a qualitative manner, could be read by naked-eye (however, the equipment may help to register the data or even quantify the concentration of analyte).

Table 1. Portfolio of relevant companies with *in vitro* diagnostic LFIA and its application field.

Company	Brand name	Reporter	Reader	Application field of the assays	Regulatory approval
SD BIOSENSOR	STANDARD F	Europium beads	STANDARD F 2400 STANDARD F 200 STANDARD F 100	Respiratory Serology Vector borne Gastrointestinal Hepatitis Blood borne Chronic disease Inflammation Cardiovascular Hormones Tumor marker	Depending on the assay RUO and/or CE
SD BIOSENSOR	STANDARD Q	Colloidal gold	No	Respiratory Serology Vector borne Blood borne Hepatitis Gastrointestinal Cardiovascular	Depending on the assay CE
Abbot	BinaxNow™	Colloidal gold	DIGIVAL™	Respiratory antigens Urine Malaria G6PD deficiency	Depending on the assay CE, FDA and/or EUA
Abbot	PANBIO™	Colloidal gold	No	Respiratory antigens Dengue Serology	Depending on the assay CE and/or FDA

Company	Brand name	Reporter	Reader	Application field of the assays	Regulatory approval
Operon	Rapid tests	Colored latex particles	OPERON Lateral Flow Reader	Respiratory Gastrointestinal Food intolerance Tumor markers Pregnancy Hepatitis	All CE
CerTest Biotec	Rapid test	Colored latex particles	No	Fecal antigens Respiratory antigens Urine Inflammatory and tumor markers	All CE
R-Biopharm AG	RIDA®QUICK	Colored latex particles or colloidal gold	RIDA®QUICK SCAN II	Enteropathogenic infections	All CE
NG-BIOTECH	NG-Test®	Colloidal gold	NG-Test® Reader	Antibiotic resistance Respiratory Pregnancy Serology	Depending on the assay CE and/or FDA
CORIS BioConcept	RESIST (for the antibiotic resistance product line)	Colored latex particles or colloidal gold	No	Respiratory Enteric Gastric Antibiotic resistance Drug Serology	All CE
QUIDEL	Sofia	Fluorescent microparticles	Sofia 2	Respiratory Gastrointestinal Lyme Pregnancy	Depending on the assay CE, FDA, EUA and/or CLIA waived
BD	Veritor™	Colloidal gold	Veritor System	Respiratory	Depending on the assay CE, FDA, EUA and/or CLIA waived

RUO: Research use only. FDA: Approved by FDA by premarket notification 510(k), De Novo or Premarket Approval Application (PMA). EUA: Emergency use authorization only by FDA. CE: European conformity, can be CE-IVDD by the Directive 98/79/EC on in vitro Diagnostic Medical Devices or CE-IVDR by the European conformity by Regulation (EU) 2017/746 (IVDR) on in vitro diagnostic medical devices. CLIA waived: Clinical Laboratory Improvement Amendments waiver, which means classified as “simple laboratory examinations and procedures that have an insignificant risk of an erroneous result” by FDA according to requirements (42 CFR Part 493).

NUCLEIC ACID AMPLIFICATION TECHNIQUES

Nucleic acid amplification technologies (NAATs) are based on sequence-specific recognition and amplification of a region of interest in the genome of targets to be detected. The most common NAAT used in *in vitro* diagnostic is the PCR, which is a laboratory technique developed by Kary Mullis in 1983 that can make millions of copies of a specific DNA region in a short time. PCR presents some advantages and drawbacks in comparison to RDT, despite being highly sensitive, it relies on qualified personnel and expensive equipment. However, during the last decade, other NAAT based on isothermal amplification (IA) have gained attention, especially for POC applications.

Polymerase chain reaction (PCR)

Technique

PCR is a powerful technique used to produce multiple copies of a nucleic acid region of interest.[4,81]

The four main components of a PCR are the DNA template, the primers, the nucleotides (deoxynucleotide triphosphates, dNTPs), and a thermostable DNA polymerase: (i) The DNA template is the sample which contains the region of DNA to be amplified; (ii) primers are short oligonucleotides DNA sequences, custom designed to be complementary to the target sequence; (iii) dNTPs are the material to synthesize new DNA strands, they are single units of Adenine (A), Thymine (T), Cytosine (C) and Guanine (G); (iv), the DNA polymerase is an enzyme which synthesizes new DNA strands complementary to the target sequence, PCR uses a thermostable polymerase, which allows the enzyme to withstand the heating required for the reaction and maintain its activity.[81–83]

PCR reaction follows three main steps repeated in cycles: denaturation, annealing, and extension (Figure 3a).[84] Denaturation takes place at 94-96 ºC and it separates the double-stranded DNA (dsDNA) template into ssDNA. Next, the temperature is lowered between 45 and 60 ºC, allowing the primers to hybridize to their complementary site on the DNA template (annealing). The union of the primers provides a place for the polymerase to attach and begin copying the template, usually at 72 ºC (extension). These steps are repeated many times in cycle using an automated thermal cycler resulting in the exponential amplification of the DNA between the primers.[,82,83] In cases where the sample is RNA, the PCR is preceded by the conversion of the RNA to a complementary DNA (cDNA) by a reverse transcriptase (**RT-PCR**), and then de cDNA enters the PCR cycles (Figure 3c).[84]

The use of more than one primer pair allows to amplify multiple DNA regions simultaneously in one PCR reaction, resulting in a multiplex PCR.[4]

Types

When the results of the PCR are analyzed at the end of the reaction it is called **end-point PCR** (or end-point RT-PCR[85,86]). Typically, this is performed using an agarose gel electrophoresis, where the samples are ran together with a ladder to check if the amplicon size (i.e. the number of base pair, bp) obtained is the one expected.[82] With the corresponding primer modification, the PCR result can also be read by a NALF o NALFIA.[78,87,88]

