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Recombinase polymerase amplification: Basics, applications and recent advances

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ABSTRACT

Recombinase polymerase amplification (RPA) is a highly sensitive and selective isothermal amplification technique, operating at 37–42 °C, with minimal sample preparation and capable of amplifying as low as 1–10 DNA target copies in less than 20 min. It has been used to amplify diverse targets, including RNA, miRNA, ssDNA and dsDNA from a wide variety of organisms and samples. An ever increasing number of publications detailing the use of RPA are appearing and amplification has been carried out in solution phase, solid phase as well as in a bridge amplification format. Furthermore, RPA has been successfully integrated with different detection strategies, from end-point lateral flow strips to real-time fluorescent detection amongst others. This review focuses on the different methodologies and advances related to RPA technology, as well as highlighting some of the advantages and drawbacks of the technique. © 2017 Elsevier B.V. All rights reserved.

1. Introduction

The 1953 discovery of the structure of DNA ushered a revolution in molecular biology, leading to an increased understanding of the central dogma and the subsequent development of invaluable molecular biology techniques, including the polymerase chain reaction (PCR), electrophoresis and automated sequencing, culminating in molecular biology techniques, including the polymerase chain reaction (PCR), electrophoresis and automated sequencing, culminating in the completion of the human genome project (HGP) in 2003. The last decade has seen an avalanche of information gleaned in the post-HGP era, such as gene assignation, identification of disease related mRNA biomarkers, as well as the discovery of the importance of single nucleotide polymorphisms (SNPs) and methylated DNA. To date, the vast majority of genotyping techniques require a previous step of amplification, routinely carried out using the robust PCR thermal cycling methodology, and more recently quantitative real-time PCR (qPCR). However, these techniques inherently require the use of thermocycler and a reliable power supply, thus restricting their use to laboratories. To address requirements of amplification for use in low-resource settings, or at the point-of-need, isothermal DNA amplification methods have been developed, including nucleic acid sequence-based amplification (NASBA), strand displacement amplification (SDA), rolling circle amplification (RCA), the loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), as well as the recombinase polymerase amplification (RPA). The characteristics of these isothermal approaches are summarised in Table 1 and the advantages and disadvantages of each technique has been extensively reviewed elsewhere [1–3]. RPA is remarkable due to its simplicity, high sensitivity, selectivity, compatibility with multiplexing, extremely rapid amplification, as well as its operation at a low and constant temperature, without the need for an initial denaturation step or the use of multiple primers. Overall, RPA positions itself very favourably for widespread exploitation in kits and assays for use at the point-of-care or point-of-need, as well as in affordable, sensitive, specific, user friendly, rapid, robust, equipment-free and delivered (ASSURED) devices, in low-resource settings.

In this review the reader will find the principles of RPA and a complete review of the majority of publications to date, detailing interesting aspects of RPA and diverse RPA approaches, covering different elements of the process, from sample pre-treatment, to amplification and detection strategies.

2. Recombinase polymerase amplification (RPA)

2.1. RPA mechanism

In 2006 Piepenburg et al. developed the RPA technology using proteins involved in cellular DNA synthesis, recombination and repair, which is currently commercialised by TwistDx (www.twistdx.com).
The RPA process starts when a recombinase protein uvsX from T4-like bacteriophages bind to primers in the presence of ATP and a crowding agent (a high molecular polyethylene glycol), forming a recombinase-primer complex. The complex then interrogates double stranded DNA seeking a homologous sequence and promotes strand invasion by the primer at the cognate site. In order to prevent the ejection of the inserted primer by branch migration, the displaced DNA strand is stabilised by single-stranded binding proteins. Finally, the recombinase disassembles and a strand displacing DNA polymerase (e.g. large fragment of Bacillus subtilis Pol I, Bsu) binds to the 3’ end of the primer to elongate it in the presence of dNTPs. Cyclic repetition of this process results in the achievement of exponential amplification (Fig. 1).

### 2.2. RPA operating parameters

#### 2.2.1. Primer design

Whilst it was initially believed that specifically designed primers of 30–35 bases in length were necessary for RPA, there are several reports demonstrating that normal PCR primers can be used and efficient amplification achieved [5,6]. Longer primers (up to 45 nucleotides) can be used, but they could lead to secondary structures and potential primer artifacts. It is also recommended to avoid long tracks of guanines at the 5’ ends while cytidines may be beneficial, whilst guanines and cytidines at the 3’ tend to improve performance. A GC content below 30% or above 70% is not recommended and, as with PCR primers, sequences that promote primer-primer interactions, secondary structures or hairpins should not be used. RPA can amplify targets up to 1.5 kb but is better suited to amplicons between 100 and 200bp. The primer selection process is thus the same as that used for PCR and involves four steps: choice of target region, design of primer candidates, experimental screening, and, if necessary, secondary and tertiary candidate screening. To date, there is no software available to design primers for RPA. The use of self-avoiding molecular recognition (SAMRs) oligonucleotides can also be employed, where natural bases are replaced by A*, T*, G* and C*, where A* pairs with T, T* with A, G* with C, and C with G, but A* does not pair with A*, T* and G* does not pair with C*, thus avoiding the formation of primer-dimers [7].

#### 2.2.2. Temperature

The reaction can operate at temperatures ranging from 22 to 45°C and does not require a narrow temperature control [8–10]; however, most published reports are optimised for temperatures between 37 and 42°C. In order to control the reaction temperature different apparatus can be employed, including incubators, heating blocks, chemical heaters [9] and body heat [11], and there are also examples of RPA working at ambient temperature in warm areas (above 30°C) [9].

#### 2.2.3. Effect of crowding agent and mixing

The crowding agent affects key biochemical process during the RPA reaction. Among them, it prevents the spontaneous recombinase-primer disassembly that occurs in the presence of the single stranded binding proteins needed for the amplification. However, the crowding agent has a negative impact on RPA performance at low target copy levels due to its viscosity, thus impeding the diffusion of reagents through the reaction mixture and inherently increasing amplification time. To minimise this effect, a mixing step is included 5 min after initiation of the RPA action, or, alternatively, mixing can be avoided by reducing the total volume of the reaction mixture to 5 μL [12]. An alternative strategy is to continuously mix the reaction solution, where an active matrix for electrowetting-on-dielectric facilitates continuous mixing of 270 nL or 750 nL of RPA cocktail, improving the limit of detection 100 times as compared to the benchtop assay [13]. The use of a phase-guided passive batch microfluidic chamber actuated by a syringe resulted in a reduction of the mixing time from hours to 1 min [14].

#### 2.2.4. Incubation time

The time required to amplify the DNA to detectable levels inherently depends on the number of starting DNA copies, but 20 min are usually adequate, although amplification times of as low as 3–4 min have been observed [15]. Long incubation times are unlikely to be beneficial in most applications, as for solution phase RPA the recombinase consumes all the available ATP within 25 min.

#### 2.2.5. Sample types

RPA can be used to amplify double stranded DNA, single stranded DNA, methylated DNA [16], cDNA generated through reverse transcription of RNA or miRNA [17] (Tables 2–6). There are several reverse transcriptases that have been used with RPA, including Transcriptor² (Roche), Sensiscript² (Qiagen), or MuLV² (Applied Biosystems), with initial reports demonstrating that Transcriptor provides the best performance. cDNA can be produced prior to RPA or in the same reaction [18,19] and RT-Freeze is also available from TwistDx.

RPA has successfully been used for different kinds of target organisms: bacteria, virus, protozoa, fungi, animals and plants, with diverse samples types, ranging from cultured microorganisms to body fluids (urine, sputum, respiratory washes, nasal, blood, plasma, saliva, vaginal and anal swabs), surgical biopsy specimens, organ tissues (skin, lymphatic nodes, liver, lungs, stomach, kidney), as well as animal and plant products (eggs, shrimps, rice, milk, fruit). Microfluidic devices incorporating a one-step digital plasma separation platform with autonomous parallel plasma separation and sample compartmentalisation for digital nucleic acid amplification have been developed for use with RPA [20]. A valveless microfluidic chip to pre-concentrate bacteria in urine using anion exchange magnetic beads prior to heat lysis has also been reported [21], as well as an isotachophoresis chip for the extraction of DNA from Listeria monocytogenes in blood samples prior to RPA [22].

