Rapid Detection of Circulating Tumour DNA using Allele Specific Mini-Loop Mediated Isothermal Amplification

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Research Article

Keywords: circulating tumour DNA, allele specific

Posted Date: January 11th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1232564/v1>

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Abstract

Isothermal amplification is an emerging approach for non-invasive, rapid and cost-effective real-time monitoring of cancer specific mutations through circulating tumour DNA (ctDNA). This study demonstrates a compact allele specific (AS) loop mediated isothermal amplification (LAMP) strategy, termed 'AS-Mini-LAMP', modelled using wild type (WT) and mutation specific reactions targeting the estrogen receptor *ESR1* c.1138G>C (p.E380Q) missense mutation. Allele selectivity, encoded at the 5'-end of the forward and backward inner primers (FIP and BIP) promotes enhanced selectivity upon self-hybridisation, loop formation and self-primed exponential amplification. Inclusion of unmodified self-stabilising (USS) primers aimed to reduce the likelihood of non-specific allele amplification through competitive inhibition and to enhance reaction velocity through an assisted strand displacement 'swarm' priming effect. The two assays were optimised using short synthetic WT and E380Q mutant DNA templates, and subsequently validated to a limit of detection of 500 mutant copies in under 25 minutes in ddPCR-confirmed positive (20.7% variant allele frequency) and negative patient plasma cfDNA samples. These results demonstrate the ability of AS-Mini-LAMP to achieve sensitive and selective amplification of actionable mutations present within plasma ctDNA.

Introduction

Current statistics provided by the World Health Organization calculate an annual worldwide cancer mortality rate at a level of 10 million. In Europe, this equates to one in every four deaths (26%) with the four most common cancer types identified as lung, breast, colorectal and prostate. In women, breast cancer is the most commonly occurring cancer accounting for 30% of all new diagnoses ¹.

The current standard for cancer diagnosis and primary therapy response relies on tumour biopsies and conventional imaging approaches. Whilst providing a valuable snapshot, there is an urgent need to profile and assess tumour evolution over time, guiding predictive, preventive, and personalized medicine to inform treatment decisions in response to changes in tumour biology at all phases of cancer care ². Liquid biopsy monitoring of plasma derived circulating tumour DNA (ctDNA) holds great promise in achieving this, with proof of principle demonstrated using approaches to target single variants by droplet digital PCR (ddPCR), real-time PCR, plus broader multigene and genome wide next-generation sequencing (NGS) approaches. Progress is greatly dependent on technological advances of highly sensitive techniques offering exquisite selectivity over high background levels of wild-type alleles in a sample. Tzanikou and Lianidou ³ comprehensively review a variety of techniques and their clinical utility in breast cancer.

To circumvent the long turnaround times and requirement for expensive thermal-cycling machinery associated with NGS, ddPCR and real-time PCR methods, isothermal amplification reactions have emerged as attractive alternatives. Loop mediated isothermal amplification (LAMP) ⁴ in particular has received significant attention as a molecular diagnostic tool due to its ability at the point of sample collection to provide highly sensitive and specific results at a significantly lower cost ⁵⁻⁸. If validated, this

approach could be ideally suited for liquid biopsy where 1 mm tumour is predicted to be associated with 1 copy of ctDNA in the circulation ⁹, ctDNA making up as little as 0.01% total cfDNA ¹⁰.

Several variations of LAMP have been successfully demonstrated. Itonaga *et al.*, ¹¹ developed a PNA-LNA mediated LAMP approach for mutant *KRAS* allele detection (helpful for patient stratification with anti-EGFR therapies), reproducibly detecting mutant alleles within samples diluted down to 0.1% mutant to wild type ratio, in contrast to 1% mutant to WT ratio when using a PNA-clamping PCR approach. Kumasaka *et al.*, ¹² developed mutation-oriented LAMP (MO-LAMP), a 5-primer reaction (FIP, BIP, F3, B3 plus loop backwards primer) plus a PNA for the sensitive detection of *KRAS* gene mutations. Arjuna *et al.*, ¹³ developed allele-specific LAMP (AS-LAMP), a typical 6-primer LAMP reaction, incorporating the T790M *EGFR* mutation or wild type counterpart in the backward inner primer (BIP). Subsequent application on cfDNA template material (termed CF-LAMP) included a pre-LAMP conventional PCR using LAMP outer primers (F3 and B3) to enrich for copies of target cfDNA prior to CF-LAMP ¹⁴. Our previous work describes a universal self-stabilising single base-LAMP method (USS-sbLAMP) ⁵, a typical 6-primer reaction encoding single base variant selectivity at the 5' end of both the forward and backward inner primers (FIP and BIP), coupled with unmodified self-stabilising (USS) competitive primers for enhanced prevention of un-specific wild type variant base amplification. Using this method, selective amplification of the *PIK3CA* c.3140 A > G (H1047R) cancer mutation was validated using DNA from patient breast tumour tissue samples and the assay successfully piloted on an ISFET (ion-sensitive field-effect transistor) based CMOS integrated Lab-on-Chip (LoC) system ¹⁵. Furthermore, this approach was applied to assays targeting the estrogen receptor gene, *ESR1* p.E380Q and p.Y537S mutations, both reproducibly detecting 1,000 copies of synthetic DNA in under 25 minutes on the LoC platform, with further optimisation ongoing towards reproducible 100 copy sensitivity ¹⁶.

