



Development of mRNA-based body fluid identification using reverse transcription loop-mediated isothermal amplification

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Abstract

Identifying body fluids from forensic samples can provide valuable evidence for criminal investigations. Messenger RNA (mRNA)-based body fluid identification was recently developed, and highly sensitive parallel identification using reverse transcription polymerase chain reaction (RT-PCR) has been described. In this study, we developed reverse transcription loop-mediated isothermal amplification (RT-LAMP) as a simple, rapid assay for identifying three common forensic body fluids, namely blood, semen, and saliva, and evaluated its specificity and sensitivity. Hemoglobin beta (HBB), transglutaminase 4 (TGM4), and statherin (STATH) were selected as marker genes for blood, semen, and saliva, respectively. RT-LAMP could be performed in a single step including both reverse transcription and DNA amplification under an isothermal condition within 60 min, and detection could be conveniently performed via visual fluorescence. Marker-specific amplification was performed in each assay, and no cross-reaction was observed among five representative forensically relevant body fluids. The detection limits of the assays were 0.3 nL, 30 nL, and 0.3 μ L for blood, semen, and saliva, respectively, and their sensitivities were comparable with those of RT-PCR. Furthermore, RT-LAMP assays were applicable to forensic casework samples. It is considered that RT-LAMP is useful for body fluid identification.

Keywords Body fluid identification · mRNA · RT-LAMP · Hemoglobin beta · Transglutaminase 4 · Statherin

Introduction

Identifying body fluids and their genetic profiles from forensic samples can facilitate crime scene reconstruction, providing valuable evidence for criminal investigations. For example, in a case of sexual assault, verifying the presence of semen in the victim's vagina and matching its DNA type with that of the suspect can provide critical evidence for a criminal trial. Therefore, these tests have become standard in forensics. To date, protein-based methods employing biochemical, serological, and immunological tests have been used for body fluid identification in actual forensic examination procedure [1–5]. However, these conventional methods are presumptive and inconclusive concerning several body fluids such as saliva

and vaginal secretions due to their low specificity [3, 5]. In addition, these tests tend to have significant time and sample demands because each sample should be tested independently.

In recent years, alternative approaches based on genetic markers, such as mRNA, microRNA, and epigenetic DNA markers, have been developed to overcome the limitations of protein-based methods [6–19]. Among these approaches, mRNA-based methods that identify body fluids by amplifying particular mRNAs expressed in cells have been well studied. Although it is generally assumed that mRNA is unstable and susceptible to degradation by ubiquitously present ribonucleases (RNases), several reports indicated that mRNA is available for body fluid identification as genetic markers [20–23]. Effective mRNA markers for body fluid identification have been already identified for almost all forensically relevant body fluids [6–14]. Because this approach employs gene amplification technology, it allows us to achieve highly sensitive identification. In addition, this strategy can reduce time and sample consumption because this method can be applied to multiplex mRNA detection for parallel body fluid assays [7, 9, 14]. Meanwhile, a DNA/RNA co-extraction method, which can extract RNA and DNA from the same sample simultaneously, has

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been utilized for both body fluid identification and DNA typing [24–27]. This method also reduces time and sample consumption in a series of tests. Some commercially available co-extraction kits have been evaluated for forensic analysis [26, 27]. Thus, mRNA-based body fluid identification has some advantages over conventional methods. However, some problems related to mRNA-based methods must be resolved. Typical mRNA-based methods employ reverse transcription into cDNA and PCR amplification. The amplified gene-specific product is detected either by real-time monitoring or by electrophoresis. This series of experiments requires multiple labor-intensive steps (time consuming), and this defect prevents the implementation of mRNA-based methods as standard for body fluid identification in forensic examination procedures.

Loop-mediated isothermal amplification (LAMP) is a well-established technique for detecting the presence or absence of DNA [28–33]. As it is also possible to detect RNA via reverse transcription (RT)-LAMP, this technique has been used in several fields, such as clinical analysis, food and environmental hygiene monitoring, and forensic science [34–41]. Because RT-LAMP can be performed in a single step including both reverse transcription and high-efficiency DNA amplification under isothermal conditions, this technique permits rapid RNA detection without tube changes or thermal cycling. In addition, special detection instruments are not needed because the amount of DNA produced by LAMP is sufficient for the product to be detected simply as white precipitation or fluorescence by the naked eye. These advantages of RT-LAMP will overcome the defects of typical mRNA-based body fluid identification. In a recently reported pioneering study, Su et al. demonstrated blood identification using hemoglobin beta (HBB) as the blood-specific marker [40], finding that the marker could be detected in as little as 1×10^{-5} ng of total RNA within 60 min.

