Reports

Visual detection of isothermal nucleic acid amplification using pH-sensitive dyes

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Nucleic acid amplification is the basis for many molecular diagnostic assays. In these cases, the amplification product must be detected and analyzed, typically requiring extended workflow time, sophisticated equipment, or both. Here we present a novel method of amplification detection that harnesses the pH change resulting from amplification reactions performed with minimal buffering capacity. In loop-mediated isothermal amplification (LAMP) reactions, we achieved rapid (<30 min) and sensitive (<10 copies) visual detection using pH-sensitive dyes. Additionally, the detection can be performed in real time, enabling high-throughput or quantitative applications. We also demonstrate this visual detection for another isothermal amplification method (strand-displacement amplification), PCR, and reverse transcription LAMP (RT-LAMP) detection of RNA. The colorimetric detection of amplification presented here represents a generally applicable approach for visual detection of nucleic acid amplification, enabling molecular diagnostic tests to be analyzed immediately without the need for specialized and expensive instrumentation.

Nucleic acid amplification is an essential tool in life sciences fields from basic laboratory research to clinical diagnostics. The use of diagnostic nucleic acid amplification has expanded outside of specialized laboratory settings as improvements in cost and amplification techniques have enabled wider access. The key technique for amplification has long been the polymerase chain reaction (PCR), which, although widely used and understood, requires thermal cycling equipment and some mechanism of amplification detection, either through real-time monitoring (e.g., fluorimetry) or post-reaction electrophoresis. Sequence-specific isothermal amplification represents a promising alternative that obviates the need for even basic equipment; examples of this approach include strand-displacement amplification (SDA) (1,2), helicase-dependent amplification (HDA) (3,4), and loop-mediated isothermal amplification (LAMP) (5,6).

LAMP in particular has been used in a number of field and point-of-care diagnostics (7–9). LAMP reactions use a strand-displacing DNA polymerase (and reverse transcriptase for RNA targets) with four to six primers, resulting in highly exponential amplification (5,10). This high degree of DNA synthesis facilitates visual detection of positive amplification based on the precipitation of magnesium pyrophosphate. Detection can be performed in real time with a specialized turbidity instrument or by direct visual assessment, although the latter typically requires a long incubation time (≥60 min), and the precipitate can be difficult to see under even ideal conditions (11–13).

Alternative detection methods for nucleic acid amplification use the color change of a metal-sensitive indicator, such as a shift from dark yellow to yellow (calcein) (14), dark blue to blue (hydroxynaphthol blue) (15), or dark blue to light blue (malachite green) (16–17). These indicators require long incubation times (typically 60 min) and have been demonstrated with only moderate sensitivity (>100–1000 copies of target) (14–18). Metal-sensitive indicators have been successfully applied in LAMP reactions; however, the concentrations of reaction cofactors and degree of amplification required have so far precluded extension to PCR and other methods.

Intercalating nucleic acid dyes can be added to the reactions for real-time and visual detection, but clear visualization requires UV illumination (15). When a DNA polymerase incorporates a deoxyribonucleoside triphosphate into the nascent DNA, the released by-products
include a pyrophosphate moiety and a hydrogen ion. The release of this proton has been the basis of previous detection methods, notably the semiconductor sequencing technology in the Ion Torrent platforms (19–21). A version of this pH sensor was recently used to monitor DNA amplification with LAMP and PCR (22). These studies also demonstrated the ability of a DNA polymerase to perform efficient synthesis in weakly buffered or nonbuffered solutions. We used this enzyme property to perform LAMP reactions in the presence of low concentrations of buffer and observed a significant change from an initial alkaline pH to a final acidic pH. Such a significant change in pH presented the possibility of detecting DNA amplification through the use of pH-sensitive indicator dyes, which we present here as a rapid, robust method for detecting LAMP amplification. We also demonstrate the universality of this approach by its detection of PCR and SDA reactions.

