




The detection and identification of saliva in forensic samples by RT-LAMP

Li-Chin Tsai¹ · Chih-Wen Su² · James Chun-I Lee³ · Yu-Sheng Lu¹ · Hsuan-Chen Chen¹ · Yu-Chih Lin⁴ · Adrian Linacre⁵ · Hsing-Mei Hsieh¹ 

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Abstract

We report on a novel method for saliva identification by reverse transcription-loop-mediated isothermal amplification (RT-LAMP). In our previous report, real-time RT-LAMP was used for blood identification by using HBB detection as a model but in this advanced study, this method was refined for the identification of the more challenging body fluid of saliva. Expression of the 18S rRNA gene was used as the internal control and the Statherin (STATH) gene as the saliva-specific marker. A turbidimeter was used for real-time detection of the RT-LAMP products, and confirmation was obtained that the real products were generated using: agarose gel electrophoresis, calcein fluorescence detection and/or enzymatic digestion. The specificity of the test was performed using 42 samples including 7 different body fluids, and the expression of STATH was only observed in all the saliva samples (6) with a threshold time of 39.4 ± 2.9 min. Sensitivity testing showed that RT-LAMP products for STATH were stably detected when the RNA template was not less than 6.25 ng. When the primer concentrations for STATH were two times that of 18S rRNA, saliva could be identified in the body fluid mixtures even at a ratio (saliva:semen) of 1:3 (without loop primer)/1:5 (with loop primer). A multiplex RT-LAMP was established to simultaneously amplify the 18S rRNA and STATH genes, and applied to the identification of saliva on ten non-probative cigarette butts. A positive result for saliva was obtained from all ten butts, even for those that returned a negative or ambiguous result using the amylase test. A direct RT-LAMP test is also reported where the RNA extraction step was omitted to speed the collection of data and all tests using either the simplex or multiplex RT-LAMP resulted in a positive response if saliva was present. Our data provide a simple and effective means to detect the presence of saliva.

Keywords Saliva identification · RT-LAMP · Statherin (STATH) · Multiplex RT-LAMP · Non-probative evidence samples

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✉ Hsing-Mei Hsieh
mei@mail.cpu.edu.tw

¹ Department of Forensic Science, Central Police University, 56 Shu-Jen Road, Kwei-San, Taoyuan 33304, Taiwan, Republic of China

² Forensic Biology Division, Criminal Investigation Bureau, National Police Administration, No.5 Lane 553, Sec. 4, Zhongxiao E. RD., Xinyi District, Taipei 11072, Taiwan, Republic of China

³ Department of Forensic Medicine, College of Medicine, National Taiwan University, No. 1 Jen-Ai Road Section 1, Taipei 10051, Taiwan, Republic of China

⁴ Taichung City Government Police Department, 588 Wenxin Road Section 2, Xitun District, Taichung 40758, Taiwan, Republic of China

⁵ College of Science & Engineering, Flinders University, Adelaide 5001, Australia

Introduction

Saliva is frequently encountered in forensic investigations on items such as cigarette butts, the edges of cups/bottles or on bite marks. Saliva can also be transferred during an alleged sexual assault. DNA profiling of samples from such samples cannot discriminate between cell types and therefore the accurate tissue assignment is necessary.

Amylase is usually considered as a suitable target for identification of saliva and is currently used by forensic analysts as a presumptive test [1–3]. Salivary amylase is abundant in human saliva but there is also a small amount in breast milk and sweat resulting in potential false positive reactions when applying a presumptive test for saliva [4]. Furthermore, when amylase is detected it is difficult to determine if there is a weak or a strong response as these are subjective judgements.

Previous reports for saliva detection have focused on the bacteria in the oral cavity. Nakanishi et al. reported a novel

PCR-based method for saliva identification by detecting oral Streptococci/Streptococcus [5]. Choi et al. provided a method to identify saliva using integrated analysis of DNA methylation and saliva-specific microbial DNA [6].

Recently, mRNA and microRNA markers have been identified in a number of studies for the forensically relevant body fluids, including saliva, based on the functionality of the cells [7–11]. Several candidate markers have been evaluated for their saliva-specific expression in these studies. For example, it was confirmed that mRNA of the STATH (Statherin) gene was present in saliva but absent in blood, semen, vaginal secretions and menstrual blood [12]. Statherin is a salivary protein encoded by the STATH gene. The protein helps to control the formation of hydroxyapatite crystals and plays an important role in maintaining the tooth enamel in the oral cavity [13, 14]. Based on several studies, STATH mRNA has been shown as a specific marker for saliva identification [15, 16].