In this study, we developed RT-LAMP assays for three forensically common body fluids, namely blood, semen, and saliva, and evaluated their specificity and sensitivity. Additionally, we examined six forensic casework samples using RT-LAMP assays.

Materials and methods

Body fluid samples

All body fluids were collected from five adult volunteers using procedures approved by the Human Genome/Gene Analysis Research Ethics Committee of the Japanese Association of Forensic Science and Technology. Written informed consent was obtained from each participant. Fresh blood, semen, saliva, urine, and sweat samples were collected from male volunteers in plastic tubes, and 50- μ L aliquots were stored at -80°C until use. Semen-free vaginal secretions were collected from women using sterile cotton swabs and stored at -80°C after

air-drying. Blood, semen, and saliva were diluted using PBS for real-time RT-LAMP and sensitivity evaluations.

Forensic casework samples

Bloodstain sample was prepared by dropping 30 μ L of blood on sterile gauze. Blood swab sample was prepared by wiping 30 μ L of bloodstain on a table using a sterile cotton swab. Semen stain sample was prepared by dropping 30 μ L of semen on tissue paper. Saliva swab samples were prepared by collection from the rim of a cup and lip of a coffee can using sterile cotton swabs. These samples and a cigarette butt were kept at room temperature for 5 days after air-drying.

Primer design for RT-LAMP

Marker genes, which were previously identified in each body fluid via RT-PCR, were selected as follows: HBB for blood, transglutaminase 4 (TGM4) for semen, and statherin (STATH) for saliva. The nucleotide sequences of HBB, TGM4, and STATH were obtained from GenBank (accession numbers: HBB, NM_000518; TGM4, NM_003241; and STATH, NM_003154). RT-LAMP primer sets for the genes were designed using PrimerExplorer V4 (<http://primerexplorer.jp/lamp4.0.0/index.html>) following its instructions, as shown in Table 1. Four primers consisting of a forward inner primer (FIP), a reverse inner primer (BIP), and two outer primers (F3 and B3) were designed for each gene. The loop primers LF and LB were designed for HBB and TGM4, and LF was designed for STATH.

At least one primer in each set was designed to overlap exon/exon junctions to avoid amplification of contaminating genomic DNA.

Total RNA extraction and DNase I digestion

Total RNA was extracted from 30 μ L of body fluid samples, vaginal cotton swabs, or forensic casework samples (cotton swabs, 1×1 cm² of bloodstain, 2×2 cm² of semen stain, and 1×2 cm² of cigarette butt rolling paper) using an RNeasy® Mini kit (QIAGEN, Hilden, Germany), and DNase I digestion was performed using an RNase-Free DNase Set (QIAGEN) according to the manufacturer's protocol. RNA was eluted in 50 μ L of RNase-Free H₂O.

Real-time monitoring of RT-LAMP

RT-LAMP was performed in a total volume of 25 μ L using a LoopAmp RNA amplification kit (Eiken, Tokyo, Japan) in accordance with the manufacturer's protocol. For each assay, a 5- μ L aliquot of RNA sample extracted from body fluid was mixed with 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 20 pmol of loop primer, 1 μ L of

