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Design, optimisation and preliminary validation of a human specific loop-mediated amplification assay for the rapid detection of human DNA at forensic crime scenes.

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Abstract

The identification of samples at a crime scene which require forensic DNA typing has been the focus of recent research interest. We propose a simple, but sensitive analysis system which can be deployed at a crime scene to identify crime scene stains as human or non-human. The proposed system uses the isothermal amplification of DNA in a rapid assay format, which returns results in as little as 30 minutes from sampling. The assay system runs on the Genie II device, a proven in-field detection system which could be deployed at a crime scene. The results presented here demonstrate that the system was sufficiently specific and sensitive and was able to detect the presence of human blood, semen and saliva on mock forensic samples.

1. Introduction

There has been increasing interest in the development of tools and technologies which can potentially detect or identify crime scene stains at or near a crime scene, and be used as a triage to establish which samples should be sent for further, in depth analysis, in a forensic laboratory. This research activity has been driven by the decreasing budgets available for the analysis of samples, alongside a requirement for a quicker response times. This is especially true in the area of DNA analysis, which has become the 'gold standard' evidence type for the criminal justice system.

Current technologies include non-destructive methods which use ultraviolet light, for example the Polight, which can detect stains under paint [1] and methods which study the chemical composition of a stain, for example SEM-EDX, which has been shown to be able to identify blood, semen, saliva, urine and sweat [2, 3]. Some promising technologies are currently little used, for example a portable x-ray fluorescence device capable of detecting the iron present in blood stains with great sensitivity [4], and a technology which uses infrared light to detect latent blood traces [5].

Alternative technologies use destructive methodologies, but despite this have perhaps greater utility. Bienvenue *et al*., [6] describe a microfluidic device which integrates the DNA extraction from complex samples with limited STR fragment analysis, and Dawney *et al*., [7] describe the ParaDNA sampling and analysis system which also performs limited STR analysis, and can be used at a crime scene. Although these two technologies are promising, the information garnered from limited STR fragment analysis is restricted, and it is unclear how the interface for searching the DNA database for the generated STR profiles will be enacted. It is difficult to envisage how the stringent security requirements of the DNA database are compatible with STR fragment analysis at the crime scene.

An alternative approach to STR-based methods is to use a loop-mediated amplification (LAMP) assay (http://loopamp.eiken.co.jp/e/lamp/), for the detection of

human stains at a crime scene, which we describe here. LAMP, first described by Notomi *et al* [8], is a novel technique for the amplification of target DNA at a single temperature, thus requiring less equipment then conventional PCR based technologies. This technology has been shown to be highly sensitive and has been used to detect pathogens [9, 10, 11, 12]. Recently it has been adapted to run on the Genie II device, a fully portable in field detection system which was proved highly effective in identifying *chalara* infected plants in a recent ash dieback outbreak in the UK in 2012. Moreover, the reagent running costs are low, in the order of £1.50 per reaction, and the Genie II device itself is equivalent in cost to a PCR machine.

Here we describe the development, optimisation and preliminary validation of a human-specific LAMP assay on the Genie II device which can be deployed at a crime scene. The experiments reported here demonstrate that the assay has the sensitivity and specificity to rapidly identify human samples at a crime scene, with confirmation that a sample is human in under 30 minutes from sampling. The assay system is straightforward, can be performed after a minimum amount of training and is very low cost. Its prime utility will be as a 'triage' system, capable of discriminating between samples of a human or non-human origin, and aiding crime scene investigators to decide whether to send the samples for further analysis or not, thus reducing the potential for backlogs at forensic laboratories.