Methods for analysis of RNA markers include: the endpoint RT-PCR (Reverse transcription-polymerase chain reaction) combined with capillary electrophoresis [4, 7]; quantitative RT-PCR (qRT-PCR) [4, 17]; a quantum dot molecular beacon test [18]; and a targeted multiplexed next generation mRNA sequencing assay [19]. In our previous study, detection of HBB mRNA was used as the model for establishment of real-time RT-LAMP for body fluid identification [20]. With the exception of this study, only four other reports [21–24] were observed on forensic applications to use LAMP- a process developed by Notomi et al. in 2000 [25]. These include using LAMP to detect *Streptococcus salivarius* for saliva identification [21], determining sex from dental pulp [22], identifying human DNA in forensic evidential samples [23], and rapidly screening for male DNA in criminal investigations [24]. This method is sensitive and has the potential for identification from body fluids that contain only few cells, such as saliva.

The initial step of RNA extraction is a practical problem for identifying body fluids at crime scenes. To overcome this obstacle, a direct RT-LAMP [26] was also tested in this study.

Materials and methods

Sample collection

Body fluids were collected from volunteers with informed consent and using the procedures approved by the Antai Medical Care Cooperation, Antai- Tian-Sheng Memorial Hospital Institutional Review Board, in Taiwan (TSMH IRB No./ Protocol No.: 14–085-B1). The body fluids used in this study included: venous blood, saliva, semen, menstrual blood, sweat, urine and vaginal secretions. All collections were performed in accordance with the ethical standards and the procedures described in our previous study [20]. The samples of

each body fluid were collected from six volunteers of between 20 and 40 years old. Ten non-probative evidence samples of cigarette butts were collected from the Criminal Investigation Bureau in Taiwan and screened by amylase test (Phadebas, Lund, Sweden).

RNA extraction and quantification

Total RNA was extracted using the RNeasy® Mini Kit (Qiagen® Ltd., CA, USA) following the manufacturer's protocol. The isolated RNA was quantified by using the Nanodrop spectrophotometer (NanoDrop® ND-1000 UV spectrophotometer, Thermo Scientific, Wilmington, USA).

Marker selection and primer design

Expression of the 18S rRNA gene was used as the internal control [20] and the STATH gene was identified as the saliva-specific marker based on previous reports [8, 12]. The primers used in RT-LAMP were designed using the software PRIMER EXPLORER V4 (<http://primerexplorer.jp/elamp4.0.0/index.html>). The recommendations for LAMP primer design were described in the previous report [20]. The primer sequences and the respective sites are shown in Table 1 and Fig. 1.

RT-LAMP reaction and real-time detection of products

Reverse transcription (RT) and LAMP reactions were conducted simultaneously using the Loopamp RNA Amplification Kit (Eiken Chemical Co. Ltd., Tochigi, Japan) following the manufacturer's suggestions. The reaction volume was 25 µL and consisted of a 12.5 µL reaction mix (40 mM pH 8.8 Tris-HCl, 20 mM KCl, 16 mM MgSO₄, 20 mM (NH₄)₂SO₄, 0.2% Tween 20, 1.6 M betaine and 2.8 mM of each dNTPs), 1.0 µL enzyme (a mixture of *Bst* DNA polymerase and AMV reverse transcriptase), and approximately 25 ng of the total RNA as the template and the appropriate primers. The working concentrations for each of the primers (F3, B3, FIP and BIP) are shown in Table 1.

A real-time turbidimeter LA500 (Eiken Chemical Co. Ltd.) was used for real-time detection of the RT-LAMP products. The reaction program was 65 °C for 60 min to amplify DNA and then 80 °C for 5 min to cease the reaction. The value of 0.1 was used as the turbidity threshold. The threshold time (T_t, min) is recorded as the time when the turbidity exceeds 0.1 following the manufacturer's suggestions. The T_t within 60 min is interpreted as the positive result.

