Analytical Biochemistry 478 (2015) 1–7

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Quantification of pyrophosphate as a universal approach to determine polymerase activity and assay polymerase inhibitors

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ARTICLE INFO

Article history:
Received 14 October 2014
Received in revised form 24 February 2015
Accepted 3 March 2015
Available online 12 March 2015

Keywords:
Pyrophosphate detection
DNA polymerase activity
Translesion DNA synthesis
Nucleotide analogs
TLS inhibitors

ABSTRACT

The importance of DNA polymerases in biology and biotechnology, and their recognition as potential therapeutic targets, drives development of methods for deriving kinetic characteristics of polymerases and their propensity to perform polynucleotide synthesis over modified DNA templates. Among various polymerases, translesion synthesis (TLS) polymerases enable cells to avoid the cytotoxic stalling of replicative DNA polymerases at chemotherapy-induced DNA lesions, thereby leading to drug resistance. Identification of TLS inhibitors to overcome drug-resistance necessitates the development of appropriate high-throughput assays. Since polymerase-mediated DNA synthesis involves the release of inorganic pyrophosphate (PPi), we established a universal and fast method for monitoring the progress of DNA polymerases based on the quantification of PPi with a fluorescence-based assay that we coupled to *in vitro* primer extension reactions. The established assay has a nanomolar detection limit in PPi and enables the evaluation of single nucleotide incorporation and DNA synthesis progression kinetics. The results demonstrated that the developed assay is a reliable method for monitoring TLS and identifying nucleoside and nucleotide-based TLS inhibitors.

DNA polymerases have been implicated in drug resistance due to their ability to perform DNA lesion bypass in a process called translesion synthesis (TLS)[1] [1–5]. Specialized DNA polymerases such as polymerases η, ζ, ι, Rev1, and κ were identified to alleviate cell cycle termination caused by blockade of DNA replication [3,4,6] and by promoting error-free [7–10] or error-prone [11–18] DNA damage bypass due to a wide active site and an extra DNA binding domain [19]. DNA polymerase η (Pol η), while protecting cells against UV radiation [10], was found to replicate over DNA single-strand breaks (ssb), DNA mono-adducts like O^6-methylguanine (O6-MeG), 3-methyladenine [15,20–22], and intrastrand crosslinks induced by cisplatin [23–25]. TLS polymerase ζ (Pol ζ) also bypasses cisplatin adducts and adducts induced by the bulky therapeutic cisplatin analogs [24,26] contributing to most mutations induced by DNA-damaging agents [4]. Pol κ, Pol ι, and Rev1 are mostly associated with bypass of N^2-guanine lesions (DNA minor-groove) induced by environmental pollutants and food carcinogens [27–31], or by the chemotherapeutic drug mitomycin C [29]. Due to the apparent contribution to drug resistance, inhibiting TLS is considered as a potential strategy for enhancing the efficacy of chemotherapeutic alkylating agents [32].

The complex dynamics of TLS polymerases and conformational changes occurring during lesion bypass and progression [33–38] make screening of small molecules the most convenient method for gaining access to polymerase inhibitors in comparison to rational drug design. Over the years, detection of DNA polymerase activity from *in vitro* primer extension assays has been approached in various ways [39], including imaging of radiolabeled products following gel electrophoresis [40] and staining with DNA intercalating fluorescent dyes coupled to spectrophotometric analysis [41]. Other methods have been based for the detection of fluorescence arising from the incorporation of fluorescently labeled nucleotides to monitor primer extensions [42] or from the polymerase-mediated displacement of a fluorescent reporter strand [43–45].

A different approach to the study of DNA polymerase activity is represented by the detection of PPi released during dNTP incorporation. A prominent example is the chemiluminescence-

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[1] [1]*Abbreviations used:* 5-COOH-dCTP, 5-carboxy-2′-deoxycytidine-5′-triphosphate; dPTP, 6*H*,8*H*-3,4-dihydro-pyrimido [1,2]oxazin-7-one-8-D-2′-deoxy-ribofuranosid-5′-triphosphate; 5-nitro-TP, 5-nitro-1-indolyl-2′-deoxyribose-5′-triphosphate; 5-OH-dCTP, 5-hydroxy-2′-deoxycytidine-5′-triphosphate; 8-oxo-dATP, 8-oxo-2′-deoxyadenosine-5′-triphosphate; O6-MeG, O^6-methylguanine; PE, primer extension; Pol, polymerase; PPi, inorganic pyrophosphate; TLS, translesion synthesis.

