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Trends in Analytical Chemistry 98 (2018) 19–35

Contents lists available at ScienceDirect

Trends in Analytical Chemistry

journal homepage: www.elsevier.com/locate/trac

Recombinase polymerase amplification: Basics, applications and recent advances

Ivan Magriñá Lobato [a], Ciara K. O'Sullivan [a, b, *]

[a] INTERFIBIO Consolidated Research Group, Departament d'Enginyeria Química, Universitat Rovira i Virgili, Països Catalans, 26, 43007, Tarragona, Spain
[b] Institució Catalana de Recerca i Estudis Avançats, Passeig Lluís Companys, 23, 08010 Barcelona, Spain

ARTICLE INFO

Article history:
Available online 26 October 2017

Keywords:
Recombinase polymerase amplification
Isothermal amplification
Solid-phase amplification
Multiplexing

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.

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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 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.

* Corresponding author. INTERFIBIO Consolidated Research Group, Departament d'Enginyeria Química, Universitat Rovira i Virgili, Països Catalans, 26, 43007, Tarragona, Spain.
E-mail address: ciara.osullivan@urv.cat (C.K. O'Sullivan).

https://doi.org/10.1016/j.trac.2017.10.015
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Table 1
Summary of isothermal nucleic acid amplification techniques.

Isothermal technique	Target	Primers needed	Initial heating	Incubation temperature (°C)	Amplification time (min)	Limit of detection (copies)	Multiplexing	Lyophilised reagents	FDA approved tests
NASBA	RNA	2	No	41	60–180	1	Yes	Yes	Yes
SDA	DNA	4	Yes	30–55	60–120	10	Yes	No	Yes
RCA	DNA/RNA	1	Yes	30–65	60–240	10	No	No	No
LAMP	DNA	4–6	Yes	60–65	60	≈5	Yes	No	Yes
HDA	DNA	2	No	65	30–120	1	Yes	No	Yes
RPA	DNA/RNA	2	No	37–42	20–40	1	Yes	Yes	No

co.uk) [4]. 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 polyethyleneglycol), 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 1, 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 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 reaction, 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 Transcriptor^{R} (Roche), Sensiscript^{R} (Qiagen), or MuLV^{R} (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

