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REASSURED Test System for Food Control—Preparation of LAMP Reaction Mixtures for In-Field Identification of Plant and Animal Species

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Featured Application: The Authors demonstrate the preparation and application of LAMP-based mobile testing at the point of care for authentication of animal and plant derived food.

Abstract: The potential of loop-mediated isothermal amplification (LAMP) assays for species identification in medical diagnostics, food safety, and authentication is indisputable. The challenge in commercialization of such DNA-based rapid test methods for resource-limited settings is the on-site availability of the required reagents and an intuitive read-out system. In this work, reaction mixtures for LAMP assays for the detection of animal (plaice) and plant food (safflower) were lyophilized and stored at room temperature for up to 24 weeks, demonstrating that refrigeration of reagents after lyophilization is not absolutely necessary. During this period, reaction mixtures were stable and the polymerase showed no loss of activity. In addition, mobile testing, including DNA isolation, using the lyophilized LAMP reaction mixtures and a handheld fluorescence detection read-out system (Doctor Vida Pocket test) was successfully performed outside of the laboratory environment in less than 40 min using a proposed standard operation procedure. The results highlight that the use of the lyophilized LAMP reaction for food control purposes has the potential to meet the WHO-proposed REASSURED criteria.

Keywords: mobile testing; food authentication; LAMP

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1. Introduction

With increasingly complex commodity flows due to the globalization of markets, cases of falsifying food products have risen simultaneously [1–3]. In these rapidly changing environments, rapid test systems for point of contact (POC) are required to closely monitor the authenticity of foodstuffs along the value chain. This applies in particular to perishable as well as high-value commodities like fish and spices, respectively.

Species identification is relevant in the field of food safety and food authenticity as price differences between species can motivate substitution of an expensive species like saffron with a similar looking but less valuable species like safflower. This can also lead to a severe health risk if the added food contains species with allergenic potential [4]. DNA-based tests allow definite species identification of biological samples. Since the development of thermal cyclers, PCR has been established as a routine procedure in analytical laboratories in all fields of application and is still considered the gold standard for DNA-based testing. However, exploiting PCR is inappropriate if a rapid answer is required directly at the POC due to the complex temperature profile of the reaction and the need for an expensive thermal cycler. Therefore, the samples are usually sent to external

analytical laboratories. As shipping suspicious samples to analytical laboratories is time-consuming and costly, detection systems that can also be used on site generally represent significant benefits for users.

In 2003, the World Health Organization (WHO) published a set of criteria for ideal point of care test systems. These criteria are known by the acronym ASSURED (**a**ffordable, **s**ensitive, **s**pecific, **u**ser-friendly, **r**apid, **e**quipment-free, **d**elivered). Recently, ASSURED criteria were updated to provide real-time quality control (R) and the ease of specimen collection (E), building REASSURED criteria [5,6]. REASSURED criteria not only can be used for development of medical POC test systems, but also represent a promising basis for the development of test systems for the surveillance of food commodities. Developed tests should be easy to perform in 2–3 steps (sampling, extraction and specific amplification coupled with intuitive read out) and should only require minimal user training with no prior knowledge of diagnostic testing. For the amplification of target DNA at the POC, the reduction in necessary equipment is especially relevant.

Since its publication by Notomi et al. in 2000, the LAMP (loop-mediated isothermal amplification) reaction has become one of the most widely used isothermal DNA-amplification methods [7]. Due to its isothermal nature, the reaction is particularly well suited for DNA analysis in resource-limited environments [8]. The advantage of LAMP is the high sensitivity and sequence specificity, although off-target effects, such as unspecific amplification or cross-reactivity, have been described [9]. Several LAMP assays have been published for use in food analysis [10–12].

In general, publications on the topic of LAMP reactions often emphasize its potential for on-site analysis due to its isothermal nature, robustness against inhibitors and the variety of visual detection methods [13]. However, the problems that can arise during on-site analysis are usually scarcely addressed. These include, in particular, the transportation and storage of the required reagents (i) and the intuitive visualization of the assays result (ii). The LAMP assays still require fresh preparation of the LAMP reaction mixtures to perform, and therefore fail to fulfill the REASSURED criteria, especially the robustness, equipment-free and delivered criteria, completely [14]. To our knowledge, only two publications on the detection of different species in food showed the performance of DNA isolation and the LAMP reaction at the POC [15,16]. However, multiple heating steps and laborious temperature control prevent user-friendly operation [16].

Ensuring an uninterrupted cooling chain when transporting a ready-to-use LAMP reaction mix to the POC is mandatory and involves considerable costs. Storing LAMP reaction mixes (i) in lyophilized form not only overcomes instability issues but also enhances the user-friendliness of the tests and therefore affects the REASSURED criteria in more than one way. Various enzymes, including Bst polymerase, retain their activity in freeze-dried form after reconstitution [17–20]. During the process of lyophilization, the protein is exposed to drying and temperature stress, resulting in the risk of (partial) denaturation of the protein and subsequent loss of enzyme activity. To protect the enzyme during the drying process, protein stabilizers (lyoprotectants and cryoprotectants) must be added. These are, for example, saccharides that interact directly with the proteins and form a protective layer on the protein surface [18]. The disaccharides sucrose and trehalose are frequently used as stabilizers. Trehalose in particular proved to be suitable due to its lower hygroscopicity and the lack of intramolecular hydrogen bonds [17].