2. Materials and Methods

2.1 Ethics

This project was assessed and approved by the Teesside University Research ethics committee

2.2 Source materials and preparation of samples.

Unless otherwise stated all chemicals and reagents were purchased from Sigma. All reagents and plastic ware was UV irradiated 3600mJ/cm^2 in a Spectrolinker XL-1500 cross linker prior to use. Whole blood was obtained from finger-pricks of consenting volunteers. Blood was used immediately either for direct detection (1μl and 10μl pipetted into microcentrifuge tubes), or was laid as fingerprints on either plastic petri dishes or freshly laundered, UV irradiated, white cotton. Other body fluids (semen and saliva) were also obtained from consenting volunteers. Semen was frozen prior to use whereas saliva was used immediately. Both semen and saliva were used for direct detection as above, for blood, or were seeded onto plastic petri dishes or freshly laundered, UV irradiated white cotton in 0.1ml aliquots. Both body fluids soaked the white cotton, but remained as a single drop on the plastic and were therefore spread into an approximate 1 x 2 cm area using a pipette tip. Samples of animal tissues were obtained from local suppliers. Shed pet fur, with retained follicles, were used for DNA extractions when tissue was not available. For a positive control, an MRC-5 cell line was obtained from the ECACC, and was maintained in continuous culture to passage 30, in RPMI 20% (v/v) FBS, 100 units/ml penicillin,

100 µg/ml streptomycin and supplemented with 2mM L-glutamine. It was sub-cultured with trypsin EDTA when 80% confluent. Pellets of 10^6 cells were stored frozen until use.

2.3 Primer design

The Human LAMP primer set was designed using the human mitochondrial cytochrome B gene sequence, retrieved from GenBank (AF254896), with the aid of Primer3 [13,14] using the parameters stipulated on the Eiken website (http://loopamp.eiken.co.jp/e/lamp/) (Table1). In particular the 3' end of primers were placed on points of mismatch between the human gene sequence and comparable sequence data retrieved from GenBank for animals which are common in the UK and which could be present at a crime scene: cat (AB004238), cow (D34635), dog (JF480119), mouse (Z96069), pig (AB015081), rat (AF295545), rabbit (HQ596486) and sheep (D84205). Additionally primers which would have formed hairpin loops, have a secondary structure or formed primer-dimers were redesigned to eliminate these features.

The primers were then combined in specific combinations to mediate the LAMP amplification. The F3, F-loop and B3, B-loop primers were used in complementary and reverse complementary orientation respectively. The FiP primer was composed of the reverse complement F1 primer combined with the complement F2 primer whereas the BiP primer was composed of the B1 primer combined with the reverse complement B2 primer (Table 2). Primers were purchased from Sigma Genosys, with HPLC purification for the FiP and BiP primers only, to ensure that these primers were full length.

2.4 Lyse and LAMP DNA template preparation

DNA template was prepared from all samples by incubation in 0.1ml 0.3M KOH at 95^{o}C for 5 min with mixing at 1000rpm using an Eppendorf Comfort Thermomixer.

2.5 LAMP assay conditions

The LAMP reactions were performed in a 25µl reaction volume, which contained 15µl of the LNL reaction buffer (Optigene, Cat no 004LNL) and a final concentration of 2µM for each of F1P and B1P, 0.3µM for each of F-loop and B-loop and 0.1µM for each of F3 and B3, and 5µl of either 0.3M KOH extracted sample, 0.3M KOH as negative control or 0.3M KOH extracts containing 1.25 x 10^4 MRC-5 cells as positive control.

LAMP amplification reactions were run in a Genie II (Optisense) at 65^{o}C for 20mins for the amplification phase and were then ramped between 91 and 81^{o}C for the annealing phase. The analysis took 30 minutes to run with results displayed in real-time. Samples were deemed positive if amplification had occurred and the annealing temperature of the amplicons conformed to 87^{o}C ± 0.5^{o}C, indicating that the amplicons were true amplification products of the human LAMP assay primers.

2.6 Optimisation of the LAMP assay conditions

An evaluation of the effects of different amplification temperatures (63-67^{o}C), the presence of the loop primers, of changing the ratio of F1P/B1P to F3/B3 and decreasing the overall concentration of the primers in the reaction was performed using 0.3M KOH extracts containing 1.25 x 10^4 MRC-5 cells.

