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Rolling Circle Amplification (RCA) On-Chip towards
COVID-19 molecular diagnostics

João Carlos Varela

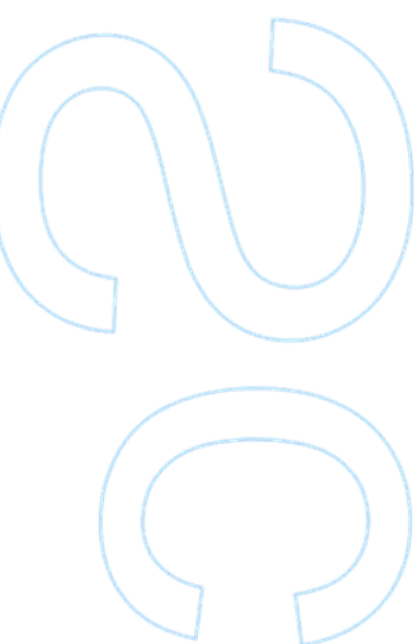


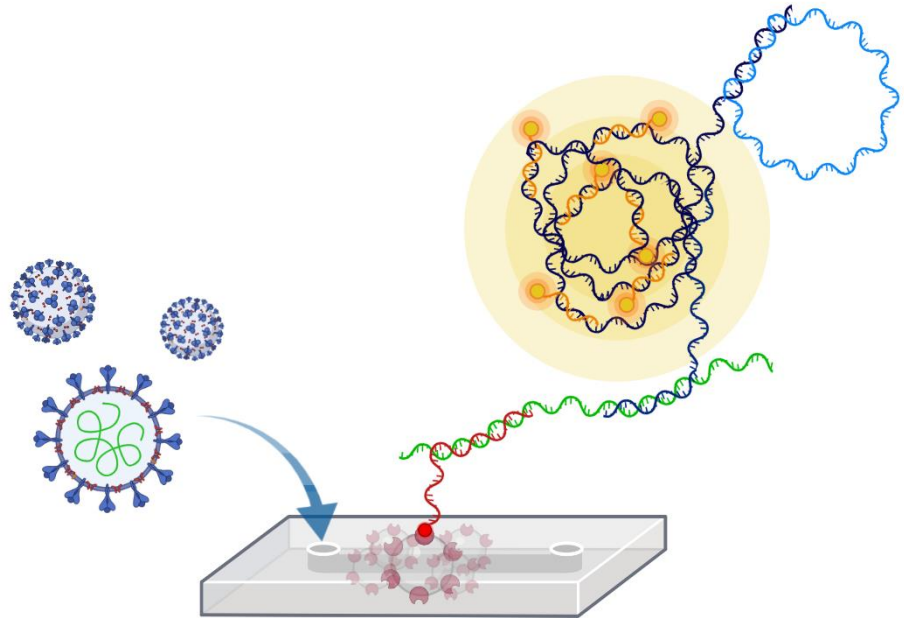
Rolling Circle Amplification (RCA) On-Chip towards COVID-19 molecular diagnostics

João Carlos Varela

Master's thesis in Biochemistry presented to
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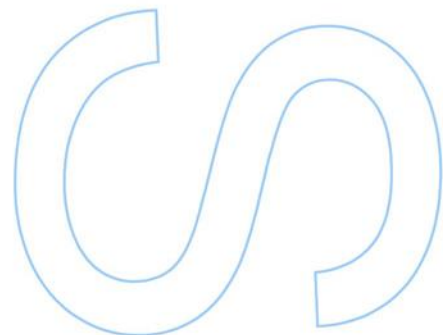
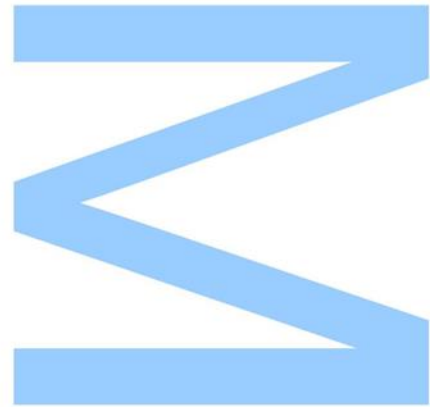
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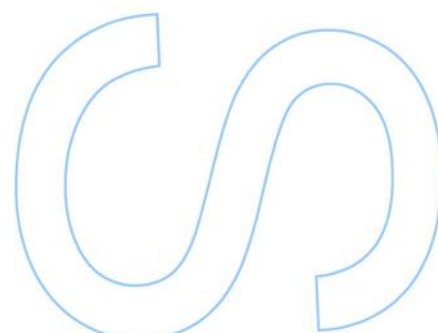
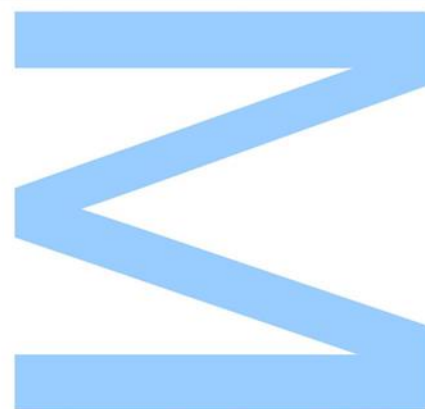




Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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“Those who pass by us, do not go alone, and do not leave us alone;

They leave a bit of themselves, and take a little of us.”

- Antoine de Saint-Exupery

ABSTRACT

The SARS-CoV-2 pandemic, that resulted in nearly 225 million confirmed infections and 4,6 million deaths all over the world one and a half years after the discovery of the virus in December 2019, clearly shows the limitations that still exist in the diagnostic and management of infectious diseases. The rapid spread of COVID-19 is fuelled by the high infectivity of the virus, combined with absent or mild flu-like symptoms in most of the population below 60 years of age, allowing infected patients to spread the virus before a confirmatory diagnostic. Current SARS-CoV-2 diagnostic tests do not yet provide an adequate balance between high-frequency screening (daily) and cost-effectiveness, while also not being accessible for routine use in resource-limited settings, hindering the necessary coordinated global response to contain the pandemic. Thus, novel cost efficient, efficient, sensitive, and rapid diagnostic methods are required to effectively reduce viral spread imposing targeted lockdown measures.

Rolling Circle Amplification (RCA) coupled with padlock probes (PLPs) is an isothermal nucleic acid amplification method that has shown significant potential for pathogen diagnostics, providing adequate sensitivity and single base pair specificity while being performed at a low constant temperature of 37°C. When combined with microfluidic detection devices, this method has already been able to reach sub-attomole sensitivity in viral detection, showing the potential implementation in cost-effective Point-of-Care platforms.

This work reports novel RCA-based methods for SARS-CoV-2 diagnostic without the need of an initial reverse transcription step, based on the hybridization of the viral RNA with biotinylated oligos and L-probes in solution, captured in a microchannel and subsequently amplified *in situ* using PLPs against the L-probes. A viral subtyping methodology was also developed, allowing direct detection of SARS-CoV-2 RNA in microfluidics, where “chimeric” PLPs hybridize directly with the RNA. Importantly, the hybridization-based RCA method allowed the detection of 1×10^3 viral copies/ μL within 90 minutes of amplification, demonstrating the potential of an alternative approach to both current COVID-19 diagnostics and isothermal amplification methods.

Keywords: Diagnostics, Rolling Circle Amplification, Padlock Probes, SARS-CoV-2, COVID-19

RESUMO

A pandemia do SARS-CoV-2, que provocou perto de 225 milhões infeções confirmadas e cerca de 4,6 milhões de mortes por todo o mundo em apenas um ano e meio após a sua descoberta em dezembro de 2019, mostra claramente as limitações ainda existentes no diagnóstico e gestão de doenças infecciosas. A rápida propagação do COVID-19 resulta da alta infetividade deste vírus, que quando combinada com a total ausência ou presença de sintomas ligeiros semelhantes à gripe na maioria da população com menos de 60 anos de idade, permite que indivíduos infetados propaguem o vírus antes de um diagnóstico positivo. Os testes atualmente usados no diagnóstico do SARS-CoV-2 não proporcionam ainda um balanço adequado entre uma testagem com elevada frequência (diária) e elevada razão custo-benefício, para além de não estarem ainda disponíveis para diagnósticos de rotina em locais com recursos limitados, dificultando a resposta coordenada necessária para combater a pandemia. Assim sendo, são necessários novos métodos de diagnóstico rápidos, baratos, eficientes e sensíveis para reduzir a propagação viral através de medidas de “lockdown” direcionadas.

A Amplificação em Círculo Rolante, ou “Rolling Circle Amplification” (RCA), quando combinada com “Padlock Probes” (PLPs), é um método de amplificação isotérmica de ácidos nucleicos que já mostrou potencial no diagnóstico de patógenos, garantindo uma sensibilidade adequada e especificidade ao nível de um único par de bases a uma temperatura baixa e constante de 37°C. Quando combinada com dispositivos de deteção microfluídicos, este método é capaz de atingir limites de deteção ao nível sub-atomolar, demonstrando a sua potencial implementação em plataformas portáteis de baixo custo.

Neste trabalho é apresentado um novo método de diagnóstico baseado no RCA sem a necessidade de um passo inicial de transcrição reversa, através da hibridização do RNA viral com oligonucleotídeos biotinilados e “L-probes” em solução, capturados num microcanal e subsequentemente amplificados *in situ* usando PLPs direcionados para os “L-probes”. Uma técnica para a identificação de variantes virais também foi desenvolvida, permitindo a hibridização direta de PLPs “quiméricas” com o RNA. Este novo método de diagnóstico com mostrou-se capaz de detetar 1×10^3 cópias virais/ μL em 90 minutos de amplificação, apresentando-se como uma potencial alternativa tanto ao diagnóstico de COVID-19 como aos métodos de amplificação isotérmica.

Palavras-chave: Diagnóstico, Rolling Circle Amplification, Padlock Probes, SARS-CoV-2, COVID-19

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LIST OF ABBREVIATIONS

ACE2 – Angiotensin I-Converting Enzyme 2

C2CA – Circle-To-Circle Amplification

cDNA – Complimentary DNA

CT – Chest Tomography

DEPC-water – Diethyl Pyrocarbonate-treated Water

DETECTR – DNA Endonuclease-Targeted CRISPR *trans* Reporter

DO – Detection Oligonucleotide

dsDNA – Dual-strand DNA

ELISA – Enzyme-Linked Immunosorbent Assay

ELOSA – Enzyme-Linked Oligosorbent Assay

ERGIC – Edoplasmatic Reticulum – Golgi Intermediate Compartment

HDA – Helicase-Dependent Amplification

HybRCA – Hybridization-based Rolling Circle Amplification

LAMP – Loop-Mediated Isothermal Amplification

LoD – Limit-of-Detection

NAAT – Nucleic Acid Amplification Testing

NASBA – Nucleic Acid Sequence-Based Amplification

nsp – Non-Structural Proteins

On-Chip RCA – On-Chip Rolling Circle Amplification

ORF – Open Reading Frame

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PDMS – Poly(dimethylsiloxane)

PLP – Padlock Probe

PoC – Point-of-Care

qPCR – Quantitative Polymerase Chain Reaction

RCA – Rolling Circle Amplification

RCP – Rolling Circle Product

RdRp – RNA-dependent RNA polymerase

RO – Restriction Oligonucleotide

RPA – Recombinase Polymerase Amplification

RT-PCR – Reverse Transcription Non-Quantitative Polymerase Chain Reaction

RT-qPCR – Reverse Transcription Quantitative Polymerase Chain Reaction

SARS-CoV-2 – Severe Acute Respiratory Syndrome Coronavirus 2

SHERLOCK – Specific High-sensitivity Enzymatic Reporter un-LOCKing

SMART – Signal-Mediated Amplification of RNA Technology

SplintR – PBCV-1 DNA ligase/Chlorella virus DNA ligase

SSC – Saline-Sodium Citrate

ssDNA – Single-strand DNA

ssRNA – Single-strand RNA

T4Rnl2 – T4 RNA Ligase 2

TMA – Transcription Mediated Amplification

TMPRSS2 – Type II Transmembrane Serine Protease

WHO – World Health Organization

χRCA – Chimeric-Padlock Probe-based Rolling Circle Amplification

INTRODUCTION

1. RELEVANCE, DEVELOPMENT AND CHALLENGES OF MODERN MEDICAL DIAGNOSTICS OF INFECTIOUS DISEASES

Diagnostics are ubiquitous in modern medicine. Identifying a certain disease accurately and at early stages is essential to initiate proper treatment, prevent spread of infectious diseases and worsening of patient state. Diagnostics play a key role in this and have evolved beyond just correlating certain symptoms to a disease [1].

Culture and microscopy-based methods have been used for a very long time to identify and treat various infectious diseases [2]. Upon growing a high enough number of pathogens for testing, various bioassays can be performed to better determine the cause of disease [3]. Although those methods are still the gold-standard techniques for diagnosis, for example, of urinary infections and tuberculosis (culture method can take up to 42 days) [4], those are very expensive and time-consuming, showing the necessity of cheaper and faster diagnostic methods [5]. Means of identifying new and different strains of a pathogen quickly and accurately are also important, since more pathogenic variants of the disease may arise due to the highly variable genome of some microorganisms.

Diagnostic techniques can detect the pathogen via surface receptors or antigen detection, a part of the pathogen (ex: proteins, DNA, RNA), host antibodies against the pathogen, or other biomarkers that may indirectly suggest infection (ex: reactive C-protein levels in the blood) [6, 7]. Detection of said biomarkers provide better insight upon the patient state of disease than the sole analysis of symptoms, while being faster and cheaper than culture and microscopy-based methods [8].

Considering the various different ways a pathogen can be detected, it is of uttermost importance to choose which patient sample (ex: saliva, blood) should be collected and tested, and which biomarker or antigen should be targeted. However, attention should be made to a variety of aspects when selecting the samples. The collected sample can contain other molecules that can interfere with the test results or might not be representative of the total pathogen load in any part of the body [9]. The quality of the sample should also be considered, since poorly collected or contaminated samples can provide false results for the diagnosis. Thus, the type and collection of the sample, along with any necessary processing or purification, are an essential part of any diagnostic test, regardless of the method [10].

1.1. Mass Spectrometry and Immunological Assays as reliable Diagnostic Methods

Detection of pathogens can be done directly, avoiding culture and microscopy methods. **Mass spectrometry**, a highly specific and sensitive method, requires minimal sample preparation [11]. This method can determine the mass to charge ratio (m/z) by relying on the production, selection, fragmentation, and detection of charged ion species in a patient sample [12]. Due to the quick sample preparation and analysis of this method, done in just a few minutes, options for analysis in resource-limited settings have been developed [13].

Immunological assays have also been developed to diagnose various diseases, and their simplicity and ability to rapidly detect a certain antigen with minimum sample preparation quickly made them one of the clinical microbiology analysis gold standards. Pathogen surface proteins/receptors or host antibodies can be detected depending on the pretended diagnosis. Direct detection of the pathogen provides unambiguous proof that a patient is infected, but a high enough concentration of the antigen needs to be present in the sample, and slight variations on the surface proteins due to mutations will result in false-negative results. On the other hand, tests targeting host antibodies are unable to detect active viral shedding during the early stages of infection, thus being more useful to understand if someone had had previous contacts with said pathogen.

One immunological assay commonly used in centralized clinical laboratories for infectious disease diagnosis is the **Enzyme-Linked Immunosorbent Assay (ELISA)**, where the target antigen is captured using specific antibodies that can provide subsequent colorimetric or chemiluminescence signal [14, 15]. Those methods may also be combined with lateral flow assays, making simple easy-to-use and possible over-the-counter diagnostic tests¹⁶, even though the miniaturization of said methods tends to hinder its sensitivities. Despite those great advantages, the observed sensitivity in immunoassays for antigen detection is limited by the quality of the antibody used. This often requires those assays to run in optimal conditions at centralized laboratories or hospitals, with high-throughput machines, to allow for highly reproducible and viable results, increasing the testing costs and limiting the accessibility to the diagnosis [17, 18].

Presented methods do provide reliable ways of patient diagnosis, but often lack the sensibility and specificity when not performed in centralized hospitals or laboratories. Not only that, but difficult accessibility to certain biomarkers at high enough

concentrations, resulting in the incapability to detect early stages of infection, require new approaches to diagnosis. As such, new diagnostic methods were developed based on the molecular fingerprinting of pathogens – each possessing unique genetic material that differentiates them from each other.

1.2. Nucleic Acid Amplification Testing (NAAT) and Polymerase Chain Reaction (PCR)

Nucleic Acid Amplification Testing (NAAT) consists in the detection of amplified genetic material from a certain pathogen, DNA or RNA, relying on sequence-specific hybridization instead of phenotypical characterizations, greatly shortening the time necessary for diagnosis [19]. Targeting the pathogen genetic material greatly improves the specificity of said methods, since genome sequences typical of certain pathogens are usually conserved across the species. Also, because NAAT requires amplification of immense amounts of nucleic acids in a very short time, sensitivity is greatly increased, allowing for pathogen detection soon after the infection, even before clinical illness is noticeable [20].

One of the most widely used NAAT methods is the **Polymerase Chain Reaction (PCR)**. This technique allows for the exponential amplification of a selected genetic target using two small 20-22 bp oligonucleotide primers complementary to the ends of the target, a thermostable DNA polymerase, and multiple high temperature heating and cooling cycling rounds. First, template denaturation occurs, then primers anneal, and finally the extension of annealed primers is done by the DNA polymerase. Multiple cycles can be performed to increase the target yield up to a million-fold in just one hour [21], greatly increasing its sensitivity. The heating and cooling cycles, requiring temperatures as high as 95°C, provide high specificity to this assay by preventing mismatch hybridization. The small oligonucleotides primers complementary to the target also contribute to this, since amplification will only occur if the annealing is successful.

Various variants of the aforementioned method have since been developed to make it more suitable for clinical diagnostics. Quantitative Polymerase Chain Reaction (qPCR) has made a break-through advancement for PCR diagnostics by allowing the real-time detection of PCR products. Through the use and hybridization of fluorescent intercalating dyes, fluorescent labelled probes, fluorescent intercalators or molecular beacons to the amplified products during the various amplification cycles, it is possible to quantify the amplified target [22]. Reverse Transcription Quantitative and Non-Quantitative Polymerase Chain Reaction (RT-qPCR and RT-PCR) are other important

variants of PCR, being able to detect RNA once a reverse transcription step originates complementary DNA (cDNA). This method is especially important since it allows the detection and quantification of RNA viruses [23]. The inclusion of degenerate bases in certain primer positions have also allowed a broader detection of genetic variants of the target pathogens [24].

PCR has been used for the diagnosis of several fungal [25], bacterial and viral infections [26, 27], mainly due to its low Limit-of-Detection (LoD) of < 1 copies/ μL [28]. The great sensitivity and rapid turn-around times when compared to other assays made PCR and its multiple forms constitute the current gold standard in the diagnosis of various infectious diseases.

Despite the mentioned advancements and advantages, the machine-heavy power-intensive thermocycling requirements of PCR-based methods, alongside the necessity of highly skilled personnel to perform and interpret the test results, severely hinders the application of this method in resource-limited settings [6, 18]. As such, many efforts have been done to develop new alternative strategies to bring NAAT to wider and broader settings, outside of centralized healthcare centres and laboratories.

1.3. Isothermal Nucleic Acid Amplification

Isothermal Nucleic Acid Amplification has emerged as an alternative to the heavy thermocycling-based methods, since isothermal amplification allows fast and efficient amplification at constant, typically not very high temperatures in the range of 37°C to 65°C , which can easily be provided by simple heat generators found in resource-limited settings (ex: water baths), thus eliminating the need of any complex instrumentation [29]. Various simple and rapid isothermal nucleic acid amplification methods have been developed, relying on distinct enzymatic activities, primers and probes, to better approach diagnostic in those broader settings. Below, specific examples of isothermal amplification methods are briefly presented and discussed.

Helicase-Dependent Amplification (HDA) is a method very similar to PCR, since it is based on the same three-cycle process: template separation, primer annealing, and extension of the primers. A schematic of HDA is shown in **Fig. 1**. This methodology excludes the temperature cycling process for the template separation step by using a DNA helicase enzyme, and single-strand binding proteins to stabilise the single-strand genetic template and facilitate primer annealing and elongation. All those steps occur at constant temperature in a continuous cycle [30], and amplified products can be

visualised, just like other PCR-based methods, in real-time by fluorescent intercalators or molecular beacons or, as an end-point, by agarose gel electrophoresis. This method has been used to detect various protozoan, bacterial and viral infections, like *Clostridium difficile*, *Trichomonas vaginalis*, and Herpes Simplex Virus, respectively. Commercial diagnostic kits for the detection of various bacterial and viral pathogens based on HDA have also been developed, namely for various Streptococcus species and Influenza A and B [31, 32]. Although this method can achieve over a million-fold amplification, it is prone to amplify non-specific products due to primer mispairing due to the low assay temperatures, although this can be partly overcome by computed primer design [32].

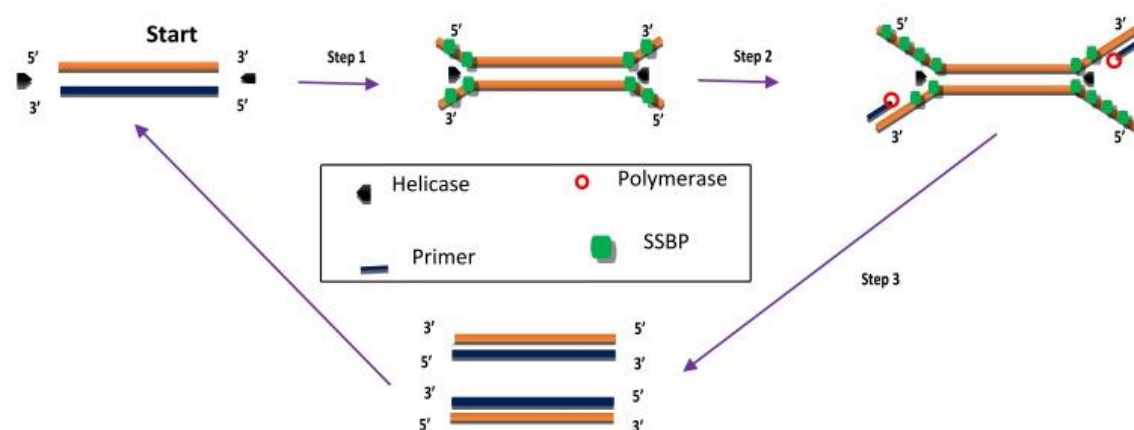


Fig. 1 – Schematic representation of Helicase-Dependent Amplification (HDA) as presented in G. A. Obande, and K. K. B. Singh (2020) [32].

Loop-Mediated Isothermal Amplification (LAMP), schematized in **Fig. 2**, is a rapid, highly sensitive and specific isothermal amplification method that uses DNA polymerases with high strand displacement activity and 5'-3' exonuclease activity, requiring a set of four to six primers. The amplification template is generated from target DNA by using inner and outer primer pairs that form dumbbell-like single-strand DNA (ssDNA) loop structures [33]. By adding a reverse transcription step to the LAMP assay (RT-LAMP), it is possible to amplify and detect RNA templates. This method has allowed detection of several RNA virus, namely HIV [34], Zika [35], and SARS-CoV-2 [36]. Products of this amplification can be detected by gel electrophoresis, fluorescence detection, or direct detection by turbidity due to the accumulation of magnesium pyrophosphate, an end product of this amplification process. The use of fluorescent dual-strand DNA (dsDNA) intercalating dyes to enhance visual detection, alongside the fast (only a few minutes in some cases) and precise diagnostic, makes this method suitable for resource-limited settings testing, and various commercial products are available for the detection of various bacterial and viral pathogens. However, the complexity of this assay that arises from the use of multiple primers makes non-specific primer

hybridization prone to happen often, and although software for LAMP primers design exist, empirical testing is still required, since designed primers can often result in no-template amplification due to primer dimers or non-specific amplification [32].

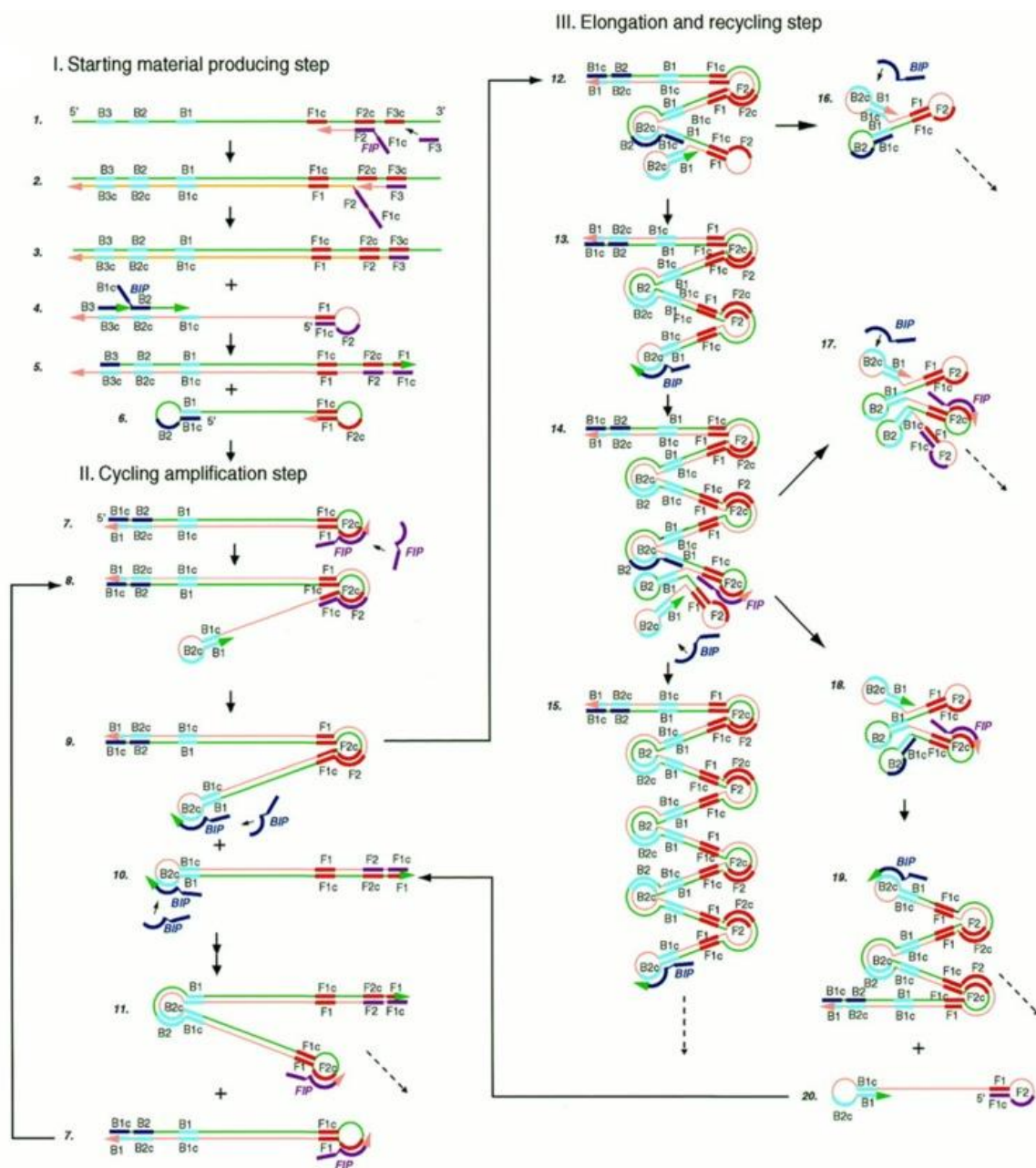


Fig. 2 – Loop-Mediated Isothermal Amplification (LAMP) schematized in G. A. Obande, and K. K. B. Singh (2020) [32].