Additionally, RPA has been also reported to indirectly detect non-nucleic acid targets, when aptamers are used as RPA template, and the first example of this was an aptamer based bio-barcode...
assay [23], which is based on the use of magnetic beads labelled with capturing antibodies and aptamers free in solution that are selective for different epitopes of the same target. In the presence of the target, a sandwich comprising magnetic beads, antibodies, target and aptamer is formed. The sandwich is then magneto-captured, the solution removed and the bound aptamers are amplified using RPA and detected using fluorescence. Another example of the combination of RPA with aptamer detection was based on the immobilisation of β-conglutin on magnetic beads and following a competition assay, aptamers bound to the magnetic bead immobilised target are eluted, amplified by RPA and detected fluorescently [24], or via lateral flow [16].

2.2.6. Solid Phase RPA
Amplification can be executed in solution, with both primers in the solution phase, or, alternatively, on a solid phase, when one primer is immobilised on a surface and the other primer is in solution. In a more challenging approach, termed bridge amplification, both forward and reverse primers are immobilised on a surface. However, the vast majority of reports describing RPA exploit solution-phase amplification [25,26]. In solution-phase, due to the unimpeded diffusion of primers and reaction reagents, amplification kinetics are favoured and the achieved limit of detection is subsequently usually better and amplification is achieved in a faster time than solid-phase. Nevertheless, solid-phase and bridge amplification present some advantages, such as the potential for spatially resolved multiplexed amplification or the possibility to couple the amplification with diverse detection techniques including ring resonators [27–29], electrochemical [30–36] and colorimetric detection [5,6,30,33,37,38]. Several methods have been developed with solid phase amplification with performances usually inferior to that achieved with solution phase amplification [5,27–30,39] as primer accessibility is more restricted impeding amplification efficiency, and future work will need to focus on strategies to decrease amplification time. Efforts to optimise the surface chemistry of the immobilised primers, exploiting vertical and horizontal spacers to enhance solid phase amplification has been reported [40]. To decrease the reaction time and improve the limit of detection, the surface-immobilised primer can also be introduced in the solution phase in an approach termed hemi-nested asymmetric solid-phase amplification [41–43]. Finally, when both primers are surface-tethered, bridge amplification can take place, but the required reaction time increases and the limit of detection can be compromised.

![Fig. 1. RPA amplification scheme. Recombinase proteins form complexes with each primer (A), which scans DNA for homologous sequences (B). The primers are then inserted at the cognate site by the strand-displacement activity of the recombinase (C) and single stranded binding proteins stabilise the displaced DNA chain (D). The recombinase then dis-assembles leaving the 3' end of the primers accessible to a strand displacing DNA polymerase (E), which elongates the primer (F). Exponential amplification is achieved by cyclic repetition of this process.](image-url)
high genomic DNA concentrations in whole blood samples. However, RPA is inhibited by serum as well as in the presence of known PCR inhibitors, such as heparin. This could be exploited to improve the achievable detection limit.

Nevertheless, bridge amplification allows multiplexing with a high number of different targets and novel labelling strategies could be exploited to improve the achievable detection limit [44].

2.2.7. Presence of Inhibitors

It has been demonstrated that RPA can be carried out directly in serum as well as in the presence of known PCR inhibitors, such as haemoglobin, ethanol and heparin [8]. However, RPA is inhibited by high genomic DNA concentrations in whole blood samples (20–100 ng/μL), but this problem has been reported to be partially solved via the use of a lateral flow-based enrichment of target DNA prior to amplification [45]. Another approach successfully implemented for the analysis of diluted crude DNA extracts from blood or swab samples consisted of heating the sample with AVL buffer and Trizol, followed by centrifugation [46].

RPA can also be carried out directly in urine [47], pleural fluids [48], seed powders [10], milk [49] and stool samples [50], only requiring heat lysis, direct lysis with nuclease free water or use of the EzWay™ Direct PCR buffer [49]. However, another study found that while 1.25% (v/v) of urine has no impact on amplification efficiency, 10% (v/v) did inhibit amplification when small amounts of target DNA were present in the sample (100 fg), but, this inhibition is not observed when the target DNA concentration is higher (10 pg), even at 10% (v/v) urine [51]. The robustness of RPA in the presence of traditional inhibitors facilitates amplification from crude extracts, which is not achievable using PCR. Whilst RPA pellets are more expensive than PCR reagents, the possibility to eliminate sample pre-treatment simplifies the assay and lowers costs [52].

2.2.8. Multiplexing

Multiplexing with RPA in the same solution is possible but is highly dependent on target sequences, amplicon size and primer design [39]. Primer, probe ratios and concentrations thus need to be carefully optimised for each multiplexing assay. Primers can compete for the recombinase proteins, with one of the reactions

### Table 2

| Organism | Target | Sample | Heat source | Amplification time (min) | Temperature (°C) | Limit of detection (LOD) | Ref. |
|----------|--------|--------|-------------|--------------------------|-----------------|--------------------------|------|
| Methicillin resistant | dsDNA | Genomic DNA | Not specified | <30 | 37 | 10 copies | [4] |
| Staphylococcus Aureus (MRSA) | dsDNA | Human plasma spiked with R. typhi cells | Heat block | 15 | 37 | 10 copies | [64] |
| HIV | dsDNA | Plasmid template | Heat block | 30 | 37 | 1–10 copies | [65] |
| Cryptosporidium | dsDNA | Human stool spiked with cryptosporidium | Body heat | 20–30 | Body heat (≥ 31–35) | 10 copies | [11] |
| Plasmodium falci| dsDNA | Genomic DNA | Body heat | 10 | 38 | 10 fg | [8] |
| Chlamydia trachomatis | dsDNA | Urine | Incubator | 10 | 38 | 50 copies | [47] |
| HIV-1 | dsDNA | Infected cell line | Incubator | 20 | 39 | 1–3 | [61] |
| HIV-1 | dsDNA | Infected cell line | Chemical heater | 20 | 10–44 | 10 | [9] |
| Orientia tsutsugamushi | dsDNA | Blood from infected patients and infected mice | Heat block | 20 | 39 | 53 copies | [68] |
| Rickettsia typhi | dsDNA | Human plasma spiked with R. typhi cells | Heat block | 20 | 39 | 20 copies | [68] |
| Plasmodium | dsDNA | Plasmid template | Hot plate | 30 | 37 | 50 | [66] |
| Giardia, cryptosporidium and Entamoeba | dsDNA | Live parasites spiked into stool | Heat block | 35 | 37 | 400 copies | [57] |
| HIV-1 | dsDNA | Plasmid template | Heat block | 30 | 37 | 100 | [45] |
| HIV-1 | dsDNA | Infected cell lines | Portable heat block | 20 | 39 | 10 | [12] |
| Giardia duodenalis | dsDNA | Stool | Incubator | 30 | 37 | 1 | [69] |
| Pork breed mangalica | dsDNA | Meat, sausages and paté | Twirla | 30 | 39 | 1 copy | [70] |
| Listeria monocytogenes | dsDNA | Genomic DNA spiked on pork, chicken, beef, fish and milk | Dry bath | 20 | 37 | 1300 CPU/mL | [71] |
| Borrelia burgferfori | dsDNA | Cell lines and serum samples | Thermoshaker | 20 | 37 | 25 copies | [72] |
| Leishmanial Vannia spp | dsDNA | Skin ulcers | Dry bath | 30 | 45 | 0,1 parasite | [73] |
| Schistosoma japonicum | dsDNA | Stool samples | Twista | 20 | 39 | 5 fg | [74] |
| Cryptosporidium | dsDNA | Feces | Thermoshaker | 25 | 37 | 0,05 oocyst | [50] |
| Caprine arthritis-encephalitis virus | dsDNA | Blood | Not specified | 30 | 37 | 10 copies | [75] |
| GM soybean | dsDNA | Seeds | Incubator | 20 | 39 | 10 copies | [10] |
| Ov virus | dsDNA | Nasal swabs, skin, lymphatic nodes, liver, lungs, stomach and kidneys | Water bath | 20 | 37 | 80 copies | [76] |