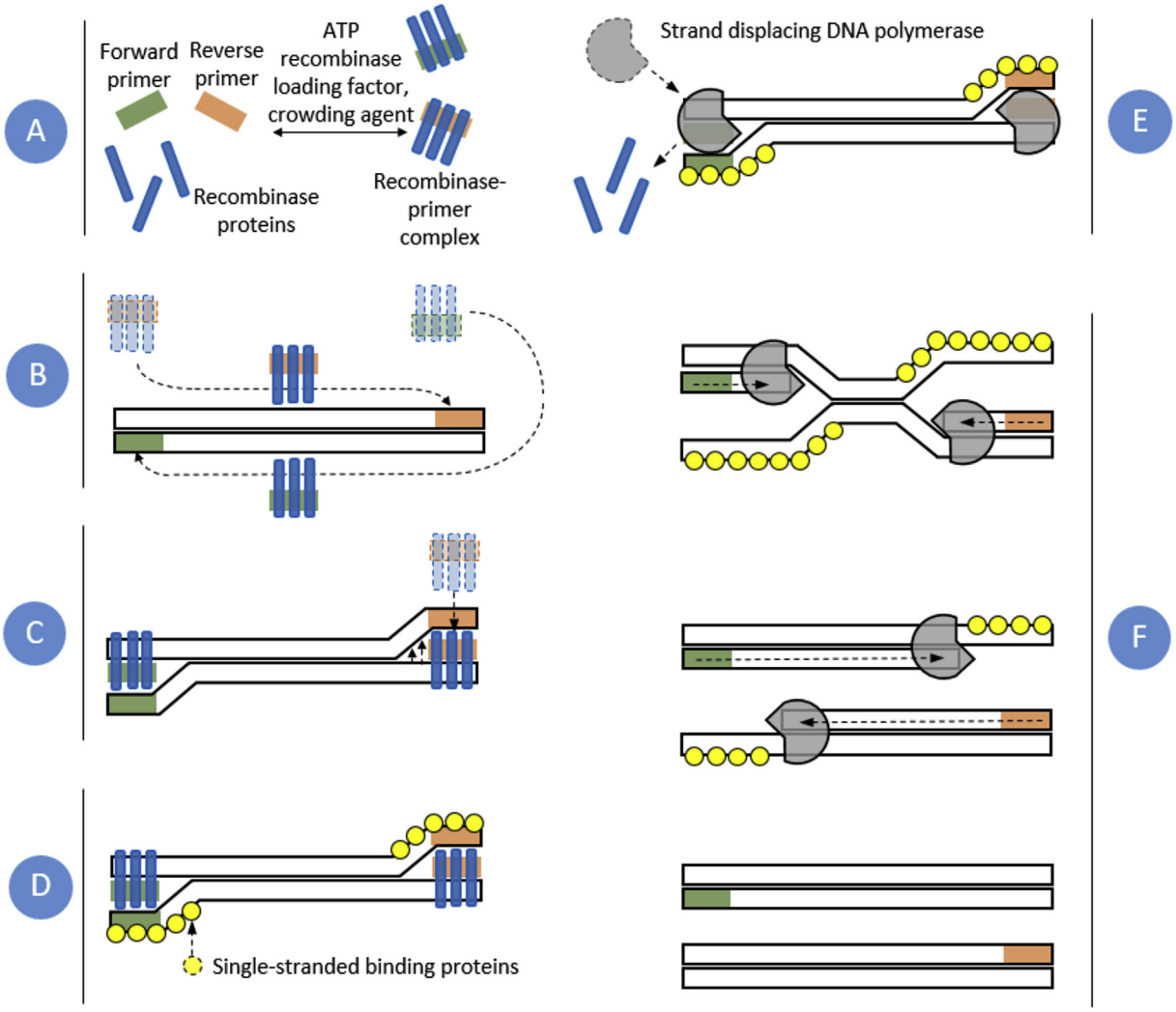

Fig. 1. RPA amplification scheme. Recombinase proteins form complexes with each primer (A), which scans DNA for homologous sequences (B). The primers are then inserted at the cognate site by the strand-displacement activity of the recombinase (C) and single stranded binding proteins stabilise the displaced DNA chain (D). The recombinase then disassembles 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.

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.

Table 2
RPA methods developed for lateral flow strip detection.

Organism	Target	Sample	Heat source	Amplification time (min)	Temperature (T) (°C)	Limit of defection (LOD)	Ref.
Methicillin resistant *Staphylococcus Aureus* (MRSA)	dsDNA	Genomic DNA	Not specified	<30	37	10 copies	[4]
HIV	dsDNA	Plasmid template	Heat block	15	37	10 copies	[64]
Cryptosporidium	dsDNA	Human stool spiked with cryptosporidium	Heat block	30	37	1–10 copies	[65]
HIV-1	dsDNA	Plasmid template with human genomic DNA background	Body heat	20–30	Body heat (≈31–35)	10 copies	[11]
Plasmodium falciparum	dsDNA	Genomic DNA	Incubator	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. typhy* cells	Heat block	20	39	20 copies	[68]
Plasmodium	dsDNA	Plasmid template	Hot plate	30	37	50	[66]
[a] *Giardia, cryptosporidium* and *Entamoeba*	dsDNA	Live parasites spiked into stool	Heat block	35	37	≈400 copies (triplex)	[57]
HIV-1	dsDNA	Plasmid template	Heat block	30	37	1000	[45]
HIV-1	dsDNA	Infected cell lines	Portable heat block	20	39	10	[12]
Giarda duodenalis	dsDNA	Stool	Incubator	30	37	10	[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	1360 CFU/mL	[71]
Borrelia burgforferi	dsDNA	Cell lines and serum samples	Thermoshaker	20	37	25 copies	[72]
Leishmania Viannia spp	dsDNA	Skin ulcera	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	0,5 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]
Orf virus	dsDNA	Nasal swabs, skin, lymphatic nodes, liver, lungs, stomach and kidneys	Water bath	20	37	80 copies	[76]
[b]Synthetic target	dsDNA	Artificial sample	Incubator	15	37	10^{-11} M	[67]
Penaeus stylirostris virus	ssDNA	Muscles of shrimps	Heat block	30	35–40	100 copies	[77,78]
[b]*B-conglutin*	ssDNA, aptamer	Artificial sample	Room temperature	15	Room temperature	0,17 amol	[79]
[c]Yellow fever virus	ssRNA	Cell culture supernatant and mosquito pools	Heat block	20	39	<21	[80]
[c]*Peste des petits rumiants virus*	ssRNA	Tissues	Thermocycler	20	39	150 copies	[81]
[d] *Little cherry virus 2*	ssRNA	Leaves, budwood, mealybugs and cherry tissue	Incubator	15	39	–	[82]
[d] Plum pox virus	ssRNA	Peach, apricot, plum, cherry tree leaves	Portable heat block	15	39	1.0 fg	[83]
[d] *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] AmplifyRP^{R} and Acceler8™