Whilst these methods show great promise, the short length and heavily fragmented nature of cfDNA ¹⁷ remains a challenge. Sequencing-based approaches identify a prominent peak at 166 bp with several factors shown to influence the recovery and quality of plasma cfDNA ¹⁸. Optimal LAMP primer design typically exceeds this target length, requiring a minimum of 200 bp sequence for a 4-6 primer reaction as typified by our own previous 214 bp and 208 bp LAMP reactions ^{15,16}. This study aims to demonstrate a more compact allele specific LAMP reaction, more likely to fit within the cfDNA template span (see Figure 1 schematic). Mini-LAMP selectivity for mutant over wild type is maintained via dual FIP and BIP 3'-mutation recognition plus competitive inhibition of non-selective allele amplification with the use of universal self-stabilising (USS) primers. Termed AS-Mini-LAMP + USS and spanning 155 bp of the *ESR1* c.1138G>C E380Q template DNA, this approach selectively amplified synthetic DNA to a sensitivity of 500 mutant copies in under 25 minutes ahead of validation via ddPCR on E380Q confirmed patient cfDNA material.

Methods

Samples and cfDNA extraction methods

A 402bp double-stranded synthetic DNA sequence containing the *ESR1* c.1138G>C p.E380Q missense mutation (Cosmic ID COSM3829320) or WT counterpart was synthesised by Integrated DNA Technologies. DNA was resuspended to 10 ng/μL in nuclease free water, stored at -20°C and diluted using nuclease free water to the desired number of copies per reaction. Patient cfDNA was isolated from 4 ml of plasma using the MagMAX™ Cell-Free DNA Isolation Kit (Thermofisher, Waltham, MA, USA) in combination with the Kingfisher Flex System automated purification system (Thermofisher, Waltham, MA, USA) according to manufacturer's instructions, providing optimal recovery of shorter cfDNA fragments (between 50 bp–200 bp) known to harbor the vast majority of ctDNA¹⁹ and minimising germline DNA contamination. Extracted cfDNA was analysed using the cfDNA ScreenTape Assay for TapeStation Systems (Agilent), confirming the recovery of short fragment cfDNA.

Patient samples and experimental protocols used in this study were approved by the Riverside Research Ethics Committee REC:12/LO/2019 and 17/WA/0161. Methods were carried out in accordance with the relevant guidelines and regulations. Informed consent was obtained from all participants.

AS-Mini-LAMP assay design: Compact 155 bp reactions were developed with the single nucleotide responsible for the E380Q mutation or WT allele encoded at the 5'-end of both FIP and BIP primers. Competitive USS primers (FB and BB) targeting the opposing variant (WT in a mutant reaction and mutant in a WT reaction) aiming to enhance AS-Mini-LAMP amplification, were designed according to the guidelines described in Malpartida-Cardenas *et al.*,⁵. In lieu of loop primers (prohibited by the compact design), poly-T linkers were placed between the F1-F1c and B1-B1c of FIP/BIP, aiding loop formation to enhance reaction velocity. Primers were analysed using the online Oligo Analyser Tool in the IDT database (<http://eu.idtdna.com/calc/analyzer>) and prepared as HPLC purified (Merck, Sigma). Primer sequences are detailed in Supplementary Table 1.