Nucleic Acid Sequence-Based Amplification (NASBA) centres around the amplification of single stranded RNA (ssRNA) in a self-sustaining system using a T7 DNA dependent RNA polymerase, RNase H, and an avian myeloblastosis virus reverse transcriptase. The method, shown in **Fig. 3**, starts by converting ssRNA into cDNA through reverse transcription using a primer with T7 promoter regions. A second primer hybridizes to the cDNA once the RNA is degraded by the RNase H, allowing it to be used as a template for the reverse transcription, producing various copies of antisense RNA with T7 RNA polymerase promoter sequences. Those can be used as cDNA template once again and be further amplified, up to a 10^9 -fold amplification within 90 mins at 41°C [37]. NASBA has been mainly used for viral detection, namely for the influenza virus [38] and HIV, with the latter being available in a commercial kit [39]. Amplification products can be detected through gel electrophoresis, enzyme-linked gel assay, and electro-chemiluminescence. DNA targets can also be amplified, requiring two denaturation cycles at 95°C , making this method not ideal for DNA detection in resource-limited settings. NASBA also requires various RNase-free conditions to ensure the integrity of the sample and can only efficiently amplify sequences with 100 to 250 bp [32], sometimes requiring a restriction step of viral sequences before amplification takes place.

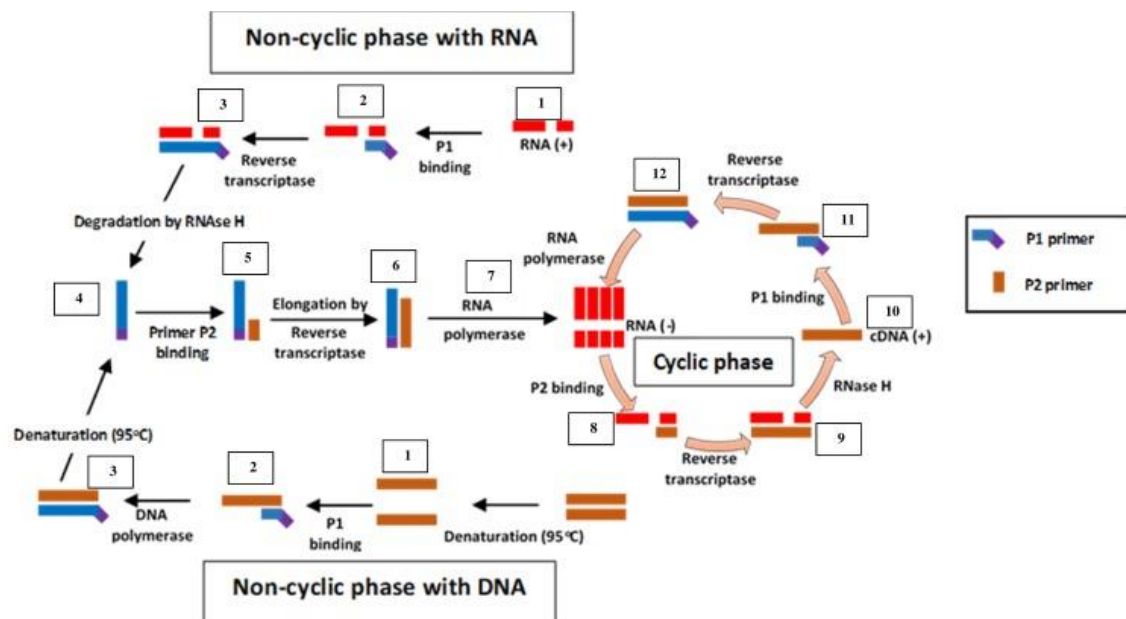


Fig. 3 – Schematic of Nucleic Acid Sequence-Based Amplification (NASBA) in G. A. Obande, and K. K. B. Singh (2020) [32].

Transcription Mediated Amplification (TMA) uses the same principles as NASBA, but makes use of the RNase reverse transcriptase activity, employing only two enzymes instead of three, thus reducing the cost of the assay [40]. As such, TMA has been employed in several commercial diagnostic kits for the detection of various bacterial and viral pathogens responsible for several sexual transmitted diseases, namely *Chlamydia trachomatis*, *Mycoplasma genitalium*, HIV-1, HBV and HCV [32].

Rolling Circle Amplification (RCA) is a linear isothermal amplification method that relies on the unique activities certain polymerases have on circular templates. The ground-breaking discovery of the Padlock Probe (PLP) – a linear oligonucleotide composed by two target complementary arms/ends (approximately 20 bp each) connected by a 40-50 bp long non-complementary backbone – allowed this assay to take advantage of the ligation reaction of this probe, thus increasing its specificity. The probe arms possess a free phosphorylated group at the 5' end and a hydroxyl group at the 3' end, while the PLP backbone has detectable sequences, non-complementary to the target, usually around 20 bp long [41].

PLP-based isothermal RCA is thoroughly explained in **Fig. 4.** and starts with PLP hybridization, followed by ligation and PLP circularization, and finally the amplification [42].

First, the PLP complementary arms hybridize to the target in a juxtaposed manner that, if correctly hybridized, allows a DNA ligase to form a phosphodiester bond between the phosphorylated group at the 5' end and the hydroxyl group at the 3' end, ligating the PLP and turning the previously linear probe into a circle. Specificity of RCA relies mainly on this step: since PLPs are sequence selective, simultaneous hybridization of both arms is necessary for ligation to take place, and any mismatches preventing ligation to occur will also result in no amplification. In the case of non-specific hybridization, the use of high-fidelity DNA ligases, such as the *Tth* DNA ligase with a high temperature activity, prevents ligation from occurring if there are mismatches around the ligation junction. The specificity offered by the hybridization combined with the ligation step is what sets PLP-based RCA from other isothermal amplification methods. The high-fidelity property of the ligase also allows the detection of single nucleotide polymorphisms [43] using various PLPs with just one single different nucleotide in one of the complementary arms, and different detectable sequences on the backbone.

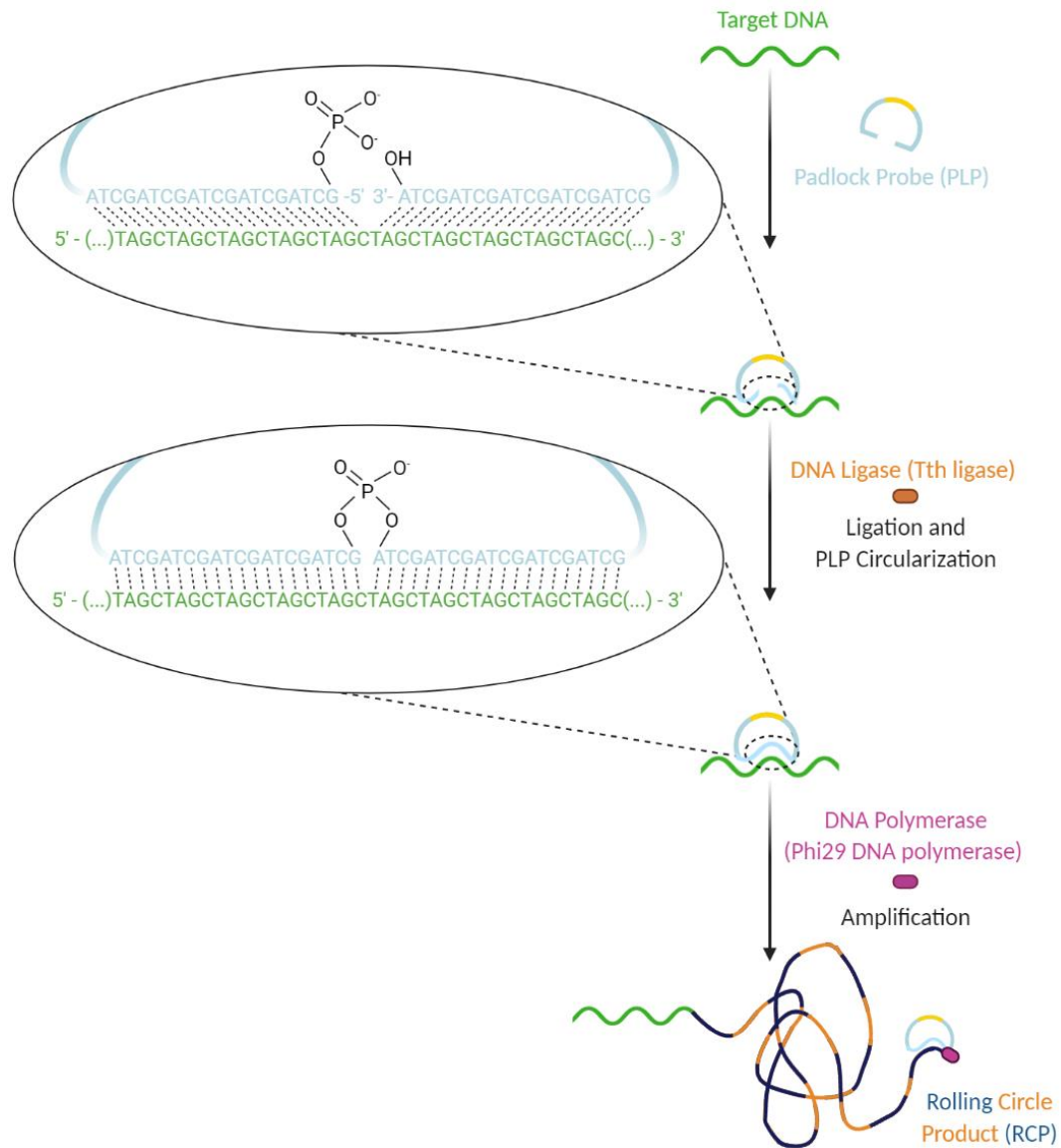


Fig. 4 – Schematic representation of Padlock Probe (PLP)-based isothermal Rolling Circle Amplification (RCA). Upon correct hybridization of the PLP with the DNA target, ligation and consequent circularization of the PLP occurs, and the circle template is amplified several times, originating a long single strand DNA product with tandem repeats of the complementary PLP sequence. The repeats of the complementary detection sequence from the PLP allow the amplification products to be efficiently labelled with detection oligonucleotides containing fluorophores or other detection molecules.

Upon proper ligation, a strand-displacing DNA polymerase can be provided to amplify the PLP several times, originating the Rolling Circle Product (RCP), a typically very long ssDNA with tandem repeats, that tends to spontaneously collapse into μ m-sized DNA coils. If the ligation is not successful, amplification cannot take place. The sensitivity of this assay is highly dependent on the processive polymerase amplification, namely of the phi29 DNA polymerase. This enzyme possesses a high processivity, with

polymerization rates that produce around 1000 copies of a circular template in one hour, while also having a 5' to 3' proofreading exonuclease activity [44].

Finally, RCPs can be labelled using small detection oligonucleotides (DOs) with the same sequences present by design on the PLP backbone, attached to molecules that allow detection of the amplification products – like fluorophores that enable microscopic detection and digital counting of RCPs or average signal quantification [32] – or molecules that aid the capture of those products – for example, biotinylated DOs to allow the capture of RCPs in streptavidin beads [45].

RCA has already been exploited for viral diagnostics and subtyping, namely of HIV [45], Zika [46], Ebola and Dengue [47] and Influenza [48], but there are still no RCA-based products commercially available for pathogen diagnostics.

RCPs are detected mainly through fluorescent microscopy, either by digital measurement directly on positively charged glass slides [49], using a digital enrichment membrane [47], or through analog enrichment in microbeads [45, 46]. RCA products may also be detected without requiring the labelling step, using gel electrophoresis [32], although such detection process is more time consuming, requires sophisticated methodologies, and is difficult to implement in a resource-limited diagnostic setting when compared to the digital and analog quantification options that arise once the labelling step is done.

Various other RCA-based methods have also been developed, achieving low and clinically relevant limits-of-detection when compared to the regular RCA, being those Cascade RCA [50], Exponential RCA [51], Hyperbranched RCA [52], and Ramified RCA [53]. Although the reaction schemes vary slightly between the methods, all of them result in branch-like amplicons and increased amplification kinetics. Another method that also typically achieves higher sensitivities when compared to classical RCA is **Circle-To-Circle Amplification (C2CA)** [46], schematized in **Fig. 5**.

In C2CA, two consecutive rounds of RCA are performed. The initial round is done just like regular RCA, but restriction motifs are present alongside the detectable sequences in the backbone of the PLPs. During the first labelling step, small oligonucleotides complementary to the restriction motifs – restriction oligos (ROs) – are provided. Upon labelling, restriction enzymes can recognize and cleave areas where ROs have hybridized, fragmenting the RCPs into several small monomers. Those small RCP monomers can re-circularize by hybridizing with an excess of ROs, and then be ligated and amplified once again. This allows for an initial target to generate several hundreds or thousands of RCA products, depending on the time of the first amplification,

increasing the sensitivity of the assay up to 1000 times per hour on the second round of amplification [46]. Multiple amplification cycles can be performed, increasing the sensitivity of the assay, but making it more time-consuming.

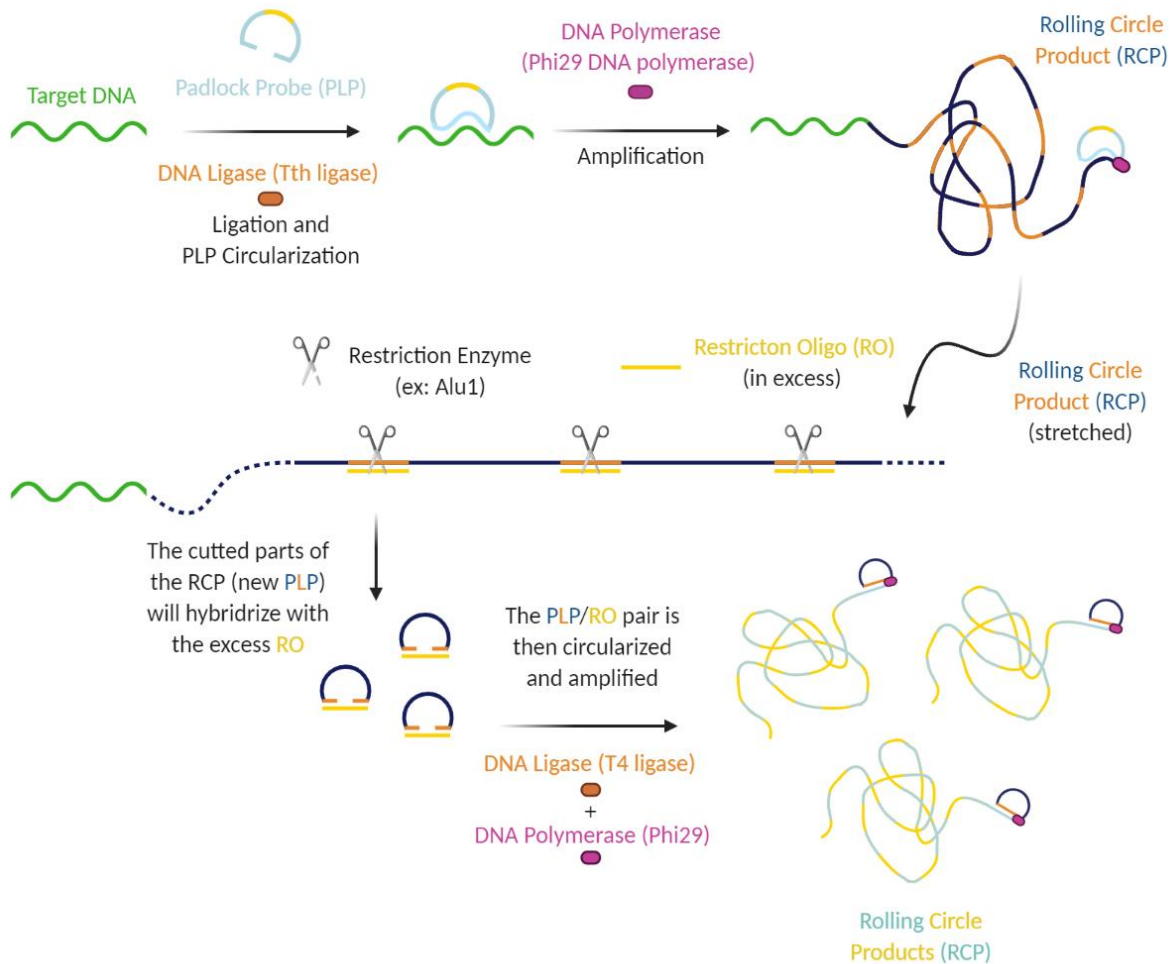


Fig. 5 – Schematic representation of Circle-to-Circle Amplification (C2CA). Upon a first round of RCA, the initial RCPs can be labelled with restriction oligonucleotides (RO) instead of detection oligonucleotides for restriction enzymes to cleave the amplification product. The small RCP monomers will then hybridize with an excess of RO in a PLP-like way and can be circularized and amplified – second round of RCA – originating even more RCPs.

RCA presents several advantages when compared to other amplification methods, namely the lack of very high strict temperature regulation requirement while still providing adequate sensitivity and single base pair specificity. This, alongside the lack of machine-heavy complex steps, makes this method amenable to implementation in cost-effective platforms for resource-limited settings diagnosis. The simplicity and versatility of this assay also allows for customization and tailoring of detection through the repeated sequences of the RCPs, and the ability to be performed in liquid or solid matrixes, and both *in vivo* and *in vitro*. This method can also easily be used for

multiplexed assays, either for the detection of multiple diseases or different pathogen strains [32, 42, 43, 48]. This is the main advantage of RCA, since no other method allows multiplexing to such an extended level without requiring Next Gen Sequencing.

However, RCA also presents several drawbacks, namely the arouse of linear multimeric by-products if the ligation step is not done properly, and the limitations in terms of degree of amplification when compared to exponential methods [32].

Recombinase Polymerase Amplification (RPA), presented in **Fig. 6**, consists of the use of a recombinase and a DNA polymerase alongside DNA-binding proteins for target amplification by catalysing primer hybridization with homologous target sequences. The recombinase enzyme can aid RPA primers to their specific site, allowing their hybridization to the target, while also separating the dsDNA into ssDNA templates. Upon proper binding of the primers and strand displacement of dsDNA, ssDNA-binding proteins ligate to the displaced DNA strand without the primer, while a DNA polymerase amplifies the primer to form new dsDNA that will act as a template for additional rounds of amplification [54]. The amplification products can be detected through gel electrophoresis, but the use of fluorescent probes allows RPA to be performed as a one-tube reaction [32]. RPA can generate millions of copies of a certain DNA target in just 40 minutes, having a very high sensitivity, and the procedure does not require high temperatures, usually between 25°C and 42°C [55]. Those advantages, alongside the easily detectable amplification products, have made RPA a suitable candidate for diagnosis in resource-limited settings, being integrated in various microfluidic devices and lateral flow assays for the detection of various bacteria, parasites and fungi [32]. Several viruses have also been detected using this method, namely Ebola, Dengue, HIV-1, and Influenza A [56]. However, similarly to other isothermal amplification methods, RPA requires optimization and is unable to detect single-base-pair differences between

target sequences, an important distinction that can have consequences on the diagnosis [57].

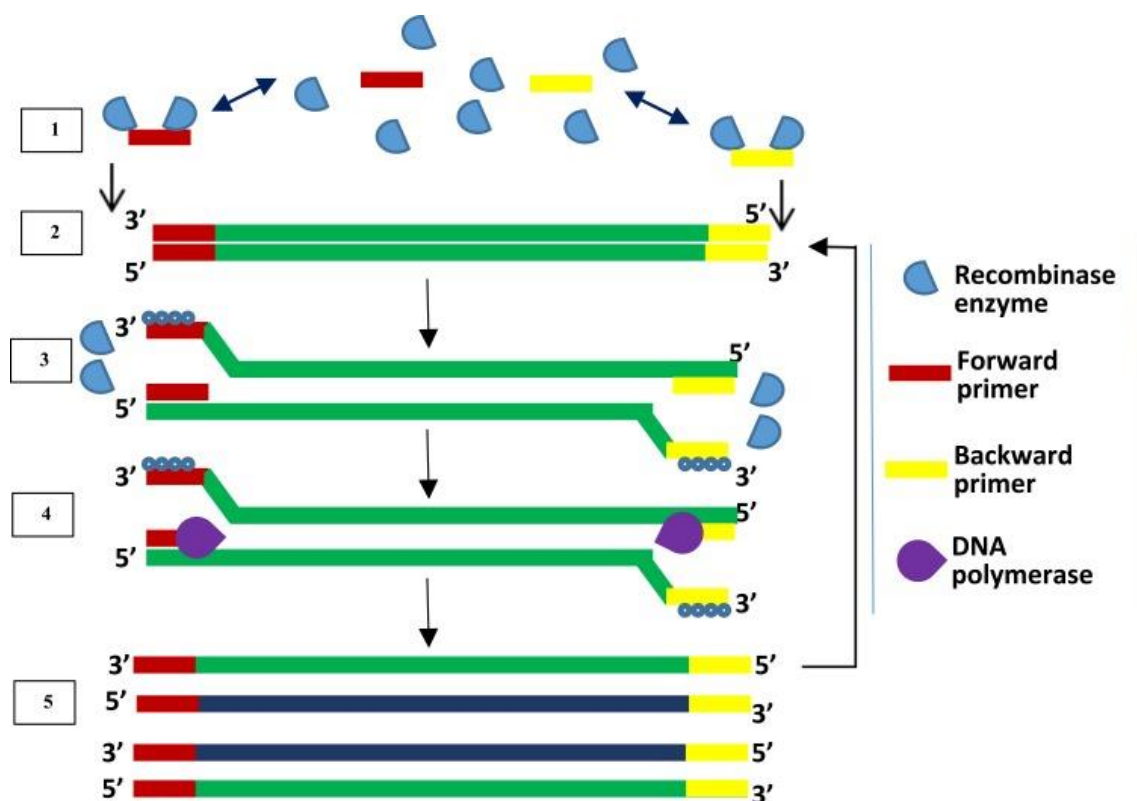


Fig. 6 – Schematic representation of Recombinase Polymerase Amplification (RPA) as presented in G. A. Obande, and K. K. B. Singh (2020) [32].

Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK) and **DNA Endonuclease-Targeted CRISPR *trans* Reporter (DETECTR)** are methods usually coupled either with RPA or RT-RPA for the detection of a pathogen DNA or RNA, respectively. The SHERLOCK assay is schematized in **Fig. 7**. Those rely on the CRISPR-Cas13 (SHERLOCK) and Cas12 (SHERLOCK and DETECTR) protein families collateral RNase activity, and fluorescent labelled RNA reporters – oligonucleotides with a fluorophore that is quenched by a quencher when intact, and that emits fluorescence when cleaved. Upon pre-amplification of either target input, the amplified targets are detected by the Cas13 or Cas12 complexes, that will activate and cleave the fluorescent RNA and/or DNA reporters, originating a fluorescent signal that will be proportional to the number of amplified targets. While Cas12 can typically cleave DNA, Cas13 can only cleave RNA and, as such, a T7 transcription step is needed. The fluorescent-based detection methods can be used to determine the target concentration on a certain sample, although single-plex colorimetric lateral flow assays have been developed, not requiring any special equipment [57, 58]. Despite not being able to achieve single

molecule detection, SHERLOCK has been able to detect as few as 2 viral copies/ μL of the ZIKA virus, and the rapid and inexpensive nature of the assay, alongside the possible multiplexing capability, makes it suitable for bedside diagnostics [59]. Regardless of its advantages, this methodology is very complex, and still involves preparation and testing of reaction components that are not widely commercially available, most of which require highly skilled personnel [58].

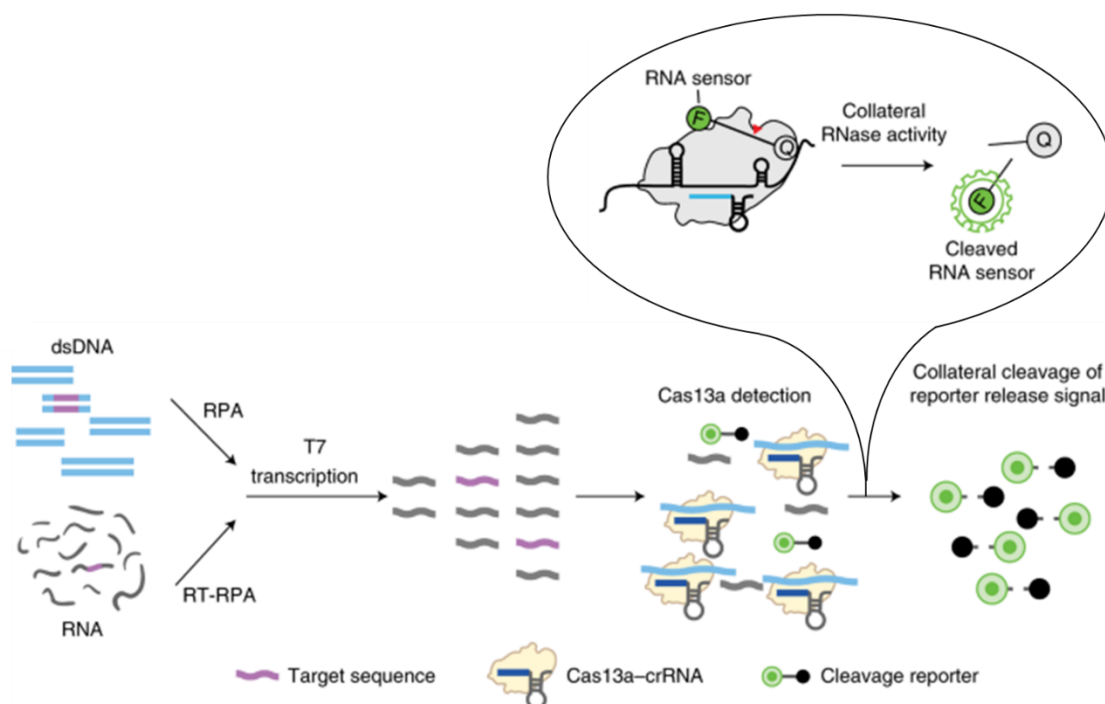


Fig. 7 – Specific High-sensitivity Enzymatic Reporter un-LOCKing (SHERLOCK) schematic adapted from M. J. Kellner *et al.* (2019) [57]. DNA Endonuclease-Targeted CRISPR trans Reporter (DETECTR) differs mainly on the use of Cas12 proteins for detection, although the same principle is used.

