

Characterization of a novel DNA polymerase activity assay enabling sensitive, quantitative and universal detection of viable microbes

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ABSTRACT

During the past 50 years, *in vitro* measurement of DNA polymerase activity has become an essential molecular biology tool. Traditional methods used to measure DNA polymerase activity *in vitro* are undesirable due to the usage of radionucleotides. Fluorescence-based DNA polymerase assays have been developed; however, they also suffer from various limitations. Herein we present a rapid, highly sensitive and quantitative assay capable of measuring DNA polymerase extension activity from purified enzymes or directly from microbial lysates. When tested with purified DNA polymerase, the assay detected as little as 2×10^{-11} U of enzyme (~ 50 molecules), while demonstrating excellent linearity ($R^2 = 0.992$). The assay was also able to detect endogenous DNA polymerase extension activity down to less than 10 colony forming units (cfu) of input Gram-positive or Gram-negative bacteria when coupled to bead mill lysis while maintaining an $R^2 = 0.999$. Furthermore, preliminary evidence presented here suggests that DNA polymerase extension activity is an indicator of microbial viability, as demonstrated by the reproducibly strong concordance between assay signal and bacterial colony formation. Together, the innovative methodology described here represents a significant advancement toward sensitive detection of potentially any microorganism containing active DNA polymerase within a given sample matrix.

INTRODUCTION

DNA polymerase activity is indispensable for genome replication and organism propagation across all biological domains (1–3). Since its initial characterization (4), the ability to harness DNA polymerase activity *in vitro* has

become a fundamental tool in the field of molecular biology research (5). Above and beyond its established importance in research, *in vitro* measurement of DNA polymerase activity potentially offers numerous useful applications within the pharmaceutical and clinical setting. For instance, since bacterial DNA polymerase is actively being targeted for the development of novel antimicrobial agents (6,7), a rapid and sensitive assay capable of measuring DNA polymerase activity is desirable. Also, loss or gain of DNA polymerase activity is intimately involved in human disease. For example, emerging links between DNA polymerase activity and genetic aberrations are designating the enzyme as a target for anticancer therapies (8,9). Deficiencies in DNA polymerase activity have also been linked to mitochondrial disorders (10). Furthermore, measurement of DNA polymerase activity has the potential to be used as a rapid and sensitive diagnostic tool, capable of detecting virtually any organism harboring active DNA polymerase within a given environmental or biological matrix where sterility is expected.

The most common method used to measure DNA polymerase activity *in vitro* depends upon the incorporation of radiolabeled nucleotides (11). However, routine use of such DNA polymerase assays is undesirable due to the inherent risks and restrictions associated with radioisotopes. Consequently, over the past few decades numerous non-radioactive *in vitro* polymerase assays have been developed. Some rely upon the measurement of fluorescence generated by DNA polymerase-mediated release of single-stranded binding protein (12) or binding of PicoGreenTM to double-stranded DNA (13,14). Other methods rely on microplate coupling and detection of fluorescently labeled nucleotides (15). More recently, molecular beacon-based (16) and electrochemical-based (17) DNA polymerase assays have been developed. Despite successfully averting the use of radioactivity, the above assays are limited by either poor sensitivity, a small linear dynamic range of measurement or the use of purified polymerase.

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Our laboratory has an ongoing interest in methodology involving enzymatic template generation and amplification (ETGA). Herein we describe the initial characterization of novel ETGA methodology based upon the measurement of DNA polymerase extension (DPE) activity coupled to a quantitative PCR (qPCR) readout. For the remainder of the article, we will refer to this assay as DPE coupled PCR. The DPE-PCR assay is used to measure low levels of purified enzyme and is capable of detecting endogenous DPE activity directly from microbial cell lysates.

MATERIALS AND METHODS

DNA substrate design and preparation

The sequences of the DNA substrate were adapted from DNA oligos previously used to measure bacterial-derived ATP via T4 DNA ligase (18). Oligo 1 (5'-gccgatatcgacaacggcgaactgggaaggcgagactgaccgaccgataagctagaacagagagacaacaac3') and Oligo 2 [5'-uaggcgucggugacaacggccagcguuguugucucu(dideoxyCytidine)-3'] were synthesized by the Integrated DNA Technologies (Coralville, IA, USA). The 'u' in Oligo 2 represents deoxyUridine. DideoxyCytidine (ddC) was included as the last base on the 3'-end of Oligo 2 to block DNA polymerase-mediated extension (see Figure 1 schematic). First, lyophilized Oligos 1 and 2 were resuspended to a final concentration of 100 μ M in sterile Tris-EDTA, pH 8.0 (Ambion). Routine pre-annealing of the substrate was performed as follows. To begin, 100 μ l of Oligo 1 (100 μ M stock) and 100 μ l of Oligo 2 (100 μ M stock) were added to 800 μ l of annealing buffer (200 mM Tris, 100 mM potassium chloride and 0.1 mM EDTA), pH 8.45, resulting in a 1-ml mixture of Oligos 1 and 2 each at 10 μ M. One hundred microliter aliquots of the 10- μ M oligo mixture were dispensed into thin walled 0.2 ml PCR tubes, capped, placed into a GeneAmp[®] 9700 thermocycler (Applied Biosystems) and the following pre-annealing program was performed: 95°C for 2 min, ramp at default speed to 25°C and incubate for 5 min, ramp at default speed to 4°C. A substrate dilution buffer was prepared by diluting oligo annealing buffer (described above) 1:10 in sterile water (Ambion, cat# AM9932). The pre-annealed DNA substrate was subsequently diluted to a final concentration of 0.01 μ M (10 \times stock) in oligo dilution buffer, aliquoted and stored at -20°C.

DPE reaction conditions

DNA Pol I (NEB cat# M0209L), Klenow (NEB cat# M0210S) and Klenow exo(-) (NEB cat# M0212S) were diluted to the indicated units per microliter stock in sterile Tris-EDTA, pH 8.0. To begin, 2 μ l of DNA polymerase stock at each concentration were placed into a 50- μ l DPE reaction mixture containing the following components: 50 μ M dNTP, 20 mM Tris, pH 8.0, 10 mM ammonium sulfate, 10 mM potassium chloride, 2 mM magnesium sulfate, 1% BSA, 0.1% Triton X-100, 0.1% Tween-20 and 0.001 μ M pre-annealed DNA substrate (described above). Two microliters of Tris-EDTA (without DNA polymerase) was routinely added to an additional tube

containing complete DPE reaction mixture and is referred to as a 'No Input Control' (NIC). Reactions containing DNA polymerase (or NICs) were vortexed briefly and placed at 37°C for 20 min. After 20 min, 3 μ l of each reaction were immediately placed into a qPCR (see below for qPCR conditions).

