Technical Note

The Utilization of the SaLux19 -Based LAMP Assay for the Rapid and Sensitive Identification of Minute Amounts of Biological Material

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The Utilization of the SaLux19 -Based LAMP Assay for the Rapid and Sensitive Identification of Minute Amounts of Biological Material

Ema Ruszova [1,4], Daniel Vanek [1,2,3,4], Walter Stühmer [5], Ziad Khaznadar [1] and Nidhi Subhashini [6,*]

[1] Bulovka University Hospital, Prague, Czech Republic
[2] Charles University Prague, Institute for Environmental Sciences, Prague, Czech Republic
[3] Department of Forensic Medicine, Second Faculty of Medicine, Charles University, Czech Republic
[4] Forensic DNA Service, Budinova 2, 180 81 Prague 8, Czech Republic
[5] Max Planck Institute of Multidisciplinary Sciences, Hermann-Rein-Str. 3, 37075 Göttingen
[6] Seratec GmbH, Ernst-Ruhstrat-Str. 5, 37079 Göttingen
* Correspondence: nidhi.subhashini@seratec.com

Abstract: We investigated the development of a simple and sensitive assay for the identification of minute amounts of animal and human biological material. The suggested system uses isothermal amplification of DNA in a rapid assay format, which returns the results in as little as 40 minutes from sampling. The assay used for the proof of concept employs the novel handheld **SaLux19** device (Max-Planck Innovation prototype, Germany). This well-established infield detection system could also be utilized for forensic scenarios. The results presented here demonstrated that the assay is sufficiently specific and sensitive and can detect the presence of Sus scrofa nucleic acids.

Keywords: species identification; LAMP; point-of-care testing; **SaLux19**; *Sus scrofa (pig)*

1. Introduction

Loop-mediated amplification (LAMP) assays are highly specific (due to the large number of primer pairs), generate much more amplicon DNA than conventional PCR, and can detect the amplification product by colorimetry or fluorescence. Fluorescent indicators (dyes) can be included in a LAMP reaction, which, as expected, provides greater sensitivity than colorimetric detection [1]. LAMP allows specific amplification of target nucleic acids with greater efficiency than typical PCR [2]and most importantly, under isothermal conditions [3,4]. The simplicity of this screening method allows for fast and straightforward DNA analysis. Second, the nature of isothermal amplification massively reduces the complexity of the instrument that must be designed and built without the need for sequential thermocycling stages and the associated expensive and specialized thermocycling equipment, most often restricted to a dedicated laboratory [5].

Current methods based on DNA analysis, which determine the presence of species-specific biological materials, are fast and have excellent sensitivity [6]. This study aimed to develop an *in-house* method that exploits a novel amplification technique called loop-mediated amplification (LAMP), particularly using the device SaLux19 (Max-Planck Innovation prototype, Germany), allowing fluorescent *real-time* control of amplification and focusing on DNA identification from *Sus scrofa* biological fluid (venous blood) from forensically sized samples (~2 µl or less, diluted 100x, 1000x, at their appropriate concentration units within the range from ng to pg of DNA [7]).

The sample for this study was selected according to the following criteria:

Pork and its derivates are religiously forbidden for some groups, such as Jews and Muslims [8,9]. Moreover, the consumption of pig tissue-related products can trigger the development of allergies in sensitive individuals [9,10].

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2. Materials and Methods

2.1. SaLux19 device

Figure 1. SaLux19 microdevice.