http://dx.doi.org/10.1016/j.ab.2015.03.002
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based luciferase assay that is widely applied in pyrosequencing, the stepwise monitoring of dNTP incorporation to determine DNA sequence [46]. While this method is highly effective for DNA sequencing and potentially adaptable for evaluating inhibitors, this is not done in practice, possibly because it can suffer from the interaction of small molecules, such as nucleotides and nucleotide analogs, with luciferase which uses the nucleotide substrate ATP [18,47]. Furthermore, several colorimetric and fluorescence-based assays for PPi quantification have been reported [48–52]; however, to our knowledge, none have been shown to be applicable to monitoring DNA synthesis. Considering the anticipated benefits of a polymerase activity measurement method that does not require the preparation of fluorescent tags for DNA substrates and that is compatible with screening of nucleotide analogs as polymerase inhibitors, we envisioned the coupling of primer extension (PE) reactions to the fluorescence-coupled measurement of pyrophosphate. An excellent measurement tool for this could be the commercial PiPer assay, which involves a chain of enzymatically catalyzed reactions that culminate in the oxidation of non-fluorescent Amplex Red to red-fluorescent resorufin (Fig. 1) [51,52]. The PiPer assay was initially developed to analyze the activity of the ATP-ase Hsp90. Reported herein is the demonstration that coupling of a primer extension reaction to the PiPer assay (PE-PiPer) allows for monitoring, on the basis of PPi release, DNA polymerase activity and is applicable for screening polymerase inhibitors with natural or modified templates in a 96-well plate format.

To establish PE-PiPer, we analyzed polymerase-mediated DNA synthesis reactions using natural DNA templates and DNA templates containing modified nucleobases corresponding to those produced by DNA alkylating agents, including DNA O^6-alkylation by temozolomide, 3-alkylation by methyl lexitropsin, and 7-alkylation by cisplatin. For validation, all fluorescence-based data were compared to those derived from conventional gel electrophoresis. DNA synthesis progression was evaluated for the TLS polymerases DPO4, Y-family DNA polymerase IV from *Sulfolobus solfataricus*, and Pol η, human Y-family polymerase. Both DNA polymerases were tested in a primer extension assay with natural dNTPs to establish the relationship between nucleotide incorporation and fluorescence readout. Further, extension was carried out in the presence of modified nucleotides to investigate whether PE-PiPer is amenable for detecting alterations in DNA synthesis efficiency.

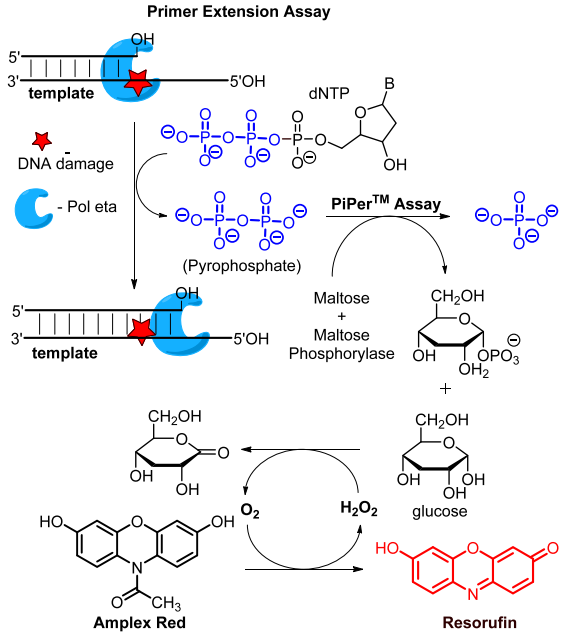

Fig.1. PE-PiPer assay for monitoring polymerase activity and DNA synthesis progression/inhibition.

Materials and methods

Reagents and proteins

The commercial PiPer kit was obtained from Life Technologies (Zug, Switzerland). Tris-HCl, glycerol, dithiothreitol (DTT), $MgCl_2$, ethylenediaminetetraacetic acid (EDTA), formamide, bromophenol blue, and cisplatin were obtained from Sigma Aldrich (St. Louis, MO, USA). SYBR Gold nucleic acid gel stain was obtained from Life Technologies. The archeal polymerase Dpo4 from *S. solfataricus* was obtained from Trevigen (Gaithersburg, MD, USA) and stored in 20 mM Tris-HCl (pH 7.8), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 50% (v/v) glycerol at a concentration of 1.2 μM. Human Pol η was expressed and purified as previously described and generously provided by Prof. Barbara Van Loon, University of Zurich [53]. Pol η was obtained in phosphate buffer, which interferes with the pyrophosphate assay; therefore, a buffer exchange to Tris-HCl (25 mM), pH 7.5, was performed with VWR centrifugal filters (Radnor, PA, USA) (molecular weight cutoff of 3 kDa). The concentration of Pol η after buffer exchange was measured with the Pierce BCA protein assay (Thermo Scientific, Waltham, MA, USA) before reconstitution to storage buffer containing DTT (150 μM) and glycerol (10%).