Heat treatment of DPE reaction components

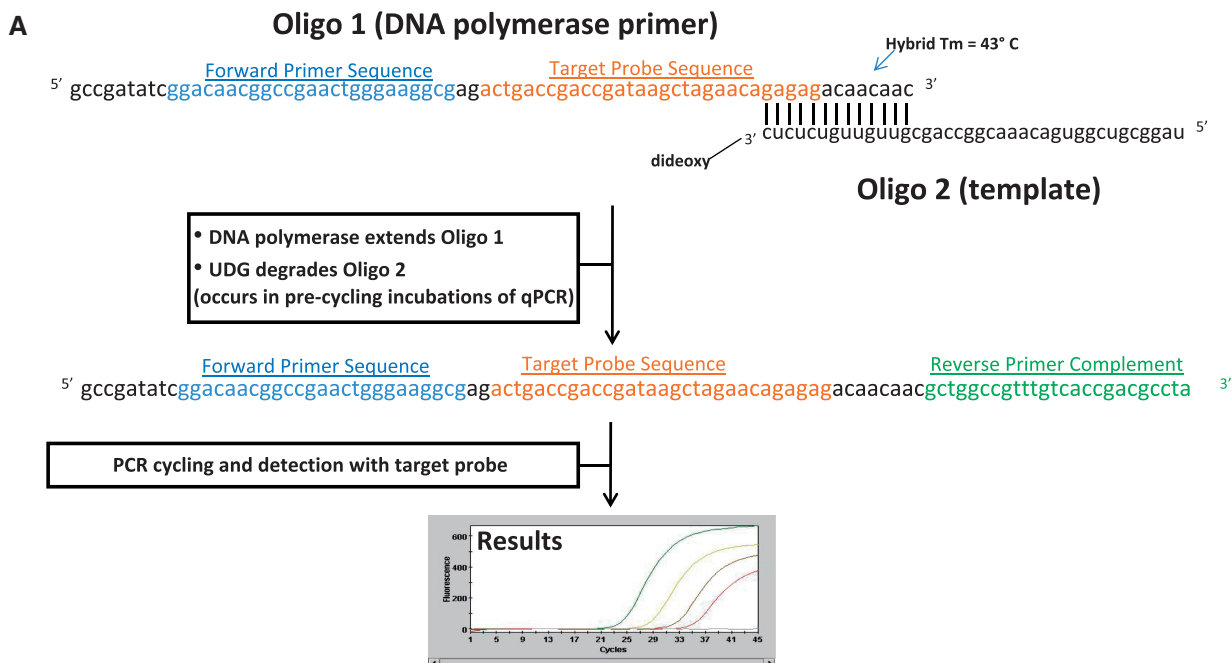
Prior to usage, DPE reaction reagent stocks (minus DNA substrate) were heat treated as follows: 10 \times dNTP mixture (500 μ M dATP, dCTP, dGTP, dTTP) was heated at 90°C for 30 min. The 10 \times core reaction mix (200 mM Tris, pH 8.0, 100 mM ammonium sulfate, 100 mM potassium chloride, 20 mM magnesium sulfate) was heated at 90°C for 30 min. The 1.43 \times BSA/Detergent mix (1.43% BSA, 0.143% Triton X-100, 0.143% Tween-20) was heated at 75°C for 45 min. Substrate annealing buffer (200 mM Tris, 100 mM potassium chloride and 0.1 mM EDTA) pH 8.45 was heated at 90°C for 30 min. Bead mill tubes were heated at 95°C for 20 min.

Quantitative PCR primers, probes and competitive internal control design

The DPE-PCR primers described here were previously used to amplify a DNA substrate modified by T4 DNA ligase (18) and are as follows: forward primer (5'-ggacaa cggcgaactgggaaggcg-3'), reverse primer (5'-taggcgtcggtagcacaacggccagc-3'). The detection probe used in this study was (5' FAM-actgaccgaccgataagctagaacagagag-IABk-FQ 3'). As a tool to monitor qPCR inhibition, a competitive internal control was generated and contains the following sequence (5'-gccgatatcgacaacggcgaactgggaaggcgagatcagcaggccacacgttaagacagagagacaacaacgctggcggttgtcaccgagccta-3'). The internal control sequence was synthesized and cloned as a 'minigene' by Integrated DNA Technologies (Coralville, IA, USA). Upon receipt, the internal control minigene plasmid was linearized using the restriction enzyme PvuI (New England Biolabs) and repurified using a PCR cleanup column (Qiagen). The purified internal control was quantified using a Nanodrop spectrophotometer (Thermo Scientific, ND-1000), diluted to the desired concentration in Tris-EDTA and stored at -20°C. A probe, specific for the internal control DNA, was synthesized by Integrated DNA Technologies (5' TX615-atcagcaggccacacgtt aaagaca-IABRQSp 3'). A detailed schematic containing the relative positioning of the primers/probes within the substrate/competitive Internal Control can also be found in Figure 1.

Quantitative PCR composition and thermocycling parameters

Each 30 μ l qPCR contained: 1X LightCycler 480 Probe Master (from 2X stock, Roche cat# 04707494001), 333 nM of forward and reverse primers, 166 nM detection probe (FAM), 166 nM internal control probe (TX Red), 1.2 U of Uracil DNA Glycosylase (abbreviated hereafter as UDG, Bioline cat# BIO-20744) and 40 copies of the



B **Competitive Internal Control Sequence (40 copies/PCR)**

5' gccgatatc **Forward Primer Sequence** **Internal Control Probe Sequence** **Reverse Primer Complement** 3'

Figure 1. Basic overview of the DPE-PCR assay. (A) DNA polymerase is incubated with a substrate consisting of pre-annealed Oligos 1 and 2. DNA polymerase extends only the 3'-end of Oligo 1 during a 20-min incubation at 37°C. Three microliters of the DPE reaction mixture is subsequently transferred into a hot start qPCR containing UDG. Prior to and during activation of Taq, UDG degrades the deoxyuridine within Oligo 2, leaving only a single-stranded product derived from DNA polymerase-mediated extension of Oligo 1. After activation of Taq, PCR-based amplification is initiated via reverse primer binding to the Oligo 1 extension product. (B) The sequence of a competitive internal control DNA is presented. The competitive internal control is present at 40 copies within each PCR.

competitive Internal Control DNA (described above). Three microliters of each DPE reaction (from purified DNA polymerase or microbial cell lysates) were added to 27 μ l of qPCR master mix and a two-step thermocycling protocol was run on a SmartCycler (Cepheid, Sunnyvale, CA, USA) as follows: initial incubation of 40°C for 10 min and 50°C for 10 min and at 95°C for 5 min (to activate Taq and complete UDG-mediated DNA backbone hydrolysis of Oligo 2), followed by 45 cycles of 5 s denaturation at 95°C and 20 s annealing/extension at 65°C. Cycle threshold (C_t) values were generated automatically by the SmartCycler software using second derivative analysis of the emerging qPCR curves. An example of a typical DPE-PCR containing all controls (including target and competitive internal control curves) is presented in Supplementary Figure S1.

Bead mill lysis tube composition

Bead mill lysis tubes are generated by pipetting 60 μ l (wet volume) of 0.1 mm glass beads (Scientific Industries cat# SI-G01) using a 100- μ l size Eppendorf tip and 50 μ l (wet volume) of 0.5 mm glass beads (Scientific Industries cat# SI-BG05) using a modified 1000 μ l size Eppendorf tip (to enable more reproducible and accurate dispensing of the

0.5-mm beads, the end of the 1000- μ l size Eppendorf tip was cut to a 1-mm inner diameter using a sterile razor blade). Once a slurry of both size beads were dispensed into a 1.5-ml tube (with screw cap), the aqueous supernatant was subsequently aspirated using a sterile gel loading pipette tip attached to a vacuum source. After aspiration, tubes were capped and heat treated prior to use (see above 'Heat treatment of DPE reaction components' section).

Contamination prevention recommendations

A sufficient aseptic working environment can be readily achieved by routinely working in a positive air pressure bench top hood/box equipped with HEPA filter feed (Sentry Air Systems) or comparable HEPA filtered PCR work station. Pipettes and work surfaces should also be frequently cleaned with a laboratory wipe that has been moistened with 10% bleach solution that has been prepared weekly.

Bacterial strains and media

Staphylococcus aureus (ATCC 25923) and *Escherichia coli* (ATCC 25922) were primarily used in this study. Cultures

were grown in/on Brain-Heart Infusion liquid media/agar (Teknova). The ATCC reference numbers and growth media for the additional 17 microorganisms tested are listed in Supplementary Figure S9.