A number of methods are available for visualization of the LAMP amplification products (ii), which can be divided into the categories of real-time reaction monitoring and endpoint detection. Despite their ease of use, techniques like the lateral flow assay (LFA), colorimetric detection by pH indicators or fluorescence-based detection by addition of calcein or Sybr Green are limited to endpoint detection, resulting in lower information content compared to real-time methods [15,21–23]. Real-time fluorescence measurements are used routinely in research and testing laboratories to carry out and monitor amplification reactions. The obtained fluorescence curves allow a reliable assessment of the reaction process and in some cases also a quantitation, or at least a relative estimation, of the target

DNA content in the sample. The combination of LAMP with a handheld device for fluorescence measurements enables real-time connectivity (real-time quality control, REASSURED criteria R). Researchers and manufacturers have developed connectivity solutions that use smartphones for the read-out of the results [24,25].

Only a few on-site LAMP-based tests systems are commercially available, none of them in the food sector. One published and commercially available on-site LAMP-based test system is the lab-on-phone COVID-19 assay by Stab Vida (Doctor Vida® pocket COVID-19 assay, Cat no. 133001001, STAB VIDA Lda, Caparica, Portugal) [26]. The authors did not provide further information on storage and transportation in the associated publication. Kits are also available for the detection of tuberculosis and malaria (Loopamp™ MTBC Detection Kit, Loopamp™ MALARIA Pv Detection Kit, Eiken Chemical Co., Ltd., Tokyo, Japan). The reagents are provided in lyophilized form, but a real-time fluorometer, a turbidimeter or incubator and a source of UV light must be provided by the user.

The aim of this work was to investigate the stability of LAMP reaction mixtures during storage prepared on a small scale. The main goal was to demonstrate and document the applicability of the method outside the laboratory environment at the point of care. Since, to our knowledge, no LAMP-based test systems are commercially available for in-field food diagnostics, this represents an interesting challenge for the development of DNA-based test systems. Without the ability to simplify the test systems so that DNA isolation, amplification and evaluation of the results can be carried out by untrained personnel, the potential of the LAMP reaction remains largely unexploited.

2. Materials and Methods

2.1. Sample Material

Plant: Fresh leaves of safflower were collected at the Botanical Garden of the University of Hamburg. A commercial sample of saffron was authenticated according to ISO 3632-2:2011 clause 7, 14, 16 and ISO 928:2009 by Husarich GmbH [27,28].

Fish: Frozen whole or filleted fish samples were collected in previous projects (Table 1). All fish samples used for assay development were authenticated by sequencing a cytochrome B gene (*Cytb*)-fragment according to DIN CEN/TS 17303:2019-06 [29].

Table 1. Information on sample material used in this study. *Carthamus tinctorius* (*C. tinctorius*); *Pleuronectes platessa* (*P. platessa*); 27: Fishing area 27—Atlantic, Northeast; 27 IV: Subarea IV, North Sea.

Species/Sample	Origin	Additional Information
C. tinctorius	Botanical Garden University of Hamburg	Fresh material
C. tinctorius	Hela GmbH	Dried petals, ground
C. sativus	Husarich GmbH	Dried, ground, Grade 3
Tea "*Ingwerzauber*"	Local market	Declaration: ginger (48%), lemongrass (29%), black pepper, licorice (10%), safflower
Sample "Saffron"	Local market	Dried, ground
P. platessa1	27 IV	Whole fish, frozen
P. platessa2	27 IV	Whole fish, frozen
S. solea1	27	Whole fish, frozen
S. solea2	27	Filleted, frozen
Sample "Plaice"	Local market	Filleted, fresh

2.2. DNA Extraction

2.2.1. DNA Extraction from Plant Tissue

High-Quality DNA Isolation from Plant Tissue

The isolation of high-quality DNA from 50 mg of dried plant material was performed using the DNeasy Plant Pro Kit according to the manufacturer's instructions (Qiagen GmbH, Hilden, Germany). Further cleanup was conducted with the Monarch® PCR and DNA Cleanup Kit (New England Biolabs, Inc., Frankfurt am Main, Germany).

Point-of-Care-Suitable DNA Isolation from Plant Tissue

Approximately 10 mg ground and dried plant material were sampled with Isohelix Buccal Swabs (Cell Projects Ltd., Kent, UK) and incubated in 1 mL of Millipore water for 5 min. To remove residual plant material, the formed supernatant was carefully pipetted to

a new reaction tube and diluted 1:10 with Millipore water. The diluted extract was used directly in the LAMP reaction.

2.2.2. DNA Extraction from Fish Tissue

High-Quality DNA Isolation from Fish Tissue

DNA was extracted according to Rehbein et al. [30]. In brief, 100 mg muscle tissue was used as the starting material and incubated for 1 h at 65 °C in 0.5 mL of buffer 1 (1.2% (*w*/*v*) CTAB, 60 mM Tris, 10 mM Na_2-EDTA, 0.8 M NaCl, 3-mercapto-1,2-propanediol 0.1% (*v*/*v*) and proteinase K (final concentration 0.5 mg/mL, pH 8.0)). After centrifugation for 10 min at 10,625× *g*, the supernatant was washed twice with 500 µL chloroform. Then, two volumes of buffer 2 (1% (*w*/*v*) CTAB, 50 mM Tris, 10 mM Na_2-EDTA, pH 8.0) were mixed with the supernatant. After centrifugation, 400 µL of buffer 3 (1 M NaCl, 10 mM Tris, 1 mM Na_2-EDTA, pH 8.0) were added to the precipitate. The extract was then incubated at 65 °C for 10 min and subsequently precipitated with 400 µL of isopropanol. After another for 10 min at 10,625× *g* centrifugation, the precipitate was washed twice with 500 µL of ethanol (70% (*v*/*v*)). The dried precipitate was dissolved in 100 µL Millipore water.