Table 1 Primer sequences for HBB, TGM4, and STATH

Gene	Primer	Position in mRNA (5' to 3')	Sequence (5' to 3')
HBB ^a	F3	280–299	CTCACCTGGACAACCTCAAG
	B3	467–484	TTGTGGGCCAGGGCATT
	FIP ^d	300–317 + 351–372	CCAGGAGCCTGAAGTTCTCAGGGGCACCTTTGCCACACTG
	BIP ^e	387–406 + 442–460	TGTGTGCTGGCCATCACTTCCAGCCACCCTTTCTGAT
	LF	318–336	TGTCACAGTGCAGCTCACT
	LB	409–428	GCAAAGAATTCACCCACCA
TGM4 ^b	F3	877–895	CTGGGTGTTTGTGGGATC
	B3	1047–1066	ATCCGTCCACACATGGAAAT
	FIP ^d	898–916 + 942–963	TCGTGAGCTGAATCGAAGCCTGGACTACAGTGTGAGAGCG
	BIP ^e	966–987 + 1026–1044	CAGAAAGGAACCTCACGGTGGACAGACAGAGTCGTGGGTCA
	LF	917–933	CGTGTGGGATGCCCAA
	LB	988–1010	CACCTATGTGAATGAGAATGGCG
STATH ^c	F3	29–51	GTAGCACATCATCTCTTGAAGCT
	B3	313–334	GCCTCAATAATCATGTCTGCA
	FIP ^d	104–126 + 145–169	TCAGCTCCAATCATGGAACCATGAGCCAACTATGAAGTTCCTTGTCT
	BIP ^e	200–224 + 242–264	TGGAAGATTCGGTTATGGGTATGGCGTTGTGGGTATAGTGGTTGTTCT
	LF	127–144	GAGCCAAGATGAAGCAA

HBB, hemoglobin beta; TGM4, transglutaminase 4; STATH, statherin

^{a,b,c} GenBank accession numbers: HBB, NM_000518; TGM4, NM_003241; and STATH, NM_003154

^{d,e} FIP and BIP contain two distinct binding sequences

Enzyme Mix, and 12.5 μ L of Reaction Mix. The reaction mixture was incubated at 63 °C in a LoopAmp Reaction Tube (Eiken) using a LoopAmp turbidimeter (LA-320-C, Eiken), and the turbidity of each sample was measured during the reaction.

Analysis of amplified products

The aforementioned reaction mixture was incubated at 63 °C for 30 min for HBB and TGM4 and at 63 °C for 60 min for STATH, followed by heating at 80 °C for 5 min to terminate the reaction, using a thermal cycler (Applied Biosystems® GeneAmp® PCR System 9700, Life Technologies by Thermo Fisher Scientific, Carlsbad, CA). A 5- μ L aliquot of the amplified product was digested with a restriction enzyme in a total volume of 20 μ L of reaction mixture as follows: 30 units of BamH I at 30 °C for 2 h for HBB and 20 units of Hinf I at 37 °C for 2 h for TGM4 and STATH. Aliquots of 2 μ L of amplified products and 8 μ L of the digested products were electrophoresed on 2% agarose gels followed by staining with ethidium bromide.

Visual fluorescence detection

One microliter of Fluorescent Detection Reagent (Eiken) was added to the aforementioned reaction mixture before the amplification. The mixtures were incubated using a thermal cycler as described in the preceding section. Fluorescence was

detected under UV (254 nm) radiation after the amplification. A positive reaction was defined as a color change from pale orange to green or green fluorescence emission.

Results

Optimization of reaction time by real-time monitoring

Blood, semen, and saliva dilution samples were prepared via 10-fold serial dilutions, and total RNA was extracted from each sample. The reaction curves of RT-LAMP were measured via real-time turbidity monitoring (Fig. 1). Turbidity initially increased proportionally with input fluids, and then reached a plateau phase. The turbidities of diluted blood and semen reached the plateau phase within 30 min (range, 1 to 1×10^{-4} and 1 to 1×10^{-2} , respectively) and that of diluted saliva reached the plateau phase within 60 min (range 1 to 1×10^{-1}). Turbidity was not increased in the other samples or negative controls. Thus, subsequent end point RT-LAMP assays were performed for 30 min for HBB and TGM4 and for 60 min for STATH.

Analysis of RT-LAMP products

A ladder-like pattern, which is characteristically observed in a positive RT-LAMP product via agarose gel electrophoresis [28], was detected for each body fluid. Moreover,

