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Lyophilised colourimetric LAMP for visual readout with dual colour indicators

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Loop-mediated isothermal amplification (LAMP) is increasingly recognised as a practical alternative to PCR for pathogen detection, offering rapid turnaround time, a constant operating temperature, and compatibility with a wide range of detection methods. Colourimetric LAMP has gained popularity due to its potential for instrument-free readout, making it suitable for molecular diagnostics in low-resource settings. Despite these advantages, its adoption at the point-of-care remains limited as it has been widely used in liquid format and therefore restricted to the availability of cold-chain storage and trained personnel. This work introduces the development and optimisation of ready-to-use lyophilised colourimetric LAMP (lyo-cLAMP) that does not require cold-chain, additional reagents, or manual intervention beyond the addition of extracted nucleic acids from a sample, which indicates the presence of a target of interest by colour change after amplification. A variety of pH and metal indicators were screened and combined to evaluate their synergy, identifying four combinations with high discrimination between positive and negative amplification. The performance of lyo-cLAMP was assessed with synthetic SARS-CoV-2 RNA, comparing it to the liquid format and achieving the same limit-of-detection. Lastly, the translation of lyo-cLAMP to diagnostic applications was demonstrated by screening positive SARS-CoV-2 residual clinical samples, achieving high accuracy. The developed lyo-cLAMP is compatible with any LAMP assay, allowing for rapid adaptation to new targets, which is particularly valuable in outbreak scenarios.

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Introduction

Loop-mediated isothermal amplification (LAMP) is an isothermal method that has emerged as a potential alternative to PCR for pathogen detection. Owing to its high sensitivity, specificity (requiring four to six primers), and rapid time to result (often less than 40 minutes), LAMP has been widely applied, including for the detection of COVID-19, malaria, and antimicrobial resistance markers.[1–4] These advantages, combined with its isothermal amplification, which forgoes the requirement for bulky and expensive thermocyclers, make LAMP especially suitable for use at the point-of-care (POC).

Nucleic acid amplification can be detected through fluorescence emission using DNA intercalating dyes or *via* end-point detection methods such as agarose gel electrophoresis, colourimetric dyes, or lateral flow strips. Notably, colourimetric LAMP (cLAMP) has gained popularity as a cost-effective solution for molecular diagnostics.[5–8] Under low or unbuffered conditions, protons released during amplification lower the pH of the reaction. In addition, pyrophosphates are generated as a byproduct, which reduce free magnesium ion levels.[9] These chemical changes affect pH and metal indicators respectively, triggering colour shifts in the reaction and allowing for visual detection without the need for specialised equipment (Fig. 1).[10,11] Most studies have focused on the development of pH-based colourimetric reactions, including the use of indicators such as phenol red and neutral red,[12] as well as novel compounds like curcumin[13] and polydiacetylene (PDA).[14] Multiple publications have also reported the use of metal indicators, such as Eriochrome Black T and Hydroxynaphthol Blue,[15] and, less frequently, chemical reactions such as copper sulphate–induced diphenylamine colourimetric reaction.[16] Nevertheless, the combined use of any of these approaches has not been fully explored, particularly regarding potential synergistic effects of pH and metal indicators, the expansion of the colour palette and increasing robustness of the visual readout. Such combinations could reduce the incidence of false negatives and false positives when the initial reaction conditions (*i.e.*, pH or salt

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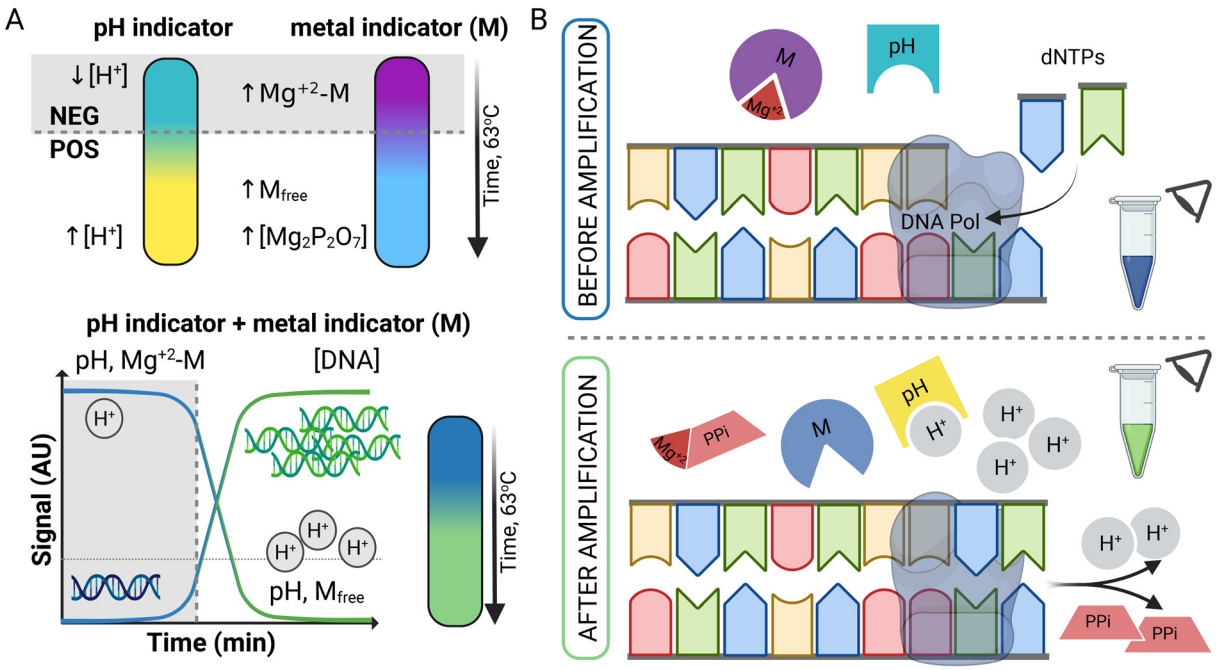

Fig. 1 Schematics of cLAMP colour change in the presence of the target of interest. (A) Top panel: Example of pH indicator and metal indicator colour change across time during amplification at 63 °C; Bottom panel: example of combining pH and metal indicator to obtain a new starting (negative, dark blue) and end colour (positive, green) during amplification. (B) Top panel: Schematics before amplification showing Mg^{2+} bounded to metal indicator (M) and free pH indicator; bottom panel: schematics after amplification showing free metal indicator, pH indicator bounded to H^+ ions, and the byproduct pyrophosphate (PPi) which captures Mg^{2+} giving rise to $Mg_2P_2O_7$.

