Published online 16 May 2011 Nucleic Acids Research, 2011, Vol. 39, No. 17 e112
doi:10.1093/nar/gkr350

A novel fluorescence-based assay for the rapid detection and quantification of cellular deoxyribonucleoside triphosphates

Peter M. Wilson[1], Melissa J. LaBonte[1], Jared Russell[2], Stan Louie[2], Andrew A. Ghobrial[1] and Robert D. Ladner[1,*]

[1]Department of Pathology, Norris Comprehensive Cancer Center and [2]Department of Clinical Pharmacy and Pharmaceutical Sciences, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089, USA

Received December 19, 2010; Revised April 15, 2011; Accepted April 26, 2011

ABSTRACT

Current methods for measuring deoxyribonucleoside triphosphates (dNTPs) employ reagent and labor-intensive assays utilizing radioisotopes in DNA polymerase-based assays and/or chromatography-based approaches. We have developed a rapid and sensitive 96-well fluorescence-based assay to quantify cellular dNTPs utilizing a standard real-time PCR thermocycler. This assay relies on the principle that incorporation of a limiting dNTP is required for primer-extension and *Taq* polymerase-mediated 5–3′ exonuclease hydrolysis of a dual-quenched fluorophore-labeled probe resulting in fluorescence. The concentration of limiting dNTP is directly proportional to the fluorescence generated. The assay demonstrated excellent linearity ($R^2 > 0.99$) and can be modified to detect between ~0.5 and 100 pmol of dNTP. The limits of detection (LOD) and quantification (LOQ) for all dNTPs were defined as <0.77 and <1.3 pmol, respectively. The intra-assay and inter-assay variation coefficients were determined to be <4.6% and <10%, respectively with an accuracy of 100 ± 15% for all dNTPs. The assay quantified intracellular dNTPs with similar results obtained from a validated LC–MS/MS approach and successfully measured quantitative differences in dNTP pools in human cancer cells treated with inhibitors of thymidylate metabolism. This assay has important application in research that investigates the influence of pathological conditions or pharmacological agents on dNTP biosynthesis and regulation.

INTRODUCTION

The coordinated regulation of intracellular deoxyribonucleoside triphosphates (dNTP) pools is critical for the fidelity of DNA synthesis during DNA replication and repair in both prokaryotic and eukaryotic organisms (1). Dysregulation of intracellular dNTP pools is observed in a large number of pathological conditions and represents a critical mechanism of action of a number of pharmacological inhibitors. The quantification of cellular dNTP levels is therefore of fundamental importance in assisting our understanding of the mechanisms of action of pharmacological agents and the biology of physiological and pathological phenomena that result in altered dNTP biosynthesis.

One evolving and potentially impactful application of fluorescence-based technology is the detection and quantification of dNTPs. dNTPs in extracts from cultured cells are frequently quantified by two independent methods; a DNA polymerase-based assay based upon the enzymatic synthesis of DNA and a high-performance liquid chromatography (HPLC)-based assay. Although protocols that employ radioisotopes in polymerase-based assays for dNTP detection are sensitive and quantitative and have undergone continued optimization, such methods can employ hazardous reagents in cellular extraction protocols and necessitate the use of radioactivity in the form of tritium-labeled dNTPs, with some protocols also requiring ^{32}P-labeled primers. Such reagents can pose health hazards and require specialized handling, dedicated equipment, storage and disposal and often necessitate safety inspections in line with government and institutional regulations (2–7). DNA polymerase-based assays represent an indirect method for quantifying dNTPs and as such various aspects of the assay from the point of cellular

*To whom correspondence should be addressed. Tel: +1 323 865 3116; Fax: +1 323 865 3099; Email: rladner@usc.edu

© The Author(s) 2011. Published by Oxford University Press.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/3.0), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

dNTP extraction right down to enzyme selection and assay conditions can influence the validity and robustness of the results. In addition DNA polymerase-based assays are also hampered by potential interference from ribonucleotides (rNTPs) and the utilization of different DNA polymerases was recently proposed for the detection of the different canonical dNTPs to circumvent this (7). Therefore, DNA polymerase-based assays require rigorous testing and optimization prior to use to ensure tangible and reproducible results.

The continued development of techniques such as HPLC and HPLC coupled with mass spectrometry (MS) have solved some of these issues and these assays represent a more direct analytical method of quantifying dNTPs (8–11). However, despite being precise and reproducible, these procedures are not as sensitive as radioisotope-based DNA polymerase assays and require larger sample sizes. In addition, these techniques are time, reagent and labor-intensive and require costly instrumentation and skilled operators which limits the overall utility of this procedure in conventional laboratories. In addition, both HPLC-based and DNA-polymerase-based assays are somewhat limited in their throughput capabilities in their current respective forms.

In the current article, we describe the design and validation of a rapid, sensitive and reproducible 96-well fluorescence-based method for measuring dNTPs that eliminates the need for radioactivity and is compatible with a simple methanol-based cellular dNTP extraction protocol. This assay eliminates the requirement for hazardous reagents and specialized HPLC and MS-based instrumentation. The assay principle is similar to technology found in a typical *Taq*Man PCR but utilizes a synthetic oligonucleotide user-supplied template, a single primer and a dual-quenched fluorophore-labeled probe. A number of templates with varying sensitivities and linear capabilities for each dNTP were designed and validated and could reproducibly detect between 0.3 and 100 pmol of corresponding dNTP depending on assay requirements and conditions. The assay was validated according to the US Food and Drug Administration ‘Analytical Procedures and Methods Validation’ guidelines and demonstrated excellent linearity ($R^2 > 0.99$) with excellent accuracy, sensitivity, recovery and reproducibility. This novel method showed good concordance to a validated LC–MS/MS-based approach when performed in comparison. Finally, the assay was successfully applied to the detection of dNTPs from human cancer cells.

MATERIALS AND METHODS

Chemicals, drugs and reagents

The oligonucelotide primer, templates and fluorophore- and quencher-labeled detection probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA), subjected to polyacrylamide gel electrophoresis purification and reconstituted in Omnipur sterile nuclease-free water (EMD Chemicals USA, Gibbstown, NJ, USA) at a stock concentration of 100 µmol/l. The two non-emissive (dark) quenching molecules incorporated into the detection probes include the Iowa black fluorescein quencher (IBFQ; absorption max = 531 nm) and ZEN (non-abbreviation; absorption max 532 nm). The fluorescent label utilized was 6-FAM (5′-carboxyfluorescein; excitation max. = 494 nm, emission max. = 520 nm). Probes were further diluted to a working stock of 10 µmol/l and aliquoted to avoid repeated freeze/thaw cycles. AmpliTaq Gold DNA Polymerase, GeneAmp 10× PCR Buffer 2, $MgCl_2$ and MicroAmp Optical 96-well Reaction Plates were purchased from Applied Biosystems (Carlsbad, CA, USA) and were sealed with ThermalSeal RT2RR sealing film from Excel Scientific (Victorville, CA, USA). dNTPs were purchased individually at stock concentrations of 100 mmol/l from New England Biolabs at HPLC-certified >99% purity (Ipswich, MA, USA). Ribonucleoside triphosphates (rNTPs) were purchased individually from Applied Biosystems at stock concentrations of 10 mmol/l. Fluorodeoxyuridine (FUdR) and 5-fluorouracil (5-FU) were obtained from Sigma (St Louis, MO, USA) and maintained in sterile double-distilled water and DMSO, respectively at stock concentrations of 50 mmol/l. Pemetrexed disodium salt (>99%) was purchased from LC Laboratories (Woburn, MA, USA) and maintained in sterile double-distilled water at a stock concentration of 50 mmol/l. Recombinant human deoxyuridine nucleotidohydrolase (dUTPase) was expressed and purified as described previously (12).

Assay components, instrumentation and real-time fluorescence conditions

Reaction mixtures contained primer, probe and template at an equimolar final concentration of 0.4 µmol/l. $MgCl_2$ was included at a final concentration of 2 mmol/l. Non-limiting dNTPs were included in the reaction mix in excess at a final concentration of 100 µmol/l (the dNTP to be assayed was excluded). AmpliTaq Gold DNA polymerase was added at 0.875 U/reaction, 2.5 µl of 10× PCR Buffer 2 added and nuclease-free ddH_20 added to a final reaction volume of 25 µl. For analysis of cell extracts, the volume of ddH_20 was modified to accommodate the addition of 2.5 µl of cell extract. For dUTP determination from cell extracts, the volume of ddH_20 was further modified to accommodate an additional 1 µl of dUTPase (10 ng/µl). Thermal profiling and fluorescence detection was performed using the ‘isothermal’ program on board an Applied Biosystems 7500 Real-Time PCR System. For analysis of dNTPs, the thermal profile consisted of a 10 min 95°C step to ‘hot-start’ the *Taq* polymerase and a primer extension time of up to 30 min at 60°C depending on the application. Raw fluorescence spectra for 6-FAM was measured using filter A at specified time intervals (typically every 5 min) to follow assay progression using Sequence Detection Software (SDS Version 1.4, Applied Biosystems) and exported and analyzed in Microsoft Excel (Microsoft, Redmond, WA, USA) and Prism 5.0 (GraphPad Software, La Jolla, CA, USA). In all cases, fluorescence values for blank reactions (limiting dNTP omitted) were subtracted to give normalized fluorescence units (NFUs) to account for background probe

fluorescence. A detailed step-by-step protocol can be found in Supplementary Figure S2.

Cell lines and cell culture conditions

The human colorectal cancer cell line HCT116 was purchased from American Type Culture Collection (Lockville, MD, USA). HCT116 cells were maintained in McCoy's 5A media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Lonza, Walkersville, MD, USA) with penicillin/streptomycin, sodium pyruvate and L-glutamine (Invitrogen). For pemetrexed analyses, cells were incubated in folate-depleted RPMI supplemented with 25 nmol/l 5-Formyltetrahydrofolate, 10% fetal bovine serum (Lonza), penicillin/streptomycin, sodium pyruvate and L-glutamine for 24 h prior to treatment. Cells were maintained in a humidified Forma incubator (Forma, Waltham, MA, USA) at 37°C with 5% CO_2 and routinely screened for mycoplasma using the MycoALERT detection kit (Lonza) and verified mycoplasma negative.

