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Isothermal multiple displacement amplification of DNA templates in minimally buffered conditions using phi29 polymerase†

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The isothermal amplification of DNA in minimally buffered conditions allows to perform and monitor nucleic acid amplification with minimal technological and operative requirements. We show in this work how phi29 can operate multiple displacement amplification in minimally buffered conditions producing, as a readout, pH shifts attaining subunits of pH.

The isothermal amplification of nucleic acids represents an ideal alternative to classical PCR for the implementation of nucleic acid testing (NAT) in limited resources scenarios (LRS) since it can be attained without precise thermal cycling and dedicated equipment. Strategies like loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA), rolling circle amplification (RCA) and recombinase polymerase amplification (RPA), to name some, have been proposed in different configurations as the working principle of proof-of-concept devices for nucleic acid amplification and detection in LRS and for point-of-care diagnostics.[1–3] These different approaches circumvent the thermal requirements of classical PCR by using proteins that operate the synthesis of DNA more similarly to what was observed *in vivo* (*i.e.* where nucleic acid amplification is attained at physiological temperatures and through complexes of enzymes that unwind, replicate and ligate DNA or RNA templates). *In vitro* isothermal methods rely on the activity of mesophilic polymerases (like phi29 or Bst polymerase) whose working temperature spans the 30–65 °C range and which possess strand displacement activity[4–7] as is the case for phi29.[8] An interesting feature of this enzyme is, in addition to the strong strand displacement activity, its optimal processivity at temperatures close to 30 °C, which makes it an ideal candidate for the amplification of nucleic acids under conditions of minimal heating (*i.e.* at environmental temperatures). The existence of commercial phi29-based DNA amplification kits that can be stored at room temperature and that are ordinarily employed in research and diagnostics[9,10] corroborates the adaptability of phi29 biochemistry to perform NAT in scenarios where a cold chain and adequate stocking conditions cannot be attained or guaranteed.

The detection of nucleic acid amplification through pH shifts generated by elongation has raised major interest for its translational potential, demonstrated by the existence of commercial solutions for pH-based DNA sequencing, amplification and detection.[11,12] The biochemistry of DNA polymerization generates a proton for each nucleotide incorporated in elongating DNA strands[13] (Fig. 1A), and this phenomenon can be exploited to monitor DNA amplification through the accumulation of protons (*i.e.* the decrease of pH) in the reaction solution (Fig. 1B).

The interest for an approach based on isothermal amplification and pH readout is highlighted by the ever-increasing
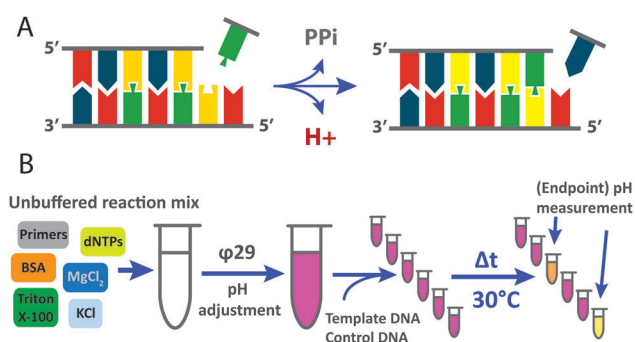

Fig. 1 By-products of polymerase-mediated elongation and explanatory diagram of the experiments performed in this work. (A) The elongation of DNA strands operated by polymerases generates an accumulation of pyrophosphate and the production of protons which can be exploited for the detection of DNA amplification. (B) The minimally buffered mixtures used for the experiments described in this work were adjusted to an initial pH value and aliquoted to perform individual amplification reactions after the addition of specific templates and control DNA. The elongation operated by phi29 resulted in the acidification of the reaction tubes where amplification was attained (blue arrows).