concentration) deviate from optimal values, causing the colour transition of a single indicator to fall outside the effective discrimination range between positive and negative reactions. These deviations could be due to a partial loss of salts or volatile counterions during lyophilisation, testing beyond the recommended shelf-life, or sample carry-over effects that alter the starting pH or ionic composition. In addition, the use of dual colour indicators could improve readout reliability for samples that are not fully transparent (*e.g.* those containing blood-derived pigmentation), where colour outputs within a similar range may otherwise lead to ambiguous interpretation. A major challenge for the translation of cLAMP-based tests outside laboratory-based settings is the need for cold-chain storage and trained personnel. Currently, New England Biolabs (NEB) commercializes a cLAMP reaction mix, "WarmStart® colourimetric LAMP Master Mix" in liquid format, therefore restricting its use to laboratory settings with cold-chain storage (*i.e.*, −20 °C). Lyophilisation is a commonly used approach in molecular diagnostics to remove the need for cold-chain, which also enables long-term stability. Only a few studies have reported the development of lyophilised LAMP combined with end-point colourimetric detection, where it is described as a two-step process. For example, García-Bernalt Diego *et al.*[17] reported the addition of SYBR Green I as visual indicator after the amplification of the rehydrated lyophilised master mix; and Chen *et al.*[18] described the use of a metal indicator, hydroxynaphtol blue, which was added during the rehydration of the lyophilised master mix. In both cases, the separation of the colourimetric dye from the lyophilised reagents limits the applicability of the approach at the POC, still requiring trained personnel and laboratory equipment (*e.g.* micropipettes). In this study, we developed and optimised a one-step lyophilised colourimetric LAMP (lyo-cLAMP) which is suitable for use in POC settings, being stable at room temperature and only requiring the addition of extracted nucleic acids from a sample. We optimised a LAMP protocol and evaluated pH and metal indicator dyes to investigate their synergistic effect to generate a wider colour palette for end-point detection, blending both types of dyes into a single test. We also validated lyo-cLAMP with 15 positive SARS-CoV-2 respiratory specimens obtained from nasal, nasopharyngeal or buccal swabs. The development of a ready-to-use, cold-chain-free cLAMP test enables rapid screening of extracted samples without the need for trained personnel or lab-based equipment apart from a heat-block to perform the test. This easily repurposed end-point test has the potential to facilitate the deployment of LAMP to detect any nucleic acid target (DNA or RNA) of interest at the POC.

Experimental section

LAMP reaction conditions

Optimisation of LAMP protocol. LAMP reactions were carried out at a final volume of 10 μL per reaction. Each mix contained the following: 1× custom isothermal solution

(10 × stock), 4–10 mM $MgSO_4$ (100 mM stock, NEB), 1–1.8 mM of dNTPs (25 mM stock, NEB), 0–1.2 mg mL^{-1} of glycerol-free or glycerol-based BSA (40 mg mL^{-1} stock, Sigma-Aldrich/Invitrogen), 0–0.8 M glycerol-free or glycerol-based betaine (5 M stock, Sigma-Aldrich/ThermoFisher),1× LAMP primer mix (10 × primer mix stock) containing 0.25 μM (each) F3/B3, 1 μM (each) LF/LB and 2 μM (each) FIP/BIP, 0.5 μM of Syto-9 dye (20 μM stock), 2 mM of NaOH (0.5 M stock, Sigma-Aldrich), 0.5 U μL^{-1} of Bst 2.0 WarmStart DNA polymerase (120 U μL^{-1} stock, NEB) or Bst 2.0 WarmStart DNA polymerase glycerol-free (120 U μL^{-1} stock, NEB) or Bst 3.0 (120 U μL^{-1} stock, NEB) or Bst Large Fragment (120 U μL^{-1} stock, NEB), 1 μL of sample and enough nuclease-free water. Additionally, for RNA targets, 0.45 U μL^{-1} of WarmStart RTx (NEB) was added. For colourimetric reactions, 25 to 200 μM of colourimetric dyes (3 mM stocks) was added. To evaluate the compatibility with lyophilisation, excipients were added at variable concentrations (0 to 10% w/v). Reactions were loaded into 96-well plates and incubated at 63 °C for 35 min using a QIAquant real-time PCR (QIAGEN) and LightCycler 96 (LC96) instrument (Roche). One melting cycle was performed at 0.1 °C s^{-1} from 63 °C up to 97 °C to validate the specificity of the amplified products. A non-template control (NTC) was included in every experiment.

Lyophilised colorimetric LAMP (lyo-cLAMP) protocol. 2× cLAMP reactions were prepared at a final volume of 10 μL per reaction before lyophilisation. Each mix contained the following: 4% w/v mannitol (20% w/v stock), 2% v/v Ficoll (20% v/v stock), 2× custom isothermal solution (10 × stock), 10 mM $MgSO_4$ (100 mM stock, NEB), 2.8 mM of dNTPs (25 mM stock, NEB), 2.4 mg mL^{-1} of glycerol-free BSA (40 mg mL^{-1} stock, Sigma-Aldrich/Invitrogen), 35 mM of NaOH (1.5 M stock, Sigma-Aldrich), 150–300 μM of colourimetric dyes (3 mM stocks), 1 U μL^{-1} of Bst 2.0 WarmStart DNA polymerase glycerol-free (120 U μL^{-1} stock, NEB), 0.9 U μL^{-1} of WarmStart RTx (75 U μL^{-1} stock, NEB) and enough nuclease-free water (Invitrogen) to bring the final volume to 10 μL. Master mixes were prepared without primers or fluorescent dye to allow for experimental flexibility, with these components added only when required. Lyophilisation was performed by Biofortuna or Cytiva. Upon receipt, the freeze-dried beads were rehydrated to a final volume of 20 μL. Then, reactions were loaded into 96-well plates and amplification was performed at 63 °C during 35 min using a QIAquant real-time PCR (QIAGEN) or LightCycler 96 (LC96) instrument (Roche). One melting cycle was performed at 0.1 °C s^{-1} from 63 °C up to 97 °C for validation of the specificity of the amplified products. A non-template control (NTC) was included in every experiment. Additionally, when assessing only colour change, amplification reactions were carried out in a portable isothermal heat block for 35 min at 63 °C.