Signal-Mediated Amplification of RNA Technology (SMART) is a method shown in **Fig. 8** that was designed to generate highly specific target-dependent signals. It involves one smaller extension probe, and one template probe with a T7 RNA promoter and a detection sequence. Both of those probes also have sequences that allow them to hybridize with the target sequence and with each other, allowing for the two probes and the target to form a three-way junction structure when this last is present. Upon correct formation of the three-way junction, the *Bst* DNA polymerase extends the shorter probe until a double-stranded promoter sequence of T7 RNA results from this amplification. Once this promoter sequence becomes double-stranded, the T7 RNA polymerase amplifies the template probe (with a detection sequence) various times, originating several RNA signals per target sequence. Detection of those RNA signals is done through an Enzyme-Linked Oligosorbent Assay (ELOSA), that originates a colour

change that is proportional to the amount of generated signal [60]. Even though this technique has been used for the detection of phage viruses of marine *Synechococcus* and methicillin-resistant bacteria *Staphylococcus aureus*, it is not nearly as sensitive as PCR [32] and has thus not yet been developed towards clinical resource-limited diagnostics.

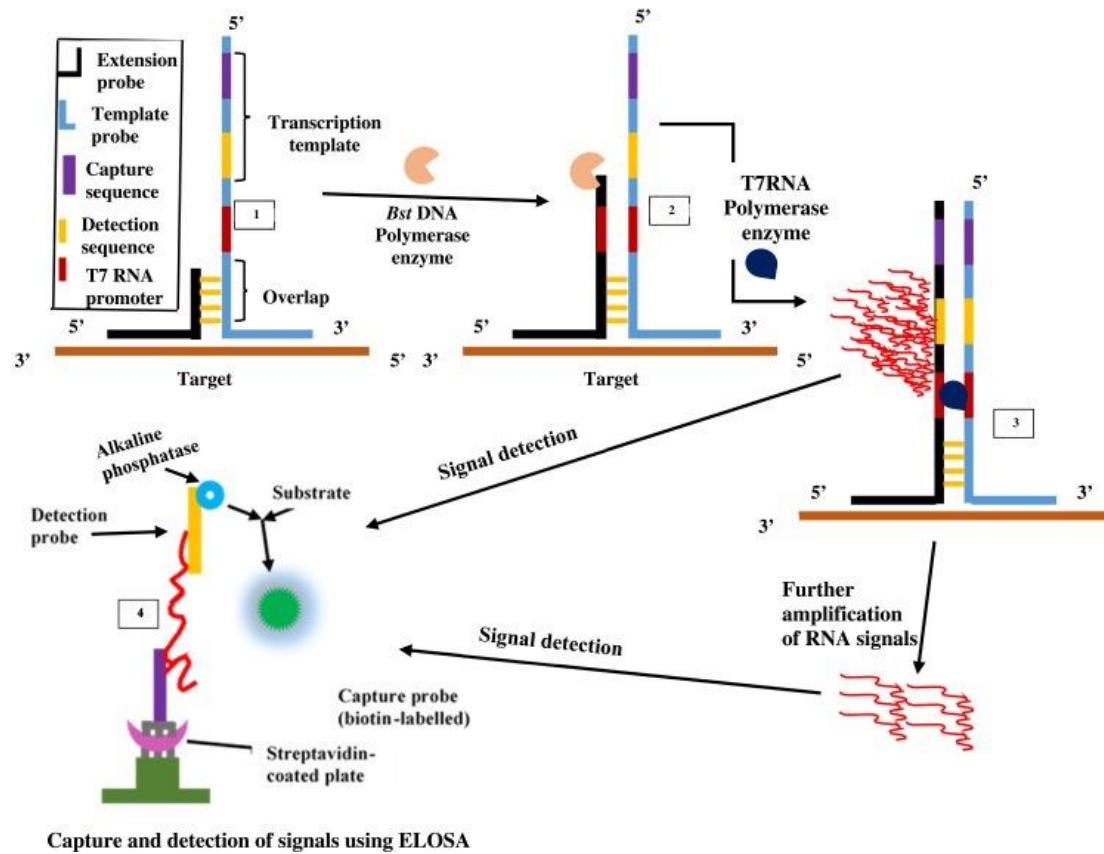


Fig. 8 – Schematic representation of Signal-Mediated Amplification of RNA Technology (SMART) as presented in G. A. Obande, and K. K. B. Singh (2020) [32].

Infectious diseases can be a particular burden in developing nations, where expensive technologies are unaffordable, and the restricted resources prevent the adoption of sophisticated instrumentation to perform various gold-standard centralized diagnostics, namely PCR-based tests. As such, presented isothermal nucleic acid amplification techniques appear as an alternative for diagnostic in resource-limited settings.

1.4. Diagnostics in resource-limited settings

Despite many diagnostic methods have been developed over the years – several of which are used daily in centralized clinics, hospitals, and laboratories – their complexity, expensive cost, use of heavy machinery and necessity of trained personnel to be performed only allows those technologies to be operated in specialized areas, thus not being widely available in many settings.

Recent technological development allow diagnosis to be performed in the place where the patient can be treated, such as clinics, instead of centralized labs or hospitals. Those are called Point-of-Care (PoC) or Bedside Diagnostics, and their better portability and rapid turn-over time allows the analysis to be done either in pharmacies or right next to the patient bed, while ensuring a quick diagnosis [3, 5, 12]. Afinion™ 2 Analyser is an example of a “compact, rapid, multi-assay analyser that provides valuable near patient testing at the point-of-care”, being able to diagnose and control various biomarkers related to diabetes and cardiovascular diseases only in a couple of minutes [61]. In the infectious disease diagnostics, the m-PIMA™ Analyser has shown to be able to perform HIV infant diagnosis and determine the HIV viral load of a patient within 70 minutes [62], while the Cepheid Xpert® Xpress SARS-CoV-2/Flu/RSV test provides rapid detection of multiple respiratory viruses that usually present the same clinical conditions as SARS-CoV-2 [63]. However, most of those methods lack the sensitivity and specificity of centralized diagnostics, while still being expensive (cost around 2000 - 5000 US\$ per equipment), somewhat bulky, and sometimes requiring trained operators [64]. PoC testing has shown that can be fully portable, not relying in any outside power source [61, 62], but the associated cost does not make them suitable for resource-limited settings yet.

According to the World Health Organization (WHO), an appropriate diagnostic test for the aforementioned settings should follow the **REASSURED** criteria: have **Real-time connectivity**, having the test connected to a reader that can easily read and provide test results with required data for decision-making; have **Ease of specimen collection**, being design for use with non-invasive specimens and samples; be **Affordable**, so that everyone can have access to the test; **Sensitive**, meaning it should be able to detect the pathogen; **Specific**, so that it detects only the wanted pathogen; **User-friendly**, so that even a 5-years-old can run the test; **Rapid** and **Robust**, in order to quickly treat the patient and prevent the spread of the disease; **Equipment-free** and **Deliverable to end-users**, which allows the diagnostic to be performed virtually

anywhere [65]. An example of a conceptual ideal diagnostic test following all the REASSURED criteria is presented in **Fig. 9**.

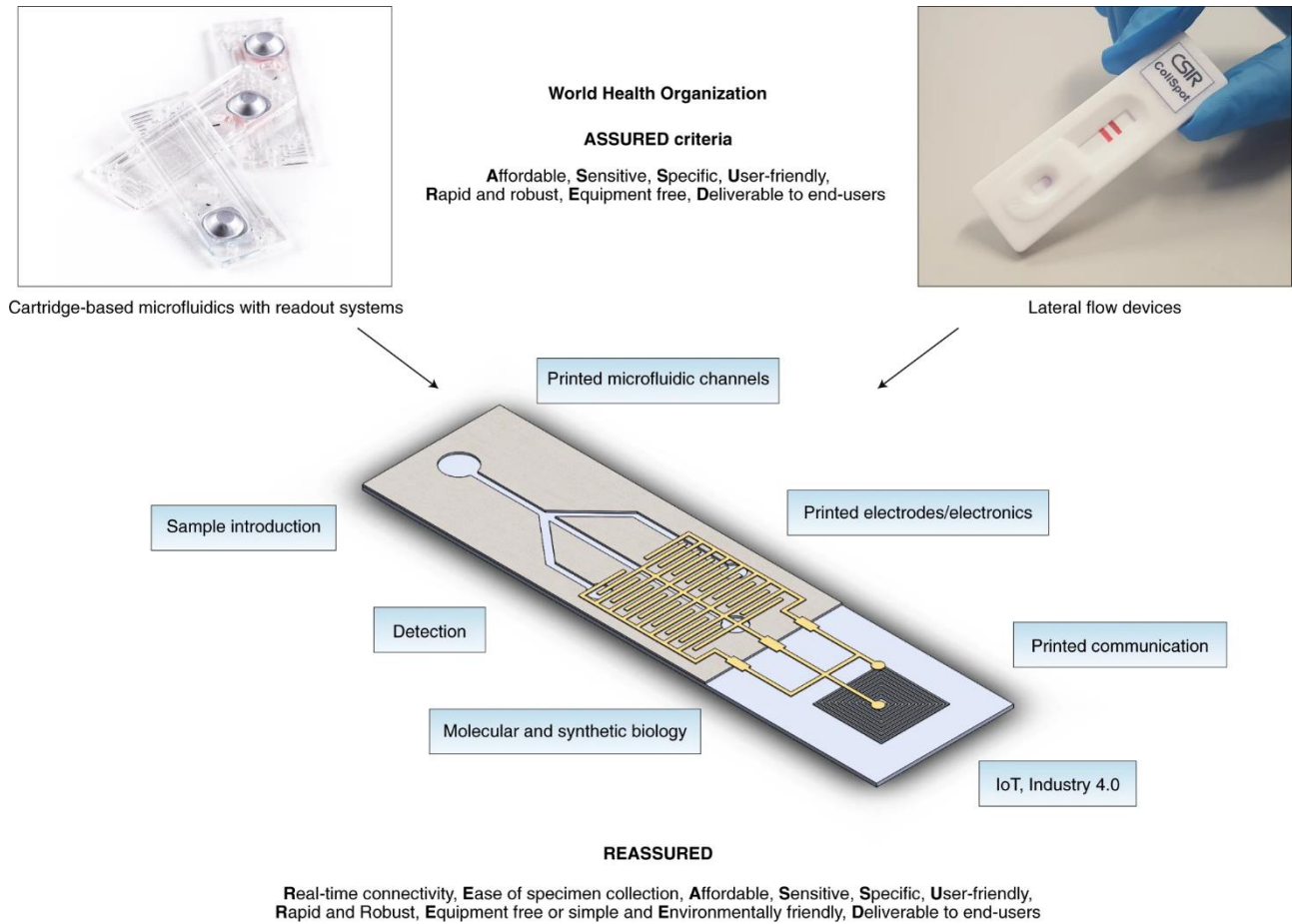


Fig. 9 – Concept for an ideal diagnostic test, according to the REASSURED criteria, as shown in K. J. Land et al. (2019) [65].

As further advances were made in science and engineering, various novel technologies emerged to try and take previously centralized diagnostics to those resource-limited settings, following the previously mentioned criteria. The growing field of microfluidics, that allowed the manipulation of extremely small volumes of fluids in microchannels, along with the emerging lab-on-a-chip concept, helped to miniaturize and reduce the cost of various methods, while trying to retain their sensitivity and specificity [66, 67]. This new technology also ensured the portability of the diagnostic test, while the possibility of automation granted that anyone was able to properly use the device. All those factors contributed to take previously lab complex diagnostics to any setting, giving birth to Home Testing. The Lucira COVID-19 All-In-One Test Kit, for example, allows for a quick diagnosis of SARS-CoV-2 in just 11 minutes, being the first COVID-19 Home Test approved by the FDA [68]. Despite this, the transition from centralized methods to

microfluidics is not always optimal, and some of the home testing diagnostics lack the specificity, sensibility, sensitivity, and reproducibility of centralized diagnostics [64].

The development of Home Testing also arouses several related ethical issues, namely the rise in disease-related discrimination, improper interpretation of test results, and acceptance of results without confirmation or follow-up testing [64, 69], the last two being especially noteworthy, since they can contribute to a wrong perception of the patient state of disease, contributing to the spread of a certain pathogen at early stages of the disease.

1.5. Taking diagnostics anywhere with microfluidic devices

Although the rise of new molecular diagnostic methods greatly contributed to the decentralization of diagnostics, the ability to transform expensive and time-consuming centralized diagnostics into smaller, faster, automated, portable devices have mainly been possible due to the recent advances in the science and technology that allows the precise manipulation of very small volumes of fluids (microlitres to picolitres) in microchannels with tens or hundreds of micrometres [70]. The development of microfluidic studies and the “lab-on-chip” concept have been the focus not only of many resource-limited molecular diagnostic assays and devices, but also in resource-rich settings alike.

The various benefits of microfluidic systems vary for each application. One universal advantage of those systems is the overall reduced use of reagents, that greatly lowers the cost of assays. Smaller environments also tend to optimize interactions while reducing reaction and sample processing time, since at the micro-scale, fluid moves in linear, easily predictable paths due to the laminar flow that typically arises from the small size of the microchannels [71]. This laminar regime inside the microchannel allows mass transportation of analysed molecules perpendicular to the flow to depend on their intrinsic diffusion coefficient alone, making this process rely solely on diffusion. However, it is important to note that in flow conditions that provide residence times inside the channel significantly smaller than the time required by the molecules to reach the intended region of the microchip by diffusion, those analytes are bound to be lost downstream [72]. Even in less laminar and more turbulent regimes (ex: when beads are present in the channels), microfluidics also have the advantage of providing a more easily controlled environment due to the inherent stable solution flow dynamics, increasing the reproducibility of conducted assays. The mixing that naturally arises from turbulent flow may also help increasing the interaction between flowing molecules [73]. Microfluidic devices can also

easily be designed for multiplexed assays, allowing different analysis to take place in the same microchip [74]. Finally, by confining a certain sample solution to a smaller area, it is possible to enrich it and more easily detect a target analyte [45, 46].

The availability of several fabrication methods to manufacture small devices on a large scale at a very low cost is another advantage of the microfluidic systems [75]. Those microdevices can be fabricated through soft lithography-based processes that are faster and cheaper than other conventional methods. Poly(dimethylsiloxane) (PDMS) is a polymer that is widely used for prototype fabrication of microfluidic devices due to its properties: it is optically transparent, allowing several detection methods to be used like fluorescence-based microscopy; nontoxic, so living cells can be the target of various assays; can be deformed reversibly; and can seal irreversibly against glass and some other materials after exposure to air plasma and formation of covalent bounds [76]. By exposing PDMS and glass to oxygen plasma, polar silanol groups (Si-OH) are introduced on the surface at the expense of methyl groups (Si-CH₃) and, upon conformal contact, a Si-O-Si covalent bond is made [77].

All those advantages make microfluidic devices the perfect platforms for development of resource-limited molecular diagnostic assays. Miniaturization of the assays is, however, not always optimal, and several optimizations are required to achieve the clinically relevant sensibilities and sensitivities while working at the microscale [64].

2. THE COVID-19 OUTBREAK

In December 2019, an unidentified coronavirus surged in Wuhan (China) and quickly expanded globally, resulting in an unknown pneumonia outbreak all over the world. Upon sequencing, this Novel 2019 Coronavirus – 2019-nCoV – was identified as being a positive-sense ssRNA β -coronavirus, phylogenetically related to the Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV), having an almost identical genetic sequence [78]. The similarities to SARS-CoV renamed this novel virus to **Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2)**, and the associated disease as COVID-19. On 11th March 2020, WHO characterized COVID-19 as a worldwide pandemic [79]. By 15th of September 2021, COVID-19 has resulted in nearly 225 million confirmed infections and 4,6 million deaths all over the world since December 2019 [80].

2.1. SARS-CoV-2 structure and infection pathway

SARS-CoV-2 is composed by four main structural proteins – spike (S) glycoprotein, small envelope (E) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein – while also having various accessory proteins and non-structural proteins [81].

The infection pathway begins when the S protein binds to angiotensin I-converting enzyme 2 (ACE2) receptors on the membrane of host cells, which is then followed by fusion of the viral membrane with the host membrane and subsequent activation of receptor-attached S proteins by the type II transmembrane serine protease (TMPRSS2), leading to conformational changes that allow the entry of the virus. Both the ACE2 receptors and TMPRSS2 are the main determinants of the entry of SARS-CoV-2 in the human cells, being expressed mostly in secretory and epithelial cells of the respiratory tract, digestive, urogenital and nervous systems, while also being present in various organs such as the heart, lungs, kidneys, and liver [81, 82]. SARS-CoV-2 has a higher ACE2 receptor binding affinity than SARS-CoV [83, 84], infecting cells more easily.

Upon entering the cytoplasm of host cells, the virus will release its genomic material in the cytoplasm. With the help of various non-structural proteins (nsp), most of the host translation mechanisms are hijacked to translate the viral RNA, suppressing the host cell defences [85]. The disruption of host translation also happens because of the high levels of viral transcripts present in the cell [86]. The viral genome contains several open reading frames (ORF) that encode a variety of viral proteins, namely two overlapping ORFs – ORF1a and ORF1b – that generate continuous overlapping polypeptides – pp1a and pp1ab – that can be cleaved by proteases into several non-structural proteins that contribute for the virulence of SARS-CoV-2 [81, 86].

Upon translation, some of the non-structural proteins form a replicase-transcriptase complex that will transcribe endogenous genome templates several times, producing progeny viral RNA that will be incorporated into the new viruses. This complex is composed by an RNA-dependent RNA polymerase (RdRP) and a helicase that correspond to non-structural proteins nsp12 and nsp9, respectively [87].

Accessory, M, S and E structural proteins are transported to the endoplasmic reticulum and then to the endoplasmic reticulum – Golgi intermediate compartment (ERGIC), undergoing N-glycosylation, disulphide bond formation, and other

posttranslational modifications. Those modifications are of uttermost importance for several processes in the virus, namely for viral tropism and host cell specificity, where it is key for the folding, assembly, and recognition of ACE2 receptors by S proteins, and are mostly conserved across SARS-CoV strains [88].

After proper replication of the endogenous viral genome, the progeny RNA joins the N protein to form the nucleocapsid, and then moves to the ERGIC, where new SARS-CoV-2 units are fully assembled, being exported out of the cell in small vesicles through exocytosis [81].

Transmission of the aforementioned pathogen is done via contact, droplet spread, and aerosols [89] typically being detected in respiratory samples (throat swab, nasopharyngeal swab, sputum, endotracheal aspirates and bronchoalveolar lavages). The virus can, however, be detected in the patient stool and blood in more severe cases of infection [90].

2.2. The COVID-19 paradigmatic change in disease perception

Various factors contributed for the fast worldwide spread of SARS-CoV-2 around the world after being first discovered in Wuhan, China. The ease of entry and infection of host cells was one of the determining factors that allowed SARS-CoV-2 to surpass other coronaviruses in efficiently infecting several people across the world [83, 84], but it was not the only one.

Just like other human coronaviruses, SARS-CoV-2 has developed several immune evasion strategies especially against the innate immune responses of the host, namely by making use of its various non-structural viral proteins. This disrupts the host immune system response due to the virus evasion, leading to typically very long incubation times (up to 11 days), where the host presents little, very mild or no symptoms at all but can spread the disease [81, 91]. Individuals who develop symptoms usually do so around 11 days after infection, and present mostly mild flu-like symptoms (namely fever, sore throat, dry cough [90], hyposmia, hypogeusia [92], and shortness of breath [93]), that are commonly associated and more frequent in other infectious diseases. Fatigue, nausea, vomiting, diarrhoea, and myalgia have also been observed in infected patients, although to a lesser extent [93]. Severe clinical manifestations account for around 10-15% of infected people and tend to manifest in older patients (aged at least 50 years), especially when also presenting other comorbidities (mostly hypertension and diabetes), other respiratory problems, or in immunosuppressed patients. Those present

higher temperatures, lower peripheral capillary oxygen saturation, respiratory failure, septic shock, multiple organ dysfunction, bleeding and coagulation dysfunction [93, 94].

Although asymptomatic carriers seem to account for up to 50% of SARS-CoV-2 infections according to the literature, there has not been any agreement on the number of truly asymptomatic cases and their infectivity, since most of them tend to develop mild symptoms at some point, and larger observational studies need to be conducted to ascertain the impact of truly asymptomatic patients in the COVID-19 pandemic [95]. Despite this, it is of overall agreement that asymptomatic carriers of the virus can spread it and play an active role in the development of the pandemic [91, 93, 95].

The high infectivity of SARS-CoV-2, combined with the absent or very mild symptoms that arouse from the infection, allowed patients to spread the virus before a confirmatory diagnostic. The impossibility to determine the infectious state of someone relying solely on symptoms (since most young, healthy looking individuals could still be infected), along with the lack of a specific therapeutic or vaccine, led many countries to adopt a “lockdown policy” to slow down the spread of the virus and ease the burden on health facilities during this worldwide pandemic [96, 97], but had a huge impact on the mental health of the population [98, 99], while also resulting in a severe economic crisis [100]. Thus, there is a pressing need for cost efficient, sensitive and rapid diagnostics methods to effectively reduce viral spread with targeted lockdown measures and better control the ongoing pandemic.

2.3. Diagnosis during the pandemic

Molecular diagnostics quickly claimed a central role in the COVID-19 outbreak due to their ability of reliably detecting SARS-CoV-2 [101]. An initial RT-PCR protocol was developed to diagnose COVID-19, targeting a sequence of the N structural protein and a consensus RdRp coding region of pan β -coronavirus on the Orf1ab polyprotein gene in lower respiratory tract samples of patients [102]. However, the test was not without its flaws: the fast mutation rates of the virus in the targeted N structural protein region provided some false negatives in the early stages of the pandemic [103, 104]. The difficult sample collection process – the nasopharyngeal swab – also contributed to this, since it was a very invasive sample to collect, and required trained personnel to be done properly [92, 105]. False positives were also observed, although to a lesser extent, due to contamination of patient samples or used reagents [93].

Since no other molecular diagnostic test were easily available during the initial outbreak, RT-qPCR tests were often combined with other methods [92], namely chest tomography (CT), to better understand the infection state of patients [90, 106, 107]. CT was used for COVID-19 diagnosis due to its specificity in identifying viral pneumonia [108, 109], but would often result in false positives, since it cannot distinguish SARS-CoV-2 from other viral infections [110, 111]. Clinical and travel history [103, 112], blood analysis [92, 94], and chest X-Ray analysis [113] also complemented the molecular methods, providing extra information that could help with the diagnosis [90].

The RT-PCR test was optimized once more conserved regions of the SARS-CoV-2 genome were precisely identified and targeted [114]. This diagnostic method is, at the time of writing, the Golden-Standard of COVID-19 diagnostics, being able to detect as low as 1 viral copies/ μ L [28]. However, this procedure requires heavy machinery and highly trained personnel [6, 18], which severely hinders the test efficiency during a pandemic outbreak, where thousands of people need to be diagnosed as quickly as possible, in various contexts and environments. As such, despite its high efficiency and specificity, RT-PCR does not meet the necessary requirements for a coordinated global response to contain the COVID-19 pandemic.

Many new NAAT diagnostics have surfaced since the beginning of the outbreak that tried to fulfil the REASSURED criteria, making COVID-19 diagnosis simpler, faster, and available even in resource-limited settings. RT-LAMP [36, 115] and colorimetric LAMP [116] assays have been developed as highly specific diagnostic tests, being able to detect SARS-CoV-2 in less than one hour, reaching a LoD of 75 viral copies/ μ L [117], and are available commercially [118]. However, those methods presented some drawback, namely the challenges associated to optimizing reaction conditions and designing primers [117]. TMA as also been used as a diagnostic method for COVID-19, presenting similar results to RT-PCR [119, 120], while providing a higher throughput than said method, and are also commercially available [121]. CRISPR-based approaches to diagnostics have also been developed, since those assays have previously demonstrated capability in detecting low quantities of viral genetic material in patient samples [59, 117], and recent results have shown a LoD of 0,05 viral copies/ μ L using an assay that could be performed in paper strips [122].

Serological antibodies and antigen-detection tests have also appeared as a way of delivering reliable diagnostic results in very short times [92, 123], usually coupled to small portable lateral flow immunoassay devices [124, 125]. However, those tests are unable to detect active viral shedding during the early stages of infection [92, 126] and

usually present lower sensitivities than molecular methods [112]. Lateral flow immune assay devices for antigen detection using respiratory samples also surfaced as a fast and easily available diagnostic. However, the high mutation rates of SARS-CoV-2, especially in the surface spike-proteins, made this test prone to many false-negatives resulting from the arise of different strains. Like previously mentioned, other blood analysis were also performed as a complementary diagnostic, since lymphopenia and inflammatory biomarkers may also indicate an active on-going infection [90, 92, 94].

Despite the vast variety of available diagnostic tests, none have fully met the WHO ASSURED criteria, and although some PoC devices have been developed, they usually do not cost less than US\$10 per test and less than US\$500 per device – prices that can be considered affordable for developing countries [127]. As such, the development of new diagnostic techniques is still of uttermost importance for controlling the worldwide COVID-19 pandemic.

3. RCA TOWARDS PORTABLE DIAGNOSTICS

At the time of writing, no RCA-based pathogen diagnostic device has been fully developed or available commercially, despite the assay's simplicity, accuracy, sensitivity, versatility, and robustness [32, 42, 43, 48]. However, various recent efforts towards portable RCA diagnostics have been made. Microfluidic devices have already been coupled with RCP detection and/or quantification [46, 47], reaching sub-attomole levels of detection in some microfluidic devices [45]. Microdevices have also been used as platforms for performing the amplification assay, both with [128] and without [129] continuous perfusion during steps that require enzymatic activity, with the continuous perfusion device showing promising results by using ultrasonic mixing and circular microchannel shapes but requiring further optimization [128]. An automated microfluidic-based RCA system for detection of DNA molecules in cells has also been developed, but the dimension and complexity of this device hinders its application in resource-limited settings [130]. Regarding COVID-19 diagnostics, RCA has already been applied to detect cDNA of SARS-CoV-2, reaching a LoD of 0,4 fM of target cDNA [130, 131].

Considering the potential RCA has shown in approaching molecular diagnostics, alongside the increasing demand of new and more accessible diagnostic devices due to the on-going COVID-19 pandemic, this work presents the development of an RCA-based method for the direct detection of SARS-CoV-2 RNA, integrated in a microfluidic flow-cell. Said methodology is intended to develop a simple, cheap, sensitive, and robust RCA microfluidic device as an alternative for COVID-19 diagnosis, with possibility of automation in the future. This work also presents a novel RNA detection method with multiplexing potential and amenability to detect different SARS-CoV-2 strains with high specificity.

MATERIAL AND METHODS

1. BIOTINYLATED cDNA SYNTHESIS AND SARS-CoV-2

SAMPLES

For initial amplification experiments, biotinylated cDNA corresponding to a small, conserved portion of the RdRP region of the SARS-CoV-2 genome was synthesised through reverse transcription. The mentioned virus was first cultured in Vero E6 cell lines by a collaborator at the Swedish Health Agency in a BSL-3 lab, and supernatants containing the virus were collected in TRIzol LS™ (Thermo Fisher). Upon collection, SARS-CoV-2 RNA was extracted from the inactivated supernatant samples using the Direct-zol™ RNA MiniPrep Plus kit (Zymo Research, CA, USA), following the manufacturer's instructions. This step was performed at room temperature, using an Eppendorf Centrifuge 5424 (Eppendorf) to process the sample through the spin columns. The purified RNA was then eluted in diethyl pyrocarbonate-treated water (DEPC-water), and reverse transcription was performed with the Superscript IV First-Strand cDNA Synthesis kit (Thermo Fisher), all according to the manufacturer's instructions. The sequences used as primers for reverse transcription, as well as the obtained cDNA synthetic targets, are presented in supplementary **Table S1**.

