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

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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.

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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.

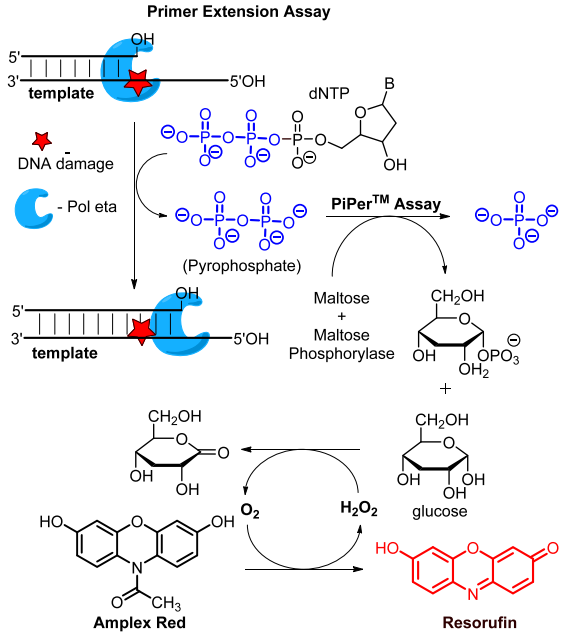

Fig.1. PE-PiPer assay for monitoring polymerase activity and DNA synthesis progression/inhibition.

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′