3. Results and discussion

3.1 LAMP assay design

The design of LAMP primers is not a trivial task. There are 8 primers to be design which ultimately result in 6 primers which mediate the LAMP reaction. There are specific requirements for these primers, furthermore a species specific assay, such as this one, must include additional design parameters to confer specificity.

There are 3 design packages available which purport to design specific LAMP assays: LAMP Designer 1.10 from Premier Biosoft, Primer Explorer V4 from Eiken (http://loopamp.eiken.co.jp/e/lamp/), and LAVA, a freeware program available on the web [15]. Each of these software packages was assessed for the design of a LAMP assay specific for human DNA using the mitochondrial cytochrome b gene sequence retrieved from GenBank. It should be noted that there are no long stretches of DNA sequence unique to human, when compared to other mammals, therefore point differences or single nucleotide polymorphisms (SNP) were targeted, which is usual when designing primers for amplification type reactions which are mediated by oligonucleotide primers.

When LAMP Designer 1.10 was assessed it was found that it could not be directed to position the primers at specific locations. Subsequently an assay designed using this software was found to amplify DNA from species other than and including human DNA, although not all species DNA amplified. For example it amplified DNA from horse, pig, pigeon and rabbit, but did not amplify sheep, turkey or chicken DNA. The positions of the primers for this assay were transferred to a CLUSTAL 1.2.0 sequence alignment containing 8 mammals which could be encountered at a crime scene (mouse, rat, rabbit, dog, cat, pig, sheep and cow) and the positions of the primers did not correspond to SNPs, either at the 3' end of the primers or at any consistent position within the primers. The program automatically checks the amplicon for specificity via a BLAST search, however depending on how this is performed, the search will report 'no match' even when most of the sequence is identical. The data for this assay is not shown and the assay itself was not taken studied any further.

The remaining two software packages were found to be unuseable for this application. Primer Explorer V4 (http://loopamp.eiken.co.jp/e/lamp/) had the facility to design specific assays using a sequence alignment uploaded onto the website. However it was found that it simply amalgamated the sequences into one sequence

and designed a set of LAMP primers which were composite between the sequences. The freeware 'LAVA' programme [15] could not be made to run and appeared to be corrupted.

The only remaining alternative for the design of human specific LAMP assay was to design the primers manually. Therefore, the CLUSTAL 1.2.0 alignment was scrutinised for SNPs across all species in comparison with the human sequence, which were then used to anchor the 3' end of each primer. The only exception to this were the B-loop primers (3' match to mouse, rat and cat) and B2 primer (3' match to dog), however, those positions are thought to be of less importance for specificity [16]. The primers were then passed through Primer3 to calculate melting temperature and to ensure that the primers in the final set did not contain secondary structure, would not form hairpin loops or primer dimers.

3.2 Assay optimisation

The optimisation of LAMP assays is straight forward. The reaction buffer is proprietary and contains all the components needed for the reaction apart from the primers and the addition of the template DNA in KOH. Therefore the optimisation of the assay focused on the amplification temperature and the ratio and amount of the primers. There was no effect on the amplification when the temperature was 63, 64, 65, 66 or 67^{o}C although there was some evidence of non-specific amplification at 62^{o}C.

The ratio of the FiP/BiP primers to either the F3/B3 or F-loop/B-loop primers was assessed for amplification success and it became apparent that a half concentration reduction in the F3/B3 primer concentration and a half concentration increase in the F-loop/B-loop primers resulted in enhanced amplification. The presence of the loop primers was found to be essential for efficient amplification although literature suggests that they are not an essential requirement for LAMP assays [17], however, when they were added to the reaction they halved the time for a positive signal: from in excess of 25 mins, to under 12 mins (Figure 1) therefore the loop primers were included in the mastermix as a mandatory addition for the remainder of the study.