| a | Synthetic target | dsDNA | Artificial sample | Incubator | 15 | 37 | 10±11 M | [67] |
| a | Penaeus stylirostris virus | ssDNA | Muscles of shrimps | Heat block | 30 | 35–40 | 100 copies | [77,78] |
| a | R-congutatin | ssDNA, aptamer | Artificial sample | Room temperature | 15 | 37 | 0,17 amol | [79] |
| a | Yellow fever virus | ssRNA | Cell culture supernatant and mosquito pools | Heat block | 20 | 39 | <21 | [80] |
| a | Peste des petits ruminants virus | ssRNA | Tissues | Thermocycler | 20 | 39 | 150 copies | [81] |
| a | Little cherry virus 2 | ssRNA | Leaves, budwood, mealybugs and cherry tissue | Incubator | 15 | 39 | – | [82] |
| a | Plum pox virus | ssRNA | Peach, apricot, plum, cherry tree leaves | Portable heat block | 15 | 39 | 1.0 fg | [83] |
| a | Tomato chlorotic dwarf viroid | ssRNA | Leaves, potato, petunia plant, seeds. | Incubator | 15 | 39 | 100 fg-1pg | [84] |

| a | Triplexing |
| b | TwistAmp Basic kit. Use of tailed primers instead of antigen-primers and antibodies/streptavidin. |
| c | TwistAmp RT nfo. |
| d | AmplifyR® and Acceler8™ |
| Organism | Target | Sample | Amplification device | Transduction | Detection Platform | Amp. LOD T Ref. |
|----------|--------|--------|----------------------|--------------|--------------------|---------------|
| Canine parvovirus type 2 | ssDNA | Fecal swabs | Water bath | Fluorescence | Agarose gel electrophoresis | 20 10 copies 38 [91] |
| Madurella mycetomatis | dsDNA | Biopsy specimens | Heat block | Fluorescence | Agarose gel electrophoresis | 20 0,47 ng 39 [92] |
| Closely related bacteria | dsDNA | Bacterial culture | Thermocycler | Fluorescence | Agarose gel electrophoresis | 20 – 39 [60] |
| Begomoviruses | ssDNA | Tomato, tobacco and bean leaves | Water bath, heating block, thermocycler | Fluorescence | Agarose gel electrophoresis | 30 9,6 pg 37 [52] |
| Canine parvovirus type 2 | ssDNA | Fecal samples | Water bath | Fluorescence | Agarose gel electrophoresis | 20 10 copies 38 [91] |
| 1Rose rosette virus | ssRNA | Leaves, stems and petals | Heating block | Fluorescence | Agarose gel electrophoresis | 20 1fg/ul. 42 [93] |
| Human cancer cells | Met-DNA | Cell cultures and whole blood | Heat block | Floculation | Eppendorf tube. Naked eye | 30 0,5 ng 37 [85] |
| Mycobacterium tuberculosis | dsDNA | Cell cultures | Incubator | Floculation | Eppendorf tube. Naked eye | 20 10 CFU 38 [86] |
| 1Pseudomonas syringae | DNA | Leaves, bovine cells, water | Incubator | Floculation | Eppendorf tube. Naked eye | 15 – 37 [87] |
| Salmonella | dsDNA | Food and clinical samples | DVD, oven | Change in reflected light intensity | DVD drive | 40 6–30 CFU/ml 37 [42] |
| GMOs, peanut, Salmonella, Campylobacter | dsDNA | Cell cultures and certified reference materials | Microstructured DVD and sealing layer, laboratory oven | Transmitted beam intensity | DVD drive | 45 50–900 fg 37 [44] |
| Franciscella tularensis | dsDNA | DNA template on DVD | Microfluidic chamber on DVD | Change in reflected light intensity | DVD drive | 40 4–9 copies or 7 µg/g 40 410 copies 37 [41] |
| Allergens, GMOs, bacteria and fungi | dsDNA | Cell cultures | Microtitre plate, incubator | Change in color | Microtitre plate reader | 40 1,2–5,2 µg/g 6–13 CFU/ml 40 [37] [5] |
| Yersinia pestis | dsDNA | Synthetic and genomic DNA | Microtitre plate, incubator | Change in color | Microtitre plate reader | 30 3,14 10^-16M 37 [5] |
| Mycobacterium tuberculosis | dsDNA | Cell cultures | Incubator | Change in color | Spectrophotometer | 20 1 cfu 38 [33] |
| Human | dsDNA | Buccal smear | 100 well array chip + oven | Color | Poly carbonate chips, desktop scanner | 40 5–10% genomic SNP 20 pg – 38 [39] |
| Human lung cancer cells | dsDNA | Lung tissue | Not specified | Visual read-out. Color change | Eppendorf tube. Naked eye | 5–10 38 [38] |
| MRSA, Neisseria gonorrhoeae, Salmonella enterica serovar | dsDNA | Genomic DNA | Programmable hybridization chamber | Fluorescence | Microarray scanner | <20 38 [39] |
| HIV, hepatitis C and B, influenza A and B | dsDNA, RNA | Whole blood | Incubator | Fluorescence | Microwell chip with QD immobilised, optics and mobile phone camera | 10–30 1000 copies 37 [89] |
| Antibiotic resistant bacteria | dsDNA | Cell cultures | Microfluidic cartridge + homemade heater | Fluorescence | Microfluidic cartridge + homemade electronic detector | 30 10 copies 37 [59] |
| Human adenovirus 41, Phi X 174 and Enterococcus faecalis | dsDNA | Plasmid template | Microarray chip | Chemiluminescence | Microarray analysis platform | 35GU/µL, 1GU/µL, 1000GU/µL 37 [43] |
| Franciscella tularensis | dsDNA | DNA template | Electrode, aluminium block | Fluorescence | Sputtered gold electrodes. Potentiostat | 60 2 10^5 copies 37 [30] |
| Piscirickettsia salmonis | dsDNA | Salmon | Electrode, aluminium block | Fluorescence | Sputtered gold electrode array. Potentiostat | 40 3000 copies 37 [31] |
| Mycobacterium tuberculosis | dsDNA | Cell cultures | Incubator | Fluorescence | Screen-printed carbon electrodes. Potentiostat | 20 1 cfu 38 [33] |
| Leishmania infantum | dsDNA | Dog blood | Not specified | Fluorescence | Screen-printed carbon electrode. Potentiostat | 10 0,8 parasites/ml 37 [32] |
| Mycobacterium tuberculosis | dsDNA | Genomic DNA from cultured cells | Incubator | Fluorescence | Screen-printed carbon electrodes. Potentiostat | 20 1 cfu 38 [34] |
| Pseudomonas syringae | dsDNA | Leaves | Thermocycler | Fluorescence | Carbon screen printed electrode. Potentiostat | 20 15 copies 37 [35] |
| Human prostate cancer cells | RNA | Urine | Incubator | Colorimetric readout electrochemical | Eppendorf/ spectrophotometer/ screen printed electrode | 20 1,000–100,000 copies 36 [36] |

(continued on next page)
consequently being suppressed [53]. Examples of successful multiplexing RPA in solution, include the detection of different MRSA alleles and an internal control [4], a fluorescent duplex RPA assay for Staphylococcal Cassette Chromosome mec and an internal control [54], and a real time fluorescent duplex RPA for C. coli and C. jejuni in chicken products [53]. A multiplex assay of three bacterial pathogens based on solid phase amplification and fluorescent detection using a reverse primer modified with a fluorescent tag has been described [39], and a similar approach detailed the use of asymmetric solid phase multiplexing RPA for the detection of two human viruses and the bacterium E. faecalis using chemiluminescence detection [43]. Further examples include duplex RPA for cancer genotyping with label free Surface Enhanced Raman Spectroscopy (SERS) detection [55] and triplex RPA for three different plant pathogens using SERS nanotags and modified primers [56]. Finally, a triplex lateral flow assay for the detection of intestinal protozoa was developed, but still requires further optimisation to improve the detection limits [57].