AS-Mini-LAMP

Unless otherwise stated, reagents were purchased from New England BioLabs. Reactions comprised of 1 x enzyme specific isothermal buffer, 8 mM MgSO₄, 1.4 mM dNTPs mix, 10 x primer master mix (final concentrations 1.6 μM FIP / BIP, 0.2 μM F3 / B3), 10 x USS primer master mix (final concentrations above) (Sigma, HPLC purified), 1 x EvaGreen dye (Biotium), 3 μl DNA template dilution and 0.32 U/μl Bst2.0 enzyme made up to a final reaction volume of 15μl with nuclease-free water. All reactions were performed at a constant temperature of 65 °C and monitored for up to 90 minutes on a RotorGene Q PCR platform (Qiagen). Experiments were prepared in quadruplicate and performed twice unless otherwise stated.

Droplet Digital PCR

Validation of the *ESR1* c.1138G>C p.E380Q missense mutation within 10 ng patient extracted cfDNA was performed using a Bio-Rad QX200 droplet digital PCR system as described previously²⁰. The assay was

designed using OligoArchitect and performed using an annealing temperature of 64°C. Primer sequences are detailed in Supplementary Table 1.

Data analysis and statistics

Amplification efficiency was quantified by the separation in 'time to positive' (TTP) between target template amplification and non-specific no template control (NTC) amplification exceeding the manually set signal threshold (point at which amplification exceeds and enters linear, maximum amplification rate) determined in a continuously monitored reaction. Data were presented as mean TTP $\pm$ S.E.M and are an average of 2 independent experiments each performed in quadruplicate unless otherwise stated.

Regression calculations determining reaction efficiency incorporated amplification TTP data from 1×10^6 down to 500 copies of template DNA.

Results

Development of an AS Mini-LAMP + USS assay for the selective amplification of the *ESR1* c.1138G>C E380Q missense mutation

Using the workflow outlined in Supplementary Figure 1 with 1×10^4 copies of synthetic template DNA, AS-Mini-LAMP_{Mut} amplification of mutant template DNA occurred at 41.5 ± 0.69 (TTP $\pm$ SEM), some 22.7 minutes later than for an equivalent number of WT template copies (64.2 ± 4.80). Addition of empirically selected (data not shown) USS_{WT} competitive primers (indicated by asterisks in Supplementary Table 1, showing enhanced prevention of un-specific wild type base amplification over other USS combinations) were tested at concentrations ranging from 2 to 4.5 μ M, with 3 μ M providing the longest delay of 17.6 minutes between AS mutant template amplification (23.3 ± 0.45) and non-selective WT template amplification (40.9 ± 4.38). Notably, this delay is 5.1 minute less than in the absence of USS primers (22.7 minutes). However, presence of USS has additionally served to enhance amplification velocity 1.8 fold (23.3 ± 0.45 versus 41.5 ± 0.69 in the absence of USS primers) for equivalent copies of mutant template DNA (Supplementary Figure 2A, Supplementary Table 2).

The AS-Mini-LAMP_{WT} reaction also demonstrated allelic discrimination but with a smaller delay of only 7.5 minutes between AS WT template amplification and non-selective mutant template amplification (57.0 ± 3.02 versus 49.5 ± 2.23 minutes) for an equivalent number of template copies (1×10^4). Addition of empirically selected (data not shown) USS_{Mut} primers (indicated by asterisks in Supplementary Table 1, showing enhanced prevention of un-specific mutant base amplification over other USS combinations) increased this delay to 13.9 minutes (32.0 ± 0.92 WT template TTP versus 45.9 ± 2.87 mutant template TTP) whilst also enhancing reaction velocity by 1.5 fold (32.0 ± 0.92 versus 49.5 ± 2.23 in the absence of USS primers) for equivalent copies of wild type template DNA (Supplementary Figure 2B, Supplementary Table 3).

Sensitivity of allelic discrimination of the AS-Mini-LAMP + USS reactions: The sensitivity of allelic discrimination of both AS-Mini-LAMP_{Mut} + USS_{WT} and AS-Mini-LAMP_{WT} + USS_{Mut} reactions were determined using serial dilutions (1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 5×10^2 , 1×10^2 and 1×10^1 copies / reaction) of synthetic WT and mutant templates independently. The AS-Mini-LAMP_{Mut} + USS_{WT} reaction demonstrated allelic discrimination across a range of template dilutions. AS mutant template amplification occurring 18.6, 36.0 and 34.1 minutes ahead of non-selective WT template amplification at 1×10^4 , 1×10^3 and 5×10^2 template copies respectively with a lower limit of selective mutation detection of 5×10^2 mutant copies at 25.7 ± 0.89 minutes. Representative amplification curves are presented in Figure 2A and 2B for mutant and WT template amplification respectively, summary data presented in Supplementary Table 4. Regression analysis (R squared) measure of reaction efficiency against AS mutant template amplification and non-selective WT template were comparable, 0.9954 and 0.9539 respectively. Mixed ratio populations of synthetic WT to mutant template DNA (totalling 1×10^4 copies) were prepared at 100:0, 75:25, 50:50, 25:75 and 0:100%. Non-selective amplification of 100% WT template occurred at 39.1 ± 1.63 (TTP $\pm$ SEM) whilst AS amplification of 100% mutant template and all WT: mutant template ratios tested occurred > 15 minutes faster (23.1 ± 0.39 , 23.1 ± 0.58 , 24.1 ± 0.57 and 24.7 ± 0.75 (TTP $\pm$ SEM) for 100% mutant, 75:25, 50:50 and 25:75% mutant : WT template ratios respectively) (Figure 3A and supplementary Table 5).