Detection of bacterial DPE activity following bead mill lysis

Staphylococcus aureus and *E. coli* cultures were grown to an OD₆₀₀ of 1.0 ± 0.2 ($\sim 1 \times 10^9$ cfu/ml). For each organism, 1 ml of culture was pelleted and washed three times in Tris-EDTA. Bacterial suspensions were serially diluted in Tris-EDTA, and 5 μ l of each stock were added to bead mill lysis tubes containing 50 μ l DPE reaction mixture (see above for composition). A titration curve of 1×10^5 to 1×10^0 cfu/reaction was performed in triplicate for each organism, including triplicate reactions without bacterial suspension (NIC). After the addition of 5 μ l bacterial stock (or NIC), reaction tubes were bead milled for 6 min at 2800 rpm using a digital Vortex Genie equipped with a disrupter head (Scientific Industries). Immediately after disruption, sample tubes were placed at 37°C for 20 min. After the 20-min incubation, sample tubes were transferred to 95°C for 5 min and removed to cool at room temperature. Sample tubes were then spun at $12k \times g$ for 30 s and 3 μ l of each reaction were placed into the qPCR portion of the DPE-PCR assay. Five microliters of each bacterial stock was plated to obtain more accurate colony forming unit input levels. Gene-specific PCR was also performed on the same lysates used for DNA polymerase detection. Primer and probe sequences for *S. aureus* and *E. coli* gene-specific PCR are listed in Supplementary Figure S15.

Dideoxy termination experiments

Termination of purified DPE activity with dideoxyCTP

DPE reactions were prepared as described above with a 50- μ M (dATP, dGTP, dTTP) mixture supplemented with either 50 μ M dCTP or 50 μ M dideoxyCTP (ddCTP) (Affymetrix cat# 77332). DPE reactions supplemented with either dCTP or ddCTP, were spiked with 2 μ l of a 1×10^{-9} U/ μ l stock of DNA polymerase I (New England Biolabs cat# M0209). Triplicate reactions were incubated at 37°C for 20 min and 3 μ l of each reaction were subsequently placed into qPCR.

Elimination of microbial detection via ddCTP

Staphylococcus aureus and *E. coli* cultures were grown, washed and diluted as described above. To demonstrate ddCTP-dependent termination of microbial DPE, 5 μ l of bacterial stock were added to bead lysis tubes containing 50 μ l of DPE reaction buffer with a 50- μ M (dATP, dGTP, dTTP) mixture supplemented with either 50 μ M dCTP or 50 μ M ddCTP. Bead mill lysis, DPE reaction and qPCR were performed as described above. Five microliters of each bacterial stock were plated to determine more accurate colony forming unit input levels. Gene-specific PCR of genomic DNA was also performed on the same lysates used for DPE-PCR.

dCTP rescue of microbial detection

Staphylococcus aureus and *E. coli* cultures were grown, washed and diluted as described above. Five microliters of bacterial stock were added to bead lysis tubes containing 50 μ l of DPE reaction buffer containing a 50- μ M (dATP, dGTP, dTTP, ddCTP) mixture. Just prior to lysis, 1 μ l of dCTP at (2.5, 0.25, 0.025 and 0.0025 mM) was added to separate ddCTP-containing reactions. Reactions containing 50 μ M (dATP, dGTP, dTTP, dCTP) alone and 50 μ M (dATP, dGTP, dTTP, ddCTP) alone were run in parallel as 'non-terminated' and 'terminated' comparators. Bead mill lysis, DPE reaction and qPCR were performed as described above. Five microliters of each bacterial stock were plated to determine more accurate colony forming unit input levels. Gene-specific PCR was also performed on the same lysates used for DPE-PCR.

Viability assessment experiments

Staphylococcus aureus and *E. coli* cultures were grown, washed and diluted as described above. Two hundred microliters of bacterial stocks at ~ 2000 cfu/ μ l (in Tris-EDTA) were incubated at 25, 45, 65, 85 and 105°C for 20 min. After heating, samples were cooled to room temperature and 5 μ l of each bacterial stock were added to bead mill lysis tubes containing 50 μ l of DPE reaction buffer. Bead mill lysis, DPE reaction and qPCR were performed as described above. Five microliters of each bacterial stock (treated at various temperatures) were also added to 1 ml of Tris-EDTA and 50 μ l were plated for colony count determination. Gene-specific PCR was also performed on the same lysates used for DPE-PCR.

RESULTS AND DISCUSSION

Overview of the DPE-PCR assay

The measurement of DPE activity could represent a useful tool with far-reaching applications such as, but not limited to, screening candidate-polymerase inhibitors *in vitro*, or detecting the presence any microbe (harboring active DNA polymerases) within a diverse range of sample types. If intended for these purposes, routine use of traditional polymerase assays that incorporate radiolabeled nucleotides is unattractive. Consequently, numerous non-radioactive DPE assays have been developed in recent decades. Despite successfully averting the use of radioactivity, current fluorescence-based DNA polymerase assays also suffer from various deficiencies. For example, detection of DNA polymerase activity via several existing non-radioactive assays is dependent upon the binding of PicoGreenTM to newly generated double-stranded DNA (13,14). If intended to analyze DNA polymerase activity from freshly lysed organisms, PicoGreenTM-based assays would likely be hampered by background fluorescence via binding of PicoGreenTM to genomic DNA. Microplate-based DNA polymerase assays have also been developed (15). Decreased sensitivity of microplate-based assays can be expected for numerous reasons, including dependence upon intermediate binding of either product or substrate to a microplate

and/or inefficient incorporation of modified dNTPs by DNA polymerase. More recently, real-time measurement of DNA polymerase activity via molecular beacons has been described (16). Despite improved sensitivity, direct measurement of molecular beacon fluorescence could also potentially be hindered by exposure to crude cellular lysates.

We set out to develop a rapid, simple, highly sensitive and quantitative assay capable of measuring DPE activity derived from purified commercial sources or freshly lysed cells. Figure 1A contains a schematic overview of the mechanisms involved in coupling DPE activity to qPCR. Notably, Oligo 2 is eliminated by UDG prior to and during Taq activation, thus preventing undesired Taq-dependent extension of the substrate just prior to PCR cycling. A microbial detection method linking T4 DNA ligase activity to PCR amplification has been previously reported (18), which contains similarities to our DPE-PCR assay and is another example of an ETGA methodology. However, in our hands a modified version of this ligase method, aimed at detecting microbial-derived NAD-dependent DNA ligase activity, suffered from a lack of sensitive and universal microbial detection, leading us to the development of the improved novel DNA polymerase-based approach named DPE-PCR described herein.

Sensitive and linear detection of purified DPE activity

To begin, we set out to determine the approximate analytical sensitivity of the DPE-PCR assay using commercially available DNA polymerase I. In this experiment, DPE-PCR signals derived from decreasing amounts of DNA polymerase I were compared with parallel reactions without input DNA polymerase (NIC). As shown in Figure 2A, detection of DNA polymerase I extension activity was achieved over a wide range of input enzyme. In fact, DNA polymerase I extension activity was distinguishable from the NIC down to as little as 2×10^{-11} U of enzyme (equivalent to ~ 50 molecules of polymerase). To our knowledge, detection of DPE activity at this level is unrivaled in existing DNA polymerase assays. In theory, this level of sensitivity could enable single microbe detection as *E. coli* has been reported to contain ~ 400 DNA polymerase I molecules per cell (11). Regression analysis also showed a strong positive linear correlation ($R^2 = 0.992$) between the DPE-PCR C_t values and Units of input commercial DNA polymerase I after graphing data from two independent limit of detection experiments (Figure 2B and Supplementary Figure S3A).

After sensitivity and linearity experiments were performed, it was important to determine if the DPE-PCR assay signal was independent of intrinsic exonuclease activity. To this end, we subsequently compared signals generated by 2×10^{-7} U of DNA polymerase I to those generated from DNA polymerase I lacking 5'→3' exonuclease activity (Klenow) and another version of the enzyme lacking all exonuclease activity (Klenow exo-). For additional specificity and background signal determination, *E. coli* DNA ligase at 2×10^{-7} U and an NIC were tested in parallel. As shown in Figure 2C, both

Klenow and Klenow exo- were detected at similar levels when compared with wild-type DNA polymerase I, providing evidence that the DPE-PCR assay signal is derived from DNA polymerase-dependent extension and not intrinsic exonuclease activity (also see Supplementary Figure S3B).