Other reports detail pseudomultiplexing platforms through parallelised single reactions, using foil based centrifugal microfluidic cartridges with stored reagents [49,58], digital versatile discs (DVD) [41,42,44], vacuum degassed microfluidic cartridges [59] or polylactic acid/polycarbonate chips [6].

2.2.9. Storage

The reagents necessary for RPA are sold in kits consisting of pellets, rehydration buffer and magnesium acetate, which is used as a reaction initiator and is thus not included in the rehydration buffer, and is provided separately. Pellets are stable for at least one year when stored in a freezer (−15°C), fridge (2–8°C) and up to 6 months when stored at room temperature (22–28°C) [10]. The preparation of “homemade” pellets containing all the reagents necessary for RPA, including magnesium acetate, primers and the components present in the rehydration buffer has been reported. However, the resulting pellets should be stored at −20°C for optimum sensitivity, and reconstituted solutions can then be stored at 4°C but the achievable limit of detection was 10-fold less when compared with fresh solutions and it was not recommended to store these homemade RPA pellets at 37°C as they degrade and no amplification can be achieved [48].

### Table 3 (continued)

| Organism Target Sample | Amplification device | Transduction | Detection Platform | Amp. LOD T Ref. |
|------------------------|----------------------|--------------|-------------------|-----------------|
| Human prostate cancer cells RNA Urine | Not specified | SERS | Portable raman microscope | 15 100 copies 41 [55] |
| Human prostate cancer cells RNA Cell cultures, tumor tissue | Not specified | SERS | Portable raman microscope | 20 100 copies 41 [90] |
| Botrytis cinerea, Pseudomonas syringae, Fusarium oxysporum dsDNA Plant and tomato tissue | Incubator | SERS | Portable raman microscope | 20 1.9 fmol 37 [56] |

ssDNA = single stranded DNA; ds DNA = double stranded DNA; met-DNA = methylated DNA.

a TwistAmp Basic RT kit.
b Fusarium oxysporum, Botrytis cinerea, cucumber mosaic virus, bovine herpes virus 1, Escherichia coli, proviral HIV, Mycobacterium tuberculosis, influenza virus H1N1.

### Table 4.1

| Organism Target Sample | Kit/extra reagents | Amp. time T LOD Ref. |
|------------------------|-------------------|---------------------|
| Human cancer cell R-HepG2 ssDNA aptamer Cell culture | TwistAmp Basic kit, EvaGreen | 15 37 10 ng/ml cyt-c [23] |
| Porcine parvovirus ssDNA Serum, liver, kidney, lymph node, spleen and duodenum | TwistAmp Exo kit | 20 38 300 copies [99] |
| Bacillus subtilis MRSA dsDNA Cell cultures | TwistAmp Basic Kit, SYBR Green I E. coli endonuclease IV (Nfo) | <30 37 <100 copies [4] |
| Listeria monocytogenes Campylobacter jejuni and campylobacter coli dsDNA Blood | TwistAmp Exo kit | <30 <45 5000–20,000 cells [22] |
| Leptospiro dsDNA Culture medium, plasma and blood | TwistAmp Exo kit | 25 38 <2 copies [100] |
| Orf virus dsDNA Nasal swabs, skin, lymphatic nodes liver, lungs, stomach and kidney | TwistAmp Exo kit | 20 40 100 copies [101] |
| S. enterica serovar enteritidis dsDNA Cell culture, eggs and chicken meat | TwistAmp Exo kit | 10 37 10–100 cfu/g [102] |
| Peste des petits tumeurs virus RNA Tissue | TwistAmp RT Exo kit, MuLV reverse transcriptase. | 20 37 14 pg/µL [103] |
| Porcine reproductive and respiratory syndrome virus dsDNA Tissue and serum | TwistAmp Exo kit | 20 40 70 copies [104] |
| Human cancer cells RNA ssDNA Stool | TwistAmp RT Exo kit TwistAmp Basic kit, ligases, SYTO 9 dye | 20 40 202 copies [105] |

Human cancer cells RNA ssDNA Stool | TwistAmp RT Exo kit TwistAmp Basic kit, ligases, SYTO 9 dye | 20 40 202 copies [105] |
Real-time RPA methods based on fluorescence detection using portable flow cytometers.

| Organism                                      | Target       | Sample                               | Kit/extramaterials                                                    | Amp. time (min) | T (°C) | LOD | Ref. |
|-----------------------------------------------|--------------|--------------------------------------|----------------------------------------------------------------------|-----------------|--------|-----|------|
| Hypodermal and hematopoietic necrosis virus   | dsDNA        | Shrimp hepatopancreas                | TwistAmp Exo kit                                                    | 20              | 39     | 4 copies | [106] |
| Francisella tularensis                       | dsDNA        | Hare and rabbit                      | TwistAmp Exo kit                                                    | 20              | 42     | <20 copies | [107] |
| Orientia tsutsugamushi                       | dsDNA        | Human blood, mice                    | TwistAmp Exo kit                                                    | 20              | 39     | 50 copies | [68]  |
| Riftia typhi                                 | dsDNA        | Human plasma spiked with R. typhi cells | TwistAmp Exo kit                                                  | 20              | 39     | 40 copies | [68]  |
| Vibrio owensii                               | dsDNA        | Shrimp hepatopancreas                | TwistAmp Exo kit                                                    | 20              | 39     | 2 copies | [108] |
| White Spot syndrome virus                    | dsDNA        | Shrimps                              | TwistAmp Exo kit                                                    | 20              | 39     | 5 copies | [15]   |
| Mycoplasma capricolum                        | dsDNA        | Pleural fluid and lung tissue        | TwistAmp Exo kit                                                    | 20              | 42     | 50–500 copies | [48] |
| Brucella                                      | dsDNA        | Serum                                | TwistAmp Exo kit                                                    | 20              | 38     | 3 copies | [109] |
| Biothreat agent panel                         | dsDNA        | Inactivated whole organisms spiked into plasma | TwistAmp Exo kit, TwistAmp Exo RT kit, | <10          | 42     | 16–21 copies | [110] |
| Rift Valley fever virus                      | RNA          | RNA isolated from cell culture       | TwistAmp Exo kit, reverse transcriptions: Transcriptor, Sensiscript, MuLV | 8              | 42     | 10 copies | [18]  |
| Foot and mouth disease virus                  | RNA          | Heart, blood, serum, milk, saliva, and vesicular materials from cattle, buffalo, and sheep | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | <10          | 42     | 1500 copies | [19]  |
| Bovine coronavirus                            | RNA          | Nasal and fecal swabs               | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit             | 10–20          | 42     | <20 copies | [111] |
| Plum pox virus                               | RNA          | Peach, apricot, plum, cherry tree leaves | AmplifyRF XRT, XRT probe | 15            | 39     | 16 fg | [83]  |
| Dengue Virus                                  | RNA          | Plasma                               | TwistAmp Exo kit                                                    | 3–8            | 42     | 14–241 copies | [112] |
| Ebola Virus                                   | RNA          | Oral swabs, plasma spiked with inactivated virus | TwistAmp Exo RT kit | 15          | 42     | 5 copies | [113] |
| Avian influenza H5N1 HA                      | RNA          | Tracheal swabs from chicks           | TwistAmp Exo RT kit | 20          | 42     | 1 copies | [114] |
| Middle East Respiratory Syndrome Coronavirus  | RNA          | Extracted RNA provided others        | TwistAmp Exo RT kit | 10          | 42     | 10 copies | [115] |
| Schmallenberg virus and Bovine viral diarrhea virus | RNA       | Serum, infected cell culture supernatant, whole blood and homogenized tissue | TwistAmp Exo kit | 20          | 42     | 50,000 copies | [116] |
| Chikungunya virus                             | RNA          | Culture supernatant and plasma       | TwistAmp RT Exo kit | 15          | 39     | 80 copies | [117] |
| Ebola virus                                   | RNA          | Blood and swabs                      | TwistAmp Exo RT kit | 20          | 42     | 10 copies | [46]   |
| Yellow fever virus                            | RNA          | Cell culture supernatant and mosquitoes | TwistAmp Exo RT kit | <20         | 39     | 21 copies | [80]   |
| GMO: rice                                     | dsDNA        | Infected mosquito cultures           | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | 20          | 40     | 4 copies | [119] |
| HIV-1                                         | dsDNA        | Blood                                | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | <20          | 39     | 3 copies | [120] |
| Mycobacterium tuberculosis                   | dsDNA        | Sputum and respiratory washes        | TwistAmp Exo kit                                                    | 20              | 39     | 6,5 fg | [121] |
| MRS, V. cholera                              | dsDNA        | Nasal and groin swabs                | TwistAmp Exo kit                                                    | 20              | 39     | 5 copies | [54]   |
| MDROs, V. cholera                            | dsDNA        | Maize, rice, cotton and soybean      | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | 15–25        | 39     | 100 copies | [122] |
| Group B streptococcus                        | dsDNA        | Vaginal swabs                        | TwistAmp Exo kit                                                    | 20              | 40     | 6–12 copies | [123] |
| Mycobacterium avium subsp. paratuberculosis  | dsDNA        | Blood, sperm, feces and tissues      | TwistAmp Exo kit                                                    | 15              | 42     | 16 copies | [124] |
| Lumpy skin disease virus                     | dsDNA        | Skin nodules and skin                | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit RR2Y (primers and probes included) | 15           | 42     | 179 copies | [125] |
| GM soybean                                    | dsDNA        | Seeds                                | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | 20           | 39     | 10 copies | [10]   |
| Leishmania donovani                           | dsDNA        | Skin                                 | TwistAmp Exo kit                                                    | 15              | 42     | 39 copies | [62]   |
| Influenza A (H7N9) virus                     | RNA          | In vitro transcribed RNA standards   | TwistAmp RT Exo kit | 10          | 42     | 10–100 copies | [94]  |
| Dengue virus                                  | RNA          | Culture supernatant, patient serum   | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | <20          | 40     | 10 copies | [126] |
| Vibrio cholera                                | dsDNA        | Shrimp, clamps and fishes            | TwistAmp Exo kit                                                    | 20              | 39     | 5 copies | [127] |
| Feline herpesvirus 1                          | dsDNA        | Nasal and ocular swabs               | TwistAmp Exo kit                                                    | 20              | 39     | 100 copies | [128] |
| Type 2 porcine reproductive and respiratory syndrome virus | RNA     | Lymph node, lung, spleen and liver   | TwistAmp Exo kit, Transcriptor and TwistAmp Exo RT kit | 20           | 40     | 690 copies | [129] |