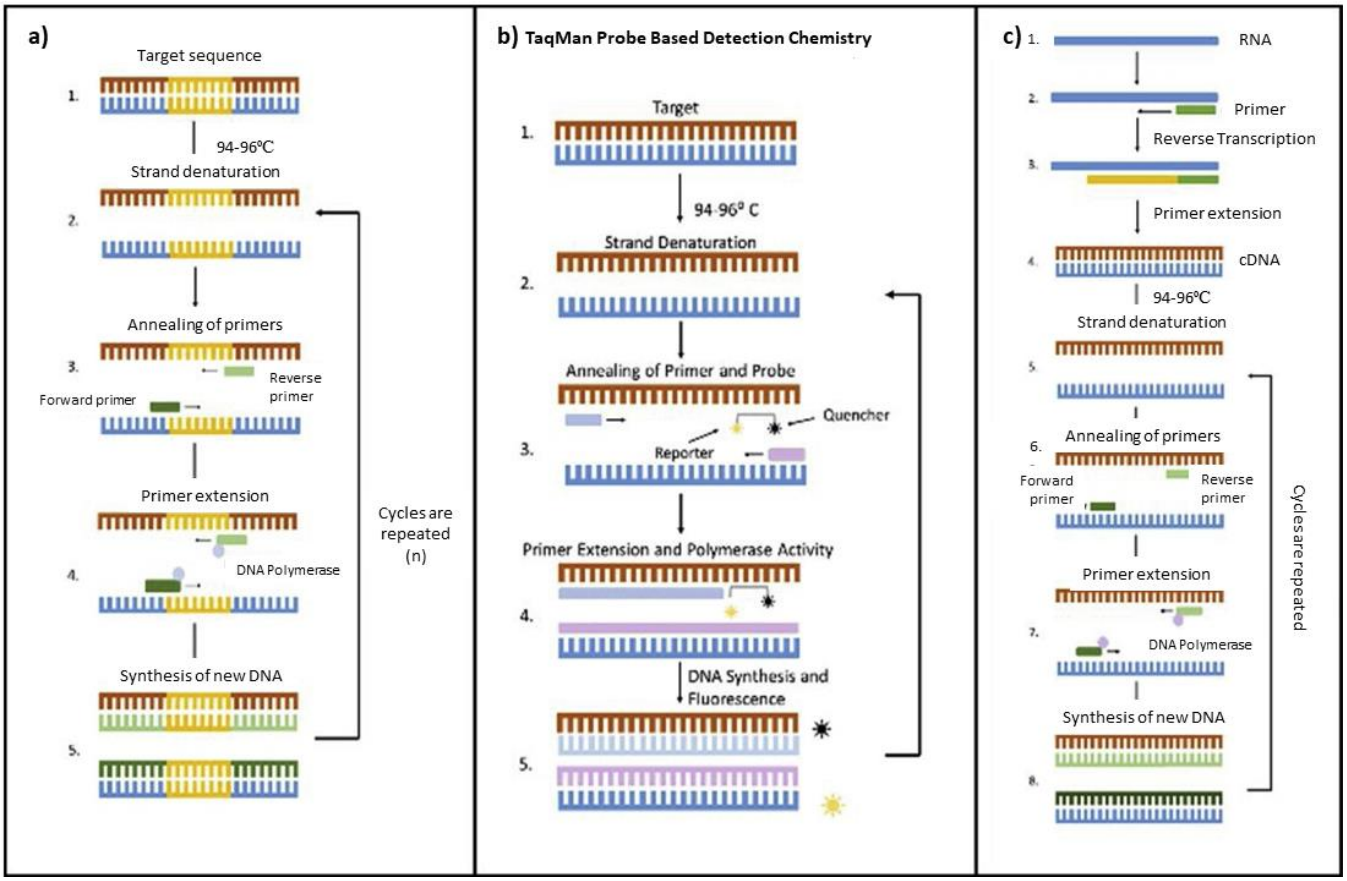

Figure 3: Schematic representation of the **a)** PCR process, **b)** TaqMan Probe Real Time PCR and **c)** RT-PCR. Modified with permission from Dunbar, S.; Das, S. Amplification Chemistries in Clinical Virology. *J. Clin. Virol.* **2019**, *115*, 18–31 (ref. 84). Copyright © (2019) Elsevier.

On the other hand, **Real Time PCR** (qPCR or RT-qPCR) enables detection and measurement of products generated as the amplification progresses. There are several chemistries used for the detection of PCR product during real-time PCR[89], being one of the most applied the use of TaqMan® probes, which are designed to hybridize within the target sequence. These probes are labelled by a fluorophore and a quencher, once they hybridize, they are cleaved by the DNA polymerase during the extension resulting in the separation of the quencher from the fluorophore, which start to emit fluorescence that is monitored by an instrument.[4,89,90]

The inclusion of a standard curve in the qPCR allows quantification of the target by extrapolating the amplification curve during the exponential phase of the reaction itself.[89,91,92] However, **digital PCR** (dPCR) is a method for absolute quantification of the DNA/RNA of interest based on end-point fluorescence measurement without standard curve.[92,93] In dPCR, first the sample is separated into many independent PCR sub-reactions and, depending on the method used for this partition, dPCR is classified as droplet based dPCR or chip-based dPCR.[4,93,94] Following the split, the PCR takes place and finally, the fluorescence signals are detected and quantified based on Poisson distribution. Although dPCR has many advantages when compared to qPCR, such as higher reproducibility, accuracy and tolerance to inhibitors, it still has major disadvantages as the high cost and complex instrumentation.[93,94]

Figure 4 summarizes the main differences, already discussed, between end-point PCR, qPCR and dPCR.[92]

Tests in the market

PCR is widely distributed in the *in vitro* diagnostic market in different setups (e.g., closed systems, automated robots, syndromic testing, etc.). Table 2 compiles some of these systems available today for *in vitro* diagnosis (IVD).

Table 2 does not include IVD PCR assays to be use with common thermocyclers such as Bio-Rad Real Time PCR detection systems, the LightCycler or the StepOne from Applied Biosystems among others. However, form its information we can observe how many PCR platforms consist of a cartridge and automated instruments, resulting in walkaway solutions where the user only needs to add the sample (sample-to-result). This trend is also reaching dPCR with the first cartridge plus automated systems receiving regulatory approvals. Some of these platforms aim at single or few pathogens detection (multiplex) while others follow a syndromic approach (simultaneously target multiple pathogens with overlapping symptoms), offering panels of several pathogens being simultaneously tested.

Isothermal era

What is isothermal amplification?

Recent advanced methods in NAATs are based on isothermal amplification (IA), which overcomes the need of thermal cycling given the ability to amplify the sequence of interest at constant temperatures.[95,96] By eliminating the need of thermocyclers, amplification techniques are set free from energy-demanding and technologically complex systems, opening the door to POCT and in-field applications.[96–100] IA techniques are thus suitable for laboratories lacking expensive PCR equipment and of low-resource settings. In addition, IA methods require less sample and reagent preparation, and the results can be read through different approaches (e.g., turbidimetry, LF), enhancing their accessibility and ease-of-use.[98,99,101]

IA methods are becoming more and more popular, in a way that in the near future we could be entering the isothermal era as IA defies PCR. IA techniques differ from one another in features such as the enzymes used, number of primers, time and temperature of amplification or the template required. Nevertheless, the future of IA methods is still dependent on fulfilling technical maturity and broader commercialization of enzymes and reagents, which will come with the expiry of the corresponding patents.[99,102–106]

As the field expands, there are several IA already patented, like the Crossing Priming Isothermal Amplification (CPA)[102] and the Helicase Dependent Amplification (HDA),[107] or under development as the Signal-Mediated Amplification of RNA Technology (SMART).[98,99] These last IA methods will not be described within this review since the available information or the predicted impact is still limited. Instead, we briefly describe the most noteworthily and currently having most potential to be part of *in vitro* diagnostic tests.

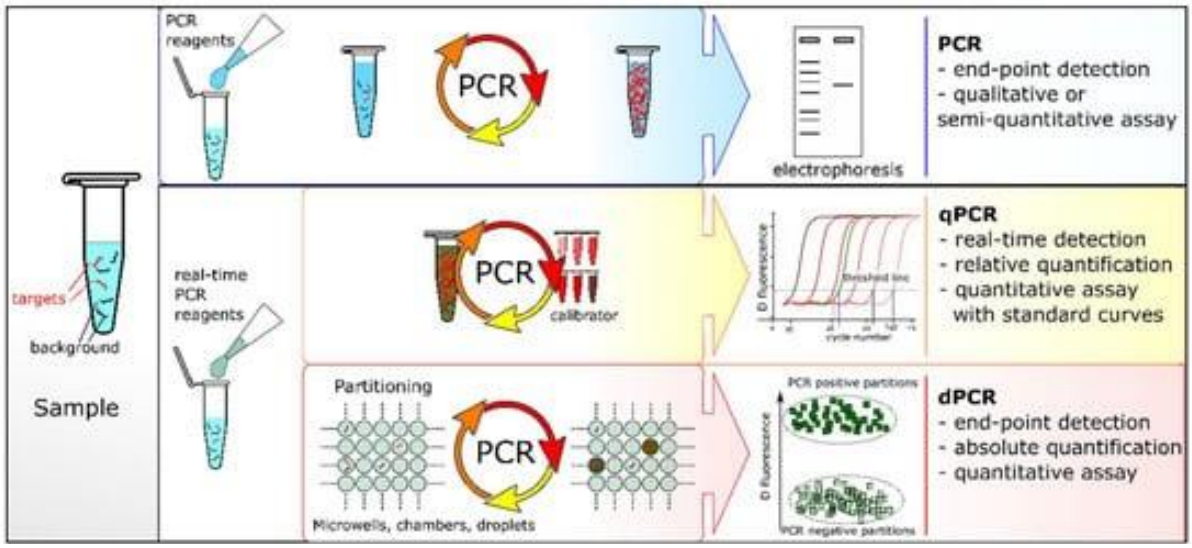

Figure 4: A comparison of end-point PCR, qPCR and dPCR. Reprinted with permission from Quan, P. L.; Sauzade, M.; Brouzes, E. DPCR: A Technology Review. *Sensors* **2018**, Vol. 18, Page 1271, 2018, 18 (4) (ref. 92). Copyright © (2018) MDPI.

Table 2. Some of the remarkable PCR platforms for IVD commercially available.

