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Original Article

A novel approach for evaluating the performance of real time quantitative loop-mediated isothermal amplification-based methods

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ABSTRACT

Molecular diagnostic measurements are currently underpinned by the polymerase chain reaction (PCR). There are also a number of alternative nucleic acid amplification technologies, which unlike PCR, work at a single temperature. These 'isothermal' methods, reportedly offer potential advantages over PCR such as simplicity, speed and resistance to inhibitors and could also be used for quantitative molecular analysis. However there are currently limited mechanisms to evaluate their quantitative performance, which would assist assay development and study comparisons. This study uses a sexually transmitted infection diagnostic model in combination with an adapted metric termed isothermal doubling time (IDT), akin to PCR efficiency, to compare quantitative PCR and quantitative loop-mediated isothermal amplification (qLAMP) assays, and to quantify the impact of matrix interference. The performance metric described here facilitates the comparison of qLAMP assays that could assist assay development and validation activities.

1. Introduction

The use of molecular approaches for clinical diagnosis has increased over the past 30 years since the development of PCR [1] resulting in a wide variety of diagnostic applications [2–4]. Alternative nucleic acid amplification (NAA) technologies, reviewed by Craw and colleagues [5–7], utilising isothermal conditions offer a range of potential advantages over PCR, including speed and simplicity, and lend themselves to near patient and point of care diagnostic testing [8,9]. Loop-mediated isothermal amplification (LAMP) [10] is an example of an isothermal NAA technology that is typically faster than PCR [11–13] and reportedly less susceptible to common biological inhibitors [14–17]. LAMP has been successfully utilised in the development of a wide range of diagnostic assays [18–20].

The application of LAMP to measure template abundance, originally established by real time quantitative PCR (qPCR), has been explored by a variety of laboratories [17,20–26]. However, real time quantitative LAMP (qLAMP) is a relatively immature technology compared with qPCR and demonstrates poor quantitation capabilities below 1000 target copies [22,23,26]. Threshold time (T_t) [26] is a real-time quantitative LAMP measurement analogous to quantification cycle (C_q) [27] and is central to quantifying template abundance using qLAMP. While T_t, allows quantification to be performed there is currently no agreed method for expressing assay performance or defining the magnitude of a difference between two results when conducting qLAMP; a role that 'PCR efficiency' has provided since the development of qPCR. Metrics for assay performance are essential to enable assay optimisation, evaluation of the impact of matrices [28–31] and to facilitate laboratory comparison.

An isothermal performance metric, analogous to PCR efficiency, has been proposed for real-time helicase dependent amplification (HDA) [32] reactions by Goldmeyer and colleagues [33], termed doubling time (t_d) and calculated by comparing the time required by different starting quantities of template to reach a uniform

Abbreviations: C_q, quantification cycle; IDT, isothermal doubling time; MIQE, minimum information for the publication of quantitative real-time PCR experiments; NAA, nucleic acid amplification; qLAMP, quantitative loop-mediated amplification; qPCR, quantitative real-time polymerase chain reaction; t_d, doubling time; T_t, threshold time.

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threshold (2-point approach). We discuss the application of a more comprehensive metric termed isothermal doubling time (IDT) that utilised a standard curve-based approach to provide an equation (based on the slope of the T_t against concentration) to define the magnitude of a difference in results and estimate assay performance which also benefits from evaluating the linear dynamic range.

Effective mechanisms for comparing the performance of NAA technologies such as qLAMP to that of established PCR-based approaches is central to the uptake of these technologies and establishing confidence in their diagnostic potential. This paper describes a methodology for assessing quantitative isothermal nucleic acid amplification performance that, similar to PCR efficiency for qPCR [34], can assign a relative value to any qLAMP assay. We investigate the application of this metric to target pathogens and assess the impact of inhibition on assay performance.

2. Materials and methods

2.1. Artificial urine matrices

A panel of artificial urine matrices (Table S1) were developed from published work [35] that contained varying levels of urea concentration (500 or 1000 mM urea) which is a known PCR inhibitor [29]. For investigations into matrix impact, reactions were supplemented with 10% (v/v) of the respective artificial matrix.

2.2. Cultured DNA extracts

Chlamydia trachomatis, serovar E (ATCC VR-348BD) genomic DNA containing co-purified cryptic plasmid DNA and *Mycoplasma genitalium* G37 (ATCC 33530D) gDNA (LGC Standards, Teddington, United Kingdom) were used as genomic templates. Ten-fold serial dilutions (~5×10^4 to ~50 copies per reaction) of each DNA template (spectrophotometrically quantified) were prepared in 6.25 ng μl^{-1} sonicated salmon sperm DNA carrier (GE Healthcare, Chalfont St Giles, United Kingdom).