Principle of real-time SaLux19 point of care (POC)–based testing

This pocket-size device has dimensions 14,5 cm x 9,5cm x 4cm (L x W x H) and is equipped with a portable Xlayer power charger QC 3.0 (Fig. 1). By nature, the SaLux19 device can detect specific DNA or cDNA genetic codes. This POC modality allows the determination of both, as previously reverse-transcribed mRNA–based pathogens or DNA pathogens and all biological tissue containing DNA specific to the corresponding species and individuals. The specificity of detection is based on the match in the sequence of a piece of DNA with synthetic "baits" (primers) that bind to the target to be analysed. The SaLux19 biosensor is manufactured for working temperature being set to 64°C. The principle exploits the presence of DNA polymerase with strand-displacement activity, newly formed stem-loop structures, and a new self-primed 3'-end creation [5]. A fluorescent dye with the primer binds to any newly formed double helix. The binding of the fluorescent dye causes a shift in the fluorescent colour of the dye. This shift in fluorescence is obtained by a light source (light-emitting diode, LED), a set of optical filters with cut-off -510nm, and the photodetectors. The method utilized by SaLux19 is known as loop-mediated isothermal amplification (LAMP). During a real-time LAMP reaction, the amount of shifted fluorescence increases exponentially with time until saturation is reached due to the limited reagent. In this sense, a chain reaction is initiated by the presence of a few molecules of the agent to be detected, and the increase in fluorescence correlates with the input amount of DNA and corresponds to the Ct value acquired by loop amplification. The biosensor display is a part of the microdevice architecture. The maximum possible observation time allowed by the SaLux19 biosensor to detect only a few molecules of the specific substance to be analysed is 40 minutes.

2.2 DNA extraction and quantitation

DNA was extracted using a column-based Quick-DNA Miniprep Kit (cat. no. D3024, ZymoResearch, USA). The nDNA amount was quantified using a standard curve constructed by amplifying known amounts of the human DNA control (cat. no. 431266, Thermo Fisher Scientific, USA).

Absolute quantification of the nDNA of Sus scrofa

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DNA extracted from nonprecipitating Sus scrofa blood was quantified using iQ SybrGreen Supermix qPCR (cat. no. 1708880, Bio-Rad, USA) targeting the TATA-binding protein (TBP) gene, described as a 2-allele single gene [11,12]. TBP genes from different species were aligned in ClustalO (https://www.ebi.ac.uk/) and primers were designed in PrimerQuestTM (Integrated DNA Technologies tool, USA) in their most homologous sequences. Each 20 µL reaction consisted of 1x Supermix, 10 µM forward: tgctgttgacaagttggtt; reverse primers: tattctgtcctgcaatactg; and 2 µL of unknown DNA. TaqMan human control DNA was used as a standard and ranged from 0.001–10 ng/µL. Samples were run in duplicate on a CFX Connect Real-Time PCR System (Bio-Rad, USA) at 95 °C for 3 min and then 40 cycles of 95 °C for 15 sec and 58 °C for 45 sec. The threshold cycle (Ct) was automatically determined by CFX Manager Dx software v. 3.1 (Bio-Rad, USA).

Relative quantification and determination of the mtDNA:nDNA ratio

Relative quantification of mtDNA was performed using the same iQ SYBR Green Supermix (cat.no. 1708880, Bio-Rad, USA). The changes in the relative quantity of mtDNA with respect to nDNA were determined as the ratio of the number of copies of the mitochondrial 16S gene to that of the TBP gene (nDNA) in the same tube. The adapted mathematical formula by Livak [13]

$2x2^{\Delta Ct\ (Ct(n)-\ Ct(mt))}$ was used for analysis. PCR was carried out in duplicate for each DNA sample. The PCR primers used in the present study were published in the study of Boessenkool et al. [14], and the PCR temperature profile was the same as that used for absolute quantification.

Two dilutions of Sus scrofa DNA were used for the LAMP assay experiments: 100x (at a concentration of ≈127 pg/µL of nDNA with a ratio of mtDNA/nDNA ≈ 8.75 and 1000x diluted DNA (at a concentration of ≈13 pg/µL of nDNA).

As a quick alternative to standard nucleic acid extraction procedures, direct lysis was performed using a DipStick DNA Extraction Kit [15].

2.3 Primers

1) LAMP primers targeting the mitochondrial cytochrome c oxidase subunit I (COI) gene of Sus scrofa (accession number MN124249.1) were obtained from Jawla et al. 2021 [16] and synthesized by Eurofins Genomics Europe Shared Services GmbH (Germany). The size of the LAMP product is forensically friendly (226 bp).

2) LAMP primers targeting the human Y-amelogenin gene (AMEL-Y) (accession number NG_008011.2) were used for monitoring male DNA amplification using TaqMan™ Control Genomic DNA (cat. no. 4312660, Thermo Fisher Scientific, USA). The primers used were acquired from the published article of Nogami et al. 2008 [17] and synthesized by Eurofins Genomics Europe Shared Services GmbH (Germany). The length of the amplification product (202 bp) is sufficient for the detection of AMEL-Y in body fluids, as demonstrated in another study [18].