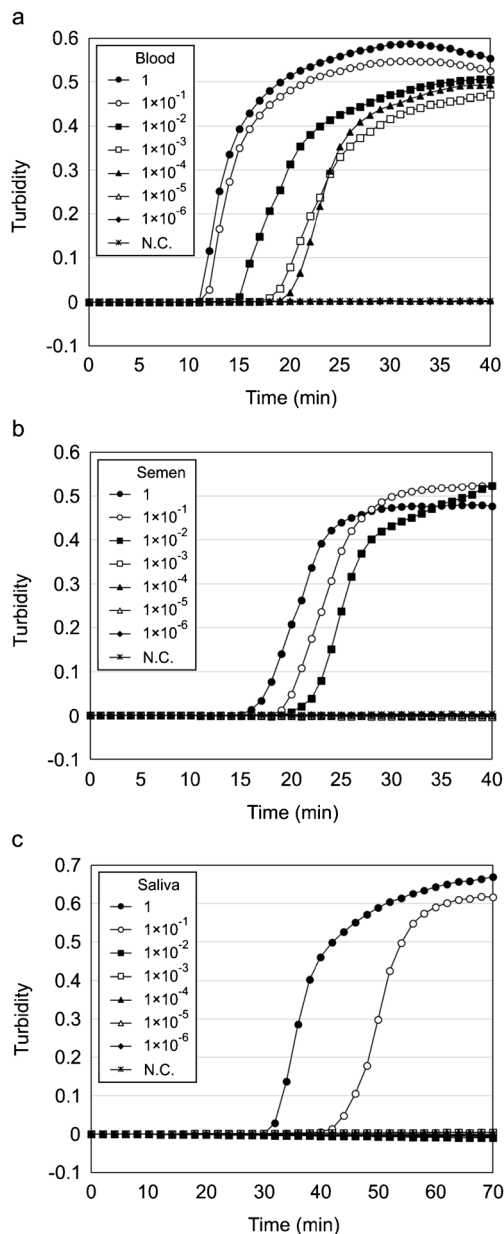


Fig. 1 Time course of turbidity in reverse transcription loop-mediated isothermal amplification (RT-LAMP). Body fluid samples were prepared via 10-fold serial dilutions, and total RNA was extracted from 30 μ L of each sample. Each RNA sample was added to the RT-LAMP reaction mixture corresponding to the target marker gene. RT-LAMP assays (a) for blood-targeting hemoglobin beta, (b) semen-targeting transglutaminase 4, and (c) saliva-targeting statherin were monitored via real-time measurements of turbidity. N.C., negative control

no amplified products were detected in the negative controls (Fig. 2). To confirm the marker-specific amplification, each amplified product was digested with restriction enzyme recognizing a target region sequence. The ladder-like pattern disappeared after the digestion (Fig. 2), indicating that RT-LAMP was performed without nonspecific amplification.

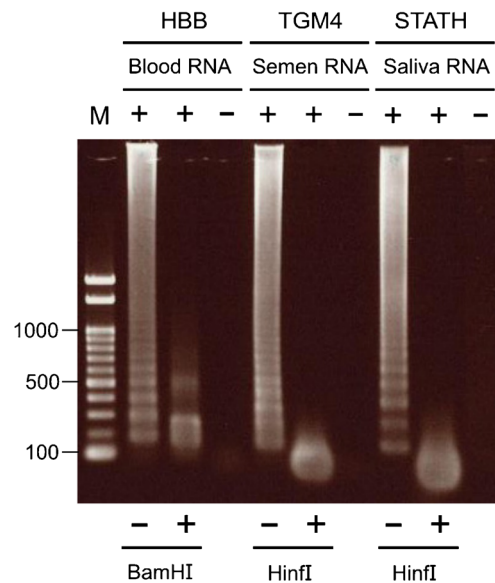


Fig. 2 Analysis of amplified products. Each total RNA extracted from blood, semen, and saliva was amplified by reverse transcription loop-mediated isothermal amplification (RT-LAMP) targeting hemoglobin beta (HBB), transglutaminase 4 (TGM4), and statherin (STATH). A part of the product was digested using BamH I for HBB and Hinf I for TGM4 and STATH. The products of RT-LAMP with (RNA+, enzyme-) and without (RNA-, enzyme-) total RNA, those digested with restriction enzyme (RNA+, enzyme+), and the 100-bp ladder marker (M) were subjected to electrophoresis on a 2% agarose gel

Visual fluorescence detection

A green color change or strong green fluorescence was observed in positive RT-LAMP reactions but not in the negative controls (Fig. 3), and a positive judgment could be more easily made using fluorescence. Additionally, the judgment could be efficiently made using a monochrome CCD camera because the fluorescence contrast between positive and negative samples could be observed more clearly. Thus, subsequent visual fluorescence observation and judgment were performed using a monochrome CCD camera.