3.3 Human LAMP assay sensitivity

The sensitivity of the assay was evaluated using a 10 fold dilution series of the MRC-5 cell line. This afforded the opportunity to accurately determine the sensitivity of the assay in terms of actual cell number since the cell line is easy to enumerate using standard tissue culture techniques. Other studies have compared the sensitivity of the newly reported assay system with quantification of the input template DNA or have STR profiled the DNA. Unfortunately this was not possible in this instance because the KOH extraction method was not compatible with either Quantifiler or SGMPlus, however, a previous unpublished study, determined the DNA content of these cells and this was used as an approximation for the input template DNA. It should be noted that this assay is based on a mitochondrial sequence and therefore

the actual copy number for this target is unknown, since mitochondrial DNA can vary between 50 and 10,000 fold when compared to genomic DNA, although the most widely quoted number appears to be approximately 100 fold greater. Nevertheless, correlating the number of cells with an approximation of the DNA quantity, the assay was able to reliably detect approximately 12 MRC-5 cells (Figure 2). Cell concentrations below this gave spurious amplification curves, as seen in Figure 2 for 1.25 cells, which was found to be not reliably detected. Detection of approximately 12 cells is comparable with other systems reported in the literature, particularly the ParaDNA system [7].

3.4 Human LAMP assay specificity

The specificity of the assay was evaluated against all other commonly available animal species likely to be encountered at a crime scene. There was no amplification observed for any of the animal species tested (Table 3).

Although a wide range of animal species were tested, the range of possible animal species at a crime scene is virtually limitless. Therefore, an *in silico* exercise was conducted to determine if any less common animal species might support amplification of this human assay. DNA sequence data for a wide range of species (badger, AB049809, chimpanzee, EF660835, chinchilla, AF283978, deer, AY118199, degu, AM407929, ferret, EF987743, fox, AB292765, golden hamster, AJ973379, guinea pig, AY228363, hare, AJ130825 and hedgehog, AF05141) was retrieved from Genbank and aligned in CLUSTAL. The primer positions were then transcribed to the alignment and an assessment made of the likely cross amplification. Although an *in silico* assessment is no substitute for empirical evidence of specificity, the data in Table 3 can be used to make an informed assessment that the assay would not amplify DNA from deer, degu or ferret which did not share any homology at the 3' end of any of the primers, would not amplify badger, chinchilla, fox, guinea pig, golden hamster, hare or hedgehog, based on the non-amplification of mouse, rat, cat and dog, which show similar 3' matches to the primers. There was however, significant homology observed for the chimpanzee sequence and it is quite likely that chimpanzee and primate DNA would be amplified by this assay. However, since the prevalence of primates is very limited, it was concluded that to all intense and purposes, the assay was specific for human, although it should be used at a zoo or animal park with caution.

3.5 Inhibitor tolerance

DNA samples recovered from exhibits and crime scenes often contain PCR inhibitors co-purified during DNA extraction [18]. There has been a great deal of effort applied to the development of DNA clean-up techniques and STR profiling kits which are more resistant to the effects of inhibitors [19, 20]. However, this assay uses a direct sampling and extraction technique with no DNA cleanup step, similar to other studies [7, 21] and therefore the effect of inhibitors on the amplification of the human LAMP

assay was assessed by the addition of common PCR inhibitors spiked directly into the reaction mixture (Table 4).

The Human LAMP assay was found to be relatively resistant to the presence of humic acid, since no effect on amplification or anneal activity was observed at any of the concentrations tested. This is in contrast to PCR based assays, which appear to be less resistant to humic acid, in one study the addition of 10ng caused a 90% reduction in the DNA detection score for the ParaDNA DNA screening test [7] but affected the new 17 plus kits to a lesser extent [19, 20].