Materials and methods

LAMP reactions

Unless otherwise indicated, LAMP reactions contained 10 mM (NH₄)₂SO₄, 50 mM KCl, 8 mM MgSO₄, 1.4 mM dNTPs, and 0.1% v/v Tween-20 and were incubated at 65°C. All components were mixed in water, and 1 M KOH was used to adjust the pH to 8.8–9.0, as measured by pH paper (colorpHast, EMD Millipore, Billerica, MA) or a pH meter. For low initial pH reactions (bromocresol purple), pH was adjusted to 7.5; for high-pH reactions (thymol blue, naphtholphthalein) pH was adjusted to 10.0. Immediately before reaction, primers (sequences in Supplementary Table S1) were added and reactions were incubated at 65°C. All controls were performed in triplicate.

Colorimetric pH detection

All indicator dyes were obtained from Sigma-Aldrich (St. Louis, MO) and made into 50 mM stocks in water (or ethanol for naphtholphthalein) pH was adjusted to 10.0. Immediately before reaction, primers (sequences in Supplementary Table S1) were added and reactions were incubated at 65°C. For the indicated time, visually inspected for color change after removal of reaction temperature, and scanned. For fluorescence measurement, 2′,7′-bis-(2-carboxyethyl)-5′-(and 6′)-carboxyfluorescin, acetoxymethyl ester (BCECF-AM) and 5′-(and 6′)-carboxy SNARF-1 (Life Technologies, Carlsbad, CA) were diluted in water to 50 µM and 250 µM, respectively, and added immediately before the reaction was initiated. Real-time fluorescence was measured using a Bio-Rad (Hercules, CA) CFX96 Touch instrument with plate reads every 15 s. Channel 1 (FAM) was used to record BCECF-AM, and channels 2 (HEX) and 3 (ROX) were used to record the low- and high-pH forms of SNARF-1. Three raw (without background subtraction) fluorescence curves for each sample were averaged and then background corrected using initial (SNARF-1 low pH) or final (BCECF-AM, SNARF-1 high pH) fluorescence values for presentation.

PCR

PCR was performed in a 50 µL reaction containing 50 mM KCl, 2 mM MgCl₂, 0.3 mM dNTPs, and 0.075 U/µL Taq DNA polymerase (New England Biolabs), with pH adjusted to ~8.5 with 1 M KOH. Reactions were incubated at 95°C for 2 min followed by 40 cycles of 95°C for 10 s, 58°C for 15 s, and 68°C for 30 s. Amplified target was a 1287 bp fragment from pAIL17 plasmid DNA (a gift from W. Jack, New England Biolabs). Amplification was detected using 100 µM phenol red and confirmed with 1% agarose gel electrophoresis.

SDA

SDA was performed in a solution containing 10 mM (NH₄)₂SO₄, 50 mM KCl, 8 mM MgSO₄, 0.4 mM dATP/dGTP/dTTP, and 0.8 mM 2′-deoxyctydine-5′-O-(1-thiotriphosphate) (dCTP-αS; Trilink Biotechnologies, San Diego, CA), with the pH adjusted to ~8.5 with 1 M KOH. Reactions contained 0.5 µM each of forward and reverse SDA and Bump primers designed to target the human BRCA1 gene (Supplementary Table S1), along with 100 ng HeLa genomic DNA, 0.32 U/µL Bst 2.0 DNA polymerase, and 0.2 U/µL BsoBI (New England Biolabs). Indicator dyes were included at 100 µM as described, and reactions were incubated at 65°C.

Results and discussion

We investigated the behavior of LAMP DNA polymerases (Bst DNA polymerase, Large Fragment and Bst 2.0 DNA polymerase) in minimal buffering conditions and observed a wide range of pH tolerance, with efficient LAMP reactions occurring within a pH range of 6.0–10.0. To determine the drop in pH caused by LAMP amplification, we measured the reaction pH before initiation and after completion and observed a significant drop in pH, from the initial value of pH 8.8 to a final value of pH 6.0–6.5 (Figure 1A). The pH tolerance of LAMP reactions and this >2 pH unit decrease enables, in theory, the use of pH indicator dyes to monitor amplification success in LAMP reactions. There are many commercially available pH indicator dyes that have easily discernable color transitions in the neutral range (Supplementary Table S2). For visual detection,
the indicator dye concentration needed to be relatively high (>20 μM), and we first determined the effect of these dye concentrations on LAMP reactions. Performing LAMP with a control lambda DNA amplicon and 5–50 μM of various indicators had minimal effect on threshold time as measured by SYTO-9 intercalation (Supplementary Figure S1).

