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# Fast and User-Friendly Detection of Flatfish Species (*Pleuronectes platessa* and *Solea solea*) via Loop-Mediated Isothermal Amplification (LAMP)

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**ABSTRACT:** The detection of a Cytochrome *b* gene (*cytb*) for species differentiation in fish is intensively used. A fast alternative to expensive and time-consuming DNA barcoding is loop-mediated isothermal amplification (LAMP) in combination with efficient readout systems. For this reason, we developed LAMP assays for rapid species detection of *Pleuronectes platessa* and *Solea solea*, two economically important flatfish species in Europe that are prone to mislabeling. Species-specific primer sets targeting *cytb* were designed, and LAMP assays were optimized. With the optimized LAMP assays, we were able to detect up to 0.1 and 0.01 ng of target DNA of *P. platessa* and *S. solea*, respectively, and in each case up to 1% (w/w) of target species in mixtures with nontarget species. For future on-site detection, a lateral flow assay and a pocket-sized lab-on-phone assay were used as readout systems. The lab-on-phone assay with the *S.* solea specific primer set revealed cross-reactivity to *Solea senegalensis*. The assay targeting *P. platessa* proved to be highly specific. Both assays could be performed within 45 min and provided rapid and easy detection of fish species.

**KEYWORDS:** *isothermal species detection, loop-mediated isothermal amplification, flatfish, seafood fraud, Pleuronectes platessa, Solea solea*



## INTRODUCTION

Over 59 million tons of live weight of seafood was internationally exported in 2020, making fish one of the most traded commodities worldwide.[1] For decades, the international trade of fish products has grown significantly from regional to global scale. Involving many actors, the supply chain in the globalized fish market is prone to illegal substitution of fish species. For consumer protection, regulations such as the European Regulation No. 1379/2013 require appropriate species traceability and labeling of the fish by giving information on the commercial denomination and the scientific names of the traded species.[2] To verify the correct specification of the fishery product, the species are traditionally checked for morphological traits. However, analysis of morphological traits is possible only to a limited extent for processed fish products if head, fins, and skin get removed. For this purpose, protein and DNA molecular markers have been developed.[3] DNA can still be analyzed even if the product has undergone intensive processing steps and is hardly influenced by exogenous factors. In addition, DNA has a high identification power at the species and variety levels.[4,5] Mitochondrial DNA genes encoding for Cytochrome *b* (*cytb*) and cytochrome c oxidase subunit I (COI) are considered reliable DNA barcodes for fish species identification[6,7] and hence have been used in technical standards like DIN CEN/TS 17303:2019-06.[8] Moreover, *cytb* has been reported as the preferred target in closely related species.[9,10] However, a major drawback of DNA barcoding is that it is time-consuming and expensive, limiting its suitability for routine analysis as it requires laboratory infrastructure and expert knowledge.[11] Instead of PCR-based detection methods, a fast and simple alternative such as the loop-mediated isothermal amplification (LAMP)[12] could be used. LAMP can be performed in less than 1 h and works at a constant temperature (e.g., *Bst* 3.0 Polymerase, 55−72 °C).[13,14] Thus, LAMP assays are independent of laboratory equipment, such as thermocyclers. Additionally, four−six specially designed primers ensure high specificity and sensitivity for the target species.[15] *Bst* polymerase, which is required for high strand displacement activity, is more resistant to inhibitors, reducing the need for high-quality DNA extracts.[16] Thus, the developed LAMP assays are much more suitable for routine analysis in the laboratory, for in-field testing, and for limited-resource settings than conventional PCR-based methods. LAMP has been successfully developed on *cytb* sequences for a wide range of animal-derived foods, including fish species such as *Salmo salar* and *Oncorhynchus mykiss*[15,16] belonging to the genus *Gadus*,[10,17,18] *Katsuwonus pelamis*,[19,20] *Thunnus albacares*,[21] and *Anguilla anguilla*.[22]

In this study, we developed a loop-mediated isothermal amplification assay for rapid species detection of *Pleuronectes*

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*platessa* and *Solea solea*, economically important fish species in Europe belonging to the order of flatfish (*Pleuronectiformes*).[5,23] Both fish are valued for their excellent organoleptic and sensory properties and high nutritional values.[5] Many flatfish species are difficult to distinguish, especially when processed.[11] For this reason and because of their large price differences to other fish, *P. platessa* and *S. solea* show global mislabeling rates of 5 and 20%, respectively,[24] although higher values have also been reported for *P. platessa*.[7,25,26] To facilitate the verification of the correct specification of *P. platessa* and *S. solea* and to uncover fraudulent activities in the fish sector, specific LAMP primers targeting the *cytb* region of the mitochondrial genome were developed for both target species. Moreover, we optimized the assay for easy sampling, rapid and simple DNA extraction, and tested two detection techniques (lateral flow assay (LFA) and hand-held fluorescence detector (Doctor Vida pocket test)) to facilitate in-field monitoring for food regulatory agencies and on-site species verification for fishing industry stakeholders. An LFA is a popular platform for on-site diagnosis[27] that has been shown to provide fast and reliable test results for untrained personnel (e.g., during the COVID-19 pandemic[28]). The LFA technique is usually based on the biochemical interaction between an antigen and an antibody, and, in a modification, can also be used to detect DNA amplicons.[27,29] The Doctor Vida pocket test was originally designed for fast and easy molecular diagnosis of SARS-CoV-2. The portable and hand-held device allows isothermal amplification of any genetic material and can measure fluorescence in real time. The progress of amplification can be monitored via a mobile app (Dr Vida Pocket PCR).[30] The isothermal lab-on-phone assay offers the advantage of real-time monitoring, simple and user-friendly operation, and more informative results than LFAs. Both platforms have the potential to enable in-field detection of fish species and thus contribute to compliance with current legislation and extend already existing in-field readout systems for *Pleuronectiformes*.[31]

## MATERIALS AND METHODS

**Sample Material.** Raw or frozen, whole, or filleted fish samples as well as fish samples in ethanol were obtained from local farmer's markets or local producers or had been collected in previous projects (Table 1). All samples were authenticated by PCR and sequencing of an approximately 415 bp *cytb* fragment according to DIN CEN/TS 17303:2019-06.[8]