Point-of-Care-Suitable DNA Isolation from Fish Tissue

The specimen was sampled with a medex cytobrush (medesign I.C. GmbH, Linden, Germany) by swiping the tissue five times. The brush was incubated in 100 µL extraction-buffer (100 mM KCl, 10 mM Tris-HCL, 0.1 mM EDTA, 1 mM DTT, 0.1% Triton X-100) for 10 min at an ambient temperature (approx. 20 °C).

2.3. Quality and Quantity Assessment

Determination of the OD_{260}/OD_{280} and OD_{260}/OD_{230} values was performed with a Nanodrop™ one^C (Thermo Fischer Scientific Inc., Waltham, MA, USA) for checking the quality of DNA. The DNA concentration was determined via photometric measurement with a Quantus™ Fluorometer and Quanti-Fluor® dsDNA System (Promega GmbH, Walldorf, Germany) and each high-quality DNA sample was diluted with water to a final working concentration of 1 ng/µL for plant-derived DNA and 10 ng/µL fish-derived DNA. The DNA isolates were either immediately used for assays or stored at −20 °C.

2.4. LAMP Assays Specific for Carthamus Tinctorius and Pleuronectes Platessa

2.4.1. Assay with Bst 3.0 DNA Polymerase from NEB

Bst 3.0 DNA Polymerase (M0374L), associated $MgSO_4$ solution, and 10x isothermal reaction buffer were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany). Designed primers were ordered from IDT DNA Inc. (Coralville, IA, USA). Primer stocks (25×) of the respective primer sets (see Table 2) contained 40 µM FIP, 40 µM BIP, 5 µM F3, 5 µM B3, and if desired 10 µM LoopF, and 10 µM LoopB. Desoxynucleosidtriphosphates (dNTPs) were purchased from Carl Roth GmbH (Karlsruhe, Germany). Fluorescent dye Syto™9 (Thermo Fisher Scientific Inc.) was used for reaction monitoring in real-time measurements in a final concentration of 2 µM. To prevent an early start of the amplification, the reactions were prepared on ice. For each LAMP assay, a total volume of 25 µL was used. The final concentration in the reaction mix of the DNA polymerase Bst 3.0 was 0.32 U/µL, 1.2 mM (Safflower-assay) or 1.4 mM (Plaice-assay) of each dNTP and 6 mM (Safflower-assay) or 10 mM (Plaice-assay) of $MgSO_4$. The safflower-specific reactions contained either 1 ng of purified DNA from safflower (positive) or saffron (negative). The plaice-specific reactions contained either 10 ng of purified DNA from plaice (positive) or common sole (negative). The no template control (NTC) contained no added DNA. The assay reaction temperature with New England Biolabs (NEB) Bst 3.0 was set to 72 °C for both safflower- and plaice-specific assays for 60 min, and these were followed by enzyme inactivation at 80 °C for 5 min if not stated otherwise.

Table 2. LAMP primer sequences [31,32].

Species	Target	Primer Binding Side	Sequence (5′–3′)
Carthamus tinctorius [32]	ITS1-2	Tin_F3	GCCTTAGCCCTACGATGCT
		Tin_F2	CGGGGTTTGTTTTTGTGCCGAC
		Tin_F1c	CATGCGTGCAAGGTGCTT
		Tin_B3	TTCATCGATGCGTGAGCC
		Tin_B2	GGTTCGTCTCGTGTTGCCCC
		Tin_B1c	CGTTGCCGAGAGTCGTTTA
		Tin_LF	GACGTCCACGATGCCTAGAGAT
		Tin_LB	TTGCGGTGTGCACACGG
Pleuronectes platessa [31]	*Cyt b*	Plat_F3	GCTTCGCAGTCCTCCTCA
		Plat_F2	CTGCACTGGCTTCACTCG
		Plat_F1c	AGGCGTGAAGTTGTCTGGGTCT
		Plat_B3	GCCAAGCTTGTTTGGGATG
		Plat_B2	AGCGGAGAATGGCGTAGG
		Plat_B1c	GTCACGCCGCCACACATCAA
		Plat_LB	GCCAGAGTGATACTTCCTCTTTG

2.4.2. Assay with Glycerol-Free Bst DNA Polymerase (GFP) from Meridian Bioscience

Glycerol-free Bst polymerase (High Conc. Glycerol-Free Bst, MDX018, Meridian Bioscience Inc.) and associated 10× Bst reaction and enzyme dilution buffer were purchased from Meridian Bioscience Inc. (Luckenwale, Germany). Highly concentrated GFP was diluted in 1× dilution buffer to a working concentration of 8 U/µL. The final concentration of the reagents in the reaction mix was identical to the reaction mix used with NEB's Bst 3.0 DNA polymerase. For the glycerol-free Bst DNA polymerase from Meridian Bioscience, 68 °C was set as the reaction temperature. After the fluorescence detection time of 60 min, the enzyme was inactivated for 5 min at 80 °C.