2.4 LAMP assay

Preliminary tests for the identification of Sus scrofa DNA were performed exactly according to the New England Biolabs (NEB, U.K.) web protocols with visual control of colour pH changes [19] (Fig. 2a) or the typical LAMP protocol [20], where the intercalating dye SYBRgreen Gel Stain (cat.no. S7563, Thermo Fisher Scientific, USA), at a dilution of 1:10, was loaded into the inner part of the tube lid (Fig. 2b). Reactions were carried out in 0.5 ml reaction microtubes within a thermoblock (Eppendorf, Germany).

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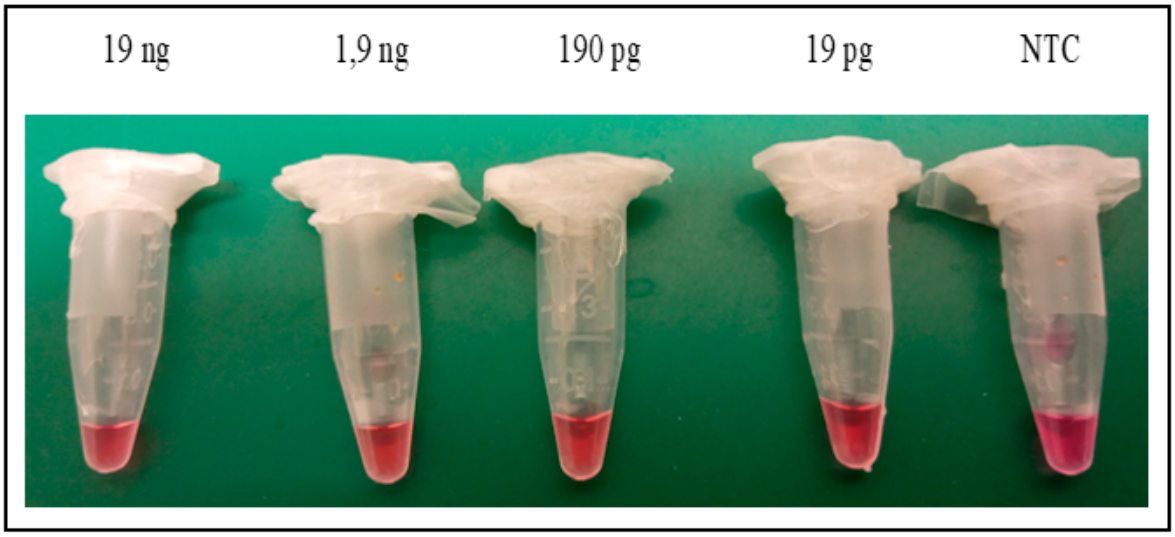


Figure 2. a: LAMP assay *Sus scrofa* - detection based on pH changes, pH indicator-phenol red; evaluation with the naked eye, NTC- no template control.