In addition to using exonuclease free polymerases, we set out to further demonstrate that DPE-PCR assay signal is derived from DNA polymerase-dependent extension of the DNA substrate prior to qPCR. Since incorporation of dideoxy nucleotides is a well-established method used for termination of DNA polymerase chain extension activities (19,20), we chose to substitute dCTP with ddCTP within our DPE reaction mix. The schematic shown in Figure 2D reveals the first possible position within the substrate that ddCTP can be incorporated by DNA polymerase. If ddCTP is incorporated into this position, the extension product of Oligo 1 would be insufficient in length for successful annealing by the qPCR reverse primer (see Figure 1 schematic). As shown in Figure 2D, substitution of dCTP with ddCTP eliminates signal generated by DNA polymerase I, thus demonstrating that the DPE-PCR assay signal is dependent upon DPE of the substrate prior to qPCR. The presence of a low copy competitive internal amplification control confirms that qPCR was not inhibited by the presence of low amounts of ddCTP that are carried over from the DNA polymerase assay reagents (Supplementary Figure S4).

In addition, we feel it is important to note that we sporadically observe a weak, but detectable signal in the absence of input DNA polymerase (NIC). Due to the exquisite sensitivity of the DPE-PCR assay, we have demonstrated that weak background noise signals can be attributed to 'contaminant' DNA polymerase activity present in the DPE stock reagents prior to reaction assembly. Consequently, pre-treatment of the DPE reagents (see 'Materials and methods' section) is routinely performed and is sufficient to eliminate the contaminant DNA polymerase signal observed (see Supplementary Figure S2A for an example). Additionally, we have demonstrated that a major potential source of unwanted Taq-dependent signal could arise from the operator's failure to add active UDG to the qPCR mastermix. For example, intentional omission of UDG from the qPCR mastermix results in a high background signal derived from Taq-dependent extension of the DNA substrate (see Supplementary Figure 2B); however, we have never observed high background signals (resulting from UDG failure) when UDG is added as described in the 'Materials and methods' section. Another hypothesized source of increased background signal could be derived from DNA polymerase introduced by the operator during experimental setup. It is, therefore, recommended that the operator exhibit good aseptic technique when preparing samples and reagents for the DPE and qPCR portions of the assay (see 'Materials and methods' section for contamination prevention recommendations). Considering the above, we feel it is very important that an NIC be run in parallel with each experiment to verify that the starting reagents are free of contamination and that UDG has been added to the qPCR mastermix.

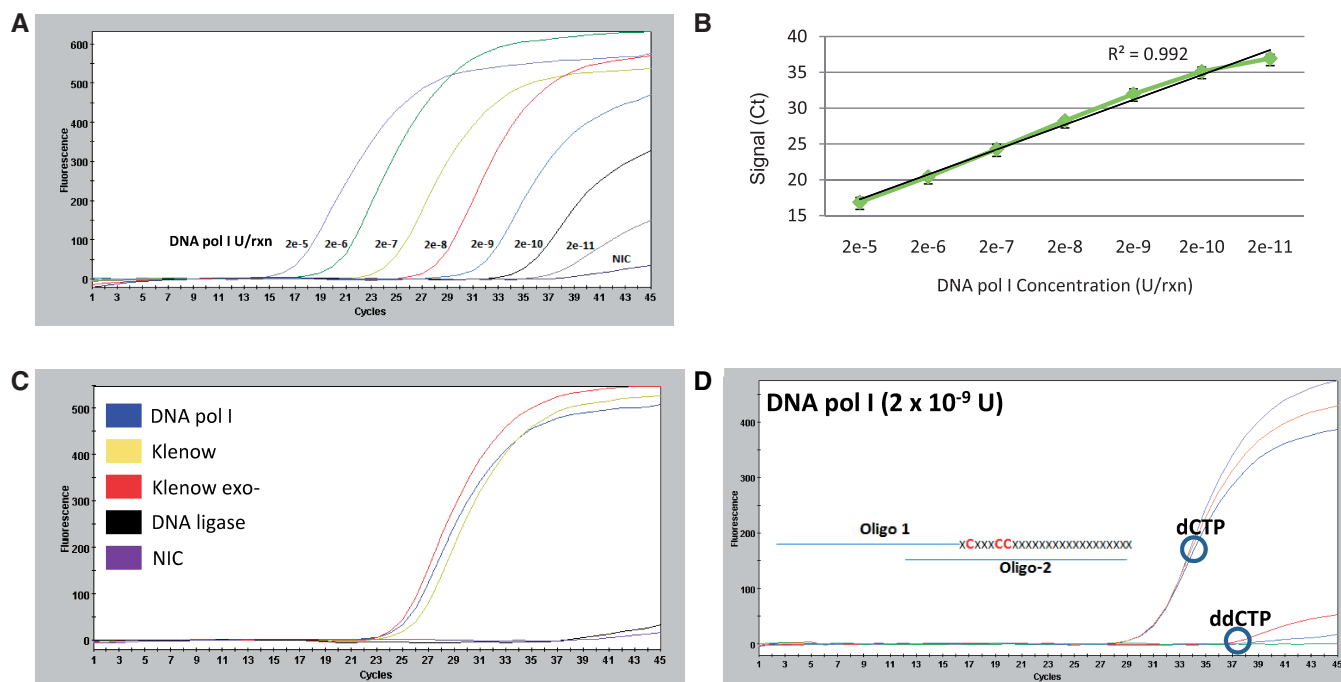


Figure 2. Sensitive detection of purified DNA polymerase using DPE-PCR. (A) A commercial source of DNA polymerase I was assayed in duplicate at 10-fold increments starting at 2×10^{-5} U down to 2×10^{-11} U per reaction. A representative DPE-PCR curve is shown for each polymerase input level and NIC. (B) A plot was constructed from $n = 4$ data points per polymerase input level, taken from two independent experiments and linear regression analysis was performed. (C) Triplicate reactions containing 2×10^{-7} U of DNA polymerase I, Klenow, Klenow (exo $-$) and *E. coli* DNA Ligase were assayed in comparison to an NIC. A representative DPE-PCR curve is presented for each of the assayed enzymes and NIC. (D) Triplicate DPE-PCR curves are shown from corresponding DPE reactions containing a 50- μ M (dATP, dGTP, dTTP) mixture supplemented with 50 μ M of either dCTP or ddCTP. A schematic representing some of the first available sites for dCTP or ddCTP incorporation within the DNA substrate is presented adjacent to the DPE-PCR curves.