Excipients

A total of six excipients were titrated into LAMP reactions. The excipients were grouped as: (i) disaccharide-based (sucrose and trehalose), titrated in the range of 0–10% w/v, (ii) polyalcohol sugar-based (sorbitol and mannitol), titrated in the range of 0–4% w/v, and (iii) polysaccharide-based (dextran and Ficoll), titrated in the range of 0–2% w/v. Excipients were stored at 4 °C until used.

Colourimetric dyes

A range of pH and metal indicators were titrated at concentrations ranging from 25 μM to 150 μM for colourimetric LAMP. The pH-sensitive dyes included: bromothymol blue (BB) (SigmaCat no. 114413-5G), thymol blue (TB) (Sigma, Cat no. 114545-5G), neutral red (NR) (Sigma, Cat no. 72210-5G) and phenol red (PR) (Sigma, Cat no. P3532-5G). Metal indicating dyes included: eriochrome black T (EBT) (Sigma, Cat no. 858390-100G), calmagite (C) (Sigma, cat no. C204-10G-A), bromocresol purple (BCP) (Sigma, Cat no. 114375-5G), and xylidyl blue (XB) (Sigma, Cat no. 248266-1G).

Lyophilisation protocol

Freeze-drying microscopy (FDM) and lyophilisation were performed by UK-based companies (Biofortuna or Cytiva). The critical temperature obtained from the FDM analysis was used to design a suitable freeze-drying protocol. The onset of collapse was determined at −38.6 °C and the total collapse at −37.9 °C under a pressure of 5.4 Pa. Detailed freeze-drying conditions are shown in Table 1. LAMP formulations were dispensed to form beads of 10 μL volume at 2× concentration.

Analytical sensitivity and specificity of LAMP reactions

Analytical sensitivity was evaluated using serial dilutions of synthetic DNA (gBlocks from Integrated DNA Technologies) ranging from 2×10^5 to 25 copies per reaction, or RNA (Twist Biosciences) ranging from 2×10^4 to 25 copies per reaction. Dilutions were performed in nuclease-free water. Each condition was run in duplicates or triplicates. Analytical specificity of the assay was evaluated experimentally using the Twist Respiratory Virus Research Panel (Cat no. 103730, Twist Bioscience) (Fig. S1). LAMP primers used in this study were previously described in Rodriguez-Manzano *et al.*,[4] primer sequences are included in Table S1.

Extraction of nucleic acids from respiratory specimens

Residual samples from nasal, nasopharyngeal or buccal swabs collected at North West London Pathology laboratory (United Kingdom) were used as described in a previous study.[19] Nucleic acids were extracted (N = 15) using the QIAamp Viral

Table 1 Freeze-drying conditions

Phase	Temperature (°C)	Step (rmp/hold)	Time (min)	Vacuum (mTorr)
Pre-cooling	−45	R	30	N/A
	−45	H	30	N/A
Primary drying	−40	R	60	60
	−40	H	1100	60
	−40	H	1000	60
Secondary drying	+20	R	120	60
	+20	H	360	60

RNA Mini Kit (Cat. No.52904, QIAGEN) following the manufacturer instructions. In brief, 140 μL of sample was used as input eluting into 80 μL nuclease-free water. Eluates were tested on the day and then stored at −80 °C.

PCR reaction conditions

Published CDC COVID-19 assay (IDT) was used as gold standard to evaluate the viral load of the clinical samples. Primer sequences are included in Table S1. RT-qPCR experiments were performed using the Promega GoTaq Probe RT-qPCR (Cat. No. A6121, Promega) master mix at a final volume of 20 μL per reaction following this protocol: 2 × GoTaq qPCR mastermix (10 μL), 50 × GoScript™ RT Mix for 1-Step RT-qPCR (0.4 μL) PCR assay mix (1.5 μL), 5 μL of sample, and nuclease-free water. The cycling conditions were: 1 cycle at 45 °C for 15 min, 1 cycle at 95 °C for 2 min, and 45 cycles at 95 °C for 3 s followed by 55 °C for 30 s. Experiments were performed using a QuantStudio5 instrument reading in FAM channel. Published human control assay targeting RNaseP from the CDC was used to verify the human origin of the sample and the quality of the extraction (Table S1).

Absorbance reading

The FLUOstar® Omega microplate reader was used to obtain the absorbance and transmittance spectra (400 to 700 nm) of positive and negative colourimetric LAMP reactions after amplification. The reactions were loaded into a 384-well microplate (40 μL each), including positive and negative controls without any dye. Transmittance data was converted to CIE XYZ colour space using the CIE XYZ 1964 10 degrees reference and the CIE standard illuminator D65 at the specific wavelength range from 400 to 700 nm. Next, the perceived difference between two colours in CIELAB space, corresponding to positive and negative reactions, was determined. Delta E (ΔE) was calculated using the following formula $\Delta E_{ab} = \sqrt{((L_2 - L_1)^2 + (a_2 - a_1)^2 + (b_2 - b_1)^2)}$, where L_1, a_1, b_1 are the Lab coordinates of the first colour, and L_2, a_2, b_2 are the Lab coordinates of the second colour. A scoring system was developed based on the ΔE values obtained and the inhibitory effects of the colourimetric indicators used. The scoring of the inhibition began at 1 and was penalised by −0.05 based on a ranking system of the dyes used (BB, PR, BCP, TB. EBT, NR, C, XB). This ranking was determined by the difference in the time-to-positive (TTP) values between reactions containing the respective indicator and a positive control (no indicator). In addition, the inhibitory effect of the concentration (50 μM and 100 μM) was taken into account and penalised by −0.2/−0.3 in the case of 100 μM.

Statistical analysis

The TTP data are presented as the mean TTP ± standard deviation (min); p-values were calculated by Student's homoscedastic t-test, with a two-tailed distribution. Statistically significant difference was considered as *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001; linear fitting and statistical tests were performed in Origin software (OriginLab, Northampton, MA).

Ethics

From October 2020 to January 2022, residual samples from nasal, nasopharyngeal or buccal swabs were collected at North West London Pathology laboratory (United Kingdom). All samples used for this study were inactivated onsite by transferring 500 μL of VTM sample to Copan eNAT at 1 : 2 ratio prior to shipping the sample to Imperial College London (Hammersmith Campus). Use of fully anonymised samples was approved by the Health Research Authority (HRA) and Health and Care Research Wales (HCRW) with NHS Research Ethics Committee (REC) reference 20/HRA/1561.