RT-LAMP product confirmation

Confirmation that the RT-LAMP products were generated was performed using three processes: agarose gel electrophoresis,

Table 1 Primers used in this study for RT-LAMP

Gene	Primer sequence (5' → 3') ^a	Working concentration (μM)
18S rRNA	F3: ATTGACGGAAGGGCACCA	0.2
	B3: TGCCAGAGTCTCGTTCGTTA	0.2
	FIP: CAATCCTGTCCGTGTCGGGAGCCTGCGGCTTAATTTGAC	1.6
	BIP: AGCTCTTTCTCGATTCCGTGGGACAAATCGTCCACCAACT	1.6
	LF: FITC-CGGGTGAGGTTTCCCGT	0.8
STATH	F3: TCATCTTGAGTAAAAGAGAACC	0.2
	B3: GCAGTTACTGATGATATAAAAGGT	0.2
	FIP: TGAATCAGCTCCAATCATGGAAACTATGAAGTTCCTTGTCTTTGC	1.6
	BIP: GAAGAGTATGGGTATGGCCCTATTGTTGGTATTGTGGTTGG	1.6
	LB: ROX-ATCAGCCAGTTCCAGAACAAC	0.8

^a FITC and ROX indicate the fluorescent dye labelled

calcein fluorescence detection and/or enzymatic digestion. For agarose gel electrophoresis, 2 μL RT-LAMP products were separated on the 3% agarose gel in 1X TBE buffer and stained with the SYBR® Green I dye (Invitrogen™, Paisley, UK). For calcein fluorescence detection, calcein (Eiken Chemical Co., Ltd.) was added to the RT-LAMP preparation to make the final concentration of approximately 2 mM and the fluorescence was excited by 365 nm UV light after the amplification. For enzymatic digestion, the restriction enzymes, *AluI* (New England Biolabs, Ipswich, Massachusetts, USA) and *Hpy188I* (New England Biolabs), were used based on the sequences of 18S rRNA (accession no. NT_167214.1 in GenBank) and STATH (accession no. NM_001009181.1 in GenBank) genes respectively. The recognition sites are highlighted in Fig. 1. The digested conditions were as the manufacturer's suggestions.

Multiplex RT-LAMP

For RT-LAMP product detection, the multiplex amplifications of 18S rRNA and STATH were performed by using dye-labelled loop primers LF (labelled with FITC and emitting the green color) and LB (labelled with ROX and emitting the red color) respectively, in addition to the other LAMP primers (F3, B3, FIP and BIP). The composition of a multiplex amplification was performed as described previously with the exception of the inclusion of both the 18S rRNA and STATH primers in this test. The primers and their concentrations are showed in Table 1. The dye labelled products were observed by adding 2 μL (0.7 μmol) PEI (polyethyleneimine) (Alfa Aesar, Ward Hill, Massachusetts, USA) and centrifuged at 900 x g for 1 min. The fluorescence was excited by UV light at 365 nm. A positive result will be observed as an orange precipitate in the multiplex amplification.

Direct RT-LAMP

To evaluate the feasibility for omitting the RNA extraction step, neat saliva (100 μL) was mixed with 5 μL DTT (1 M). The solution was heated to 100 °C for 5 min. After this pre-treatment, 1 μL, 3 μL and 5 μL of the solutions were used in RT-LAMP following the above mentioned conditions.

Results

Gene selection and primer design

The expression of the 18S rRNA housekeeping gene was used as the internal control as previously reported [20]. Initially 5 genes (AMY1A, SPRR3, STATH, KRT6A and MUC7) with saliva-specific expressions were selected after reviewing previous reports [8, 9, 12, 27, 28] and used in the real-time RT-LAMP for saliva identification. Based on the preliminary results, the genes with the greatest saliva-specific expression, STATH, was adopted and further analyzed in this study. The primer sequences used and their respective sites are shown in Table 1 and Fig. 1.

Specificity analysis

Specificity for saliva was performed using 42 samples including 7 different body fluids (venous blood, saliva, semen, menstrual blood, sweat, urine and vaginal secretions) (Table 2). The average and standard deviation of Tt ranged from 26.8 ± 2.2 (for venous blood) to 44.7 ± 5.6 (for menstrual blood) for 18S rRNA. A positive STRATH result was obtained from all 6 saliva samples (with a Tt of 39.4 ± 2.9 min). These are the results expected if expression of the STATH gene is specific for saliva. To confirm this result three further tests were performed: agarose gel