**DNA Extraction.** DNA was extracted according to Rehbein et al.[32] Muscle tissue (100 mg wet weight) was mixed with 0.5 mL of buffer 1 (1.2% (w/v) CTAB, 60 mM Tris, 10 mM $Na_2$-EDTA, and 0.8 M NaCl, pH 8.0). Directly before use, 3-mercapto-1,2-propanediol 0.1% (v/v) and proteinase K (final concentration 0.5 mg/mL) were added to buffer 1. The reaction-mix was incubated for 1 h at 65 °C, cooled to room temperature, and centrifuged for 10 min at 10,625*g*. 500 μL of chloroform was added to the supernatant. The two phases were mixed by inverting for 30 s and centrifuged for 10 min at 10,625*g*. The step was repeated once. Then, two volumes of buffer 2 (1% (w/v) CTAB, 50 mM Tris, 10 mM $Na_2$-EDTA, pH 8.0) was added to the supernatant, centrifuged, and 400 μL of buffer 3 (1 M NaCl, 10 mM Tris, 1 mM $Na_2$-EDTA, pH 8.0) was added to the precipitate. The mixture was heated at 65 °C for 10 min, and 400 μL of isopropanol was added. The mixture was incubated for 10 min at room temperature. After centrifugation, the precipitate was washed twice with 500 μL of ethanol (70% (v/v)). The dried precipitate was dissolved in 100 μL of buffer 4 (10 mM Tris, 1 mM $Na_2$-EDTA, pH 8.0). DNA quality (260/280 nm and 260/230 nm ratios) was determined with the Nanodrop One (Thermo Fisher Scientific Inc., Waltham, MA). DNA quantity was determined fluorometrically via the QuantiFluor dsDNA System on a Quantus Fluorometer (both from Promega GmbH, Walldorf, Germany). All samples were stored at −20 °C.

**Table 1. Overview of Fish Samples with FAO Fishing Area and Processing Status**

| sample | species | FAO fishing area/origin | processing |
|---|---|---|---|
| Pplat | *P. platessa* | 27 IV | whole fish |
| Pplat1 | *P. platessa* | 27 IV | whole fish |
| Pplat2 | *P. platessa* | 27 IV | whole fish |
| Pplat3 | *P. platessa* | 27 IV | whole fish |
| Pplat4 | *P. platessa* | 27 IV | whole fish |
| Ssol | *S. solea* | 27 | whole fish |
| Ssol1 | *S. solea* | 27 | filleted |
| Ssol2 | *S. solea* | 27 | filleted |
| Ssol3 | *S. solea* | 27 | filleted |
| Lasp | *Limanda aspera* | 61 | filleted |
| Ssen | *Solea senegalensis* | aquaculture (Iceland) | whole fish |
| Pfles | *Platichthys flesus* | 27 IIIc | whole fish |
| Pqua | *Pleuronectes quadrituberculatus* | 67 | filleted |
| Llim | *Limanda limanda* | 27 IIIc | whole fish |
| Mkitt | *Microstromus kitt* | 27 IV | whole fish |
| Phyp | *Pangasius hypophthalmus* | Vietnam | filleted |
| Saeg | *Solea aegyptiaca* | Italy | DNA isolate |
| Gmor | *Gadus morhua* | 27 | filleted |
| Smax | *Scophthalmus maximus* | 27 | filleted |
| Rhip | *Reinhardtius hippoglossoides* | 27 | filleted |
| Hhipp | *Hippoglossus hippoglossus* | 27 | filleted |

**Simplified Sampling and DNA Extraction.** To be independent of the laboratory infrastructure, various DNA extraction buffers were tested. Centrifugation steps were omitted for all of the extraction methods. The optimized LAMP assay was tested with DNA isolates extracted with (i) KOH-based lysis solution and related protocol,[33] (ii) 100 μL of *Bst* 3.0 polymerase storage buffer (New England Biolabs Inc., Ipswich, MA), (iii) Kapa Express Extract protocol (F. Hoffmann-La Roche Ltd., Basel, Switzerland), (iv) 100 μL of 1× TE-Buffer (Promega GmbH, Walldorf, Germany), and (v) 100 μL of ultrapure water for 10 min, respectively. Isohelix Buccal Swabs (Cell Projects Ltd., Kent, U.K.) and medex cytobrushes (medesign I.C. GmbH, Linden, Germany) were used to facilitate tissue collection.

**LAMP Primer Design.** Cytb sequences of *P. platessa* (EU492113.1, EU492112.1, EU224075.1, EU492292.1, EU492291.1, EU224076.1, AY164472.1), *S. solea* (EU492072.1), *S. senegalensis* (FJ528353.1, DQ198002.1, EF427602.1), *Buglossidium luteum* (EU492126.1, EU492268.1), *Microchirus azevia* (DQ197963.1, EF392600.1, EF392599.1, AB125329.1, AY164464.1, FJ515650.1), *Synaptura lusitanica* (AB125333.1, AY164468.1), *Dagetichthys lusitanica lusitanica* (EF439605.1), *Cynoglossus senegalensis* (DQ197938.1), *Glyptocephalus cynoglossus* (EU492157.1, EU492156.1, EU492258.1, EU492257.1), and *Atheresthes stomias* (MH031802.1, MH031801.1) were retrieved from GenBank (NCBI) and aligned using Clustal Omega.[34] The obtained multiple sequence alignment (MSA) in NEXUS format was then used to generate a haplotype file with DnaSP 6 software.[35] Sequences of the same haplotype were reduced to one surrogate sequence. All haplotype sequences were realigned to obtain an MSA for LAMP primer oligonucleotide design by PrimerExplorerV5. Specific primer sets for *P. platessa* and *S. solea* consisted of outer primers (F3 and B3), forward inner primers FIP (F1c and F2), backward inner primers BIP (B1c and B2), and if applicable, two loop primers (LF and LB). At least two primer sets for each species were designed. Table 2 and Figures S1 and S2 show the primer sets, which generated the most robust results.