Evaluation of specificity for target body fluids

The specificities of RT-LAMP for blood, semen, and saliva were examined via visual fluorescence detection. In all RT-LAMP assays, a change in fluorescence was only observed in the target fluids after the reaction (Fig. 4). In addition, this specificity for target body fluids was observed in all five samples for each fluid type, suggesting high reproducibility between individual variations (Table 2).

Sensitivity of RT-LAMP

The sensitivities of RT-LAMP for blood, semen, and saliva were examined via visual fluorescence detection and 10-fold

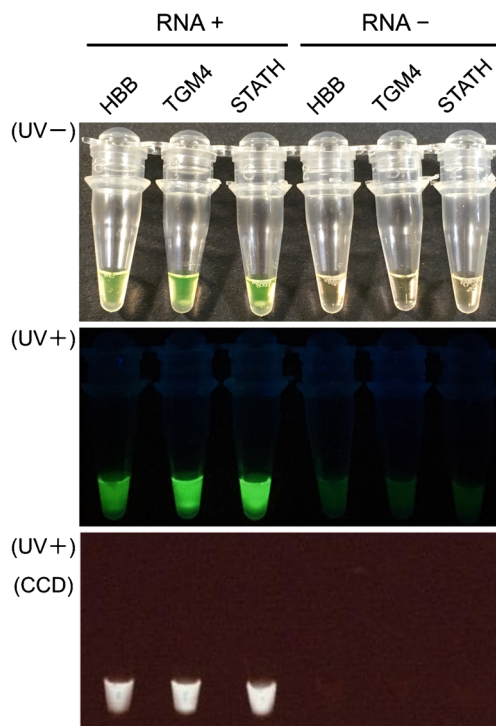


Fig. 3 Detection of amplified samples with and without UV. Total RNA was extracted from 30 μL of each body fluid sample. Each RNA sample was added to the RT-LAMP reaction mixture corresponding to the target marker gene. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) using Fluorescent Detection Reagent was performed with (RNA+) and without (RNA-) total RNA, and their products were observed with (UV+) and without (UV-) UV light (254 nm) irradiation. The samples were observed by the naked eye and using a monochrome CCD camera (CCD)

serial dilutions. Fluorescence changes were observed in RT-LAMP assays over the dilution ranges of 1 to 1×10^{-4} for blood, 1 to 1×10^{-2} for semen, and 1 to 1×10^{-1} for saliva (Fig. 5).

Identification of forensic casework samples

Forensic casework samples were examined by RT-LAMP for blood, semen, and saliva via visual fluorescence detection. Fluorescence changes were observed in RT-LAMP for blood in bloodstain on gauze and swab from a bloodstain on a table, for semen in semen stain on tissue paper, and for saliva in swab from the rim of a cup, swab from the lip of a coffee can and a cigarette butt. In all RT-LAMP assays, positive results were only observed in the target fluids after the reaction (Table 3).

Discussion

Forensic laboratories encounter a large number of samples, and they are expected to produce results in a timely manner due to the increasing importance of objective evidence in the

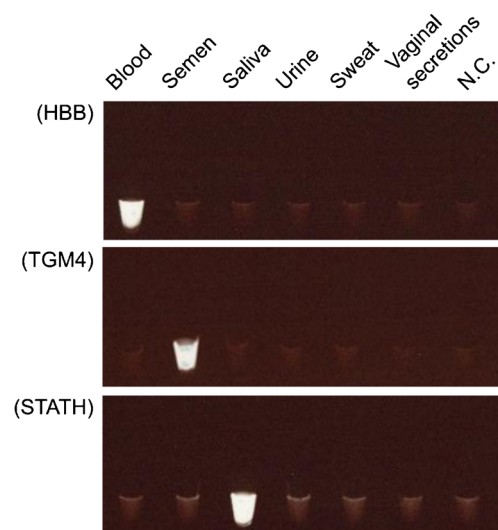


Fig. 4 The specificity for each target body fluid. Total RNA was extracted from blood, semen, saliva, urine, sweat, and vaginal secretions; reverse transcription loop-mediated isothermal amplification targeting hemoglobin beta (HBB), transglutaminase 4 (TGM4), and statherin (STATH) was performed. The products were detected via visual fluorescence

criminal justice system. Therefore, simple and rapid assays are especially important in forensics. LAMP and RT-LAMP are useful for developing forensic tests involving nucleic acid amplification because they can be performed easily and rapidly. In fact, several forensic tests targeting genetic markers have been developed using LAMP or RT-LAMP [37–41]. Recently, Kitamura et al. reported a LAMP assay for the rapid screening of male DNA targeting multi-repeat sequences of the Y chromosome [41]. They developed a male DNA assay with a detection limit of 1 pg of male DNA within 20 min, and the assay was considered useful for the rapid screening of samples from males.