Platform name	Company	PCR technology	Detection technology	Setup	Singleplex/Multiplex/Syndromic	Assays	Regulatory approval
ePlex	GenMark	PCR RT-PCR	Electrochemical	Sample-to-result Cartridge and instrument	Syndromic	Respiratory Pathogen Panel 1 Respiratory Pathogen Panel 2 BCID-GP Panel BCID-GN Panel BCID-FP Panel	CE and FDA CE and EUA CE and FDA CE and FDA CE and FDA
ARIES®	Luminex	RT-qPCR qPCR	Real Time fluorescence	Sample-to-result Cartridge and instrument	Multiplex	SARS-CoV-2 *Bordetella* *C. difficile* Flu A/B & RSV Group A Strep Group B Strep HSV 1&2 MRSA Norovirus	EUA and CE CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA FDA CE
STANDARD M10	SD BIOSENSOR	RT-qPCR qPCR LAMP	Real Time fluorescence	Sample-to-result Cartridge and instrument	Multiplex	MPX/OPX MDR-TB MTB/NTM HPV *C. difficile* Arbovirus panel Flu/RSV/SARS-CoV-2 SARS-CoV-2 SARS-CoV-2 turbo	RUO CE CE CE CE CE CE CE CE
GeneXpert	Cepheid	RT-qPCR qPCR	Real Time fluorescence	Sample-to-result Cartridge and instrument	Multiplex	Xpress CoV-2/Flu/RSV plus Xpress CoV-2 plus Xpress SARS-CoV-2/Flu/RSV Xpress SARS-CoV-2 Xpress Strep A Xpress Flu/RSV Xpress Flu MRSA NxG SA Nasal Complete	CE and EUA CE and EUA CE and EUA CE and EUA CE, FDA and CLIA waived CE, FDA and CLIA waived FDA and CLIA waived CE and FDA CE and FDA

Platform name	Company	PCR technology	Detection technology	Setup	Singleplex/Multi-plex/Syndromic	Assays	Regulatory approval
						MRSA/SA BC	CE and FDA
						MRSA/SA SSTI	CE and FDA
						Carba-R	CE and FDA
						Norovirus	CE and FDA
						EV	CE and FDA
						C. difficile BT	CE
						C. difficile/Epi	FDA
						vanA/vanB	CE
						vanA for VRE	FDA
						MTB/RIF	CE and FDA
						MTB/RIF Ultra	CE
						MTB/XDR	CE
						Ebola	CE and EUA
						CT/NG	CE and FDA
						HPV	CE
						GBS	CE and FDA
						Trichomonas vaginalis	CE and FDA
						Resistance Plus® MG FleXible	CE
						HBV Viral Load	CE
						HCV Viral Load	CE
						HCV VL Fingerstick	CE
						HIV-1 Qual XC	CE
						HIV-1 Viral Load XC	CE
						Bladder Cancer Detection	CE
						Bladder Cancer Monitor	CE
						Breast Cancer STRAT4	CE
						BCR-ABL Ultra	CE and FDA
						FII & FV	CE and FDA
						BCR-ABL Ultra p190	CE
FilmArray	bioMérieux	PCR RT-PCR	Fluorescence (melting curve)	Sample-to-result Cartridge and instru-ment	Syndromic	Respiratory Panel 2.1	CE and FDA
						Respiratory Panel 2.1 plus	CE
						Respiratory Panel EZ	FDA and CLIA waived
						Respiratory Panel 2.1 EZ	EUA
						Blood Culture Identification	CE and FDA

Platform name	Company	PCR technology	Detection technology	Setup	Singleplex/Multiplex/Syndromic	Assays	Regulatory approval
						Gastrointestinal Panel Meningitis/Encephalitis Panel Pneumonia Panel Pneumonia Panel Plus Joint Infection Panel	CE and FDA CE and FDA CE and FDA CE CE and FDA
QIAstat-Dx	Qiagen	RT-qPCR	Real Time fluorescence	Sample-to-result Cartridge and instrument	Syndromic	Respiratory Panel Respiratory SARS-CoV-2 Viral Vesicular Panel Gastrointestinal Panel SARS-CoV-2/FluA/B/RSV Meningitis/Encephalitis Panel	CE and FDA CE and EUA RUO CE CE CE
Savanna®	Quidel	RT-qPCR	Real Time Fluorescence	Sample-to-result Cartridge and instrument	Syndromic	Respiratory Viral Panel-4	CE
Accula™	Mesa Biotech (now ThermoFisher)	RT-PCR (Oscar™)	LF	Sample-to-result Automated and self-contained system with cassettes and dock station	Singleplex or Multiplex	FluA/FluB RSV Strep A SARS-CoV-2	CE, FDA and CLIA waived CE, FDA and CLIA waived FDA and CLIA waived EUA
cobas® Liat®	Roche	RT-qPCR qPCR	Real Time Fluorescence	Assay tubes and automated instrument	Multiplex	Influenza A/B & RSV Strep A SARS-CoV-2 Influenza A/B & SARS-CoV-2 *C. difficile*	CE and FDA CE and FDA EUA EUA CE and FDA
cobas® 6800/8800	Roche	RT-qPCR qPCR	Real Time Fluorescence	Sample-to-result Huge, fully integrated, and highly automated instruments for high throughput Flexible workflow and the ability to test multiple assays in each run	Multiplex	Babesia BKV CHIKV/DENV CMV CT/NG DPX EBV HBV HCV HEV HIV-1	CE and FDA CE and FDA CE CE and FDA CE and FDA CE CE and FDA CE and FDA CE and FDA CE CE and FDA

Platform name	Company	PCR technology	Detection technology	Setup	Singleplex/Multiplex/Syndromic	Assays	Regulatory approval
						HIV-1/HIV-2 Qualitative HPV Influenza A/B & RSV UC MAI MPX MTB MTB-RIF/INH SARS-CoV-2 SARS-CoV-2 and Influenza A/B SARS-CoV-2 variant TV/MG WNV Zika	CE and FDA CE and FDA CE CE CE and FDA CE CE CE and EUA CE and EUA RUO CE and FDA CE and FDA CE and FDA
QXDx AutoDG ddPCR	Bio-Rad	RT-ddPCR	Fluorescence	Automated Droplet Generator system consisting of QXDx Droplet Reader and QXDx Automated Droplet Generator Previous NA extraction and thermocycler needed	Multiplex	BCR-ABL SARS-CoV-2	CE and FDA EUA
Dr. PCR™	Optolane Technologies	Digital RT-PCR	Fluorescence	Cartridge + LOAA analyzer	Multiplex	BCR-ABL1	CE
FastPlex Triplex SARS-CoV-2 Detection Kit	PreciGenome	RT-ddPCR	Taqman fluorescent probe	DropX-2000 digital PCR system and the associated microfluidic cartridge Previous NA extraction	Multiplex	SARS-CoV-2	EUA

RUO: Research use only. FDA: Approved by FDA by premarket notification 510(k), De Novo or Premarket Approval Application (PMA). EUA: Emergency use authorization only by FDA. CE: European conformity, can be CE-IVDD by the Directive 98/79/EC on in vitro Diagnostic Medical Devices or CE-IVDR by the European conformity by Regulation (EU) 2017/746 (IVDR) on in vitro diagnostic medical devices. CLIA waived: Clinical Laboratory Improvement Amendments waiver, which means classified as “simple laboratory examinations and procedures that have an insignificant risk of an erroneous result” by FDA according to requirements (42 CFR Part 493).

Loop-mediated isothermal amplification (LAMP)

LAMP was first published in 2000,[108] after being patented,[105] and since then it has been successfully applied to detection of a variety of pathogens, food control and cancer related mutations.[99,100,109] One of its most relevant acknowledgements has been its approval by the WHO as an alternative molecular diagnostics test for pulmonary tuberculosis (TB).[99,110]

LAMP consists of a single amplification period from 60 to 65 ºC with a turnaround time (TAT) of less than 1 hour. LAMP assays require 4 or 6 primers (these include inner and outer primers), that recognize 6-8 different regions of the target. To do so, it makes use of a strand-displacing DNA polymerase from *Bacillus stearothermophilus* (Bst), which initiates the DNA synthesis (Figure 5a).[98,111] The results of

the amplification can be interpreted by naked eye visualizing the turbidity of the medium[97] or by other methods such agarose gel, electrochemistry, real-time fluorescence or LF strips.[112–115]

Features like the possibility of being performed in a heating block and interpreting the results by naked eye make LAMP suitable as a POCT.[116] In addition, it is reliable for low resource clinical settings that lack minimal expertise and equipment. However, the complex primer design, the chance to obtain false positives (due to hybridization by primers) and the difficulty to multiplex (due to the number of primers required) still need to be addressed before LAMP is routinely applied as an *in vitro* diagnostic test.[97]

Rolling Circle Amplification (RCA)

RCA is inspired on the rolling circle replication that happens in plasmids and viruses *in vivo*, is an enzyme-based IA that can be performed from room temperature to 60 ºC with a TAT of maximum 90 min.[99] RCA needs a strand-displacement DNA polymerase, a primer (RNA or DNA), a target (circular template) and dNTPs. RCA employs enzymes such as Bst, Phi29, and Vent exo-DNA polymerases, but Phi29 DNA polymerase is the most used.[98] Briefly, RCA is performed by a strand-displacement DNA polymerase when a specific primer binds to a circular template in presence of dNTPs and