Assay validation, data analysis and statistics

The dNTP detection assay described herein was rigorously tested in accordance with the US Food and Drug Administration 'Analytical Procedures and Methods Validation' and 'Bioanalytical Method Validation' guidelines available at http://www.fda.gov. The relationship between assay response and known concentrations of the analytes (dNTPs) was analyzed by a 5-point (minimum) calibration curve. Calibration curves were obtained by assaying serial dilutions of dNTP standards diluted with ultra-pure nuclease-free water to the desired concentration and evaluated using regression coefficients (R^2). Samples were assayed in triplicate, and experiments were repeated on three independent occasions as appropriate. The limit of detection (LOD) was defined as the mean value of the negative blind controls plus 3 SDs of the mean, i.e. the concentration with a signal:noise ratio of 3:1. The limit of quantification (LOQ) was defined as the mean value of the negative controls plus 5 SD determined from three independent assays (signal:noise ratio of 5:1). Coefficient of variation (%CV) values were calculated from the cumulative mean and SD of replicates. Inter-assay %CVs were calculated from three independent experiments performed on different days. The intra-assay %CV represents the mean ± SD %CV obtained from assaying replicate samples within three identical yet independent assays performed on the same day. The accuracy was assessed by comparing the nominal dNTP concentrations with the corresponding calculated values based on the calibration curve and presented as a percentage. Recoveries were determined by obtaining the result from a known quantity of a dNTP standard spiked into an unknown extract and comparing this to the sum of the individual results obtained for the dNTP standard and the cell extract. All dNTP quantities are expressed as picomoles or picomoles per 10^6 cells as appropriate.

Extraction of intracellular dNTPs

Cells were plated in 10-cm tissue culture dishes (TPP, Trasadingen, Switzerland) at 1×10^6 and allowed to adhere and enter log-phase growth for 48 h. Cells were then treated with vehicle or FUdR (1 µmol/l) for 4 h after which the medium was aspirated and cells were rinsed with phosphate-buffered saline (PBS) to remove residual media. The adherent cells were detached by trypsin, resuspended gently in 10 ml of ice-cold PBS and and a 100 µl aliquot removed to determine cell number via haemocytometer. The samples were centrifuged for 5 min at 3000*g* at 4°C, the supernatant discarded and cell pellets were then resuspended in 500 µl of ice-cold 60% methanol, vortexed vigorously to resuspend, placed at 95°C for 3 min and sonicated for 30 s in a Branson Sonifier 450 (Branson, Danbury, CT, USA). The extracts were centrifuged (16 000*g* for 5 min at 4°C) to remove cell debris, precipitated protein and DNA. The resultant cell extract supernatants were passed through pre-equilibrated Amicon Ultra-0.5-ml centrifugal filters at 4°C to remove macromolecules >3 kDa according to the manufacturer's directions (Millipore, Billerica, MA, USA). The filtrate was evaporated under centrifugal vacuum at 70°C and the resultant pellet was resuspended in 25 µl nuclease-free water ready to assay or stored at −80°C until use.

Liquid chromatography MS

A previously validated LC–MS/MS approach was utilized to determine dNTP concentrations (13). Standard solutions of dATP, dTTP, dCTP and dGTP at a concentration of 100 µmol/l were utilized to construct a 9-point calibration curve consisting of 0, 50, 75, 100, 250, 500, 750, 1000 and 2500 ng/ml standards. Each standard was spiked with 100 µl of a mixture of 500 ng/ml dideoxyCTP (ddCTP), 1000 ng/ml of dideoxyGTP (ddGTP), 500 ng/ml of 2-chloroadenosine triphosphate (2-ClA) and 500 ng/ml of ADV-DP to serve as internal quality control standards. Calibration standards were processed simultaneously. Least-square linear regression using a weighting of $1/x^2$ was performed to establish a linear calibration curve between the area ratios of analyte to internal standard and the concentrations of analyte. The linearity was established by the back calculate concentration for each calibration standard. The comparison of the actual concentration to the expected theoretical value established the precision and accuracy of the assay. Samples were vacuum dried using an SPD SpeedVac and suspended in 0.5 ml of 0.01% formic acid in HPLC H_2O which contained two units of acid phosphatase/ml (Sigma) and incubated for 30 min at 37°C, to dephosphorylate and yield the corresponding deoxynucleoside. The dephosphorylated standards and quality controls were vacuum dried in a SPD SpeedVac and reconstituted with 50 µl of 7% methanol in deionized water and 30 µl of the sample was injected into an Agilent 1100 (Agilent, San Jose, CA, USA) high performance liquid chromatography (HPLC) system running an ACE C18 column 2.0 × 50 mm with 3 µm packing (Advanced Chromatography Technologies; Aberdeen, Scotland), coupled to an Sciex API 3000 triple

quadrupole tandem mass spectrometer (Applied Biosystems). The operating software was Analyst 1.4.2. A step gradient program was applied to separate all the analytes with a flow rate of 300 µl/min. The mobile phase consisted of methanol as component A and 20 mmol/l ammonia acetate buffer at pH 4.5 as component B. After separation, the analytes in the HPLC efferent were introduced into the mass spectrometer through a TurboIonspray interface coupled with a heated turbo nitrogen stream to evaporate solvents and to increase ionization efficiency. The mass spectrometer operated in distinct periods: the first period had 8-min scan time for the detection of deoxycytidine (dC), dideoxycytidine (ddC), deoxyguanosine (dG) and dT; the second period lasted 9 min and scanned for deoxyadenosine (dA), dideoxyguanosine (ddG), and 2-ClA. The following mass transitions were monitored—dA: 252→136, retention time of 7 min; dT: 243→127, retention time of 4 min; dG: 268→152, retention time of 3.5 min; dC: 228→112, retention time of 1.5 min; ddC: 212→112, retention time of 3 min; ddG: 252→152, retention time of 7.5 min; ADV: 274→162, retention time of 2 min; 2-ClA: 302→170, retention time of 11 min.

RESULTS

Assay concept overview

The goal of the current study was to develop a rapid and sensitive fluorescence-based assay with throughput capability to detect and measure dNTPs as an alternative to currently employed approaches that utilize radioisotope-based DNA polymerase assays or HPLC and/or LC–MS/MS methodology. The DNA polymerase-based assay described previously by Sherman and Fyfe (4) was utilized as a platform to develop a non-radioactive fluorescence-based assay to detect the limiting dNTP. The aforementioned studies relied upon the incorporation of a radiolabeled nucleotide into a template–primer assay during DNA polymerase-catalyzed enzymatic synthesis of DNA. Upon depletion of the dNTP being measured, the DNA polymerase stalls and the incorporation of radiolabeled nucleotide ceases, resulting in extension delay and/or chain termination. The amount of incorporation of the radiolabeled nucleotide at the completion of the reaction is directly proportional to the concentration of the limiting dNTP being assayed. We employed a similar DNA polymerase-based approach utilizing a modified oligonucleotide template with three distinct regions: a 3′ primer-binding region, a mid-template dNTP detection region and a 5′ 6-FAM-labeled probe-binding region. During the reaction, the probe and primer hybridize to the oligonucleotide template to form the template:primer:probe complex (TPP complex). When *Taq* polymerase binds to the primer in the TPP complex and the dNTP to be measured is present, successful extension of the nascent strand occurs and the inherent 5′ to 3′ exonuclease activity of *Taq* polymerase cleaves and displaces the 6-FAM-labeled probe in a 5′ to 3′ direction, releasing the 6-FAM fluorophore from its proximity to the two quenchers. This displacement effectively disrupts the Förster resonance energy transfer (FRET) and the resulting fluorescence detected upon excitation is directly proportional to the amount of the limiting dNTP available in the assay for incorporation (Figure 1). Conversely, when the limiting dNTP becomes exhausted and is no longer available for incorporation, *Taq* polymerase stalls and extension delay and/or chain termination of the nascent strand occurs. In this instance, probe hydrolysis/degradation does not occur and the probe remains dark as fluorescence remains quenched via FRET.

Assay development and design considerations

One key advantage of the most recent modifications to the radioisotope DNA polymerase-based assays was the high signal:noise ratio that facilitates the detection of extremely low picomole quantities of dNTP (0.1 pmol) with great accuracy. This is possible through the removal of unincorporated radioactivity via numerous wash steps prior to scintillation counting, thus significantly reducing background noise. The current assay utilizes an enclosed system where the generated fluorescence signal is detected in the presence of any remaining intact fluorophore-labeled probe which, even in its intact and quenched state, will demonstrate some residual background fluorescence. The goal therefore was to create a detection system that remains as dark as possible in the absence of analyte-generated signal and thus maintains a high signal:noise ratio that will facilitate the detection of low picomole quantities of dNTP required for most applications.