Nucleoside triphosphates

Natural deoxyribonucleotide (dNTPs) solution mix (10 mM each) was obtained from New England Biolabs. 5-Hydroxy-2′-deoxycytidine-5′-triphosphate (5-OH-dCTP), 5-carboxy-2′-deoxycytidine-5′-triphosphate (5-COOH-dCTP), 8-oxo-2′-deoxyadenosine-5′-triphosphate (8-oxo-dATP) and 5-nitro-1-indolyl-2′-deoxyribose-5′-triphosphate (5-nitro-TP) were obtained from Trilink Biotechnologies (San Diego, CA, USA). 6*H*,8*H*-3,4-Dihydropyrimido [1,2]oxazin-7-one-8-D-2′-deoxy-ribofuranosid-5′-triphosphate (dPTP) was obtained from Jena Bioscience (Jena, Germany). All nucleotide analogs were dissolved in deionized water (Merk Millipore).

Oligonucleotides synthesis

The PAGE-purified 18-mer used as DNA primer was obtained from VBC Biotech (Vienna, Austria). DNA templates were synthesized by solid phase chemical DNA synthesis on a Mermade 4 DNA synthesizer (Bioautomation). Phosphoramidites were obtained from Glen Research (Sterling, VA, USA). DNA templates were 30 nucleotides long and were either natural or carrying DNA damage in position +25 from the 3′-end (Table 1). Templates with O^6-methylguanine (30mer-O^6-MeG) and 3-deaza-3-methyladenine (30mer-3d-3MeA) were synthesized with

Table 1
DNA substrates to study DNA polymerase activity.

Substrate name	Substrate sequence
30mer-GG	3′-CTATACTCACACTCTACTACACTC**GG**CATC-5′ 5′-AGTGTGAGATGATGTGAG-3′
30mer-O^6-MeG	3′-CTATACTCACACTCTACTACACTC**X**GCATC-5′ 5′-AGTGTGAGATGATGTGAG-3′
30mer-Pt	3′-CTATACTCACACTCTACTACACTC**XX**CATC-5′ 5′-AGTGTGAGATGATGTGAG-3′
30mer-3d-3MeA	3′-CTATACTCACACTCTACTACACTC**X**GCATC-5′ 5′-AGTGTGAGATGATGTGAG-3′

O^6-Me-dG-CE phosphoramidite (Glen Research) and 3-deaza-3-methyl-dA-CE phosphoramidite (Berry & Associates, Dexter, MI, USA), respectively. The 30-mer DNA template carrying an intrastrand cisplatin crosslink at adjacent guanines (30mer-Pt) was obtained by reacting the unmodified 30-mer-GG DNA template with activated cisplatin as described previously [23]. The synthesized oligonucleotides were purified by HPLC and characterized by MS analysis (Electronic Supplementary Material, Figs. S1–4). The 30mer-GG and 30mer-O^6-MeG DNA templates were purified by HPLC (Agilent 1100 Series) with an Agilent Eclipse XDB-C18 5 μm 4.6 × 150 mm column. The chromatographic mobile phases were 50 mM triethylammonium acetate and acetonitrile. A gradient of acetonitrile from 8 to 12% over 30 min was used for 30mer-GG and from 9.5 to 12% over 49 min for 30mer-O^6-MeG. For 30mer-Pt, a Phenomenex Luna C18 5 μm 250 × 4.6 mm column was used with an acetonitrile gradient from 5 to 12% over 75 min. The eluted fractions containing DNA templates were concentrated to dryness in a MiVac centrifugal evaporator (GeneVac), resuspended in deionized water, and analyzed by direct injection into an Agilent MSD SL ion trap mass spectrometer with electrospray ionization. The template 30mer-3d-3MeA was purified by PAGE electrophoresis with a 20% (w/v) acrylamide/urea 7M gel followed by solid phase extraction with a Sep-Pak C18 Classic cartridge (Waters) as described previously [54].