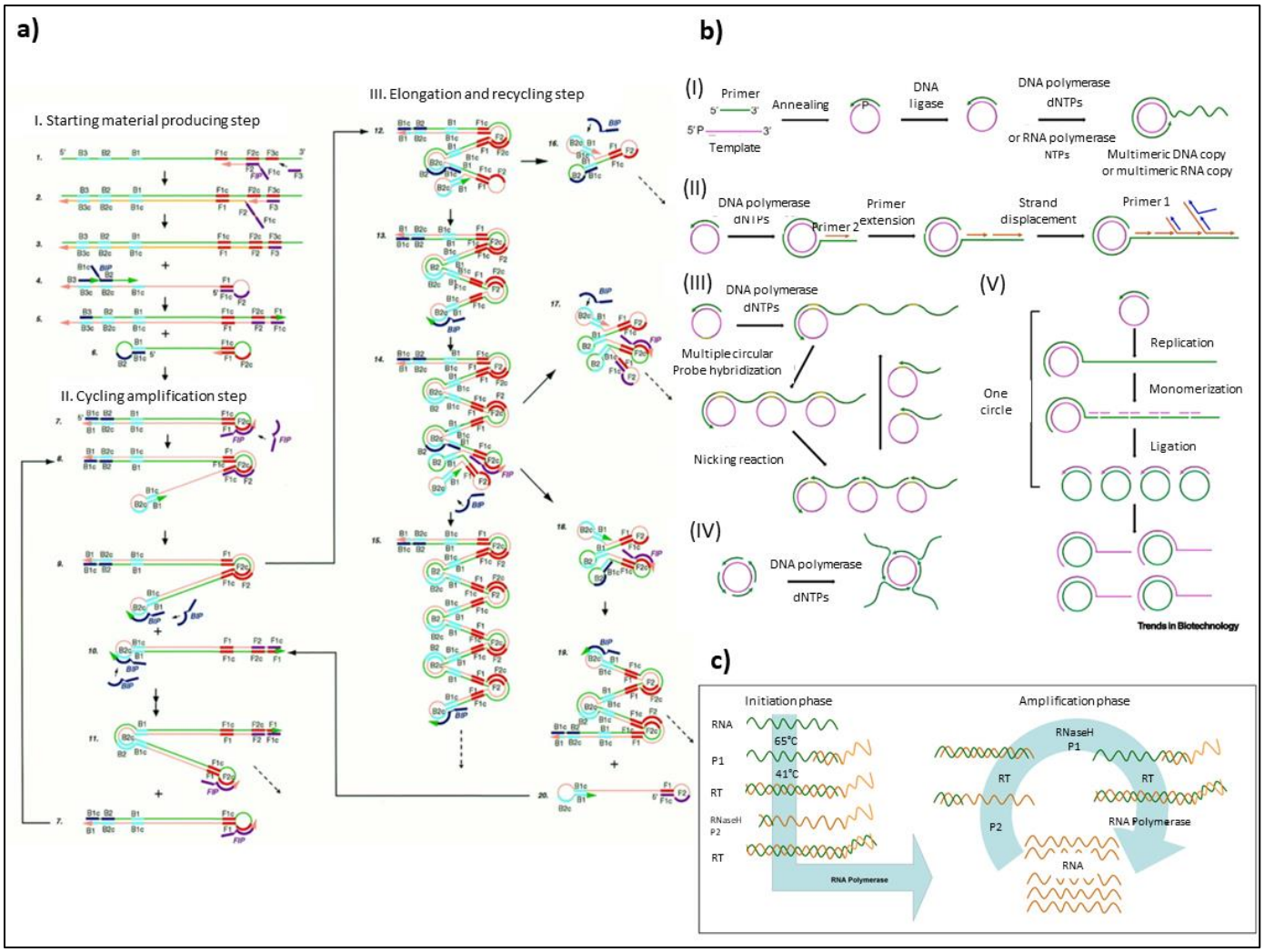

Figure 5: Working principle of some Isothermal Amplification techniques **a) LAMP**, adapted with permission from Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* 2000, 28 (12), e63–e63. (ref. 111). Copyright © (2000) Oxford University Press. **b) (I) RCA, (II) HRCA, (III) PG-RCA, (IV) multiply primed RCA, (V) C2CA**, modified with permission from Yue, S.; Li, Y.; Qiao, Z.; Song, W.; Bi, S. Rolling Circle Replication for Biosensing, Bioimaging, and Biomedicine. *Trends Biotechnol.* 2021, 39 (11), 1160–1172. (ref. 120). Copyright © (2021) Elsevier. **c) NASBA**, adapted with permission from Zanoli, L. M.; Spoto, G. Isothermal Amplification Methods for the Detection of Nucleic Acids in Microfluidic Devices. *Biosens*. 2013, Vol. 3, Pages 18-43 2012, 3 (1), 18–43. (ref. 122). Copyright © (2013) MDPI.

generates a long ssDNA or ssRNA molecule with tandem repeats (Figure 5b).[98,99,117] In order to be able to amplify a linear template, padlock probes were introduced. Padlock probes[118] are linear oligonucleotides that become circularized when the ends are joined together by ligase enzyme, which happens when they hybridize with a specific sequence. RCA results can be interpreted using agarose gel electrophoresis, visually using fluorescence spectroscopy, microscopy and flow cytometry[97,98] or with LF strips.[114,119,120]

RCA method results in a linear amplification of the target. Several modifications of RCA have been developed: hyperbranched rolling circle amplification (HRCA), primer generation-rolling circle amplification (PG-RCA), multiply primed RCA and circle-to-circle amplification (C2CA),[99,120] (Figure 5b) allowing exponential amplification.

The low temperature profile of RCA makes it interesting for POCT and it has been used for biosensing applications such detection of bacteria or miRNA[99,119,120,121,117], however, no RCA based diagnostic test have yet reached the market.

Nucleic Acid Sequence Based Amplification (NASBA)

NASBA is an isothermal transcription-based technique, that mimics the retroviral RNA replication, with a TAT of 1.5 to 2 hours. The method requires two primers (P1 and P2) and three thermolabile enzymes (T7 RNA polymerase, RNase H, and reverse transcriptase). NASBA is more suitable for RNA targets, but it can be applied to DNA targets adding two denaturation steps at 95 °C.

NASBA mechanism involves two phases: (i) Initiation phase (non-cycling), where the target RNA is converted to dsDNA by reverse transcription; and (ii) amplification phase (cycling), where the dsDNA molecules are actively transcribed into RNA products (Figure 5c).[99] The standard NASBA protocol starts with an incubation step (for RNA at 65 ºC and for DNA at 95 ºC) to denature the target and then the following amplification takes place at 41 ºC. In the initiation phase, the forward primer (P1), which includes the promoter sequence for T7 RNA polymerase, hybridizes to any RNA target present in the sample and is extended by the reverse transcriptase. Next, the RNA strand of the resulting RNA:DNA hybrid is destroyed by RNase H, and the reverse primer (P2) hybridizes to the complementary sequence following production of dsDNA by the reverse transcriptase, obtaining a dsDNA with the target sequence and a T7 promoter. Then, the T7 RNA polymerase produces multiple RNA copies that are complementary to the target RNA. In the amplification phase, each newly synthesized RNA can be copied, resulting in an exponential amplification of RNA complimentary to the target.[98,122]

Once amplification is complete, the amplified products can be detected by several ways, such as: molecular beacons, fluorescence markers, gel electrophoresis, enzyme-linked gel assay, electro-chemiluminescence, LF or electrochemical detection.[98,99,114,122]

NASBA assays have been developed for detection of a diversity of virus, bacteria, and parasites.[99,123–125] However, the method is limited by the need of thermolabile enzymes after the denaturation step, and the difference between the initial temperature for denaturation (65 °C for RNA and 95 °C for DNA) and the temperature for the amplification reaction (41 °C).[98]

Recombinase Polymerase Amplification (RPA)