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**Table 2. LAMP Primer Sets Used for Specific Amplification of *P. platessa* and *S. solea***

| species | target | primer | sequence (5′−3′) | $T_m$ (°C) |
|---|---|---|---|---|
| *P. platessa* | *cytb* | Plat_F3 | GCTTCGCAGTCCTCCTCA | 60.65 |
| | | Plat_F2 | CTGCACTGGCTTCACTCG | 59.80 |
| | | Plat_F1c | AGGCGTGAAGTTGTCTGGGTCT | 65.79 |
| | | Plat_B3 | GCCAAGCTTGTTTGGGATG | 59.01 |
| | | Plat_B2 | AGCGGAGAATGGCGTAGG | 60.80 |
| | | Plat_B1c | GTCACGCCGCCACACATCAA | 65.45 |
| | | Plat_FIP_LFA | 5′Biotin/AGGCGTGAAGTTGTCTGGGTCT-CTGCACTGGCTTCACTCG | 70.30 |
| | | Plat_BIP_LFA | 5′FAM/GTCACGCCGCCACACATCAA-AGCGGAGAATGGCGTAGG | 70.70 |
| | | Plat_LB | GCCAGAGTGATACTTCCTCTTTG | 60.44 |
| *S. solea* | *cytb* | Sol_F3 | GCCTCTGCCTAATTACCCAA | 59.09 |
| | | Sol_F2 | GTCACAGGACTCTTACTCGC | 59.14 |
| | | Sol_F1c | ACGACGGAGGAAAATGCGGTAG | 64.50 |
| | | Sol_B3 | TCCTCGTCCGATGTGAAGG | 60.43 |
| | | Sol_B2 | AAGAATGATGCGCCGTTGG | 60.68 |
| | | Sol_B1c | GCACATCTGCCGGGACGTTA | 64.46 |
| | | Sol_LF | AGGCATCTGCTGTATAGTGCA | 61.07 |
| | | Sol_LB | GGATGATTAATCCGAAATGTCCACG | 61.88 |

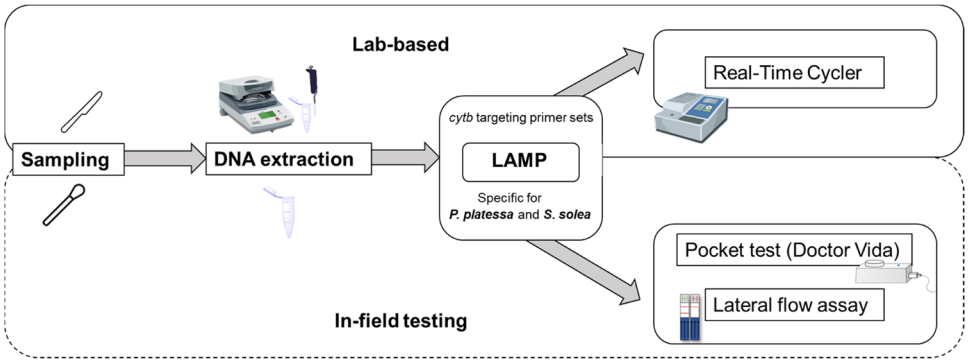


**Figure 1.** Overview of the implemented workflow. The lab-based workflow uses a scalpel for sampling and an elaborated DNA extraction method to detect successful LAMP amplification with the Real-Time Cycler. In-field testing uses simplified sampling and DNA extraction combined with straightforward readout systems.

**LAMP Reaction.** LAMP reaction parameters were optimized to maximize the time gap between successful amplification of the target sequence (time-to-positive) and unspecific amplifications. In a design of experiment approach, the influence of temperature (60−72 °C), final concentration of *Bst* 3.0 polymerase (0.04−1.4 U/μL), $MgSO_4$ (4−10 mM), and dNTPs (0.4−1.4 mM) on the reaction efficiency were tested, and the results were analyzed with the JMP software version 16.2 (SAS Institute Inc., North Carolina). The optimized LAMP reaction-mix consisted of 1 μL of DNA extract, 2.5 μL of 10× isothermal reaction buffer, 0.5 μL of $MgSO_4$ solution, 0.32 U/μL *Bst* 3.0 Polymerase (all from New England Biolabs Inc., Ipswich, MA), and 1 μL of 25× primer-mix (40 μM FIP, 40 μM BIP, 5 μM F3, 5 μM B3, if applicable 10 μM LF and 10 μM LB; Integrated DNA technologies Inc., Coralville). When used for fluorescence measurements, 1 μL of SYTO 9 Green (1 μM, Thermo Fisher Scientific Inc., MA) was added. Ultrapure water was used to reach a reaction-mix volume of 25 μL. LAMP reaction was run on a CFX96 Touch Real-Time PCR System (BioRad Laboratories Inc., Hercules, CA). The temperature was set at 72 °C and the fluorescence was recorded after 30 s incubation steps plus fluorescence measurement (average 6 s). To create comparability between assays, we refer to this as one amplification time unit (approx. 36 s). The reactions were stopped by raising the temperature to 80 °C for 5 min.

**Detection via LFA.** For detection via lateral flow assay, the Milenia HybridDetect lateral flow stripes (Milenia Biotec GmbH, Gießen, Germany) were used. For successful isothermal amplification of the *cytb* target sequence, 5′-biotin-labeled FIP and 5′-FAM-labeled BIP were used in the 25× primer-mix (IDT Integrated DNA Technologies Inc., Coralville, IA). LAMP assays were carried out as described above. Then, 10 μL of the LAMP reaction mixture was added to the sample pad of the lateral flow stick, and the assay was carried out as described in the manufacturer's instructions. A positive control and a negative control were each included in triplicate in each series of experiments. In addition, two no template controls (NTC) were used in which water instead of DNA was added to the LAMP master mix.

**Detection via Doctor Vida Pocket Device.** In parallel with the LAMP assay on the real-time cycler, we used the reusable, portable, and hand-held pocket device Doctor Vida Pocket Test (STAB VIDA Lda, Caparica, Portugal) for isothermal amplification and simultaneous fluorescence measurement. The composition of the LAMP reaction mixture for the pocket device measurements was identical with the reaction mixture used on the real-time cycler. To prevent condensation in the reaction tube, 50 μL of mineral oil was added on top of the reaction mixture. The results of the Doctor Vida pocket test are displayed as a report in the associated app. The intensity of the fluorescence is shown as a function of the duration [minutes]. All measurements on the Doctor Vida pocket test device were repeated using the real-time cycler (CFX96 Touch Real-Time PCR System, BioRad Laboratories Inc., Hercules, CA) to confirm positive or negative amplification, respectively.