Primer extension reactions

DNA substrates for primer extension reactions were prepared by annealing 30-mer DNA templates (11 μM) to the 18-mer primer (10 μM) in a total volume of 200 μl 1X NEBuffer 2 (New England Biolabs, Ipswich, MA, USA). The reaction mixture was heated to 94 °C for 4 min and allowed to cool slowly to room temperature for 1 h. Primer extension reactions were carried out in DNA LoBind microcentrifuge tubes (Eppendorf) by combining DNA substrate, dNTPs, and DNA polymerase in a total volume of 8 μl in 25 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$. The DNA polymerase was added last to start the reaction. The resulting solution was placed in a PCMT Thermoshaker (Grant-Bio) at 37 °C and DNA synthesis was stopped by the addition of EDTA solution (8 μl, 20 mM). This reaction solution was used for parallel analysis of primer extension with PE-PiPer assay and gel electrophoresis as described below. The concentrations of DNA, DNA polymerases, and dNTPs varied according to the specific enzyme and DNA substrate present in the reaction as follows. Dpo4 single nucleotide incorporation reactions were performed with 4 nM Dpo4, 3.2 μM 30mer-GG, and 300 μM dCTP (Fig. 2a) or with 5.6 nM Dpo4, 4.5 μM 30mer-GG, and variable concentrations of dCTP (0–300 μM). Reactions to assess the influence of nucleotide analogs on Dpo4 activity were performed with 4 nM Dpo4, 1.6 μM 30mer-GG, 300 μM dNTPs, and 30 μM nucleotide analog (10 min reaction time) or with 14 nM Dpo4, 1.6 μM 30mer-O^6-MeG, 600 μM dNTPs, and 60 μM nucleotide analog. A longer (60 min) reaction time was needed for primer extension (PE) over a modified template containing O^6-MeG, consistent with the 6-fold slower kinetics of Dpo4 over O^6-MeG vs G, as reported previously [13]. Reactions to assess the influence of nucleotide analogs on Pol η activity contained 20 nM Pol η, 1.6 μM DNA substrate, 600 μM dNTPs, and 60 μM nucleotide analogs. Reaction times were 30 min with 30mer-GG and 90 min with the damaged DNA templates.

PE-PiPer assay

To quantify released PPi, solutions resulting from primer extension reactions performed as described above (8 μl) were quenched by adding EDTA (8 μl, 20 mM) to the reaction tube. Then 13 μl of the quenched reaction solution was transferred to a half-area black 96-well plate (Greiner Bio One). To each well was added the commercial PiPer (LifeTechnologies) solution: assay buffer (12 μl of 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$), followed by a freshly prepared working solution (25 μl) containing glucose oxidase (8 U/ml), maltose phosphorilase (4 U/ml), maltose (0.4 mM), Amplex Red (100 μM), horseradish peroxidase (0.4 U/ml), and inorganic pyrophosphatase (4 U/ml) resulting in a total volume of 50 μl in each well [52]. The plate was incubated in a plate reader (Infinite 200 PRO series, Tecan) at 37 °C for 60 min, and fluorescence was measured (λ_{exc} 533 nm, λ_{em} 590 nm). Fluorescence data were corrected for background fluorescence with control samples containing EDTA prior to the addition of the DNA polymerase resulting in a reaction quenched at time zero.

Analysis of PE by gel electrophoresis

Extent of primer extension for each reaction was monitored in parallel by denaturing gel electrophoresis for comparison with PE-PiPer data. Two microliters of the quenched primer extension reaction was combined with 3 μl loading buffer (95% formamide, 18 mM EDTA, 0.1% bromophenol blue) and electrophoresis was performed with a 20% (w/v) acrylamide/urea 7M gel with an XCell SureLock mini-cell electrophoresis system from Life Technologies at 300 V for 90 min. The gel was then stained in 1X SYBR Gold solution at room temperature for 20 min. Gel bands were quantified with a Molecular Imager Gel Doc XR + Imaging System from Bio-Rad. The percentage product formation was determined with the equation $\sum I_{(n+x)}/(\sum I_{(n+x)} + I_n)$, where $I_{(n+x)}$ represents the intensity of all bands above the primer and I_n represents the band intensity of the nonextended primer.