RPA requires three different enzymes (recombinase, single stranded binding protein (SSB), and DNA polymerase) and two primers.[97,98] Depending on the detection method, exo, nfo and fpg probes can be used.[98] Adding more sets of primers, RPA offers the feasibility of multiplex detection of more than one target in a single reaction.[97] The reaction starts with the recombinase, which binds to single stranded nucleic acids, the primers, in the presence of ATP, and targets the complementary double strand sequence in the template. When the primer hybridizes with the template, a D-loop structure is formed that allows a strand displacement. The displaced DNA strand is then coated by SSB while primer extension by DNA polymerase forms a new dsDNA which acts as a template for the next cycle of amplification (Figure 6a).[98,99,126] The primers are longer than the standard PCR primers, between 30-36 bp,[95,97] the amplicon size is recommended to be less than 500 bp, the temperature for the whole protocol is 37-42 ºC, and amplification time from 10 to 20 minutes with a TAT of 20 to 40 min. Gel electrophoresis, fluorescence methods for real-time and LF strips can be used to detect the amplified product.[61,62,98,127]

RPA has been applied for detection of human, animal and plant pathogens, including virus, bacteria and parasites, in singleplex and multiplex assays.[98,99] The ease of the procedure, the low working temperature and the short time required for amplification makes RPA a suitable candidate technique for integration into *in vitro* diagnostic devices through microfluidics as POCT or in-field testing.[98,126–128]

The main drawback of this method is that the RPA reagents are only sold by one company, TwistDx™ Limited (Abbot), who owns the patent.[103,129] Other limitations are that it is prone to produce unspecific signals due to primer-dimer formation and that real time RPA is dependent on RPA kinetics.[129]

Strand Displacement Amplification (SDA)

This IA technique needs an exonuclease-deficient DNA polymerase (typically Bst DNA polymerase or Klenow fragment), a restriction endonuclease HincII and four different primers (one pair, E1 and E2, with single-stranded restriction site overhangs, and a second pair known as "bumper primers" to support the displacement of the amplification product from the first set of primers).[95,98] The protocol starts with the annealing of a primer in a DNA breathing site of the template[95,130] or by denaturing the dsDNA (~95 ºC) to allow hybridization with the template of E1 and E2, which have recognition sites for the restriction enzyme and are extended to produce a strand containing those sites. These strands are next displaced by the bumper primers, resulting in dsDNA and displaced single strands from E1 and E2 extensions (Figure 6b). The new nicking sequences are recognized and cleaved by HincII, generating a nick with an extendable 3′ end. The strand-displacing polymerase extends from the nick, causing the displacement of the single-stranded template sequence, where the primers will hybridize again.[95] The exponential amplification is accomplished by coupling sense and antisense reactions, in such a way that the displaced sense strand serve as template for the antisense reaction and vice versa.[99,131] The TAT is around 2.5 h and the results are interpreted through gel electrophoresis, fluorescence methods, molecular beacons[98,99] or LF.[114,130]

The low temperature required for the isothermal amplification (between 30 and 55 °C) makes it prone to unspecific products due to unspecific primer-binding,[99] in line with the other IA methods presented. Nevertheless, SDA has been successfully applied for the diagnosis of tuberculosis (TB), *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG) and herpes simplex virus (HSV) from urogenital samples.[98,99] One of the limitations of the technique is the capability to amplify sequences only between 50 bp to 120 bp long,[95] but on the other hand this feature makes it suitable for miRNA profiling. [98,99,132]

Nicking Enzyme Amplification Reaction (NEAR)

NEAR,[106] also called nicking enzyme assisted amplification (NEAA) or nicking enzyme mediated amplification (NEMA),[133] combines the use of a strand-displacing DNA polymerase and a nicking endonuclease. The endonuclease creates a nick that enables the polymerase to initiate amplification, rapidly producing many short nucleic acids from the target sequence.[134] This process is extremely fast and sensitive, allowing detection of small DNA or RNA fragments in minutes (Figure 6c). The nicking, polymerization, and strand displacement is repeated at 60 °C resulting in linear amplification,[95] but there is also an exponential version of the method (EXPAR), where the product also acts as a primer.[99] The products generated are ssDNA and can be detected using molecular beacons or other probes based methods and LF.[84,135]

The main limitation of the NEAR technique is the formation of a large number of non-specific products, which inhibit the progression of the forward reaction and compromise the detection sensitivity, so it therefore may need more specific amplification analysis methods.[84,133,135] Other shortcoming is the primer design, which needs to include a restriction site, a stabilizing region, and a target-binding region that is complementary to the template.[133,135]

Tests in the market

This increasing interest in the IA techniques is also being reflected in the market, as some *in vitro* diagnostic tests based on IA are reaching commercialization. Table 3 presents these new acquisitions of the market.

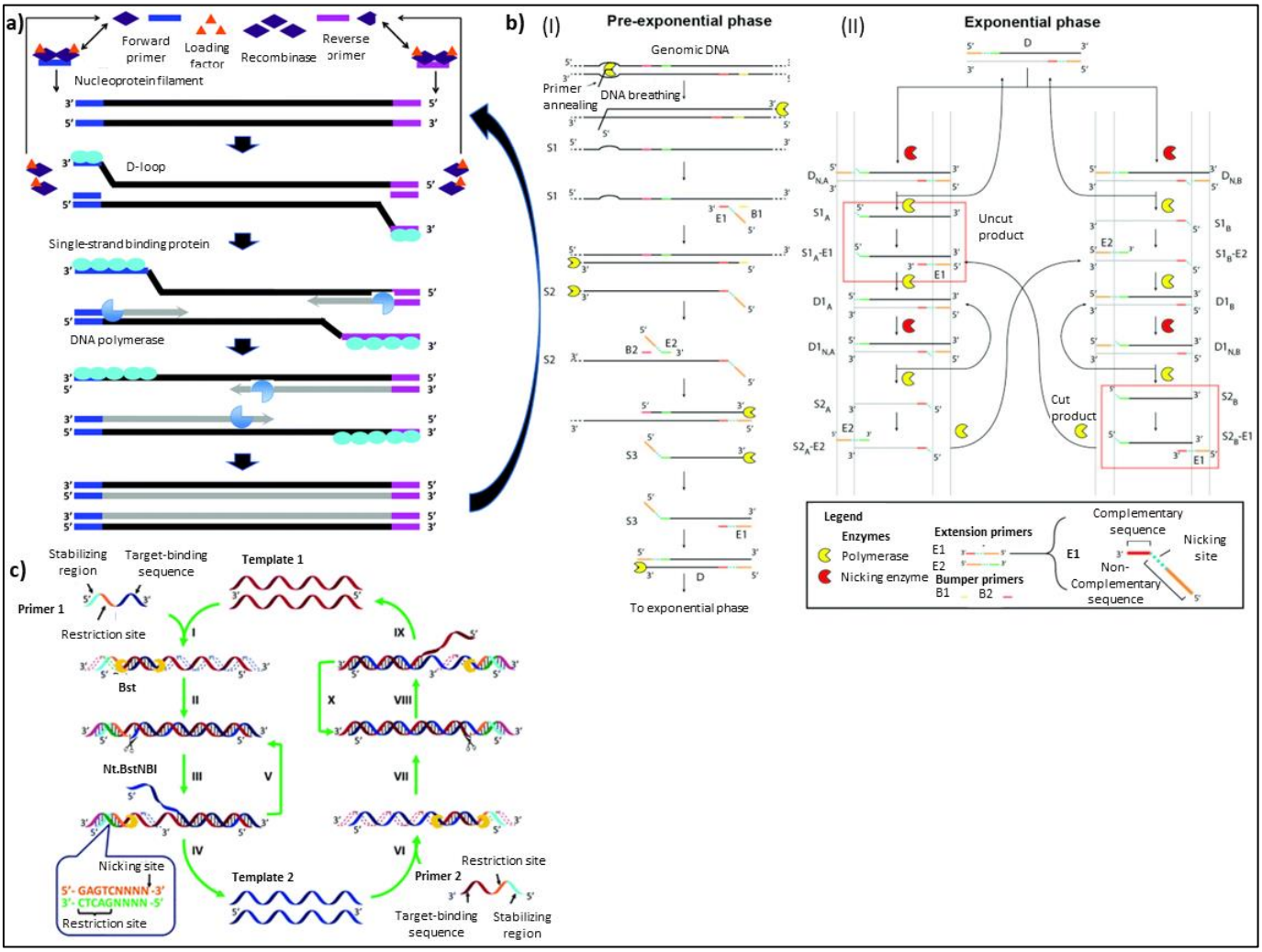

Figure 6: Working principle of some Isothermal Amplification techniques. **a) RPA**, adapted with permission from Li, J.; Macdonald, J.; Von Stetten, F. Review: A Comprehensive Summary of a Decade Development of the Recombinase Polymerase Amplification. *Analyst* 2019, 144 (1), 31–67. (ref. 126). Copyright © (2019) Royal Society of Chemistry. **b) SDA**, modified with permission from Toley, B. J.; Covelli, I.; Belousov, Y.; Ramachandran, S.; Kline, E.; Scarr, N.; Vermeulen, N.; Mahoney, W.; Lutz, B. R.; Yager, P. Isothermal Strand Displacement Amplification (ISDA): A Rapid and Sensitive Method of Nucleic Acid Amplification for Point-of-Care Diagnosis. *Analyst* 2015, 140 (115), 7540–7549. (ref. 130). Copyright © (2015) Royal Society of Chemistry. **c) NEAR**, adapted with permission from Wang, L.; Qian, C.; Wu, H.; Qian, W.; Wang, R.; Wu, J. Technical Aspects of Nicking Enzyme Assisted Amplification. *Analyst* 2018, 143 (6), 1444–1453. (ref. 133). Copyright © (2018) Royal Society of Chemistry.