In contrast both hematin and indigo caused significant inhibition of the LAMP assay, although interestingly, they inhibited the anneal phase of the assay to a greater extent than the amplification phase. This phenomenon could have been due to the colour of the solutions themselves, rather than a chemical inhibition of the reaction. The stock hematin and indigo solutions were brown and blue respectively and even after dilution and spiking into the reaction mixtures, significant colour remained. The observed inhibition was most probably due to the colour of the reaction mixture interfering with the optics of the detection system rather than the inhibition of the LAMP reaction. The tolerance of the LAMP reaction to these inhibitors was therefore reduced in comparison with other systems [7, 20] however the level of inhibitors used in this and other studies is far greater than would be encountered during the recovery of exhibits and therefore the Human LAMP assay was deemed fit for purpose in relation to inhibitor tolerance.

3.6 Direct sampling and mock case work samples

The recovery of DNA from evidence and at a crime scene usually relies on swabbing the area in question, with a wet and dry swab being the currently favoured method [7, 22]. Additionally, there are a wide range of swab types available which are used for different applications [23, 24, 25, 26]. The use of swabs for recovery of evidence was evaluated for compatibility with the LNL extraction buffer, which is highly caustic. The head of a variety of swab types were added to cell pellets in microcentrifuge tubes, prior to the addition of 0.2ml 0.3M KOH and incubation at 95^{o}C for 5 mins with shaking. The resultant extract was then used as template for the Human LAMP assay. It was found that the wooden handled Copan swab and flocked swab extracts completely inhibited the Human LAMP assay amplification and in both these cases there was neither amplification nor anneal activity. The IsoHelix and OmniSwabs had less of an effect on the assay, but nevertheless they did cause some inhibition, as evidence by an increase in the time before amplification was observed (data not shown).

Alternative sampling devices were therefore assessed for efficacy at recovering evidence and compatibility with the extraction method. These included a pipette tip, a disposable micropestle, the ParaDNA sampling device, a blue microbiological loop (blue loop), a clear microbiological loop (clear loop) and a purple microbiological

needle (purple needle). The sampling devices were assessed using either dried blood fingerprints or dried semen on plastic petri dishes, chosen as exemplars of samples which could be found at a crime scene, although not particularly challenging because it is a non-porous substrate.

After sampling, the devices were incubated in 0.1ml 0.3M KOH at room temperature for 5 mins to recover the cellular material. The resultant solutions were then processed exactly as before. These extracts were then used as template for the Human LAMP assay, with varying success (Table 5). Preliminary data for the extracts from the purple needle, the clear loop, the pipette tip and micro-pestle indicated that they were not efficient at recovering cellular material from plastic, when compared to either the blue loop or the ParaDNA sampling device and were therefore not investigated any further.

The blue loop and the ParaDNA sampling device were then further assessed on samples which included blood and semen seeded onto cloth and saliva seeded onto plastic and cloth, as exemplars of human crime scene samples on both porous and non-porous substrates. The ParaDNA sampling device produced the strongest amplification, across all sample types, in comparison to the blue loop. It should be noted that comparable results were obtained from sampling from plastic for both of these sampling devices, however the blue loop was not efficient at recovering samples from cloth. This may have been due to the construction of these sampling devices. The blue loop was a relatively flexible structure and it was difficult to apply any pressure to the surface whilst sampling, whereas the ParaDNA device has 4 extremely hard prongs upon which it collects the sample by adsorption [7]. One drawback of the ParaDNA sampling device was that the 4 prongs of the sampling device could not be separated and recovery of the sample necessitated holding the sampling device vertically in a 0.1ml pool of 0.3M KOH in a petri dish. After 5 mins the ParaDNA device was removed, and the KOH transferred to a microcentrifuge for processing. This technique is obviously not compatible with a crime scene detection system and a better sampling device, which incorporates a type of prong similar to the ParaDNA extraction device, needs to be identified or developed for this application to move forward.