2.2.10. Specificity

RPA has been described as highly specific, with 100% specificity for the target sequence in most cases. However, RPA has been reported to be dependent on the number and distribution of mismatches in the sequence of closely related DNA molecules, where 1 or more mismatches cannot be differentiated, depending on their distribution. However, more than 1 mismatch at the 3' end of primers has been observed to effectively prevent or reduce amplification, which has also been observed for 3 mismatches at both the 5' and 3' ends, or at the centre of the primer [60]. Whilst this may limit RPA's usefulness in using sequence specific primers, its tolerance to mismatches can be exploited to develop methods to determine the presence of emerging variant pathogens, when it is not necessary to discriminate from the wild-type target, exemplified by a method developed to detect HIV-1 proviral DNA, where even 9 changes across the primer and probe binding sites is not necessary to discriminate from the wild-type target as its tolerance to mismatches can be exploited to develop methods to determine the presence of emerging variant pathogens when it is not necessary to discriminate from the wild-type target [61]. However, this tolerance to mismatches can also lead to cross-reactivity as demonstrated by an RPA assay developed to detect the three different genotypes of Chikungunya virus that was
observed to have cross-reactivity with another related alphavirus, the O’nyong’nyong virus, based on 4 to 7 mismatches in the primers. A further example is an assay to determine Leishmania donovai that was observed to also amplify other Leishmania spp [62]. However, a method to detect EGFR mutations in lung cancer cells with specificity of just one base mismatch or single nucleotide polymorphism has been developed. Background amplification was reduced via the use of peptide nucleic acids, as PNA-DNA interactions are stronger than DNA-DNA, and one single mismatch is more destabilising than a normal DNA-DNA mismatch, thus improving specificity. However, an extra step is required to allow genomic DNA – PNA hybridization, heating to 99 °C and then cooling down to 66 °C, moving away from the attractive isothermal nature of RPA [38].

An alternative approach exploiting the use of shorter primers (19–21mer) to decrease the stability between primers and targets and increase specificity towards SNPs has also been reported, where a mismatch in the 3’- of the primer was included to increase the specificity. Furthermore, similar to the use of PNA, the use of natural dNTPs vs locked nucleic acids was compared. However, a loss of specificity was observed when multiplexing in the same reaction mixture was pursued, which was attributed to a competition between primers and amplicon [6].

3. Detection of RPA amplicons

RPA can be monitored by end point detection (following amplification) or in real time (during amplification) and probes may be used depending on the detection strategy.

3.1. End point detection

Several detection techniques can be used following amplification to determine the presence or absence of targeted nucleic acid

| Table 4.3 | Real-time RPA methods based on fluorescence detection using alternative devices. |
|-----------|---------------------------------------------------------------------------------|
| Organism  | Target               | Sample       | Kit/extra reagents | Amplification device, Detection platform | Amp. time (min) | T (°C) | LOD       | Ref. |
| Klebsiella pneumoniae | dsDNA | Urine | TwistAmp Exo kit | Microplate reader | 20 | 39 | 1000 UFC/ml | [21] |
| Chlamidia trachomatis | dsDNA | Synthetic DNA | TwistAmp Fpg kit | Homemade heating block and optical system | 40 | 44 | 100,000 copies | [130] |
| MRSA | dsDNA | PCR amplicon | TwistAmp Exo kit | Microfluidic lab on a foil, Real-time rotatory analyser | <20 | 37 | <10 copies | [58] |
| Group B Streptococci and B. atrophus | dsDNA | Vaginal and anal | TwistAmp Exo kit | Real-time rotatory analyser | <20 | 39 | 20 copies | [131] |
| Clostridium difficile | dsDNA | Cell cultures | TwistAmp Exo kit | Slip-chip, Real time thermocycler | <20 | 39 | 1000 copies | [96] |
| Antibiotic resistance Escherichia coli | dsDNA | Milk | TwistAmp Exo kit | Digital microfluidic on an AM-EWOD device | 20 | 39 | 4 cells | [49] |
| Salmonella enterica, Escherichia coli O157:H7, vibrio parahaemolytics | dsDNA | Vaginal and anal | TwistAmp Exo kit | Centrifugal microdevice, Custom made portable genetic analyser with a miniaturized optical detector | 15 | 39 | 1 copy | [13] |
| Zika Virus | RNA | Urine | TwistAmp RT Exo kit | Modified 3D printer, Blue laser, mobile phone camera and filter | 12 | 40 | 5 PFU/ml | [97] |

| Table 5 | Alternative real-time detection approaches. |
|----------|---------------------------------------------------------------------------------|
| Organism  | Target               | Sample       | Kit/extra reagents | Amplification device | Transduction | Detection Platform | Amp. time (min) | T (°C) | LOD       | Ref. |
| Mycobacterium tuberculosis | dsDNA | Sputum | TwistAmp Basic kit | Silicon microring resonator | Wavelength shift | IR sensor | 20 | 37 | 26 pg/mm2 | [29] |
| Francisella tularensis | dsDNA | DNA template | TwistAmp Basic kit | Silicon microring resonator | Wavelength shift | IR sensor | 60 | 37 | 600,000 copies/ul | [28] |
| Human cancer cells | dsDNA | Bladder cancer cells | TwistAmp Basic kit | Silicon microring resonator | Wavelength shift | IR sensor | 20–30 | 37 | 500fg/ul | [27] |
| Plasmodium falciparum | dsDNA | Whole blood | TwistAmp Basic kit | Sensor chip + heating plate | Phase change | Mach-Zehnder interferometer | 30 | 37 | 1 parasite/ul | [132] |

| Table 6 | Absolute quantification strategies. |
|----------|---------------------------------------------------------------------------------|
| Organism  | Target               | Sample       | Kit/extra reagents | Amplification device | Transduction | Detection Platform | Amp. time (min) | T (°C) | LOD       | Ref. |
| MRSA | dsDNA | Genomic DNA | TwistAmp Exo kit | SlipChip + plate reader | Fluorescence | Plate reader | 30 | 39 | 300 copies/ml | [134] |
| MRSA | dsDNA | Genomic DNA spiked in blood | TwistAmp Exo kit | Microfluidic chip, digital plasma separation, incubator | Fluorescence | Fluorescence microscope | 30 | 37 | 1000 copies/ml | [20] |
| Listeria monocytogenes | dsDNA | Certified DNA | TwistAmp Basic kit | Centrifugal heater with an integrated fluorimeter | Fluorescence | Modified fluorescence microscope | 30 | 39 | – | [133] |
| Listeria monocytogenes | dsDNA | Genomic DNA | TwistAmp Exo kit | Picoliter array chip | Fluorescence | Modified fluorescence microscope | 15 | 39 | 4 · 10^-3 copies/well | [135] |
sequences. In general, end-point detection requires less instrumentation than real-time detection, decreasing the overall cost of the test, and thus could be more appropriate for low resource settings.