In this study, we demonstrated that three forensically common body fluids, namely blood, semen, and saliva, could be identified simply and rapidly via RT-LAMP specifically amplifying mRNA marker genes. We specifically developed RT-LAMP assays for semen and saliva for the first time. RT-LAMP assays for these body fluids could be performed by employing common laboratory equipment within 60 min. In particular, blood and semen could be amplified within 30 min because two loop primers were successfully designed for HBB and TGM4. It is generally known that loop primers are not necessary for RT-LAMP, but they can enhance the reaction rate and specificity of amplification [31]. The effect of loop primers for HBB amplification was also evaluated in our developed RT-LAMP assays, and the rate of amplification was increased by approximately twofold (data not shown). In fact, the reaction rate of RT-LAMP for HBB was faster than that of a previously reported RT-LAMP assay for HBB featuring no loop primers [40]. Thus, loop primers should be designed and

Table 2 RT-LAMP assays in five individual body fluid samples

Target marker gene	Body fluids					
	Blood	Semen	Saliva	Urine	Sweat	Vaginal secretions
RT-LAMP ^a						
HBB	5/5	0/5	0/5	0/5	0/5	0/5
TGM4	0/5	5/5	0/5	0/5	0/5	0/5
STATH	0/5	0/5	5/5	0/5	0/5	0/5

RT-LAMP, reverse transcription loop-mediated isothermal amplification; HBB, hemoglobin beta; TGM4, transglutaminase 4; STATH, statherin

^a Positive number/tested number

prepared for rapid assays whenever possible. In addition to its rapidity, RT-LAMP could be performed in a single step including reverse transcription and amplification under isothermal conditions, and detection could be performed via the naked eye as a color change or fluorescence, thus requiring less liquid handling and basic laboratory equipment.

These characteristics should reduce the risks of artificial and environmental body fluid contamination and simplify the test. Thus, RT-LAMP is appropriate for mRNA-based body fluid identification.

The specificity of targeted mRNA marker gene amplification for representative body fluids was demonstrated for RT-LAMP as well as previously reported RT-PCR methods [7, 10–14]. Regarding other forensically relevant body fluids, it has been reported that menstrual blood also expresses HBB and that nasal secretions express STATH [11, 13, 14]. Therefore, the possibilities of cross-reaction with HBB and STATH in these fluids were presumed. Although some reports have indicated that TGM4 is also expressed in adult male urine and that STATH is expressed in vaginal secretions and menstrual blood, negative reactions were observed for urine and vaginal secretion samples with our developed assay [11–13]. TGM4 expression is lower in adult male urine than in semen, and STATH expression is lower in vaginal secretions than in saliva; thus, they were suggested that amplification would not be detected in urine and vaginal secretions in the limited reaction time of the assay. Several reports regarding mRNA-based identification employing RT-PCR illustrated that histatin 3 (HTN3) is the most suitable for conclusive