Results and discussion

Optimisation of a LAMP protocol

Each component of a conventional LAMP reaction was evaluated to develop an optimised protocol. In addition, homologous glycerol-free versions were tested to study the impact of glycerol in the reaction. In particular, we investigated the role of $MgSO_4$, dNTPs, betaine, BSA, glycerol and four Bst DNA polymerases:

(1) Two core reagents in LAMP are $MgSO_4$ and dNTPs, the former as the source of Mg^{2+} cations which are essential cofactors in the activity of the DNA polymerase, and the latter needed as building blocks for new DNA strands.[20,21] $MgSO_4$ was titrated at ranging concentrations between 4 mM to 10 mM, and dNTPs between 1 mM to 1.8 mM. Selected concentrations were 5 mM and 1.4 mM, respectively, shown in Fig. 2A and B.

(2) The use of Betaine and BSA as part of the LAMP formulation has been described in other studies.[6,22] Betaine is a dsDNA destabilising agent which decreases non-specific interactions and lowers the T_m of the primers. Its effect in the reaction was negligible except for higher concentrations where an increase in the TTP values was observed, in agreement with previous study from Ma *et al.*[23] The glycerol-free version had a similar behavior (Fig. 2C). BSA has been commonly used in molecular biology as an enzyme stabiliser.[24] The incorporation of BSA improved assay reproducibility and precision (lower standard deviation) as well as speed of the reaction (Fig. 2D). BSA at a concentration of 1.2 mg mL^{-1} reported the lowest TTP value, with this value being even lower when using the glycerol-free version.

(3) Glycerol is well known for its properties as an enzyme-stabilising agent.[25] However, as a hygroscopic agent, its concentration must be minimised or completely removed when translating formulations to lyophilised format. The overall glycerol percentage in standard LAMP reactions can vary from 0% to 10%, 10% being the maximum possible with all the corresponding reagents stored in glycerol-based solutions. A glycerol titration in LAMP was performed to assess the impact on the reaction kinetics while minimising the glycerol content for lyo-

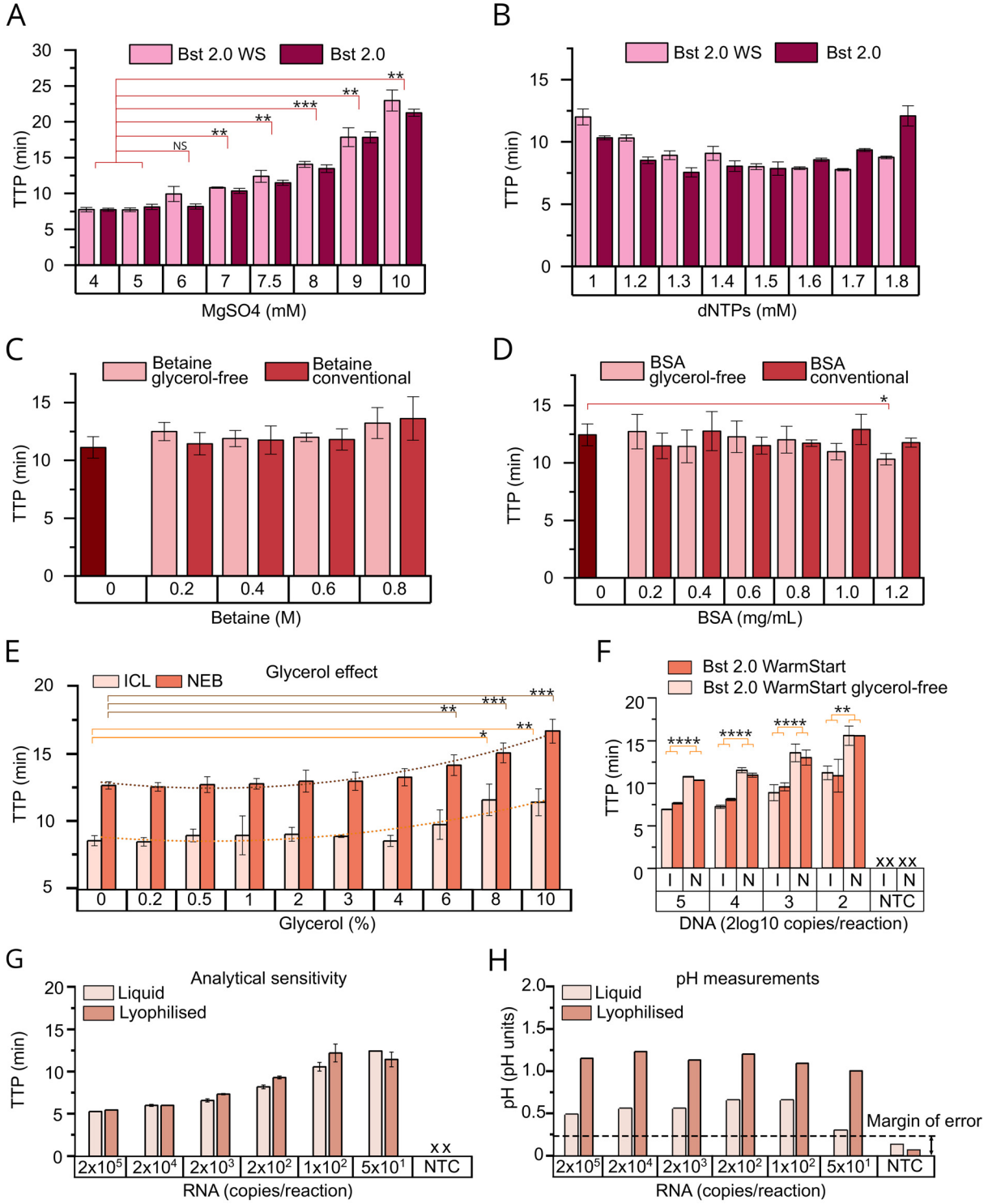

Fig. 2 Optimisation of LAMP and its compatibility to undergo lyophilisation. (A) Effect of the concentration of $MgSO_4$ using Bst 2.0 WarmStart (Bst 2.0 WS) and Bst 2.0. DNA polymerase (Bst 2.0). An additional concentration is included in the reaction (the isothermal solution already contains 2 mM). (B) Effect of the concentration of dNTPs using Bst 2.0 WS and Bst 2.0. (C) Effect of incorporating Betaine (50% glycerol) and a glycerol-free version. (D) Effect of incorporating BSA (50% glycerol) and a glycerol-free version. (E) Impact of the addition of glycerol at various final concentrations (% in the reaction) in a glycerol-free LAMP raction mix. (F) Comparison of the NEB protocol and the proposed glycerol-free protocol using Bst 2.0 WS and its glycerol-free version. (G) Analytical sensitivity of the lyo-LAMP beads in comparison to the liquid format using a serial 10-fold dilution of SARS-CoV-2 RNA. (H) ΔpH values of lyo-LAMP and liquid LAMP before and after amplification across various concentrations of SARS-CoV-2 RNA.

philisation. Results demonstrated a negative impact of glycerol, with increasing TTP values observed as the overall percentage increased. A steady behaviour was observed up to 4% glycerol, however the TTP values significantly increased above this concentration (Fig. 2E). Contrary to other studies,[26] the increase in the viscosity of the reaction seemed to have a negative effect in the diffusion and rate of amplification.