Probe design

A 23-bp oligonucleotide hybridization probe was designed that incorporates two similar napthyl-azo structure-based quenching molecules to maximize the signal:noise ratio. The IBFQ quencher was incorporated at the 3′-end of the probe and an additional phosphoramidite internal ZEN quencher was incorporated 9 bp from the 5′ 6-FAM fluorophore (Table 1). Dark quenchers that have maximum absorption wavelengths in the 531–534 nm range such as IBFQ and ZEN and other functionally-similar and commercially available dark quenchers including black hole quencher 1 (BHQ-1, Sigma; absorption max: 534 nm) and Eclipse (Glen Research, Sterling, VA, USA; absorption max: 530 nm) have demonstrated efficient FRET when coupled with fluorescent dyes that emit in the green to pink spectral range including the 6-FAM fluorophore (14,15). The incorporation of two quenching molecules also serves two purposes; two quenchers will ensure maximal FRET to both quenchers minimizing the quantum yield and background fluorescence generated in the absence of *Taq*-mediated probe hydrolysis. In addition, the close proximity of the internal ZEN quencher reduces the physical distance between the reporter fluorophore and the quencher to only 9 bp which is reportedly a key determinant that increases the efficiency of the FRET mechanism (16). Attempts to develop the assay using an identical 23-bp single IBFQ-quenched probe were unsuccessful and the incorporation of the ZEN internal quencher was critical for the sensitivity in this assay (data not shown). In addition, quenching in linear

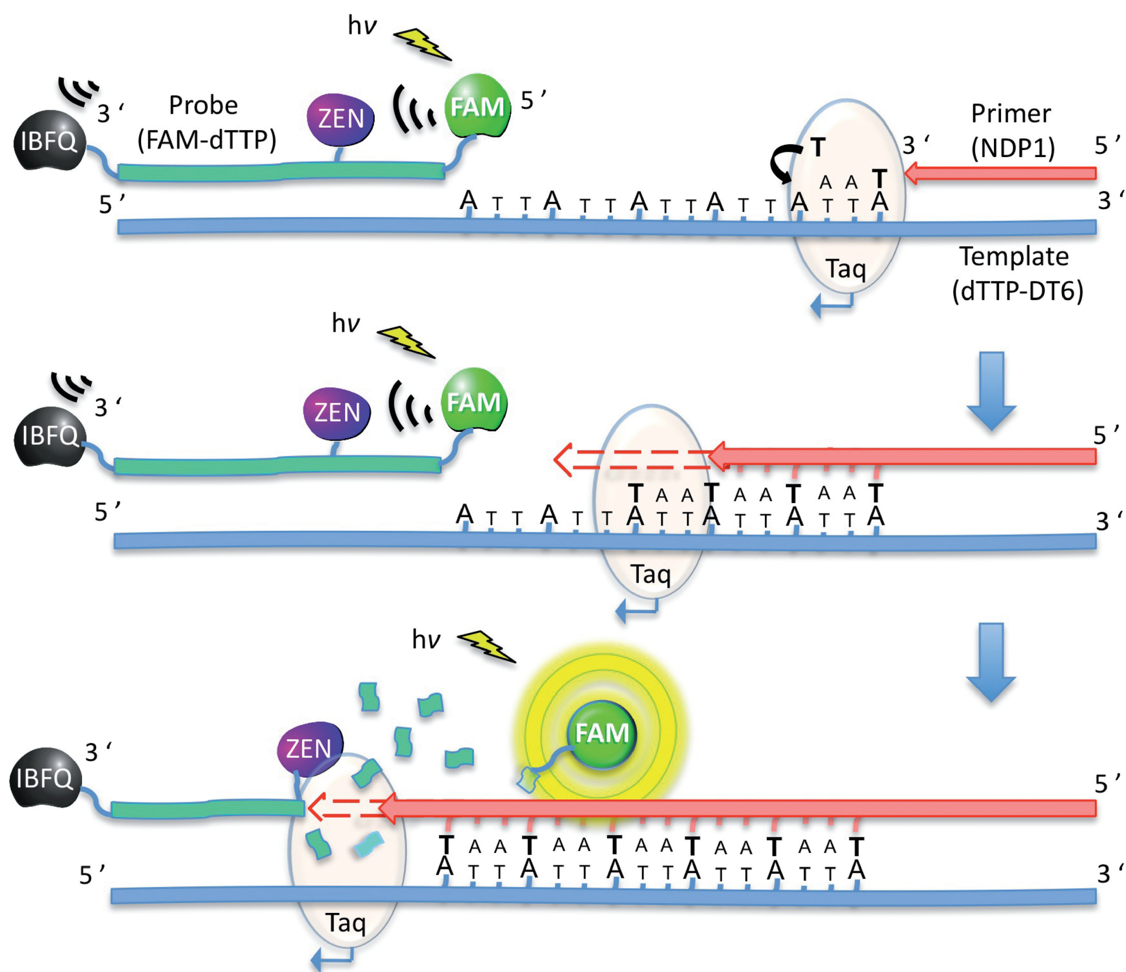

Figure 1. Simplified schematic illustrating the principle mechanism involved in the fluorescence-based assay for measuring dNTP concentrations. Detection of dTTP using template DT6 is given as the example. The template is depicted in blue, the FAM-dTTP probe in green and primer NDP1 in red. Briefly, as the temperature declines from the 95°C hot-start, the probe anneals to the template first (65–70°C), followed by the primer at 60°C to form the TPP complex at which point *Taq* polymerase begins extension of the nascent strand. In the presence of a sufficient concentration of limiting dNTP (six dTTP molecules in the example of dTTP-DT6), successful primer extension occurs through the mid-template dNTP detection region and *Taq* polymerase cleaves the terminal nucleotide labeled with the 6-FAM fluorophore via its 5′-3′ exonuclease activity releasing it from the dual-quenched (ZEN and IBFQ) probe resulting in disruption of FRET and generation of a fluorescence signal in response to excitation-induced photon energy (hν). When the dNTP being measured (dTTP) is not present or becomes exhausted, *Taq* polymerase stalls, extension is inhibited/terminated, fluorescence remains quenched via FRET and the probe remains dark. In any given reaction, the level of fluorescence generated is directly proportional to the concentration of the limiting dNTP. The dAMP molecules enlarged in the template strand represent the nucleotides opposite which the limiting dTTP nucleotides (also enlarged and in bold) will base pair. Only the nucleotide sequence found in the mid-template dNTP detection region is given for simplicity. The complete sequences of all templates (including primer- and probe-binding regions), primer NDP1 and detection probes are given in Table 1. Template, primers and probes were prepared as described in ‘Materials and Methods’ section.

probes incorporating dark quenchers and fluorescein-based probes such as FAM also occurs via the non-FRET-based mechanism of static quenching which may further limit assay background and improve signal:noise ratio (17).

dNTP template and primer design

The first oligonucleotide detection template (DT) designed and evaluated for the detection of dTTP was dTTP-DT6 depicted in Figure 1. This template requires six dTTP nucleotides to be available for incorporation into the nascent strand as dTMP (each dTMP event is separated by two dAMP incorporation events) by *Taq* polymerase before the exonuclease activity of *Taq* cleaves the 6-FAM fluorophore resulting in fluorescence. The requirement for six near-sequential dTTP incorporations in the nascent strand greatly limits the probability of fluorescence generated as a result of dNTP misincorporation by *Taq* polymerase which reportedly can occur in the presence of severely imbalanced dNTP concentrations (18). However, the requirement for six dTTP molecules per probe degradation-induced fluorescence event also has the potential to limit the sensitivity of the assay. To evaluate this, two additional oligonucleotide DTs were designed to test the sensitivity capabilities, one requiring two dTTPs for incorporation (dTTP-DT2) and a template requiring only a single dTTP for incorporation during primer extension to facilitate probe hydrolysis (dTTP-DT1).

Table 1. Primer, probe and templates utilized in the assay

Name	Classification	Sequence (5′–3′)	Size (bp)	T_m (°C)[a]
NDP-1	Primer	CCGCCTCCACCGCC	14	56
FAM-dTTP	Probe[b]	***6FAM***/AGGACCGAG/***ZEN***/GCAAGAGCGAGCGA/***IBFQ***	23	70
dTTP-DT6	Template[c]	TCGCTCGCTCTTGCCTCGGTCCTT***A***TT***A***TT***A***TT***A***TT***A***TT***A***GGCGGTGGAGGCGG	54	69
dTTP-DT2	Template[c]	TCGCTCGCTCTTGCCTCGGTCCTTT***A***TTT***A***TTTGGCGGTGGAGGCGG	47	72
dTTP-DT1	Template[c]	TCGCTCGCTCTTGCCTCGGTCCTTT***A***TTTGGCGGTGGAGGCGG	43	73
FAM-dATP	Probe[b]	***6FAM***/TGGTCCGTG/***ZEN***/GCTTGTGCGTGCGT/***IBFQ***	23	68
dATP-DT2	Template[c]	ACGCACGCACAAGCCACGGACCAAA***T***AAA***T***AAAGGCGGTGGAGGCGG	47	73
dATP-DT1	Template[c]	ACGCACGCACAAGCCACGGACCAAA***T***AAAGGCGGTGGAGGCGG	43	74
FAM-dCTP	Probe[b]	***6FAM***/AGGATTGAG/***ZEN***/GTAAGAGTGAGTGG/***IBFQ***	23	63
dCTP-DT2	Template[c]	CCACTCACTCTTACCTCAATCCTTT***G***TTT***G***TTTGGCGGTGGAGGCGG	47	70
dCTP-DT1	Template[c]	CCACTCACTCTTACCTCAATCCTTT***G***TTTGGCGGTGGAGGCGG	43	70
FAM-dGTP	Probe[b]	***6FAM***/ACCATTCAC/***ZEN***/CTCACACTCACTCC/***IBFQ***	23	64
dGTP-DT2	Template[c]	GGAGTGAGTGTGAGGTGAATGGTTT***C***TTT***C***TTTGGCGGTGGAGGCGG	47	71
dGTP-DT1	Template[c]	GGAGTGAGTGTGAGGTGAATGGTTT***C***TTTGGCGGTGGAGGCGG	43	71

[a] T_m calculated in presence of 50 mmol/l NaCl.
[b] Letters in bold and italics denotes the type and location of probe modifications.
[c] Bases in bold and italics represent the dNTP to which the limiting dNTP will base pair opposite.

The primer utilized (Nucleotide Detection Primer 1; NDP1) was designed complementary to the template 3′ region and incorporated a GC clamp. NDP1 was also designed to have a T_m of 56°C which is 5–10°C lower than the T_m of the probes at 63–70°C. This facilitates the specific binding order of the probe to the template, followed by the primer to form a template:probe:primer complex (TPP complex) and thus ensures that primer extension and template completion does not occur in the absence of template-bound probe whereby no fluorescence would be generated. The sequences of all templates, primers and probes are given in Table 1.