**Specificity and Sensitivity of LAMP Assays.** To assess the specificity of the two LAMP assays, DNA from all species listed in Table 1 was tested in triplicate. For the sensitivity of both established LAMP assays, a serial dilution of target DNA from 10 ng to 0.01 pg was used. Each dilution was run in triplicate. Additional sensitivity


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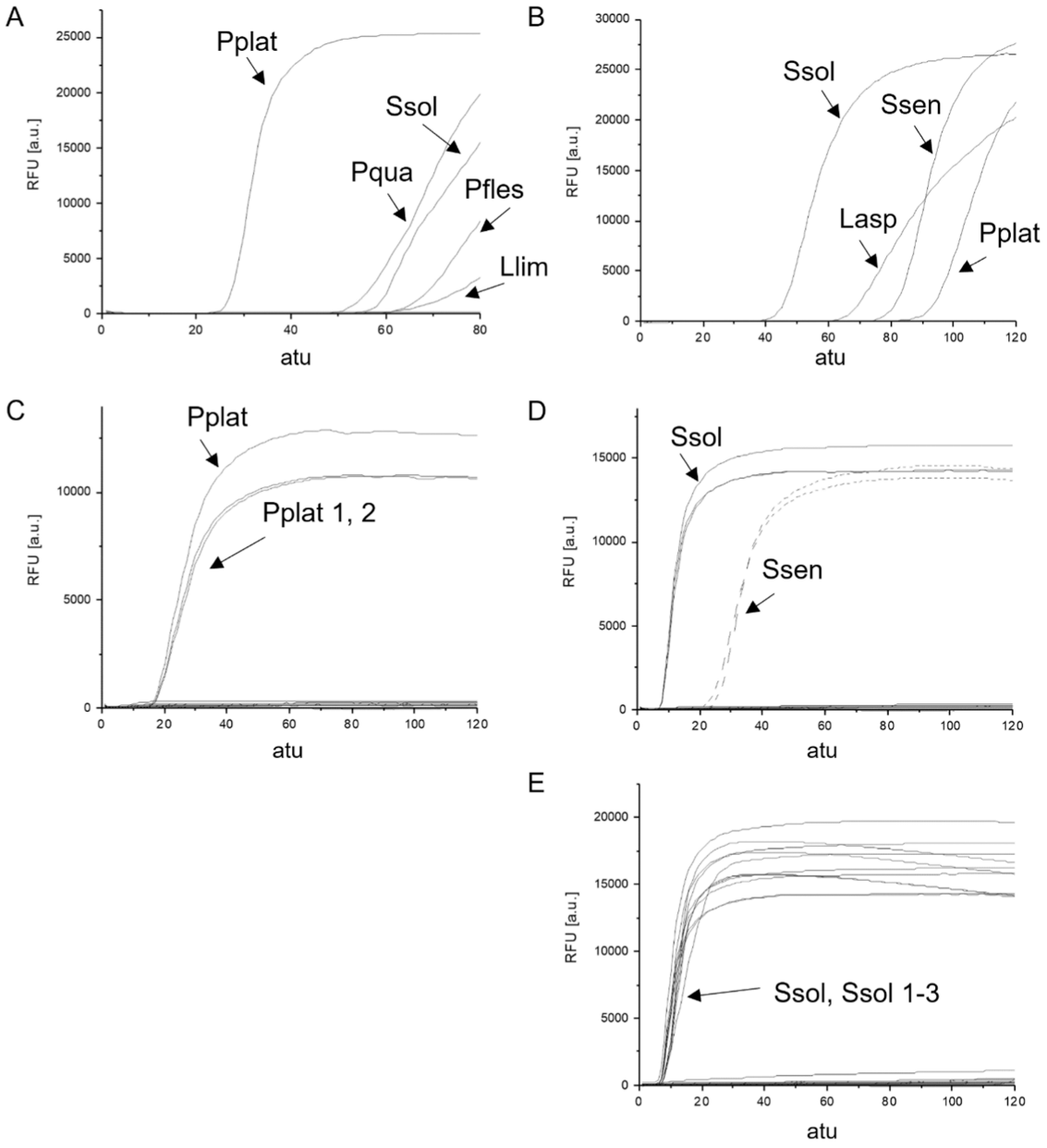


**Figure 2.** Amplification profiles of real-time fluorescence LAMP assays. (A) Initial *P. platessa* LAMP assay before optimization; DNA input amount, 10 ng. (B) Initial *S. solea* LAMP assay before optimization; DNA input amount, 10 ng. (C) Optimized *P. platessa* LAMP assay with three different *P. platessa* samples. All other samples described in Table 1 were negative; DNA input amount, 10 ng. (D) Optimized *S. solea* LAMP assay. Three of 3 Ssol and 2 of 3 Ssen measurements were amplified. The amplification of Ssen was approximately 10 atu later than for Ssol; DNA input amount, 10 ng. (E) *S. solea* LAMP assay with reduced DNA input amount of 1 ng. Four different Ssol samples were successfully amplified, while all other samples described in Table 1 were negative. atu = amplification time unit.

tests were carried out by measurements of DNA extracts from fresh homogenized *S. solea* sample spiked with 10, 1, 0.1, and 0.01% (w/w) fresh homogenized *P. platessa* sample.

## RESULTS AND DISCUSSION

Rapid and easy verification of the correct species in fish depends on user-friendly sampling, rapid DNA extraction, specific but easy to perform amplification of the target sequence, and visualization methods that are independent of laboratory infrastructure. DNA amplification in this study was performed using an isothermal amplification method (LAMP, loop-mediated isothermal amplification). For this purpose, specific LAMP primer sets were designed for the *cytb* sequence of *P. platessa* or *S. solea*. Optimized assays were tested with isolates obtained by different DNA extraction methods to combine user-friendly sampling, DNA extraction, and specific amplification. To enable on-site testing, two different readout systems (lateral flow assay or Doctor Vida pocket test) were used to demonstrate successful formations of LAMP amplicons (see Figure 1).

**LAMP Assay.** In this study, we developed LAMP primer sets targeting a partial sequence of *cytb* to detect two flatfish species (*P. platessa* and *S. solea*). A multiple sequence alignment (MSA) was created for mitochondrial *cytb* sequences of most common haplotypes of target and nontarget fish species. MSA was then used to generate target-specific LAMP primer sets for *P. platessa* and *S. solea*. The primer sets were first checked using the NEB's typical LAMP protocol for


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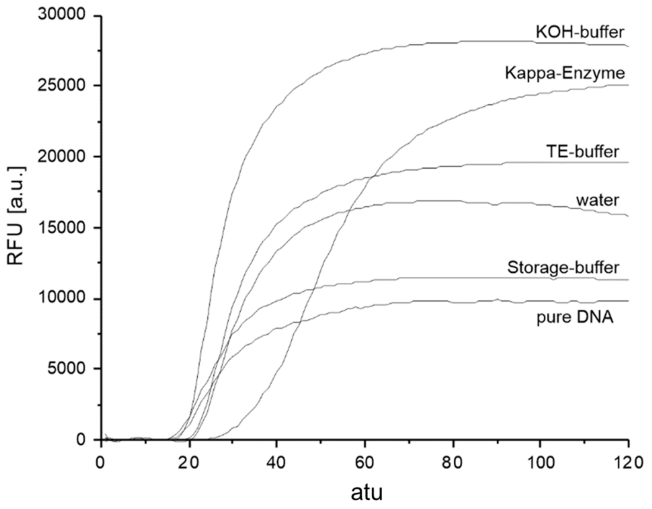