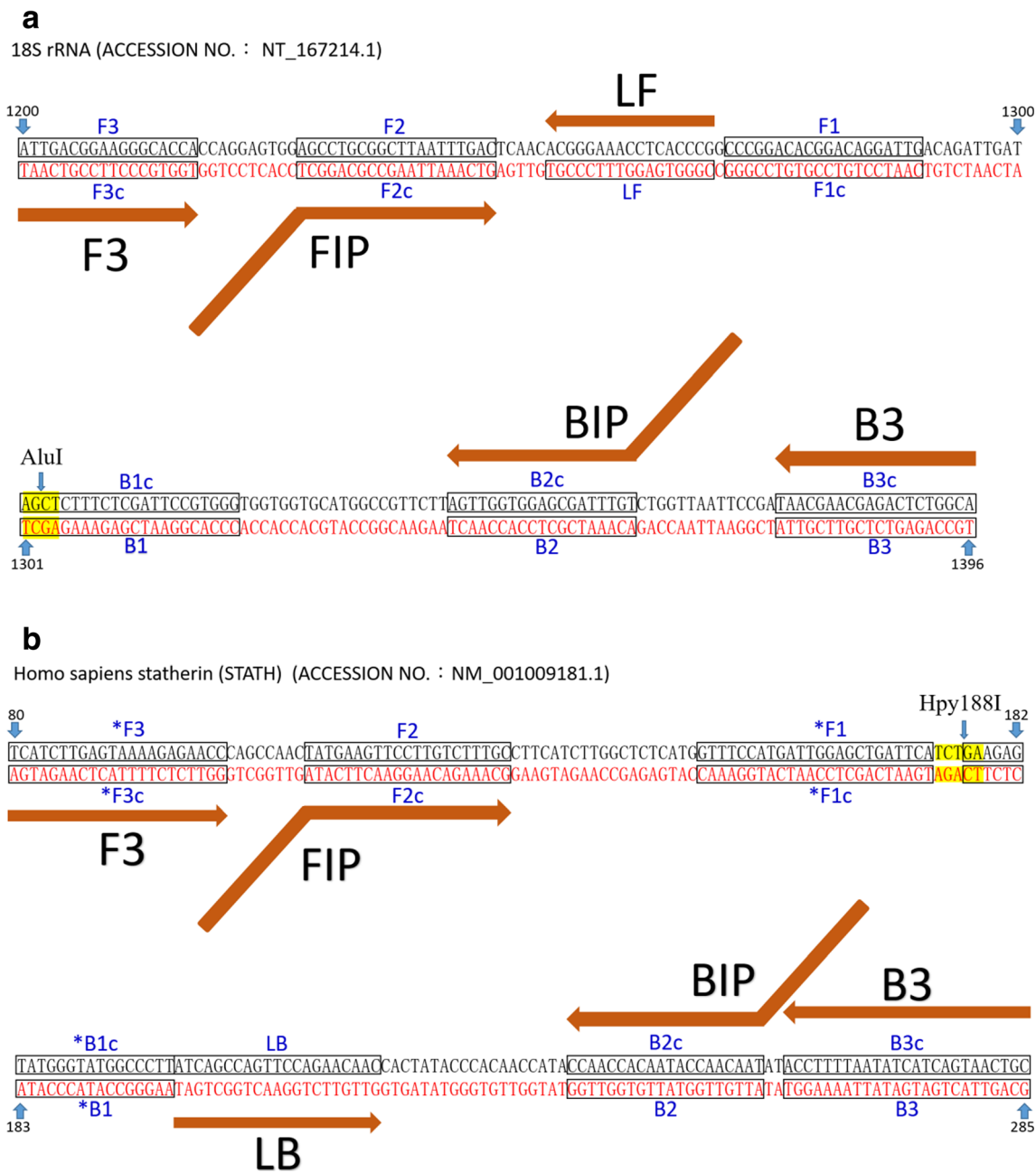


Fig. 1 Sequences and their respective sites of the RT-LAMP primers used in this study. DNA sequences of 18S rRNA (a) and STATH (b) were extracted from GenBank (accession nos. NT_167214.1 and NM_001009181.1 respectively). The symbol “*” indicates the primer spanning the exon-exon junction and used to prevent the amplification

from contaminating DNA. The restriction enzyme sites (*AluI* and *HPY1881*) are indicated as yellow marks. LF and LB are loop primers and dye labelled (FITC and ROX respectively) for fluorescent detection of RT-LAMP products

electrophoresis, calcein fluorescence detection and/or enzymatic digestion. An example is shown in Fig. 2 of agarose gel electrophoresis and calcein fluorescence detection indicating that these results support the initial findings that the expression of 18S rRNA was observed in all body fluids, but STATH was observed only in the saliva samples. Further confirmation was obtained by enzymatic digestion (*AluI* and *Hpy1881*) of the RT-LAMP products from 18S rRNA and STATH genes respectively. An

example is shown (Fig. 3) with the two products as predicted (84 bp and 103 bp for 18S rRNA; 94 bp and 104 bp for STATH) [25].