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 efficacy, 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 3
RPA methods with end point detection other than lateral flow assays.

Organism	Target	Sample	Amplification device	Transduction	Detection Platform	Amp. Time (min)	LOD	T (°C)	Ref.
Canine parvorius 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]
[a]Rose 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	Flocculation	Eppendorf tube. Naked eye	30	0,5 ng	37	[85]
Mycobacterium tuberculosis	dsDNA	Cell cultures	Incubator	Flocculation	Eppendorf tube. Naked eye	20	10 CFU	38	[86]
[b]*Pseudomonas syringae* …	dsDNA, RNA	Leaves, bovine cells, water …	Incubator	Flocculation	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]
GMOs	dsDNA	Food products	Microfluidic chamber on DVD. Oven	Change in reflected light intensity	DVD drive	40	4–9 copies or 7 μg/g	37	[41]
Francisella tularensis	dsDNA	DNA template	Microtitre plate, incubator	Change in color	Microtiter plate reader	40	$4 \cdot 10^6$ copies	37	[30]
Allergens, GMOS, bacteria and fungi	dsDNA	Cell cultures	Microtiter plate, oven	Change in color	Microtiter plate reader. Visual detection	40	1,3–5,3 μg/g 6–13 CFU/ml	40	[37] [5]
Yersinia pestis	dsDNA	Synthetic and genomic DNA	Microtiter plate, incubator	Change in color	Microtitre plate reader	30	$3{,}14 \cdot 10^{-16}$M	37	[5]
Mycobacterium tuberculosis	dsDNA	Cell cultures	Incubator	Change in color	Spectrophotometer visual detection	20	1 cfu	38	[33]
Human	dsDNA	Buccal smear	100 well array chip + oven	Color	Polycarbonate chips, desktop scanner	40	5–10% genomic SNP	37	[6]
Human lung cancer cells	dsDNA	Lung tissue	Not specified	Visual read-out. Color change	Eppendorf tube. Naked eye	5–10	20 pg	–	[38]
MRSA, *Neisseria gonorrhoeae*, *Salmonella enterica*	dsDNA	Genomic DNA	Programmable hybridization chamber	Fluorescence	Microarray scanner	<20	10–100 cfu	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	40	35GU/μL, 1GU/μL, 1000GU/μL	37	[43]
Francisella tularensis	dsDNA	DNA template	Electrode, aluminium block	Electrochemical. Chronoamperometry	Sputtered gold electrodes. Potentiostat	60	$2 \cdot 10^5$ copies	37	[30]
Piscirickettsia salmonis	dsDNA	Salmon	In house heater	Electrochemical. Chronoamperometry	Sputtered gold electrode array. Potentiostat	40	3000 copies	37	[31]
Mycobacterium tuberculosis	dsDNA	Cell cultures	Incubator	Electrochemical. Chronoamperometry	Screen-printed carbon electrodes. Potentiostat	20	1 cfu	38	[33]
Leishmania infantum	dsDNA	Dog blood	Not specified	Electrochemical. Chronoamperometry	Screen-printed carbon electrode. Potentiostat	10	0,8 parasites/ml	37	[32]
Mycobacterium tuberculosis	dsDNA	Genomic DNA from cultured cells	Incubator	Electrochemical. Differential pulse voltammetry	Screen-printed carbon electrodes. Potentiostat	20	1 cfu	38	[34]
Pseudomonas syringae	dsDNA	Leaves	Thermocycler	Electrochemical. Differential pulse voltammetry	Carbon screen printed electrode. Potentiostat	20	15 copies	37	[35]
[a]Human prostate cancer cells	RNA	Urine	Incubator	Colorimetric readout/ electrochemical	Eppendorf/ spectrophotometer/ screen printed electrode	20	1.000–100.000 copies	43	[36]