Detection of SARS-CoV-2 RNA was first performed on a small 226 bp fragment (Integrated DNA Technologies, USA) corresponding to a conserved region encoding for RdRP (sequence presented in **Table S1**), and then using Synthetic Control 2 SARS-CoV-2 RNA (GISAID ID: MN908947.3, Twist BioScience).

SARS-CoV-2 saliva samples were collected from one anonymous COVID-19 negative individual, using the same method as described by Hung, D. L. L., *et al.* (2020) [132], collecting the samples only once per day, around 11:00 am (Central European Summer Time), two hours after the previous meal, and before lunch. Positive samples were obtained by spiking the saliva samples with 1:10 ratio (V/V) of Synthetic Control 2 SARS-CoV-2 RNA, reaching a final concentration of 10^5 viral copies/ μ L in the saliva solution, and 10^4 viral copies/ μ L inside the microfluidic device. Nuclease-free water (Integrated DNA Technologies, USA) was added in a 1:10 (V/V) ratio to the saliva samples to simulate the negative samples.

2. PADLOCK PROBES, DETECTION OLIGONUCLEOTIDES, BIOTINYLATED ANCHORS, L-PROBES AND PLP-PRIMER DESIGN

Different Padlock Probes (PLPs) were designed for each on-chip method, with one PLP directly targeting the biotinylated cDNA, another PLP targeting the L-Probes overhang, and a “chimeric” PLP aiming to hybridize directly with the SARS-CoV-2 RNA, modified with a ribose base at the 3'-end. Aside from the complementary target regions, all the PLPs were ordered with 5'-phosphorylation, and included a backbone sequence that, upon amplified, could be recognized by small oligonucleotides (approximately 20 bp long) with a detectable fluorophore molecule – Detection Oligonucleotides (DOs). The “chimeric” PLP backbone also allowed primer hybridization.

Three Biotinylated Anchors – ssDNA oligonucleotides with a biotinylated poly-A tail at the 5'-end – and twelve L-Probes – L-shaped ssDNA oligonucleotides – were designed targeting a conserved Orf1ab genomic region of SARS-CoV-2 RNA that encodes for RdRp. L-Probes were designed with one half targeting this RdRp region, close to the Biotinylated Anchors, and with an overhang sequence for PLP ligation.

All PLPs, Biotinylated Anchors, DOs L-Probes and PLP-Primers were ordered from Integrated DNA Technologies (USA), and their sequences can be found in **Table S1**.

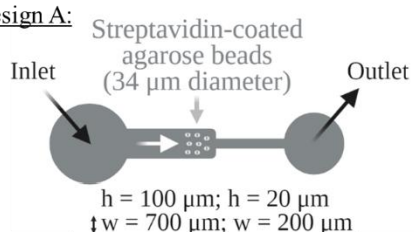
3. MICROFLUIDIC DEVICE FABRICATION, DESIGN AND BEAD PACKING

The microfluidic devices were fabricated in the same way as described in previous works [45], using standard mold replication and soft lithography techniques. PDMS elastomer was first prepared by mixing 10:1 (w/w) of PDMS pre-polymer to curing agent mixture (Sylgard 184, Dow Corning), degassed for 30 minutes, and then poured into the two-level SU-8 mold for the design of choice. The pre-polymer was then cured at 65°C for 90 minutes in a convection oven. Once cured, the PDMS was peeled from the mold, access holes were punched using a blunt 18-gauge syringe tip, and the microchannels were subsequently sealed against 700 µm thick glass slides (Corning microscope slides, plain) by exposing both surfaces to an oxygen plasma treatment for 30 s (Femto Science CUTE, 100 W, 80 Pa).

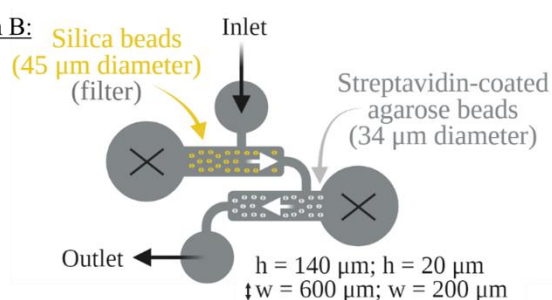
Three different flow cell designs were used in this work – Design A, Design B and Design C – all schematized in **Fig. 10**.

TOP VIEW:

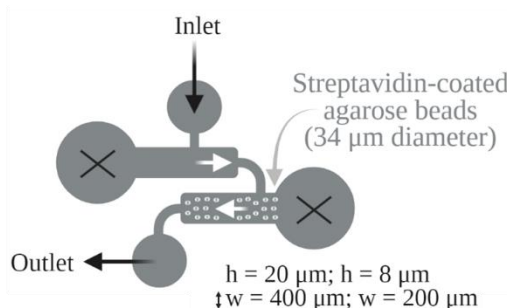
Design A:



Design B:



Design C:



SIDE VIEW:

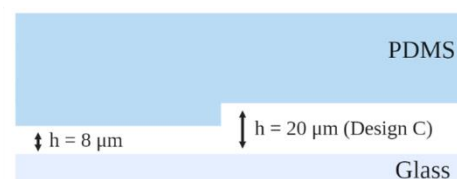
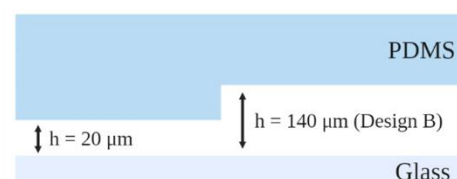
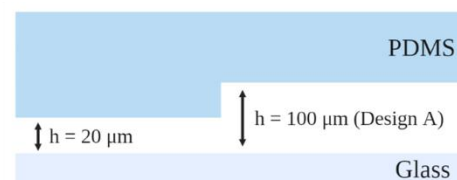


Fig. 10 – Top and side views of the single and dual-column microfluidic channel designs A, B and C. White arrows represent the flow direction inside the microfluidic devices.

Design A, reported by I. F. Pinto *et al.* (2017) [133], consists of straight 350 nL flow cells with a 700 x 100 μm (width x height) cross-section and an interface with a 200 x 20 μm (width x height) cross-section.

Design B and C, initially developed for multiplexed protein monitoring [134], were adopted here for molecular diagnostics, comprising two parallel taller columns, interconnected by channels with smaller cross-sections. Design B consists of 600 x 140 μm (width x height) cross-section columns and 200 x 20 μm (width x height) microchannels. Design C is similar to B, with changes only to the width and height of the columns and microchannels: 400 x 20 μm (width x height) cross-section columns and 200 x 8 μm (width x height) microchannels.

Bead packing was done differently for each of the device's design. For Design A, streptavidin-coated agarose beads with an average of 34 μm diameter (Streptavidin

Sepharose High Performance, GE Healthcare) were mixed in phosphate buffered saline (PBS) (137 mM NaCl, 10 mM phosphate, pH 7.4) at a ratio of 1:100 (V/V), and subsequently packed by flowing 25 μ L of solution through the microchannels by applying negative pressure at a flow rate of 10 μ L/min, using a New Era Pump Systems NE-1200 12-channel syringe pump. The height difference between the channels allowed those agarose beads with an average diameter bigger than the smaller channels height to be physically trapped at this intersection.

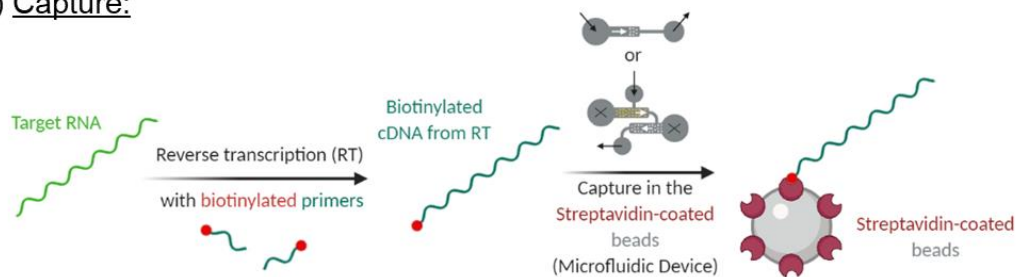
In designs B and C, the second closest column to the inlet was entirely packed with the same streptavidin-coated agarose beads used for Design A, mixed with PBS at a ratio of 10:100 (V/V). Those were packed by flowing 75 μ L of solution in the respective column through a dedicated inlet, while applying positive pressure at a flow rate 10 μ L/min, subsequently sealing the inlet with a metal plug, confining the beads inside the column, and preventing their movement thorough the assay. The principle used for Design A in physically trapping the beads at the intersection also applies to this design. For Design B, the closest column to the inlet is packed beforehand with silica beads, having a diameter around 45 μ m (Flash Silica Beads, Sigma-Aldrich), mixed with PEG8000 (20%, w/w; diluted from a 40% stock solution, Sigma Aldrich) at a ratio of 10:100 (V/V). 75 μ L of this solution is flowed at 10 μ L/min (positive pressure) through the respective inlet, and the column is then sealed with a metal plug. In Design C this column is not packed.

Before all the experiments, 1% BSA blocking solution in PBS (Thermo Fisher) was flowed into the microchannels at 5 μ L/min for 10 min.

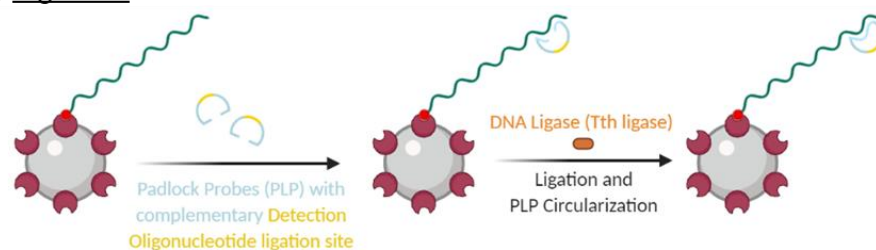
4. ON-CHIP ROLLING CIRCLE AMPLIFICATION

On-Chip Rolling Circle Amplification (On-Chip RCA) is summarized in **Fig. 11**.

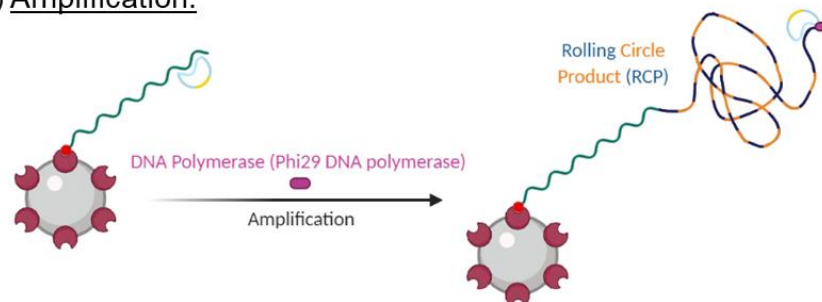
1) Capture:



2) Ligation:



3) Amplification:



4) Labelling, Imaging and Quantification:

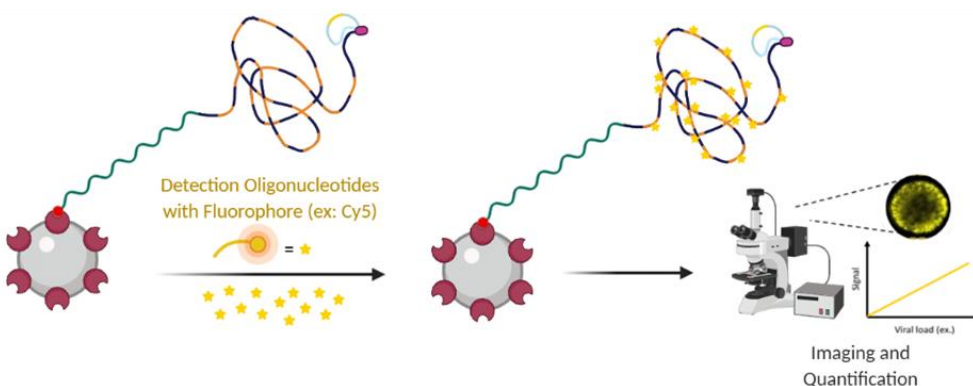


Fig. 11 – Schematic of On-Chip Rolling Circle Amplification (On-Chip RCA), consisting of four main steps: capture, ligation, amplification, and labelling, imaging, and quantification.

After a reverse transcription step of SARS-CoV-2 RNA using biotinylated primers, different concentrations of synthesized biotinylated SARS-CoV-2 cDNA were first captured in the streptavidin-coated agarose beads inside the microfluidic device by flowing 37,5 μL at 2,5 $\mu\text{L}/\text{min}$ of a 50 μL capture solution mix containing 1,5 μL of the sample, 40,75 μL of capture buffer (5x saline-sodium citrate (diluted from a SSC Buffer 20x stock, Invitrogen); 30% formamide; 0,1 mg/mL salmon sperm DNA) and nuclease-free water.

Upon capture of the cDNA inside the microfluidic device, two wash steps are performed, first with washing solution 1 (5x SSC; 30% formamide; 0,5% Tween20), and then with PBS, both flowed at 10 $\mu\text{L}/\text{min}$ for 5 minutes.

PLP hybridization and ligation was then performed on-chip at 45°C, by flowing at 0,5 $\mu\text{L}/\text{min}$ for 30 min the first reaction mix containing 10 nM PLPs, 20% formamide, 200 $\mu\text{g}/\mu\text{L}$ BSA, 0,5 U/ μL Tth DNA ligase (Blirt, Gdansk) in the respective ligation buffer (200 mM Tris-HCL pH 8.3, 250 mM KCl, 100 mM MgCl_2 , 5 mM NAD, and 0,1% Triton X-100), and nuclease-free water.

Amplification was subsequently carried out at 37°C, where the second reaction mix, that was flowed at 0,5 $\mu\text{L}/\text{min}$ for 90 min, consisted of 5% glycerol, 250 μM dNTPs, 200 $\mu\text{g}/\mu\text{L}$ BSA, 1 U/ μL phi29 DNA polymerase (Blirt, Gdansk) in 1x polymerase buffer (500 mM Tris-HCL pH 8.3, 100 mM MgCl_2 , and 100 mM $(\text{NH}_4)_2\text{SO}_4$), and nuclease-free water.

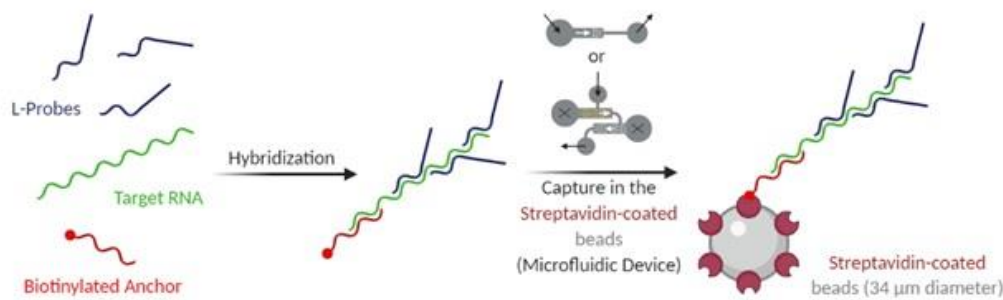
Labelling of amplified RCPs was done at room temperature, flowing the third and final reaction mix at 5 $\mu\text{L}/\text{min}$ for 10 min, containing 2x SSC, 20% formamide, nuclease-free water, and 0,1 μM of detection (Cy3) oligonucleotides.

Finally, a washing step was done by flowing washing solution 2 (2x SSC, 20% formamide) at 10 $\mu\text{L}/\text{min}$ for 5 minutes. Imaging and quantification of amplified RCPs was performed immediately after the final washing step.

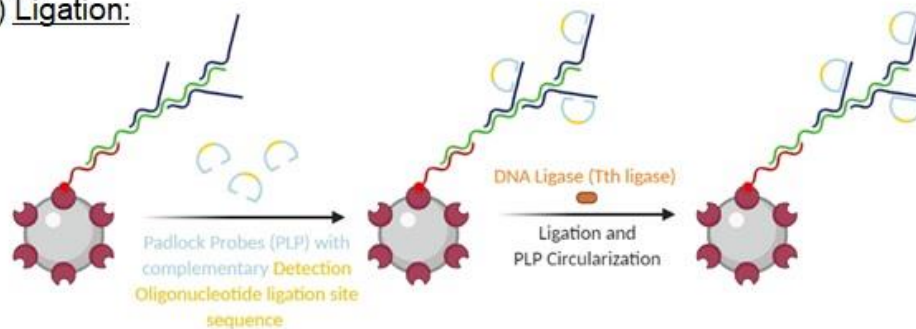
5. HYBRIDIZATION-BASED ROLLING CIRCLE AMPLIFICATION

A schematic of Hybridization-based Rolling Circle Amplification (HybRCA) is shown in **Fig. 12**.

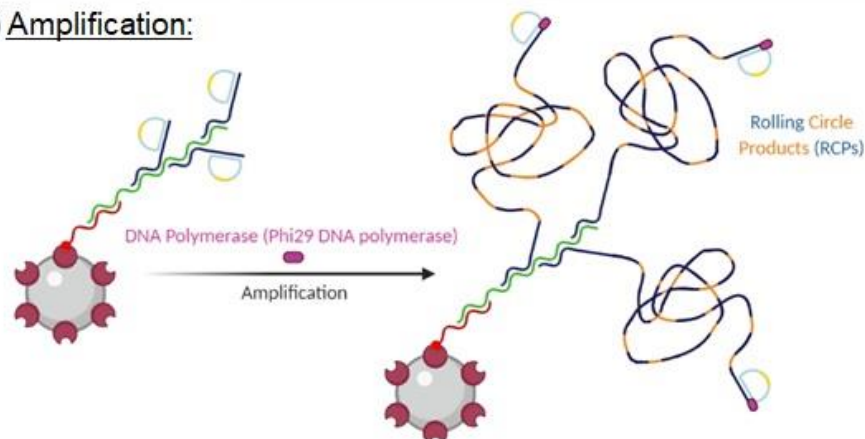
1) Hybridization and Capture:



2) Ligation:



3) Amplification:



4) Labelling, Imaging and Quantification:

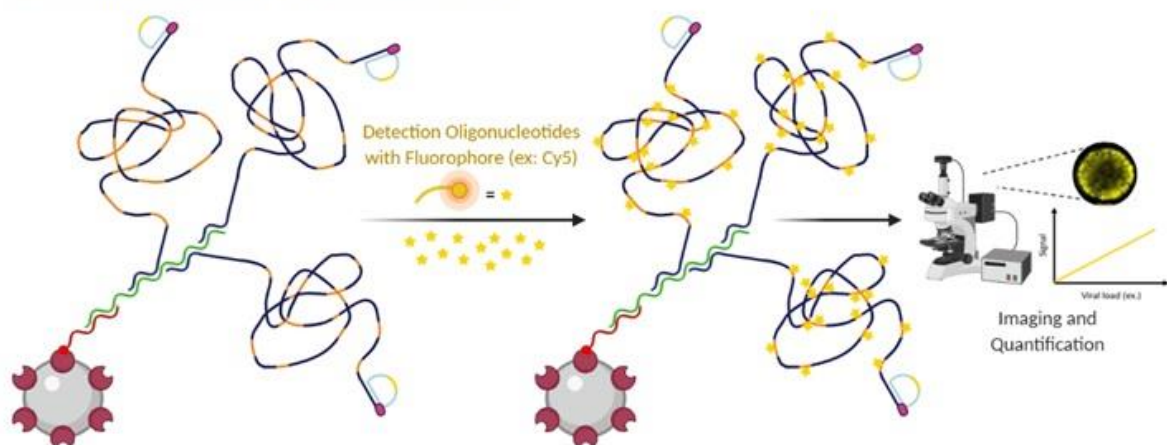


Fig. 12 – Schematic of Hybridization-based Rolling Circle Amplification (HybRCA), divided in its four steps: hybridization and capture, ligation, amplification, and labelling, imaging, and quantification.

First, various concentrations of SARS-CoV-2 RNA fragments or full RNA hybridized with 3 nM of 3 (RNA fragment), 8 or 12 (full RNA) different L-Probes, and 10 nM of 1 (RNA fragment) or 3 (full RNA) different Biotinylated Anchors, inside 50 μL of capture solution mix containing also 1,5 μL of this RNA sample, 40,75 μL of capture buffer (5x SSC; 30% formamide; 0,1 mg/mL salmon sperm DNA), 1 U/ μL RiboProtect (Blirt, Gdansk), and nuclease-free water. This solution was incubated outside of the chip, without RiboProtect, at 65°C for 5 min and, after adding the mentioned RNase inhibitor, was then flowed at 2,5 $\mu\text{L}/\text{min}$ for 15 min, at 45°C.

Similarly to On-Chip RCA, two washing steps were performed after the capture step, both at 10 $\mu\text{L}/\text{min}$ for 5 minutes, first with washing solution 1 (5x SSC; 30% formamide; 0,5% Tween20), and then with PBS.

PLP hybridization and ligation was, once again, performed on-chip at 45°C, flowing at 0,5 $\mu\text{L}/\text{min}$ for 30 min the first reaction mix consisting of 10 nM PLPs, 20% formamide, 200 $\mu\text{g}/\mu\text{L}$ BSA, 1 U/ μL RiboProtect, 0,5 U/ μL Tth DNA ligase in the respective ligation buffer (200 mM Tris-HCL pH 8.3, 250 mM KCl, 100 mM MgCl_2 , 5 mM NAD, and 0,1% Triton X-100), and nuclease-free water.

Upon ligation, amplification was carried out at 37°C using the second reaction mix, that was flowed at 0,5 $\mu\text{L}/\text{min}$ for 90 min, containing 5% glycerol, 250 μM dNTPs, 200 $\mu\text{g}/\mu\text{L}$ BSA, 1 U/ μL RiboProtect, 1 U/ μL phi29 DNA polymerase in 1x polymerase buffer (500 mM Tris-HCL pH 8.3, 100 mM MgCl_2 , and 100 mM $(\text{NH}_4)_2\text{SO}_4$), and nuclease-free water.

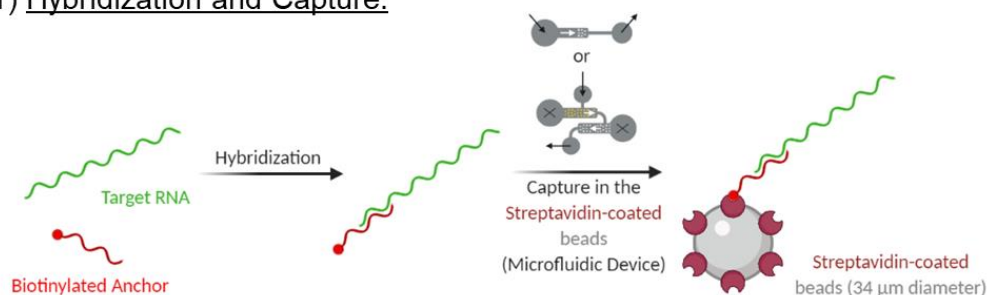
Labelling was once again performed at room temperature, flowing the final reaction mix at 5 $\mu\text{L}/\text{min}$ for 10 min, containing 2x SSC, 20% formamide, nuclease-free water, and 0,1 μM of detection (Cy5) oligonucleotides.

The final washing step was done by flowing washing solution 2 (2x SSC, 20% formamide) at 10 $\mu\text{L}/\text{min}$ for 5 minutes. Imaging and quantification of amplified RCPs was performed immediately after the final washing step, similarly to On-Chip RCA.

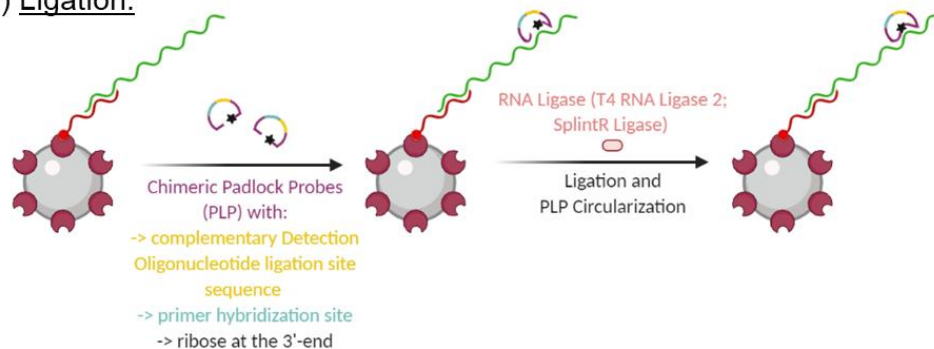
6. CHIMERIC-PLP-BASED ROLLING CIRCLE AMPLIFICATION

A summarized schematic of Chimeric-PLP-based Rolling Circle Amplification (χ RCA) is presented in **Fig. 13**.

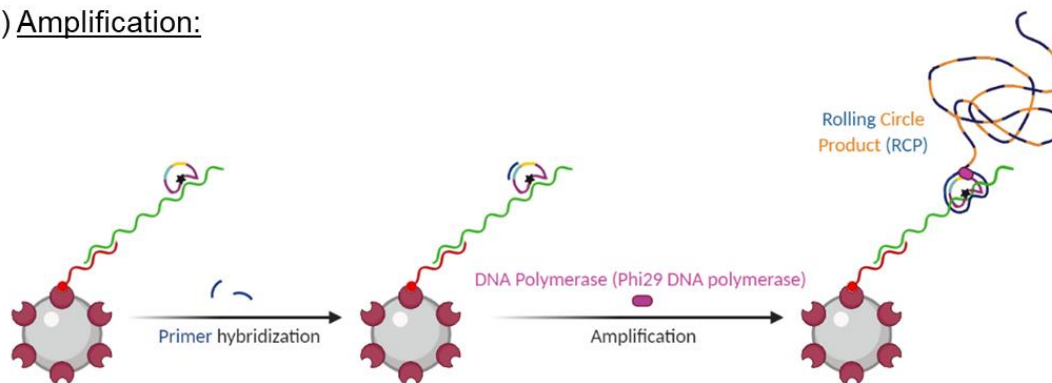
1) Hybridization and Capture:



2) Ligation:



3) Amplification:



4) Labelling, Imaging and Quantification:

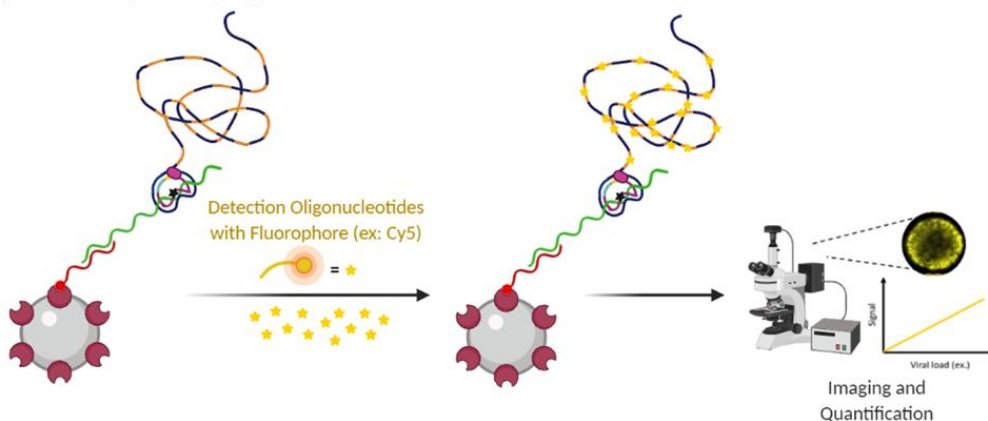


Fig. 13 – Schematic representation of Chimeric-PLP-based Rolling Circle Amplification (χ RCA), divided in its four main steps: hybridization and capture, PLP hybridization and ligation, primer hybridization and amplification, and labelling, imaging, and quantification.