**Figure 3.** Amplification profiles of real-time fluorescence *P. platessa* LAMP assay tested with different extraction buffers, namely, (i) KOH-based lysis solution and related protocol,[32] (ii) Bst 3.0 polymerase storage buffer, (iii) Kapa Express Extract protocol, (iv) 1× TE-Buffer, and (v) ultrapure water compared to pure DNA extracts. Pplat extract was used as sample. One microliter of lysate was used as input volume. atu= amplification time unit.

*Bst* 3.0 (1× Isothermal Amplification Buffer II, 6 mM $MgSO_4$, dNTP Mix (1.4 mM each), 1.6 $\mu$M of each inner primer, 0.2 $\mu$M of each outer primer, 0.4 $\mu$M of each loop primer, 320 U/mL *Bst* 3.0, 65 °C for 60 min). If no or only minor differences between the positive and negative control were found, the primer set was discarded. For both target species, a total of three primer sets was designed (see Table S1 for sequences). The LAMP primer sets shown in Table 2 generated the best results in initial testing (see Figure 2A,B). The optimization of reaction parameters was performed using a real-time cycler in which the temperature was set on 65 °C. To compare the different approaches, fluorescence measurements were performed after each 30 s incubation at this temperature. One incubation time (30 s) plus one fluorescence measurement (average 6 s) is referred to as amplification time unit (atu) in the following. *P. platessa cytb* specific LAMP primer set amplified target DNA after approximately 25 amplification time units (atu) and *S. solea cytb* specific LAMP primer set amplified target DNA after approximately 45 atu. Nontarget species were amplified after approximately 50 atu for the *P. platessa* primer set and after 60 atu for the *S. solea* primer set. The initial LAMP assay parameters did not allow straightforward species testing. The observed nonspecific amplification is a core problem of the LAMP technique[36] and probably arises from mechanisms like linear target isothermal multimerization and amplification (LIMA),[37] unusual isothermal multimerization and amplification (UIMA),[38] or from the extension of double-stranded products initiated by homodimerization of the BIP.[36] The presence of nonspecific amplicons can negatively

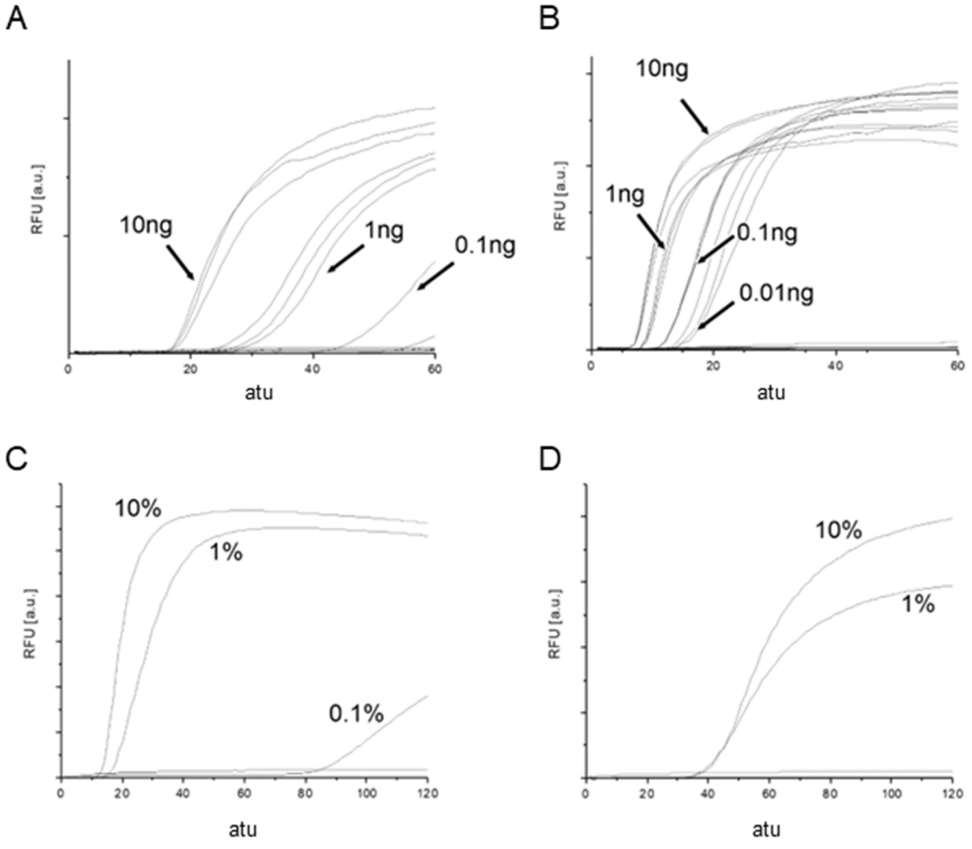


**Figure 4.** Amplification profiles of real-time fluorescence LAMP assays. (A) Limit of detection of the *P. platessa* LAMP assay with Pplat DNA extracted according to Rehbein.[32] Dilution series was tested in 3-fold with optimized assay parameters. (B) Limit of detection of the *S. solea* LAMP assay with Ssol DNA extracted according to Rehbein.[32] Dilution series was tested in 3-fold with optimized assay parameters. (C) Mixtures of *P. platessa* in *S. solea* of 10, 1, 0.1, and 0.01% (w/w) were tested with the *P. platessa* specific LAMP primer set. (D) Mixtures of *S. solea* in *P. platessa* of 10, 1, 0.1, and 0.01% (w/w) were tested with the *S.* solea specific LAMP primer set. atu = amplification time unit.