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number of proof-of-concept solutions, often relying on minimal thermal cycling (*e.g.* a single-step initial denaturation and isothermal elongation) or on incubation at temperatures higher than or equal to 37 °C.[3,14–17]

We tested in this work the feasibility of multiple displacement amplification (MDA) of DNA templates through phi29 using reaction formulations bearing only micromolar amounts of buffering agents and at constant low temperatures (*i.e.* 30 °C), demonstrating how phi29 can be successfully employed in minimally buffered and isothermal conditions to catalyse the amplification of DNA with template specificity.

We elaborated a reaction mixture devoid of buffering components normally contained in polymerase reaction buffers (*i.e.* Tris–HCl), yet maintaining similar ionic strengths and Mg^{2+} concentrations to the ones recommended for the enzymes used in these experiments – phi29 polymerase from New England Biolabs – Ipswich, MA, USA (product #M0269) or phi29 polymerase from Thermo Fisher Scientific – Waltham, MA, USA (product #EP0092).

We used as a specific target template the circular genome of the human papillomavirus 16 (HPV16) virus (plasmid p1203 pML2d HPV-16 Addgene catalog #10869 – 10.1 kb, a gift provided by Peter Howley). The non-relevant template used as a negative control for non-specific amplification was human genomic DNA (hgDNA) extracted from immortalised T cells (clone J-19 – a kind gift of R. Genolet, UNIL, Lausanne, Switzerland) using the DNeasy Blood and Tissue kit and according to the instructions of the manufacturer (Qiagen, Düsseldorf, Germany). We performed MDA experiments using two different sets of primers previously reported in the literature[18,19] and using different template amounts resuspended in Milli-Q water (to avoid the presence of buffers) pre-emptively quantified by UV spectrophotometry (NanoDrop 2000 – Thermo Fisher Scientific – Waltham, MA, USA).

The unbuffered reaction mixture was composed of 400 mM betaine, 10 mM $MgCl_2$, 66 mM KCl, 500 μM dNTPs (each), 0.1% Triton-X100, and 1 mg ml^{-1} BSA. The MDA was at first performed using a set of 13 different primers annealing on the + strand and the – strand of the HPV16 genome[19] and which is referred to as primer set 1 (Fig. 2A and Table S1, ESI†). The final concentration of the primers in the reaction mixture was 0.25 μM for each oligo. The initial pH of the mixture was adjusted to pH 7.5 using 0.1 M NaOH so to replicate the pH value of the standard reaction buffer provided with the phi29 enzyme used for the experiment. The reactions were initiated by the addition of 2 units of phi29 per single reaction tubes. In general, the employment of commercial enzymes (which are resuspended in solutions containing Tris–HCl) corresponded to the addition of sub-millimolar concentrations of Tris in the reaction mixture (in the 40 μM–1 mM range, depending on the units of enzymes used for every experiment).

We established a template-specific reaction by adding 10 ng of the HPV16 plasmid in a final reaction volume of 50 μl (corresponding to a concentration of 60.5 pM) and two different negative controls to account for the pH drift of the sole reaction mixture (no-template control) and the pH shift generated by the non-specific amplification of a non-relevant template through primer set 1 (*i.e.* hgDNA – 10 ng).
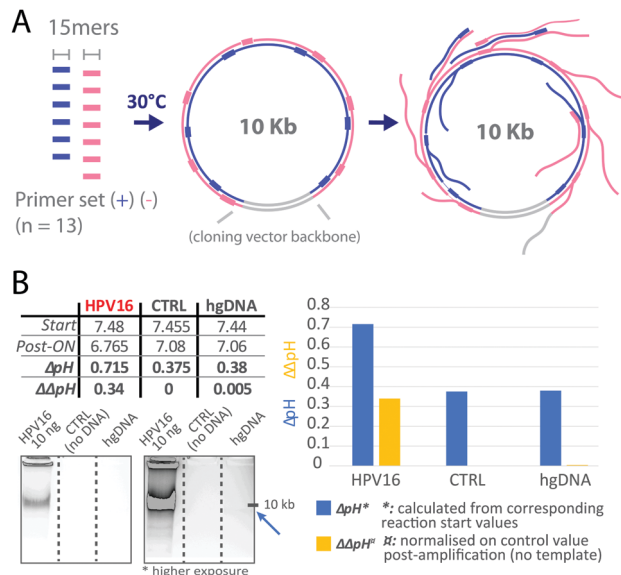