2.5. Lyophilization and Storage of LAMP Reaction Mixtures

LAMP reaction mixtures were prepared according to Section 2.4 as sevenfold reaction mixtures for long-term storage in order to perform negative and positive controls as triplicate determinations after storage and in single reaction tubes. The reaction mixes additionally contained a trehalose solution in a final concentration of 0.3 M or 10% *w*/*w*. The trehalose solution was prepared as 100% *w*/*w* (~3 M) stock solution from trehalose dihydrate and Millipore water. The reaction mixes were first shock frozen in liquid nitrogen, lyophilized for 14 h and then stored on silica gel in a desiccator at room temperature until use. A Beta 2-8 LSCplus freeze-dryer was used for preparation of the sevenfold reaction mixtures and an Alpha 2-4 LSCplus freeze-dryer (both from Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) device was used for the single reaction mixtures. To perform the reactions, the appropriate amount of Millipore water or DNA isolate (see Sections Point-Of-Care-Suitable DNA Isolation from Plant Tissue and Point-of-Care-Suitable DNA Isolation from Fish Tissue) was added to the lyophilized reaction mix to recondition the reagents.

2.6. Doctor Vida Pocket Test

For POC testing and preliminary testing of the POC-suitable assay in the lab, the Doctor Vida Pocket device was used. Real-time fluorescence and temperature parameters were created with the assay creator at stabvida.com and scanned with the Dr Vida app in R&D mode prior to assay start. The freeze-dried reaction mixtures were reconditioned with sample according to Section 2.5 and 50 µL mineral oil (8042-47-5, Merck KGaA, Darmstadt, Germany) was added to prevent evaporation. Reactions were carried out for 30 min at 68 °C (GFP) or at 72 °C for Bst 3.0 (NEB). If necessary, a baseline correction was applied by subtraction of a linear function, as the LAMP reaction is prone to a linear baseline shift due to cumulation of unspecific amplicons [9].

2.7. Statistical Analysis

Statistical analysis was performed using OriginPro 2022 (OriginLab Corporation, Northampton, MA, USA). The Shapiro–Wilk test was used to assess the normality of the data. For normally distributed data, analyses were performed using an ANOVA (Analysis of Variance) to identify significant differences between the groups. Pairwise comparisons were conducted using the Scheffe test. When normality was not confirmed, the Kruskal–Wallis ANOVA was applied instead. A significance level of $\alpha = 0.05$ was used for all statistical tests.

3. Results and Discussion

3.1. Preliminary Experiments on Glycerol Removal and Freeze-Drying

The NEB polymerase Bst 3.0 contains a non-negligible glycerol content of 50 v/v% in its storage buffer. Despite the low volume of polymerase used (final glycerol concentration in the LAMP reaction mixture 2 v/v%), the hygroscopic properties of glycerol can lead to incomplete drying or a reduced shelf life of the enzyme when lyophilizing the reaction mixtures [19]. Therefore, a comparison was first made between the removal of glycerol from the polymerase storage buffer by dialysis or by spin filtration with subsequent lyophilization. In addition, GFP was used and conducted according to the same procedure regarding lyophilization. The procedure is described in the Supplementary Material (Figure S1, Supporting Information).

Both spin filtering the polymerase and dialyzing led to satisfactory results, but the results with spin filtering were much more reproducible. This is possibly due to the longer duration of dialysis (18 h) compared to spin filtering (maximum 2 h), as the activity of the polymerase can be attacked despite reduced temperatures (6 °C in both cases). The probability of this increases with the duration of the process.

The reduction in the stabilizer concentration (trehalose) from 0.3 M to 0.15 M led to a significant loss of activity. No activity was detected without the addition of trehalose. Therefore, all further experiments were carried out with the spin-filtered Bst 3.0 or the GFP and 0.3 M trehalose in the reaction mixture.

The effect of different stabilizers on different proteins and the optimal concentration differs from protein to protein with 0.3 M being the proposed minimal concentration for effective stabilization [33]. Using the example of maltose and catalase, Tanaka et al. were able to show that it is not the absolute concentration of the stabilizer but the ratio of stabilizer to protein that is decisive. The minimum concentration must be sufficient to cover the protein surface with a monomolecular layer [34].

3.2. Long-Term Stability at Room Temperature

To investigate the stability of the LAMP reaction mixtures over time, the glycerol-free Bst 3.0 polymerase (spin-filtered) and the GFP were used. The reaction mixtures were prepared with two different primer sets, lyophilized and stored for up to 24 weeks (168 days) before real-time measurements were performed. The results using the plaice-specific primer set are shown in Figure 1A and the results of the safflower-specific primer set in Figure 1B.

Both assays demonstrated that the LAMP reaction can be carried out with both polymerases even close to 6 months of storage time at room temperature if the reaction mixtures have been suitably dried (freeze-drying with 0.3 M trehalose as stabilizer).

For the safflower-specific primer set, the spin-filtered Bst polymerase showed an increase in reaction time (hereon: time to positive, ttp) of 10 min after seven days of storage compared to the first day of storage. A further increase of 10 min in reaction time was observed for storage up to 168 days. No significant differences in reaction times were observed for the different storage days compared to the mean reaction time over all storage days. Moreover, no significant difference was observed between the first and the following days of storage.