Results and discussion

Monitoring single nucleotide incorporation by PE-PiPer

To test the feasibility of a PE variant of the PiPer assay, i.e., PE-PiPer, we measured the release of PPi from polymerase-mediated single nucleotide incorporation reactions with the PiPer assay. Steady-state primer extension reactions were carried out with an unmodified oligonucleotide template and the Y-family DNA polymerase Dpo4 from *S. solfataricus*. Nucleotide incorporation opposite adjacent guanines in position +25 and +26 from the 3′ end was monitored by PiPer and the magnitudes of fluorescence, expressed as percentage extension, were compared to those derived from gel-based assay. Products of primer extension reactions were separated by gel electrophoresis and visualized by SYBR Gold staining. In a time- and dCTP concentration-dependent manner, two new bands formed, corresponding to single (n + 1) or double (n + 2) incorporation of dCTP (Fig. 2a and b). The time-dependent increase in product formation was similar whether monitored by PE-PiPer or gel-based assays (Fig. 2c). Also by both methods, we derived Michelis-Menten parameters on the basis of following the extent of primer extension as a function of increasing dCTP concentration (Fig. 2d). The kinetic parameters obtained by PiPer were V_{max} 467.7 nM/min, K_m 7.5 μM, and K_{cat}/K_m 11.14 min^{-1} nM^{-1}, and by gel electrophoresis V_{max} 479.9 nM/min, K_m 8.01 μM, and K_{cat}/K_m 10.70 min^{-1} nM^{-1}, suggesting that the fluorescence readout is effective for evaluating polymerase activity.

Influence of nucleotide analogs on Dpo4 activity

We used PE-PiPer to screen the capacity of nucleotide analogs to reduce polymerase activity. For this purpose we performed primer extensions in the presence of modified nucleotide analogs

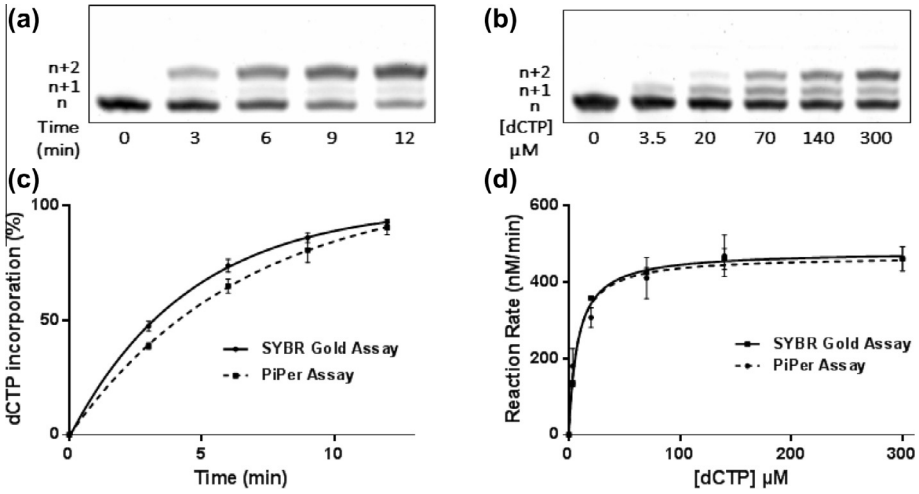

Fig.2. Single nucleotide incorporation catalyzed by Dpo4 monitored by PE-PiPer or standard gel electrophoresis: (a) Dpo4 (4 nM) with 30merGG (3.2 μM) and dCTP (300 μM); (b) Dpo4 (5.6 nM) with 30mer-GG (4.5 μM) and increasing concentrations of dCTP (0, 3.5, 20, 70, 140, 300 μM); (c) nonlinear regression analysis of data obtained for the samples in a analyzed by both methods; (d) Michaelis-Menten analysis of data obtained for the samples in b analyzed by both methods. Graphs represent the average independent replicates ($n = 3$ in c and $n = 2$ in d) and their standard deviation.

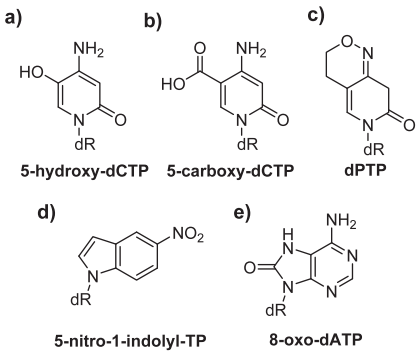

Fig.3. Structures of nucleotide analogs tested as PE inhibitors: (a) 5-hydroxy-2′-deoxycytidine-5′-triphosphate, (b) 5-carboxy-2′-deoxycytidine-5′-triphosphate, (c) 6*H*,8*H*-3,4-dihydro-pyrimido[4,5-c][1,2]oxazin-7-one-8-D-2′-deoxy-ribofuranosid-5′-triphosphate, (d) 5-nitro-1-indolyl-2′-deoxyribose-5′-triphosphate, (e) 8-oxo-2′-deoxyadenosine-5′-triphosphate. dR indicates 2′-deoxyribosetriphosphate.