Fig. 2 Non-buffered MDA of circular dsDNA *in vitro* using phi29. (A) Primer strategy for the MDA of the HPV16 genome (cloned in a circular dsDNA backbone plasmid) consisting a mixture of 13 oligonucleotides hybridising the + and − strands of the template. (B) Summary table of pH shifts observed after ON amplification and the relevant bar plot. The electrophoretic run (1% agarose – 21.4 V cm^{-1} constant) of the MDA products underlines how traces of hgDNA amplification could be detected after amplification (blue arrow).

The initial pH values for the different experimental points were measured using a commercial ISFET pH-meter (Sentron SI600 – Leek, the Netherlands) and the reactions were incubated for 16 h (ON) at 30 °C in a PCR thermal cycler (Esco Healthcare Swift MaxPro, Singapore). Notably, no denaturation of the DNA was necessary to attain amplification, as evident from the electrophoretic run in Fig. 2B. This could possibly result from the strong displacement activity of the enzyme coupled to partial denaturation of dsDNA template molecules at 30 °C. The pH of the no-template reaction showed a spontaneous drift towards neutrality. We reckon that this could be due to the possible buffering action of bovine albumin[20,21] resulting from its significant concentration in the primary formulation of the reaction mixture.

We characterized the amplification at lower starting pH values (pHs 7.2 and 7.0) by performing time-resolved MDA experiments (1 h, 4 h and ON incubation) at constant 30 °C. The results of these are recapitulated in Fig. 3.

Concomitantly, we performed the reaction using the standard buffer provided with phi29 polymerase, so as to verify whether the extended amplification could overcome the buffering power of the Tris–HCl present in the commercial reaction mixture. The pH values of all experimental samples were measured at the different time points (Fig. 3A) and an aliquot of each MDA product was loaded on a 1% agarose gel to check for the effective amplification of templates. Fig. 3B shows the result of the electrophoretic run of the samples after ON incubation at either lower exposure times (Fig. 3B, top) or higher exposure times (Fig. 3B, bottom). The amplification of
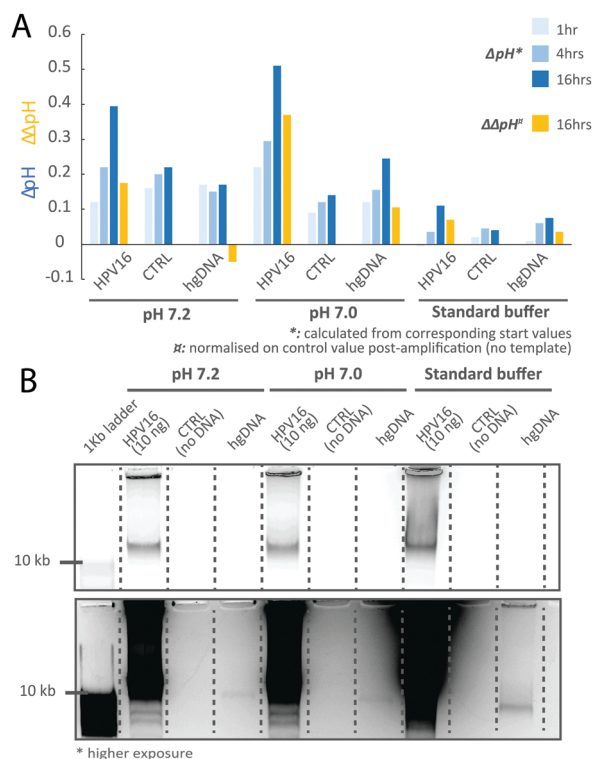
Fig. 3 Time-course amplification of HPV16 and hgDNA under non-buffered conditions with phi29. (A) Bar plot reporting the pH drifts in different amplification reactions (HPV16 template, no-template control and hgDNA) performed for 1 h, 4 h and overnight (16 h) – starting from two different starting pH values (pH 7.0 and pH 7.2). (B) Electrophoretic run performed with an aliquot of the ON amplification product (1% agarose gel – 21.4 V cm^{-1} constant). The different gel exposures are provided to emphasize the presence of background amplification in the case of the non-specific template.