Sensitivity analysis

Saliva left at a crime scene is usually at trace levels, hence it is necessary to determine the limit of detection of the real-time RT-LAMP assay. Total RNA extracted from the saliva of three

Table 2 Analysis of the specificity for expressions of 18S rRNA and STATH by RT-LAMP with the real-time turbidimeter

Gene	Ave \pm SD of Tt value (min) ^a							
	Venous blood	Saliva	Semen	Menstrual blood	Sweat	Urine	Vaginal secretions	Negative control
18S rRNA	26.8 \pm 2.2	44.2 \pm 2.3	33.2 \pm 2.2	44.7 \pm 5.6	44.7 \pm 2.5	32.9 \pm 0.4	38.2 \pm 2.8	-
STATH	-	39.4 \pm 2.9	-	-	-	-	-	-

^aThe average and standard deviation (Ave \pm SD) of Tt for 6 samples of each body fluid are shown. The symbol of “-” represents the turbidity not exceeding 0.1 within 60 mins of reaction time

individuals was serially diluted in triplicate from 25 ng down to 0.39 ng using a 4-fold dilution series. The results showed that it was stably detected when the RNA template was not less than 6.25 ng; however, the expressions of these two genes were occasionally detected even using only 0.39 ng (Online

Resource 1). When the RNA was at such trace levels, the random effect of amplifications and individual variation were observed.

To investigate the sensitivity of the RT-LAMP system for saliva identification in mixed body fluid samples, different ratios (from 1:5 to 3:1) of saliva and semen mixtures were tested to simulate evidential samples from sexual assaults. When the primer concentrations of 18S rRNA and STATH were the same, as shown in Table 1, the saliva could not be identified in any of the mixtures. When the primer concentrations for STATH were increased to be two times greater than those for the 18S rRNA, saliva could be identified in almost all of the body fluid mixtures, even at a ratio of 1:3 (saliva:semen) (Online Resource 2A).

Multiplex RT-LAMP

A multiplex RT-LAMP was performed to reduce labor costs and minimize the use of template. To differentiate products from different genes, additional loop primers, LF and LB, for 18S rRNA and STATH respectively were used and labelled with different dyes (FITC or ROX).

Firstly, to analyze the specificity of the loop primers, comparisons of the products for RT-LAMP with/without loop primers were performed (Fig. 4). The results in Fig. 4a showed that the RT-LAMP products for STATH were observed only for the saliva samples whether using the loop primer (lane

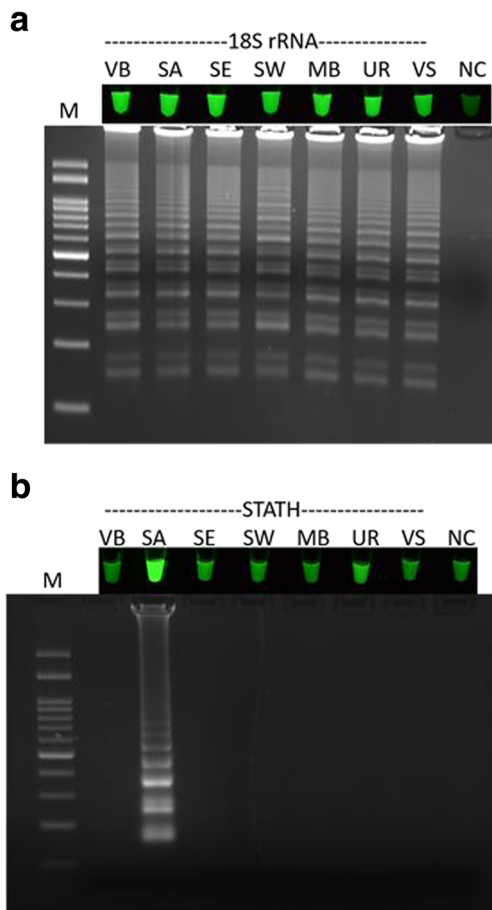


Fig. 2 An example for confirmation of the real-time RT-LAMP products for 18S rRNA (a) and STATH (b) with calcein fluorescence and electrophoresis on 3% agarose gel. The abbreviations VB, SA, SE, SW, MB, UR, VS and NC represent venous blood, saliva, semen, sweat, menstrual blood, urine, vaginal secretion and a negative control respectively. M represents the 100 bp DNA ladder. The calcein fluorescence was detected under the yellow filter and excitation by UV light (365 nm). Conditions of the photography are ISO6400, F/7.1 and 1/400 s for exposure (a), and ISO2500, F/7.1 and 1/80 s for exposure (b)