(Fig. 3) varying in size, H-bonding, or π-stacking interactions, features that may alter DNA synthesis progression [55,56]. The PE reactions were carried out in the presence of a modified dNTP and the four natural dNTPs over a natural DNA template (30-mer-GG) and a DNA template containing a drug-induced lesion (O^6-methylguanine, 30mer-O^6-MeG), known to be bypassed by Dpo4 with low fidelity [13]. The concentration of the modified dNTP was 10 times lower than the natural dNTPs in order to mimic the conditions of inhibitor screening. PE in the presence of natural nucleosides only was used as a control for evaluating the impact of modified nucleosides on DNA synthesis.

The data obtained from PE over a natural vs modified DNA template indicated that PE-PiPer can sense diminished DNA synthesis rates in the presence of candidate inhibitors, and that inhibitory properties of modified dNTPs alter with respect to the template. Thus, addition of a modified dNTP to a PE reaction over a natural template (30mer-GG) in the presence of natural dNTPs for 10 min led to a 20–40% decrease in the formation of full-length DNA product (Fig. 4a), suggesting an inhibitory role of modified nucleosides in PE by Dpo4. In contrast to PE with 30mer-GG, the presence of modified nucleoside analogs did not impact the efficiency of DNA synthesis over 30mer-O^6-MeG, suggesting that inhibitory

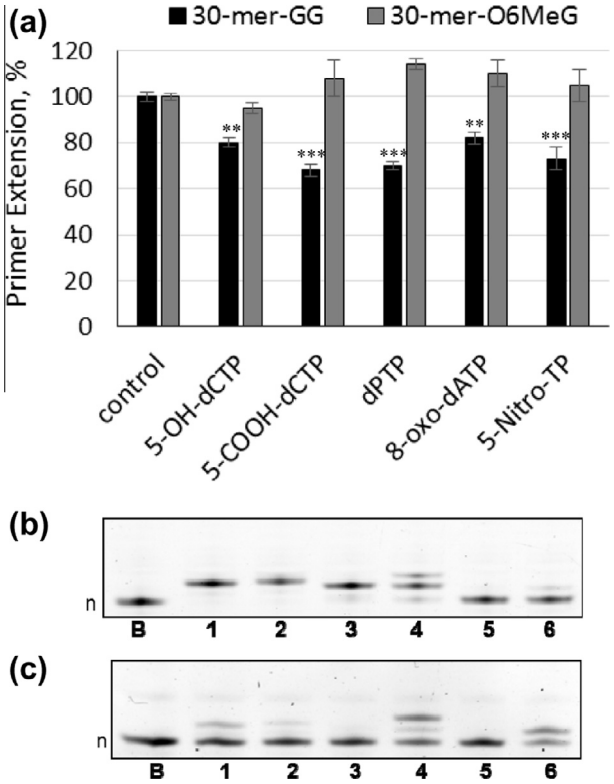

Fig.4. Influence of nucleotide analogs on Dpo4-mediated primer extension and single nucleotide incorporation. (a) Primer extension by Dpo4 (4 nM) on 30mer-GG and 30mer-*O*6-MeG (1.6 μM) in the presence of natural dNTPs and modified nucleotide analogs. Primer extension in the presence of natural dNTPs only is taken as a control. Average values of primer extension and standard deviation of three runs are reported. Statistical analysis was performed with one-way ANOVA for multiple comparisons with Dunnett's correction ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). (b) Single nucleotide incorporation by Dpo4 on 30mer-GG. (c) Single nucleotide incorporation by Dpo4 on 30mer-*O*6-MeG. Lanes: B, no dNTP; 1, dCTP; 2, 5-OH-dCTP; 3, 5-COOH-dCTP; 4, dPTP; 5, 8-oxo-dATP; 6, 5-nitro-TP. Graph bars represent the average of three independent replicates and their standard deviation.