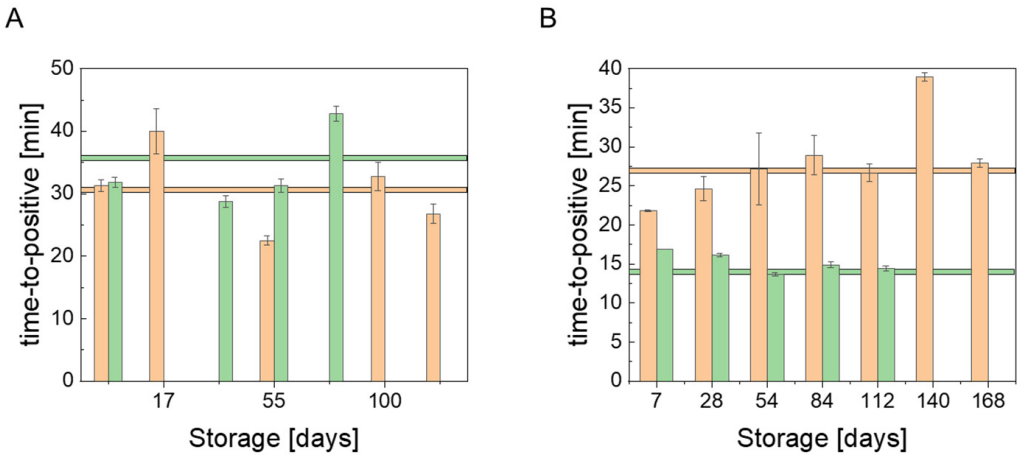

Figure 1. Time-to-positive values of real-time LAMP reactions performed after up to 24 weeks of storage at room temperature for the plaice-specific assay (**A**) and the safflower-specific assay (**B**). Orange: Spin-filtered Bst 3.0 polymerase, Green: Glycerol-free Bst Polymerase (GFP).

For the plaice-specific primer set reaction, a relatively high variability in assay ttp was observed over the period of 150 days, although there was no actual loss of activity of the freeze-dried reaction mix. The ttp after a seven-day storage at room temperature was an average of 31.29 ± 0.99 min compared to 26.80 ± 1.48 min after 150 days. The reaction for 28 and 84 days of storage failed to generate results. Due to the lack of stored reaction mixtures, it was not possible to repeat the measurements. Although significant differences between individual storage days were observed, due to the high variability of the assay ttp, statistical tests revealed no significant differences between the individual storage days and the mean reaction time over all storage days.

The GFP also showed no significant differences between the individual storage days and the mean reaction time over all storage days. Furthermore, the extension of the reaction time after storage for the safflower-specific primer set for 7 days (16.93 min) and 112 days (14.46 min) revealed no significant difference.

The plaice-specific primer set reaction showed relatively constant reaction times (statistically no significant differences) in the first 54 days after lyophilization (7 days: 32.71 ± 1.18 min; 28 days: 28.73 ± 0.94 min; 54 days: 31.31 ± 1.03 min). However, after 72 days, the reaction time increased dramatically to 42.80 ± 1.18 min. The ttp after 72 days of storage was significantly different compared to the mean reaction time over all storage days. In order to determine whether the ttp on day 72 was an outlier, further storage days would have to be tested.

The conducted tests were stored at room temperature for the experiments. Hence, observing similar reaction dynamics after two months for both assays is a promising result to ensure robustness for REASSURED testing. In contrast to our results, Wan et al. did observe a significant increase in the time-to-positive rate after only 12 days of storage of LAMP reaction mixes at room temperature, even though trehalose and glycine were used as stabilizers. Therefore, they recommend storage of lyophilized reaction mixtures at 4 °C. It was not discussed by the authors if glycerol in the storage buffer might have led to loss of activity. Moreover, a lyophilized RT-PCR assay specific for *Myobacterium tuberculosis* stored at room temperature showed poor performance already after two weeks, despite the different cryoprotectants that were used. Only when stored at −20 °C and 4 °C did the assay show a shelf life of up to 6 months [35]. Storage temperature has a significant impact on shelf life. However, to be fully independent of cooling and freezing, the storage tests were conducted at 20 °C only, as the need for a freezer is not desirable for resource-limited settings. The presented results fulfill the robustness requirement of the REASSURED criteria regarding shelf life at room temperature, which at the same time contributes to fulfilling the user-friendliness criterion. No special equipment such as a

freezer is required for storage and transportation. Dry storage can be ensured, for example, by storing with desiccant silica gel, which also sufficiently fulfills the criterion of freedom from equipment in connection with transport and storage.

As already described in Section 3.1, additional steps, even if carried out under cooling, place a strain on the polymerase and can lead to a loss of activity and thus to fluctuating values and longer reaction times. In the commercial production of rapid test systems, an additional step for glycerol removal would not be practicable; instead, a glycerol-free polymerase would be used directly.

For both large-scale industrial applications and pilot projects by research and development departments that want to test their assays in practice, it is desirable to carry out assay preparation in as few steps and with as few highly specialized devices as possible. Lyophilization can be a hurdle for initial applications, as it is associated with relatively high one-time costs if such a device is not already available. To circumvent this, commercially available LAMP reaction mixtures that can be air-dried can be used as an alternative. However, a precision oven (temperature control of ±1.5 °C) is required and the components of the reaction mixture cannot be adjusted, except for the primer concentration [36].