4. Summary

We report here the design, optimisation and preliminary validation of a rapid and portable analysis system for the identification of human samples at a crime scene. The technology is well established in the arena of portable in-fieled diagnostics but this is the first time that this technology has been applied to the identification of human body fluids.

The Human LAMP analysis system, comprised a rapid DNA sampling strategy, rapid DNA extract preparation and a Human LAMP assay, is simple to perform, requires the minimum of equipment and returns results within 30 mins of sampling.

The data presented in this report demonstrates that the Human LAMP analysis system is sensitive and specific and moreover was able to identify human blood semen and saliva, recovered from mock case samples.

This human LAMP DNA analysis system is designed to facilitate the triage of samples to be sent for full STR analysis and as such would help to identify samples which would not be suitable for further analysis ie were of non-human origin.

The provision of the primer sequences and the full experimental detail is designed to facilitate the uptake of the Human LAMP analysis system by the forensic science community. The assay is designed for use at a crime scene and the costs per sample are below £1.50 for reagents. The Human LAMP DNA analysis system needs the development of a sampling device compatible with the extraction buffer, the system will then be ready for full validation and implementation at a crime scene.

Conflict of interest

None

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Acknowledgements

The authors would like to thank Fera for supporting this project and Optigene for helpful advice. They are also grateful to all the volunteers who donated their samples for this project, to Jenny Tomlinson who provided invaluable advice and to James Chisholm for technical assistance with enumerating the DNA concentration of the MRC-5 cell line and Gaynor Johnson for tissue culture of the MRC-5 cell line.

Highlights

- Rapid, sensitive, robust system for the identification of human DNA bearing samples at crime scenes
- Use of proven in-field detection system using LAMP technology coupled to the Genie II
- Results returned within 30 minutes of sampling using low cost reagents ~£1.50 consumable cost per sample
- Provision of all experimental detail to encourage further development of sampling device and future validation.

Table

Design, optimisation and preliminary validation of a human specific loop-mediated

Table 1 Design specifications for LAMP assay primers

Attribute*	Effect on primer design*
Melting temperature	Primer melting temperature F1 and B1 64-66^{o}C; F2, B2, F3 and B3 59-61^{o}C; F-loop and B-loop 64-66^{o}C
G/C content	40-60%
Distance between primers	Distance between: end F2 and end of B2 120-160 bp 5' end F2 to 5' end F1 40-60 bp, F2 and F3 0-60 bp
2^{o} structure	No secondary structure in primers acceptable

*Adapted from Eiken Chemical Company Ltd (no date)

Table 2. Human LAMP primer set

Primer name	Sequence 5' – 3'
F1P (F1c+F2)	cggagttggcggaaaagtagttCTGCCTGATCCTCCAAATCAC
B1P (B1c+B2)	gcccacatcactcgagacgtaaattCAGATAAAGAATATTGAGGCGCCA
F3	TGAAACTTCGGCTCACTCCTT
B3	TCGCCCGATGTGTAGGAAG
F-loop	GAGTAGTGCATGGCTAGGAATAGT
B-loop	ATCCGCTACCTTCACGCC

Table 3. Amplification success of the Human Lamp assay on DNA extracts of possible species encountered at crime scenes. + denotes successful amplification or anneal activity, the number of + denotes the strength of amplification, - denotes no observed amplification or anneal activity

Species	Amplification success
Human	+++
Cat	-
Chicken	-
Cow	-
Dog	-
Duck	-
Horse	-
Partridge	-
Pheasant	-
Pig	-
Pigeon	-
Rabbit	-
Sheep	-
Turkey	-

Table 4. Effect of common PCR inhibitors on the amplification success and annealing activity of the Human LAMP assay. + denotes successful amplification or anneal activity, the number of + denotes the strength of amplification, - denotes no observed amplification or anneal activity

Inhibitor	Concentration	Amplification success	Anneal activity
Indigo	0.5mM	+	-
	0.25mM	+	-
	0.125mM	+	+
Haematin	40μM	-	-
	20μM	+	-
	10μM	+	-
Humic acid	20ng/μl	+	+
	10ng/μl	+	+
	5ng/μl	+	+