Next, we demonstrated the use of indicator dyes to detect amplification. LAMP reactions were performed using a pH 8.8 solution containing only a small amount of Tris-HCl carried over from the DNA polymerase storage buffer (10 mM Tris-HCl in the enzyme storage buffer, 25–400 μM the in final reaction). Figure 1B shows reactions at setup, with the initial reaction color dependent on the specific dye and the starting pH. After a 30 min incubation at 65°C, reactions containing DNA polymerase displayed color changes, such as red to yellow (phenol red, cresol red) or faint orange to pink (neutral red). These results showed that indicator dyes were amenable to standard LAMP conditions with minimal buffering, indicating strongly at initial pH 8.8 and changing to the low-pH color rapidly in reactions. Many other indicators are available, but their color transitions occur in pH ranges not well suited for DNA polymerase reactions. Examples are shown in Supplementary Figure S2, with dyes that require a higher (>pH 9.5) initial pH for clear indication (e.g., naphtholphthalein, thymol blue) or a lower initial pH (pH 7.5) to reach the necessary pH transition zone. These dyes were usable but required longer reaction times, likely because of the larger pH changes needed to reach transition and suboptimal polymerase performance at the pH range extremes. Thus, we preferred the dyes shown in Figures 1–3 (phenol red, cresol red, neutral red, m-cresol purple). Reactions without DNA polymerase remained at the high-pH indicator color, demonstrating that, although not buffered or degassed, the solution’s pH is sufficiently stable during a lengthy incubation at 65°C to retain the initial indicator color. We observed that a small reaction volume (25 μL), sealed vessels, and weak buffering (26 μM Tris) were sufficient to maintain pH without enzyme through at least a 2 hour 65°C incubation followed by overnight storage at room temperature.

Having demonstrated that pH indicator dyes are compatible with LAMP reactions, we next examined the effect of background or nontemplate amplification. LAMP, although highly specific in regards to template sequence recognition, can result in substantial nontemplate amplification depending on reaction conditions and primers (25). Figure 2A shows the specificity of the pH change during amplification using two amplicons that display nontemplate amplification when assayed using other detection methods. Both primer sets for the detection of C. elegans lec-10 and human BRCA1 showed a pH drop in positive reactions in under 15 min, but negative control reactions maintained the initial reaction color. Extending the incubation time to 60 min resulted in some color change that we attribute to nontemplate amplification, with an intermediate color change visible in some control reactions. These results demonstrate that pH detection performs similarly to other methods in regards to nontemplate amplification, but even when primer design and optimi-
Figure 2. Specificity and sensitivity of colorimetric detection. (A) Specificity of pH amplification detection. Loop-mediated isothermal amplification (LAMP) reactions with either lec-10 primers and Caenorhabditis elegans DNA or BRCA1 primers and HeLa genomic DNA. All reactions contained 8 U Bst 2.0 DNA polymerase, were pH 8.8 (26 µM Tris) at initiation, and were incubated for 60 min at 65°C. At 15 min, a pH-induced color change was apparent in all positive reactions, but nontemplate controls (NTCs) remained the starting color. After 60 min, positive reactions showed increased color change, and some intermediate change was observed in NTCs. (B) Sensitivity of pH amplification detection. LAMP reactions with cystic fibrosis transmembrane conductance regulator gene (CFTR) primers and various amounts of HeLa genomic DNA as indicated were incubated for 30 min at 65°C. At 15 min, a pH-induced color change was clearly seen in reactions with higher template concentrations or those containing m-cresol purple (excluding the nontemplate control). By 30 min, reactions at all template concentrations exhibited color changes indicative of amplification. For all dyes, the nontemplate control remained at the initial high pH color after 30 min of incubation.

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zation cannot be performed, the amplification time differential in the positive and negative reactions is sufficient to allow discrimination.