Polymerase selection

In contrast to previous template-based polymerase assays that quantify dNTPs, the design of this assay necessitates the use of a DNA polymerase with inherent 5′ to 3′ exonuclease activity to cleave the fluorophore-labeled probe during successful polymerization and nascent strand synthesis. In addition, one major drawback in previous polymerase-based assays using Klenow polymerase was the potential for misincorporation of rNTPs leading to artificially elevated measurements particularly for dGTP and dCTP (7). This was an important assay design consideration as rNTPs can be present in cell extracts (quiescent cells in particular) in molar ratios 1000-fold greater than their corresponding dNTP. *Taq* polymerase is reported to be 30 000-fold more efficient at discriminating beween ribo- and deoxyribonucleotides (300 000-fold in the case of dTTP and UTP) primarily due to a single Glu-615 residue that can sterically exclude the 2′-OH of an incoming rNTP (19). A recent report identified *Taq* polymerase as having a 10-fold higher capacity to discriminate dNTPs from rNTPs than the commonly utilized Klenow DNA polymerase and demonstrated no significant interference from rNTPs even at the extremes of physiological rNTP concentrations (7). In addition to its dNTP discriminatory capabilities, the utilization of AmpliTaq Gold DNA polymerase that requires a 'hot-start' has a number of important advantages in this assay. The inactivity of the enzyme before the hot-start allows flexibility in the reaction setup, including pre-mixing of the reagents (including *Taq* polymerase addition) at room temperature. This was confirmed empirically by performing plate setup on ice (3°C) versus room temperature (22°C) with no significant variation in assay performance observed (data not shown). Second, the hot-start also serves as an efficient DNA denaturation step after which the ramp down to 60°C facilitates the sequential binding (based on calculated T_m) of the probe followed by the primer to form the functional TPP complex. In addition, *Taq* polymerase inactivity during set-up and the first temperature ramp of the assay ensures that the timing of the reaction can be tightly controlled for all individual reactions and replicates and that mis-primed primers are not extended at temperatures where sub-optimal primer annealing may occur. At the 60°C temperature in this assay and under optimum conditions, *Taq* polymerase demonstrates ~50% maximum polymerization activity with 1 U catalyzing the incorporation of 5 nmol of dNTP into acid insoluble product in 30 min at 60°C.

Validation of assay principle and performance

Initially, dTTP-DT6, -DT2 and -DT1 were all evaluated in the assay for their ability to detect dTTP. The raw fluorescence units obtained after 10-min incubation were normalized to account for background probe fluorescence that was determined from controls lacking the limiting dNTP. The resulting NFUs obtained from serial dilutions of dTTP were utilized to generate calibration curves. Consistent with the design hypothesis, dTTP-DT6 which requires six dTTP incorporations per TPP complex to yield fluorescence demonstrated excellent linearity and facilitated robust detection in the assayed range between ~10 and 100 pmol of dTTP. dTTP-DT2 which requires two dTTP incorporation events for extension also demonstrated excellent linearity in the assayed range

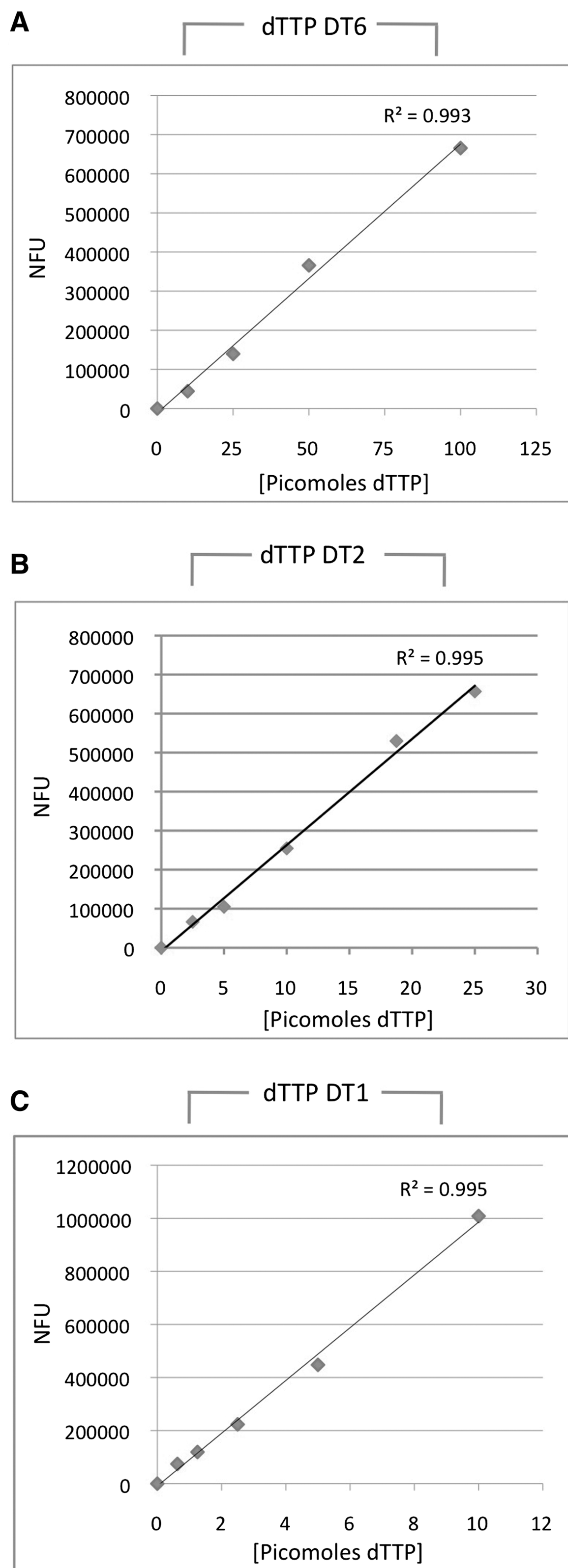

Figure 2. Validation of dTTP templates with varying detection sensitivities and linear range. Three specific oligonucleotide templates were initially generated and tested for their ability to detect dTTP and tested by calibration curve as described in 'Materials and Methods' section. (**A**) dTTP-DT6 requires the incorporation of six dTTPs for fluorescence generation and yielded a linear range of 0–100 pmol. (**B**) dTTP-DT2 requires the incorporation of two dTTPs and yielded a linear range of 0–25 pmol. (**C**) Finally, dTTP-DT1 requires only a single dTTP for incorporation per TPP complex to yield fluorescence and had a linear range of 0.6–10 pmol. Calibration curves for all three templates demonstrated R^2 of >0.993. In all cases, fluorescence values for blank reactions (limiting dNTP omitted) were subtracted to give NFU.

between ~2.5 and 25 pmol of dTTP. Finally, dTTP-DT1 which requires only a single dTTP incorporation event to yield fluorescence, demonstrated excellent linearity in the assayed range between 0.6 and 10 pmol (~31–500 nmol/l) of dTTP. Calibration curves for all three dTTP DTs demonstrated excellent linearity with $R^2 > 0.99$ in every case (Figure 2). Subsequent analysis of the remaining dNTPs (dATP, dCTP and dGTP) was performed using their corresponding DT1 and DT2. DTs for all dNTPs evaluated resulted in $R^2 > 0.99$ in every case and each yielded similar detection ranges and NFU under the same assay conditions (Figure 3).

Assay sensitivity, precision, accuracy and recovery

The mean LOD, which represents the smallest concentration or quantity of an analyte that can be reliably shown to be present or measured under assay conditions was investigated (20). The LOD was calculated from the standard curve as the mean value of the negative control plus 3 SD from three identical, inter-day assays and was determined to be 0.46 ± 0.02 pmol for dTTP, 0.77 ± 0.5 for dATP, 0.36 ± 0.16 for dCTP and 0.38 ± 0.1 for dGTP (Table 2). The LOQ, defined as the mean value of the negative cosntrols plus 5 SD from three independent assays, was determined by standard curve to be 0.88 ± 0.15 pmol for dTTP, 1.3 ± 0.1 for dATP, 0.77 ± 0.2 for dCTP and 0.81 ± 0.01 for dGTP (Table 2).

The variability of the assay was evaluated by calculating the inter- and intra-assay coefficients of variation (%CV) as described in the 'Materials and Methods' section. The intra-assay coefficients of variation (%CVs) were determined to be 3.4 ± 0.85 for dTTP, 3.64 ± 1.2 for dATP, 4.04 ± 0.63 for dCTP and 4.58 ± 0.77 for dGTP. The interassay CV for dTTP was 8.5 ± 6.3 for dTTP, 6.5 ± 2.6 for dATP, 9.6 ± 2.3 for dCTP and 4.1 ± 3.5 for dGTP (Table 2).

Accuracy was determined in the low- and high- assay range and was within 100 ± 15% for all dNTPs (Table 2). Recoveries were determined by obtaining the result from an undetermined cell extract spiked with a known quantity of a dNTP standard and comparing that to the sum of the individual results obtained for the dNTP standard and the extract determined separately. The assay method gave high recoveries within 100 ± 7% for all dNTPs (Table 2).

Assay optimization for the quantification of intracellular dNTPs

Progression of the polymerase reaction. Having demonstrated the feasibility of a fluorescence-based approach to detect and quantify low picomole quantities of dNTPs, the assay was optimized to determine the best conditions for the detection and quantification of