Sensitive universal detection of microbes via measurement of endogenous DPE activity directly from cell lysates

In addition to detecting purified polymerase activity a simple, sensitive and universal method that measures microbial-derived DNA polymerase activity would be highly desirable. For instance, measurement of DPE activity could be used to screen environmental or biological samples for the presence of any microorganism harboring active DNA polymerase. To this end, we developed a simple method that couples microbial lysis to our DPE-PCR assay. As shown in Figure 3, a liquid sample known to contain, or suspected of containing, microbes is added to a bead mill lysis tube, disrupted and immediately transitioned into the DPE-PCR assay. We initially chose one Gram-negative bacteria (*E. coli*) and one Gram-positive bacteria (*S. aureus*) to demonstrate the ability of our assay to measure microbial-derived DPE activity in crude cellular lysates. As shown in Figure 4A, when linked with bead mill lysis, the DPE-PCR assay is capable of detecting a wide dynamic range of input *E. coli*, down to and below 10 cfu per lysis tube. Linear regression analysis of *E. coli* detection was also performed down to 10 cfu of input bacteria and showed a strong positive linear correlation between input colony forming unit and DPE-PCR signal as indicated by an R^2 value of 0.999 (Figure 4B). Colony count plating and *E. coli* gene-specific qPCR (gsPCR)

were run in parallel, confirming both the input level of colony forming unit per reaction and the ability to monitor intact genomic DNA from the exact same lysates. DPE activity from *S. aureus* lysates was detected to a similar input level (Figure 4C). *Staphylococcus aureus* detection was plotted down to 10 cfu of input bacteria and also showed a strong linear correlation between input colony forming unit and DPE-PCR signal ($R^2 = 0.999$, Figure 4D). Colony count plating and gsPCR were performed in parallel to confirm the amount of *S. aureus* present in each bead lysis tube, as well as the presence of directly analyzable genomic DNA. Complete tables of plating, gsPCR and DPE-PCR results for both *E. coli* and *S. aureus* can be found in Supplementary Figures S5 and S6. We subsequently tested the ability of the DPE-PCR assay to measure DNA polymerase activity from 17 additional clinically relevant microorganisms. As shown in Table 1, we were able to detect DNA polymerase activity from all 17 additional organisms including six Gram-negative bacteria, six Gram-positive bacteria and five *Candida* species. Detection of the 17 additional microbes exhibited a strong positive linear correlation to input colony forming unit with impressive low limits of detection. The upper linear dynamic ranges have yet to be fully characterized. More comprehensive results containing parallel plating data and DPE-PCR results for each of the 17 additional microbes are

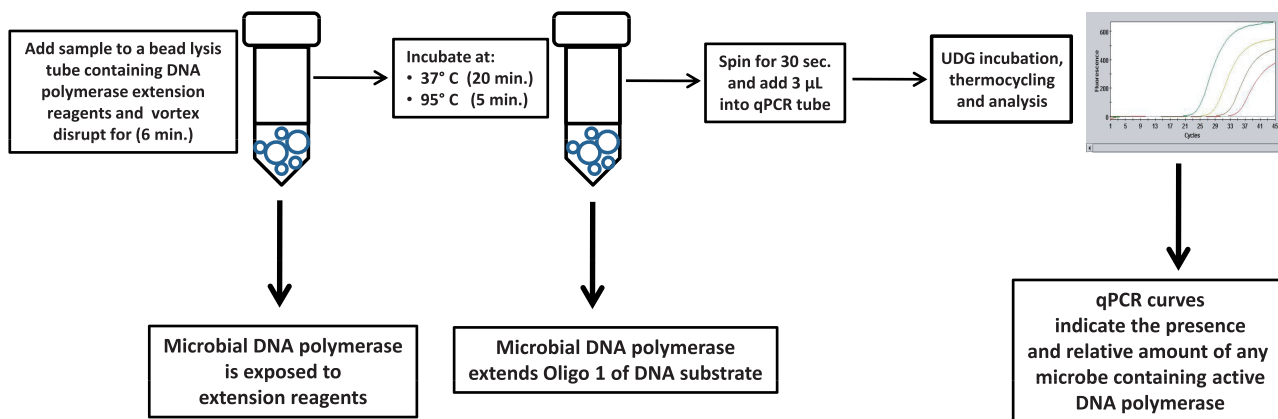


Figure 3. Schematic overview of coupling bead lysis to DPE-PCR.

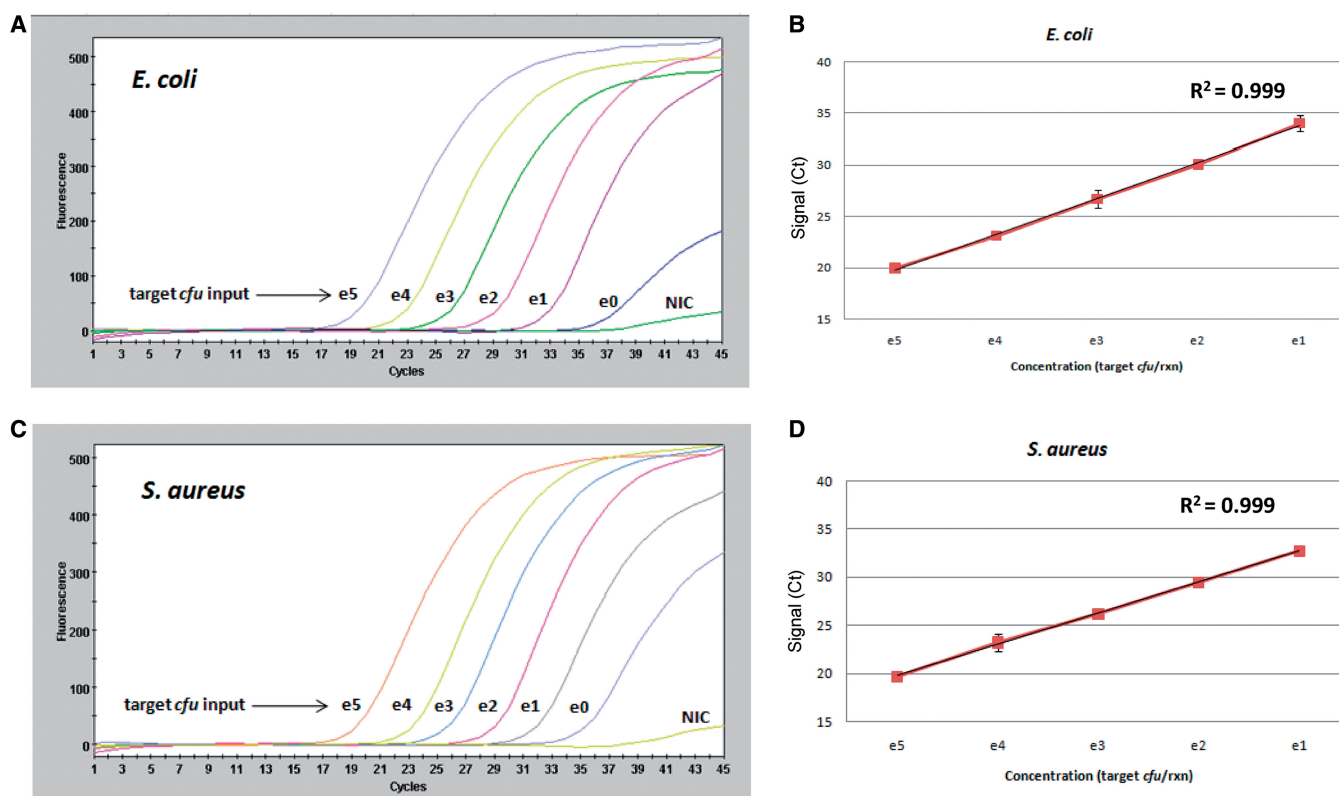


Figure 4. DPE-PCR enables sensitive and quantitative detection of Gram-negative and Gram-positive bacteria via measurement of DPE activity in crude lysates. (A) Decreasing amounts of *E. coli* colony forming unit were spiked into bead lysis-coupled DPE-PCR. NIC were also included to monitor reagent background levels. All colony forming unit spikes and NICs were performed in triplicate. A representative DPE-PCR curve is shown below for each level of bacterial input. Colony count plating and gsPCR were performed in an effort to obtain a better estimate of the actual colony forming unit placed into each reaction and is presented in Supplementary Figure S5 (B) A plot of *E. coli* DNA polymerase activity and linear regression analysis is presented. Graphs were generated using the average C_t values obtained from triplicate reactions of bacterial spikes ranging from 1×10^5 to 1×10^1 input colony forming unit. (C and D) Colony forming unit titration experiments were performed for *S. aureus* exactly as described above for *E. coli*. Colony count plating and gsPCR were performed in an effort to obtain a better estimate of the actual colony forming unit placed into each reaction and is presented in Supplementary Figure S6.

presented in Supplementary Figures S10–S14. Together, these data support the notion that DPE-PCR has the potential to be useful as a universal ‘pan’ test for the sensitive detection of any microbe in a normally sterile environment.