The potential reaction sensitivity when using pH indicator dyes is shown in Figure 2B, where a range of HeLa DNA amounts (0.01–100 ng; ~3–30,000 copies) was detected using primers for the cystic fibrosis transmembrane conductance regulator gene (CFTR). After 15 min, all template concentrations above 10 pg (~3 copies) were clearly detected with an intermediate change seen for the reactions containing 10 pg of DNA. After 30 min, all concentrations indicated amplification, but the nontemplate controls (0 ng input DNA) remained at the starting reaction color. This 5 order-of-magnitude dynamic range covers typical test DNA amounts and demonstrates a clear, robust amplification signal visible by eye for samples containing as little as 2–3 copies of the target in under 30 min and under 15 min for template amounts above 10 copies.

Although LAMP has useful characteristics and is a reliable amplification method, numerous other technologies are widely used, including the most popular method, PCR. Therefore, we tested the pH indicator dye method for applicability to PCR and SDA reactions. Supplementary Figure S3A shows a pH indicator dye color change in low-buffer PCR amplifying a 1287 bp fragment from plasmid DNA in the presence of phenol red. Supplementary Figure S3B shows a color change in low-buffer SDA reactions amplifying a region of the BRCA1 gene with three pH dyes. As shown, both methods produced clear changes in color because of a drop in pH. However, PCR reactions were prone to a background pH drop caused by thermal cycling, but using cap-sealed PCR tubes and 25 µL of mineral oil mitigated this problem and enabled consistent colorimetric pH detection. SDA required >30 min to observe a clear color change, and PCR needed >20 cycles even with ~10^6 copies of input target DNA compared with LAMP reactions, which displayed a pH drop in <10 min. We observed for PCR a dependence of color change on amplicon length: amplification of products <500 bp did not produce a color change even with 40 cycles of PCR, whereas amplification of longer (1.2–10 kb) products produced a robust color change (data not shown). This is evidence that the amount of DNA synthesis, and thus proton release, determines the magnitude of the drop in pH in weakly buffered amplification reactions. These additional constraints and considerations for PCR reactions indicate that our detection method is best suited to isothermal amplification.

We also sought to demonstrate colorimetric pH detection of amplification from an RNA template, using RT-LAMP as an example method. Using primers for human hydroxymethylbilane synthase gene (Supplementary Table S1), Bst 2.0 DNA polymerase, and the reverse transcriptase RTx, we ran one-step RT-LAMP reactions with 10 ng Jurkat total RNA. Reactions were heated directly to 65°C and incubated for 30 min, and color change was detected in the presence of neutral red and RNA but not in reactions lacking RNA (Supplementary Figure S3C). This indicates that RTx was active in low-buffer reactions and further
demonstrates the applicability of colorimetric pH detection to various amplification methods, whether RNA or DNA templates are used as amplification targets.