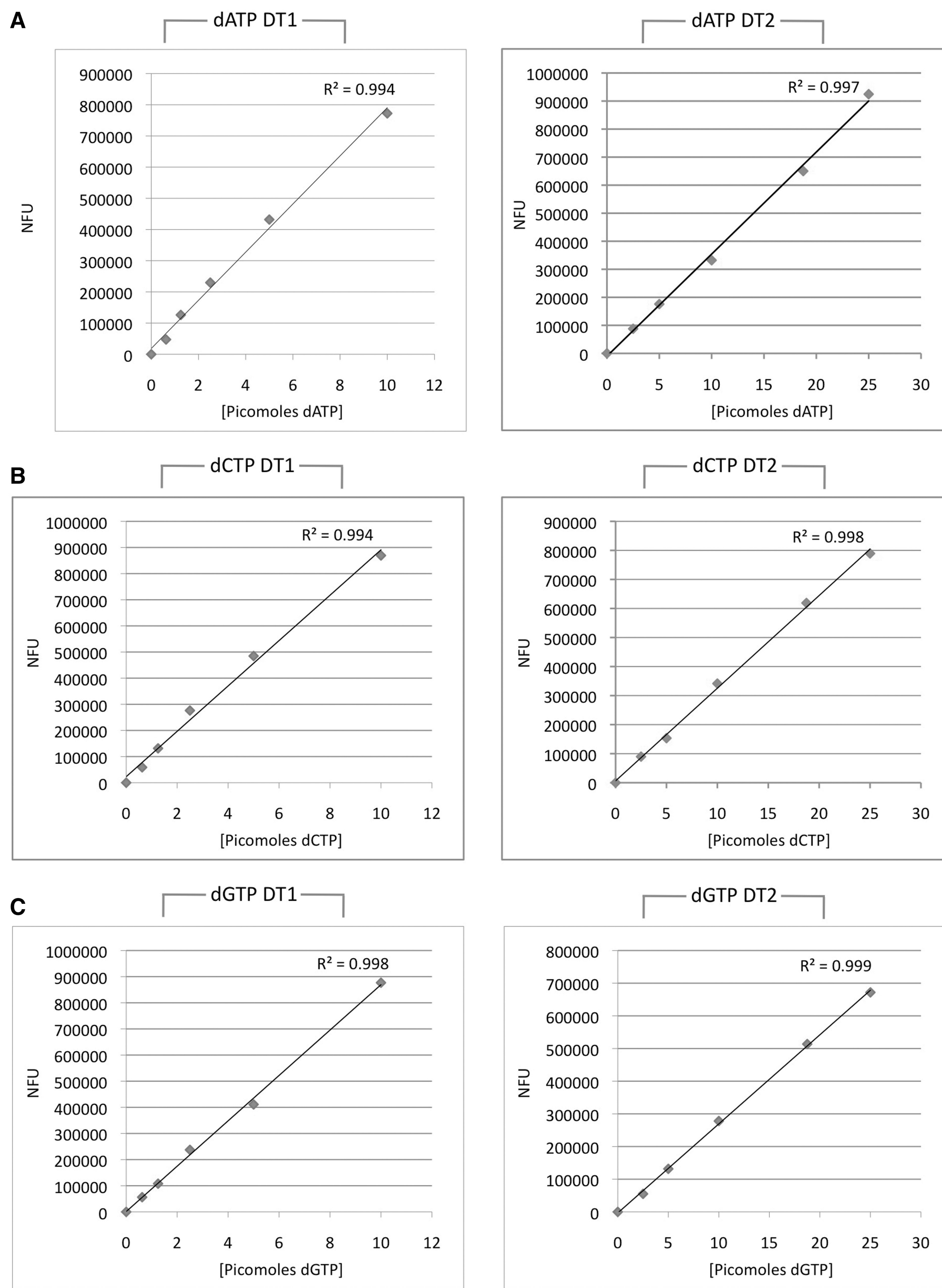

Figure 3. Validation of dATP, dCTP and dGTP detection templates. Calibration curves were generated for dNTPs using dNTP-specific templates (DT1 and DT2) and probes (Table 1) and were performed as described in 'Materials and Methods' section. In all cases, fluorescence values for blank reactions (limiting dNTP omitted) were subtracted to give NFU. All curves demonstrated $R^2 > 0.99$. (**A**) dATP, (**B**) dCTP, (**C**) dGTP. Left, DT1; Right, DT2.

Table 2. Assay performance results for each dNTP determined from DT1

dNTP	Regression coefficient	Accuracy[a] Low, High (%)	LOD (pmol)	LOQ (pmol)	Interassay CV (%)	Intrassay CV (%)	Recovery (%)
dTTP	>0.995	114.9 ± 6, 101.4 ± 5.3	0.46 ± 0.02	0.88 ± 0.15	8.5 ± 6.3	3.4 ± 0.85	103.6
dGTP	>0.998	98.9 ± 11.7, 95.6 ± 8.2	0.38 ± 0.1	0.81 ± 0.01	4.1 ± 3.57	4.58 ± 0.77	103.3
dATP	>0.994	100.5 ± 13.3, 101.6 ± 7.7	0.77 ± 0.5	1.3 ± 0.1	6.5 ± 2.6	3.64 ± 1.2	93.2
dCTP	>0.994	101.6 ± 14.7, 100.3 ± 5.26	0.36 ± 0.16	0.77 ± 0.2	9.6 ± 2.3	4.04 ± 0.63	96.4

[a]Accuracy was calculated in the low-mid and mid-high assay ranges.
LOD, limit of detection; LOQ, limit of quantification.

intracellular dNTPs. As the assay was performed on an Applied Biosystems 7500 Real-Time PCR System, the real-time monitoring and fluorescence data-capture over specified 5-min time intervals was evaluated to monitor the progress of the polymerase reaction to completion. The generation of fluorescence with DT1 was rapid and robust with an incubation time of as little as 5 min necessary for the generation of calibration curves with good signal:noise ratio and excellent linearity ($R^2 > 0.99$). All subsequent time-points analyzed also showed excellent linearity indicating that the polymerase reaction is progressing in a linear manner and that substrate saturation has not occurred and the polymerase is below V_{max}. However, while linear calibration curves could be generated for all dNTPs with a 5 min incubation period, the polymerase reaction was not driven to completion (maximum DNA synthesis) until approximately 15 min for dGTP, dCTP and dTTP and 20 min for dATP (Figure 4). However, for all dNTPs the NFUs obtained, and subsequent calibration curves generated at the later time-points of 20 and 25 min remained virtually identical to those obtained at 15 min with $R^2 > 0.99$ in every case (Figure 4). Considering the potential for the presence of inhibitory molecules in cell extracts that may reduce assay kinetics, these later time-points should facilitate the accurate quantification of dNTPs from cell extracts where the assay has reached completion.

Evaluating assay interference from ribonucleotides. It has been well established that DNA polymerases have imperfect dNTP selectivity both *in vitro* and *in vivo* (21,22). Therefore one potential source of interference in the detection of intracellular dNTPs is from endogenous rNTPs. Although kinetic studies have revealed that *Taq* polymerase in particular is reported to be highly efficient at discriminating between rNTP and dNTPs (7,19), the potential for rNTP misincorporation resulting in overestimation of dNTP recovery in the present assay was tested. We measured the recovery of 5 pmol of dNTP in the presence and absence of 100- and 1000-fold molar excess of the corresponding rNTP and followed the assay in 5-min intervals to completion. A 100-fold molar excess of rNTP did not result in any significant increase in the recovery of the corresponding dNTP. Similarly, a 1000-fold molar excess of rNTP did not induce any increases in dNTP recovery (Figure 5). Interestingly, while no evidence of significant rNTP misincorporation was evident, the presence of a 1000-fold molar excess of rNTPs demonstrated a mild inhibitory effect on assay kinetics. Specifically, the analysis of dGTP and dTTP recovery at the earlier time-points of 5 and 10 min indicated the presence of a weak competitive effect of a 1000-fold rNTP molar excess and reduced assay kinetics resulting in a reduced recovery of dGTP and dTTP calculated at those time points. However, the effect was transient and no evidence of rNTP-mediated assay inhibition (or activation) was observed for any of the dNTPs at the later time-points of 15 and 20 min upon assay completion.

Determination of intracellular dNTP pools. The current assay was used to analyze the nucleotide pool content of the human colon cancer cell line HCT116 in log-phase growth and following treatment with anti-neoplastic agents known to perturb dTTP biosynthesis. The fluoropyrimidines fluorodeoxyuridine (FUdR) and 5-fluorouracil (5-FU) and the anti-folate pemetrexed are anti-neoplastic chemotherapeutics that inhibits DNA synthesis through inhibition of thymidylate synthase (TS), resulting in depletion of dTMP an essential precursor to dTTP synthesis. Cells were incubated with vehicle or 1 μmol/l FUdR, 2.5 μmol/l pemetrexed, or 5 μmol/l 5-FU for 4 h and processed for analysis as described in 'Materials and Methods' section. All dNTPs were successfully detected and quantified from cell extracts during normal log-phase growth (Table 3). Treatment with all three chemotherapeutics depleted dTTP pools as expected. Specifically, 1 μmol/l FUdR depleted dTTP >4-fold from 20.4 pmol in vehicle-treated cells to 4.5 pmol per 10^6 cells while pemetrexed and 5-FU depleted dTTP down to 3.9 and 1.6 pmol per 10^6 cells, respectively. In addition, while dATP showed no significant change, depletion of dGTP from 4.7 to 0.43, 1.1 and 0.9 pmol per 10^6 cells following treatment with FUdR, pemetrexed and 5-FU, respectively was observed. Reductions in dCTP levels from 10.5 to 8 and 8.7 pmol per 10^6 was observed following treatment with FUdR and 5-FU respectively (Table 3).

Comparison to a validated LC–MS/MS assay for detecting dNTPs. Our enzymatic assay for quantifying cellular dNTPs was directly compared with results obtained from a previously validated LC–MS/MS-based assay performed by the Department of Clinical Pharmacy and Pharmaceutical Sciences at the University of Southern California (13). A known concentration of each dNTP was provided from which calibration curves were generated and the concentration of three biological