Elimination of DPE-PCR detection of microbes via ddCTP substitution

As previously shown in Figure 2D, substitution of dCTP with ddCTP in the DPE reaction mix represents a powerful tool for blocking extension of Oligo 1 within

Table 1. Sensitive and linear detection of 17 additional clinically relevant microbial species

Bacterial panel	Lower limit colony forming unit detected by DPE-PCR	R^2 (1e4-1e1 cfu)
<i>Klebsiella pneumoniae</i>	<10	0.9957
<i>Pseudomonas aeruginosa</i>	<10	0.9860
<i>Enterobacter cloacae</i>	<10	0.9995
<i>Acinetobacter baumannii</i>	<10	0.9980
<i>Haemophilus influenzae</i>	<10	0.9996
<i>Serratia marcescens</i>	<10	0.9956
<i>Enterococcus faecalis</i>	<10	0.9963
<i>Enterococcus faecium</i>	<10	0.9899
<i>Streptococcus pyogenes</i>	<10	0.9945
<i>Streptococcus agalactiae</i>	<10	0.9969
<i>Streptococcus pneumoniae</i>	<10	0.9999
<i>Staphylococcus epidermidis</i>	<10	0.9990
Candida panel	Lower limit colony forming unit detected by DPE-PCR	R^2 (1e5-1e3 cfu)
<i>Candida albicans</i>	~20	0.9945
<i>Candida tropicalis</i>	~20	0.9969
<i>Candida glabrata</i>	~40	0.9111
<i>Candida parapsilosis</i>	~20	0.9950
<i>Candida krusei</i>	~15	0.9868

our assay. To demonstrate that the signal derived from bacterial spikes was dependent upon their DPE activity, and not the other endogenous bacterial enzyme activities present in the lysates, we set up an experiment to compare DPE-PCR signals obtained from *E. coli* and *S. aureus* using a standard DNA polymerase reaction mix containing (dATP, dTTP, dGTP, dCTP) versus a reaction mix containing (dATP, dTTP, dGTP, ddCTP). As shown in Figure 5A, when compared with the standard reaction mix, substitution of ddCTP blocked the generation of signal derived from *E. coli* colony forming unit spikes (Figure 5A). A dCTP rescue experiment was subsequently performed by comparing DPE activity from bacteria lysed in a DPE reaction mix containing 50 μ M (dATP, dTTP, dGTP, ddCTP only), to those containing increasing amounts of supplemented dCTP (see 'Materials and methods' section for a detailed description of rescue experiments). Figure 5B demonstrates the rescue effect that increasing amounts of supplemented dCTP has on quantifiable DPE activity derived from *E. coli* lysates. In addition to measuring microbial DPE activity, gsPCR was run in parallel to verify that equivalent amounts of *E. coli* were present in each of the assayed lysates. A graphical comparison of DPE activity versus presence of genomic DNA is presented in Figure 5C. Signal termination (via ddCTP) and dCTP rescue experiments were subsequently repeated with *S. aureus* and similar results were obtained (Figure 5D–F). Tables containing DPE-PCR and gsPCR data for both *E. coli* and *S. aureus* can be found in Supplementary Figures S7 and S8. qPCR competitive internal control values are provided to demonstrate that low levels of ddCTP carried over into

qPCR are not inhibitory, and thus are not responsible for the disappearance of DPE-PCR signal (highlighted in Supplementary Figures S7A and S8A). Together, the data presented in Figure 5 strongly support the claim that the DPE-PCR assay is specifically detecting microbial DPE activity and signal is not derived from substrate modification via enzymatic activities other than DNA polymerase.

Measurement of DPE activity as an indicator of bacterial viability

Traditional methods for determining bacterial viability are dependent upon growth and visualization of a particular microbe on solid medium (21). Although bacterial growth and visualization is the current industry gold standard, the traditional colony forming unit viability determination methods are undesirable due to the length of time required for colony forming unit formation. Furthermore, the ability to grow on solid media or in liquid culture can vary dramatically from one microbe to another, thus potentially limiting the detection of certain fastidious organisms (22). Due to the aforementioned limitations of traditional methods, there is a growing need in a wide variety of pharmaceutical (23), environmental, food processing and clinical testing arenas for the rapid assessment of microbial viability. Consequently, numerous molecular methods have been developed in an effort to quickly assess microbial viability status within a given matrix (24). Despite being rapid and sensitive, molecular methods that detect the presence of nucleic acid often fall short of representing an accurate measurement of cell viability. For example, amplification of endogenous DNA or RNA is a poor indicator of bacterial viability, due to the persistence of nucleic acid after cell death (25,26). We set out to determine the feasibility of using DPE activity as an indicator of bacterial viability. To this end, an experiment was designed to compare detection of DPE activity and PCR-mediated detection of genomic DNA as indicators of bacterial viability following various amounts of heat treatment. To begin, *E. coli* suspensions were treated at increasing temperatures for a fixed period of time. After heat treatment, bacteria were subsequently assayed for the presence of both DPE activity and genomic DNA. Heat treated and non-heat treated bacterial stocks were also plated in parallel to monitor bacterial viability via the presence of visible colony forming unit. Figure 6A represents the levels of *E. coli* DPE activity measured after the indicated amounts of heat treatment. Notably, a significant drop in *E. coli* DPE activity was observed after incubation of bacterial suspensions between 45°C and 65°C (Figure 6A). In contrast, gsPCR signal obtained from the same lysates remained relatively constant at all temperatures and is graphically compared with DPE activity in Figure 6B. Plating results presented below the graph further demonstrate that increasing levels of heat treatment are sufficient to prevent colony forming unit formation and are paralleled by a dramatic loss of DNA polymerase activity; however, dead cells still contribute genomic DNA levels very close to their original input levels confirming that