As an alternative to visual detection, we examined real-time detection of the nonbuffered LAMP reactions using a 96-well plate format amenable to quantitative or high-throughput applications. We used fluorescent pH indicators that were observed to have pH-dependent spectral shifts at LAMP temperatures (BCECF-AM and SNARF-1). These dyes have pK<sub>a</sub> values of pH 6.98 and 7.5, respectively, which are close to neutral pH, and exhibit robust fluorescence changes (>10-fold) through pH 6.0–9.0 (Supplementary Figure S4). BCECF-AM exhibits high fluorescence at high pH (λ<sub>ex/λ</sub> at pH 9 500/530 nm), which decreases with decreasing pH, but SNARF-1 shifts its excitation/emission maxima from 575/650 nm (pH 9) to 525/590 nm (pH 6), enabling both a loss- and gain-of-signal fluorescent detection. We measured BCECF-AM fluorescence using the SYBR/FAM channel of a CFX-96 real-time PCR instrument and SNARF-1 using the HEX/TET (low-pH) and ROX/TexasRed (high-pH) channels. Performing low-buffer LAMP with 2 μM BCECF-AM resulted in a fluorescence decrease of >10,000 counts, with a rapid drop in signal at ~10 min (Figure 3A), matching the threshold times observed using SYTO-9 or turbidity detection. Reactions with SNARF-1 gave a fluorescence decrease of >15,000 counts in the ROX (high-pH) channel and a gain of ~100 counts in the HEX (low-pH) channel. Normalizing curves to maximum fluorescence shows a concomitant change in the two signals (Supplementary Figure S5), indicating that either channel or pH form can be used for SNARF-1. Figure 3A shows negative control reactions, which showed only background pH changes compared with the rapid and significant change of the positive reactions. The sensitivity of this detection is shown in Figure 3B, with 10 pg of HeLa DNA (~3 copies) detected in <15 min (CFTR primers), consistent with the visual detection shown in Figure 2B. Together, these data demonstrate that the pH change of LAMP reactions can be monitored in real time. For real-time detection, we used the lower stock concentration (8000 U/mL) of DNA polymerase that is commercially available, resulting in carryover of 0.4 mM Tris. Reactions with 100 ng HeLa DNA also carried over 0.4 mM Tris; thus, the high template input condition contained 0.8 mM Tris but still demonstrated a robust pH change at ~10 min consistent with a linear response to input amount. Although buffer concentration is important for detecting amplification in these reactions and should be kept at a minimum, these results demonstrate flexibility of input to buffered components, where the amount of carried-over buffer can exceed 0.5 mM.

Isothermal amplification techniques such as LAMP offer the promise of nucleic acid diagnostics with simplified instrumentation, or none at all (7). One advantage of LAMP is the ability to detect reactions by eye, facilitating field, point-of-care, and even basic rapid laboratory detection (8,9). However, previously published methods have required long incubation times using either difficult-to-see precipitation or subtle changes in the color of an indicator (11,13–18). As demonstrated here, a robust and rapid alternative for visual detection is monitoring the pH drop in the reaction mixture with indicator dyes. LAMP reactions are capable of producing a 2–3 pH unit drop without loss of reaction efficiency in very weakly buffered solutions.
This pH change is sufficient to enable the use of neutral-range transition pH indicator dyes, which are low-priced, simple to use, and exhibit easy-to-see color changes. Because this detection method responds directly to the release of protons, a natural consequence of any polymerase-catalyzed amplification reaction, it can be applied to many amplification methods, as demonstrated here with the isothermal methods LAMP, RT-LAMP, and SDA; detection in PCR is possible but is more limited compared with the isothermal techniques. Detection of amplification through pH change may not be suitable for all methodologies; for example, in helicase-dependent amplification (HDA), which includes enzymes that catalyze nucleotide hydrolysis, a drop in pH may occur because of helicase activity decoupled from amplification.

In general, isothermal amplification reactions have characteristics well suited for facilitating point-of-care and field applications, such as no requirement for a thermocycler, rapid amplification, and tolerance of inhibitors. However, the methods of detection in current use limit this potential to laboratory settings or require instrumentation for reliable analysis. The low cost, ease of visualization without instruments, and flexibility of the pH-sensitive indicator dyes may allow the promise of isothermal amplification technologies to be fully realized. The method presented here enables easy visual detection of an amplification reaction without any requirement beyond heating to reaction temperature; for room temperature reactions (e.g., recombinase-polymerase amplification) (26), no equipment would be required at all. Future studies will investigate the feasibility of using minimally processed samples with low-buffer LAMP reactions, but the variety of indicator dyes available and the tolerance of our assay for up to ~1 mM of a wide pH range buffer (such as Tris) already provides flexibility in designing compatible nucleic acid amplification reactions. In summary, the use of colorimetric visualization of amplification by detecting pH changes could further the use of amplification as a molecular diagnostic tool, enabling field diagnostics with clear, rapid, and robust visual detection.

Author contributions
N.T. and T.C.E. conceived the method and designed the experiments. N.T. and Y.Z. performed the experiments. N.T., Y.Z., and T.C.E. wrote the manuscript.

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Competing interests
The authors are employed by New England Biolabs, manufacturer of the amplification reagents described in this manuscript, and have filed a patent application describing this method.

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