2.3. Clinical purified DNA samples

All initial clinical sample purification and molecular screening activities were performed at University College London Hospitals (UCLH), London, United Kingdom. 24 pre-screened DNA samples with defined *M. genitalium* and *C. trachomatis* content (mix of positives and negatives) were provided blind by UCLH for comparative analysis at the approval of the Chair of the Camden and Islington Community Research Ethics Committee.

C. trachomatis testing was performed as part of a routine clinical diagnostic protocol. Testing process overview: cervical swabs, self-taken vaginal swabs or urine samples were collected and transported in 3 or 4 ml of APTIMA transport medium (Gen-Probe Incorporated, San Diego, USA) mixed with urine 1:1 (v/v) for routine *C. trachomatis* testing. The test sample (400 μl) was analysed using the APTIMA CT assay on the TIGRIS® platform (Gen-Probe Incorporated, San Diego, USA).

For initial *M. genitalium* analysis, 200 μl samples were taken from materials previously tested for *C. trachomatis*, purified using a BioRobot 9604 automated workstation using the QIAamp® Virus BioRobot® 9604 Kit (QIAGEN, Hilden, Germany) and eluted in 50 μl elution buffer (QIAGEN, Hilden, Germany). The eluate (7 μl, equivalent to 28 μl of the original volume) was analysed by a qPCR assay adapted from Jensen et al. [36] incorporating a mouse CMV (mCMV) internal control system [37].

2.4. qPCR assay design and conditions

qPCR assays were designed to detect the *C. trachomatis* cryptic plasmid (GenBank Accession #X07547), and the *M. genitalium* partially sequenced MgPa gene (GenBank Accession #X91072) from strain M2300. The real-time PCR assays (Fig. 1, Table 1) were designed using Primer Express 2.0 (Life Technologies Ltd, Paisley, UK) using default design parameters and the sequences screened for homology using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/) that revealed no database alignments likely to cause cross reactivity, other than the region of interest. HPLC purified oligonucleotide primers and FAM/TAMRA hydrolysis probes were synthesised by Eurofins Genomics (Ebersberg, Germany). HPLC purified FAM/NFQ TaqMan® MGB probes were provided by Life Technologies Ltd (Paisley, UK). Preliminary primer/probe optimisation was conducted to determine optimal PCR conditions.

The qPCR reactions (20 μl volume) were performed comprising 1× TaqMan® Universal PCR Master Mix (Life Technologies Ltd, Paisley, UK), 900 nM forward and reverse primers, 200 nM 6-carboxyfluorescein (6-FAM) labelled hydrolysis probe, sample template (10 fold dilution series from ~5×10^4 to ~50 copies)

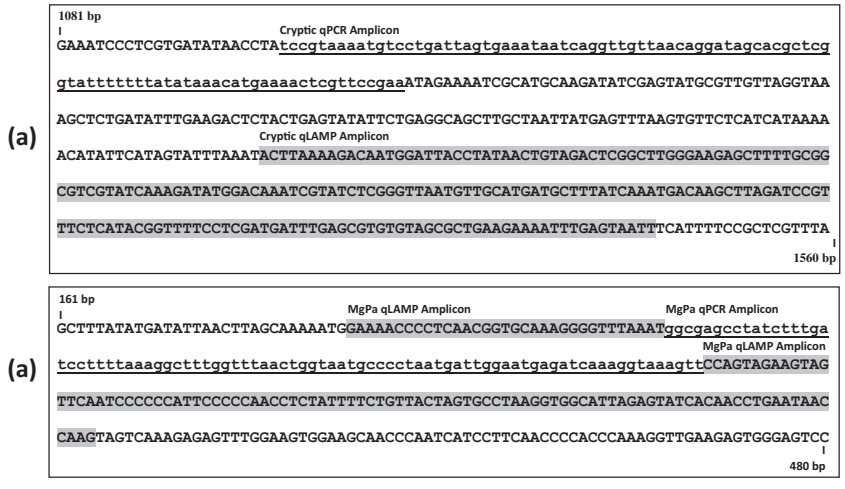

Fig. 1. Target sequences with corresponding amplicon regions for qLAMP (shaded sequence) and qPCR assays (underlined sequence). (a) *Chlamydia trachomatis* plasmid DNA for growth within mammalian cells (GenBank Acc#X07547) 1081–1560 bp target region for qPCR and qLAMP assay. (b) *M. genitalium* partial MgPa gene (strain M2300) (GenBank Acc#X91072) 161–480 bp target region for qPCR and qLAMP assay.