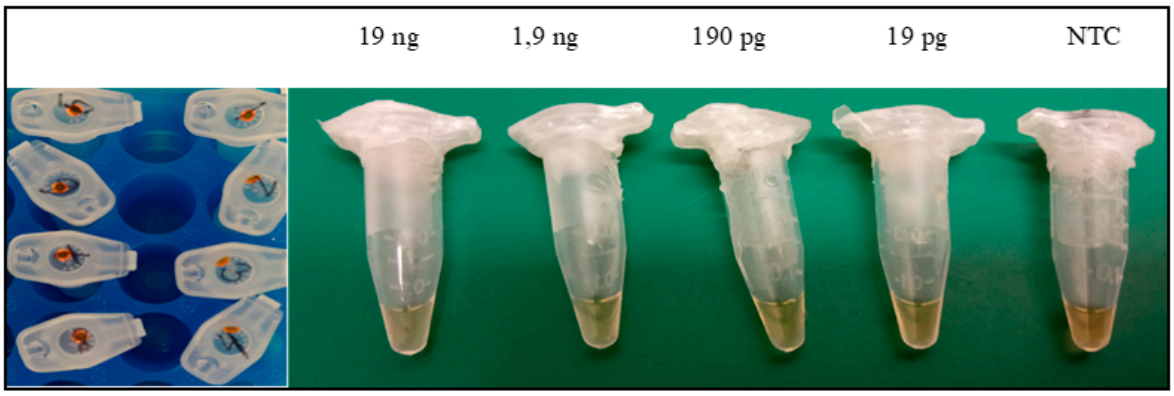
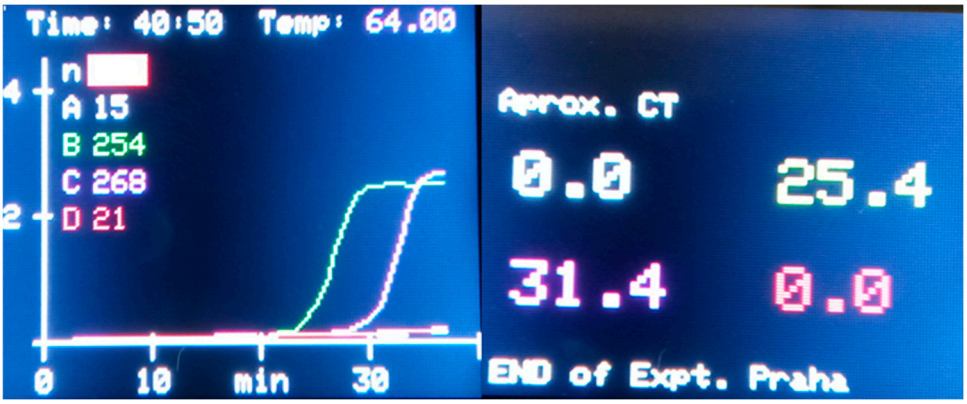


Figure 2. b: LAMP assay for *Sus scrofa* detection based on colorimetric changes; colour indicator, SYBR Green Gel Stain; evaluation with the naked eye, NTC- no template control.

Own *real-time* LAMP assay optimization was performed on both a real-time PCR system (CFX Connect PCR system, Bio-Rad, USA) and the SaLux19 device (MPI-SERATEC, Germany). The protocol used was identical to the protocol used on the NEB website [19] and included the same concentration of primer mixture and the addition of 10 000x diluted SYBR Green Gel Stain (cat. no. S7563, Thermo Fisher Scientific, USA) or recommended fluorescent LAMP dye (cat. no. B1700S, NEB, U.K.). LAMP was performed according to the written instructions on the NEB website [19–21] (Fig. 3).



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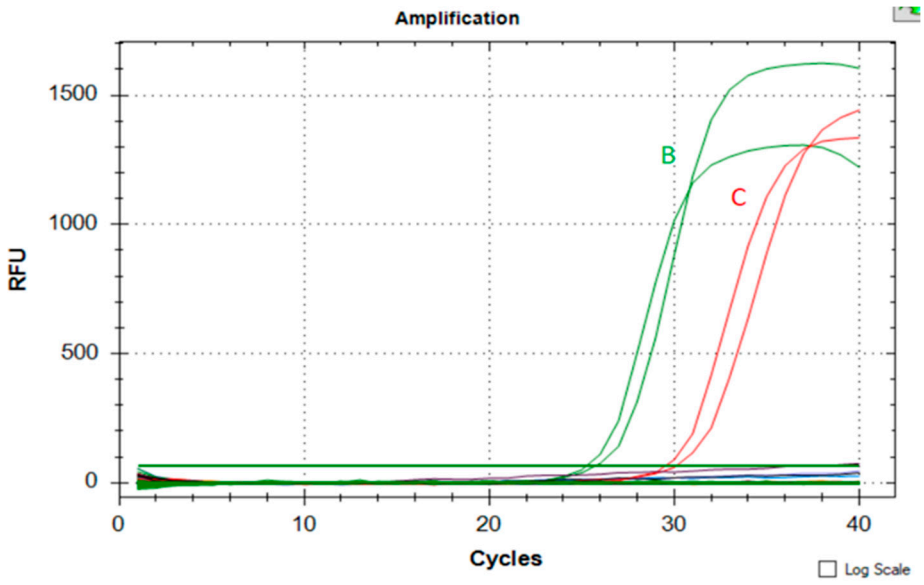

Figure 3. Real-time SALUX19 assay, 100x diluted ≈ 127 pg/µl of nDNA (position B), 1000x diluted ≈ 13 pg/µl of nDNA (position C). Two other positions (A and D) were used as NTC-no template controls. The fluorescent dye SYBR Green Gel Stain was used. An analogous test with the same concentrations of *Sus scrofa* DNA was performed using a CFX Connect real-time PCR system (Bio-Rad, USA).