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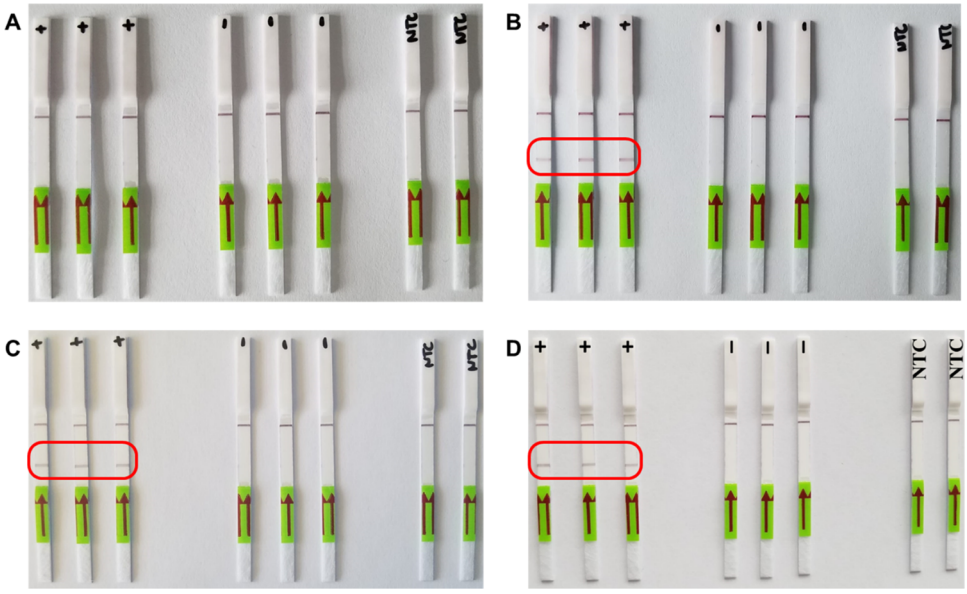


**Figure 5.** Detection of *P. platessa* specific *cytb* sequence LAMP amplicon via lateral flow dipstick. + = *P. platessa*, − = *S. solea*, NTC = no template control. (A) LAMP incubation for 15 min at 72 °C, without LB primer. (B) LAMP incubation for 30 min at 72 °C, with LB primer. (C) LAMP incubation for 20 min at 72 °C, with LB primer. (D) LAMP incubation for 30 min at 72 °C, with LB primer and storage buffer isolates.

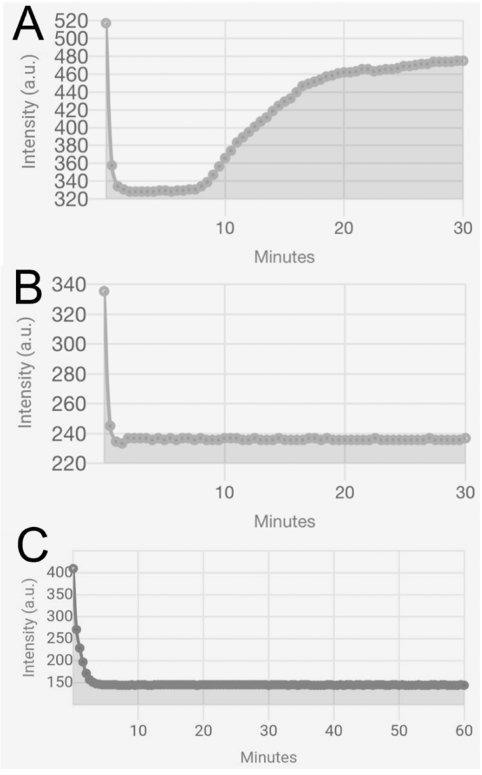


**Figure 6.** Amplification profiles of real-time fluorescence LAMP assays via the Doctor Vida pocket test. (A) *P. platessa cytb* specific LAMP primer set tested with Pplat (*P. platessa*) DNA extracted with the storage buffer for 10 min at ambient temperature. (B) Negative control measurement: *P. platessa cytb* specific LAMP primer set tested with Ssol (*S. solea*) DNA extracted with the storage buffer for 10 min at an ambient temperature. (C) No template control measurement.

affect the analytical sensitivity and specificity of the assay.[36] Potential dimer structures, for example, may be destroyed by temperatures above 70 °C.[39] Hence, to maximize the difference between the amplification time of the target and nontarget species, the effect of temperature (60−72 °C), and additionally the final concentration of *Bst* polymerase (0.04−1.4 U/μL), $MgSO_4$ (4−10 mM), and dNTPs (0.4−1.4 mM) on the reaction efficiency were empirically tested with a design of experiment. Optimization resulted in 72 °C as the most selective reaction temperature and a final concentration of *Bst* 3.0 Polymerase and dNTPs of 0.32 U/μL (Figure S3), and 1.4 mM for each dNTP (Figure S4). The $MgSO_4$ concentration did not have a high impact on the successful LAMP reaction, but 4 mM gave the most reproducible results. Interestingly, other groups found a significant effect of $Mg^{2+}$.[14,40] $MgSO_4$ concentration from 2 to 8 mM significantly improved the LAMP reaction.[40] Under optimized reaction conditions, no nonspecific amplification was observed in the 120 atu range for the *P. platessa* specific LAMP primers (Figure 2C), providing a viable test system with high specificity toward the intended target sequence. For the *S.* solea specific LAMP primers (Figure 2D), an off-target amplification (2 of 3 measurements) for the closely related *S. senegalensis* was observed. The amplification of *S. senegalensis* was approximately 10 atu later than the amplification for the *S. solea* samples. In other studies, delayed nonspecific amplification of closely related nontarget species was described as negligible.[41−43] However, when we reduced the initial nucleic acid concentration from 10 to 1 ng per batch, no off-target amplification was detectable within the 120 atu range (Figure 2E). Hence, preferably 1 ng target DNA should be used for the LAMP assay described in this study.