Hybridization of SARS-CoV-2 RNA with 100 nM of 3 different biotinylated anchors was done in 50 μ L of capture solution mix containing also 5 μ L of this RNA sample, 40,75 μ L of capture buffer (5x SSC), 1 U/ μ L RiboProtect, and nuclease-free water. Similarly to HybRCA, this solution was incubated outside of the chip, without RiboProtect, at 65°C for 5 min and, was then flowed at 2,5 μ L/min for 15 min, once the mentioned RNase inhibitor was added.

Once the RNA was captured inside the microfluidic device, a washing step using washing solution 1 (5x SSC; 30% formamide; 0,5% Tween20) is done at 10 μ L/min for 5 minutes.

PLP hybridization was performed outside of the ligation step, at 37°C, by flowing an initial hybridization solution for 30 min, at 1,0 μ L/min, consisting of 100 nM of “chimeric” PLP, 2x SSC, 5% Ethylene Carbonate, 15 mM magnesium chloride (MgCl_2 , New England Biolabs), 0,1% Tween20, 1 U/ μ L RiboProtect, and nuclease-free water.

Two different ligation steps were tested using different enzymes – T4 RNA Ligase 2 and Chlorella virus DNA ligase – PBCV-1 DNA ligase (SplintR Ligase). For the T4 RNA Ligase 2 ligation procedure, a ligation reactional mix (10% PEG8000, 1x T4 RNA Ligase 2 buffer (New England Biolabs), 8 mM MgCl_2 , 500 μ M ATP (New England Biolabs), 10% dimethyl sulfoxide (DMSO, diluted from a 99% stock, Sigma-Aldrich), 0,25 U/ μ L T4 RNA Ligase 2 enzyme (New England Biolabs), 1 U/ μ L RiboProtect, and nuclease-free water) was flowed at 0,5 μ L/min for 120 min, at 37°C. As an alternative ligation step, SplintR RNA Ligase was used on a different reactional mix (5% glycerol, 1x SplintR Ligase buffer (New England Biolabs), 0,5 U/ μ L Splint R RNA ligase (New England Biolabs), 1 U/ μ L RiboProtect, and nuclease-free water), being flowed at 0,5 μ L/min for 60 min, at 37°C.

Once the ligation step is finished, primer hybridization targeting the PLP is done using a second hybridization solution with 100 nM of PLP-Primer, 2x SSC, 5% Ethylene Carbonate, 15 mM MgCl_2 , 0,1% Tween20, 1,25% Glycerol, and nuclease-free water. This solution was then flowed 1,0 μ L/min for 30 min, at 37°C.

Upon proper primer hybridization, RCA will take place inside the microfluidic device, flowing the amplification reactional mix (containing 5% glycerol, 250 μ M dNTPs, 200 μ g/ μ L BSA, 1 U/ μ L RiboProtect, 1 U/ μ L phi29 DNA polymerase in 1x polymerase buffer (500 mM Tris-HCL pH 8.3, 100 mM MgCl_2 , and 100 mM $(\text{NH}_4)_2\text{SO}_4$), and nuclease-free water.

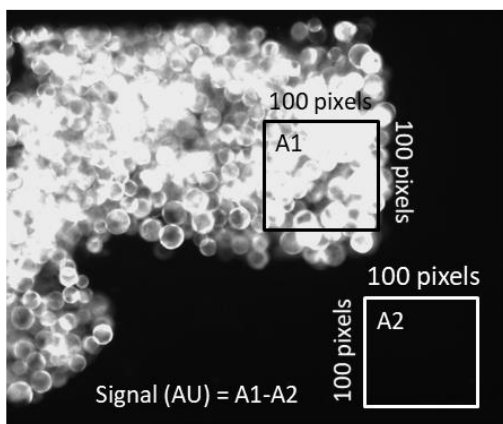
Similarly to the previous methods, labelling was performed at room temperature, flowing the final reaction mix containing 2x SSC, 20% formamide, nuclease-free water,

and 0,1 μM of detection (Cy5) oligonucleotides, at 5 $\mu\text{L}/\text{min}$ for 10 min. This is followed by the final washing step, flowing washing solution 2 (2x SSC, 20% formamide) at 10 $\mu\text{L}/\text{min}$ for 5 minutes, with imaging and quantification of amplified RCPs taking place immediately after this final step.

7. ROLLING CIRCLE PRODUCTS IMAGING AND QUANTIFICATION

Imaging and quantification of RCPs inside the microfluidic chip were performed as described elsewhere [45]. Images were acquired using an inverted Nikon Ti-Eclipse fluorescence microscope (10x objective) equipped with a SOLA SE light engine and either a Cy5 or TRITC filter cube, and quantification was performed using ImageJ software (NIH, USA) by comparing the average greyscale intensity of a given 100 pixels x 100 pixels area on the beads-empty channel interface for design A, and on the beads closest to the smaller microchannel cross-section for design B, with the fluorescence background of a 100 pixels x 100 pixels area on the channel, as shown in **Fig. 14**.

Design A:



Design B:

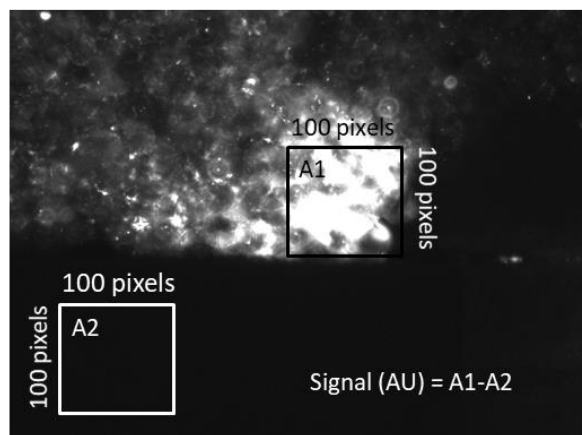


Fig. 14 – The registered fluorescence signal was obtained by measuring the mean grey value of 100 pixels x 100 pixels images in ImageJ of the beads (A1) and the empty-channel (A2) and calculating their difference (A1-A2). Images obtained using a fluorescent microscope (40x amplification).

RESULTS AND DISCUSSION

1. CHARACTERIZATION OF AMPLIFICATION EFFICIENCY THROUGH DETECTION OF BIOTINYLATED cDNA

As mentioned previously, the key concept explored in this work is the development of a microfluidic device with a simple isothermal nucleic acid amplification method (RCA), able to efficiently translate a certain concentration of a pathogen RNA (or DNA) present in a sample into a specific and easily readable optical signal.

To assert the efficiency of RCA in microfluidics with continuous perfusion, having the target immobilized in a solid phase, different concentrations of biotinylated cDNA ranging from 30 aM to 30 pM (approximately 30 copies/ μ L to 3×10^7 copies/ μ L) were tested. This was done since those conditions differ significantly from in-solution RCA, and thus such changes could prevent amplification from taking place. The used protocol was adapted from previous in-solution RCA works [45], with optimizations made based on *in-situ* sequencing methods using this amplification method [135], mainly due to the similar conditions between this sequencing method and the microfluidic environment inside the used devices. The used cDNA corresponded to a conserved Orf1ab region of the SARS-CoV-2 genome, encoding for RdRp, that is used to identify SARS-CoV-2 [87, 102]. Because the target is biotinylated, it can easily be captured in streptavidin-coated beads inside the microfluidic device, ensuring that it is amplified and remains in the device during the whole process. Representative images of each of the cDNA concentrations detected in the microfluidic device design A are shown in **Fig. 15**.

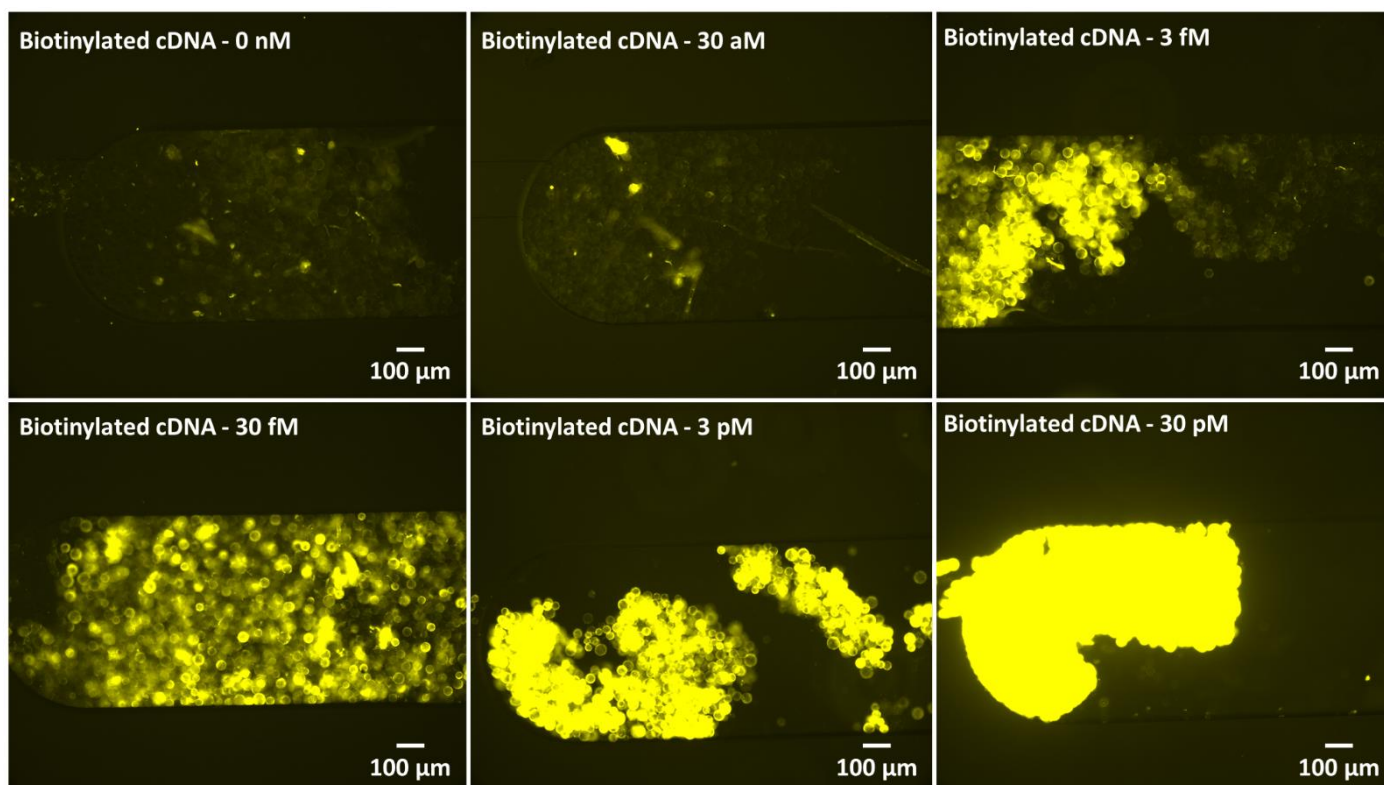


Fig. 15 – Representative images of the microchannels for different biotinylated cDNA concentrations, using the microfluidic device design A. Flow direction from the right side to the left side (←) of the images. Images obtained using a fluorescent microscope (40x amplification).

It is possible to see that the biotinylated target was amplified, and a significant fluorescent signal could be observed for concentrations down to 3 fM (approximately 3×10^3 copies/ μL), while 30 aM of target (approximately 30 copies/ μL) presented fluorescence very similar to the negative control. This shows that PLP-based RCA can be performed in a microfluidic environment with continuous perfusion, showing that fluid dynamics inside the device do not interfere significantly with the outcome of the amplification when compared to the in-solution alternative. Furthermore, the successful miniaturization of this amplification method allows further innovation and development of RCA-based on-chip RNA detection.

2. SARS-CoV-2 RNA DETECTION THROUGH BIOTINYLATED ANCHOR-BASED CAPTURE AND in-L-PROBE PLP AMPLIFICATION (HybRCA)

Because of the difficult challenge of performing PLP-based RCA directly on RNA reliably, mainly due to the lack of highly specific and efficient enzymes that can ligate the PLP in a DNA-RNA interface, a reverse transcription step of the target is usually required, not only increasing the time but also the cost of the amplification and, subsequently, of the diagnostic. If hybridized correctly, L-Probes allow PLPs to hybridize indirectly to the RNA in a DNA-DNA interface that can easily be recognized by high-fidelity DNA ligases, allowing PLP ligation and amplification to occur without the need for reverse transcription of the target RNA.

Biotinylated anchors were used to capture SARS-CoV-2 RNA in the streptavidin-coated beads inside the device, relying on a similar strategy used in previous works to successfully capture amplification products [45].

2.1. RNA fragment detection using Microfluidic Device Design A

Initial HybRCA experiments were performed targeting a small SARS-CoV-2 RNA fragment of the RdRp coding sequence (226 bp long), using one biotinylated anchor to capture the RNA inside the microfluidic chip, and three L-Probes to allow detection through PLP-based RCA inside the device. Dilution series of viral RNA with concentrations ranging from 6×10^7 copies/ μL down to 3×10^3 copies/ μL (inside the device) were tested for device design A and obtained microscope images were processed and analyzed as mentioned in the previous section. Representative images of the RNA detected in the microfluidic device design A are presented in **Fig. 16**, and the established

correlation between the target concentration inside the microfluidic device and the observed fluorescent signal is shown in **Fig. 17**.

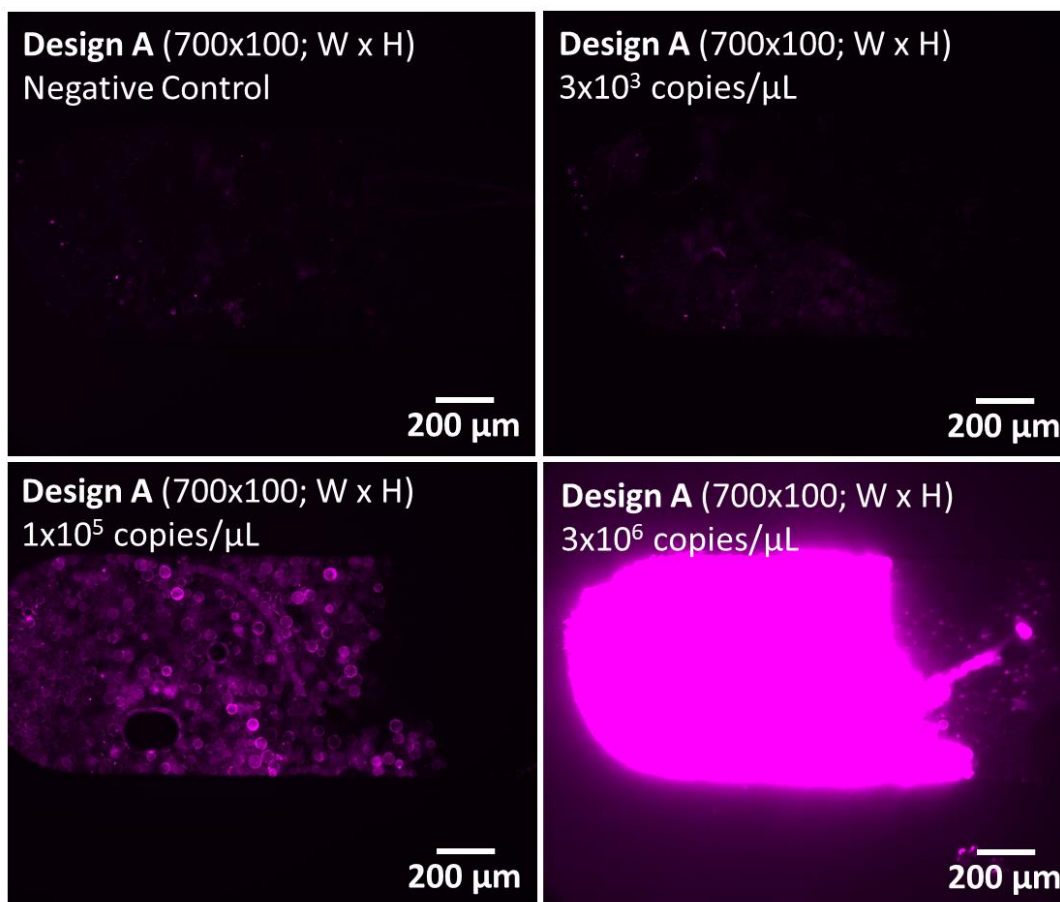


Fig. 16 – Representative images of the microchannels for different SARS-CoV-2 RNA fragment concentrations (viral copies/ μL), using the microfluidic device design A. Flow direction from the right side to the left side ($\leftarrow$) of the images. Images obtained using a fluorescent microscope (40x amplification).

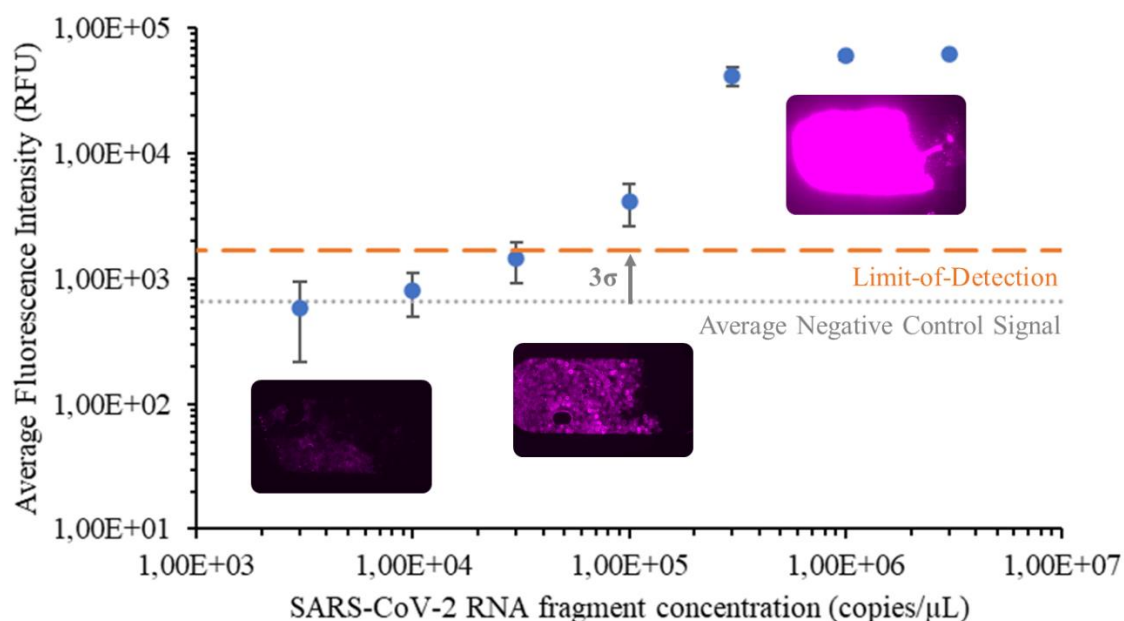


Fig. 17 – Average Fluorescence Intensity (RFU) measured on the beads as a function of SARS-CoV-2 RNA concentration (viral copies/ μL) – Blue. Limit-of-Detection for the used method corresponding to approximately 3×10^4 viral copies/ μL –

Orange. The measurements were performed using fluorescence microscopy followed by grey scale intensity image analysis.

Microscope images of the microchannels confirm that not only the RNA fragment is captured in the streptavidin-coated beads upon prior hybridization with the biotinylated anchors, but the L-Probes allow PLP-based amplification to occur. This originates RCPs that, upon being labelled with detection oligonucleotides with Cy5 fluorophore, make possible the detection of RNA without relying on a reverse transcription step. Since HybRCA allows the formation of one RCPs per hybridized L-Probe, the signal provided by one single RNA target will increase proportionally to the number of used L-Probes (in this case, the use of three different L-Probes increases provides a fluorescent signal corresponding, in theory, to three RCPs per SARS-CoV-2 RNA fragment). It should be noted, however, that increasing the number of L-Probes may also increase the background fluorescence due to non-specific hybridization, having a negative impact upon the LoD of the method.

Average fluorescence intensity seems to increase with the RNA fragment concentration in a sigmoidal curve with a linear part in the middle, and a fluorescence saturation level can be observed for concentrations above 3×10^5 copies/ μL . This, however, is not problematic in the design of rapid yes/no qualitative diagnostic platform, since high RNA levels are detected regardless, indicating accurately if the patient is infected or not.

Although uncertainty still surrounds the exact viral load threshold estimates the infectivity of a COVID-19 patient, it has been observed that infectious individuals tend to present a viral load between 100 and 1000 viral copies/ μL on collected samples [136]. The determined LoD for this method – corresponding to the average observed signal of the negative control plus three times its standard deviation (σ) – was approximately 3×10^4 viral copies/ μL , around three times above the clinically relevant threshold. Nevertheless, initial HybRCA experiments proved that said method can reliably detect RNA inside the microfluidic device, and the same methodology was applied for detection using the whole SARS-CoV-2 Control 2 RNA.

2.2. Detection of SARS-CoV-2 Control 2 RNA using Microfluidic Device Design A

Given the performance observed in the detection of the RNA fragment, HybRCA was further tested and optimized for detection of SARS-CoV-2 Control 2 RNA, using the whole RNA instead of just a small fragment.

Considering the different sizes between the previously used RNA fragment (226 bp) and Synthetic Control 2 SARS-CoV-2 RNA (approximately 29000 bp), optimizations were made to the HybRCA protocol to ensure the detection of this large RNA.

The number of used biotinylated anchors and L-Probes was increased to three and eight, respectively, all targeting the Orf1ab region encoding SARS-CoV-2 RdRp, no more than 70 bp apart from each other. This was done to minimize the effect of RNA degradation that could occur due to the typical instability of this molecule, that could result only in the capture of RNA without any L-Probe (thus not providing any signal), in the case anchors and probes were scattered through the RNA and far away from each other. This increase in number of anchors and probes guarantee a more efficient capture while also providing more fluorescent signal for each target RNA.

Hybridization of biotinylated anchors and L-Probes was performed at 65°C to prevent the formation of any RNA secondary structures that would negatively affect the efficiency of this step.

Dilution series of Synthetic Control 2 SARS-CoV-2 RNA with concentrations ranging from 6×10^6 copies/ μL down to 6×10^3 copies/ μL were tested using the microfluidic device design A, and obtained microscope images were processed and analyzed, with representative images of said detection shown in **Fig. 18**. Average fluorescence intensity observed in beads is represented in **Fig. 19** as a function of SARS-CoV-2 RNA concentration inside the device.

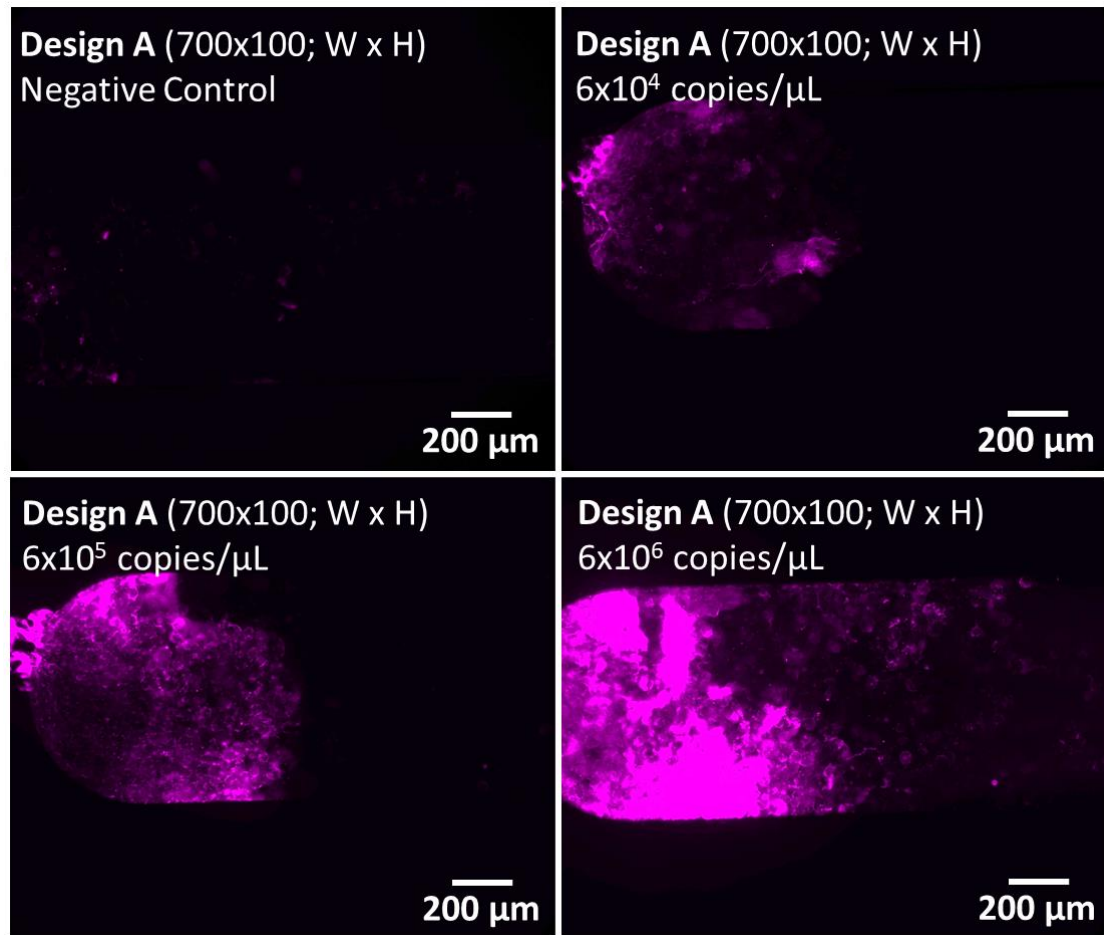


Fig. 18 – Representative images of the microchannels for different SARS-CoV-2 Control 2 RNA concentrations (viral copies/ μL), using the microfluidic device design A. Flow direction from the right side to the left side ($\leftarrow$) of the images. Images obtained using a fluorescent microscope (40x amplification).