GFP is used in the point-of-care experiments because of the shorter reaction time and easier handling (no spin filtering required), which probably also led to the lower variability of the measured values. Nevertheless, removal of glycerol by spin filtration is a suitable alternative for experiments on a smaller scale, as no new reaction conditions need to be established for the LAMP assay.

3.3. Reproducibility

The reaction time of the LAMP reactions varies between the individual measurements, although the reaction mixtures were prepared at the same time. It was therefore necessary to check whether the fluctuations were due to storage or to individual effects during lyophilization (Figure 2). In order to verify the cause of the fluctuations, 20 individual reaction mixtures were prepared for each primer set and each polymerase, lyophilized and measured after seven days of storage. Reaction tubes with a volume of 0.2 mL were used for this purpose. Instead of the previously used Beta 2-8 LSCplus freeze-dryer, the smaller Alpha 2-4 LSCplus freeze-dryer was used, which enabled better performance for small quantities and small volumes. All prepared single tubes were stored at an ambient temperature (20 °C ± 2 °C) in a desiccator under exclusion of light for seven days. After one week, all reaction tubes were resuspended with 24 µL of ultrapure water. A total of 15 reactions were performed in replicates with the target DNA (1 ng or 10 ng) and 5 reactions were performed in replicates with non-target DNA.

As shown in Figure 2, both the GFP and the spin-filtered Bst 3.0 had a low standard deviation. For the lyophilized reaction mix specific for *Pleuronectes platessa* (*P. platessa)*, the average time to positive was 15 min ± 0.82 min for GFP and 17.69 min ± 0.99 min for Bst 3.0. For the lyophilized reaction mix specific for *Carthamus tinctorius* (*C. tinctorius)*, the average time to positive was 11.43 min ± 2.10 min for GFP and 12.12 min ± 1.52 min for Bst 3.0. Both assays showed low variation in amplification time and, thus, enabled robust POC testing with no refrigerated storage needed. Interestingly, both assays revealed overall faster times to positive when compared to the long-term stability tests described above. This is likely due to the variable performance of the device used for lyophilization.

The LAMP technique is known to produce reproducible results over a wide range of temperatures, elongation times and pH values. However, as described above, most of the published research has used freshly prepared reaction mixtures. For user-friendly POC application, cooling the reaction mix prevents ease of use. It has been demonstrated that lyophilized reaction mixtures can be stored for up to 24 weeks. The reproducibility of single lyophilized tubes is, to the best of our knowledge, only described in detail for colorimetric LAMP assays. Song et al. (2022) designed a test kit for SARS-CoV-2 RNA based on lyophilized colorimetric RT-LAMP. Their test kit remained stable for at least 30 days at 4 °C and 10 days at room temperature (20–22 °C). Song et al. achieved high

sensitivity and a >99% specificity with a reproducible LoD of 100 RNA copies per reaction under both storage conditions [37]. Recently published results of Prado et al. (2024) support the fact that lyophilized colorimetric RT-LAMP assays show high stability at room temperature for up to 6 weeks. The authors described those standard thermal reactions with SARS-CoV-2-positive RNA as showing a functional and stable reaction [38].

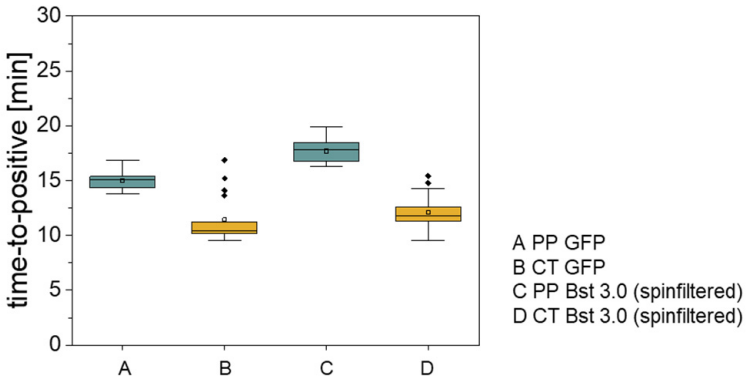

Figure 2. Comparison of time-to-positive values of GFP and spin-filtered Bst 3.0 of individual reaction mixtures. Boxplots of time-to-positive values of *P. platessa*-specific assays (PP) are shown in green and those of *C. tinctorius* (CT)-specific assays are shown in orange. ♦ indicate outliers.

3.4. Fluorescence Measurements with Doctor Vida Pocket Test

The Doctor Vida Pocket test is a hand-held device for isothermal amplification for temperatures from 25 °C to 95 °C and enables simple and rapid measurement of fluorescence on-site. Compared to other portable or smartphone-based fluorescence detection devices, the Doctor Vida Pocket test offers temperature control and real-time monitoring of the fluorescence profile. Therefore, this technique is suitable for on-site monitoring of LAMP assays. Assays for COVID-19 and lactose intolerance have been published and highlight the ease of use [24,39].

The more affordable the developed test system is, the more tests can be performed at the various steps along the value chain, leading to greater integrity of the goods. Since the acquisition costs are spread over the number of assays performed, the cost per test is reduced with each test performed. With a list price of under USD 400, the Doctor Vida Pocket test device is very affordable [40].

Despite the potential for a POC application, preliminary tests were carried out in a laboratory environment. The method suitable for POC was used to prepare the DNA extract, and the individually lyophilized reaction mixtures were directly reconditioned with the crude extract. The tests were performed for both primer sets with reaction mixtures stored for one week or 2 months. Figure 3 shows the real-time fluorescence curves for both assays.