(*continued on next page*)

Table 3 (*continued*)

Organism	Target	Sample	Amplification device	Transduction	Detection Platform	Amp. Time (min)	LOD	T (°C)	Ref.
[a]Human prostate cancer cells	RNA	Urine	Not specified	SERS	Portable raman microscope	15	100 copies	41	[55]
[a]Human prostate cancer cells	RNA	Cell cultures, tumor tissue, urine	Not specified	SERS	Portable raman microscope	20	100 copies	41	[90]
Botrys 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 cineres*, cucumber mosaic virus, bovine herpes virus 1, *Escherichia coli*, proviral HIV, *Mycobacterium tuberculosis*, influenza virus H1N1.

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 significant 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 4.1
Real-time RPA methods based on fluorescence detection using a conventional real time thermocycler.

Organism	Target	Sample	Kit/extra reagents	Amp. time (min)	T (°C)	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	dsDNA	Cell cultures	TwistAmp Basic Kit, SYBR Green I	<30	37	<100 copies	[4]
MRSA	dsDNA	Cell cultures	TwistAmp Exo probe *E. coli* endonuclease IV (Nfo)	<30	37	<10 copies	[4]
Listeria monocytogenes	dsDNA	Blood	TwistAmp Exo kit	25	40	5000–20.000 cells	[22]
Campylobacter jeuni and campylobacter coli	dsDNA	Eggs, chicken meat, chicken broth	TwistAmp Exo kit	20	45	1CFU–1000CFU/ml	[53]
Leptospira	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 tumiants virus	RNA	Tissues	TwistAmp RT Exo kit	20	40	100 copies	[81]
Yam mosaic virus	RNA	Leafs	TwistAmp Exo kit, MuLV reverse transcriptase.	<30	37	14 pg/uL	[103]
Porcine reproductive and respiratory síndrome virus	RNA	Tissue and serum	TwistAmp Exo kit	20	40	70 copies	[104]
Coxsackievirus A6	RNA	Stool	TwistAmp RT Exo kit	20	40	202 copies	[105]
Human cancer cells	RNA/ssDNA	urine	TwistAmp Basic kit, ligases, SYTO 9 dye	15	37	1000 copies	[98]

Table 4.2
Real-time RPA methods based on fluorescence detection using portable fluorometers.

Organism	Target	Sample	Kit/extra reagents	Amp. time (min)	T (°C)	LOD	Ref.
Hypodermal and hematopoietic necrosis virus	ssDNA	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]
Rickettsia typhi	dsDNA	Human plasma spiked with R. typhy 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 and RNA	Inactivated whole organisms spiked into plasma	TwistAmp Exo kit, TwistAmp Fpg kit, Transcriptor	<10	42	16–21 copies	[110]
Rift Valley fever virus	RNA	RNA isolated from cell culture	TwistAmp Exo kit, reverse transcriptases: 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	10–20	42	<20 copies	[111]
Plum pox virus	RNA	Peach, apricot, plum, cherry tree leaves	AmplifyRP XRT, XRT probe	15	39	16 fg	[83]
Dengue Virus	RNA	Plasma	TwistAmp Exo kit, Transcriptor	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 kit, Transcriptor	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, Transcriptor	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 kit	20	42	10 copies	[46]
Yellow fever virus	RNA	Cell culture supernatant and mosquitos	TwistAmp Exo RT kit	<20	39	21 copies	[80]
GMO: rice	dsDNA	Rice	TwistAmp Exo kit	20	39	500 copies	[118]
Streptococcus pneumoniae	dsDNA	Blood	TwistAmp Exo kit	20	40	4 copies	[119]
HIV-1	dsDNA	Infected peripheral blood mononuclear cells	TwistAmp Exo kit	<20	39	3 copies	[120]
Mycobacterium tuberculosis	dsDNA	Sputum and respiratory washes	TwistAmp Exo kit	20	39	6,5 fg	[121]
MRSA	dsDNA	Nasal and groin swabs	TwistAmp Exo kit	20	39	–	[54]
GMOs	dsDNA	Maize, rice, cotton and soybean	TwistAmp Exo 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	15	42	179 copies	[125]
GM soybean	dsDNA	Seeds	TwistAmp Exo kit RR2Y (primers and probes included)	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 RT Exo kit	<20	40	10 copies	[126]
Vibrio cholerae	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 síndrome virus	RNA	Lymph node, lung, spleen and liver	TwistAmp RT Exo 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 as exemplified by a method developed to detect HIV-1 proviral DNA, where even 9 changes across the primer and probe binding sites are tolerated by RPA, allowing the detection of different virus strains [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