**Fast DNA Extraction Methods.** Isothermal amplification such as LAMP is preferred for in-field testing because no thermocycler is required. Furthermore, for in-field testing, rapid DNA extraction is essential for the efficient application of the assay. Hence, time-consuming and labor-intensive purification steps should be avoided whenever possible. The *Bst* 3.0 polymerase used for LAMP assays is more resistant to PCR-inhibitors than, for example, Taq polymerase, which offers the advantage that DNA isolates from simple extraction protocols can also be used.[44] In the present study, several extraction protocols were tested and evaluated for performance


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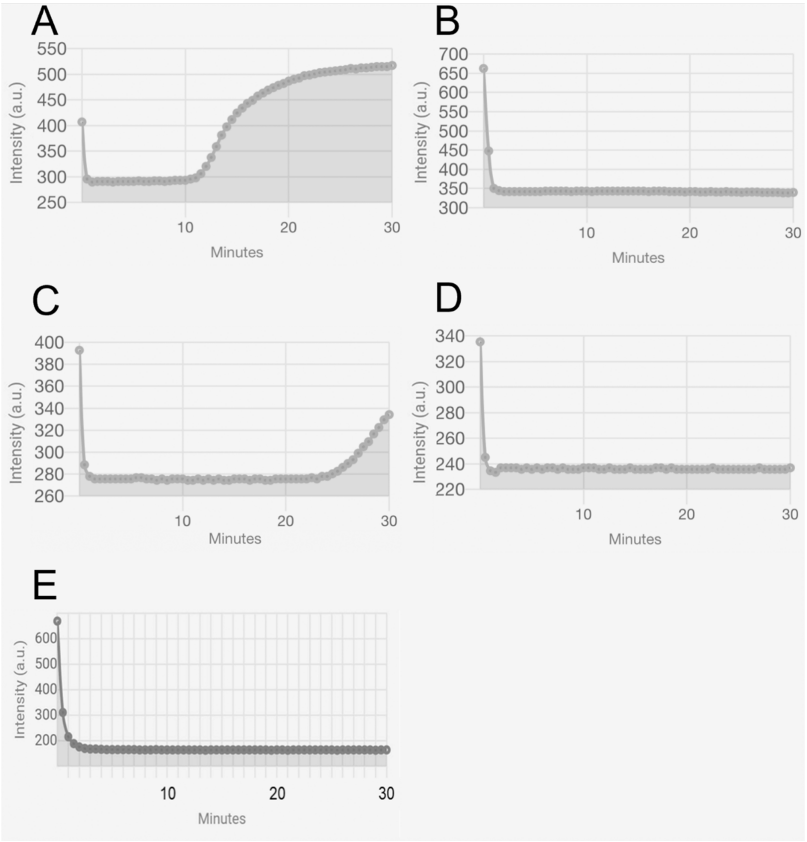


**Figure 7.** Amplification profiles of real-time fluorescence LAMP assays via the Doctor Vida pocket test. (A) *S. solea cytb* specific LAMP primer set tested with Ssol DNA extracted with the storage buffer for 10 min at an ambient temperature. (B) Negative control measurement: *S. solea cytb* specific LAMP primer set tested with Pplat (*P. platessa*) DNA extracted with the storage buffer for 10 min at an ambient temperature. (C) Negative control measurement: *S. solea cytb* specific LAMP primer set tested with Ssen (*S. senegalensis*) DNA extracted with the storage buffer for 10 min at an ambient temperature. (D) Negative control measurement: *S. solea cytb* specific LAMP primer set tested with Saeg (*S. aegyptiaca*) DNA. (E) No template control measurement.

using the optimized *P. platessa* specific LAMP assay (Figure 3). Two of the tested extraction methods (i) and (iii) consisted of a two-step protocol and were performed at either 90 °C (i) or 75 and 95 °C (iii). The DNA lysate of the KOH-buffer protocol showed the same amplification performance as pure DNA isolates did. Lysing the sample material and adding additional enzymes (KAPA Express Extract Enzyme) to the lysate resulted in an amplifiable DNA isolate but with a reduced efficiency, as evidenced by a higher time-to-positive. In the next three extraction protocols tested, TE-buffer, ultrapure water, and *Bst* 3.0 polymerase storage buffer were used as lysis solutions. The sample material was incubated in the respective solution for 10 min at room temperature, and 1 $\mu$L of the extract was then used for the LAMP assay as template DNA. Extracts of all three DNA lysates were successfully amplified using the optimized LAMP assay (Figure 3). The amplification efficiency was comparable to that of pure DNA isolates. Since samples were not homogenized and TE-buffer and ultrapure water did not contain any lysis components, the sampling step must be sufficient to disrupt the cells and bring enough DNA molecules into solution. Sample components that were coisolated did not significantly affect amplification success, as described above.[45] The storage buffer contained 0.1% Triton X-100, which acts as a surfactant active agent and thus improves the lysis activity of the buffer. Extracted DNA using the storage buffer was slightly faster to amplify overall, as a higher DNA concentration was obtained using this method (Figure 3).

**Sensitivity.** To evaluate the sensitivity of the optimized *P. platessa* and *S.* solea specific LAMP assays, 10-fold dilutions of 10, 1, 0.1, and 0.01 ng target DNA were prepared from pure DNA isolates and amplification was measured on the real-time cycler. Sensitivity of the assays was set at the lowest concentration at which a fluorescence signal was detectable. For the *P. platessa* specific LAMP assay, the lowest detectable level was 0.1 ng template DNA and for *S. solea* the lowest detectable level was 0.01 ng (Figure 4A,B). The higher sensitivity of the *S. solea* primer set is due to the use of both the LF and LB primers. Loop primers have been shown to significantly improve amplification sensitivity and speed.[45] For the *P. platessa* primer set, only a LB primer could be designed, which could explain the slightly lower LOD of the *P. platessa* assay. Compared to the recently published results of Deconinck et al.,[31] the LOD of the *S. solea* assay of this study is lower than 0.1 ng because instead of a colorimetric readout, a more sensitive method has been used. To sum up, the *P. platessa* specific LAMP assay detected up to 0.1 ng of target DNA, and the *S. solea* specific LAMP assay detected 0.01 ng. Both assays successfully detected up to 1% (w/w) of target species in mixtures with nontarget species. In addition to measuring a 10-fold dilution series, mixtures of target fish samples (either *P. platessa* or *S. solea*) were prepared in nontarget fish samples of 10, 1, 0.1, and 0.01% (w/w) (Figure


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4C,D). DNA was extracted with 100 mL of storage buffer for 10 min at room temperature. 10, 1, and 0.1% *P. platessa* were detected in nontarget fish samples (*S. solea*). The high sensitivity of the assay is not surprising and has been previously achieved in similar fish authentication studies.[10,14,22,46] However, for in-field testing, oversensitive test methods tend to amplify even trace levels of contamination, leading to "false-positive" results. For example, the UK Food Standards Agency (FSA) recommends a limit of detection of 1% (w/w) for meat speciation assays to prevent amplification of trace amounts.[18] Hence, the goal was to decrease the sensitivity of the assay. Increasing the volume of the extraction buffer from 100 $\mu$L to 1 mL decreased the concentration of the lysate. Consequently, the nucleic acid concentrations of the contaminants are also diluted. After adjusting the extraction volume, an increase in fluorescence was observed for 10 and 1% (w/w) lysates during the first 20 atu, while a negligible increase in fluorescence was observed for 0.1% lysates after 80 atu (Figure 4C). For 10, 1, and 0.1% (w/w) *S. solea* tissue in nontarget fish samples (*P. platessa*), only 10 and 1% (w/w) lysates were successfully amplified (Figure 4D). Thus, both specific assays have sufficient sensitivity and meet the FSA criteria described above.