(4) Lastly, we evaluated four Bst DNA polymerases using the recommended protocol from the manufacturer (NEB) and our optimised protocol. Significantly lower TTP values were obtained with the optimised LAMP protocol across all the enzymes tested (Fig. 2F and Fig. S2). The performance of each enzyme in the NTC was of particular interest. Only Bst 2.0 WarmStart DNA polymerase and its homolog stored under glycerol-free conditions showed resilience to non-specific amplification, whereas Bst 2.0, Bst 3.0 and Bst Large Fragment produced positive NTC signals (Fig. S2). Previous studies have also reported unspecific amplification in the absence of template when employing Bst 3.0 DNA polymerase.[27] Notably, storage in a glycerol-free solution did not adversely affect enzyme performance in our experiments. This observation is consistent with supplier-reported data indicating that glycerol is not required to preserve enzyme activity over limited freeze–thaw cycles.[28]

In summary, the optimised LAMP protocol consisted of 0% glycerol, 5 mM $MgSO_4$, 4 mM dNTPs, 1.2 mg mL^{-1} BSA glycerol-free, and 0.5 U μL^{-1} Bst 2.0 WarmStart DNA Polymerase.

Development of lyophilised LAMP

For efficient lyophilisation, hygroscopic components such as glycerol are required to be removed or minimised, whereas the addition of excipients aid in the lyophilisation process, protecting proteins through crystallisation formation.[29–31] The optimised glycerol-free LAMP protocol was subsequently employed for formulating LAMP in a lyophilised format. Various excipients were titrated and analysed by real-time LAMP to evaluate their impact on amplification (Fig. S3A), and by freeze-drying microscopy (Fig. S3B). Previous studies[17,32,33] have demonstrated that formulations containing trehalose, or combinations of trehalose and dextran supported successful lyophilisation but slow down amplification. These findings are consistent with the results found in this work. The best formulation identified in this study was a combination of 2% dextran or Ficoll, and 4% mannitol, yielding an onset collapse temperature of −38.6 °C, and total collapse at −37.9 °C under 5.4 Pa. A freeze-drying protocol was defined based on the freeze-drying microscopy data, with the primary drying phase maintained below the glass transition temperature (T_g) of the product at −40 °C (Table 1).

A comparison between LAMP in liquid format and lyophilised format was conducted. Results shown in Fig. 2G and H demonstrate that lyophilised LAMP (lyo-LAMP) achieved comparable analytical sensitivity and higher change in pH. We hypothesise that the increased ΔpH results from a reduced effective buffering capacity due to the loss of salts (*i.e.*, ammonium salts) during the lyophilisation process, as well as lower background buffering resulting from reduced exposure to atmospheric CO_2. The performance of the lyo-LAMP beads showed that the developed formulation is compatible with freeze-drying without negatively impacting on analytical sensitivity and specificity.

Evaluation of colourimetric dyes compatible with LAMP

A total of four pH and four metal indicators were titrated at varying concentrations ranging from 25 μM to 150 μM, including phenol red (PR), neutral red (NR), thymol blue (TB), bromothymol blue (BB), eriochrome black T (EBT), calmagite (C), xylidyl blue (XB), and bromocresol purple (BCB). Each indicator dye was evaluated at a fixed RNA concentration (10^3 copies per reaction) in real-time LAMP using a conventional thermal-cycling instrument to monitor fluorescence, in addition to visual readout by colourimetric detection at the end of the run. Results are shown in Fig. 3A and B. A cross-titration of pH indicators with metal indicators was carried out to identify combinations that exhibited a different colour change. The concentration of pH indicators was fixed, while two concentrations of metal indicators were tested (Fig. 3C and Fig. S4). To evaluate whether $MgSO_4$ concentration should be further adjusted in the presence of the metal indicator, we compared the performance of master mixes with (i) a pH indicator, (ii) a metal indicator, (iii) both indicators, and (iv) no indicator, across various $MgSO_4$ concentrations. As shown in Fig. 3D, the initial choice of an additional 5 mM $MgSO_4$ provided the lowest TTP across all mixes. Additionally, UV-Vis spectroscopy was performed to evaluate the perceived difference between positive and negative amplification for each colourimetric reaction. Adjusted ΔE values were calculated (Fig. S5) and the following combinations PR100, PR100/EBT75, BB100/EBT50, BB100/C50, and TB150/EBT100 were selected to be lyophilised. Among them, four were pH-metal combinations and one was a pH indicator.

Development and evaluation of lyophilised cLAMP with clinical samples

To preserve the required initial pH (~8.0) after rehydration, several conditions were tested which involved increasing the salt concentration in the master mix prior to lyophilisation. The optimal condition was determined to be 35 mM of NaOH in the 2x cLAMP reaction mix.[34] Five master mixes (MM1 to MM5) were lyophilised (lyo-cLAMP) using the optimised LAMP formulation with the top five colourimetric dye combinations. Analytical sensitivity was assessed against their liquid counterparts in real-time and endpoint (Fig. 4A–E), using a serial dilution of synthetic RNA of SARS-CoV-2 ranging from 2×10^4 to 25 copies per reaction. The results showed high analytical sensitivity achieving a limit of detection (LOD) of 50 copies per reaction, a drop of pH of 1.2 ± 0.1 pH units on average (Fig. 4F), and the absence of non-specific amplification (negative NTCs). Although quantification of ΔpH is not clinically relevant for the outcome of a qualitative binary assay, these data demonstrate that the amplification-induced pH change is consistent across all formulations, irrespective of the indicator