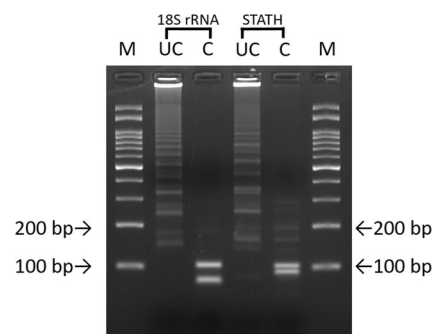


Fig. 3 An example for confirmation of the RT-LAMP products by the enzymatic digestion with 3.5% agarose gel electrophoresis. UC: uncut; C: cut with *AluI* (for 18S rRNA) or *Hpy188I* (for STATH); M: marker (100 bp DNA ladder)

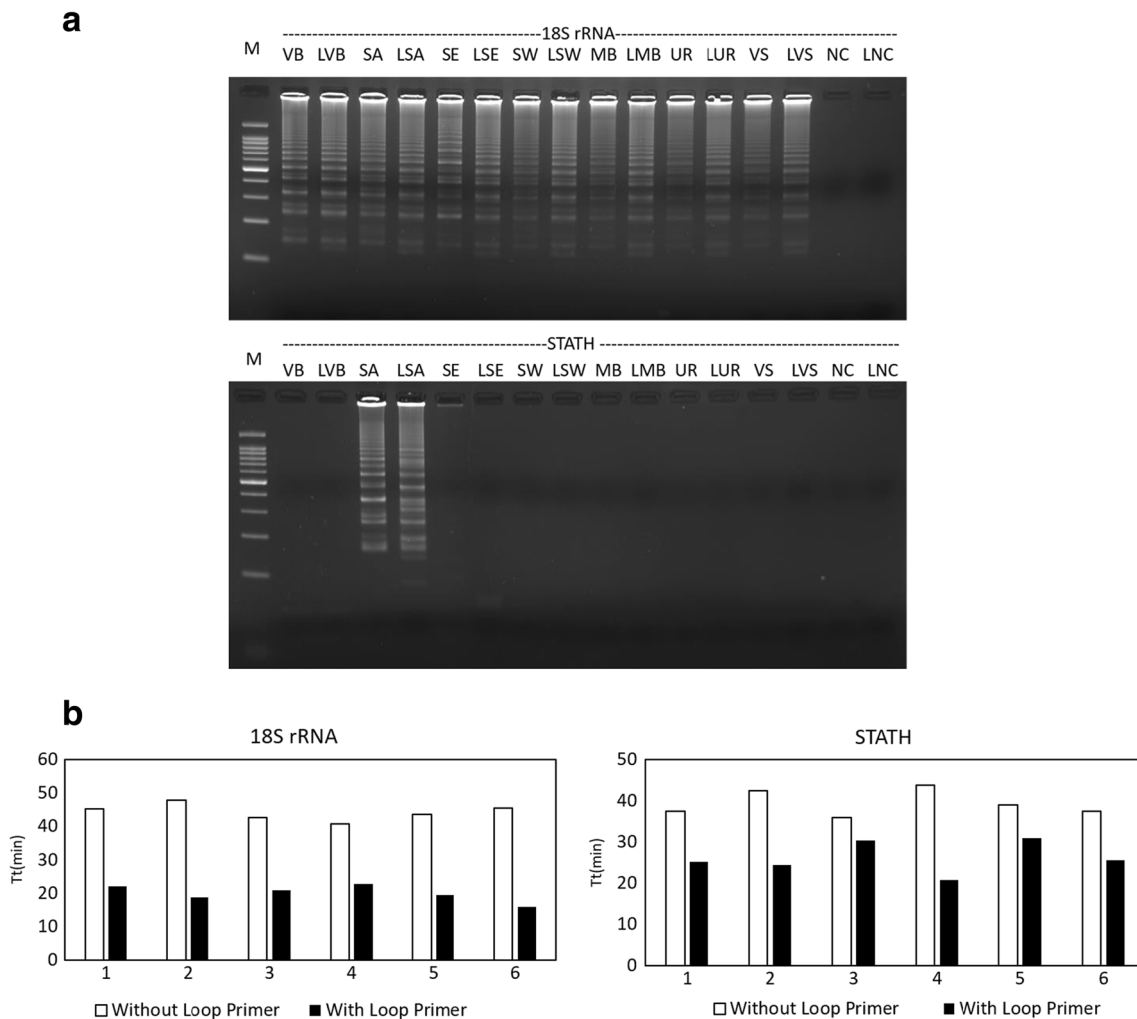


Fig. 4 Comparison of the RT-LAMP products with and without loop primer by using the 3% agarose gel electrophoresis (**a**) and real-time turbidimeter detection (**b**). “L” in (**a**) represents that the loop primers are used. The abbreviations VB, SA, SE, SW, MB, UR, VS and NC

represent venous blood, saliva, semen, sweat, menstrual blood, urine, vaginal secretion and a negative control respectively. The number of cross axis in (**b**) represents different individual