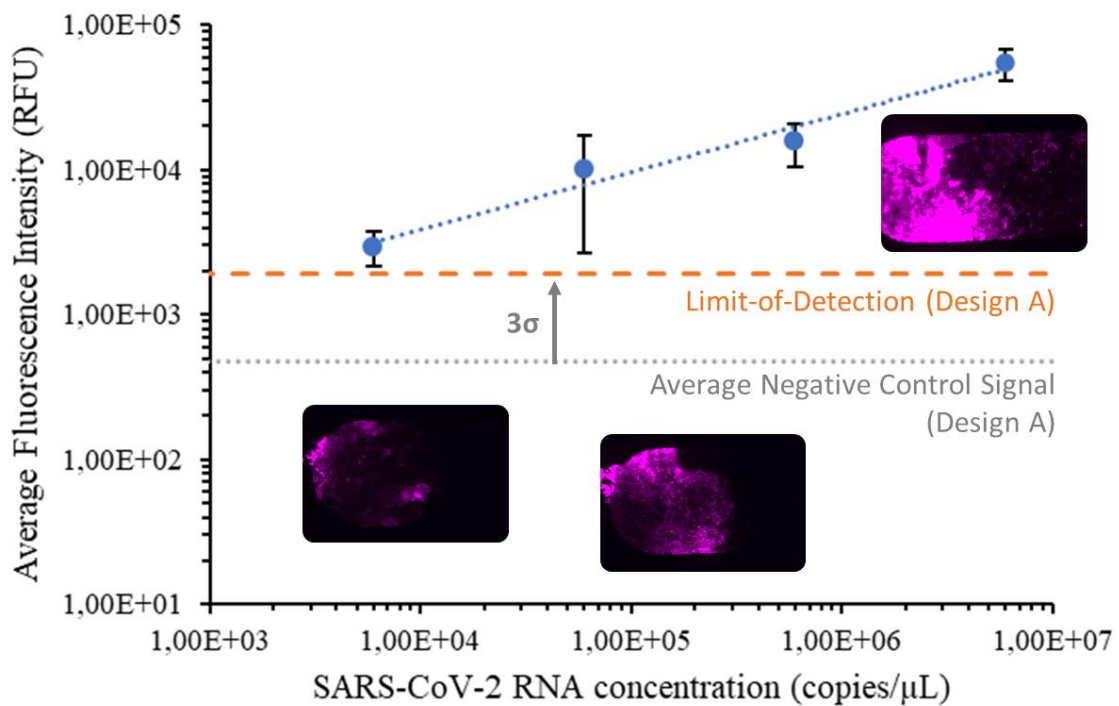


Fig. 19 – Average Fluorescence Intensity (RFU) measured on the beads as a function of SARS-CoV-2 RNA concentration

(viral copies/ μL) – Blue. Limit-of-Detection for the used method corresponding to approximately 6×10^3 viral copies/ μL – Orange. The measurements were performed using fluorescence microscopy followed by grey scale intensity image analysis.

Just like the previous experiments, it is possible to conclude that proper capture of RNA and amplification of L-Probes is happening inside the microfluidic chip by observing obtained fluorescent microscope images.

The determined LoD for SARS-CoV-2 Control 2 RNA was lower when compared to the RNA fragment detection, reaching an approximate value of 6×10^3 viral copies/ μL , closer to the clinically relevant detection range. By hybridizing an increased number of L-Probes with the pretended RNA target, more RCPs are going to be amplified, providing more fluorescent signal per target molecule. An increase in used biotinylated anchors also contributes for a more efficient capture of the RNA on the streptavidin-coated beads inside the device. It should be noted, however, that an exaggerated use of L-Probes and biotinylated anchors can also provide an increase in the background fluorescence, and the optimal probe-per-target ratio still requires further studies.

The microscopy images also show that, especially for the lower tested concentrations, the observed fluorescent signal is very heterogeneous across the microfluidic channel. This is especially problematic considering the used image analysis methodology, since the beads-empty channel interface would sometimes not present a grey scale intensity value that was representative of the rest of the microchannel. The observed heterogeneity resulted from the movement of the streptavidin-coated beads due to the plugging and unplugging of the inlet when switching flowed solutions. Another problem that also contributed to the arise of a heterogeneous signal was the flow of debris into the microchannels, that would not only disrupt the placement of the beads, but also provide non-specific fluorescence.

Considering that a reliable diagnostic test should not rely on the analysis of a heterogeneous fluorescent signal, new microfluidic devices were used to surpass this problem.

2.3. Microfluidic Device optimization – Design B and Design C

Since the heterogeneous signal arises mainly from the movement of the streptavidin-coated beads throughout the assay, it was necessary to find a way of confining the beads to prevent their movement. To do this, the microfluidic design from I. F. Pinto *et al.* (2021) [134] was chosen to be used and adapted for this novel diagnostic method. This new design presented taller parallel columns interconnected by microchannels with a cross-section smaller than the bead size, thus confining the beads in all directions and preventing their movement throughout the assay. Having several parallel columns also allowed for the possibility of creating a filter to prevent debris from reaching the inside of the channels with the streptavidin-coated beads. Two different microfluidic devices were designed having this idea in mind – Design B and Design C – with the main difference between the two being the dimensions, that would vary between the two due to the different debris flow prevention strategy used. A summary of each strategy is presented in **Fig. 20**.

For Design B, two parallel 600 x 140 μm (width x height) cross-section columns were used, interconnected by 200 x 20 μm (width x height) microchannels. The first column, closest to the inlet, was packed with Flash silica beads that would work as a filter, while the second column was fully packed with streptavidin-coated beads, confined in all direction to prevent their movement throughout the assay. Silica beads were chosen to fulfil the filter role in the first column due to not being very reactive with other reagents in the used conditions, while also presenting a very low ability of capturing nucleic acids, as shown in previous works [45].

Design C consisted of two smaller parallel 400 x 20 μm (width x height) columns connected by 200 x 8 μm (width x height) cross-section microchannels. For this design, the column closest to the inlet, the first one, was left unpacked, while the second column was packed with streptavidin-coated beads, also confined in all directions. Due to its very small dimensions, the small column would physically trap the bigger debris inside, preventing it from reaching the column with the streptavidin-coated beads.

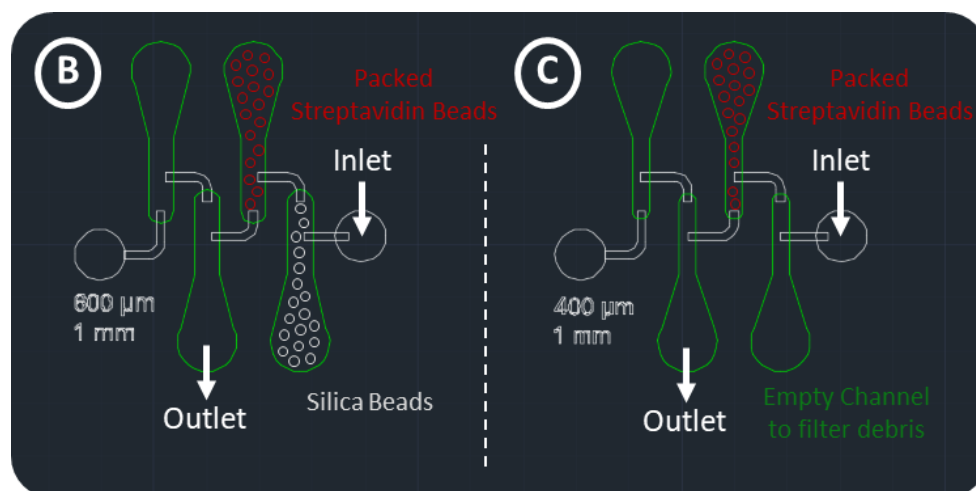


Fig. 20 – Top view of microfluidic devices design B and design C, inspired and adapted from I. F. Pinto et al. (2021). Design B (B) uses silica beads to filter debris and prevent its flow to inside the column with the streptavidin-coated beads, while design C (C) relies on a smaller column to trap bigger debris.

Brightfield images comparing the flow of debris into the streptavidin-beads inside the various microfluidic device designs are shown in **Fig. 21**.

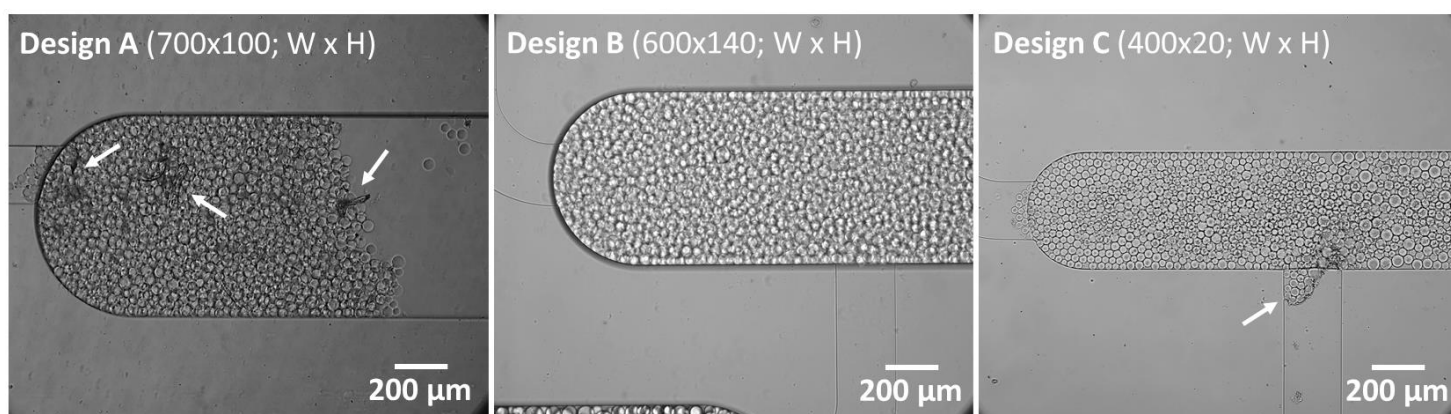


Fig. 21 – Representative brightfield images of the streptavidin-coated beads inside each microfluidic device design – A, B and C – with visible debris identified using a white arrow. Flow direction from the right side to the left side ($\leftarrow$) of the images. Images obtained using a fluorescent microscope (40x amplification).

By analysing the brightfield images presented above, it is possible to see that Design A, having no filter, has debris in-between the streptavidin-coated beads, disrupting the detection of RNA on those areas, while neither Design B nor C have debris among the beads. However, in the case of Design C, debris tends to accumulate at the cross-section of the column, which also corresponds to the area of the microfluidic device where most of the RNA is going to be captured, affecting the analysis of the observed fluorescent signal. This proves that, despite the small dimensions of the cross-section column and channels (400 x 20 μm and 200 x 8 μm , width x height, respectively), debris

still manages to reach the column containing the streptavidin-coated beads. Design B presented the cleanest results, with no visible debris reaching the column where RNA capture and detection is going to take place, showing that silica beads provide a reliable filter preventing debris from getting inside the microfluidic device. It should be noted, however, that heterogeneous fluorescence could be observed in the silica beads. This happened mainly due to the retention of debris in those beads, that would sometimes capture non-specifically L-Probes (resulting in the amplification of non-specific RCPs) and DOs. Because the signal observed in the silica beads column did not follow any trend and had no impact upon the detection of SARS-CoV-2 RNA in the subsequent experiments, such fluorescence was considered irrelevant in the context of this work. Other filter options should, however, be considered in the future, and the phenomenon observed in the aforementioned column should be further studied.

Due to its ability to confine the streptavidin-coated beads in all directions, preventing their movement, alongside the ability to block debris from reaching the streptavidin-beads channel, Design B was used for the remaining experiments, since it provided a less heterogeneous and more reliable fluorescent signal for RNA detection.

2.4. Effect of increased L-Probe usage in HybRCA RNA detection

Considering that theoretically, the number of RCPs and, subsequently, the observed fluorescent signal, increases proportionally with number of L-Probes hybridized to a certain target, one possible solution to reduce the LoD of the method would be increasing the number of used L-Probes. It should be noted, however, that an excessive increase of probes will not only increase the complexity of the assay, but also possibly enable non-specific interactions that could result in an increase of background fluorescence.

To better understand the effect of using an increased number of different L-Probes, detection of SARS-CoV-2 Control 2 RNA was performed using a set of 8 L-Probes and 12 L-Probes, targeting the Orf1ab region encoding for SARS-CoV-2 RdRp. The concentrations of each individual L-Probe were the same as the previous experiments. A single concentration of RNA was tested for those experiments – 6×10^4 copies/ μL – above previously determined LoD, to have a better overview upon a possible increase in observed signal. A negative control was also used, similarly to previous experiments, without any RNA. Obtained images for each experiment are presented in **Fig. 22**.

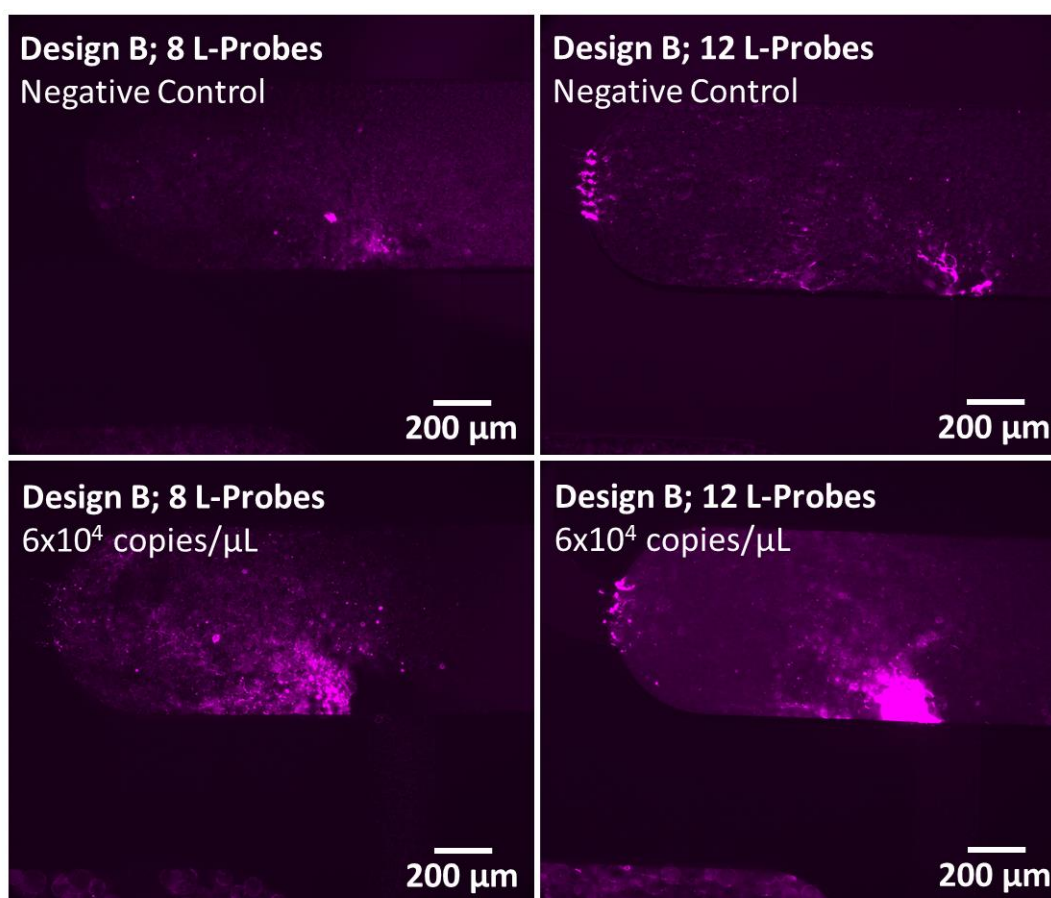


Fig. 22 – Representative images of the SARS-CoV-2 Control 2 RNA detection inside microfluidic device design A, using either 8 L-Probes (left) or 12 L-Probes (right) targeting the detected RNA. Flow direction from the right side to the left side (←) of the images. Images obtained using a fluorescent microscope (40x amplification).

Obtained microscope images show a clear increase of the observed fluorescent signal in the detection of 6×10^4 copies/ μL SARS-CoV-2 Control 2 RNA. This was to be expected, since the use of more L-Probes results in more RCPs per target, thus increasing the detected signal.

A slight increase in the background fluorescence was also observed when increasing the number of L-Probes from 8 to 12. This might have happened due to any non-specific interactions between L-Probes and biotinylated anchors, that resulted in the amplification of non-specific RCPs, thus providing fluorescent signal. It should be noted, however, that the increase of the specific fluorescent signal (RNA detection) was much more than the non-specific fluorescence. As such, the increased usage of L-Probes proved to be beneficial for the RNA detection, and 12 L-Probes were used in the subsequent experiments.

2.5. Detection of SARS-CoV-2 Control 2 RNA using Microfluidic Device Design B

Dilution series of SARS-CoV-2 Control 2 RNA ranging from 3×10^4 copies/ μL to 3×10^2 copies/ μL were tested using the new microfluidic device design B optimized protocol, with the changes mentioned in sections 2.3. and 2.4. Obtained microscope images are shown in **Fig. 23**, with the average fluorescence intensity observed in beads represented as a function of SARS-CoV-2 RNA concentration inside the device presented in **Fig. 24**.

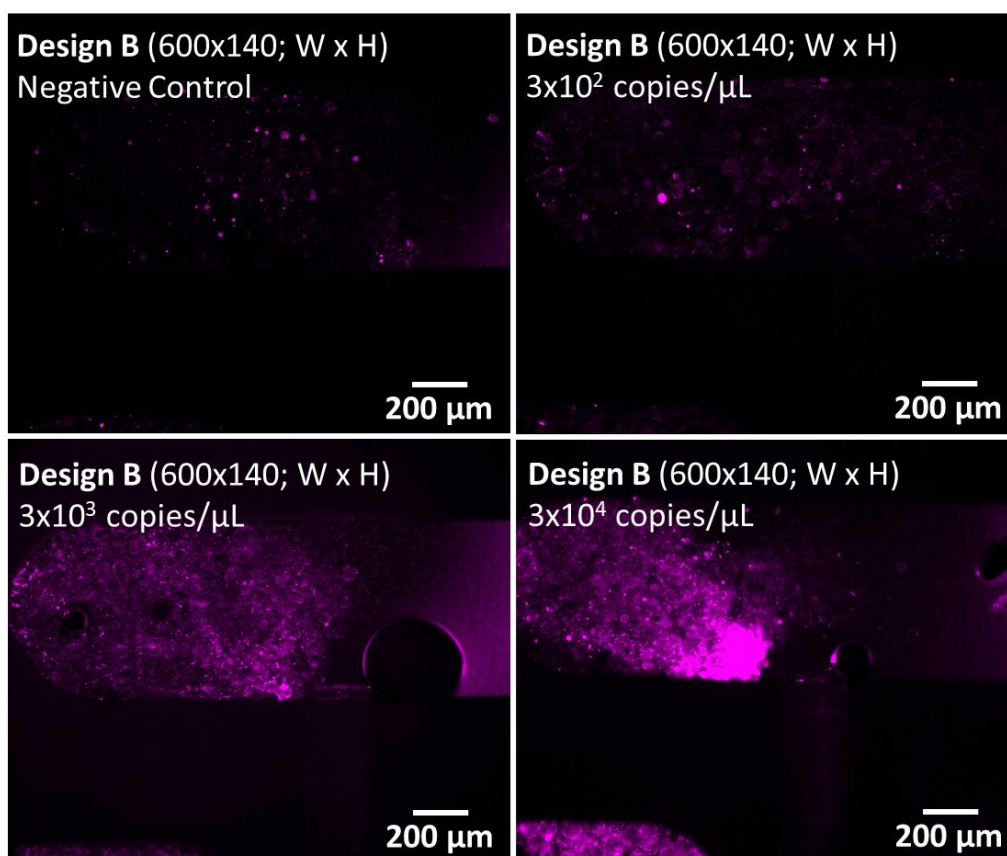


Fig. 23 – Representative images of the microchannels for different SARS-CoV-2 Control 2 RNA concentrations (viral copies/ μL), using the microfluidic device design B. Flow direction from the right side to the left side ($\leftarrow$) of the images. Images obtained using a fluorescent microscope (40x amplification).

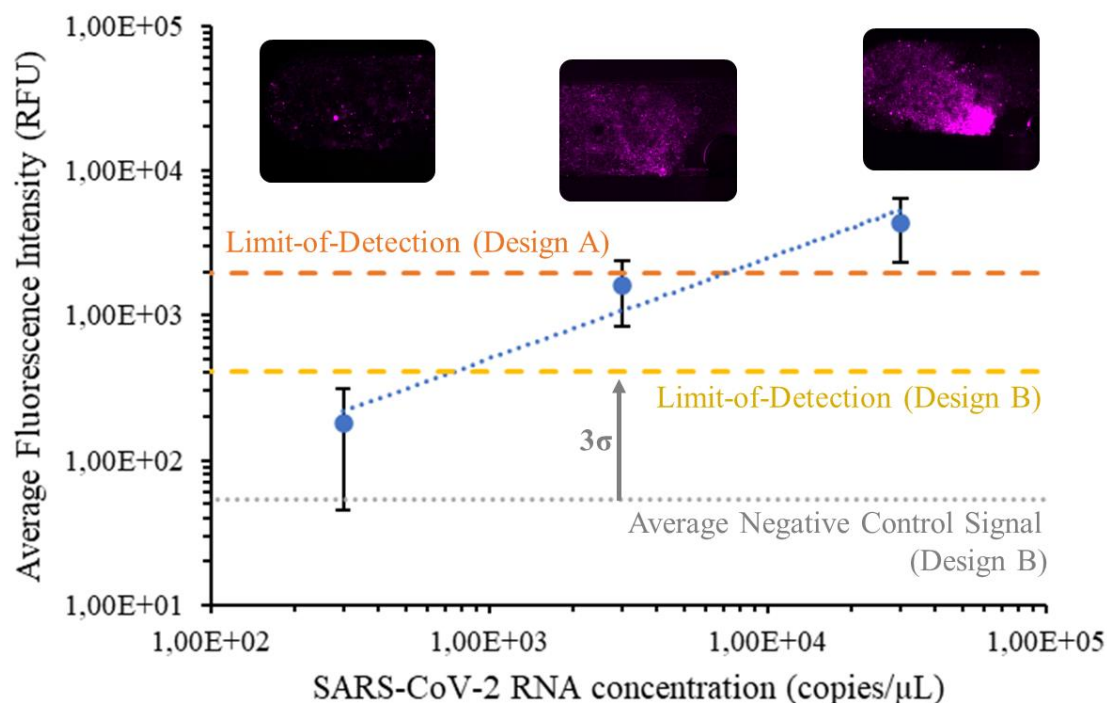


Fig. 24 – Average Fluorescence Intensity (RFU) measured on the beads as a function of SARS-CoV-2 RNA concentration (viral copies/ μL) – Blue. Limit-of-Detection for the method using microfluidic device design A, corresponding to approximately 6×10^3 viral copies/ μL – Orange. Limit-of-Detection for the used method using microfluidic device design B, corresponding to approximately 1×10^3 viral copies/ μL – Yellow. The measurements were performed using fluorescence microscopy followed by grey scale intensity image analysis.

By analysing the obtained results, it is possible to see that a much more homogeneous fluorescent signal is observed inside the microchannels of microfluidic device design B when compared to design A, facilitating the quantification of RNA captured in the beads. The silica beads filter also greatly lowered the fluorescence observed for the negative samples by preventing debris from reaching the streptavidin-beads column inside the microfluidic device. Those optimizations, combined with the L-Probe usage increase that allowed amplification of more RCPs per target RNA, allowed the LoD to reach a value slightly under the clinically relevant limit of 1000 viral copies/ μL .

Considering those results, HybRCA as shown the potential to provide quick reliable detection of SARS-CoV-2 RNA without the need of a reverse transcription step, and although further method optimization can still be done, current methodology can already provide a reliable qualitative yes/no answer for the patient state of infection.

2.6. Detection of SARS-CoV-2 Control 2 RNA in a complex matrix (saliva samples)

To study the effect of complex matrixes on HybRCA efficiency, saliva samples from an anonymous COVID-19 negative individual were collected and tested upon being spiked with 1:10 ratio (V/V) of Synthetic Control 2 SARS-CoV-2 RNA – positive sample (10^4 viral copies/ μL) – and after nuclease-free water was added in the same ratio – negative sample. Images were obtained using a fluorescent microscope with a Cy5 filter and a FITC filter for comparison, to better detect and identify RCPs amongst the complex saliva samples. Obtained images are shown in **Fig. 25**.

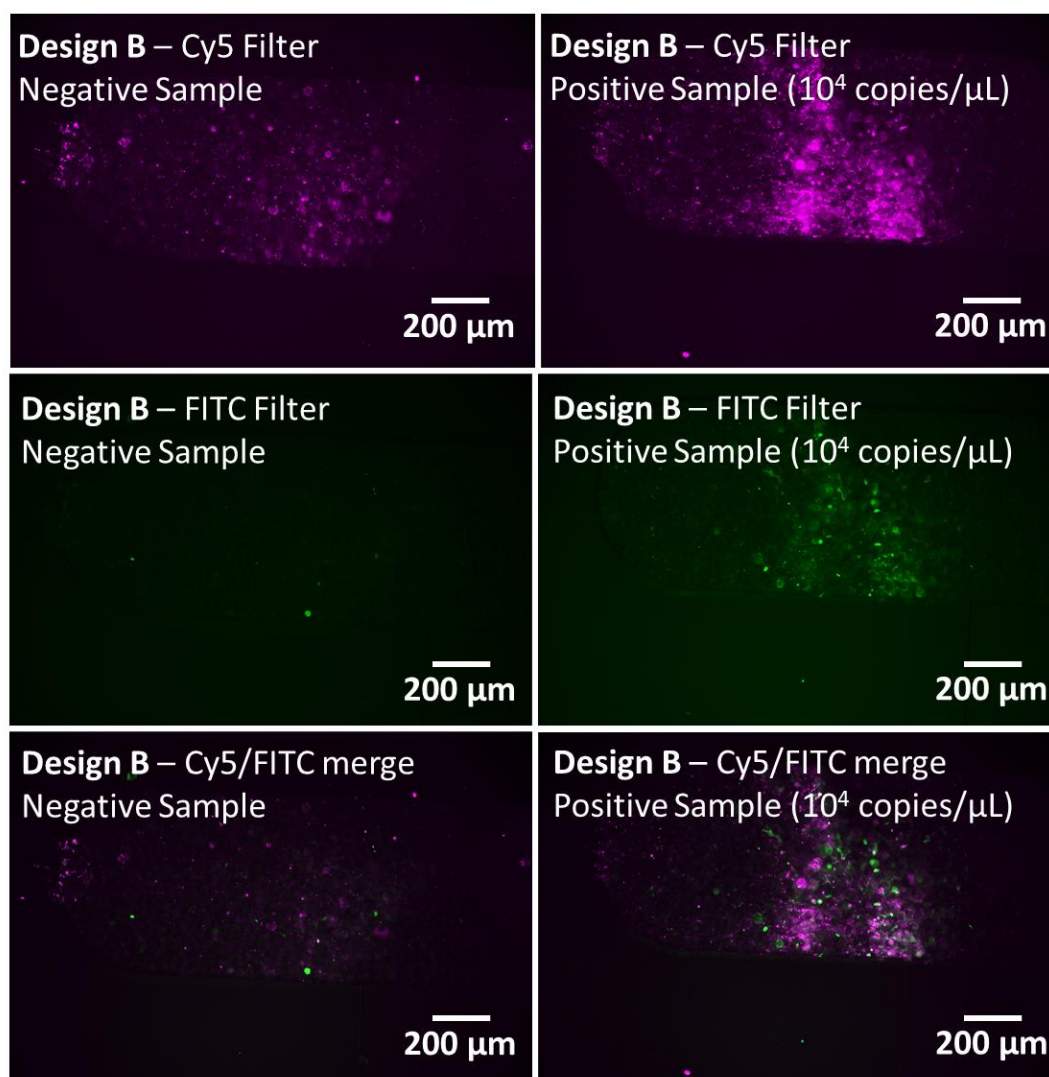


Fig. 25 – Representative images of the microchannels for the positive and negative saliva samples, using a Cy5 filter for RCP detection and identification, and a FITC filter for comparison. Flow direction from the right side to the left side (←) of the images. Images obtained using a fluorescent microscope (40x amplification).