2.5. Assay validation

Here, we explored a method based on isothermal amplification to rapidly identify DNA originating from S*us scrofa* on the SaLux19 device. Assay validation reactions were prepared in a final reaction volume of 25 µl with nuclease-free water and incubated at 64 °C for 40 minutes on CFX Connect PCR system (Bio-Rad, USA) or SALUX19 (MPI-SERATEC, Germany). Nerbe-Plus 0,1-ml microtubes (cat. no. 04-032-0350 Nerbe plus GmbH & Co. KG, Germany) have proven useful for both real-time devices.

DNA samples from both eukaryotic and prokaryotic species, e.g., *Panthera tigris, Panthera leo, Panthera pardus, Caracal serval,* and *Bos taurus "Salers bull"*, the latter mentioned as a representative of phylogenetically related *Artiodactyla,* further *Homo sapiens* and the ZymoBIOMICS® Microbial Community Standard (ZymoResearch, USA), were cross-tested for assay specificity. A specificity test revealed no amplification signal for any of the examined species. When SYBR Green Gel Stain was replaced with a fluorescent dye for LAMP assays, there was a significant increase in Y-axis-based relative fluorescent units (RFUs), as shown in Figures 3 and 4.

Positive control for amplification

Human male TaqMan™ Control Genomic DNA (cat. no. 431266; Thermo Fisher Scientific, USA) was used as a positive control for amplification. We prepared a dilution series exactly according to the Thermo Fisher application notes [22]. Once de-frosted, the standard was diluted into the needed aliquots, and stored at –20 °C, to prevent repeated freeze-thaw. When assessing sensitivity, successful amplification of the human AMEL-Y target was achieved by repeated use of at least 10 copies of this DNA control (data not shown).

Test arrangement

The standardized SaLux19 run aiming for species identification should include both LAMP diagnostic reagents with primer cocktails in all positions and an examined sample in one device position, next to a suitable target that serves as a control for a successful LAMP reaction (positive control). For this purpose, human male DNA focused on the Y-amelogenin gene was selected. Finally,

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one device position is occupied with no template control (NTC), allowing the room´s purity to be checked.

3. Results

The final, standardized run on **SaLux19** included 10 µl of S*us scrofa* whole nonprecipitating blood lysed in direct DipStick extraction buffer (position **A**), *Sus scrofa* DNA at a concentration of 127 pg/µl nDNA as a positive control (position **B**), human male DNA at 30 copies of the AMEL-Y gene for checking out of own amplification (position **D**), and no template control (NTC, position **C**). In addition to the possibility of *real-time* run monitoring, the addition of fluorescent dye directly to the colorimetric master-mixes allows us to assess the results with the naked eye, as shown in Figure 4.