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. atrophaeus*	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	Cell culture	TwistAmp Exo kit	Digital microfluidic on an AM-EWOD device	15	39	1 copy	[13]
Salmonella enterica, Escherichia coli O157:H7, vibrio parahaemolyticus	dsDNA	Milk	TwistAmp Exo kit	Centrifugal microdevice, Custom made portable genetic analyser with a miniaturized optical detector	20	39	4 cells	[49]
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 Nfo kit	Centrifugal heater with an integrated fluorimeter	Fluorescence	Centrifugal heater with an integrated fluorimeter	30	39	–	[133]
Listeria monocytogenes	dsDNA	Genomic DNA	TwistAmp Exo kit	Picoliter array chip	Fluorescence	Modified fluorescence microscope	15	39	$4 \cdot 10^{-3}$ copies/well	[135]

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 donovi* 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

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 TwistAmp® 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.

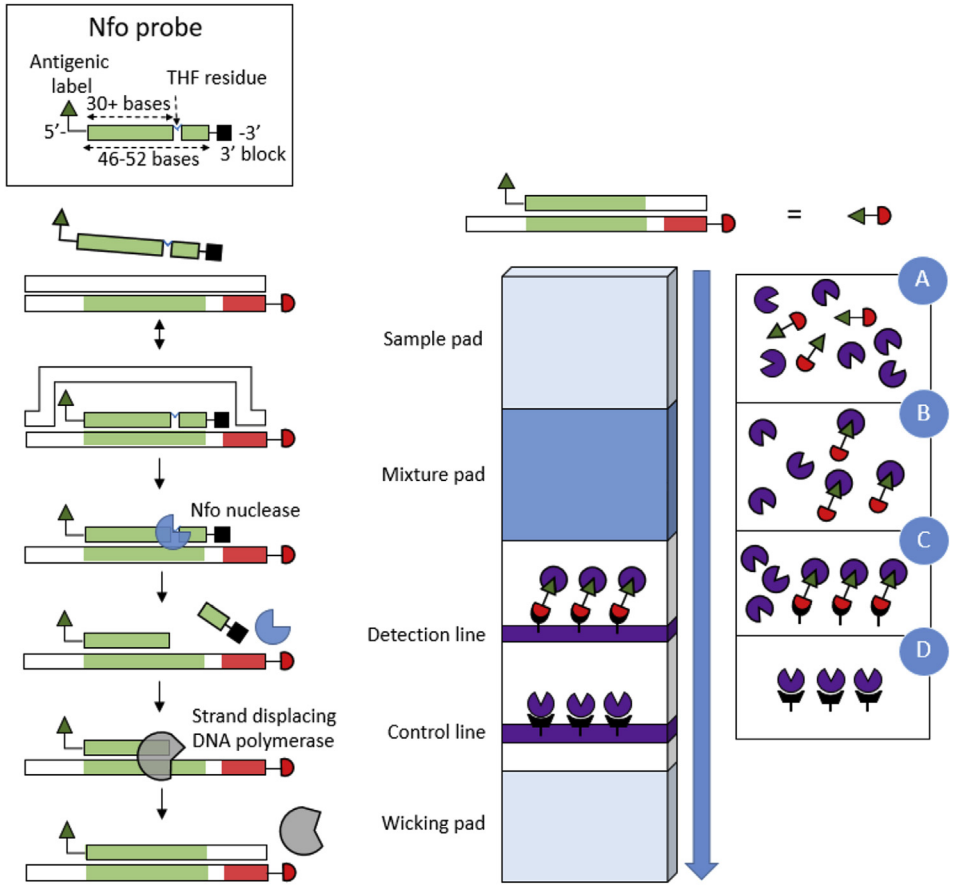

Fig. 2. *Nfo probe and lateral flow strip.* The Nfo probe is exchanged at the cognate site by recombinase proteins and nfo nuclease cleaves the probe on the THF residue. The blocking group is released and the probe is thus converted into a primer. The double labelled amplicon obtained from amplification 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).