Table 3. Main *in vitro* diagnostic systems based on IA.

Platform name	Company	IA technology	Detection technology	Setup	TAT	Assays	Regulatory approval
PortNAT®	Ustar Biotechnologies	CAP	LF	all-in-one test cassette	40 min	COVID-19	CE
Veros™	Sense Biodetection	Isothermal exponential amplification	LF	all-in-one test cassette	15 min	COVID-19	CE
ID NOW™	Abbott	NEAR	Real time fluorescence	Cartridges + instrument	Max. 13 min	FluA/FluB RSV Strep A 2 COVID-19	CE and EUA CLIA waived CLIA waived CE and EUA
Solana	Quidel	HDA	Fluorescence	Pre heat sample lysis Cartridges + instrument	25 to 60 min depending on the assay	*Bordetella* *C. difficile* Group A Strep Group B Strep HSV 1+2/VZV Influenza A+B RSV + hMPV SARS-CoV-2 *Trichomonas*	CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and EUA CE and FDA
NUCLISENS® EASYQ®	bioMérieux	Real time NASBA	Beacon probes	Big instrument (8 to 48 samples at the same time)	60 to 150 min depending on the sample number	HIV HPV Enterovirus MRSA	CE and FDA CE CE and FDA CE and FDA
eazyplex®	Amplex Diagnostics GmbH	LAMP	Real time fluorescence	Sample pretreatment Freeze dried ready to use amplification reaction to be used with instrument GENIE® II	15 to 30 min depending on the assay	SARS-CoV-2 *Candida auris* BloodScreen STDs *Pneumocystis jirovecii* PneumoBug expert CSF direct SuperBug CRE SuperBug complete SuperBug expert SuperBug Acineto	All CE

Platform name	Company	IA technology	Detection technology	Setup	TAT	Assays	Regulatory approval
						SuperBug AmpC SuperBug basic MRSA VRE EHEC TyphiTyper *C. difficile*	
ProbeTec™ ET System	BD Diagnostics	SDA	Fluorescence	Big instrument	Up to 46 patient samples in 3 hours	CT/GC *Chlamydia trachomatis* *Neisseria gonorrhoeae* CT/GC *Trichomonas vaginalis* HSV	All CE and FDA
Alethia®	Meridian Bioscience	LAMP	Turbidity	Sample heat pretreatment Meridian Alethia Incubator / Reader	Less than 1 hour	HSV type 1 & 2 *C. difficile* CMV Group A Strep Group B Strep *Mycoplasma pneumonia* *Bordetella pertussis* Malaria *Chlamydia trachomatis* *Neisseria gonorrhoeae*	CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE and FDA CE CE CE
Loopamp™	Eiken Chemical	LAMP	Visual fluores-cence	Strip tubes with lyophilized reagents	40 min	Malaria *Mycobacterium tuberculosis* SARS-CoV-2	CE CE CE
mfloDx™ MDR-TB	EMPE Diagnostics	RCA	LF	Amplification kit + LF kit	75 min approx.	*Mycobacterium tuberculosis* complex and mutations in the rpoB, katG and inhA genes	Under clinical evaluation

FDA: Approved by FDA by premarket notification 510(k), De Novo or Premarket Approval Application (PMA). EUA: Emergency use authorization only by FDA. CE: European conformity, can be CE-IVDD by the Directive 98/79/EC on in vitro Diagnostic Medical Devices or CE-IVDR by the European conformity by Regulation (EU) 2017/746 (IVDR) on in vitro diagnostic medical devices. CLIA waived: Clinical Laboratory Improvement Amendments waiver, which means classified as "simple laboratory examinations and procedures that have an insignificant risk of an erroneous result" by FDA according to requirements (42 CFR Part 493).

As can be seen in table 3, LAMP is, for the moment, the IA method most used in the commercialized tests. Other worthy observation is that half of these platforms are still instrument-dependant, mostly because the detection technology chosen is by fluorescence.

NEW TRENDS

Recently, molecular assays and LF tests have intersected at the diagnostic field, particularly for POCT, with LF being used as the readout technology for nucleic acid testing.[49] The Accula™ system from Mesa Biotech (now ThermoFisher),[136,137] the PortNAT® system from Ustar Biotechnologies[138,139] and the Veros™ test from Sense Biodetection[140] prove it, as they are the first integrated molecular LF *in vitro* diagnostic tests in the market. The three of them use NAAT, integrated in a cassette, and the results are display in a LF strip with a simple workflow of one/two steps from the sample, being suitable as a POCT.

The Accula™ system has assays for FluA/FluB, respiratory syncytial virus (RSV), Group A *Streptococcus* (strep A) and SARS-CoV-2 detection. Accula™ Flu A/Flu B, RSV and Strep A tests have received 510(k) clearance and Clinical Laboratory Improvements Amendments (CLIA) waivers from the FDA. Flu A/Flu B and RSV tests have also obtained CE Mark in the EU. Additionally, the Accula™ SARS-CoV-2 test has obtained EUA from the FDA. Accula™ system uses a PCR NAAT named Oscar™ read by hybridization-based visual detection in a completely automated and self-contained system.[141] The results of Accula™ are displayed in around 30 min but it requires a dock that controls the fluidic and temperatures of the cassette.[136] Video from reference 137 can provide a more clear visualization of the workflow. Accula™ also proved that assays for these kinds of diagnostic tests can be rapidly developed, produced and deployed. On January 23rd, 2020, the company decided to develop an assay for the Accula™ platform for SARS-CoV-2 detection, and by February 14th they had a working assay with synthetic targets. After performing verification and validation studies, the company submitted for the FDA EUA application, which was granted on March 20, 2020.[136] That means a window time of just two months for developing and deploying an *in vitro* diagnostic test for an emerging infectious disease.

The PortNAT® system from Ustar Biotechnologies is an all-in-one test cassette with a reusable incubator which uses CAP isothermal amplification.[95,102] It has a CE marked COVID-19 RNA Test that has a TAT of 40 min (35 min of amplification and 5 min for reading the results in the LF strip).[138,142]

The Veros™ test from Sense Biodetection[140] is also for COVID-19 diagnosis and has received CE mark. The test is based on a patented exponential IA[104] and NA detection methods,[143] all self-contained in a single-use cassette with a TAT of about 15 min. The test has an electrochemical design, lyophilized chemicals and carbon-based readout particles for the LF strip, but needs to be powered with a 1.5 V direct-current battery.

In agreement with this trend, several LF tests for detection of amplified nucleic acid products have been commercialized in the last years, including AmpliVue (Quidel), Amodia EasyFLow® (Amodia Bioservice), HybriDetect (Milenia Biotec), and DCNovations NALF kit (DC Diagnostics).[49] These tests are used as a readout for nucleic acid amplification assays of the corresponding company or as a readout for assays being developed by other companies or research centres.

Coupling an IA technique with a LF platform as a readout allows developing POCT with the benefits of paper-based biosensors and the sensitivity of the molecular assays. Present and future research on these molecular LF tests will focus on the integration of the whole testing process into a microfluidic system,[77,144–146] novel nanomaterials as labels (a.k.a. transducers or reporters)[39,52] and amendment of the specificity of the IA methods.

As previously said, AuNPs clearly stand out as labels for LF tests due their easiness to be integrated in the assays. However, fluorescent nanomaterials may help reaching lower LOD and higher sensitivities, which would permit to quantify the results with improved resolution, but on exchange of the dependence of an external reader. Carbon nanoparticles, as the ones employed in Veros™ (Table 3), have demonstrated improved performance compared with AuNPs,[52,55] and their synthetic routes may be more environmental friendly and sustainable, however the conjugation with bioreceptors is more complicated and the scalability is still very challenging.