Fig. 4 Serial dilution experiment and quantity-dependent proficiency of the phi29 minimally buffered amplification – primer set 1. (A) MDA was performed using primer set 1 and the products of amplifications (performed for 18 h) were analysed after SYBR Safe incorporation (A – left) and electrophoresis (A – right; 1% agarose, 21.4 V cm^{-1} constant). Similarly to what was previously observed, the reaction produced only minimal and non-specific amplification of the hgDNA control. The different quantities of the HPV16 template resulted, instead, in the proportional accumulation of products and in a correlated pH drift, as highlighted in (B). Error bars report the standard deviation of the three replicates produced for each reaction.

HPV16 templates and the minor, non-specific amplification of the control hgDNA resulted in the accumulation of high molecular weight DNA. As expected, the pH values of MDA reactions performed using the standard buffer showed limited variation (less than 0.1 units of pH after 16 h), independently from the template and from the amount of product generated. In the case of minimally buffered reactions, the absolute pH shift from the respective initial values (plotted as ΔpH in Fig. 3A) reached almost 0.4 and 0.5 units of pH for the HPV16 template – respectively for the pH 7.2 and pH 7.0 reaction mixtures. This corresponded to a maximum ΔΔpH value (calculated from the ΔpH of the specific amplification minus the ΔpH of the no-template control – Fig. 3A) of 0.4 units of pH when using the pH 7.0 mixture after 16 h.