Conversely, the AS-Mini-LAMP_{WT} + USS_{Mut} reaction displayed reduced allelic discrimination across a range of template dilutions. AS amplification of serially diluted WT template occurring 12.4, 6.0 and 4.9 minutes ahead of non-selective mutant template amplification at template copies of 1×10^4 , 1×10^3 and 5×10^2 . The lower limit of selective WT detection when applying a reaction time cut off of 40 minutes was 1×10^4 WT copies (or 33 ng) (TTP 32.5 ± 0.80 and 44.9 ± 2.79 respectively), maintaining an allele selective delay of >10 minutes. Representative amplification curves are presented in Supplementary Figure 3A and 3B for WT and mutant template amplification respectively, summary data presented in Supplementary Table 6. Regression analysis (R squared) measure of reaction efficiency against AS WT template amplification and non-selective mutant template were lower but comparable (0.7078 and 0.7043 respectively) reflecting the lower efficiency of this reaction.

Rapid Detection of ctDNA using AS-Mini-LAMP

AS-Mini-LAMP_{Mut} + USS_{WT} selectivity and sensitivity was successfully validated using *ESR1* E380Q positive and negative patient cfDNA samples, ddPCR confirmed to contain variant allele frequencies of 20.7% and 0.0% respectively (Supplementary Figure 4A). Sample profiling using cfDNA ScreenTape identified average size DNA at 214 bp and 236 bp for the E380Q positive and negative sample respectively (Supplementary Figure 4B), composed of 97.5% and 96.1% cfDNA relative to the total DNA sample. AS-Mini-LAMP_{Mut} + USS_{WT} successfully detected E380Q within the positive patient cfDNA in 23.6 ± 0.47 (TTP $\pm$ SEM), > 20 minutes ahead of non-selective amplification of the E380Q negative patient cfDNA at 47.0 ± 5.67 (TTP $\pm$ SEM) and NTC amplification (> 60 minutes) (Figure 3B).

Discussion

Here we demonstrate a compact AS-Mini-LAMP + USS mutant specific reaction that consists of 6-primers (FIP, BIP, F3, B3, USS FB, USS BB) spanning 155 bp, within the typical range of plasma cfDNA. In contrast to alternative mutation-selective LAMP design strategies (PNA-LNA-LAMP¹¹ and AS-LAMP/CF-LAMP^{13,14}) selectivity is encoded at the terminal 5' position of both the FIP and BIP primers, promoting selective self-primed exponential amplification upon self-hybridisation and loop formation from the FIP and BIP initiated reactions. Using synthetic template models, the mutant selective reaction (AS-Mini-LAMP_{Mut} + USS_{WT}) demonstrated analytical sensitivity of 500 copies of mutant DNA within 25 minutes, > 20 minutes ahead of non-selective WT template. However, allelic discrimination was less evident within the WT reaction (AS-Mini-LAMP_{WT} + USS_{Mut}) thereby preventing its intended use as reverse confirmation of the AS-Mini-LAMP_{Mut} + USS_{WT} reaction. With only a single base pair difference at their 5' terminal FIP and BIP primers, our data reinforce the intricacies of LAMP and requirement for design and testing of multiple primer sets. Arjuna *et al.*,¹³ similarly observed that only one of six primer sets could accurately distinguish between wild type and mutated DNA for detection of the EGFR T790M point mutation.