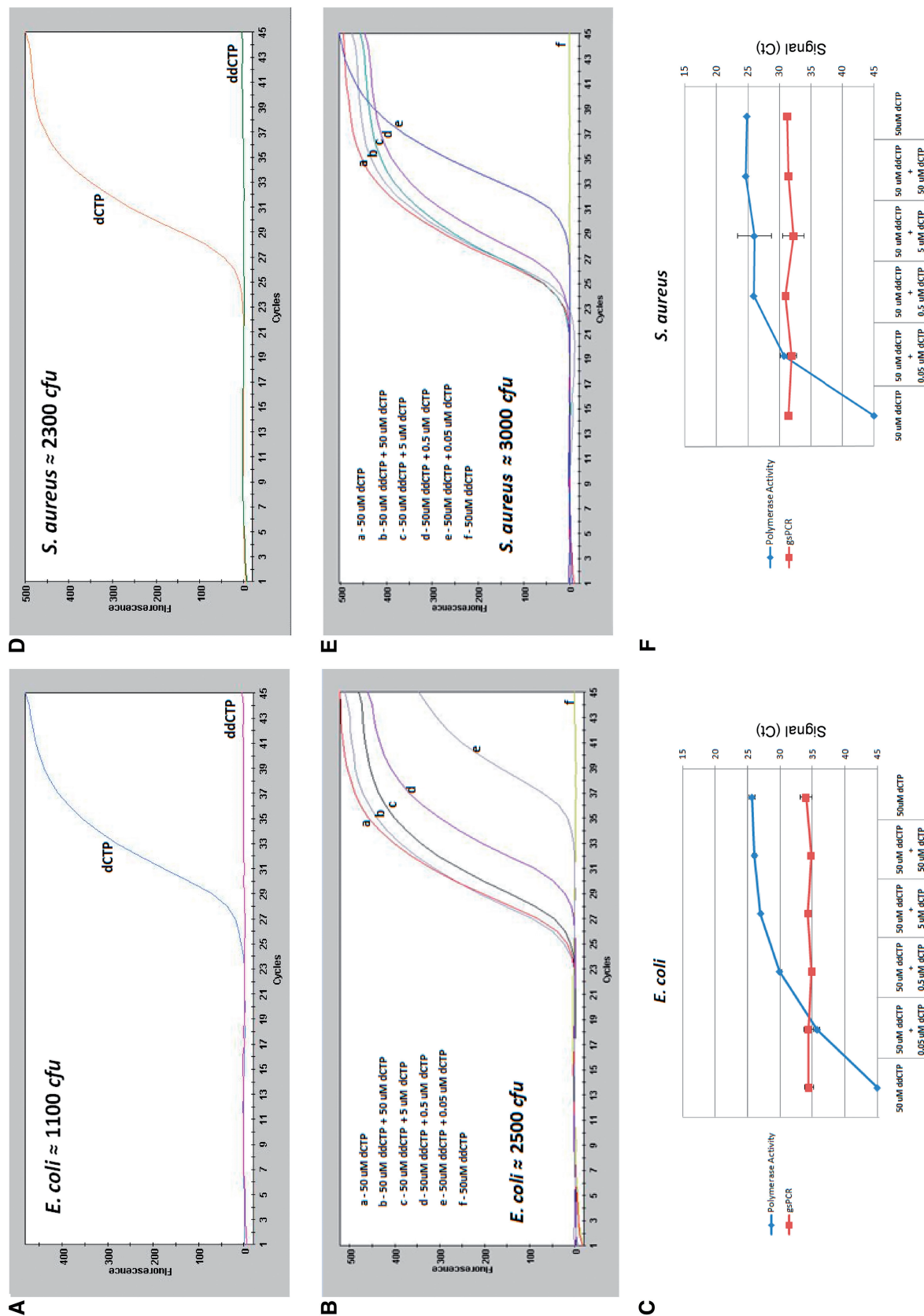


Figure 5. Detection of bacteria by DPE-PCR is blocked by ddCTP and rescued with dCTP. (A) *E. coli* suspensions were added to bead lysis-coupled DNA polymerase assays composed of a 50 μ M dATP, dGTP, dTTP) mixture supplemented with either 50 μ M dCTP or 50 μ M ddCTP. DPE-PCR curves representing *E. coli*-derived DNA polymerase activity is presented. Approximate colony forming unit input as determined by plating is presented in the upper left region of the qPCR graph (B) *E. coli* suspensions were added to bead lysis tubes containing 50 μ l reaction buffer with 50- μ M dATP, dGTP, dTTP, ddCTP). Prior to lysis, 1 μ l of dCTP (2.5, 0.25, 0.025 and 0.0025 mM) was added to selected ddCTP-containing reactions. Reactions containing 50 μ M dATP, dGTP, dTTP, dCTP) alone or 50 μ M dATP, dGTP, dTTP, ddCTP) alone were run in parallel as 'non-terminated' and 'terminated' comparators. The resultant DPE-PCR curves representing *E. coli*-derived DNA polymerase activity is presented. Approximate colony forming unit input as determined by plating is presented in the lower left region of the qPCR graph. (C) *Escherichia coli* gene-specific PCR was also performed on the same lysates used for DNA polymerase detection presented in Figure 2B. Linear plots of dCTP-dependent rescue of bacterial DNA polymerase detection versus gsPCR of genomic DNA are shown. Plots were generated using the average qPCR C_t values from triplicate reactions at the indicated conditions. (D-F) ddCTP termination and dCTP rescue experiments were performed for *S. aureus* exactly as described above for *E. coli*.

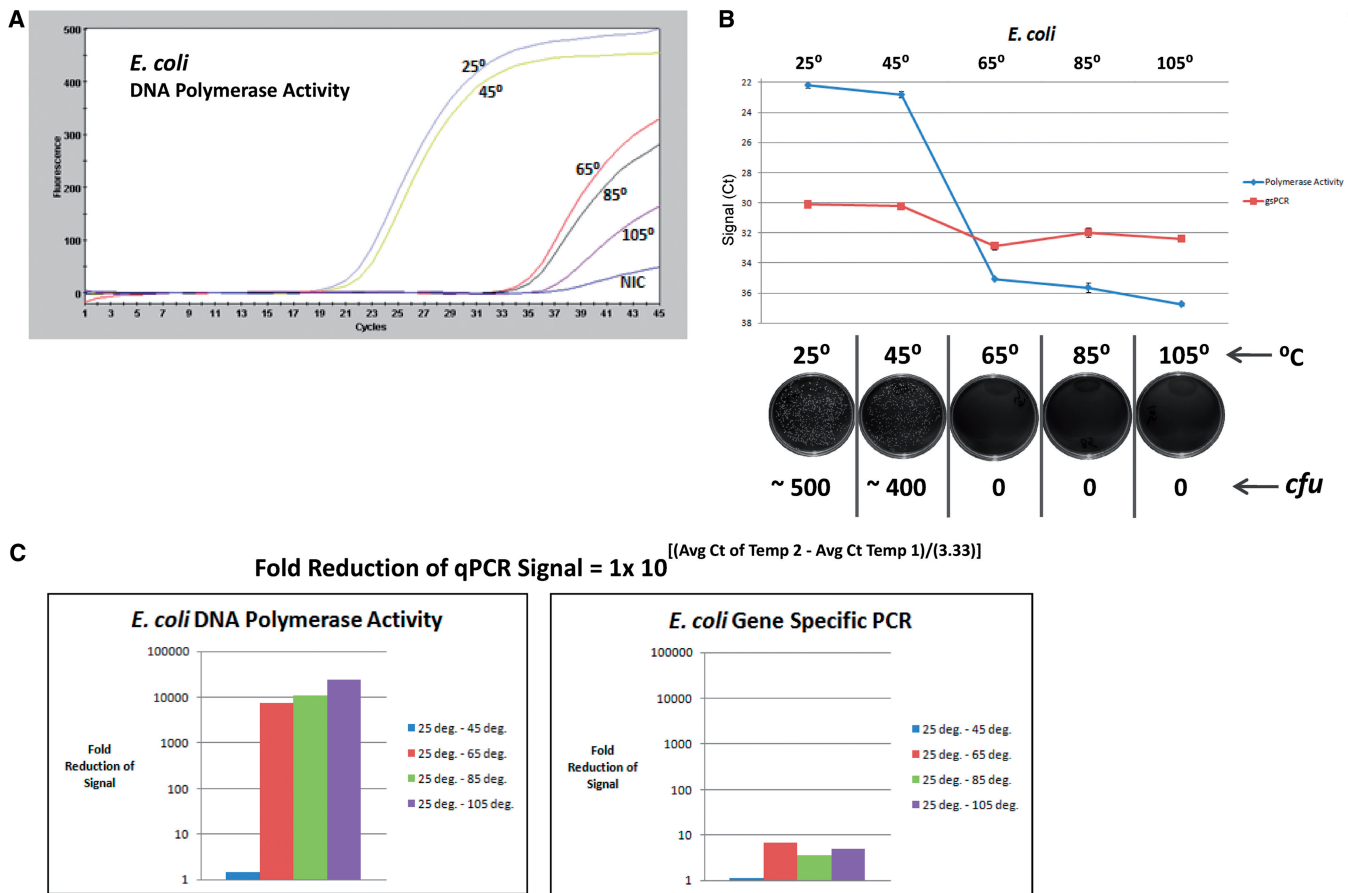


Figure 6. DPE-PCR as an indicator of *E. coli* viability in response to heat treatment. (A) Aliquots of an *E. coli* suspension (~2000 cfu/ μ l) were incubated at 25°C, 45°C, 65°C, 85°C and 105°C for 20 min. After heating, each bacterial stock was cooled to room temperature and 5 μ l were transferred to the bead lysis-coupled DPE-PCR assay. DPE-PCR curves representing *E. coli*-derived DNA polymerase activity following each of the indicated temperature treatments are presented. (B) Plots were generated from triplicate DPE-PCRs and gSPCR of genomic DNA (from the same lysates) after the indicated temperature treatments of *E. coli* suspensions. Parallel plating was also performed in triplicate for each of the treated *E. coli* suspensions. Representative colony forming unit monitoring plates are presented below the graph, revealing bacterial viability status after treatment at each temperature. (C) DPE-PCR is compared with gSPCR of genomic DNA in response to the various temperature treatments. 'Fold Reduction of qPCR Signal' was calculated using the indicated equation and the values obtained were used to generate comparative bar graphs.