Because RCPs are being labelled with DOs containing a Cy5 fluorophore, it is to be expected that those amplicons only present a fluorescent signal when using the Cy5 filter. As such, the FITC filter was used as an attempt to visualize other autofluorescent non-solid components of the saliva that could pass through the silica beads filter and provide some residual fluorescence.

Although results seem to look quite promising at first when looking to the Cy5 filter images, a more attentive look upon the FITC filter images show us that some residual fluorescence overlaps with the signal observed when using the Cy5 filter. This may indicate that a more viscous part of the saliva with residual fluorescence might ended up trapped in the region alongside the SARS-CoV-2 RNA, making a proper analysis more difficult. This is to be expected, since no prior treatment or dilution was

made to the saliva sample, and its viscous contents affected the performance of the assay. As such, dilution of the saliva before the analysis should be considered for future experiments. Non-specific amplification can also be observed for the negative sample, meaning that more stringency measures should also be included in the protocol.

3. DIRECT SARS-CoV-2 RNA DETECTION WITH χ RCA

Multiplexing is one of biggest assets of PLP-based RCA, the only isothermal amplification method able to detect up to 200 targets in a single sample [135] without relying on Next Gen Sequencing. This can be done by implementing a barcode-like detection system in the PLP backbone, that when combined with the single nucleotide specificity provided by the PLP hybridization and ligation step allows the identification of SNP. A schematic of this concept is presented in **Fig. 26**.

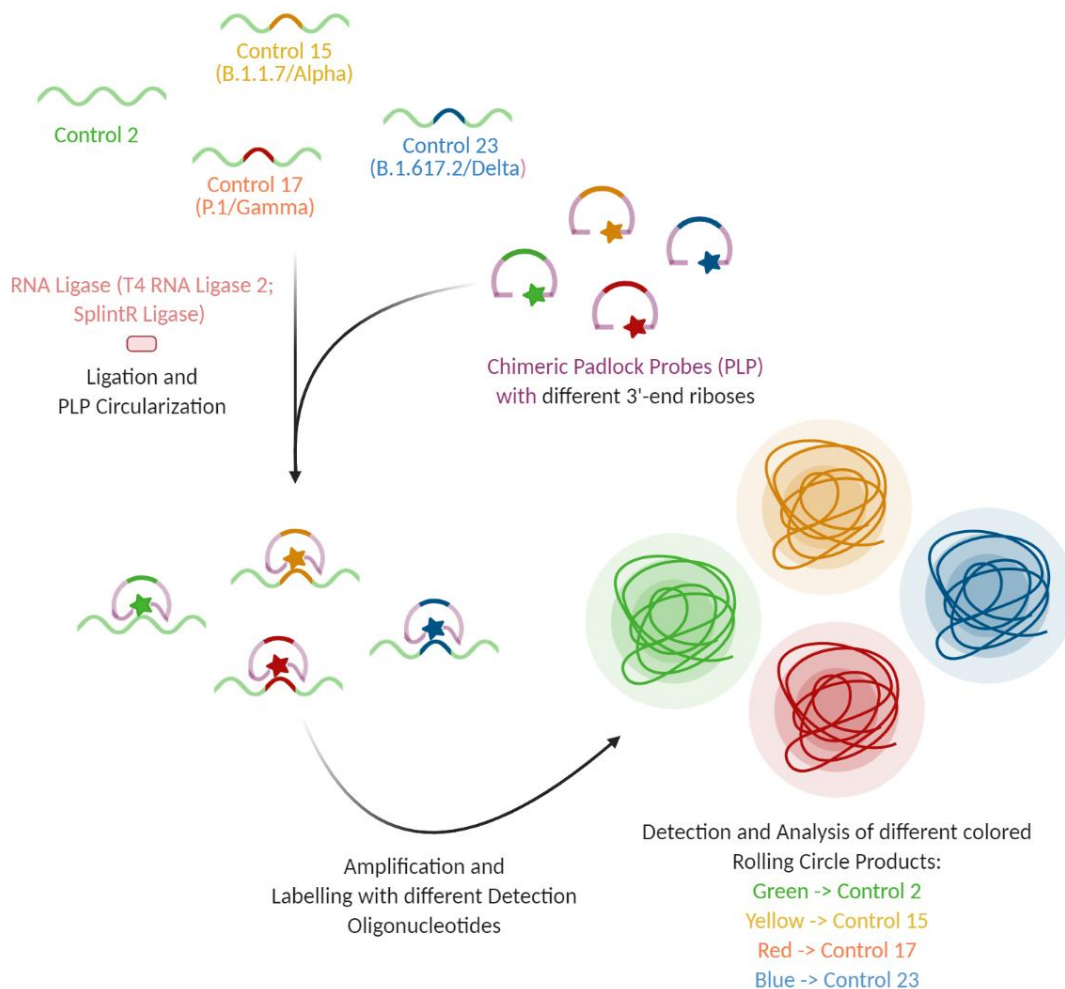


Fig. 26 – Schematic representing the multiplexing potential of χ RCA for SARS-CoV-2 detection and genotyping. By providing various chimeric PLPs with different 3'-end riboses (alongside the detectable sequence on each backbone), all targeting the same sequence, it is possible to identify the single nucleotide mutation and thus identify the COVID-19 variant of the patient.

The increasing concern with the different COVID-19 variants, alongside the pressing need of identifying them, fuelled the need for a quick diagnostic test that was able to diagnose and subtype the strain of an infected patient.

Considering its multiplexing capabilities and single nucleotide specificity, PLP-base RCA presents a good theoretical potential for this diagnosis. However, since L-Probes do not provide the single base pair specificity wanted for the detection of COVID-19 strains and considering that only few highly specific and efficient ligases able to ligate DNA-PLPs to RNA are currently known, a different approach was needed to successfully identify and distinguish the different variants.

A concept designed by Krzywkowski T. *et al.* (2019) [137], the “chimeric” PLP – a regular PLP with a ribose at the 3'-end instead of a deoxyribose – is able to hybridize directly with RNA and has been described to be ligated with end-joining fidelity (if properly hybridized) by two different RNA ligases: the T4 RNA Ligase 2 (T4Rnl2), and the Chlorella virus DNA ligase (PBCV-1 DNA ligase; SplintR Ligase).

By adapting this “chimeric” PLP usage and ligation to the developed HybRCA assay, Chimeric-PLP-based Rolling Circle Amplification (χ RCA) was used to attempt a reliable detection of SARS-CoV-2 Control 2 RNA inside microfluidic device design B, using either T4Rnl2 or SplintR for the ligation step.

Similarly to HybRCA, χ RCA uses the biotinylated anchor strategy to capture the RNA in the streptavidin-coated beads inside the microfluidic device. PLP hybridization is done before ligation, to increase its efficiency. A PLP-primer is also provided and hybridized on the PLP backbone before amplification takes place in order to facilitate amplification without using the 3'-end of the long RNA target as initiation site.

3.1. Performance comparison of T4 RNA Ligase 2 and SplintR Ligase in χ RCA

Due to the importance of the ligation step for the specificity of the detection method, initial experiments focused mainly on comparing the performance of T4Rnl2 and SplintR ligase in the circularization of “chimeric” PLPs. For this, two different χ RCA protocols were developed, with the only difference being the ligation step, that was adapted for either ligase. To ensure that the different ligation conditions did not affect PLP hybridization, this step was done separately prior to the ligation. Representative images of the obtained results are presented in **Fig. 27**.

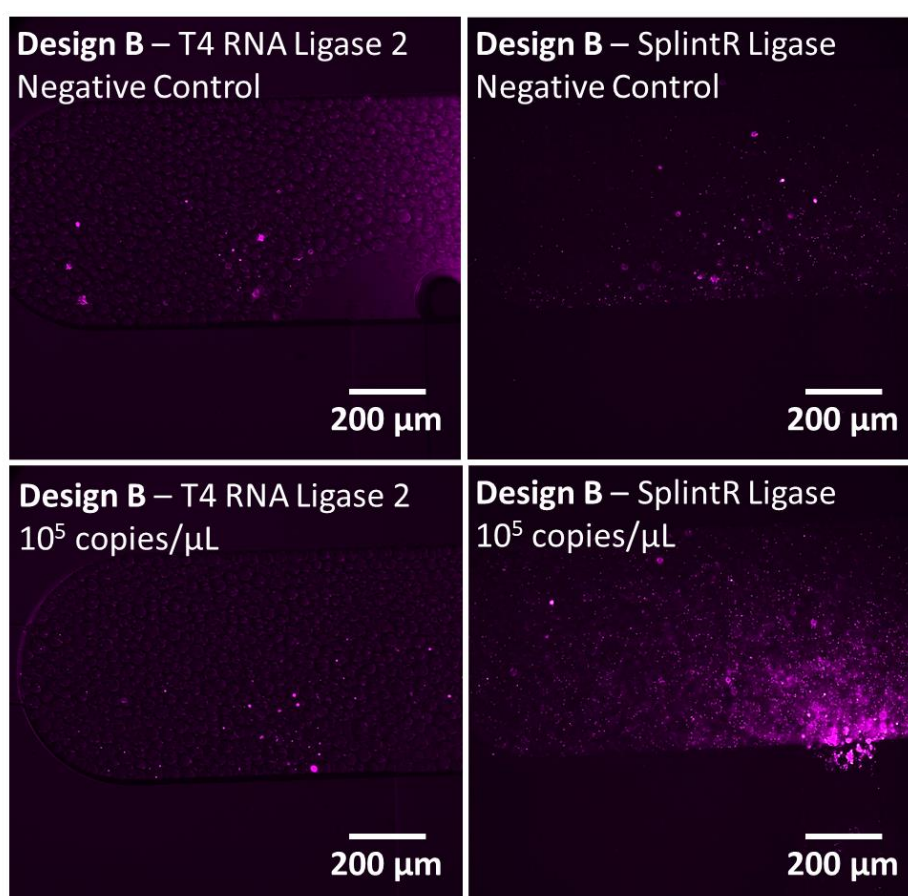


Fig. 27 – Representative images of the microchannels for the positive and negative samples with and without SARS-CoV-2 RNA, respectively, when ligation is performed using either T4 RNA Ligase 2 (left) or Chlorella virus DNA ligase (SplintR Ligase; right). Flow direction from the right side to the left side (←) of the images. Images obtained using a fluorescent microscope (40x amplification).

By analysing the images obtained through the fluorescent microscope, it is possible to understand that although both enzymes can ligate the “chimeric” PLPs, they do it with different efficiency and specificity.

For T4Rnl2, barely any significant fluorescent signal can be observed, both in the positive and negative control. As such, a digital measurement approach to image analysis could be made instead of the analog measurements made until this point, as reported in previous works [45] – instead of determining the average fluorescence in a certain area of the microchannel, the fluorescent dots must be counted, each corresponding to one RCP. T4Rnl2 overall showed a very low efficiency, just as described in the literature [137], despite providing great end-joining fidelity for the ligation.

SplintR ligase, on the other hand, efficiently circularized many “chimeric” PLPs in just half of the time of T4Rnl2, which was reflected in the big fluorescent signal that could be observed inside the microchannel with the SARS-CoV-2 RNA. However, this ligase does not present an end-joining fidelity as good as T4Rnl2 [137], resulting in the amplification of “chimeric” PLPs captured in the anchors due to mismatch hybridization of the probes, which can be observed by the fluorescence presented in the negative control. This problem can be surpassed by designing new biotinylated anchors that would have less tendency to mismatch with the PLPs, while also increasing the stringency of the hybridization and capture step (for example, by adding formamide) to match the conditions used for HybRCA.

It should be noted that, unlike T4Rnl2, SplintR does not strictly require “chimeric” PLPs, being able to ligate regular DNA-PLPs. Also, since this experiment was performed using only one “chimeric” PLP, thus obtaining only one RCP per target, it should be expected that an increase of used probes targeting the RNA should result in an increase in the observed fluorescence, further contributing to an increase of sensitivity of this method. Further testing is required to verify this.

Overall, despite its lower end-joining fidelity when compared to T4Rnl2, SplintR ligase provided a fast, efficient way of ligating the “chimeric” PLPs hybridized directly to the SARS-CoV-2 RNA, and it is expected that an increase in the stringency of the hybridization and capture conditions will reduce non-specific hybridization of probes, thus reducing the observed background signal. Future experiments should then be focused on the aforementioned optimization, followed by testing detection of SARS-CoV-2 Control 2 RNA and another strain using two different “chimeric” PLPs, with different detection sequences on the probe backbone.

CONCLUSION

In this work, novel RCA-on-chip methods for direct detection of SARS-CoV-2 RNA are presented that do not require a reverse transcription step. For HybRCA, a LoD as low as 1×10^3 viral copies/ μL was achieved in 90 minutes of isothermal amplification, with a single round of RCA at 37°C . Although further optimization is required to achieve even better sensitivity, the current methodology can already provide a qualitative, yes/no answer for a patient current state of infection. Furthermore, the sub-cm-sized, robust microfluidic device as the potential of being fully automated, while also allowing optical coupling and fluorescence detection using setups already reported for RCP detection [138], demonstrating great potential in approaching diagnostics both in resource-rich and resource-limited settings alike.

χ RCA is presented as a promising multiplexing option for SARS-CoV-2 detection and genotyping, but this direct RNA detection method is still severely hindered by the lack of efficient ligases with high end-joining fidelity. Despite this, results using SplintR showed the potential of this method, and further experiments with increased stringency conditions should be conducted. The effect of an increased use of “chimeric” PLPs should also be studied in the future, since the signal increase resulting of this can help achieving better sensitivities. The use of other ligases should also be considered – the Archaeal RNA ligase from *Thermococcus kodakarensis*, for example, has shown excellent end-joining fidelity [139], and could also help increasing the sensitivity of this multiplexing assay.

Aside from method optimization, sample preparation should also be addressed in the future considering the results observed for detection experiments in saliva samples, since the matrix complexity resulted in an increase of non-specific fluorescence. Consideration should also be given to new materials for the microfluidic device, since although PDMS is a good material for prototyping due to its properties, it is not cheap for mass fabrication, with thermoplastics appearing as a possible option.

REFERENCES

- [1] Y. Kawakita, *History of Diagnostics*, 1984.
- [2] J. Ince, and A. McNally, "Development of rapid, automated diagnostics for infectious disease: advances and challenges", *Expert Review of Medical Devices*, 6(6), 641-651, 2009.
- [3] S. T. Cowan, *Cowan and Steel's manual for the identification of medical bacteria*. Cambridge university press, 2004.
- [4] F. Drobniewski, M. Cooke, J. Jordan, N. Casali, T. Mugwagwa, A. Broda, *et al.*, "Systematic review, meta-analysis and economic modelling of molecular diagnostic tests for antibiotic resistance in tuberculosis", *Health technology assessment (Winchester, England)*, 19(34), 1-viii, 2015.
- [5] A. Vasala, V. P. Hytönen, and O. H. Laitinen, "Modern Tools for Rapid Diagnostics of Antimicrobial Resistance", *Frontiers in Cellular and Infection Microbiology*, 10, 308, 2020.
- [6] D. J. Isaacman, and B. L. Burke, "Utility of the serum C-reactive protein for detection of occult bacterial infection in children", *Archives of pediatrics & adolescent medicine*, 156(9), 905-909, 2002.
- [7] V. Gubala, L. F. Harris, A. J. Ricco, M. X. Tan, and D. E. Williams, "Point of care diagnostics: status and future", *Analytical chemistry*, 84(2), 487-515, 2012.
- [8] J. Dong, J. P. Olano, J. W. McBride, and D. H. Walker, "Emerging pathogens: challenges and successes of molecular diagnostics", *The Journal of Molecular Diagnostics*, 10(3), 185-197, 2008.
- [9] S. Skibsted, M. K. Bhasin, W. C. Aird, and N. I. Shapiro, "Bench-to-bedside review: future novel diagnostics for sepsis-a systems biology approach", *Critical care*, 17(5), 1-15, 2013.
- [10] H. Jayamohan, C. J. Lambert, H. J. Sant, A. Jafek, D. Patel, H. Feng, *et al.*, "SARS-CoV-2 pandemic: a review of molecular diagnostic tools including sample collection and commercial response with associated advantages and limitations", *Analytical and Bioanalytical Chemistry*, 1-23, 2020.
- [11] D. J. Swiner, S. Jackson, B. J. Burris, and A. K. Badu-Tawiah, "Applications of mass spectrometry for clinical diagnostics: the influence of turnaround time", *Analytical chemistry*, 92(1), 183-202, 2019.
- [12] A. W. Fung, V. Sugumar, A. H. Ren, and V. Kulasingam, "Emerging role of clinical mass spectrometry in pathology", *Journal of clinical pathology*, 73(2), 61-69, 2020.

- [13] D. Sow, B. Fall, M. Ndiaye, B. S. Ba, K. Sylla, R. Tine, *et al.*, "Usefulness of MALDI-TOF mass spectrometry for routine identification of *Candida* species in a resource-poor setting", *Mycopathologia*, 180(3), 173-179, 2015.
- [14] B. Mehra, S. Bhattar, P. Bhalla, and D. Rawat, "Rapid tests versus ELISA for screening of HIV infection: our experience from a voluntary counselling and testing facility of a tertiary care centre in North India", *International Scholarly Research Notices*, 2014.
- [15] K. Iha, M. Inada, N. Kawada, K. Nakaishi, S. Watabe, Y. H. Tan, *et al.*, "Ultrasensitive ELISA developed for diagnosis", *Diagnostics*, 9(3), 78, 2019.
- [16] H. Kim, D. R. Chung, and M. Kang, "A new point-of-care test for the diagnosis of infectious diseases based on multiplex lateral flow immunoassays", *Analyst*, 144(8), 2460-2466, 2019.
- [17] T. R. Kozel, and A. R. Burnham-Marusich, "Point-of-care testing for infectious diseases: past, present, and future", *Journal of clinical microbiology*, 55(8), 2313-2320. DOI: 10.1128/JCM.00476-17, 2017.
- [18] B. S. Parekh, C. Y. Ou, P. N. Fonjungo, M. B. Kalou, E. Rottinghaus, A. Puren, *et al.*, "Diagnosis of human immunodeficiency virus infection", *Clinical microbiology reviews*, 32(1), e00064-18. DOI: 10.1128/CMR.00064-18, 2018.
- [19] P. Craw, and W. Balachandran, "Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review", *Lab on a Chip*, 12(14), 2469-2486, 2012.
- [20] M. A. Sperança, R. B. Suzuki, A. D. Cabral, and A. D. S. Carmo, "Nucleic acid-based diagnosis and epidemiology of infectious diseases", *Nucleic Acids-From Basic Aspects to Laboratory Tools*, IntechOpen, 59-74, 2016.
- [21] R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, *et al.*, "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase", *Science*, 239(4839), 487-491, 1988.
- [22] I. M. Mackay, K. E. Arden, and A. Nitsche, "Real-time PCR in virology", *Nucleic acids research*, 30(6), 1292-1305, 2002.
- [23] M. G. Wise, D. L. Suarez, B. S. Seal, J. C. Pedersen, D. A. Senne, D. J. King, *et al.*, "Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples", *Journal of Clinical Microbiology*, 42(1), 329-338, 2004.
- [24] T. Briese, G. Palacios, M. Kokoris, O. Jabado, Z. Liu, N. Renwick, *et al.*, "Diagnostic system for rapid and sensitive differential detection of pathogens", *Emerging infectious diseases*, 11(2), 310, 2005.

- [25] T. Avni, L. Leibovici, and M. Paul, "PCR diagnosis of invasive candidiasis: systematic review and meta-analysis", *Journal of clinical microbiology*, 49(2), 665-670, 2011.
- [26] M. J. Espy, J. R. Uhl, P. S. Mitchell, J. N. Thorvilson, K. A. Svien, A. D. Wold, and T. F. Smith, "Diagnosis of herpes simplex virus infections in the clinical laboratory by LightCycler PCR", *Journal of Clinical Microbiology*, 38(2), 795-799, 2000.
- [27] W. K. Roth, M. Weber, and E. Seifried, "Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting", *The Lancet*, 353(9150), 359-363, 1999.
- [28] IDT - CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel.
- [29] J. Li, J. Macdonald, "Advances in isothermal amplification: novel strategies inspired by biological processes", *Biosensors and Bioelectronics*, 64, 196-211, 2015.
- [30] M. Vincent, Y. Xu, and H. Kong, "Helicase-dependent isothermal DNA amplification", *EMBO reports*, 5(8), 795-800, 2004.
- [31] S. Ciftci, "Padlock Probe-Based Nucleic Acid Amplification Tests: Point-of-care Diagnostics of Infectious Diseases", Doctoral dissertation, Department of Biochemistry and Biophysics, Stockholm University, 2019.
- [32] G. A. Obande, and K. K. B. Singh, "Current and future perspectives on isothermal nucleic acid amplification technologies for diagnosing infections", *Infection and drug resistance*, 13, 455, 2020.
- [33] T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino, and T. Hase, "Loop-mediated isothermal amplification of DNA", *Nucleic acids research*, 28(12), e63-e63, 2000.
- [34] D. L. Rudolph, V. Sullivan, S. M. Owen, and K. A. Curtis, "Detection of acute HIV-1 infection by RT-LAMP", *PloS one*, 10(5), e0126609, 2015.
- [35] M. Sabalza, R. Yasmin, C. A. Barber, T. Castro, D. Malamud, B. J. Kim, *et al.*, "Detection of Zika virus using reverse-transcription LAMP coupled with reverse dot blot analysis in saliva", *PloS one*, 13(2), e0192398, 2018.
- [36] W. E. Huang, B. Lim, C. C. Hsu, D. Xiong, W. Wu, Y. Yu, *et al.*, "RT-LAMP for rapid diagnosis of coronavirus SARS-CoV-2", *Microbial biotechnology*, 13(4), 950-961, 2020.
- [37] L. M. Zanolli, and G. Spoto, "Isothermal amplification methods for the detection of nucleic acids in microfluidic devices", *Biosensors*, 3(1), 18-43, 2013.
- [38] L. T. Lau, J. Banks, R. Aherne, I. H. Brown, N. Dillon, R. A. Collins, *et al.*, "Nucleic acid sequence-based amplification methods to detect avian influenza

- virus”, *Biochemical and biophysical research communications*, 313(2), 336-342, 2004.
- [39] T. Mourez, C. Delaugerre, M. Vray, V. Lemée, F. Simon, and J. C. Plantier, “Comparison of the bioMérieux NucliSENS EasyQ HIV-1 v2. 0–HIV-1 RNA quantification assay versus Abbott RealTime HIV-1 and Roche Cobas TaqMan HIV-1 v2. 0 on current epidemic HIV-1 variants”, *Journal of Clinical Virology*, 71, 76-81, 2015.
- [40] P. Singleton, “Nucleic Acid Amplification II: NASBA, TMA, SDA”, in *DNA Methods in Clinical Microbiology* (pp. 126-151). Springer, Dordrecht, 2000.
- [41] M. Nilsson, H. Malmgren, M. Samiotaki, M. Kwiatkowski, B. P. Chowdhary, and U. Landegren, “Padlock probes: circularizing oligonucleotides for localized DNA detection”, *Science*, 265(5181), 2085-2088, 1994.
- [42] M. M. Ali, F. Li, Z. Zhang, K. Zhang, D. K. Kang, J. A. Ankrum, J. A., *et al.*, “Rolling circle amplification: a versatile tool for chemical biology, materials science and medicine”, *Chemical Society Reviews*, 43(10), 3324-3341, 2014.
- [43] J. Tong, W. Cao, and F. Barany, “Biochemical properties of a high fidelity DNA ligase from *Thermus* species AK16D”, *Nucleic acids research*, 27(3), 788-794, 1999.
- [44] J. Baner, M. Nilsson, M. Mendel-Hartvig, and U. Landegren, “Signal amplification of padlock probes by rolling circle replication”, *Nucleic acids research*, 26(22), 5073-5078, 1998.
- [45] R. R. G. Soares, J. C. Varela, U. Neogi, S. Ciftci, M. Ashokkumar, I. F. Pinto, *et al.*, “Sub-attomole detection of HIV-1 using padlock probes and rolling circle amplification combined with microfluidic affinity chromatography”, *Biosensors and Bioelectronics*, 166, 112442, 2020.
- [46] R. R. G. Soares, A. Pettke, A. Robles-Remacho, S. Zeebaree, S. Ciftci, M. Tampere, *et al.*, “Circle-to-circle amplification coupled with microfluidic affinity chromatography enrichment for in vitro molecular diagnostics of Zika fever and analysis of anti-flaviviral drug efficacy”, *Sensors and Actuators B: Chemical*, 129723, 2021.
- [47] S. Ciftci, F. Neumann, S. Abdurahman, K. S. Appelberg, A. Mirazimi, M. Nilsson, and N. Madaboosi, “Digital rolling circle amplification–based detection of Ebola and other tropical viruses”, *The Journal of Molecular Diagnostics*, 22(2), 272-283, 2020.
- [48] F. Neumann, I. Hernández-Neuta, M. Grabbe, N. Madaboosi, J. Albert, and M. Nilsson, “Padlock probe assay for detection and subtyping of seasonal influenza”, *Clinical chemistry*, 64(12), 1704-1712, 2018.

- [49] P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, and D. C. Ward, "Mutation detection and single-molecule counting using isothermal rolling-circle amplification", *Nature genetics*, 19(3), 225-232, 1998.
- [50] D. C. Thomas, G. A. Nardone, and S. K. Randall, "Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction", *Archives of pathology & laboratory medicine*, 123(12), 1170-1176, 1999.
- [51] H. Cao, X. Zhou, Y. Zeng, "Microfluidic exponential rolling circle amplification for sensitive microRNA detection directly from biological samples", *Sensors and Actuators B: Chemical*, 279, 447-457, 2019.
- [52] S. V. Hamidi, H. Ghourchian, and G. Tavoosidana, "Real-time detection of H5N1 influenza virus through hyperbranched rolling circle amplification", *Analyst*, 140(5), 1502-1509, 2015.
- [53] T. P. Beals, J. H. Smith, R. M. Nietupski, and D. J. Lane, "A mechanism for ramified rolling circle amplification", *BMC molecular biology*, 11(1), 1-10, 2010.
- [54] O. Piepenburg, C. H. Williams, D. L. Stemple, and N. A. Armes, "DNA detection using recombination proteins", *PLoS Biol*, 4(7), e204, 2006.
- [55] Y. Zhao, F. Chen, Q. Li, L. Wang, and C. Fan, "Isothermal amplification of nucleic acids", *Chemical reviews*, 115(22), 12491-12545, 2015.
- [56] R. K. Daher, G. Stewart, M. Boissinot, and M. G. Bergeron, "Recombinase polymerase amplification for diagnostic applications", *Clinical chemistry*, 62(7), 947-958, 2016.
- [57] M. J. Kellner, J. G. Koob, J. S. Gootenberg, O. O. Abudayyeh, and F. Zhang, "SHERLOCK: nucleic acid detection with CRISPR nucleases", *Nature protocols*, 14(10), 2986-3012, 2019.
- [58] M. I. Mustafa, and A. M. Makhawi, "SHERLOCK and DETECTR: CRISPR-Cas systems as potential rapid diagnostic tools for emerging infectious diseases", *Journal of Clinical Microbiology*, 59(3), 2021.
- [59] J. S. Gootenberg, O. O. Abudayyeh, J. W. Lee, P. Essletzbichler, A. J. Dy, J. Joung, et al. "Nucleic acid detection with CRISPR-Cas13a/C2c2". *Science*, 356(6336), 438-442, 2017.
- [60] S. D. Wharam, P. Marsh, J. S. Lloyd, T. D. Ray, G. A. Mock, G. R. Assenberg, et al., "Specific detection of DNA and RNA targets using a novel isothermal nucleic acid amplification assay based on the formation of a three-way junction structure", *Nucleic acids research*, 29(11), e54-e54, 2001.