Table 5. Direct sampling success of various sampling devices. + denotes successful amplification or anneal activity, the number of + denotes the strength of amplification, - denotes no observed amplification or anneal activity

Sampling device	Blood on plastic	Blood on cloth	Semen on plastic	Semen on cloth	Saliva on plastic	Saliva on cloth
ParaDNA sampling device	+++	+++	+++	+++	+++	+++
Blue loop	+++	+/-	+++	+++	+++	-
Purple needle	-	ND	+	ND	ND	ND
Clear loop	+	ND	+	ND	ND	ND
Pipette tip	-	ND	+	ND	ND	ND
Micropestle	-	ND	+	ND	ND	ND

Figure

Design, optimisation and preliminary validation of a human specific loop-mediated
Figure 1. Effect of the presence/ absence of loop primers using KOH extracts of the MRC-5 cell line as template DNA.

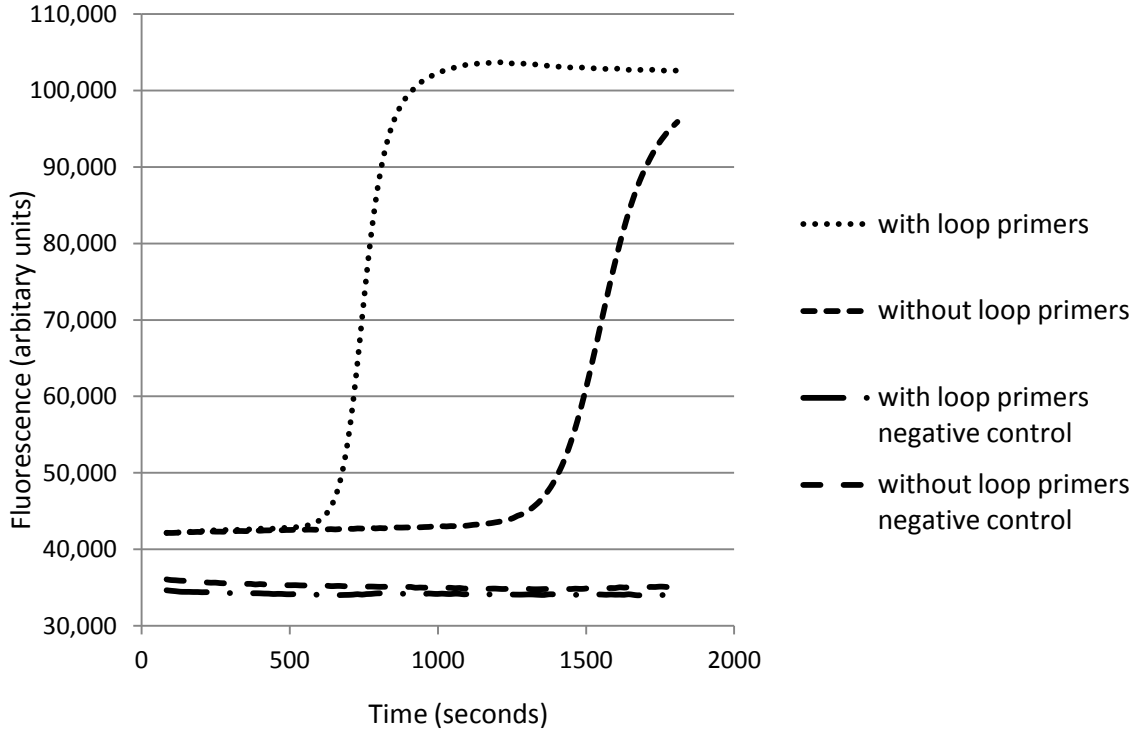

Figure 2. Amplification of MRC-5 cell DNA by the human LAMP assay.