Analytical Biochemistry 545 (2018) 20–30

Contents lists available at ScienceDirect

Analytical Biochemistry

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

Multiplexed isothermal nucleic acid amplification

Olena Mayboroda[a], Ioanis Katakis[a,*], Ciara K. O'Sullivan[a,b,*]

[a] Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona, Spain
[b] ICREA, Passeig Lluís Companys 23, 08010 Barcelona, Spain

ARTICLE INFO

Keywords:
Isothermal amplification
Multiplexing
Advantages and limitations

ABSTRACT

Multiplexed isothermal amplification and detection of nucleic acid sequences and biomarkers is of increasing importance in diverse areas including advanced diagnostics, food quality control and environmental monitoring. Whilst there are several very elegant isothermal amplification approaches, multiplexed amplification remains a challenge, requiring careful experimental design and optimisation, from judicious primer design in order to avoid the formation of primer dimers and non-specific amplification, applied temperature as well as the ratio and concentration of primers. In this review, we describe the various approaches that have been reported to date for multiplexed isothermal amplification, for both "one-pot" multiplexing as well as parallelised multiplexing using loop-mediated isothermal amplification, strand-displacement amplification, helicase-dependent amplification, rolling circle amplification, nucleic acid sequence-based amplification, with a particular focus on recombinase polymerase amplification.

Introduction

Molecular or nucleic acid-based diagnostic testing has grown significantly in recent years and has been largely influenced by different breakthroughs stemming from the knowledge gleaned from the Human Genome Project (HGP) and post-HGP technologies including Next-Generation Sequencing. Molecular diagnostics itself combines laboratory medicine with the knowledge and technology of molecular genetics and has moved from complicated and manual to rapid and automated due to improvements in sample extraction and target amplification combined with sensitive and specific detection techniques. Whilst the polymerase chain reaction (PCR) [1] has revolutionised molecular diagnostics, facilitating the rapid amplification of DNA, producing billions of copies of a specific DNA fragment or gene, that can either be quantitatively detected in real-time, or subsequently analysed by any of a number of techniques including detection via hybridisation and gel visualisation, there are several limitations of PCR for implementation in a point-of-need device [2,3]. These include the need for thermal cycling, risk of contamination, the relatively high cost of equipment and inherent requirement for a power source/electricity and trained personnel.

To address the true implementation of nucleic acid amplification tests (NAAT) at the point-of-care/need, isothermal amplification techniques have been developed, including loop mediated isothermal amplification (LAMP), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), strand displacement amplification (SDA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA), thus avoiding the need for thermal cycling apparatus [4,5].

Although singleplex isothermal assays (*i.e.* assays to measure a single analyte) are robust and represent the majority of isothermal amplification applications reported to date, it is also of great interest to simultaneously amplify and detect more than one target, especially for ASSURED diagnostics purpose (Affordable, Sensitive, Specific, User-friendly, Rapid, Robust, Equipment-free and Delivered to those who need it). The focus of this review is to describe reports of multiplexed isothermal amplification methodologies and critically analyse the advantages and disadvantages of the different approaches with a focus on recombinase polymerase amplification.

Overview of isothermal techniques exploiting multiplex detection

Loop-mediated isothermal amplification (LAMP)

LAMP is a specific, relatively simple, rapid and cost-effective isothermal nucleic acid amplification method first described by Notomi et al. [6] in 2000. LAMP takes advantage of the strand-displacing *Bacillus stearothermophylus* (Bst) DNA polymerase and 4-6 specifically designed primers (2 of which are 'fold back' primers for stem-loop formation) that recognise 6 distinct sequences on the target DNA [7]. The target amplicon is generated in a one-step amplification reaction using isothermal conditions (60-65 °C) in a reasonably short time (45-

* Corresponding authors. Interfibio Research Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Avinguda Països Catalans 26, 43007 Tarragona, Spain.
E-mail addresses: ioanis.katakis@urv.cat (I. Katakis), ciara.osullivan@urv.cat (C.K. O'Sullivan).