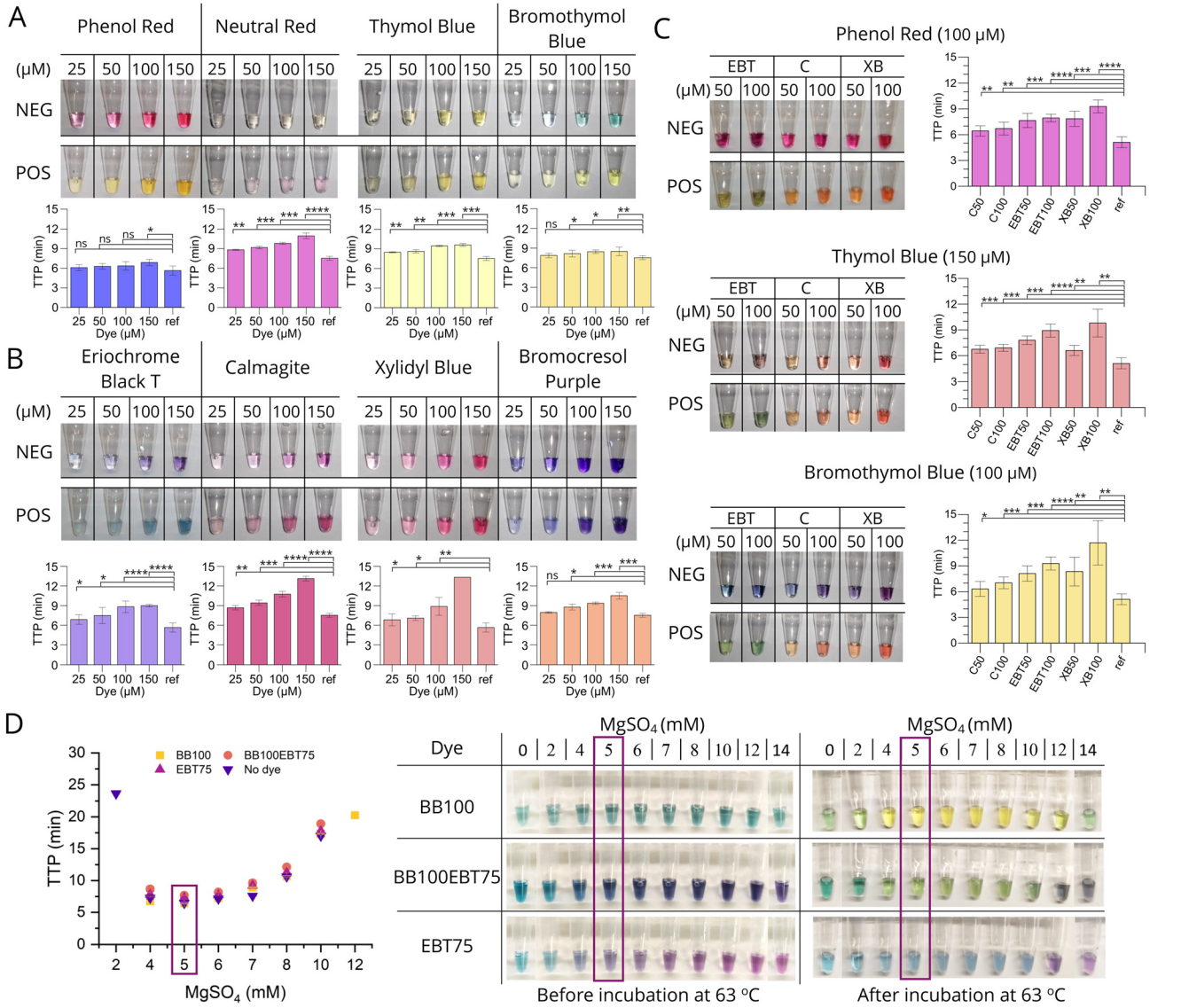

Fig. 3 Evaluation of colourimetric dyes. (A) Pictures of cLAMP reactions using pH sensitive dyes, showing negative (NEG) and positive (POS) amplification after incubation at 63 °C. Real-time data showing TTP values against dye concentration, including as reference (ref) a reaction without dye. (B) Pictures of cLAMP reactions using metal indicators, showing negative (NEG) and positive (POS) amplification after incubation at 63 °C. Real-time data showing TTP values against indicator concentration, including as reference (ref) a reaction without dye. (C) Cross-titration of pH sensitive dyes with metal indicators eriochrome black T (EBT), calmagite (C), and xylidyl blue (XB). Pictures of cLAMP reactions, showing negative (NEG) and positive (POS) amplification after incubation at 63 °C. Real-time data showing TTP values against dye concentration, including as reference (ref) a reaction without dye. (D) $MgSO_4$ titration (0–14 mM) in cLAMP comparing its effect in the presence of pH and metal indicators and the combination of both.

used. This confirms that observed differences in colourimetric performance reflect indicator properties rather than differences in amplification efficiency. Furthermore, ΔpH measurements may be informative when investigating suspected false-negative results, particularly when the pH change falls outside the effective working range of a given indicator. The colour remained consistent between the liquid and lyophilised formats after rehydration, with an estimated shelf-life of 90 days. Accelerated and real-time ageing studies were performed at two temperatures using 250 copies per reaction of extracted SARS-CoV-2 RNA to determine the long-term stability of lyo-cLAMP, data is shown in Table S2.

Next, extracted nucleic acids from SARS-CoV-2 positive nasal, nasopharyngeal or buccal swabs (N = 15) were used to confirm clinical performance of the optimised lyo-cLAMP formulation. These samples were characterised by RT-qPCR using the CDC assays targeting SARS-CoV-2 (N1, N2), and the human housekeeping gene RNAseP, with the C_q values distribution shown in Fig. 5F and complete C_q data in Table S3. The five lyo-cLAMP formulations were rehydrated with the eluted