3.1.1. Lateral flow

The majority of reports detailing end-point detection of RPA products reported to date, rely on lateral flow assays, where results are obtained extremely rapidly in a visual read-out format. 3 different oligonucleotides (2 primers and 1 probe) and the Twist-Amp® nfo kit are typically used for assay designs compatible with lateral flow strip detection [63]. The probe is recommended to be a 46–52 oligonucleotide modified at the 5′ end with an antigenic label at the 3′ end, with a polymerase extension blocking group and an internal abasic nucleotide analogue that substitutes one nucleotide found in the target sequence. The antigenic label is usually a carboxyfluorescein group (FAM), but others, including Alexa fluor488 or digoxigenin are also good candidates [57]. The abasic nucleotide (a tetrahydrofuran residue that replaces a conventional nucleotide, also called a dSpacer), is placed at least 30 nucleotides from the 5′ end and 15 nucleotides from the 3′ end. This dSpacer can be cleaved by an nfo nuclease, but only when the probe forms double stranded DNA. The cleavage produces a new 3′ hydroxyl group in the probe, thus transforming the probe into a primer. In addition to the probe, an opposing amplification primer labelled at the 5′-end with another label (e.g. biotin) is required. The second primer used is a conventional primer equidirectional to the probe. The amplicon produced in the presence of the probe and the two primers will include the two labels on one DNA amplicon, ready to be detected in a sandwich assay format by antibodies or antibody/streptavidin (Fig. 2).

Table 2 summarises reports detailing the combination of lateral flow and RPA. In all cases, the amplification and detection is performed in less than 1 h, achieving limits of detections as low as 1–10 DNA copies. There are also some reports detailing further innovations in lateral flow strip detection such as the use of inexpensive paper, glass fibre, as well as a plastic device in an origami format, which both stored lyophilised enzymes and facilitated mixing steps [64], and was applied to the detection of Cryptosporidium, with a similar analytical performance to RPA in solution [65]. The same group reported another example of a paper and plastic microfluidic device that was self-sealing and self-contained once all reagents were loaded and only required a heat source, bringing the implementation of nucleic acid testing in a low-resource setting closer to reality [66]. An alternative RPA-lateral flow assay used tailed primers (primer containing a carbon stopper to generate double stranded DNA flanked by single stranded tails), to generate double tailed amplicons. Oligo-functionalised AuNPs were used as reporter probes and oligonucleotides as capture probes in the test and control line, instead of the conventional antigen label and antibody capture approach [67], decreasing the cost of the strip.

Fig. 2. Nfo probe and lateral flow strip. The Nfo probe is exchanged at the cognate site by recombinase proteins and nfo nuclease cleaves the probe on the THF residue. The blocking group is released and the probe is thus converted into a primer. The double labelled amplicon obtained from amplification is mixed with a dye and loaded onto the sample pad of a lateral flow strip (A). The dye binds to the amplicon in the mixture pad (B) and the dye-amplicon complex is captured by antibodies immobilised on the detection line (C). The excess of dye is captured by antibodies in the control line (D).
3.12. Other end-point detection strategies

Apart from lateral flow detection, other end point strategies can be exploited as summarised in Table 3. Agarose gel electrophoresis is a widely used technique for visualisation of amplification products, but post-amplification it is necessary to purify the amplicons to avoid smeared bands on the gel due to the presence of the proteins and the crowding agent present in the amplification mix.

Bridge flocculation assay is an equipment free assay that provides a binary naked eye visual read out, suitable for low-resource settings. The assay is based on the reversible flocculation of carboxyl-functionalised magnetic beads, which is dependent on the salt concentration, pH and length of DNA. A minimum DNA length of 100bp is needed for the crosslinking, amplicons can be easily distinguished from primers. To execute the assay, a bead solution is added to the amplification products and following an ethanol wash, the beads are re-suspended in a low pH buffer and a positive answer is obtained if the beads remain flocculated [85–87].

DVDs and low reflectivity DVDs [41] are suitable substrates for the immobilisation of primers for solid phase or bridge amplification, facilitating multiplexing through parallelisation in individual readers of the DVD. Once amplification is achieved a DVD reader can be used to read out the results in reflection [42] or transmission mode [44]. Additionally, the DVD drives provide centrifugal force to actuate microfluidics for aliquoting and mixing [41].

Colorimetric detection can also be implemented with RPA. Primers modified with biotin, or biotin modified dNTPs can be used to produce labelled amplicons followed by addition of streptavidin-HRP and subsequently 3,3',5,5'-Tetramethylbenzidine (TMB) and H2O2, to produce a change in color, the intensity of which can be correlated to the concentration of the amplicons.

In some strategies RPA is carried out in solution and the product captured by magnetic beads [33] or on a microtitre plate following denaturation of the duplex RPA amplicon [37]. Other strategies involve immobilising one of the primers on a substrate and performing solid phase amplification [5,30] followed by denaturation, hybridization with enzyme labelled reporter probe and optical/electrochemical detection. In an alternative approach, chemiluminescence detection is achieved via the use of a biotinylated primer, and post-amplification incubation with streptavidin-horseradish peroxidase, luminol and H2O2 [43].

Fluorescence detection has also been employed in end-point detection approaches. Multiplexing can be achieved exploiting forward primers immobilised onto array spots, and fluorophore modified reverse primers. Following completion of RPA, the amplified product can be spatially resolved and visualised by laser scanner measurements [39].

Quantum Dot (QD) barcodes are used as an alternative to traditional fluorophores for multiplexed fluorescence detection. One approach consists of polystyrene beads loaded with different types of QDs, which were functionalised with barcodes specifically designed for each of the targets, with one QD type used for each barcode. The beads are then distributed on microfabricated slides and the location of each QD detected using a Smartphone. Following RPA, single stranded DNA is generated and hybridised between the QD-barcode and an Alexa Fluor 647 labelled reporter probe, and the fluorescent signal again measured with the Smartphone. Correlation on the location of each QD-containing bead and the final fluorescent signal facilitated multiplexed detection [88], and the strategy was validated using clinical samples [89].

The TwistAmp Exo kit is normally used for real time-RPA with fluorescence detection but it has been used as an end point detection strategy in a multiplexed format, using a low cost, easy-to-use, portable microfluidic cartridge system [59].

Electrochemical transduction for the detection of RPA products via capture of single stranded DNA generated from the amplicon between a surface immobilised complementary probe, and an enzyme labelled reporter probe was described [30,33,34]. An alternative approach uses forward primers labelled with magnetic beads and reverse primers labelled with gold nanoparticles (AuNPs). The double tagged amplification product is captured by a magnet onto a working electrode and the AuNPs are detected directly through electrocatalytic hydrogen evolution [32]. The use of biotin-dUTPs to produce tagged amplicons was developed, where streptavidin – AuNPs bind to the amplicons on an electrode surface, and gold is oxidized to AuCl4, which can be detected by differential pulse voltammetry [34]. An alternative approach is based on a solid phase RPA assay where one of the primers was tethered on a gold electrode surface and the other primer contained a biotin in the 5’, with post-amplification detection achieved using streptavidin-HRP in the presence of a precipitating TMB substrate [31].