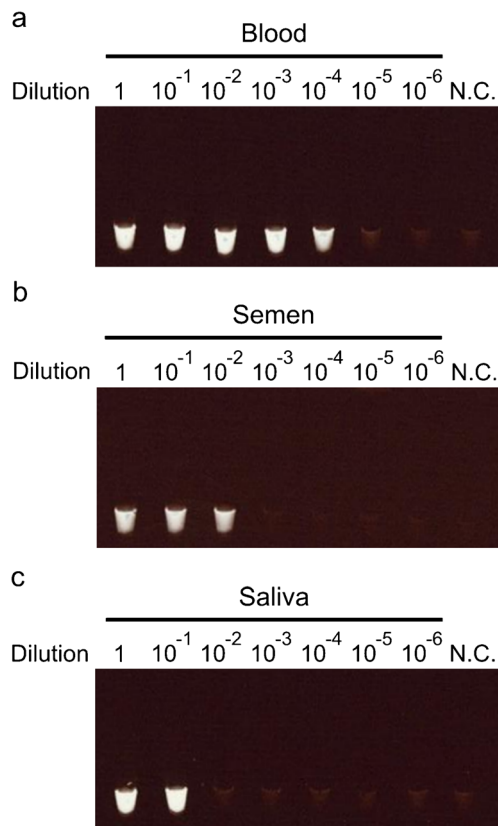


Fig. 5 The sensitivities of reverse transcription loop-mediated isothermal amplification (RT-LAMP) for blood, semen, and saliva. Diluted blood, semen, and saliva samples were prepared via 10-fold serial dilutions, and then total RNA was extracted from each sample. (a) Total RNA samples extracted from blood were subjected to RT-LAMP targeting hemoglobin beta. (b) Total RNA samples extracted from semen were subjected to RT-LAMP targeting transglutaminase 4. (c) Total RNA samples extracted from saliva were subjected to RT-LAMP targeting statherin. Detection was performed via visual fluorescence. N.C., negative control

Table 3 Identification of six forensic casework samples

Forensic casework samples ^a	Target maker gene		
	HBB	TGM4	STATH
Bloodstain on gauze	+	–	–
Swab from a bloodstain on a table	+	–	–
Semen stain on tissue paper	–	+	–
Swab from the rim of a cup	–	–	+
Swab from the lip of a coffee can	–	–	+
Cigarette butt	–	–	+
N.C.	–	–	–

HBB, hemoglobin beta; TGM4, transglutaminase 4; STATH, statherin; N.C., negative control; +, positive result; –, negative result

^a Five-day-old samples

saliva detection because it is expressed only in saliva. In our preliminary study, two HTN3 primer sets were designed, but no amplification was observed in this assay. Further detailed examinations concerning design parameter and the target region will be required for primer sets for HTN3.

The lower limits of detection for our RT-LAMP were 1×10^{-4} for blood, 1×10^{-2} for semen, and 1×10^{-1} for saliva. Equivalent volumes of body fluids added to reaction were calculated as 0.3 nL for blood, 30 nL for semen, and 0.3 μ L for saliva. These detection limits were comparable with the sensitivities of quantitative RT-PCR (qRT-PCR) methods. Matsumura et al. reported that a 1-nL bloodstain could be detected via qRT-PCR targeting HBB [15], and Sakurada et al. found that 0.1 μ L of semen and saliva could be detected using qRT-PCR targeting protamine 2 and semenogelin 1 for semen and STATH and HTN3 for saliva [13].

Targeted marker mRNA genes were successfully detected from six forensic casework samples via RT-LAMP assays. The samples were practical items, which are often examined in forensic laboratory. Although the samples were 5 days old, target marker genes were detected by our developed RT-LAMP assays as well as RT-PCR or qRT-PCR methods previously reported [10, 13, 14]. Thus, RT-LAMP assays were applicable to forensic casework samples.

RT-LAMP can be an elemental technology for microfluidic devices because of its simple reaction and detection processes. No expensive hardware is required because thermal cycling is not required and the high signal intensity due to the considerable level of DNA production. Several handheld microfluidic devices have been developed using RT-LAMP in clinical fields [42–45]. For example, Damhorst et al. developed a microchip that could measure human immunodeficiency virus loads in whole blood using a consumer smartphone [44]. The microchip was only 1 cm² in size, and it contained 36 wells. Additionally, the reaction droplet was less than 60 nL. Point-of-care testing and cost reduction could be accomplished using this small device. Our RT-LAMP is also applicable to a microchip, and the device will enable us to perform on-site testing with a low cost, less sample consumption, and parallel body fluid identification.

In summary, we developed RT-LAMP as a simple, rapid assay for identifying three common forensic body fluids, namely blood, semen, and saliva. RT-LAMP could be performed in a single step including both reverse transcription and DNA amplification under an isothermal condition within 60 min, and detection could be conveniently performed via visual fluorescence. Marker-specific amplification was performed in each assay, and no cross-reaction was observed among five representative forensically relevant body fluids. The sensitivities were comparable with those of RT-PCR. Furthermore, RT-LAMP assays were applicable to forensic casework samples. It is considered that RT-LAMP is useful for body fluid identification.

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Compliance with ethical standards

All participants in this study provided informed consent, and the study design was approved by the Human Genome/Gene Analysis Research Ethics Committee of the Japanese Association of Forensic Science and Technology. Written informed consent was obtained from each participant.

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

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