Exciting developments have been made in the integration of LF and IA technologies[147] by means of microfluidics. To quote some examples, E. A. Phillips et al.[148] developed a microfluidic rapid and autonomous analysis device (microRAAD) for HIV RNA detection from whole blood. The microRAAD consist of a microfluidic paper-based analytical device (μPAD) with vitrified amplification reagents for RT-LAMP, thermally actuated valves, and a temperature control circuit in a reusable plastic cassette. The RT-LAMP products were visualized in a LFIA (Figure 7a). N. M. Rodriguez et al.[144] created a paper fluidic foldable chip for nucleic acid extraction, IA, and LF detection of human papillomavirus (HPV) 16 DNA (Figure 7b). B. H. Park et al.[149] proposed a rotatory microfluidic system able to integrate DNA extraction, LAMP and LF results readout (Figure 7c).

Lately, paper-based microfluidics and chip-based microfluidics have enabled NAAT to be completed in an enclosed chamber, which avoids manipulation and cross contamination. Advances in dry chemistry to lyophilized reagents eliminate the need for cold-chain transportation and improve stability. The lyophilized enzymes and primers, plus the liquid buffers and the readout technology are being integrated with microfluidics and combined with portable heaters or readers for implementing these integrated molecular LF as a POCT.[114,128,144]

Additionally, the not in vain awarded with the Nobel Prize in Chemistry in 2020,[150] Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technique, has been applied to diagnostic testing.[151,152] CRISPR is known as the "next-generation molecular diagnostic technology" because of its advantages such as easy design and specific identification, thus it is a natural next step to use the LF platform as a readout to bring this highly specific and sensitive technique to the POCT.[49] CRISPR/Cas relies on pre-amplification of target sequences, which are next detected by Cas proteins. Combining the CRISPR/Cas system with an IA technique can improve the sensitivity and solve the specificity issues of the IA.[79,153] The Specific High-sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) and the DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) system are typical combinations of CRISPR and IA. Many assays

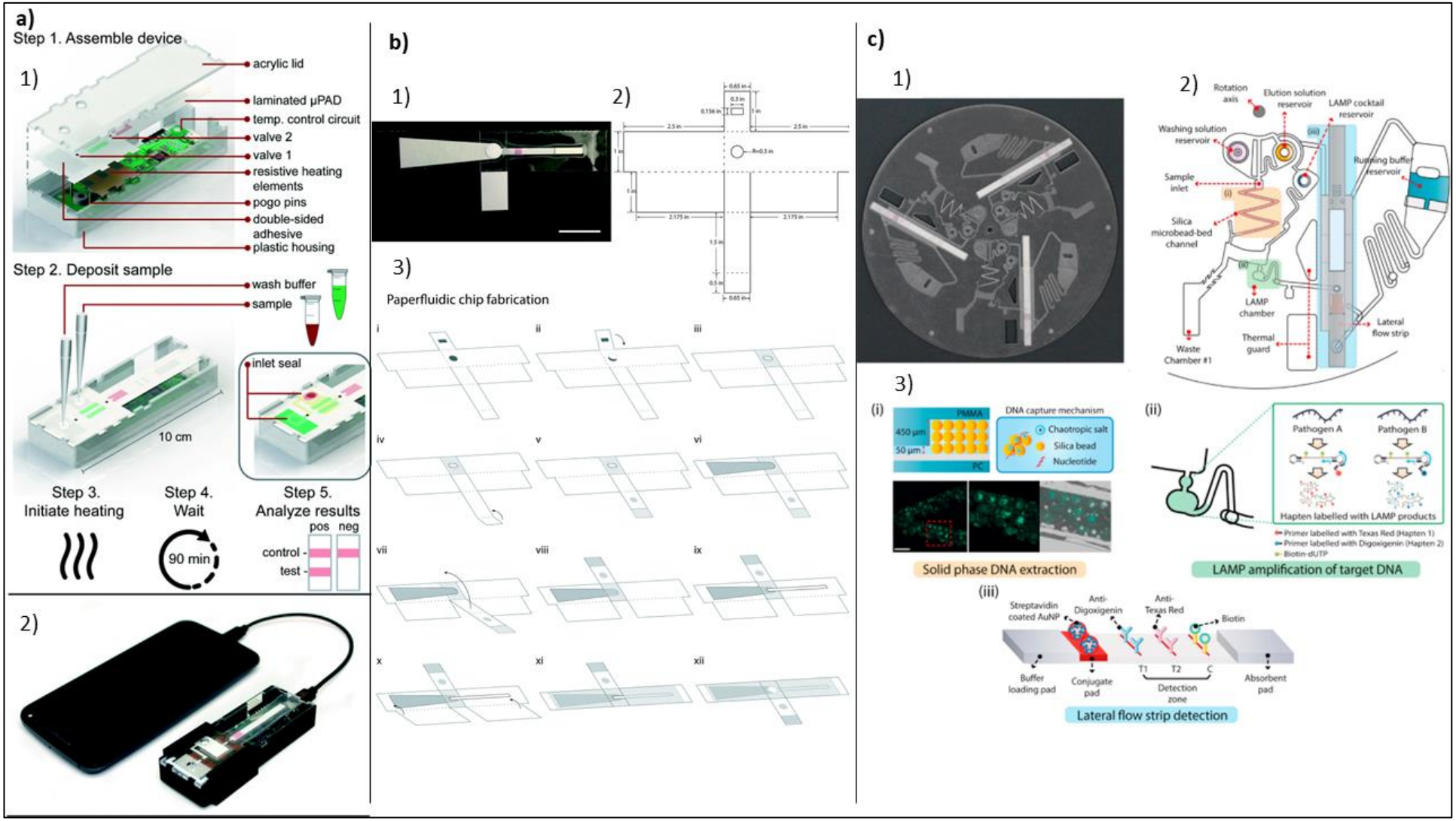

Figure 7: a) 1) Schematic of the workflow of the microRAAD for HIV testing. 2) Photo of microRAAD connected to phone to power the temperature control circuit. Reproduced with permission from Phillips, E. A.; Moehling, T. J.; Ejendal, K. F. K.; Hoilett, O. S.; Byers, K. M.; Basing, L. A.; Jankowski, L. A.; Bennett, J. B.; Lin, L. K.; Stanciu, L. A.; Linnes, J. C. Microfluidic Rapid and Autonomous Analytical Device (MicroRAAD) to Detect HIV from Whole Blood Samples. *Lab Chip* **2019**, *19* (20), 3375–3386 (ref. 148) Copyright © (2019) the Royal Society of Chemistry. **b)** 1) Image of the paper fluidic chip. 2) Drawings and dimensions for the adhesive base of the chip. 3) Schematic of chip fabrication steps. Reproduced with permission from Rodriguez, N. M.; Wong, W. S.; Liu, L.; Dewar, R.; Klapperich, C. M. A Fully Integrated Paperfluidic Molecular Diagnostic Chip for the Extraction, Amplification, and Detection of Nucleic Acids from Clinical Samples. *Lab Chip* **2016**, *16* (4), 753–763. (ref. 144). Copyright © (2016) Royal Society of Chemistry. **c)** 1) A digital image of the rotary device. 2) Schematic of the integrated rotary microdevice for DNA extraction, LAMP, and LF strip detection. 3) (i) Schematic design of the solid phase DNA extraction with microbeads, (ii) Schematic image of the LAMP amplification (iii) Schematics of the LF strip with streptavidin coated AuNPs as a reporter. Reprinted from Park, B. H.; Oh, S. J.; Jung, J. H.; Choi, G.; Seo, J. H.; Kim, D. H.; Lee, E. Y.; Seo, T. S. An Integrated Rotary Microfluidic System with DNA Extraction, Loop-Mediated Isothermal Amplification, and Lateral Flow Strip Based Detection for Point-of-Care Pathogen Diagnostics. *Biosens. Bioelectron.* **2017**, *91*, 334–340 (ref. 149). Copyright © (2017) Elsevier.

have been developed based on the SHERLOCK/DETECTR method with IA such as RPA, LAMP or RCA, being the results interpreted by different signal output methods, including LF.[153,154] It is expected that the union of these combined technologies could be applied in the field of POCT diagnosis in the future.