- [61] *Afinion™ 2 Analyser Abbott Product Information* [Online]. Available: <https://www.globalpointofcare.abbott/en/product-details/afinion2-analyzer.html>. [Accessed: 10-Feb-2021].
- [62] *m-PIMA™ Analyser Abbott Product Information* [Online]. Available: <https://www.globalpointofcare.abbott/en/product-details/m-pima-analyser.html>. [Accessed: 10-Feb-2021].
- [63] *Xpert® Xpress SARS-CoV-2/Flu/RSV test Cepheid Product Information* [Online]. Available: https://www.cepheid.com/en_US/tests/Critical-Infectious-Diseases/Xpert-Xpress-SARS-CoV-2-Flu-RSV. [Accessed: 10-Feb-2021].
- [64] L. Bissonnette, and M. G. Bergeron, “Diagnosing infections—Current and anticipated technologies for point-of-care diagnostics and home-based testing”, *Clinical Microbiology and Infection*, 16(8), 1044-1053, 2010.
- [65] K. J. Land, D. I. Boeras, X. S. Chen, A. R. Ramsay, and R. W. Peeling, “REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes”, *Nature microbiology*, 4(1), 46-54, 2019.
- [66] H. Zhu, Z. Fohlerová, J. Pekárek, E. Basova, and P. Neužil, “Recent advances in lab-on-a-chip technologies for viral diagnosis”, *Biosensors and Bioelectronics*, 153, 112041, 2020.
- [67] S. Arshavsky-Graham, and E. Segal. *Lab-on-a-Chip Devices for Point-of-Care Medical Diagnostics*, 2020.
- [68] U.S. Food and Drug Administration (FDA), “Coronavirus (COVID-19) Update: FDA Authorizes First COVID-19 Test for Self-Testing at Home”, *FDA NEWS RELEASE*, 2020 [Online]. Available: <https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-covid-19-test-self-testing-home>. [Accessed: 10-Feb-2021].
- [69] S. Campbell, and R. Klein, “Home testing to detect human immunodeficiency virus: boon or bane?”, *Journal of clinical microbiology*, 44(10), 3473-3476, 2006.
- [70] G. M. Whitesides, “The origins and the future of microfluidics”, *Nature*, 442(7101), 368–373. DOI: 10.1038/nature05058, 2006.
- [71] A. Burklund, A. Tadimety, Y. Nie, N. Hao, and J. X. Zhang, “Advances in diagnostic microfluidics”, *Advances in clinical chemistry*, 95, 1-72, 2020.
- [72] T. M. Squires, R. J. Messinger, and S. R. Manalis, “Making it stick: convection, reaction and diffusion in surface-based biosensors”, *Nature biotechnology*, 26(4), 417-426, 2008.
- [73] H. A. Stone, “Introduction to fluid dynamics for microfluidic flows”, in *CMOS biotechnology* (pp. 5-30). Springer, Boston, MA, 2007.

- [74] I. F. Pinto, R. R. G. Soares, M. E. L. Mäkinen, V. Chotteau, and A. Russom, "Multiplexed Microfluidic Cartridge for At-Line Protein Monitoring in Mammalian Cell Culture Processes for Biopharmaceutical Production", *ACS sensors*, 2021.
- [75] H. Jayamohan, H. J. Sant, B. K. Gale, "Applications of microfluidics for molecular diagnostics", *Microfluidic Diagnostics*, 305-334, 2013.
- [76] J. C. McDonald, D. C. Duffy, J. R. Anderson, D. T. Chiu, H. Wu, O. J. Schueller, and G. M. Whitesides, "Fabrication of microfluidic systems in poly (dimethylsiloxane)", *ELECTROPHORESIS: An International Journal*, 21(1), 27-40, 2000.
- [77] L. Xiong, P. Chen, and Q. Zhou, "Adhesion promotion between PDMS and glass by oxygen plasma pre-treatment", *Journal of Adhesion Science and Technology*, 28(11), 1046-1054, 2014.
- [78] P. Zhou, X. L. Yang, X. G. Wang, B. Hu, L. Zhang, W. Zhang, *et al.*, "A pneumonia outbreak associated with a new coronavirus of probable bat origin", *nature*, 579(7798), 270-273, 2020.
- [79] World Health Organization (WHO), *Rolling Updates on coronavirus disease (COVID-19)* [Online]. Available: <https://www.who.int/emergencies/diseases/novel-coronavirus-2019/events-as-they-happen>. [Accessed: 24-Mar-2021].
- [80] World Health Organization (WHO), *Coronavirus disease (COVID-19) pandemic* [Online]. Available: <https://www.who.int/emergencies/diseases/novel-coronavirus-2019>. [Accessed: 15-Sep-2021].
- [81] I. Astuti, "Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): An overview of viral structure and host response", *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*, 14(4), 407-412, 2020.
- [82] Y. Zhang, X. Geng, Y. Tan, Q. Li, C. Xu, J. Xu, *et al.*, "New understanding of the damage of SARS-CoV-2 infection outside the respiratory system", *Biomedicine & Pharmacotherapy*, 110195, 2020.
- [83] J. He, H. Tao, Y. Yan, S. Y. Huang, and Y. Xiao, "Molecular mechanism of evolution and human infection with SARS-CoV-2", *Viruses*, 12(4), 428, 2020.
- [84] J. Shang, Y. Wan, C. Luo, G. Ye, Q. Geng, A. Auerbach, and F. Li, "Cell entry mechanisms of SARS-CoV-2", *Proceedings of the National Academy of Sciences*, 117(21), 11727-11734, 2020.
- [85] A. K. Banerjee, M. R. Blanco, E. A. Bruce, D. D. Honson, L. M. Chen, A. Chow, *et al.*, "SARS-CoV-2 disrupts splicing, translation, and protein trafficking to suppress host defenses", *Cell*, 183(5), 1325-1339, 2020.

- [86] Y. Finkel, O. Mizrahi, A. Nachshon, S. Weingarten-Gabbay, D. Morgenstern, Y. Yahalom-Ronen, "The coding capacity of SARS-CoV-2", *Nature*, 589(7840), 125-130, 2021.
- [87] Y. Wang, V. Anirudhan, R. Du, Q. Cui, and L. Rong, "RNA-dependent RNA polymerase of SARS-CoV-2 as a therapeutic target", *Journal of medical virology*, 93(1), 300-310, 2021.
- [88] D. Sicari, A. Chatziioannou, T. Koutsandreas, R. Sitia, and E. Chevet, "Role of the early secretory pathway in SARS-CoV-2 infection", *Journal of Cell Biology*, 219(9), 2020.
- [89] S. Tang, Y. Mao, R. M. Jones, Q. Tan, J. S. Ji, N. Li, *et al.*, "Aerosol transmission of SARS-CoV-2? Evidence, prevention and control", *Environment international*, 144, 106039, 2020.
- [90] T. Singhal, "A review of coronavirus disease-2019 (COVID-19)", *The indian journal of pediatrics*, 87(4), 281-286, 2020.
- [91] S. Kumar, R. Nyodu, V. K. Maurya, and S. K. Saxena, "Host immune response and immunobiology of human SARS-CoV-2 infection", in *Coronavirus Disease 2019 (COVID-19)* (pp. 43-53). Springer, Singapore, 2020.
- [92] C. R. Carpenter, P. A. Mudd, C. P. West, E. Wilber, and S. T. Wilber, "Diagnosing COVID-19 in the emergency department: a scoping review of clinical examinations, laboratory tests, imaging accuracy, and biases", *Academic Emergency Medicine*, 27(8), 653-670, 2020.
- [93] W. J. Wiersinga, A. Rhodes, A. C. Cheng, S. J. Peacock, and H. C. Prescott, "Pathophysiology, transmission, diagnosis, and treatment of coronavirus disease 2019 (COVID-19): a review", *Jama*, 324(8), 782-793, 2020.
- [94] S. Liu, H. Luo, Y. Wang, L. E. Cuevas, D. Wang, S. Ju, and Y. Yang, "Clinical characteristics and risk factors of patients with severe COVID-19 in Jiangsu province, China: a retrospective multicentre cohort study", *BMC infectious diseases*, 20(1), 1-9, 2020.
- [95] Y. Wang, H. Kang, X. Liu, and Z. Tong, "Asymptomatic cases with SARS-CoV-2 infection", *Journal of medical virology*, 92(9), 1401-1403, 2020.
- [96] A. Wilder-Smith, and D. O. Freedman, "Isolation, quarantine, social distancing and community containment: pivotal role for old-style public health measures in the novel coronavirus (2019-nCoV) outbreak", *Journal of travel medicine*, 2020.
- [97] S. Muhammad, X. Long, and M. Salman, "COVID-19 pandemic and environmental pollution: A blessing in disguise?", *Science of the total environment*, 728, 138820, 2020.

- [98] R. Rossi, V. Socci, D. Talevi, S. Mensi, C. Niolu, F. Pacitti, *et al.*, "COVID-19 pandemic and lockdown measures impact on mental health among the general population in Italy", *Frontiers in psychiatry*, *11*, 790, 2020.
- [99] A. Kumar, and K. R. Nayar, "COVID 19 and its mental health consequences", *Journal of Mental Health*, *180*(6), 817-8, 2020.
- [100] A. Atalan, "Is the lockdown important to prevent the COVID-19 pandemic? Effects on psychology, environment and economy-perspective", *Annals of medicine and surgery*, *56*, 38-42, 2020.
- [101] C. Zhang, J. Gu, Q. Chen, N. Deng, J. Li, L. Huang, and X. Zhou, "Clinical and epidemiological characteristics of pediatric SARS-CoV-2 infections in China: A multicenter case series", *PLoS medicine*, *17*(6), e1003130, 2020.
- [102] N. Zhu, D. Zhang, W. Wang, X. Li, B. Yang, J. Song, *et al.*, "A novel coronavirus from patients with pneumonia in China, 2019", *New England journal of medicine*, 2020.
- [103] Y. Wang, H. Kang, X. Liu, and Z. Tong, "Combination of RT-qPCR testing and clinical features for diagnosis of COVID-19 facilitates management of SARS-CoV-2 outbreak", *Journal of medical virology*, *92*(6), 538-539, 2020.
- [104] G. B. Barra, T. H. Santa Rita, P. G. Mesquita, R. H. Jácomo, and L. F. A. Nery, "Analytical Sensitivity and Specificity of Two RT-qPCR Protocols for SARS-CoV-2 Detection Performed in an Automated Workflow", *Genes*, *11*(10), 1183, 2020.
- [105] V. M. Moreira, P. Mascarenhas, V. Machado, J. Botelho, J. J. Mendes, N. Taveira, and M. G. Almeida, "Diagnosis of SARS-Cov-2 Infection by RT-PCR Using Specimens Other Than Naso-and Oropharyngeal Swabs: A Systematic Review and Meta-Analysis", *Diagnostics*, *11*(2), 363, 2021.
- [106] X. Ren, Y. Liu, H. Chen, W. Liu, Z. Guo, C. Chen, *et al.*, *Application and optimization of RT-PCR in diagnosis of SARS-CoV-2 infection*, 2020.
- [107] W. C. Dai, H. W. Zhang, J. Yu, H. J. Xu, H. Chen, S. P. Luo, *et al.*, "CT imaging and differential diagnosis of COVID-19", *Canadian Association of Radiologists Journal*, *71*(2), 195-200, 2020.
- [108] W. Yang, and F. Yan, "Patients with RT-PCR-confirmed COVID-19 and normal chest CT", *Radiology*, *295*(2), E3-E3, 2020.
- [109] E. Bischof, G. Chen, and M. T. Ferretti, "Understanding COVID-19 new diagnostic guidelines—a message of reassurance from an internal medicine doctor in Shanghai", *Swiss medical weekly*, *150*(0910), 2020.
- [110] T. Ai, Z. Yang, H. Hou, C. Zhan, C. Chen, W. Lv, *et al.*, "Correlation of chest CT and RT-PCR testing for coronavirus disease 2019 (COVID-19) in China: a report of 1014 cases", *Radiology*, *296*(2), E32-E40, 2020.

- [111] S. Neveu, I. Saab, S. Dangeard, S. Bennani, M. Tordjman, G. Chassagnon, and M. P. Revel, "Incidental diagnosis of Covid-19 pneumonia on chest computed tomography", *Diagnostic and interventional imaging*, 101(7-8), 457-461, 2020.
- [112] Y. Han, and H. Yang, "The transmission and diagnosis of 2019 novel coronavirus infection disease (COVID-19): a Chinese perspective", *Journal of medical virology*, 92(6), 639-644, 2020.
- [113] A. R. Sahin, A. Erdogan, P. M. Agaoglu, Y. Dineri, A. Y. Cakirci, M. E. Senel, *et al.*, "2019 novel coronavirus (COVID-19) outbreak: a review of the current literature", *EJMO*, 4(1), 1-7, 2020.
- [114] J. F. W. Chan, C. C. Y. Yip, K. K. W. To, T. H. C. Tang, S. C. Y. Wong, K. H. Leung, *et al.*, "Improved molecular diagnosis of COVID-19 by the novel, highly sensitive and specific COVID-19-RdRp/HeI real-time reverse transcription-PCR assay validated in vitro and with clinical specimens", *Journal of clinical microbiology*, 58(5), 2020.
- [115] G. S. Park, K. Ku, S. H. Baek, S. J. Kim, S. I. Kim, B. T. Kim, and J. S. Maeng, "Development of reverse transcription loop-mediated isothermal amplification assays targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)", *The Journal of Molecular Diagnostics*, 22(6), 729-735, 2020.
- [116] Y. Zhang, N. Odiwuor, J. Xiong, L. Sun, R. O. Nyaruaba, H. Wei, and N. A. Tanner, "Rapid molecular detection of SARS-CoV-2 (COVID-19) virus RNA using colorimetric LAMP", *MedRxiv*, 2020.
- [117] B. Udugama, P. Kadhiresan, H. N. Kozlowski, A. Malekjahani, M. Osborne, V. Y. Li, *et al.*, "Diagnosing COVID-19: the disease and tools for detection", *ACS nano*, 14(4), 3822-3835, 2020.
- [118] J. Rödel, R. Egerer, A. Suleyman, B. Sommer-Schmid, M. Baier, A. Henke, "Use of the variplex™ SARS-CoV-2 RT-LAMP as a rapid molecular assay to complement RT-PCR for COVID-19 diagnosis", *Journal of Clinical Virology*, 132, 104616, 2020.
- [119] L. J. Carter, L. V. Garner, J. W. Smoot, Y. Li, Q. Zhou, C. J. Saveson, *et al.*, *Assay techniques and test development for COVID-19 diagnosis*, 2020.
- [120] A. J. Gorzalski, H. Tian, C. Laverdure, S. Morzunov, S. C. Verma, S. VanHooser, and M. W. Pandori, "High-Throughput Transcription-mediated amplification on the Hologic Panther is a highly sensitive method of detection for SARS-CoV-2", *Journal of Clinical Virology*, 129, 104501, 2020.
- [121] P. Trémeaux, S. Lhomme, F. Abravanel, S. Raymond, C. Mengelle, J. M. Mansuy, and J. Izopet, "Evaluation of the Aptima™ transcription-mediated

- amplification assay (Hologic®) for detecting SARS-CoV-2 in clinical specimens”, *Journal of Clinical Virology*, 129, 104541, 2020.
- [122] Z. Huang, D. Tian, Y. Liu, Z. Lin, C. J. Lyon, W. Lai, *et al.*, “Ultra-sensitive and high-throughput CRISPR-powered COVID-19 diagnosis”, *Biosensors and Bioelectronics*, 164, 112316, 2020.
- [123] D. Jacofsky, E. M. Jacofsky, and M. Jacofsky, “Understanding antibody testing for COVID-19”, *The Journal of arthroplasty*, 35(7), S74-S81, 2020.
- [124] M. Franzén, *Blue Paper: COVID-19 Quick-Test*. Available: SSRN 3551647, 2020.
- [125] E. R. Adams, M. Ainsworth, R. Anand, M. I. Andersson, K. Auckland, J. K. Baillie, *et al.*, “Antibody testing for COVID-19: a report from the National COVID Scientific Advisory Panel”, *Wellcome Open Research*, 5, 2020.
- [126] H. Esakandari, M. Nabi-Afjadi, J. Fakkari-Afjadi, N. Farahmandian, S. M. Miresmaeili, and E. Bahreini, “A comprehensive review of COVID-19 characteristics”, *Biological procedures online*, 22, 1-10, 2020.
- [127] H. D. De Paz, P. Brotons, and C. Muñoz-Almagro, “Molecular isothermal techniques for combating infectious diseases: towards low-cost point-of-care diagnostics”, *Expert review of molecular diagnostics*, 14(7), 827-843, 2014.
- [128] Y. Ishigaki, and K. Sato, “Effects of microchannel shape and ultrasonic mixing on microfluidic padlock probe rolling circle amplification (RCA) reactions”, *Micromachines*, 9(6), 272, 2018.
- [129] K. Sato, A. Tachihara, B. Renberg, K. Mawatari, K. Sato, Y. Tanaka, *et al.*, “Microbead-based rolling circle amplification in a microchip for sensitive DNA detection”, *Lab on a Chip*, 10(10), 1262-1266, 2010.
- [130] A. Reem, M. Almezgagi, F. Al-Shaebi, W. A. AL-Shehari, J. P. P. Kumal, “Application of rolling circle amplification (RCA) to detect the pathogens of infectious diseases”, *Infectious Diseases Diagnosis & Treatment*, 2020.
- [131] B. Tian, F. Gao, J. Fock, M. Dufva, and M. F. Hansen, “Homogeneous circle-to-circle amplification for real-time optomagnetic detection of SARS-CoV-2 RdRp coding sequence”, *Biosensors and Bioelectronics*, 165, 112356, 2020.
- [132] D. L. L. Hung, X. Li, K. H. Y. Chiu, C. C. Y. Yip, K. K. W. To, J. F. W. Chan, *et al.*, “Early-morning vs spot posterior oropharyngeal saliva for diagnosis of SARS-CoV-2 infection: implication of timing of specimen collection for community-wide screening”, in *Open forum infectious diseases* (Vol. 7, No. 6, p. ofaa210). US: Oxford University Press, 2020.
- [133] I. F. Pinto, C. R. F. Caneira, R. R. G. Soares, N. Madaboosi, M. R. Aires-Barros, J. P. Conde, *et al.*, “The application of microbeads to microfluidic systems for

- enhanced detection and purification of biomolecules”, *Methods*, *116*, 112-124, 2017.
- [134] I. F. Pinto, R. R. G. Soares, M. E. L. Mäkinen, V. Chotteau, and A. Russom, “Multiplexed Microfluidic Cartridge for At-Line Protein Monitoring in Mammalian Cell Culture Processes for Biopharmaceutical Production”, *ACS sensors*, *6*(3), 842-851, 2021.
- [135] D. Gyllborg, C. M. Langseth, X. Qian, E. Choi, S. M. Salas, M. M. Hilscher, *et al*, “Hybridization-based in situ sequencing (HybISS) for spatially resolved transcriptomics in human and mouse brain tissue”, *Nucleic acids research*, *48*(19), e112-e112, 2020.
- [136] K. A. Walsh, K. Jordan, B. Clyne, D. Rohde, L. Drummond, P. Byrne, *et al.*, “SARS-CoV-2 detection, viral load and infectivity over the course of an infection: SARS-CoV-2 detection, viral load and infectivity”, *Journal of Infection*, 2020.
- [137] T. Krzywkowski, M. Kühnemund, and M. Nilsson, “Chimeric padlock and iLock probes for increased efficiency of targeted RNA detection”, *RNA*, *25*(1), 82-89, 2019.
- [138] R. R. G. Soares, F. Neumann, C. R. Caneira, N. Madaboosi, S. Ciftci, I. Hernández-Neuta, *et al.*, “Silica bead-based microfluidic device with integrated photodiodes for the rapid capture and detection of rolling circle amplification products in the femtomolar range”, *Biosensors and Bioelectronics*, *128*, 68-75, 2019.
- [139] L. Zhang, and A. Tripathi, “Archaeal RNA ligase from *Thermococcus kodakarensis* for template dependent ligation”, *RNA biology*, *14*(1), 36-44, 2017.

APPENDIX A: SUPPLEMENTARY DATA

Table S1 – List of sequences for biotinylated cDNA, SARS-CoV-2 RNA fragment, Padlock Probes (PLP), Detection Oligonucleotides (DO), Biotinylated Anchors, L-Probes, and PLP-primer. **Colour codes** highlight complementary regions in the case of cDNA, PLPs, Biotinylated Anchors, L-Probes and PLP-primer, and matching sequences in the case of PLPs and DOs. **Green sequences** correspond to parts of the sequence that hybridize directly with SARS-CoV-2 Control 2 RNA, with the underlined part corresponding to part of the sequence also present in the SARS-CoV-2 RNA fragment. **Underlined sequences** of the PLP correspond to the 5'-end “arm”, with the underlined matching colour representing the region where this arm is going to hybridize. In the **“chimeric” PLP**, **underlined bases** correspond to the region of the PLP-Primer hybridization that match part of the DO sequence. **Ribose bases** are identified in the “chimeric” PLP using a lower case “r” before the mentioned base.

Sequence Name	Experiments used	Sequence (5'-3')
Biotinylated cDNA (Bio-cDNA)	On-Chip RCA	Biotin-CTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCT <u>CGGTGTTGACAAAGCTACAACACGTTGTATGTTTGCAGCAA</u>
PLP1 - Bio-cDNA	On-Chip RCA	<u>TGTTGTAGCTTGTCACACCG</u> GTGTATGCAGCTCCTCAGTAATA GTGTCTTACGGCATCACTGGTTACGTCTGT <u>TGCTCGCAACAT</u> ACAACG
DO - PLP1 (Cy3)	On-Chip RCA	TCTCAGTAATAGTGTCTTACTTTTT-Cy3
SARS-CoV-2 RNA frag.	HybRCA (RNA frag.)	TCAATAGCCGCCACTAGAGGAGCTACTGTAGTAATTGGAACAAG CAAATCTATGGTGGTTGGCACACATGTTAAAACTGTTTATAG TGATGTAGAAAACCCTCACCTTAGGGTTGGGATTATCCTAAAT GTGATAGAGCCATGCTTAACATGCTTAGAATTATGGCCTCACTT GT <u>TCTTGCTCGCAACATACAACGTTGTAGC</u> TTGTACACCC TTTCT
Biotinylated Anchor 1	HybRCA (RNA frag.)	GCTTGTTCCAATTACTACAGTAGCTCCTCTAGTGGCGGCT AAAAAAAAA-Biotin
Biotinylated Anchor 580	HybRCA (Control 2 RNA)	Biotin-AAAAAAAAAATAGTGAACCGCCACACATGACCATT CACTCAATACTTGAGCACACTCAT
Biotinylated Anchor 684	HybRCA (Control 2 RNA); xRCA	Biotin-AAAAAAAAAAATGTTACGCAAAATATGCGTAAACT CATTCACAAAGTCTGTGTCAACA
Biotinylated Anchor 817	HybRCA (Control 2 RNA)	Biotin-AAAAAAAAAAGCTTCAGACATAAAAACATTGTTTGTAT AATAAGAAGTGACTTAAAGTT
L-Probe 43	HybRCA (Control 2 RNA)	ACATGTTGTGCCAACCCCATAGAATTGGCTTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 72	HybRCA (Control 2 RNA)	CATTTGAGTTATAGTAGGGATGACATTACGTTTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 134	HybRCA (Control 2 RNA)	CAGATAGAGACACCAGCTACGGTGCGAGCTCTTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 140	HybRCA (RNA frag.; Control 2 RNA)	<u>GCCATAATTCTAAGCATGTTAGGCATGGCTC</u> TTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 180	HybRCA (RNA frag.; Control 2 RNA)	<u>GCTACAACACGTTGTATGTTTGCGAGCAAGA</u> TTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 214	HybRCA (Control 2 RNA)	CAATTACTACAGTAGCTCCTCTAGTGGCGGCTTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>
L-Probe 298	HybRCA (RNA frag.; Control 2 RNA)	<u>CCCCAACCATAAGGTGAGGGTTTTCTACATC</u> TTTTtagtGT <u>TACGATGTTGACCCTGTATTAGGGATGCGGGTAGTA</u>

(continuation of Table S1)		
Sequence Name	Experiments used	Sequence (5'-3')
L-Probe 503	HybRCA (Control 2 RNA)	GTGGCATCTCCTGATGAGGTTCCACCTGGTTTTTAGTGT TACGATGTTGACCCCTGTATTAGGGATGCGGGTAGTA
L-Probe 637	HybRCA (Control 2 RNA)	GACACTCATAAAGTCTGTGTTGTAATTGCGTTTTAGTGT TACGATGTTGACCCCTGTATTAGGGATGCGGGTAGTA
L-Probe 744	HybRCA (Control 2 RNA)	GAAACACACAACAGCATCGTCAGAGAGTATCTTTTAGTGT TACGATGTTGACCCCTGTATTAGGGATGCGGGTAGTA
L-Probe 874	HybRCA (Control 2 RNA)	GAGGTCCTTTAGTAAGGTCACTCTCAGTCCATTTTAGTGT TACGATGTTGACCCCTGTATTAGGGATGCGGGTAGTA
L-Probe 969	HybRCA (Control 2 RNA)	GCCGGCCCCCTAGGATTCTTGATGGATCTGGGTTTTAGTGT TACGATGTTGACCCCTGTATTAGGGATGCGGGTAGTA
PLP2 - L-Probe overhang	HybRCA (RNA frag.; Control 2 RNA)	TACAGGGGTCAACATCGTATGCGTCTATTTAGTGAGCCTT AGTAGCCGTGACTATCGACTACTACCCGCATCCCTAA
DO - PLP2 (Cy5)	HybRCA (RNA frag.; Control 2 RNA)	AGTAGCCGTGACTATCGACT-Cy5
"Chimeric" PLP	χRCA	CAAGAACAAAGTGAGGCCAGTGTATGCAGCTCCTCAGTAATAGTG TCTTACCCAGTCGATAGTCACGGCTACACGTTGTATGTTTGGC A-rG
PLP-primer	χRCA	TACTGAGGAGCTGCATACAC
DO - "Chimeric" PLP (Cy5)	χRCA	TCCTCAGTAATAGTGTCTTACTTTTT-Cy5