properties of TLS-targeting molecules may be template dependent. The observed loss in fluorescence observed with PE over 30mer-GG template appears to be caused by competition among 5-OH-dCTP, 5-COOH-dCTP, and dPTP with natural dCTP for incorporation

opposite GG, as can be concluded from the efficient incorporation of these analogs by Dpo4 monitored by gel electrophoresis (Fig. 4b). On the other hand, 8-oxo-dATP and 5-nitro-TP do not appear to be incorporated by the polymerase opposite GG. Nevertheless, both dNTPs are mildly inhibitory, suggesting that they either compete with Dpo4 binding or impact DNA synthesis at a later step. The presence of O^6-MeG in the DNA template, however, appears to limit incorporation of modified dNTPs by Dpo4, which is reflected by the lack of Dpo4 inhibition observed in PE-Piper screen.

Influence of nucleotide analogs on Pol η activity

We further evaluated PE-PiPer as a means to monitor the influence of nucleotide analogs on Pol η-mediated TLS. In analogy to the previous experiments with Dpo4, Pol η activity was assessed for replication of natural and damaged DNA under steady-state kinetic conditions, in the presence of four natural dNTPs (control) or with the addition of a modified dNTP. To study Pol η activity on damaged DNA we employed the 30mer-O^6-MeG template and two additional DNA templates, one containing a 1,2-cisplatin intrastrand crosslink (30mer-Pt) and one containing 3-deaza-3-methyladenine (30mer-3d-3MeA) (Table 1), a depurination-resistant model for 3-methyladenine [22].

We observed that the nature of the DNA adduct influenced the inhibitory capacity of each nucleotide analog. For example, 5-OH-dCTP significantly reduced Pol η activity on 30mer-O^6-MeG and 30mer-Pt but not on other DNA substrates (Fig. 5). Likewise, 5-COOH-dCTP selectively inhibited TLS over 30mer-O^6-MeG and 30mer-3d-3MeA, while 5-nitro-TP inhibited TLS over 30mer-O^6-MeG. Primer extension reactions were stopped after 10 min for the natural DNA substrate and after 60 min for modified DNA substrates. PiPer data correlate with the data obtained after visualizing and analyzing PE reactions by gel electrophoresis (Electronic Supplementary Material, Fig. S5), showing that the assay is useful for quickly evaluating the activity of human Pol η.

PE-PiPer in a 96-well plate

The convenience, reproducibility, and sensitivity of PE-PiPer established in this study supported its potential for use in a high-throughput setting to screen chemical libraries for novel inhibitors. As a key step toward high-throughput adaptation, we implemented a combined version of the assay where not only the quantification step but also the primer extension reactions were performed in a 96-well plate with simultaneous start and quenching of the reactions followed by PiPer quantification. To assess the feasibility of this format we performed test reactions with Dpo4 and Pol η over 30mer-GG and 30mer-Pt, respectively. The nucleotide analogs used were 5-COOH-dCTP and dPTP for Dpo4, and 5-OH-dCTP and dPTP for Pol η, under reaction