3.1.2. 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 facilely 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 reactors 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 H_2O_2, 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 H_2O_2 [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 $AuCl_4^-$, 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 instrumentless 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

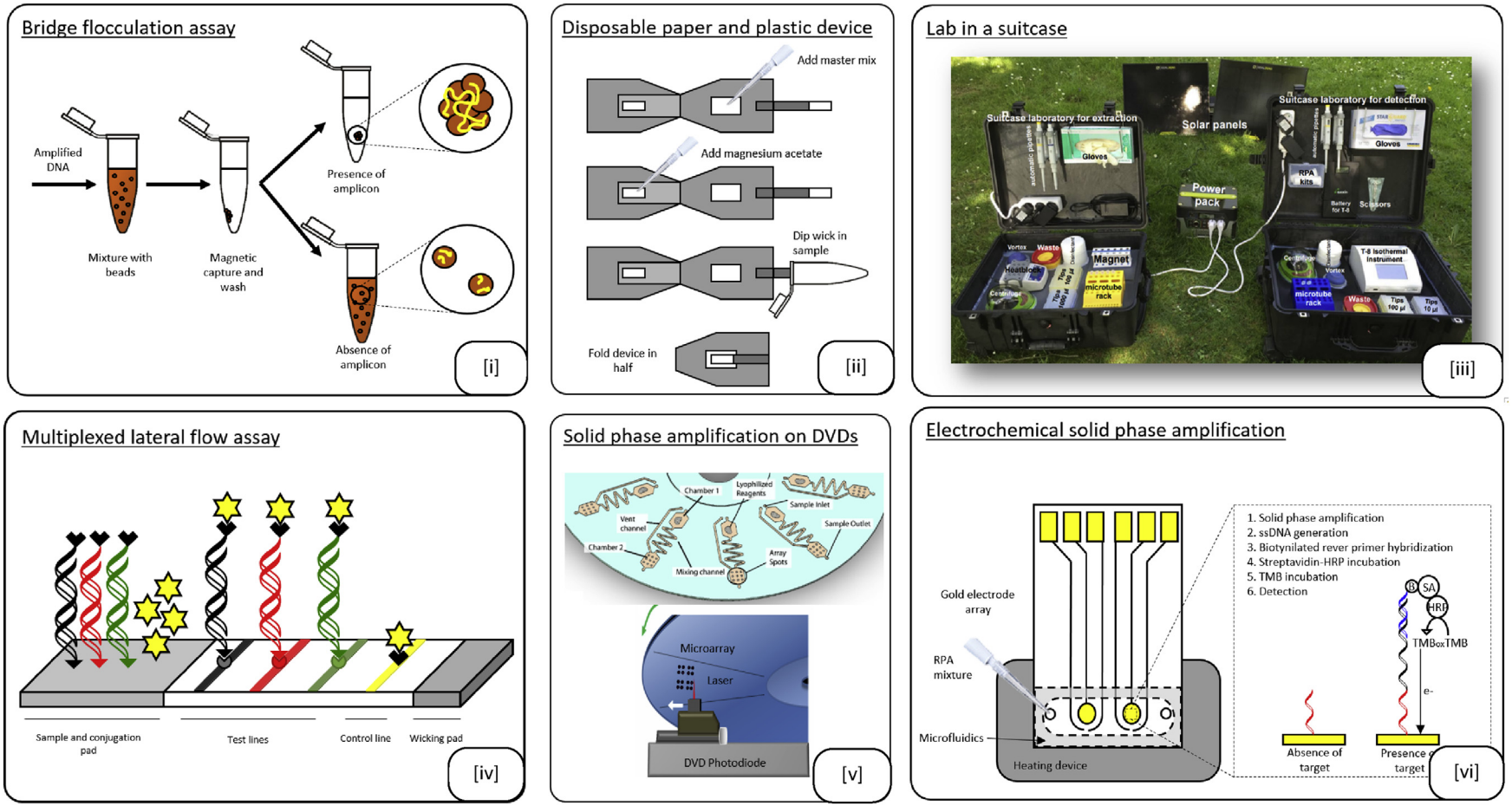

Fig. 3. Schematic representations of biosensors/POC devices using RPA: (i) Bridge flocculation assay [87]; (ii) a disposable plastic and paper device (64); (iii) lab in a suitcase [62]; (iv) multiplexed lateral flow assay [57)]; (v) solid phase amplification on DVDs [41]; (vi) electrochemical solid phase amplification [30] (Figures modified from original publications cited).