An electrochemical biosensor has also been reported for plant pathogen detection using modified primers to generate double tagged amplicons with biotin at one end and an oligonucleotide overhang at the other. Biotin was used to purify the amplicon using streptavidin magnetic beads, and the capture probe was used to bind to AuNP labelled with a complementary capture probe. Following purification, the amplicons were dropcast on screen printed carbon electrodes and the gold of the AuNP was measured using differential pulse voltammetry (DPV) [35].

SERS has been exploited for the detection of RPA amplicons. A triplex assay to determine plant pathogens in vegetal tissues was developed using biotinylated reverse primers, tailed forward primers, and AuNPs functionalised with SERS nanotags and oligos complementary to the tails of the primers [56]. The same strategy was also used to develop a rapid multiplexed reverse transcription – RPA (RT-RPA) for the genotyping of prostate cancer tumor and urine samples, using SERS nanotags for a highly sensitive one-pot readout [90]. The same group furthered this work, describing multiplex RT-RPA, with label-free SERS detection, where purified amplicons are incubated with silver nanoparticles prior to SERS detection. The technology was applied to the analysis of 43 patient urinary samples, achieving very good sensitivity, specificity and accuracy [55].

Schematic representations of different lateral flow assays, biosensors and POC devices developed using RPA are shown in Fig. 3. The bridge flocculation assay [87], and lateral flow approaches including a multiplexed lateral flow assay [57] and a disposable plastic and paper device (64) for RPA prior lateral flow assay are particularly suited to point of care devices due to the instrument-less naked eye read-out nature of the methods. Other approaches such as lab in a suitcase [62], combine all the components needed to perform RPA in situ, using a portable fluorometer for the amplification read-out and portable solar panels and batteries as power sources. Other approaches such as electrochemical solid phase amplification [30] or solid phase amplification on DVDs [41] have potential for multiplexed detection of target at the point of need, but further research is required to reduce the number of steps or to automate the whole process.

3.2. Real time detection

RPA can be also monitored in real-time using fluorescent probes and a fluorimeter, facilitating quantification of DNA (Tables 4.1, 4.2 and 4.3). To make this approach accessible to low resource settings, portable and rechargeable fluorimeters have been developed, including the ESE Quant Tube scanner device (Qiagen), Genie III (OptiGene) and the Twista (TwistDx). These fluorimeters can be
Fig. 3. Schematic representations of biosensors/POC devices using RPA: (i) Bridge flocculation assay [87]; (ii) a disposable plastic and paper device [64]; (iii) lab in a suitcase [62]; (iv) multiplexed lateral flow assay [57]; (v) solid phase amplification on DVDs [41]; (vi) electrochemical solid phase amplification [30] (Figures modified from original publications cited).
incorporated in a lab-in-a-suitcase or diagnostics-in-a-suitcase [62,94], where all instruments and disposables necessary to perform RPA in-field are packaged in a portable format. Non-specific intercalating fluorophores such as SYBR Green [4] or Eva Green [23] can be employed for real-time detection, but, as in the case of real-time PCR, these dyes cannot discriminate between amplicons and primer-dimer artefacts, thus giving rise to false positive results.

To obviate this problem, the use of specific probes, namely Exo probes and Fpg probes (Fig. 4) are recommended. Other PCR conventional probes such as Taq-Man probes are not compatible with RPA because the Taq-Man polymerases digest the displaced strand during the strand displacing process due to the 5'→3' exonuclease activity, thus preventing the DNA amplification.

The Exo probe is an oligonucleotide with homology to the target amplicon that is blocked at the 3' to prevent probe elongation. The probe also has a dT-fluorophore and a dT-quencher flanking a tetrahydrofuran residue (dSpacer), which are separated by a maximum of 2–4 bases. The fluorophore signal is thus quenched when the single stranded DNA probe is in solution. However, when the Exo probe is annealed to a complementary DNA target, the DNA repair enzyme Exonuclease III, cleaves the probe at the dSpacer site, producing two probe fragments, separating the fluorophore from the quencher, and thus facilitating the generation of fluorescence [63].

The Fpg probe, similar to the Exo probe, is an oligonucleotide with homology to the target amplicon that is blocked at the 3' to avoid probe elongation, and additionally contains a quencher and a fluorophore, separated by 4–5 nucleotides (7 at maximum). The quencher is placed at the 5' of the probe and the fluorophore is linked to an abasic nucleotide through a C–O–C linker, termed a dR-group. In the absence of target, the fluorophore signal is quenched but when the Fpg probe is annealed to a complementary DNA target, the fpg enzyme cleaves the probe at the dR position, liberating the fluorophore, resulting in emission of fluorescence [63].

It has been observed that the Exo probes provide higher sensitivity than nfo probes [61], however, Exo probes can result in the exonuclease mediated degradation of DNA and therefore are not compatible with agarose gel electrophoresis [25]. qRPA can be achieved if reactions are protected from heat and light to avoid loss of enzyme activity and the photobleaching of probes, and magnesium acetate should be added immediately prior to fluorescence detection [95].

Whilst real-time assays are routinely carried out in Eppendorf tubes, the use of a SlipChip platform for amplification has been described. The chip consists of plates clamped together and
contains 3 lanes used to place sample, RPA master mix and magnesium acetate, separately. Once each lane is loaded, the plates can slip in order to mix all the components, and amplification is followed using a real time machine [96]. The use of a programmable digital microfluidic platform based on an active matrix electrowetting-on-dielectric (AM-EWOD) for real time detection has also been described. The automated platform incorporates 16,800 electrodes that can be controlled independently to simultaneously manipulate several droplets of around 45 nL. The system allows the continuous movement and heating of droplets achieving an improved detection limit (>2 orders of magnitude) as compared to benchtop assays [13]. In another report, a commercial 3D printer was modified and coupled with blue LEDs and a mobile phone camera to construct a robotic device for DNA/RNA extraction, amplification and real time detection in a multiplex format (up to 12 samples), and applied to ZIKA spiked urine samples [97]. An alternative approach combining ligation based assays with qRPA for the detection of fusion gene mRNAs was described. Right hand and left hand side ligation probes were designed to contain universal reverse and forward primer specific sequences incorporated at either side of the ligation site. Following ligation, the probes are amplified in separate reactions, and the signal due to intercalation of the SYTOQ fluorescent dye was measured, allowing simultaneous detection of three targets in 60 min [98].

3.2.1. Alternative real time detection strategies
Real time detection is mainly restricted to fluorescence detection, however, there are some reports of alternative real-time strategies (Table 5). Real-time, label-free and highly sensitive detection of RPA can be achieved using ring-resonator technology [29], where primers are immobilised on a silicon ring resonator and the shift in the resonant wavelength is measured continuously during amplification. This approach has been demonstrated to have a sensitivity 100 times higher than benchtop RPA and conventional PCR methods and can be used to distinguish single point mutations [27]. Further examples of alternative real-time detection strategies include a label-free method that combines a dimethyl adipimidate agent facilitates the performance of up to 27,000 reactions in picoliter sized wells [135].

3.3. Absolute quantification
Fluorescence is the principle transduction technology that has been used to develop methods for absolute quantification in which the sample and reaction components are compartmentalised into several individual and parallelised reactions so that each reaction contains one or no copy of the target DNA (Table 6). The compartmentalisation approaches developed include digital plasma separation [20], centrifugal step emulsification [133], SlipChip technology [134] and picoliter array based technology [135]. In digital plasma separation, the compartmentalisation and plasma separation is carried out passively using microfluidic chips with a microcrill structure that is actuated by passive degassed driven flow, inertia and sedimentation [20]. In centrifugal step emulsification, the compartmentalisation is achieved in droplets, produced by centrifugation using an inlet chamber. One channel is connected to a chamber by a nozzle and droplet production, and read-out of the amplification with a Smartphone-based device takes place in the same chamber [133]. As described previously, in SlipChip technology two plates are clamped together to create channels and wells for the creation of individual compartments [134], and finally, picoliter-array based chips on fabricated silicon and passivated with methoxy-PEG-silane agent facilitates the performance of up to 10 target copies in less than 20 min even in the presence of some known PCR inhibitors or in crude extracts. The technique has been successfully used to amplify both RNA and DNA targets in different kinds of organisms, in both the solution and solid phase. A wide variety of detection strategies are compatible with RPA, and some of these have been tested with real samples with performances similar or better than PCR.