LSA) or not (lane SA). This is consistent with the above specificity analysis. The results indicated that the RT-LAMP reaction was accelerated by using loop primers (Fig. 4b and Online Resource 2) and were consistent with a previous report [29]. The Tt value decreased when the loop primer was used in the RT-LAMP reaction. Furthermore, the sensitivity was also enhanced by using loop primers (Online Resource 2). When the primer concentrations for STATH were two times that of 18S rRNA, saliva could be identified in body fluid mixtures at a ratio (saliva:semen) of 1:3 (without loop primer), however, even at 1:5 when the loop primer was used. The previous report also described that the LAMP reaction with the loop primers provided higher sensitivity [29].

Different ratios of primers for 18S rRNA and STATH in the multiplex system were also tested. The results showed that the orange precipitate (as predicted) was observed at a ratio of 1:2 (18S rRNA:STATH) (Online Resource 3). The RT-LAMP

products for multiplex were also confirmed to include those from both 18S rRNA and STATH using enzymatic digestion (Online Resource 4). This primer ratio was therefore adopted in the following study.

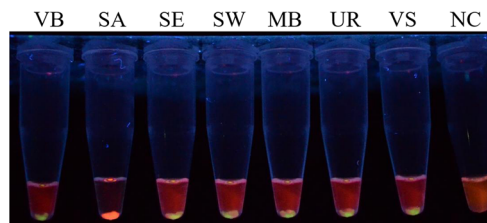


Fig. 5 Detection of the specificity for the Multiplex RT-LAMP system. The abbreviations VB, SA, SE, SW, MB, UR, VS and NC represent venous blood, saliva, semen, sweat, menstrual blood, urine, vaginal secretion and a negative control respectively. The primer ratio is 1:2 (18S rRNA:STATH). Conditions of the photography are ISO6400, F/7.1, focal length 36 mm and 1/8 s for exposure

Table 3 Tests for ten non-probative evidence samples

Evidence	RNA conc. (ng/μL)	Amylase test ^a	Real-Time RT-LAMP (Tt, min)		
			18S rRNA	STATH	Multiplex
1	5.1	+	22.4	19.9	22.1
2	4.8	±	20.2	19.3	20.5
3	6.0	+	19.8	20.6	21.1
4	6.6	–	19.6	22.8	21.6
5	5.9	+	21.4	28.2	23.0
6	6.7	±	23.1	30.5	26.2
7	5.1	+	19.3	21.4	22.3
8	7.0	–	27.4	19.8	19.6
9	15.0	±	25.4	22.2	19.8
10	34.7	+	23.7	22.3	30.6

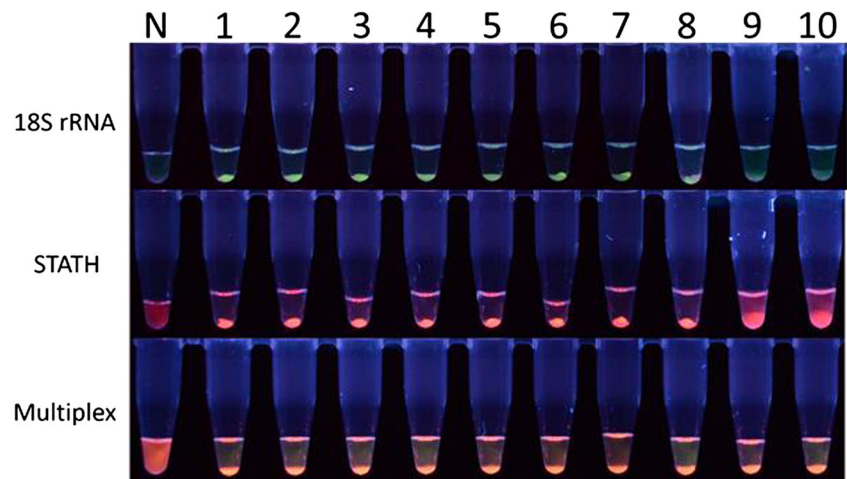
^a The symbols of ‘+’, ‘–’ and ‘±’ represent positive, negative and ambiguous results respectively

The specificity of the multiplex RT-LAMP using a range of different body fluids was analyzed. Figure 5 showed that expression of STATH was detected only in saliva (SA).