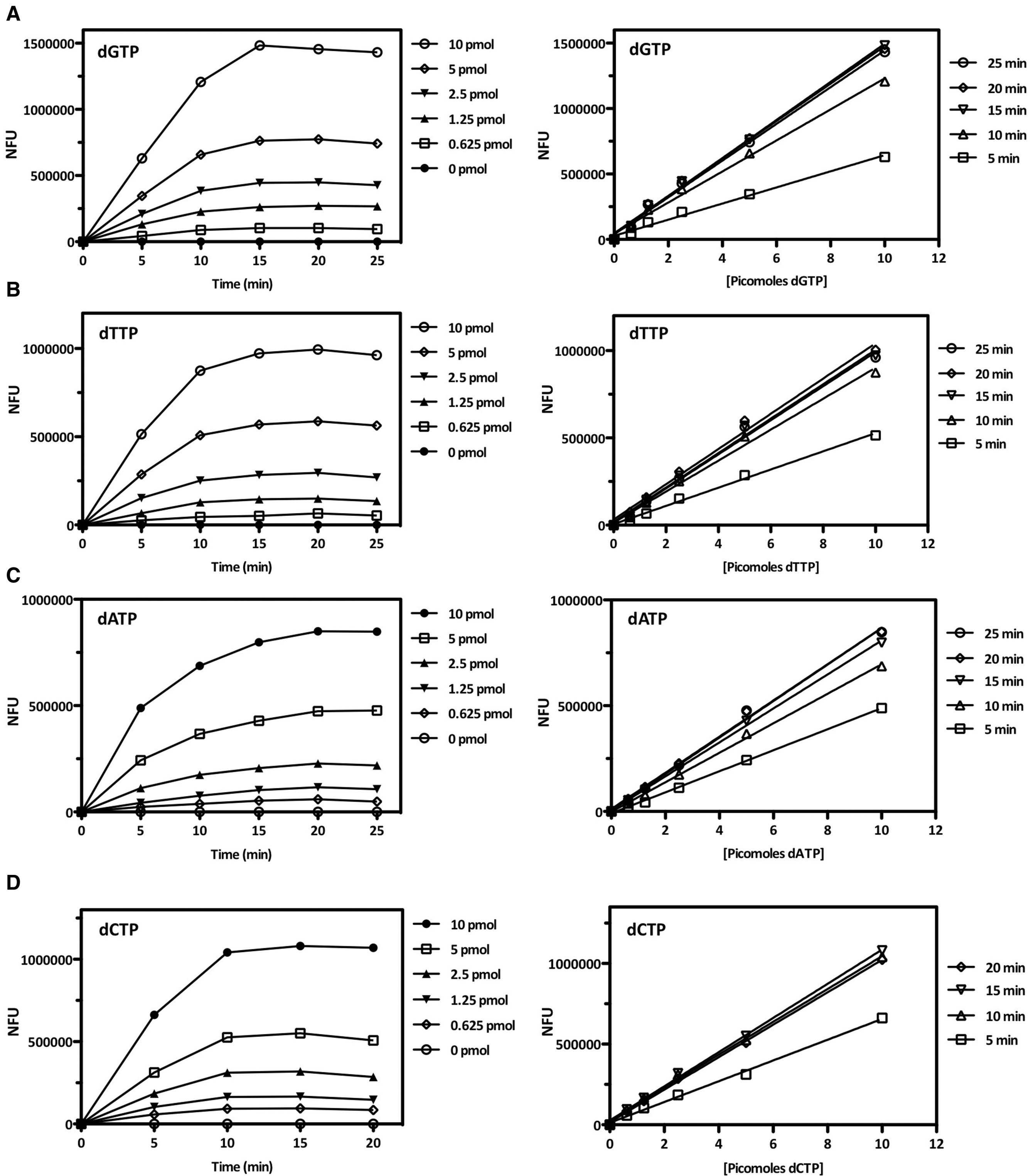

Figure 4. Time-course and calibration curve analysis of the polymerase reaction. Time course showing fluorescence generated by the dNTP-dependent *Taq* DNA polymerase-mediated hydrolysis of a dual-quenched fluorescent-labeled probe. Left. Known pmole quantities of dNTP were detected using DT1 and fluorescence was analyzed at 5-min intervals on board an Applied Biosystems 7500 Real-Time PCR System. Right. Calibration curves were generated and plotted from the NFU obtained at the specified time intervals and analyzed by linear regression. All calibration curves demonstrated R^2 of >0.99. (**A**) dGTP. (**B**) dTTP. (**C**) dATP. (**D**) dCTP. Additional details of the assay are described in the 'Materials and Methods' section.

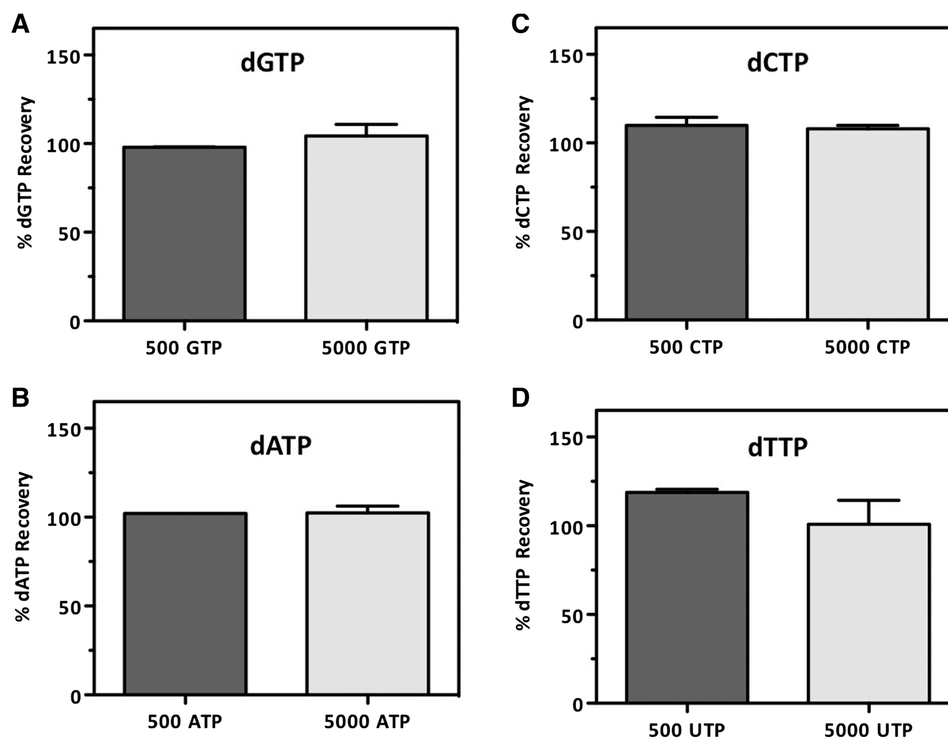

Figure 5. Effect of a 100- and 1000-fold molar rNTP excess on the recovery of dNTPs in the fluorescence-based assay with *Taq* polymerase. (**A**) The recovery of 5 pmol of dGTP was determined in the presence of both a 100- and 1000-fold molar excess of GTP at assay completion (20 min). The same analysis was applied to the recovery of (**B**) dATP, (**C**) dCTP and (**D**) dTTP in the presence of their corresponding rNTP. Bars represent the mean + SD of three individual analyses. The assay was performed as described in 'Materials and Methods' section. In all cases, fluorescence values for blank reactions (limiting dNTP omitted) were subtracted to give NFUs.

Table 3. Intracellular dNTP concentrations determined from HCT116 human colorectal cancer cells treated with fluoropyrimidine-based thymidylate synthase inhibitors FUdR and 5-FU and the anti-folate pemetrexed

dNTP	pmol × 10^6 cells[a]			
	Control	1 µmol/l FUdR	2.5 µmol/l PTX	5 µmol/l 5-FU
dATP	13.6 ± 1.17	14.5 ± 0.13	13.4 ± 1.68	13.9 ± 0.33
dCTP	10.5 ± 0.2	8 ± 0.04	11.1 ± 2.5	8.7 ± 0.24
dGTP	4.7 ± 0.8	0.43 ± 0.11	1.14 ± 0.3	0.88 ± 0.1
dTTP	20.4 ± 0.77	4.5 ± 0.71	3.93 ± 0.8	1.64 ± 0.16

[a]Values represent the mean ± SD determined from two independent isolations analyzed in duplicate. Cells were treated for 4 h with vehicle (sterile ddH_20 or DMSO) or with the specified concentrations of PTX; pemetrexed, FUdR; fluorodeoxyuridine, 5-FU; 5-fluorouracil.

extracts were determined by both assays in a blinded manner. The LC–MS/MS determination was performed as described in the 'Materials and Methods' section. A chromatograph illustrating the intensity measured in counts per second (cps) and retention time for each dN (dephosphorylated dNTPs) and internal standards is given in Supplementary Figure S1B. The results obtained for the unknowns by LC–MS/MS were directly compared to those determined in the current fluorescence-based polymerase assay and the difference between the two methodologies expressed as percent difference. Despite the differing methodologies, reagents and instrumentation involved, the assay results were in good agreement with mean ± SD percent differences of −11 ± 6% for dCTP, −14 ± 5.9% for dGTP, −5.2 ± 11.6% for dTTP and −4.5 ± 13.4% observed for dATP between the two assays.

Assay modification to detect dUTP. The nucleotide intermediate dUTP can be incorporated into newly synthesized DNA opposite dAMP in place of dTTP, therefore an additional application of this assay is the ability to measure dUTP. In applications measuring dTTP pool imbalance such as those that employ chemotherapeutics targeting thymidylate metabolism, intracellular dUTP pools can accumulate in parallel with acute dTTP depletion and provide inaccurate results in polymerase-based assays (23–26). Therefore, in order to accurately determine the concentrations of dTTP, the presence of any contributing dUTP needs to be accounted for. The presence of dUTP can be efficiently determined and accounted for by performing parallel reactions with and without a pre-incubation with the enzyme dUTPase which catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate and removes its availability for nascent strand incorporation. The dUTP pool concentration can thus be determined by subtracting the results of cell extracts treated with dUTPase from the untreated extracts. Importantly, the AmpliTaq Gold polymerase buffer II was conducive for

excellent dUTPase enzymatic activity and complete hydrolysis of 25 pmol of dUTP was observed with a 10 min incubation with 5 ng of dUTPase at 37°C prior to initiation of the normal assay program. The inclusion of dUTPase in the assay reaction mix did not interfere with assay performance as evidenced by parallel reactions measuring dTTP with and without dUTPase that demonstrated excellent linearity ($R^2 > 0.99$) and were virtually identical (Figure 6A). When dUTP was measured as the rate-limiting dNTP, excellent linearity was also recorded, however, partial digestion resulting in intermediate fluorescence was observed with 2.5 ng of dUTPase. Increasing the amount of enzyme to 5 ng of dUTPase per reaction completely hydrolyzed 25 pmol yielding no fluorescence signal above background (Figure 6B). This confirmed the specificity of the assay for the limiting dNTP (dUTP in this instance) and confirmed that the assay has the potential to be modified to accurately measure dUTP in addition to the canonical dNTPs.