CONCLUSIONS

In addition to being cheap and easy to manufacture, LF assays (nowadays the most known type of RDT) are rapid, user-friendly, and require no equipment nor battery to work. Although questioned by their sensitivity, they are suitable for POCT, at-home testing, high-frequency testing, low-resource settings, and emergency use. On the other hand, PCR is a powerful technique used to produce multiple copies of a nucleic acid region of interest, but eliminating the need of thermocyclers by means of IA methods, freeing amplification technologies of energy-demanding and technologically complex systems, opens the door also to POCT and in-field applications. Consequently, the application and progresses of IA methods are growing, in a way that in the near future IA could rival PCR.

Shyly, new medical devices that combine the accuracy and sensitivity of a PCR with the speed and ease of RDT are emerging, allowing to overcome the shortcomings of both techniques and bringing together the best of the two methods. However, technical maturity of the IA methods needs to be achieved and, solutions for the unspecific products must be found. In addition, advances in microfluids are rising to the occasion to integrate both in a single device, allowing to take the integrated molecular LF test to the POC.

Lastly, the inclusion of nanomaterials (rather than AuNPs) in the LF platforms allows achieving lower LOD and their properties grant new applications. However, these advances in nanomaterials are still not being reflected in the commercial tests, as the vast majority uses still AuNPs or latex beads. Could be that some limitations still need to be overcome, such as large-scale production or reproducibility/repeatability of the fabrication due to the very complex assays/platforms.

AUTHOR INFORMATION

Corresponding Authors

Daniel Quesada-González - Paperdrop Diagnostics S.L., MRB, Campus UAB, 08193 Bellaterra, Spain; orcid.org/0000-0003-3064-2146; Email: dquesada@paperdropdx.com

Arben Merkoçi - Paperdrop Diagnostics S.L., MRB, Campus UAB, 08193 Bellaterra, Spain; Nanobioelectronics and Biosensors Group, Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC, Barcelona, Spain; The Barcelona Institute of Science and Technology (BIST), Campus UAB, 08036 Bellaterra, Barcelona, Spain; ICREA, Institució Catalana de Recerca i Estudis Avançats, Pg. Lluis Companys 23, 08010, Barcelona, Spain; orcid.org/0000-0003-2486-8085 Email: arben.merkoci@icn2.cat

Present Addresses

†**Daniel Quesada-González** - Nanobioelectronics and Biosensors Group, Catalan Institute of Nanoscience and Nanotechnology (ICN2), CSIC, Barcelona, Spain; orcid.org/0000-0003-3064-2146; Email: daniel.quesada@icn2.cat

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors work for or collaborate with Paperdrop Diagnostics, a for-profit small-medium enterprise. However, all the authors declare neutrality, objectivity, and no conflicts of interest with the publication of this review.

Biographies

Ana Rubio-Monterde works as an R&D Scientist at Paperdrop Diagnostics, where she is an Industrial PhD candidate, and her thesis is being directed by Prof. Dr. Arben Merkoçi and Dr. Daniel Quesada-González. She is a Biologist specialized in Health Biology by Universitat Autonoma de Barcelona and she obtained her MSc in Clinical Analysis Laboratory by Universitat Pompeu Fabra in 2014. She has experience in clinical laboratories in the areas of microbiology, molecular biology, and serology, as well as in the development and verification of *in vitro* diagnostic tests for CE marking and FDA approval (510K). Her research focuses on the design and optimization of molecular rapid diagnostic tests on paper for antimicrobial resistance determinants and emerging diseases.

Daniel Quesada-González, PhD in Chemistry, master in Nanotechnology and Materials Science, graduated in Chemistry. Daniel is the Head of Product Development at Paperdrop Diagnostics, a small-medium enterprise in which he has been working since its inception. He has more than 10 years of experience in the development of LFTs, having published 5 reviews that cover this topic in the last years. Since 2017 he has received more than 1300 citations. Also, he has experience in the synthesis, characterization, and conjugation of nanomaterials, especially for their application in LF tests.

Arben Merkoçi

Prof. Arben Merkoçi is an ICREA Research Professor and leader of the ICN2 Nanobioelectronics and Biosensors Group. He is also the Co-Editor in Chief of the Biosensors and Bioelectronics journal and member of the editorial board of Electroanalysis, Microchimica Acta. Prof. Merkoçi has published 319 articles (H-index/citations: Google Scholar 87/25890; WOS 70/17682) and supervised 35 Ph.D. theses. He serves also as a scientific evaluator and member of panels of experts of various international governmental and nongovernmental agencies (EU-FP and EU-ERC panels and other panels in Europe, USA, and other countries) and is the cofounder of two spin-off companies: GraphenicaLab, devoted to graphene patterning, and Paperdrop Diagnostics, dedicated to clinical diagnostics.

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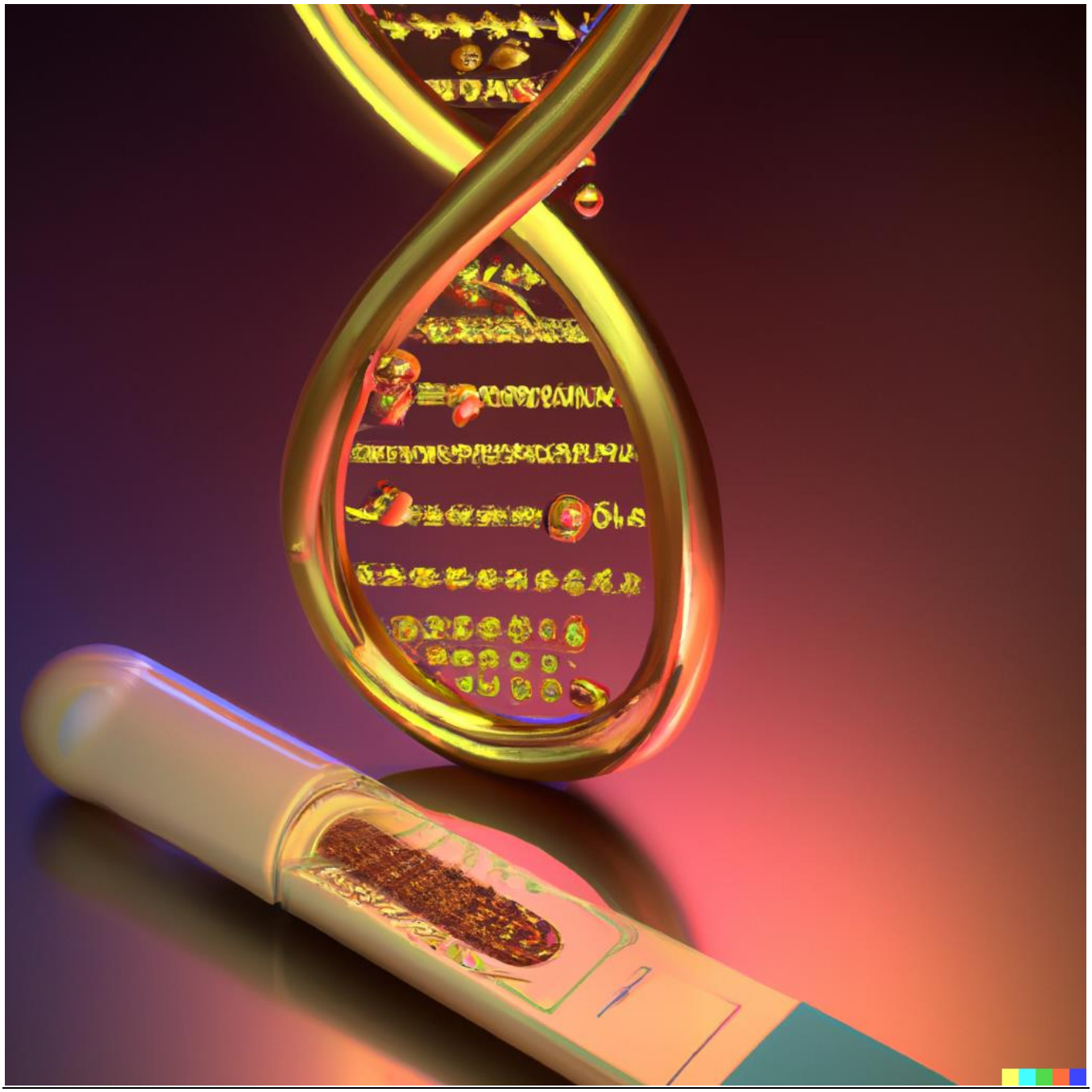

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