**Detection of Successful *P. platessa* DNA Amplification via LFA.** To provide a rapid and user-friendly detection method of specific amplification of mitochondrial *cytb* sequences, a lateral flow analysis approach was used as the LAMP readout platform. Since no LF primer was available, we used labeled FIP and BIP primers with 5′-biotin and 5′-FAM, respectively. This combination has been used several times and is characterized by high specificity and the ability to be used for multiplexing.[47−49] Figure 5A shows the assay without an added loop primer. The LAMP reaction was incubated at 72 °C for 15 min. All dipsticks showed a red control band. The test band did not show any color change for any dipstick; thus, all dipsticks were considered negative. Figure 5B shows the results of the LAMP reaction at 72 °C for 30 min. A clear test band was observed for all three positive control dipsticks, while the negative controls and NTC showed only the red control band. Thus, all three dipsticks have positively detected the *cytb* partial sequence of *P. platessa* after 30 min. To further reduce the time for positive detection by LFA, loop backward primer LB was added to the primer-mix. As previously described by Nagamine et al. (2002), the addition of the LB primer to the primer-mix results in the reduction of the total reaction time.[45] In this case, the addition resulted in a reduction of approximately 10 min. Figure 5C shows the results of LFA with LB primer and incubation at 72 °C for 20 min. The negative control and NTC were negative. In the positive control, the test bands were clearly visible. Additional additives such as guanidine chloride could shorten the LAMP amplification time if faster detection times are desired.[50] Figure 5D shows the application of DNA isolates obtained by rapid extraction methods (e.g., storage buffer) to the lateral flow dipstick. As in the previous two experiments, only the control band was visible for the negative control and for the no template control. For the positive control, the test band was clearly visible. This demonstrates the applicability of rapidly extracted isolates for LFA tests, supporting the user-friendly implementation of in-field analysis.

**Detection via Doctor Vida.** The Doctor Vida pocket test is a pocket-sized device for isothermal amplification and allows for easy and rapid on-site measurement of fluorescence. The device has been used successfully for on-site detection of SARS-CoV-2.[30] Compared to other portable or smartphone-based fluorescence detection instruments,[51,52] the Doctor Vida pocket test offers control of the temperature and real-time monitoring of the fluorescence profile. Thus, this technique should be generally suitable for the detection of LAMP assays in the field. In the present study, the applicability of the Doctor Vida pocket test device was tested with developed assays for *P. platessa* and *S. solea*. Several preliminary tests were conducted to optimize the application of the LAMP assay for the Doctor Vida pocket test. Since the instrument does not have a heating lid, mineral oil was used as a reaction-mix overlayer to prevent evaporation. For the *P. platessa cytb* specific LAMP primer set, a significant increase of the fluorescence was observed after 15 min for the positive control extracted with the storage buffer (Figure 6A). The assay with the *P. platessa* specific LAMP protocol was repeated in each case with negative control samples and NTC. No amplification was detected in the corresponding measurements using the Doctor Vida pocket instrument (Figure 6B,C). The results are congruent with the measurements made using the real-time cycler. Thus, the *P. platessa* specific LAMP assay could be successfully applied to the Doctor Vida pocket test and thus represents a potential detection device for future on-site testing for fish species. To test whether the optimized volume and $MgSO_4$ conditions also work for the *S.* solea specific LAMP primer assay, the tests were repeated with specific positive (*S. solea*) and negative (*P. platessa*, *S. senegalensis*, and *S. aegyptiaca*) controls for the *S. solea* assay. The results of the measurement of *S. solea* with the Doctor Vida pocket test are shown in Figure 7. There was little difference in the amplification of the *cytb* partial sequence of *S. solea*. In Figure 7A, the measurement of the *S. solea* storage buffer isolate showed a time-to-positive value of 13.5 min. For nontarget fish species, *P. platessa*, *S. senegalensis*, and *S. aegyptiaca* were also analyzed. The fluorescence for *P. platessa* DNA remained on the baseline level (Figure 7B). DNA from *S. senegalensis* showed an increase in the fluorescence intensity after 25 min (Figure 7C) when DNA was extracted using the storage buffer method. The fluorescence for *S. aegyptiaca* DNA was similar to that of NTC fluorescence.

Remarkably, all negative controls simultaneously measured on the BioRad real-time cycler matched the NTC fluorescence intensity (Figure S5). Although inhibition of *S. senegalensis* amplification would be desirable, a 11.5 min difference to the positive control is sufficient for species detection.[40−42]

Like the LFA-based assay, the pocket-sized lab-on-phone assay was successfully performed in a total time of 45 min and provided rapid and simple fish species detection for the two commercially important flatfish species, *P. platessa* and *S. solea*.

## ■ ASSOCIATED CONTENT

### Data Availability Statement

Fluorescence data can be accessed via the following link: 10.5281/zenodo.7876076.

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c03917.

All designed LAMP primer sets for *P. platessa* and *S. solea* (Table S1); overview of primer binding sites of *P. platessa* specific primer set on a partial *cytb* sequence (Figure S1); overview of primer binding sites of *S.* solea specific primer set on partial *cytb* sequence (Figure S2);


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difference of cq values of positive and negative control from the design of experiment (Figure S3); influence of dNTP concentration on amplification success of *P. platessa* specific LAMP (Figure S4); and amplification profiles of real-time fluorescence LAMP assays simultaneously to Doctor Vida pocket Test (Figure S5) (PDF)

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## ABBREVIATIONS

atu, amplification time unit; B3, backward outer primer; BIP, backward inner primer; COI, gene encoding for cytochrome c oxidase I; *cytb*, gene encoding for Cytochrome *b*; F3, forward outer primer; FIP, forward inner primer; FSA, UK Food Standards Agency; LAMP, loop-mediated isothermal amplification; LF, forward loop primer; LFA, lateral flow assay; LB, backward loop primer; LIMA, linear target isothermal multimerization and amplification; MSA, multiple sequence alignment; NTC, no template control; PCR, polymerase chain reaction; RFU, relative fluorescence unit; UIMA, unusual isothermal multimerization and amplification

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