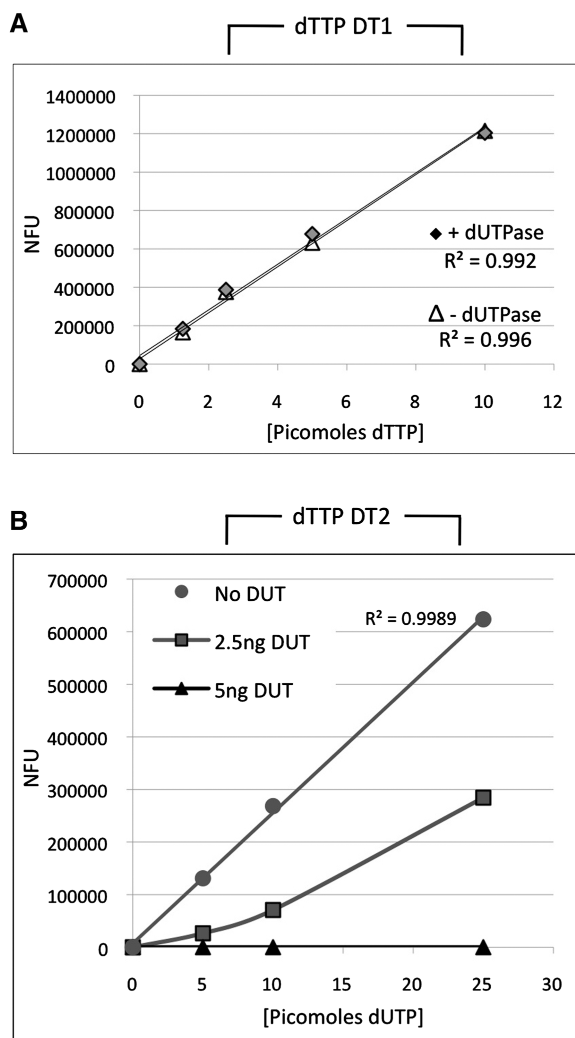

Figure 6. Detection of dUTP. The ability of the assay to detect dUTP and distinguish dTTP from dUTP in the presence and absence of the dUTP-hydrolyzing enzyme dUTPase was analyzed. (**A**) The effects of including recombinant human dUTPase (DUT) and a 5 min pre-incubation at 37°C were first analyzed. Inclusion of 5 ng of dUTPase had no significant impact on the assay performance and detection of dTTP ($R^2 > 0.99$). (**B**) dTTP was replaced with dUTP and the reaction performed in the absence of dUTPase and in the presence of 2.5 and 5 ng of recombinant human dUTPase. In the absence of dUTPase, dUTP detection was robust and yielded an excellent calibration curve ($R^2 > 0.99$). Five nanograms of dUTPase was sufficient to eliminate dUTP as the source of fluorescence in the assay, whereas 2.5 ng resulted in partial hydrolysis and intermediate fluorescence. The assay was performed as described in 'Materials and Methods' section. In all cases, fluorescence values for blank reactions (limiting dNTP omitted) were subtracted to give NFUs.

DISCUSSION

The development of fluorescence-based assays is continuing to improve the sensitivity, specificity and throughput of an increasing number of assays for the measurement of biological molecules and for bioimaging molecular processes. The current assay is a novel fluorescence-based approach utilizing fluorescence technology to provide a rapid, sensitive and cost-effective alternative to current methodologies that necessitate the incorporation of radioactivity and/or HPLC and MS instrumentation for the detection of dNTPs. This assay was successfully applied to the detection of dATP, dTTP, dGTP, dCTP and dUTP in *in vitro* assays and in the quantification of intracellular dNTP pools from human cancer cells.

The current assay was validated in line with current FDA guidelines for analytical assays and demonstrated excellent accuracy, sensitivity, reproducibility and recovery within accepted guidelines. The current assay has several inherent qualities that make it more attractive and efficient than radiolabeling or HPLC and LC–MS/MS methods that are still currently in use. One limitation with conventional template-based DNA polymerase assays is the limited linear range up to 10 pmol. To address this, we have designed and validated additional detection templates that allow detection of up to 60 pmol of dNTP at assay completion thereby significantly expanding the linear detection range. For example, in assays were maximum sensitivity is required such as dNTP detection from low numbers of prokaryotic and eukaryotic cells, DT1 which requires only a single limiting dNTP incorporation event for fluorescence generation, would provide the most sensitive detection of low picomole quantities of dNTP. However, if an *in vitro* enzymatic screening assay is being performed and extreme sensitivity is not required, DT6 which requires six limiting dNTP incorporation events to yield fluorescence, would provide a broader range of linear detection (~5–60 pmol at assay completion) and may be more suitable. It should be noted that while the LOD of the current assay ranges from 0.3 to 0.7 pmol depending on the dNTP, current radiolabeling methods report the ability to accurately measure dNTP quantities down to 0.1 pmol (7). However, the current assay has sufficient sensitivity for the majority of cell-based applications without the requirement for excessive or impractical cell numbers. Previous analyses of human intracellular dNTP concentrations report ranges of 2–80 pmol of dNTP per $10e^6$ (3,4,9,27,28), with microbiological applications

reporting approximate ranges of 5–50 pmol per 10^7 cells [*Saccharomyces cerevisiae* (29)]. However, the analysis of dNTP pools in cells not undergoing DNA replication, or the quantification of mitochondrial dNTP pools may represent a challenge for the current assay as these values may approach and exceed the limit of detection from an initial sample size of 10^6 cells. Such analyses would thus require a larger initial sample size to facilitate accurate quantification (30). It is also plausible that different probe designs and/or combinations of reporter and quencher molecules in the probe may facilitate an increase in sensitivity in the assay and this is currently being explored. Although the fluorophore detection capabilities of real-time PCR platforms vary, numerous commercially available fluorophore and quencher combinations are available to suit the requirements of most platforms and applications (14) and could be adapted to the FRET technology that this assay is based upon.

One additional advantage of the current assay over the conventional radiolabeled dNTP polymerase assays is that there is no interference in the current assay from the endogenous cellular dNTP corresponding to the radiolabeled dNTP substrate ([3H]dNTP) as previously reported (4). An additional issue with the radiolabeled assay is that the specific radioactivity of dNTPs in radioisotope experiments cannot be measured. The current assay circumvents this issue by avoiding the need for a radiolabeled dNTP and employing a fluorescence-labeled probe that demonstrates robust linearity. One key attribute of the current assay is the straightforward and uncomplicated assay set up. All reagents can be added and mixed at room temperature and the use of a 96-well-format real-time PCR instrument facilitates the simultaneous analysis of all dNTPs with the ability monitor assay kinetics in real-time by acquiring fluorescence readings at multiple user-defined time-points to follow progression of the polymerase reaction and ensure analysis occurs at the most appropriate interval without the need to irreversibly terminate the polymerase reaction and extract the sample for downstream detection. In addition, recent optimization of the radiolabeled DNA polymerase assay identified the potential for rNTP misincorporation for dCTP and dGTP and recommended that either *Taq* or Sequenase DNA polymerase be used for accurate analysis of dCTP and dGTP whereas analysis of dTTP and dATP could be performed with Klenow DNA polymerase. The current assay provides the ability to measure all four canonical cellular dNTPs on a single 96-well plate under identical assay detection conditions with the same DNA polymerase. In addition, consistent with previous observations, the use of *Taq* polymerase in this assay also appears to significantly limit the potential for dNTP overestimation as a result of rNTP misincorporation (7).

The requirement for specialized equipment is limited to a 96-well fluorescence plate reader or preferably a real-time PCR thermocycler, neither of which require a high degree of specialized training and are significantly less costly to purchase, operate and maintain than HPLC and LC–MS/MS-based instrumentation. In addition, the current assay is extremely rapid with the final detection step requiring $\sim$30 min. If extraction of intracellular nucleotides is required, the entire protocol can be completed in <3 h including extraction and concentration of dNTPs, plate setup and final detection. The small reaction volume, low enzyme and probe requirement per reaction also make this a relatively inexpensive assay. The current assay was also directly compared to a validated LC–MS/MS-based approach for detecting and quantifying dNTPs and despite the distinct contrast in techniques, the results obtained for unknown samples were in good agreement.

The assay was also modified to measure the nucleotide intermediate dUTP (which can be utilized as a substrate by DNA polymerase) with excellent results. By performing the reaction with dUTP in the presence and absence of the dUTP-hydrolyzing enzyme dUTPase, both the flexibility of the assay and the substrate specificity was confirmed. As expected, a short pre-incubation with sufficient dUTPase completely eliminated fluorescence compared to the no-enzyme control for dUTP but not dTTP confirming the specificity of the limiting dNTP (dUTP) as the source of fluorescence generation in the assay.

The determination of intracellular dNTP levels is of fundamental importance in understanding the underlying biology of a number of genetic diseases and in determining the mechanisms of action of a wide range of pharmacological agents designed to perturb dNTP metabolism and DNA replication in both prokaryotic and eukaryotic organisms. This assay therefore has important and broad application in research measuring dNTPs and/or the activity and inhibition of enzymes directly or indirectly involved in dNTP biosynthesis. The accurate quantification of dNTP pools in response to anti-cancer agents that target dNTP biosynthesis represents one important application and was the primary motivating factor that led to the development of this assay. We confirmed this application by successfully measuring perturbations in dTTP pools in human tumor cells as a result of inhibiting a key enzyme involved in dTTP biosynthesis. Treatment with FUdR, 5-FU and pemetrexed resulted in rapid perturbations in dNTP pools similar to those previously reported for both these agents. Specifically, measurable decreases in both dTTP and dGTP concentrations were observed (31–33). The intracellular dNTP concentrations obtained for tumor cells with the current assay were within 15–20% of previously published studies using similar models and conditions (3,10,11,25,34).

One critical attribute of this assay is its potential for adaptation to high-throughput applications (384-well and beyond), making it particularly amenable to high sample volumes or to the screening of pharmacological molecules that perturb dNTP metabolism either from cell-based assays or *in vitro* screening.

In summary, the fluorescence-based dNTP detection assay described in this manuscript represents a rapid, sensitive, reproducible and cost-effective alternative to current radiolabeling and HPLC and MS-based methodology to detect and quantify dNTPs.