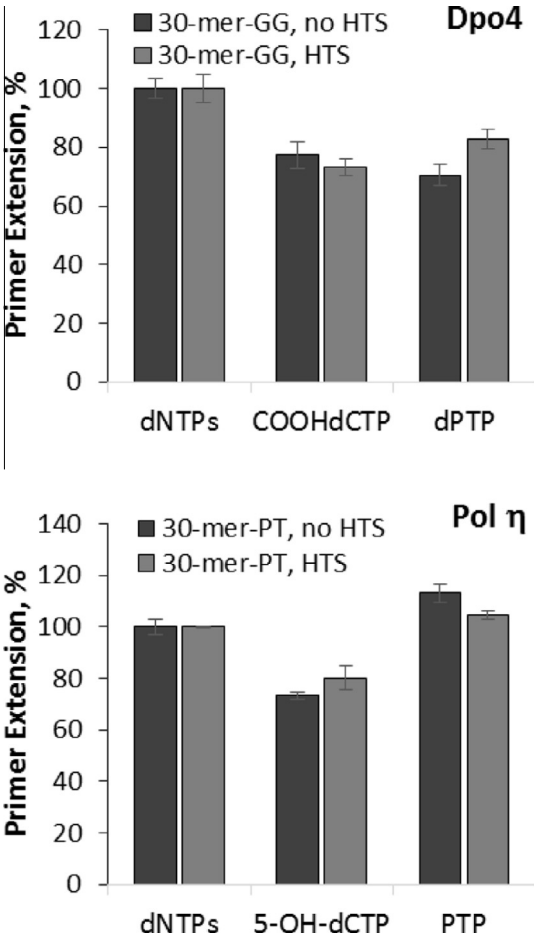

Fig.6. Implementation of PE-PiPer in high-throughput settings for Dpo4 and Pol η activity. (a) Dpo4-mediated primer extension on 30mer-GG in the presence of natural dNTPs (control), natural dNTPs plus 5-COOH-dCTP, and natural dNTPs plus dPTP ($n = 3$). (b) Pol η-mediated primer extension on 30mer-Pt in the presence of natural dNTPs (control), natural dNTPs plus 5-OH-dCTP, and natural dNTPs plus dPTP ($n = 3$). Graph bars represent the average of independent replicates and their standard deviation.

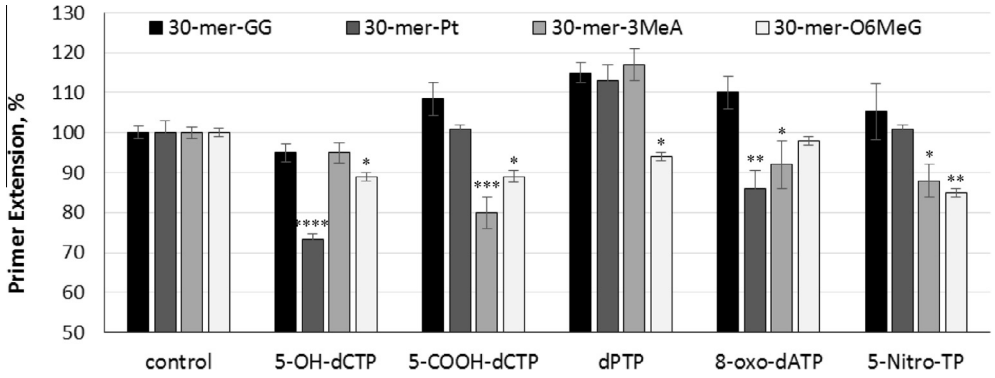

Fig.5. Influence of nucleotide analogs on Pol η-mediated primer extension. Reactions were carried out with 1.6 μM DNA template (30mer-GG, 30mer-O6-MeG, 30mer-Pt, or 30mer-3d-3MeA), 20 nM Pol η, 600 μM natural dNTPs, and 60 μM nucleotide analog. Significant alterations of primer extension from the control reaction were determined by one-way ANOVA for multiple comparisons and with Dunnett's correction ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). Bars represent the average of three independent replicates and their standard deviation.

conditions maintained as in previous experiments. The data obtained when we performed the assay in this format were similar to those obtained when the two steps were performed separately (Fig. 6). Thus, we observed that adding 5-COOH-dCTP and dPTP reduced Dpo4 activity with a 30mer-GG template, and that Pol η activity on 30mer-Pt was reduced by 5-OH-dCTP but not dPTP. The inhibitory efficiencies of dNTPs were similar regardless of analysis settings. These data support the suitability of the assay for high-throughput screening of large compound libraries, which will be pursued in future studies.

Conclusions

PE-PiPer was developed as a convenient and fast fluorescence-based assay to evaluate DNA polymerase activity by monitoring the release of PPi during DNA synthesis. The high sensitivity of the assay (0.4 μM of PPi) and miniaturization allow for the use of small amounts of enzyme and DNA substrates, in the nanomolar and micromolar range, respectively, to evaluate single nucleotide incorporation and full-length primer extension. Analysis occurs with no restrictions regarding DNA sequence and polymerase, since the fluorescence signal is proportional to released PPi rather than the formation of DNA product. We demonstrated that the PE-PiPer approach is suitable for steady-state analysis of Y-family DNA polymerase-mediated TLS over oligonucleotides with different DNA adducts and that it is feasible to screen candidate inhibitors. Furthermore, the results obtained with Pol η replicating over natural vs modified DNA templates show for the first time that the alteration of DNA polymerase activity by nucleotide analogs depends on the specific DNA adduct present in the DNA substrate. The data suggest that modified nucleotides may prove efficacious in damage-selective incorporation and inhibition of Y-family polymerases during the lesion bypass process, thus opening new opportunities to a more specific anticancer therapy.

Acknowledgments

We gratefully acknowledge Dr. Barbara van Loon (University of Zurich) and Antonia Furrer (University of Zurich) for providing mammalian polymerase η and helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2015.03.002.

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