The results show amplification of the target DNA after a maximum of 15 min for the *C. tinctorius* assay and a maximum of 20 min for the *P. platessa* assay. The results for the safflower and sole assays are therefore consistent with the results of the long-term stability test. A linear baseline shift can be observed for all tested positive, negative and no template control samples. The LAMP reaction tends to cause a linear baseline shift due to accumulation of non-specific amplicons [9]. As the R&D software (Dr Vida Pocket PCR, Version 3.2.0) is currently not able to perform baseline correction, we performed it ourselves by subtraction of a linear function when necessary. The raw data was not edited by any other means.

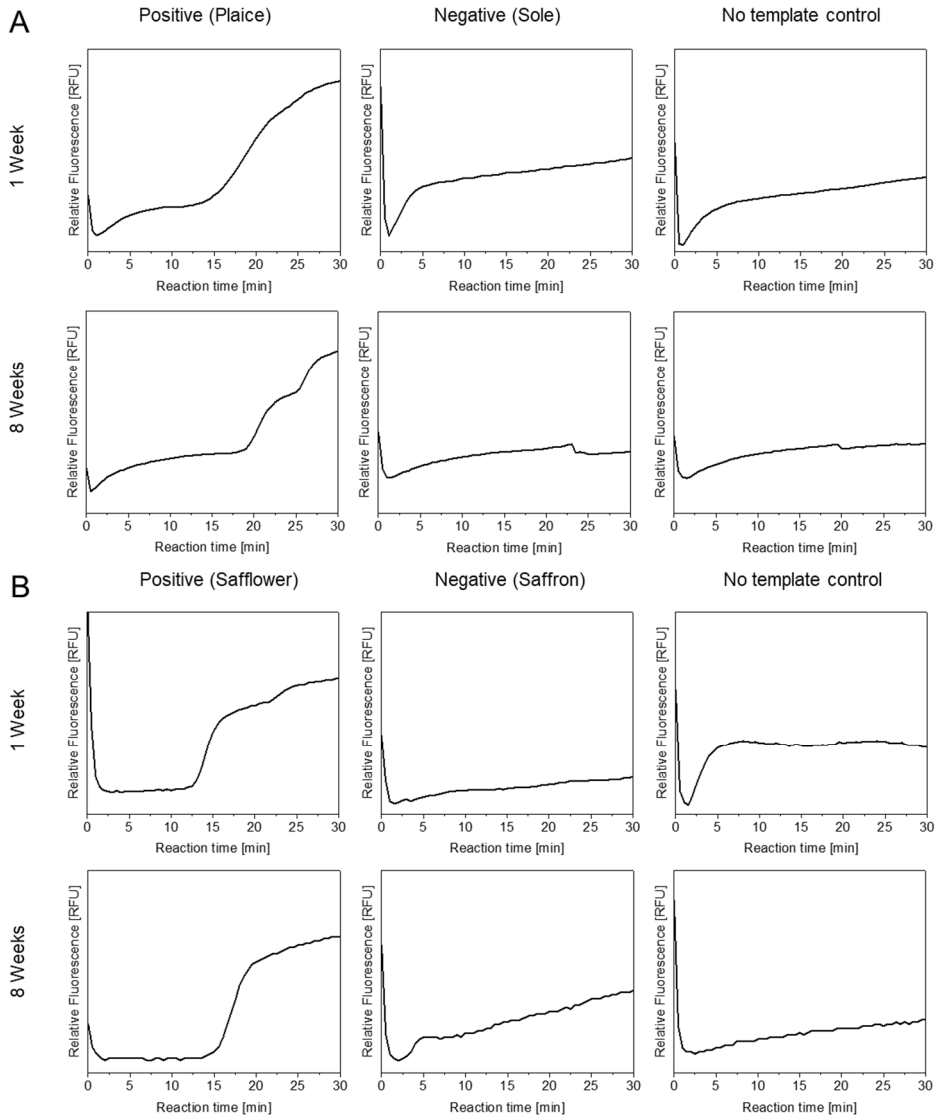

Figure 3. Real-time fluorescence curves obtained with the Doctor Vida Pocket device for the plaice-specific assay (**A**) and the safflower-specific assay (**B**) after one and eight weeks of storage of the reaction mix. The results were verified by agarose gel electrophoresis.

3.5. Point-of-Care Testing with Standard Operating Procedure and Evaluation of REASSURED Criteria

Up to this point, the obtained results confirm that both assays enable user-friendly food controlling of plant- and animal-based food. Due to the lyophilization of the reaction mixture, the assays do not need to be prepared in-field and do not require refrigeration. To verify whether the tests described here are suitable for actual POC testing, a proof-of-concept field test was conducted and a local farmers market was visited for sample acquisition. Both assays were performed at the POC according to a proposed standard operating procedure (SOP, see Figure S4 for detailed step description). Figure 4 shows that the exemplarily tested food items showed the expected results. When using the *P. platessa*-specific assay for purchased fresh plaice, plaice DNA from the sampled item was detected, as evidenced by an exponential increase in fluorescence after 10 min. The faster amplification performance of the assay may be attributed to the fresh and not frozen sample material. Hence, simplified DNA extraction could be performed more efficiently. When the *C. tinctorius*-specific assay was used for a purchased tea sample, the assay detected DNA from *C. tinctorius* in the sampled item. For a non-target purchased saffron spice, the POC test showed no amplification.