gSPCR is a poor indicator of cell viability (Figure 6B). In Figure 6C, the bar graphs further highlight the relative abilities of DPE-PCR and gSPCR to monitor the disappearance of colony forming unit in response to lethal amounts of heat treatment. Subsequently, we wanted to test whether the measurement of DPE activity could be used to indicate the viability status of a Gram-positive organism as well. The previous *E. coli* experiments were repeated with *S. aureus* under the same conditions. Figure 7A–C, show similar results obtained from heat treatment experiments repeated with *S. aureus*. Collectively, the strong concordance between the presence of colony forming unit and DPE activity shown in Figures 4, 6, 7 and Table 1 demonstrates that DPE-PCR has the potential to be used as a general indicator of cell viability. Additional experiments are underway to measure relative DPE activity from microbes exposed to other clinically or pharmaceutically relevant agents (bacteriostatic and bactericidal) aimed at reducing cell proliferation or viability.

CONCLUSIONS

In summary, we have developed a novel, highly sensitive, quantitative and rapid DPE-PCR assay. In addition to quantitative detection of extremely low levels of purified enzyme, we have demonstrated the ability of DPE-PCR to reproducibly measure DPE activity from <10 cfu of bacteria via coupling to bead lysis. We have also demonstrated the potential for DPE-PCR to universally detect microbes by testing a panel of microorganisms composed of seven Gram-negative bacteria, seven Gram-positive bacteria and five *Candida* species. Furthermore, preliminary evidence that the DPE-PCR assay can be used to assess bacterial viability was provided via the reproducibly strong correlation between DPE activity and proliferation as indicated by the presence of colony forming unit. Considering the data presented here, we strongly believe that ETGA methodology such as our DPE-PCR assay has the potential to become a useful tool for a wide range of research and testing applications within pharmaceutical, environmental, food and clinical settings.

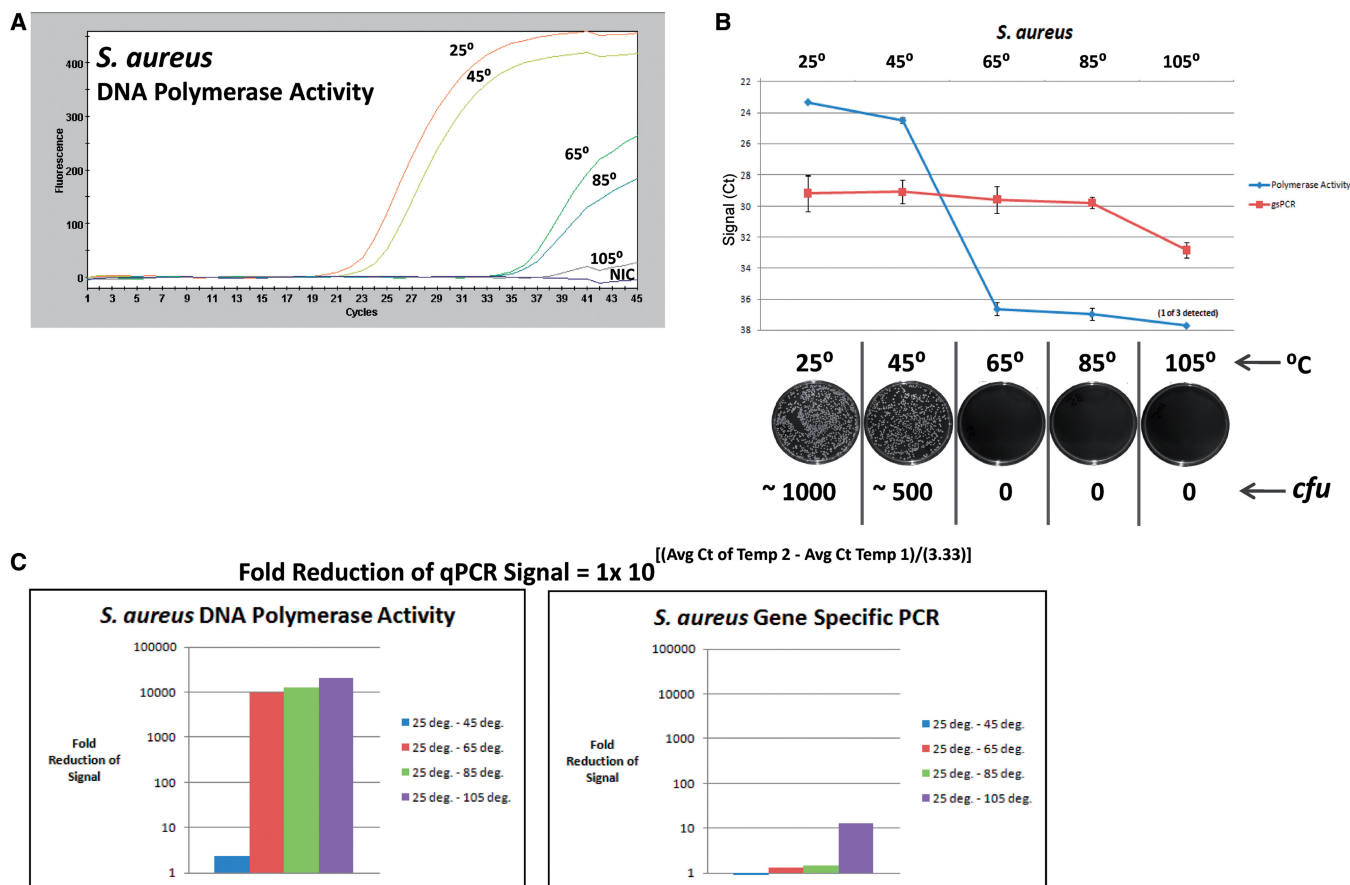


Figure 7. DPE-PCR as an indicator of *S. aureus* viability in response to heat treatment. (A) Aliquots of an *S. aureus* suspension (~2000 cfu/μl) were incubated at 25, 45, 65, 85 and 105°C for 20 min. After heating, each bacterial stock was cooled to room temperature and 5 μl were transferred to the bead lysis-coupled DPE-PCR assay. DPE-PCR curves representing *S. aureus*-derived DNA polymerase activity following each of the indicated temperature treatments are presented. (B) Plots were generated from triplicate DPE-PCRs and gPCR of genomic DNA (from the same lysates) after the indicated temperature treatments of *S. aureus* suspensions. Parallel plating was also performed in triplicate for each of the treated *S. aureus* suspensions. Representative colony forming unit monitoring plates are presented below the graph, revealing bacterial viability status after treatment at each temperature. (C) DPE-PCR is compared with gPCR of genomic DNA in response to the various temperature treatments. 'Fold Reduction of qPCR Signal' was calculated using the indicated equation and the values obtained were used to generate comparative bar graphs.

SUPPLEMENTARY DATA

Supplemental Data are available at NAR Online: Supplementary Figures 1–15.

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