Table 1 outlines the properties of RPA as compared to other isothermal amplification techniques. Whilst most other isothermal amplification methods (e.g. NASBA, RCA and SDA only requires 2 primers per target, which could position it to be more compatible with multiplexed amplification. RPA is also a very rapid method of amplification, markedly faster than other isothermal amplification methods and even though 15–25 min is recommended, efficient amplification can even be achieved in less than 5 min, depending on the target. RPA reagents are provided in a lyophilised form and are stable at ambient temperature for at least 6 months, whilst the reagents for all the other isothermal techniques require refrigeration, and this again positions RPA as being highly suited to implementation in point-of-need/care and ASSURED devices.

However, RPA does have some limitations, the principle one being that RPA kits are only sold by one company, which could have an impact on pricing, and the user also has limited flexibility in the kit formulation and whilst tailor-designed kits are available (e.g. without polymerase, without dNTPs), they are costly at low volumes. RPA normally requires purification/protein digestion following amplification, or will result in smearing or impaired flow in the cases of agarose gel electrophoresis and lateral flow, respectively. RPA, like PCR can be inhibited by high concentrations of genomic DNA, and as is the case with real time PCR, the use of SYBR Green [4] or Eva Green [23] cannot discriminate between amplicons and primer-dimer artefacts. Furthermore, real-time PCR conventional probes such as Taq-Man probes are not compatible with RPA because the Taq-Man polymerses digest the displaced strand during the strand displacing process due to the 5′–3′ exonuclease activity, thus preventing amplification. In fact, real-time amplification using RPA is not straightforward as it is based a time threshold instead of a cycle threshold, which is dependent on RPA kinetics. This time threshold is dictated not only by the initial target concentration but also by the temperature and mixing step. It is advisable to slow down the RPA reaction rate in order to have a better control during real-time RPA and this can be achieved by decreasing the magnesium acetate concentration. However, as

4. Conclusions and future trends
RPA is a relatively new isothermal amplification technology that has experienced an exponential growth in terms of publications, popularity and applications since its first report in 2006. The majority of reports since then have focused on a wide range of different applications of RPA, but there are an increasing number of publications that detail methodologies to improve the performance of RPA and to further its capabilities. RPA is remarkable among isothermal amplification techniques due to its simplicity, high sensitivity, selectivity, compatibility with multiplexing, rapid amplification, as well as its operation at a low and constant temperature, without the need for an initial denaturation step or the use of multiple primers. RPA can amplify as low as 1–10 target copies in less than 20 min even in the presence of some known PCR inhibitors or in crude extracts. The technique has been successfully used to amplify both RNA and DNA targets in different kinds of organisms, in both the solution and solid phase. A wide variety of detection strategies are compatible with RPA, and some of these have been tested with real samples with performances similar or better than PCR.

Table 1 outlines the properties of RPA as compared to other isothermal amplification techniques. Whilst most other isothermal amplification methods (e.g. NASBA, RCA and SDA only requires 2 primers per target, which could position it to be more compatible with multiplexed amplification. RPA is also a very rapid method of amplification, markedly faster than other isothermal amplification methods and even though 15–25 min is recommended, efficient amplification can even be achieved in less than 5 min, depending on the target. RPA reagents are provided in a lyophilised form and are stable at ambient temperature for at least 6 months, whilst the reagents for all the other isothermal techniques require refrigeration, and this again positions RPA as being highly suited to implementation in point-of-need/care and ASSURED devices.

However, RPA does have some limitations, the principle one being that RPA kits are only sold by one company, which could have an impact on pricing, and the user also has limited flexibility in the kit formulation and whilst tailor-designed kits are available (e.g. without polymerase, without dNTPs), they are costly at low volumes. RPA normally requires purification/protein digestion following amplification, or will result in smearing or impaired flow in the cases of agarose gel electrophoresis and lateral flow, respectively. RPA, like PCR can be inhibited by high concentrations of genomic DNA, and as is the case with real time PCR, the use of SYBR Green [4] or Eva Green [23] cannot discriminate between amplicons and primer-dimer artefacts. Furthermore, real-time PCR conventional probes such as Taq-Man probes are not compatible with RPA because the Taq-Man polymerses digest the displaced strand during the strand displacing process due to the 5′–3′ exonuclease activity, thus preventing amplification. In fact, real-time amplification using RPA is not straightforward as it is based a time threshold instead of a cycle threshold, which is dependent on RPA kinetics. This time threshold is dictated not only by the initial target concentration but also by the temperature and mixing step. It is advisable to slow down the RPA reaction rate in order to have a better control during real-time RPA and this can be achieved by decreasing the magnesium acetate concentration. However, as
the time of adding the magnesium and the effectiveness of mixing will have a strong impact on RPA kinetics, and ideally real-time RPA should be completely automated. Whilst RPA seems particularly suitable for multiplexed amplification, this requires extensive optimisation of primer concentrations as primers compete for the recombinase proteins and ratios of each need to be tested experimentally as primers for one target can suppress the amplification of another target. Furthermore, to date there is no software available for the design of primers specific for RPA and this can result in lengthy optimisation of the primer sequences. Different DNA targets, even with the same GC content, primer melting temperature and amplicon length, can be amplified with extremely different efficiencies and the basis for this is still not well understood.

Given the tremendous advantages of RPA, as well as some of the current limitations of the technique, it can be expected that there will be exponential growth in the applications of RPA as well as improving and extending its performance. Recently RPA reagents have become available in a liquid format and it can be envisaged that increased flexibility in the kit formulation will allow an improved optimisation of assay conditions and facilitate a better understanding of the RPA mechanism. Currently “optimisation” depends on using a pellet, half pellet, quarter pellet etc., and as mentioned above, different targets are amplified with different efficiencies, and whilst RPA does show the potential to be particularly amenable to multiplexed detection, quite a laborious optimisation is currently required, but with more control of the amplification mix, this could become more simplified. Indeed, with the increasing interest in the simultaneous detection and sometimes also quantification of biomarkers, it is expected that there will be an exponential increase in the number of reports detailing parallelised amplification in solution-phase, in separate reservoirs in microfluidic systems, or on separate electrodes of an electrode array for solid-phase amplification, where multiplexing can be facilitated by spatial separation. Real-time RPA also requires extensive optimisation to truly control the amplification rate and to define properly the time threshold. To date real-time RPA has been achieved using fluorescent and ring-resonator detection, and other detection methodologies may further enhance the possibilities of real-time RPA, possibly even achieving highly multiplexed real-time quantitative RPA. The use and optimisation of RPA for differentiating single base differences (SNPs/mutation) or for the amplification of a family of species needs to be further explored as very few reports addressing this theme exists to date.

The focus of a large number of RPA related publications details the use of RPA in lateral flow formats, but to date there is no report of a completely integrated paper analytical diagnostic device, which only requires end-user addition of blood/saliva/urine/food/environment sample. Innovative approaches for the application of temperature to facilitate efficient execution of RPA at the point-of-need/care have been reported and cost-effective, efficient solutions are available. As yet RPA has not been approved by the FDA and is destined for research only applications and it can be expected that the technique will be validated and approved for medical diagnostics in the near future, facilitating the true implementation of RPA in lateral flow assays for companion diagnostics or as ASSURED devices in low resource settings.

In summary, RPA is a fascinating isothermal amplification technique that has already garnered a huge amount of attention due to its very attractive properties, having widespread application. Whilst to date the majority of interest has been the use of RPA in diverse areas, there is expanding interest in a deeper understanding of the underlying mechanisms of the technique, with the objective of a complete optimisation for real-time and multiplexed applications. RPA is exploited for laboratory-based analysis, portable analysis in laboratory-in-a-suitcase, analysis at the point-of-need/care with biosensors, lateral flow assays and microfluidic devices, and its exploitation in a range of commercial devices for molecular diagnostics, food quality control, environmental analysis and detection of biowarfare agents, amongst others, can clearly be anticipated in the near future.

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