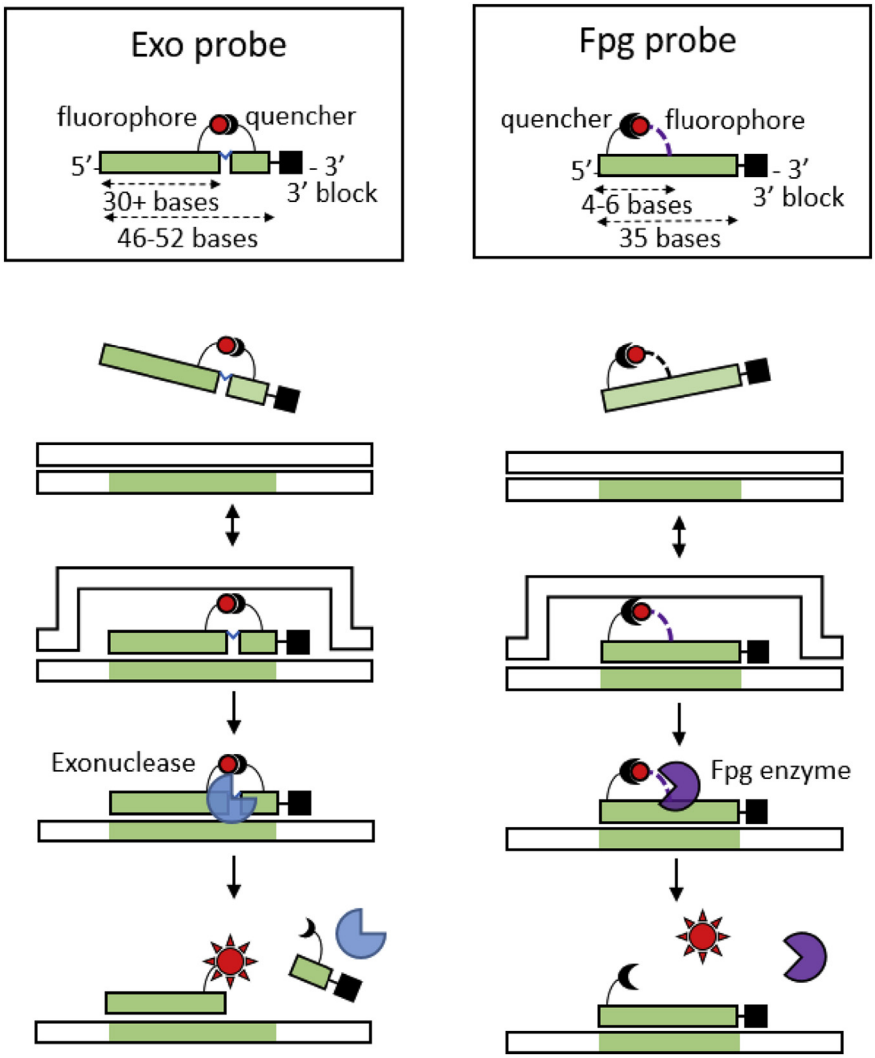

Fig. 4. Exo probe and Nfo probe for fluorescent real-time detection.

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 supported on a thin film for the extraction and purification of DNA, and solid phase-RPA integrated with a Mach-Zehnder interferometer for combined amplification and detection [132].

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 microcliff 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 27,000 reactions in picoliter sized wells [135].

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 operate between 30 and 65°C, RPA takes advantage of enzymes and crowding agents to work at a low temperature ranging between 37 and 42°C and there is no requirement for a tight control of the temperature within this range, which is a particularly positive attribute not possessed by the other isothermal techniques. RPA, as well as NASBA and HDA do not require an initial denaturation step to generate ssDNA from the dsDNA target, in contrast to SDA, RCA and LAMP, highlighting its suitability for use in the field. In addition, RPA, in common with 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 format 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 polymerases 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 appear 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.

Acknowledgements

The authors wish to thank Dr. Luis A. Tortajada-Genaro and Dr. Ahmed Abd El-Wahed for generously providing figures for Fig. 3. The authors acknowledge support from the Ministry of Economy and Competitiveness, Spain for partial financing from the project Seasensing, Ref. BIO2014-56024-C2-1-R.

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