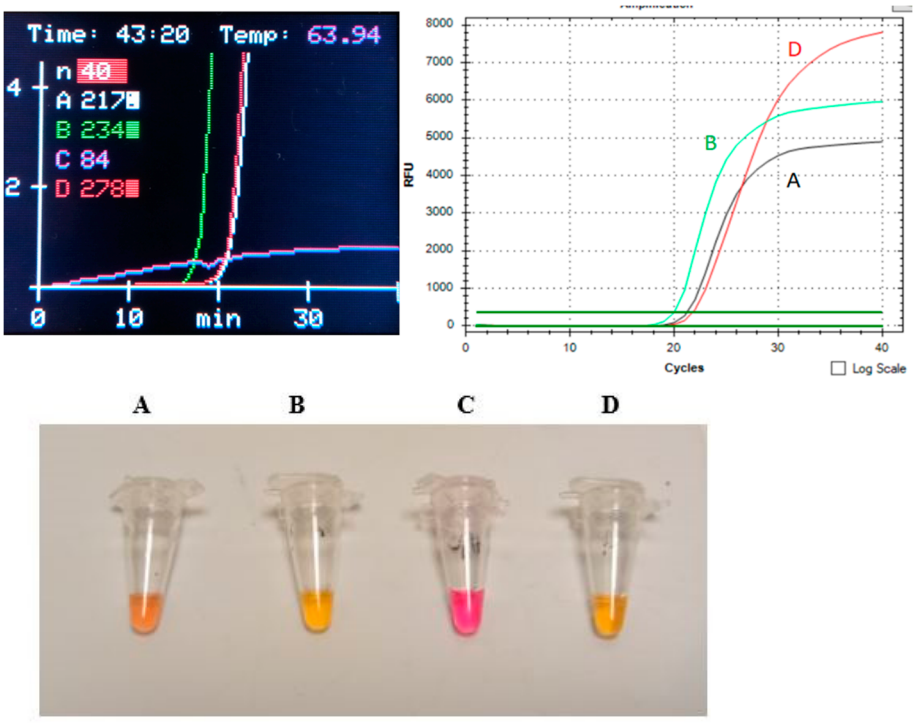

Figure 4. The standard LAMP assay run on SALUX19 Sus scrofa cells. An analogous test with the same concentrations of Sus scrofa DNA was performed using a CFX Connect real-time PCR system (Bio-Rad, USA).

4. Discussion

Here, we report the design, optimization, preliminary tests, and validation of a rapid and portable analysis system for identifying DNA from body fluids of S*us scrofa* origin on the SaLux19 device. The SaLux19 LAMP-based point of care (*POC*) testing includes fluorescent LAMP assay reagents, requires only this pocket-size equipment, and gives results in a short time. The data presented in this report demonstrated that the LAMP analysis system is sensitive enough. Tests of the specificity of the LAMP assay were successfully performed on a real-time the CFX Connect PCR System (Bio-Rad, USA), allowing the testing of more than 4 samples. The developed LAMP assay efficiently excluded 8 other prokaryotes (*Pseudomonas aeruginosa, Escherichia coli, Salmonella enterica, Lactobacillus fermentum, Enterococcus faecalis, Staphylococcus aureus, Listeria monocytogenes,* and *Bacillus subtilis*) and 8 eukaryotic species (members of *Big cats* and *Bovidae* mentioned in the assay validation chapter, *Homo sapiens* and two yeasts: *Saccharomyces cerevisiae* and *Cryptococcus neoformans*).

In addition to the apparent advantages of monitoring one's reaction in *real-time*, another benefit of our system is the double evaluation: when the system enables both fluorescent and colorimetric detection and reading based on the color change of the test tubes. Two steps facilitated the dual evaluation: both the direct addition of the fluorescent dye to the colorimetric master-mix and the

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transfer of reaction mixtures from 0.5 ml thermoblock tubes to thin-walled 0.1 ml tubes, which permits more efficient heat transfer and start-up in quantitative PCR instruments or SaLux19. Moreover, the closed wells eliminated cross-contamination to ensure accuracy, and a less time–consuming procedure was achieved when blood was directly lysed with sodium-hydroxide –based DipStick buffer.

The need for the rapid detection of biological analytes at their *points of care (POC)* led to the development of portable instruments enabling either nucleic acid amplification tests or antigen detection. LAMP assays, when coupled with user-friendly instruments. can deliver the results in tens of minutes (SMART-LAMP [23], DNAiTECH Gen2 [24], GENIE II (OptiGene Ltd., UK) [25]. The SaLux19 instrument offers the following advantages: besides the ability to monitor the progress of the run on the built-in display, there is another benefit of portability and the option of recharging via a USB adapter or even a small power bank.

It is appropriate to discuss the suitable sizes of amplified targets for processed meat products here. Potential degradation of DNA during food processing can adversely impact the amplification of longer amplicons. It has been reported that small amplicons with fragments less than 150 bp are recommended due to their increased sensitivity (LOD) in qPCR assays compared to longer fragments exceeding 200 bp [26]. On the other hand, several studies have been published in which the LAMP assay was shown to have the same detection capability as qPCR [27-30].

How the presented research pipeline, where whole nonprecipitating blood was examined, would work on heat-processed meat products remains to be elucidated. In the meantime, this methodology represents a rational, simple, robust, and low-cost approach for identifying *Sus scrofa* body fluids.

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Conflicts of Interest: W.S. is a co-founder of the SaLux U.G., which produces the SaLux19 device.

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