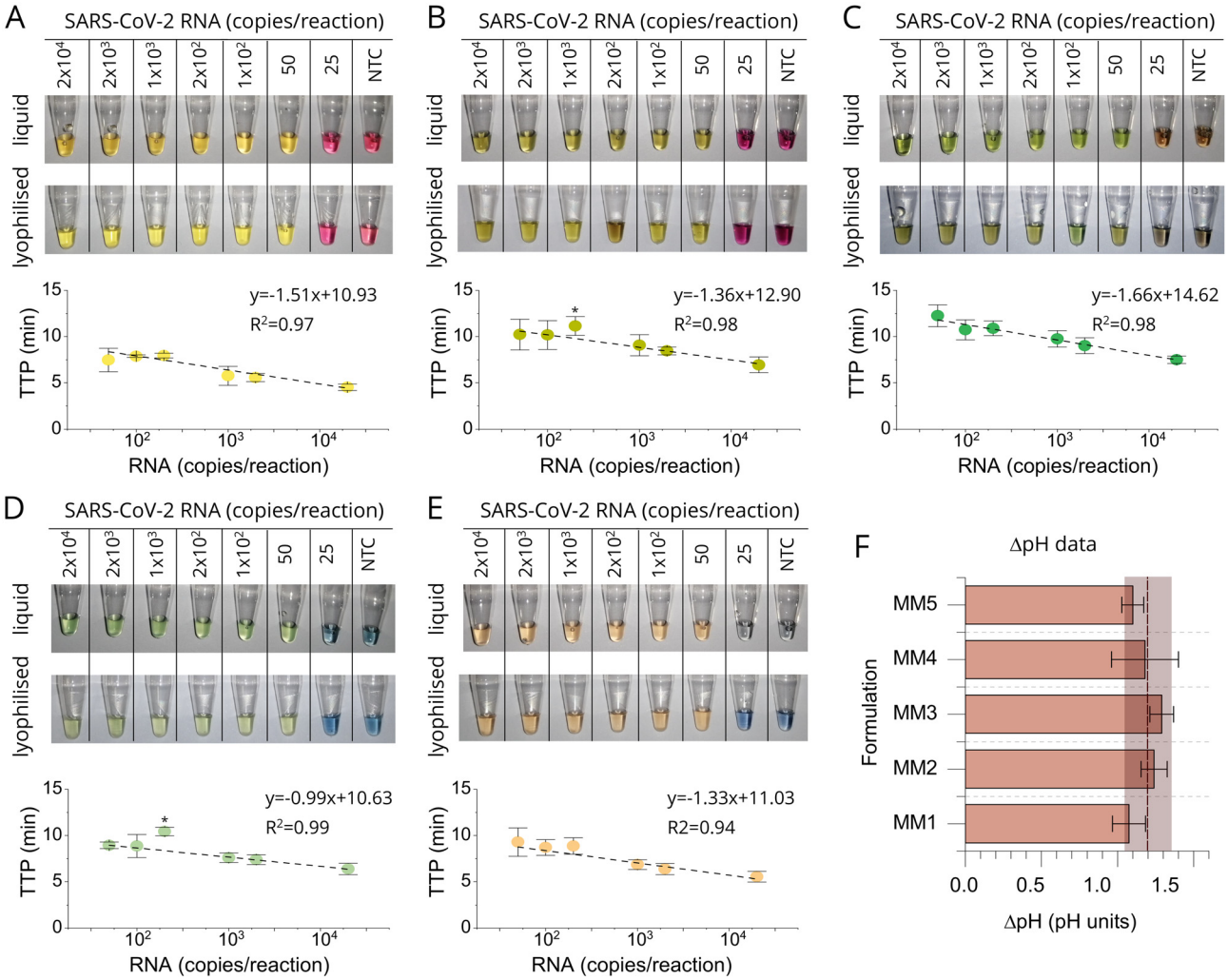

Fig. 4 Analytical sensitivity of colourimetric lyophilised reactions. Pictures and standard curves (TTP against RNA concentration) of lyo-cLAMP reactions with the following indicators: (A) phenol red 100 μM, (B) phenol red 100 μM with eriochorme black T 75 μM, (C) thymol blue 150 μM with eriochorme black T 100 μM, (D) bromothymol blue 100 μM with eriochorme black T 75 μM, and (E) bromothymol blue 100 μM with calmagite 50 μM. Pictures showing negative (NEG) and positive (POS) amplification after incubation at 63 °C, including liquid cLAMP as reference. (F) ΔpH data obtained from lyo-cLAMP (MM1 to MM5) positive amplification reactions before and after amplification. Vertical line denotes the average, and the standard deviation is shadowed.

nucleic acids and incubated for 35 min in a heat block. Results (Fig. 5A and E) showed that one sample ("±") was not clearly detected by the formulations. The highlighted sample (C_q of 26.94 ± 0.01) is considered positive for MM1 and MM5, although a transition-colour is observed for both; orange for MM1, and orange-grey for MM5. Similar transition-colours are also observed with MM2, MM3 and MM4 where the difference to the NTC control is less evident. MM5 missed another sample (C_q of 21.91 ± 0.04), which could have been an outlier as samples with higher C_q were successfully detected with MM5. A limitation of this study is the small sample size; larger datasets are needed to evaluate dual colour indicators. This proof-of-concept study shows that combining pH and metal indicators is a promising alternative to single-dye tests, producing a wider colour palette with comparable performance. Increased resilience to inadequate starting reaction conditions is showed in Fig. S6, where only one dye would be within the working range in certain cases. This could be particularly advantageous if lyophilisation leads to loss of salts or altered pH, or if extracted samples present a pH outside the working range of the pH indicator. In tests with 12-month-old lyo-cLAMP strips, the indicator colours after rehydration were out of range but could be restored by adjusting pH with NaOH (Fig. S7). Including a metal indicator could address this issue if the colour change after amplification differs from the starting colour. Overall, these results support the successful translation of cLAMP into a lyophilised format with a wider colour palette that is stable at room-temperature and requires minimal handling, as only the sample eluant needs to be added.