https://doi.org/10.1016/j.ab.2018.01.005
Received 28 November 2017; Received in revised form 5 January 2018; Accepted 9 January 2018
Available online 17 January 2018
0003-2697/

60 min). Two additional performance enhancement strategies have been reported to increase the speed and analytical sensitivity of LAMP via the use of an additional set of loop primers and/or an initial melting step (95 °C) [8,9]. Even though LAMP requires a careful design of multiple complex primers, it is still one of the most widely used and studied isothermal amplification techniques due to its high-level of specificity, sensitivity (six DNA copies) and tolerance to various inhibitors present in clinical samples [6,8].

Despite the requirement of multiple primers, one of the most exploited isothermal techniques for multiplexing is LAMP. Even though multiplex LAMP (mLAMP) detection is difficult because LAMP amplicons are quite complicated in structure, a mLAMP method for the simultaneous detection of bovine *Babesia* parasites using a set of four specific primers in which a restriction enzyme cleavage site was inserted into two pairs of species-specific primers has been described. The system resulted in eight primers in one mixture. The amplicons were simultaneously differentiated via subsequent restriction enzyme analysis. This method facilitated the detection of two parasites in blood samples, however, the detection system is limited to laboratory environment [10]. Multiplex microfluidic LAMP [11] was reported for qualitative and quantitative detection of three influenza A subtypes. An octopus-like microfluidic system allowed parallel detection of the chosen targets with a detection limit of less than 10 copies/μl within 30 min. The first report of real-time multiplex LAMP [12] described the detection of 1–4 target sequences in a single LAMP reaction tube utilising a standard real- time fluorimeter using a DARQ probe, with a 5′-quencher on the forward interior primer annealed to a 3′-fluorophore on the complementary probe. Four pathogens (hepatitis B and C virus, HIV and *Treponema pallidum*) were successfully detected by a 4-plex barcoded LAMP coupled with NEase mediated pyrosequencing in a single tube [13] and a multiplex LAMP assay for the detection of influenza A/H1, A/H3 and influenza B has also been reported where six primers were designed targeting the matrix genes of the targets with further real-time fluorescent detection [14] and assay length of 40 min achieving a 1 genome equivalent limit of detection. Recently, a multiplex fluorogenic LAMP assay for the simultaneous detection and identification of 6 different viral pathogens in human plasma was demonstrated [15]. This assay was based on fluorogenic hybridisation and autocycling strand-displacement DNA synthesis that was loop-mediated under isothermal conditions. The method used 3 pairs of virus-specific primers that targeted 8 different sequences. In the alternative approach, Wang et al. [16] reported real-time LAMP combining LAMP amplification with endonuclease restriction, where the LAMP forward or backward inner primers contained 5′ end short sequences recognised by the restriction endonuclease (Nb.*BsrDI*), and the new forward or backward inner primers were modified at the 5′ end with a fluorophore and in the middle with a dark quencher. Nb.*BsrDI* digested the newly synthesised double-stranded terminal sequences, which released the quencher resulting in fluorescent signal. The assay permitted real-time detection of single or multiple target sequences in a single tube in just 12 min. An eight-chamber lab-on-a-chip system [17] with integrated magnetic bead-based sample preparation and parallelised LAMP amplification for the detection of *Salmonella* spp. in food samples was also developed, and all steps of DNA isolation, isothermal amplification, real-time detection were carried out in just 40 min. Recently, a very interesting approach using a paper-based POC diagnostic platform incorporating LAMP was reported [18], allowing simultaneous real-time detection of multiple DNA sequences associated with bacterial meningitis, in 60 min at 63 °C. Several other LAMP multiplex protocol were published recently, including the real-time detection of *Salmonella* spp. and *Vibrio parahaemolyticus* in a single reaction [19] as well as multiplex reverse-transcription LAMP for detection of the influenza A virus (subtypes A/H5, A/H7 and 2009A/H1), where LAMP was combined with a cascade invasive reaction and nanoparticle hybridisation to differentiate the samples, achieving a detection limit of just 10 copies of RNA [20]. Recently, there have been several reports of the combination of multiplex LAMP with lateral flow detection. Simultaneous detection of the *sea* and *seb* genes of *Staphylococcus aureus* was achieved by labelling the *sea* gene amplicon with digoxigenin and biotin and the *seb* gene amplicon with fluorescein isothiocyanate and biotin. The amplicons were further detected using a multiplex lateral flow assay, where antibodies against digoxigenin and flourescein isothiocynate were immobilised at two separate test lines, to capture each of the *sea* and *seb* amplicons, respectively. NeutrAvidin-tagged gold nanoparticles were then used to complete the sandwich and allow visualisation and quantification of any *sea* or *seb* genes present in the sample. The detection limit of the optimised assay was 10 times lower than of a multiplex PCR (10^2 CFU ml^{-1}) [21]. A similar approach was also used for the detection of Pseudomonas *aeruginosa* and its toxin genes [22]. A visual multiplex detection and differentiation of *Ef0027* gene (specific gene for *Enterococcus faecalis*) and *nuc* gene (specific for *Staphylococcus aureus*) based on multiplex LAMP and lateral flow assay using fluorescein- and digoxogenin-modified primers in the amplification process. In the presence of biotin- and fluorescein -/digoxin-modified primers, numerous biotin- and fluorescein -/digoxin-attached duplex products were detected by lateral flow via immunocapture of the fluorescein/digoxogenin hapten label on the duplex amplicon by anti-FITC/digoxin on the test lines, and affinity interaction between the biotin hapten label and streptavidin on the AuNPs [23]. The variety of developed "one-pot" and parallelised LAMP assays shows that with the proper primer design and optimisation of conditions, multiplexed LAMP can combine very high selectivity and sensitivity with short amplification time (less than 60 min).