Addition of USS primers failed to extend (via competitive inhibition) the time delay between selective and non-selective allele amplification. However, they acted to significantly enhance AS-Mini-LAMP reaction velocity by 1.8 and 1.5 fold (WT and mutant selective reactions respectively). Their role, akin to 'swarm primers', appears to be an assistance of strand displacement (hence reaction efficiency) by their ability (at relatively high concentrations) to target regions on growing amplicons that do not exist as single strand for any appreciable amount of time²¹. Swarm primers target a sequence region upstream of, and on the opposing strand of the FIP/BIP primer recognition sequences, substantially overlapping F1/B1 sites (see Figure 1 and Supplementary Figure 1). Identical in design to USS primers, swarm primers are complementary to their target template sequence whereas USS primers target the opposing allele (for competitive inhibition of its amplification). Along with this USS 'swarm effect', the efficiency of shorter LAMP reactions may in fact be higher than reactions performed on longer templates, whose amplification is rate limited by strand displacement polymerase activity⁴.

Importantly, analytical sensitivity of the AS-Mini-LAMP assays was successfully validated using 10 ng of E380Q positive (VAF 20.7%; 2.07 ng ctDNA) and negative patient cfDNA samples. Selective amplification of E380Q in the positive cfDNA patient sample was detected in under 25 minutes and > 20 minutes ahead of non-selective WT amplification, a two-fold improved sensitivity within the same time frame over our previous report (1,000 copies)¹⁶. Whilst the level of sensitivity is currently too low for the low number of mutant molecules (less than 10 copies) as might be required in a liquid biopsy setting, our data clearly demonstrate the ability of AS-Mini-LAMP + USS to selectively amplify actionable mutations present within ctDNA, as we have shown previously for tumour tissue DNA¹⁵. Continued development of a panel of AS-Mini-LAMP assays targeting actionable mutations therefore has the potential to detect ctDNA within liquid biopsy with the potential for LoC approaches for rapid and cost effective point of care testing in the post pandemic era.

Declarations

Competing interests:

The authors declare no competing interests.

Acknowledgements:

This work was supported by the Cancer Research UK - Multidisciplinary Award [C54044/A25292] and the Science Committee Programme Award [C14315]. We thank the Imperial Cancer Research UK Centre, Experimental Cancer Medicine Centre (ECMC) and the Imperial College Tissue Bank for supporting patient recruitment and sample collection.

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Figures

Figure 1.

Figure 1

Schematic comparison of AS-Mini-LAMP + USS to classic LAMP: **A)** Classic 6-primer LAMP design (FIP, BIP, F3, B3, loop F and loop B primers) targeting 8 regions of template, spanning approximately 200 bp. **B)** Compact AS-Mini-LAMP + USS design comprising 4 main LAMP primers (FIP, BIP, F3 and B3) with single nucleotide variant (SNV) selectivity encoded at the 5'-position of both the FIP & BIP primers. Poly-T linker incorporation between the F2-F1C and B2-B1C of FIP / BIP aids loop formation, enhancing reaction velocity in lieu of the loop primers. Further addition of USS_{WT} primers aim to further enhance amplification of the desired mutant allele by competitively binding to any WT sequences.

Figure 2. AS-Mini-LAMP_{Mut} + USS_{WT} *ESR1* E380Q selectivity and limit of detection. (A) Reaction selective mutant template DNA and **(B)** non-selective WT template DNA was serially diluted to include 1x10⁶ (○), 1x10⁵ (△), 1x10⁴ (□), 1x10³ (▽) and 5 x10² (◇). NTC shown in grey (*). Time to positive (TTP) is the time (minutes) at which amplification exceeds the manually set, reaction consistent threshold (red dotted line) and enters the rapid linear, exponential phase. Linear regression analysis demonstrates the reactions efficiency against both mutant and WT templates. Data represents the average of two experiments each performed in quadruplicate.

Figure 2

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Figure 3. AS-Mini-LAMP_{Mut} + USS_{WT} *ESR1* E380Q selective amplification within a mixed WT and mutant template population. (A) Representative amplification curves from mixed ratio WT to mutant synthetic DNA template. WT to mutant ratios (totalling 1×10^4 copies per reaction, equivalent to 33 ng) were prepared at 100:0 (○), 75:25 (▽), 50:50 (◇), 25:75 (-), 0:100 (+), plus NTC (*). Two individual experiments were performed in quadruplicate. **(B)** Representative AS-Mini-LAMP_{Mut} + USS_{WT} amplification of 10 ng E380Q positive (+) and negative (○) patient plasma extracted cfDNA, plus NTC (*). Two individual reactions were performed in triplicate. Time to positive (TTP) is the time (minutes) at which amplification exceeds the manually set, reaction consistent threshold (red dotted line) and enters the rapid linear, exponential phase.

Figure 3

Supplementary Files

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