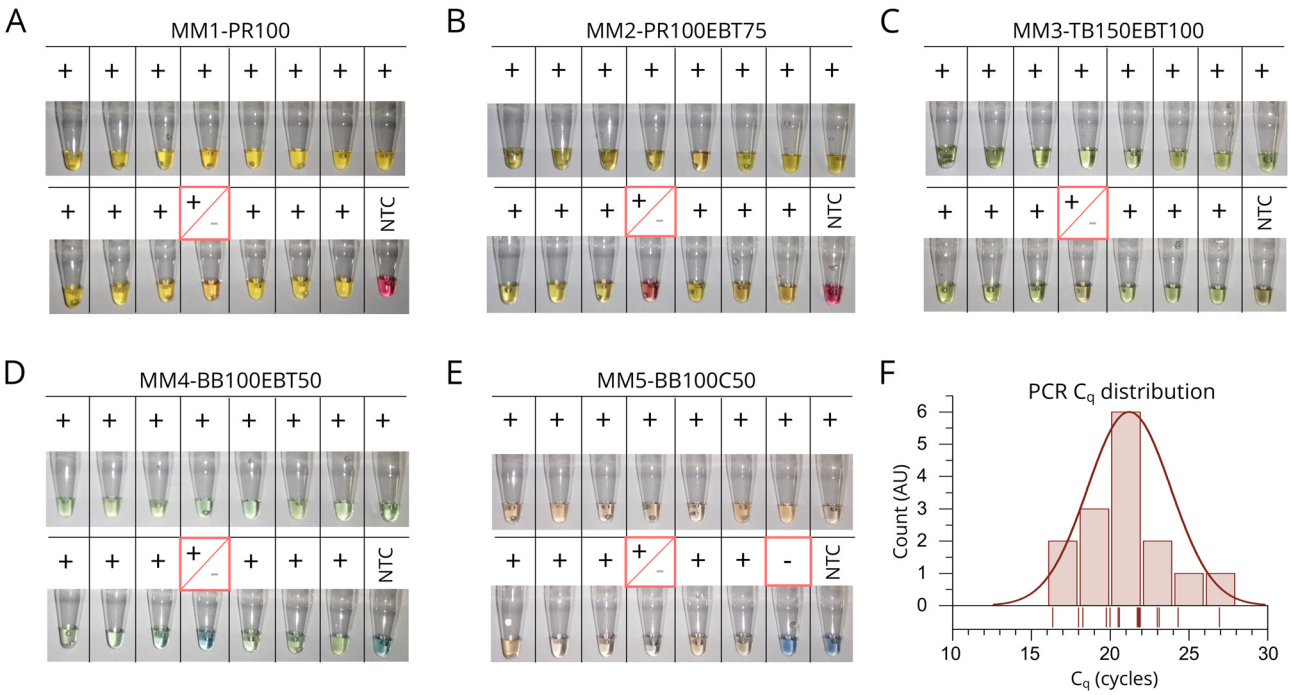

Fig. 5 Evaluation of lyo-cLAMP with residual respiratory specimens from nasal, nasopharyngeal or buccal swabs. Pictures of lyo-cLAMP reactions after 35 min incubation at 63 °C using: (A) lyo-cLAMP MM1 (phenol red 100 μM), (B) lyo-cLAMP MM2 (phenol red 100 μM with eriochorme black T 75 μM), (C) lyo-cLAMP MM3 (thymol blue 150 μM with eriochorme black T 100 μM), (D) lyo-cLAMP MM4 (bromothymol blue 100 μM with eriochorme black T 75 μM), and (E) lyo-cLAMP MM5 (bromothymol blue 100 μM with calmagite 50 μM). (F) PCR C_q values distribution using CDC assays targeting SARS-CoV-2 (N1). Extracted nucleic acids from nasal, nasopharyngeal or buccal swabs (N = 15).

Conclusion

Lyophilised colourimetric LAMP (lyo-cLAMP) presents a practical solution for translating molecular testing to POC settings. By eliminating the need for cold-chain storage and simplifying the testing process to a single sample-loading step after nucleic acid extraction, lyo-cLAMP significantly facilitates the deployment of molecular tests in the field. To the best of our knowledge, there are no lyo-cLAMP master mixes on the market, and only a liquid colorimetric master mix is available.[35,36] Compared to other studies that rely on this commercial formulation, we present an in-house developed lyo-cLAMP protocol using off-the-shelf reagents. This approach provides greater flexibility in assay optimisation and test development.

Through optimisation of the LAMP reaction mix, we identified that glycerol, commonly used as a stabiliser for reagents such as BSA, betaine, and enzymes, inhibits reaction efficiency. Reducing glycerol content improved amplification speed and enabled successful lyophilisation without compromising enzyme performance which was confirmed by comparing Bst polymerase with and without glycerol, showing comparable results. Additionally, while magnesium sulphate is essential as a cofactor for Bst, we observed that excess of $MgSO_4$ delayed amplification. A minimal but sufficient concentration was therefore selected to ensure robust performance. We systematically analysed the contribution of individual LAMP components and evaluated several reported enhancers (Fig. S8). However, none of the tested additives yielded a significant improvement in reaction efficiency.[6,22]

Colourimetric LAMP has gained attention in recent years due to its potential for field-deployable molecular diagnostics. Most studies have focused on either pH or metal ion indicators in isolation, overlooking potential synergistic effects. We tested various pH and metal indicators, as well as their combinations, and demonstrated that their combination is not only feasible but advantageous. As these combinations generate a broader palette of detectable colours, improving assay robustness against variations in sample pH or magnesium concentration, an important consideration for field applications. Among the blended dyes, the combination of bromothymol blue (100 μM) and calmagite (50 μM) showed the best performance both analytically and with clinical samples. Additionally, phenol red (100 μM) stood out as an individual dye exhibiting high sensitivity and accuracy. Although the sample size was limited, testing with clinical samples in this proof-of-concept study demonstrated the potential of lyo-cLAMP for clinical translation. There exists a limitation in sensitivity at low viral loads, but this should be further explored with a larger sample size and with a complete sample-to-result workflow.

This work introduces ready-to-use lyo-cLAMP[34] that requires no additional reagents or manual intervention beyond the addition of extracted sample elution. The developed master mix is in principle compatible with any LAMP assay, allowing

for rapid adaptation to new targets, particularly valuable in disease outbreak scenarios. A recent example of this adaptability is the integration of the developed lyo-cLAMP with SmartLid[19] in the Dragonfly diagnostic platform for the rapid diagnosis of monkeypox and malaria.[8,37] Lyo-cLAMP addresses one of the main limitations of molecular diagnostics, cold-chain dependency and manual handling, by offering a one-step, robust, and easily adaptable solution for POC testing.

Author contributions

Study concept and design: KMC, OWS, and JRM. Acquisition, analysis, and interpretation of data: KMC and OWS. Drafting the manuscript: KMC and OWS. Critical revision of the manuscript: KMC, OWS, MLC, AH, and JRM. All authors have given approval to the final version of the manuscript. KMC and OWS contributed equally.

Conflicts of interest

The authors declare the following competing financial interest (s): KMC, OWS, MLC and JRM have or had financial interest on ProtonDx Ltd, which currently has exclusive license to intellectual property linked to lyophilised colourimetric LAMP. All authors declare that they have no other conflict of interest related to this work. All the authors declare that they do not have any other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting this article have been included in the main manuscript or as part of the supplementary information (SI). Supplementary information: LAMP assay specificity; optimisation of Bst polymerases; evaluation of excipients and colourimetric dyes including adjusted ΔE values; robustness of combining dual colour indicators; shelf-life of lyo-cLAMP; effect of additives in LAMP performance; primer sequences and PCR data from respiratory specimens. See DOI: https://doi.org/10.1039/d5an01238d.

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