Nucleic acid sequence-based amplification (NASBA)

Nucleic acid sequence-based amplification, NASBA, and the similar methods, transcription mediated amplification (TMA) [24] and self-sustained sequence replication (3SR) [25] are alternative isothermal transcription-based amplification systems. NASBA is specifically designed for the detection of RNA targets but in some cases can be applied to DNA targets. Amplification occurs at a constant temperature of 41 °C with an initial strand separation step of 95 °C in the case of dsDNA and 65 °C in the case of RNA amplification [26]. The system uses three enzymes (avian myeloblastosis virus reverse transcriptase, RNase H and T7 DNA dependent RNA polymerase) producing single-stranded RNA as the main amplification product [27]. The amplification time varies from 90 min to 3 h. Transcription-mediated amplification (TMA) differs very slightly from NASBA and 3SR: instead of using three separate enzymes, the role of RNase H is carried out by the reverse transcriptase, so that only two enzymes are involved [28–30].

Multiplex NASBA was first reported in 1999, detailing the quantification of two individual mRNAs in a single NASBA reaction using biotin and ECL labels [31]. Multiple multiplex real-time NASBA assays have been reported exploiting numerous molecular beacons in one reaction [32,33]. Different fluorophores can be measured simultaneously using defined excitation and emission filters. The commercially available HIV-1 assay utilises a simple multiplex reaction with molecular beacons specific for wild-type and calibrator amplicons having the different labels, FAM and ROX, respectively. In 2002 a multiplex format of NASBA was developed for the detection of hepatitis A virus and rotavirus in cell culture lysates [34], and the same team also proposed a multiplex NASBA assay for the simultaneous detection of the most common food borne viruses, such as genogroup I and genogroup II noroviruses and hepatitis A virus [35]. Several other real-time [36–38] and end-point [39,40] multiplex reactions have also been described. NASBA has an advantage of being able to detect both RNA and DNA sequences. However, the requirement for an initial pre-melting step does not position it as suitable for use at the point-of-need, and indeed there are very few reports of multiplexing using these types of isothermal techniquies.