ADDENDUM

Since online publication of their article, the authors have made some modifications to the method described in their article resulting in significantly improved performance. The new method supersedes the one described in the main text of the article. The new method has been uploaded as a Supplementary File and contains three new figures. The new method does not influence the validity of the results and conclusions of the article.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Michael R. Lieber (University of Southern California), Dr Joshua Morris (Azusa Pacific University) and Dr Mark A. Behlke (Integrated DNA Technologies, Inc.) for discussion which assisted in the development of this assay and Michael Sandoval from the University of Southern California DNA Core Facility for assistance in oligo synthesis and reagent acquisition.

FUNDING

National Institutes of Health (5 P30CA14089-27I); Margaret Early Trust. Funding for open access charge: Institutional Funds.

Conflict of interest statement. None declared.

REFERENCES

1. Kunz,B.A., Kohalmi,S.E., Kunkel,T.A., Mathews,C.K., McIntosh,E.M. and Reidy,J.A. (1994) International Commission for protection against environmental mutagens and carcinogens. Deoxyribonucleoside triphosphate levels: a critical factor in the maintenance of genetic stability. *Mutat. Res.*, **318**, 1–64.
2. Smid,K., Van Moorsel,C.J., Noordhuis,P., Voorn,D.A. and Peters,G.J. (2001) Interference of gemcitabine triphosphate with the measurements of deoxynucleotides using an optimized DNA polymerase elongation assay. *Int. J. Oncol.*, **19**, 157–162.
3. Horowitz,R.W., Zhang,H., Schwartz,E.L., Ladner,R.D. and Wadler,S. (1997) Measurement of deoxyuridine triphosphate and thymidine triphosphate in the extracts of thymidylate synthase-inhibited cells using a modified DNA polymerase assay. *Biochem. Pharmacol.*, **54**, 635–638.
4. Sherman,P.A. and Fyfe,J.A. (1989) Enzymatic assay for deoxyribonucleoside triphosphates using synthetic oligonucleotides as template primers. *Anal. Biochem.*, **180**, 222–226.
5. Skoog,L. (1970) An enzymatic method for the determination of dCTP and dGTP in picomole amounts. *Eur. J. Biochem.*, **17**, 202–208.
6. Roy,B., Beuneu,C., Roux,P., Buc,H., Lemaire,G. and Lepoivre,M. (1999) Simultaneous determination of pyrimidine or purine deoxyribonucleoside triphosphates using a polymerase assay. *Anal. Biochem.*, **269**, 403–409.
7. Ferraro,P., Franzolin,E., Pontarin,G., Reichard,P. and Bianchi,V. (2010) Quantitation of cellular deoxynucleoside triphosphates. *Nucleic Acids Res.*, **38**, e85.
8. Chi,J., Jayewardene,A., Stone,J., Gambertoglio,J.G. and Aweeka,F.T. (2001) A direct determination of thymidine triphosphate concentrations without dephosphorylation in peripheral blood mononuclear cells by LC/MS/MS. *J. Pharm. Biomed. Anal.*, **26**, 829–836.
9. Hennere,G., Becher,F., Pruvost,A., Goujard,C., Grassi,J. and Benech,H. (2003) Liquid chromatography-tandem mass spectrometry assays for intracellular deoxyribonucleotide triphosphate competitors of nucleoside antiretrovirals. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **789**, 273–281.
10. Chen,P., Liu,Z., Liu,S., Xie,Z., Aimiuwu,J., Pang,J., Klisovic,R., Blum,W., Grever,M.R., Marcucci,G. *et al.* (2009) A LC-MS/MS method for the analysis of intracellular nucleoside triphosphate levels. *Pharm. Res.*, **26**, 1504–1515.
11. Arezzo,F. (1987) Determination of ribonucleoside triphosphates and deoxyribonucleoside triphosphates in Novikoff hepatoma cells by high-performance liquid chromatography. *Anal. Biochem.*, **160**, 57–64.
12. Ladner,R.D., McNulty,D.E., Carr,S.A., Roberts,G.D. and Caradonna,S.J. (1996a) Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. *J. Biol. Chem.*, **271**, 7745–7751.
13. Goicoechea,M., Jain,S., Bi,L., Kemper,C., Daar,E.S., Diamond,C., Ha,B., Flaherty,J., Sun,S., Richman,D. *et al.* (2010) Abacavir and tenofovir disoproxil fumarate co-administration results in a nonadditive antiviral effect in HIV-1-infected patients. *AIDS*, **24**, 707–716.
14. Marras,S.A., Kramer,F.R. and Tyagi,S. (2002) Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. *Nucleic Acids Res.*, **30**, e122.
15. Marras,S.A. (2006) Selection of fluorophore and quencher pairs for fluorescent nucleic acid hybridization probes. *Methods Mol. Biol.*, **335**, 3–16.
16. Rudert,W.A., Braun,E.R., Faas,S.J., Menon,R., Jaquins-Gerstl,A. and Trucco,M. (1997) Double-labeled fluorescent probes for 5′ nuclease assays: purification and performance evaluation. *Biotechniques*, **22**, 1140–1145.
17. Johansson,M.K. (2006) Choosing reporter-quencher pairs for efficient quenching through formation of intramolecular dimers. *Methods Mol. Biol.*, **335**, 17–29.
18. Innis,M.A., Myambo,K.B., Gelfand,D.H. and Brow,M.A. (1988) DNA sequencing with Thermus aquaticus DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA. *Proc. Natl Acad. Sci. USA*, **85**, 9436–9440.
19. Patel,P.H. and Loeb,L.A. (2000) Multiple amino acid substitutions allow DNA polymerases to synthesize RNA. *J. Biol. Chem.*, **275**, 40266–40272.
20. Rudy,J.L. (1989) Differentiating between sensitivity and limit of detection. *Clin. Chem.*, **35**, 509.
21. Nick McElhinny,S.A., Kumar,D., Clark,A.B., Watt,D.L., Watts,B.E., Lundstrom,E.B., Johansson,E., Chabes,A. and Kunkel,T.A. (2010) Genome instability due to ribonucleotide incorporation into DNA. *Nat. Chem. Biol.*, **6**, 774–781.
22. Nick McElhinny,S.A., Watts,B.E., Kumar,D., Watt,D.L., Lundstrom,E.B., Burgers,P.M., Johansson,E., Chabes,A. and Kunkel,T.A. (2010) Abundant ribonucleotide incorporation into DNA by yeast replicative polymerases. *Proc. Natl Acad. Sci. USA*, **107**, 4949–4954.
23. Wilson,P.M., Fazzone,W., LaBonte,M.J., Lenz,H.J. and Ladner,R.D. (2009) Regulation of human dUTPase gene expression and p53-mediated transcriptional repression in response to oxaliplatin-induced DNA damage. *Nucleic Acids Res.*, **37**, 78–95.
24. Wilson,P.M., Fazzone,W., LaBonte,M.J., Deng,J., Neamati,N. and Ladner,R.D. (2008) Novel opportunities for thymidylate metabolism as a therapeutic target. *Mol. Cancer Ther.*, **7**, 3029–3037.
25. Koehler,S.E. and Ladner,R.D. (2004) Small interfering RNA-mediated suppression of dUTPase sensitizes cancer cell lines to thymidylate synthase inhibition. *Mol. Pharmacol.*, **66**, 620–626.
26. Webley,S.D., Hardcastle,A., Ladner,R.D., Jackman,A.L. and Aherne,G.W. (2000) Deoxyuridine triphosphatase (dUTPase) expression and sensitivity to the thymidylate synthase (TS) inhibitor ZD9331. *Brit. J. Cancer*, **83**, 792–799.
27. Huang,D., Zhang,Y. and Chen,X. (2003) Analysis of intracellular nucleoside triphosphate levels in normal and tumor cell lines by

high-performance liquid chromatography. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **784**, 101–109.
28. Bobin-Dubigeon,C., Amiand,M.B., Herrenknecht,C. and Bard,J.M. (2009) Development and validation of an improved liquid chromatography-mass spectrometry method for the determination of pemetrexed in human plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **877**, 2451–2456.
29. Tinkelenberg,B.A., Hansbury,M.J. and Ladner,R.D. (2002) dUTPase and uracil-DNA glycosylase are central modulators of antifolate toxicity in Saccharomyces cerevisiae. *Cancer Res.*, **62**, 4909–4915.
30. Ferraro,P., Pontarin,G., Crocco,L., Fabris,S., Reichard,P. and Bianchi,V. (2005) Mitochondrial deoxynucleotide pools in quiescent fibroblasts: a possible model for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *J. Biol. Chem.*, **280**, 24472–24480.
31. Yoshioka,A., Tanaka,S., Hiraoka,O., Koyama,Y., Hirota,Y., Ayusawa,D., Seno,T., Garrett,C. and Wataya,Y. (1987) Deoxyribonucleoside triphosphate imbalance. 5-Fluorodeoxyuridine-induced DNA double strand breaks in mouse FM3A cells and the mechanism of cell death. *J. Biol. Chem.*, **262**, 8235–8241.
32. Newman,E.M. and Santi,D.V. (1982) Metabolism and mechanism of action of 5-fluorodeoxycytidine. *Proc. Natl Acad. Sci. USA*, **79**, 6419–6423.
33. Chen,V.J., Bewley,J.R., Andis,S.L., Schultz,R.M., Iversen,P.W., Shih,C., Mendelsohn,L.G., Seitz,D.E. and Tonkinson,J.L. (1998) Preclinical cellular pharmacology of LY231514 (MTA): a comparison with methotrexate, LY309887 and raltitrexed for their effects on intracellular folate and nucleoside triphosphate pools in CCRF-CEM cells. *Br. J. Cancer*, **78(Suppl. 3)**, 27–34.
34. Decosterd,L.A., Cottin,E., Chen,X., Lejeune,F., Mirimanoff,R.O., Biollaz,J. and Coucke,P.A. (1999) Simultaneous determination of deoxyribonucleoside in the presence of ribonucleoside triphosphates in human carcinoma cells by high-performance liquid chromatography. *Anal. Biochem.*, **270**, 59–68.