Applications on non-probative evidence samples

The application of the RT-LAMP system established in this study to detect saliva on mock crime scene exhibits was demonstrated using ten non-probative cigarette butts. These cigarette butts were initially screened using the Phadebas amylase test and confirmation of the presence of DNA was done using STR typing. The results showed that saliva was detected on all the cigarette butts whether by the simplex method or by multiplex RT-LAMP (Table 3 and Fig. 6). This was even for those cigarette butts with negative (evidence samples 4 and 8) and ambiguous results (evidence samples 2, 6 and 9) in amylase tests.

Fig. 6 RT-LAMP for ten non-probative evidence samples. Conditions of the photography are ISO6400, F/7.1, focal length 38 mm and 1/2 s for exposure



Direct RT-LAMP

A form of direct RT-LAMP testing was developed to speed the assay and save on resources. This was achieved by omitting the RNA extraction step. The results are shown in Fig. 7. The positive results were obtained using either the simplex or multiplex RT-LAMP assay.

Discussion

Current forensic practice uses a presumptive test for the presence of saliva. The test can however result in false positives/negatives and therefore improvements in the detection of saliva are required to increase the sensitivity and specificity of any test.

This study used STATH either in a simplex or multiplex RT-LAMP for the detection of saliva. An advantage of RT-LAMP is that it can be conducted just by using simple

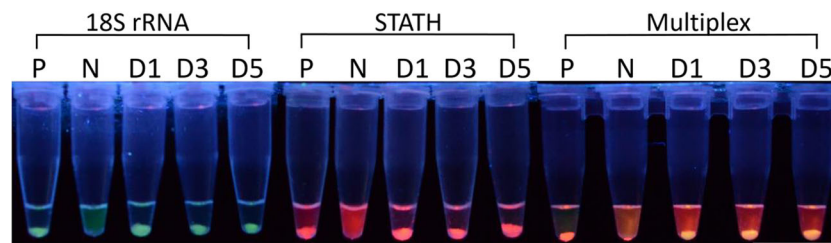


Fig. 7 Detection of the products from direct RT-LAMP for simplex and multiplex. P represents the positive control (with isolated RNA as the template) and N the negative control. The symbols of D1, D3 and D5

represent 1 μ L, 3 μ L and 5 μ L of template solution used in RT-LAMP respectively. Conditions of the photography are ISO6400, F/7.1, focal length 36 mm and 1/8 s for exposure

heating equipment rather than dedicated or expensive machine. In comparison to our previous system for the effective detection of blood using HBB [20], this present study included the loop primers to enhance the specificity and accelerate the reaction (Fig. 4b and Online Resource 2). Additionally dye-labelled loop primers were used to differentiate different gene products. The results obtained also showed that the sensitivity was enhanced by using dye-labelled loop primers (Online Resource 2). A simplex RT-LAMP was established in our HBB assay, however in this study we establish a multiplex system for the simultaneous amplifications of 18S rRNA and STATH. In response to recent applications of direct PCR applied to human saliva [30–32], a simplified assay omitting the isolation of RNA was also reported.

Conclusions

We reported on a novel forensic saliva identification by simplex and multiplex RT-LAMP and applied the method to non-probative cigarette butts. The results showed that this method was sensitive and specific. Direct RT-LAMP rapidly sped the assay. The process established in this study has the real potential to be highly valuable when the detection of saliva is central to a forensic investigation.

Key points

1. The identification of saliva for forensic applications has been developed using RT-LAMP.
2. The expression of STATH was highly specific for saliva.
3. The expression of both 18S rRNA and STATH were stably detected when the RNA was not less than 6.25 ng and saliva could be identified in the body fluid mixtures even at a ratio (saliva:semen) of 1:3 (without loop primer)/1:5 (with loop primer) by altering the primer concentration.
4. Saliva was detected on ten non-probative cigarette butts.

5. A direct RT-LAMP process for the effective detection of saliva was reported based on both a simplex or multiplex amplification.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The human body fluids used in this study were collected from the volunteers using procedures approved by Antai Medical Care Cooperation Antai- Tian-Sheng Memorial Hospital Institutional Review Board in Taiwan (TSMH IRB No./ Protocol No.: 14-085-B1).

Informed consent Informed consent was obtained from all individual participants included in the study.

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