We subsequently tested a new formulation of the reaction mixture where we reduced the quantity of BSA to 0.2 mg ml^{-1} in order to reduce the potential buffering effect of the protein[20,21] while keeping the initial pH of the mixture at pH 7 and the other ingredients unvaried. We performed MDA with primer set 1 on triplicated serial dilutions of a specific HPV16 template and control hgDNA, spanning the range 10 ng; 1 ng; 1 pg and 1 fg (corresponding to 60.5 pM; 6 pM; 6 fM, and 6 aM respectively) per single reaction (Fig. 4). We verified the effective amplification of
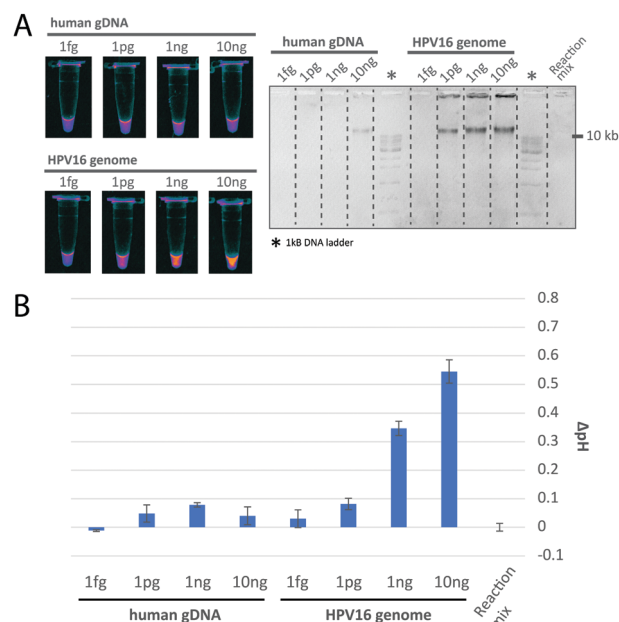
DNA by incubating the products of MDA with SYBR Safe (Thermo Fisher, Waltham, MA, USA) and by performing electrophoresis of sample aliquots on a 1% agarose gel (Fig. 4A). The new formulation of the mixture enabled the specific amplification of the HPV16 template down to the unit of picogram of DNA per single reaction. The pH of each triplicate was measured before the addition of SYBR Safe to the samples and, as reported in the bar plot of Fig. 4, the shift in pH against the *no-template* control (indicated as "ΔpH" in the plot of Fig. 4B) correlated with the quantity of product obtained from the different templates. To further corroborate these results, we performed MDA under similar experimental conditions but using an alternative priming strategy for the rolling circle amplification of human papillomavirus genomes based on 23 different shorter and partially degenerated oligos (9-mers to 12-mers)[18] and indicated hereupon as *primer set 2* (Fig. 5A and Table S1, ESI†). We increased the amount of enzyme to 10 units per reaction for this experiment to test whether the yield of amplification could be increased by coupling increased amounts of enzymes in the mix with the use of partially degenerated oligos, bringing the final concentration of Tris in this mixture to 1 mM. Similarly to what was observed using primer set 1, the amplification of DNA could be attained avoiding the denaturation of the dsDNA template. The incubation at 30 °C overnight (18 h) resulted in the amplification of the specific HPV template, as indicated by the ethidium bromide incorporation assay of products and the
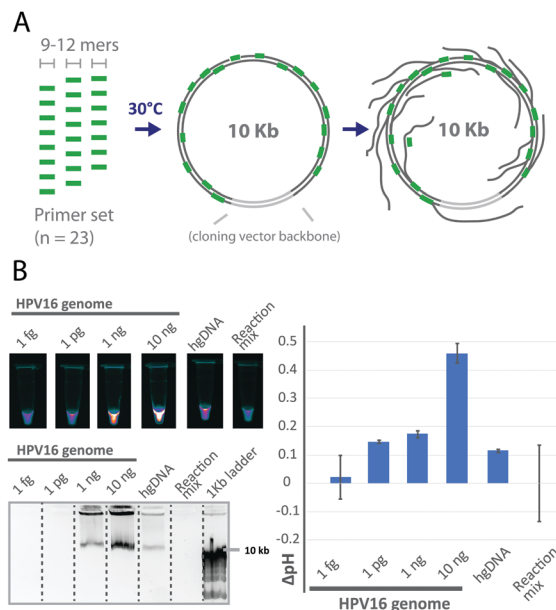
Fig. 5 Serial dilution experiment and template-dependent proficiency of the phi29 minimally buffered amplification – primer set 2. (A) Primer set 2 is composed of 23 partially degenerated sequences hybridising the + and − strands of the template. (B) The products of MDA performed using primer set 2 for 18 h were incubated with ethidium bromide and analysed by fluorescence and electrophoresis (1% agarose – 21.4 V cm^{-1} constant). The reaction produced incremental quantities of the HPV16 product proportional to the amounts of the starting template, which are indicated on the plot (B). Error bars indicate the standard deviation for the pH values of the three replicates measured for each reaction.

electrophoresis runs reported in Fig. 5B. The increased acidity observed after amplification attained the 0.5 units of pH, consistent with that observed using primer set 1, and suggesting how the buffering power of Tris at micromolar concentrations is overcome by the amplification reaction and how, despite increased enzyme concentration and less stringent priming, the exhaustion of the enzymatic activity after long-term incubation dictates the dynamics of the amplification and the final pH shift.

Overall, the results shown in this work underline how phi29 polymerase can be adapted for the amplification of templates using customized minimally-buffered reaction mixtures and in full isothermal conditions requiring minimal heating. The possibility to perform MDA with phi29 in non-buffered conditions, with a reduced thermal input and at a constant temperature, combined with the possibility to use reagents that can circumvent cold-chain requirements (*i.e.* lyophilised reaction mixtures) would enable DNA amplification readouts (as the colorimetric detection of pH shifts or the direct measurement of pH changes through appropriate miniaturised strategies) to put in place as alternatives to conventional methodologies (like fluorescence-based detection) in low resource-compatible implementations and for cost-effective point-of-care diagnostics.

Conflicts of interest

There are no conflicts to declare.

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