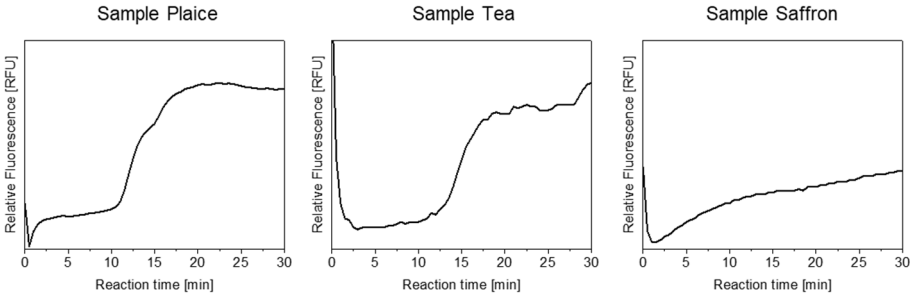

Figure 4. Real-time fluorescence curves obtained with the Doctor Vida Pocket device for the plaice-specific assay (**left**) and the safflower-specific assay (**middle** and **right**) in realistic settings at the POC.

Thus, both assays were successfully conducted at the POC and the proposed SOP could build a good baseline for future in-field food-control testing to meet the REASSURED criteria (see Table 3). Combining a lyophilized LAMP reaction mix with the Doctor Vida Pocket test can ensure real-time monitoring of the quality of the ongoing test, both in the laboratory and on-site, with just a smartphone and a power bank.

The strength of both proposed assays is obvious: Due to simplified sample collection and DNA extraction, our proposed SOP allows user-friendly and rapid preparation prior to the specific assay in 10 min. The previously published LAMP assays were sensitive and specific in laboratory settings [31,32]. The results of the proof-of-concept field test revealed no difference in target specificity although the reaction mix was lyophilized and stored at ambient temperature for one week. Preliminary experiments suggest that the reaction mixture will still work with minimal loss of polymerase activity after 24 weeks. Further tests are needed to confirm the robustness of the performance in actual, non-controlled environments. Since the results are available in under 30 min, the tested assays can be performed rapidly. These characteristics of the proposed assays may enable close monitoring of the integrity of the value chain and may combat food fraud and ensure food safety for global markets. However, in order to deliver the test system to the relevant user, the commercialization of a complete test kit is necessary and remains a pending task. Following the example of the test systems marketed by Eiken Chemical Co., the inclusion of positive and negative controls in the kit should be considered. Developing combination devices like the PEBBLE to measure multiple samples at once would also be an advantageous development [25]. A possible limitation of the proposed SOP is the risk of contamination, due to the requirement to pipette the crude DNA isolate at the POC. All-in-one devices which facilitate the entire process including DNA extraction and amplification could help to reduce the risk of false positive results and improve user-friendliness by a pipette-free approach [41].

Table 3. Overview of REASSURED criteria and evaluation of the proposed standard operating procedure.

REASSURED Criteria	Is this Criterion Met?
Real-time quality control	Doctor Vida enables real-time quality control via displaying fluorescence data in app
Ease of specimen collection	Using swab or small brush to collect specimen
Affordable by those in need	Reagent costs: EUR ~0.50Instrument cost: EUR 330
Sensitive (few false negatives)	No false negatives were detected in both assays
Specific (few false positives)	High specificity for both assays [18,19]
User-friendly	See SOP (Figure S3), initial POC testing revealed high user-friendliness
Rapid and robust	Both assays can be conducted in less than 60 min: DNA extraction: <10 min LAMP assay: 30 min Robust: Storage capacity is high at temperatures around 20 °C. See Figures 1 and 2 for detailed description.
Equipment-free	Test kit: Brush or swab for specimen collection; tube containing extraction buffer; reaction tube with lyophilized reaction mix; Doctor Vida Pocket test; and smartphone
Delivered to those who need it	Pending, future application of food controlling at institutions and industry realistic.

4. Conclusions

The aim of the research presented was to meet as many of the REASSURED criteria as possible by reducing the dependency on storage capacity and enabling real-time quality control via LAMP without the need for sophisticated equipment. By using a lyophilized reaction mix and the handheld fluorescence detector (Doctor Vida Pocket test), the described results address the shortcomings of existing LAMP protocols in the food sector. Although the presented assays still have room for improvement (e.g., analyzing multiple samples simultaneously), the results emphasize practicality at the point of care with minimized hands-on time and a simple and rapid standard operating procedure. It is important to keep in mind that a rapid test system must make compromises when it comes to meeting all criteria. To enable accurate monitoring of the integrity of the value chain, the test system should be affordable, fast and easy to use. Further development of the proposed assay towards more sophisticated equipment will lead to higher costs and counteract the affordability of the test system.

In summary, the results provide valuable insights into the transferability of the large number of published LAMP publications from the laboratory infrastructure to the POC.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app142310946/s1, Figure S1: Measurement of NMR spectrum to verify successful glycerol removal; Figure S2: Comparison of two different methods for glycerol removal and their effect on polymerase activity after freeze-drying and the effect on freeze-drying on the glycerol-free polymerase. Figure S3: Influence of trehalose conc. on polymerase activity after freeze-drying; Figure S4: SOP; Table S1: Cost estimation of LAMP assay.

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