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Fast and sensitive HPLC-MS/MS method for direct quantification of intracellular deoxyribonucleoside triphosphates from tissue and cells

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Abstract

Deoxyribonucleoside triphosphates (dNTPs) are used in DNA synthesis and repair. Even slight imbalances can have adverse biological effects. This study validates a fast and sensitive HPLC-MS/MS method for direct quantification of intracellular dNTPs from tissue. Equal volumes of methanol and water were used for nucleotide extraction from mouse heart and gastrocnemius muscle and isolated cardiomyocytes followed by centrifugation to remove particulates. The resulting supernatant was analyzed on a porous graphitic carbon chromatography column using an elution gradient of ammonium acetate in water and ammonium hydroxide in acetonitrile with a run time of just 10 minutes. Calibration curves of all dNTPs ranged from 62.5–2,500 femtomole injections and demonstrated excellent linearity ($r^2 > 0.99$). The within day and between day precision, as measured by the coefficient of variation (CV (%)), was <20% for all points, including the lower limit of quantification (LLOQ). The inter-day accuracy was within 12% of expected concentration for the LLOQ and within 7% for all other points on the calibration curve. The intra-day accuracy was within 22% for the LLOQ and within 11% for all points on the curve. Compared to existing methods, this study presents a faster and more sensitive method for dNTP quantification.

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Keywords

HPLC-MS/MS; dNTP pools; ATP

1. Introduction

Deoxyribonucleoside triphosphates (dNTPs) are required for nuclear and mitochondrial DNA replication and repair. dNTP pools are tightly controlled and an imbalance of dNTPs is observed in a variety of disease conditions such as cancer and mitochondrial diseases[1,2]. Both imbalanced dNTP pools as well as irregularly large amounts of all dNTPs have been shown to be associated with increased errors in nucleotide selectivity combined with inefficient proofreading during DNA replication and eventual oncogenesis, likely attributed to this buildup of mutations[3,4]. During the cell cycle, dNTP pools are augmented for DNA synthesis and are generally elevated during proliferation [1,5]. However, in non-dividing cells, the total dNTP pool is about 1–10 pmol/million cells and less than 1 pmol in the mitochondria[6]. Due to the exceedingly low levels combined with the mutagenic effect of even slight perturbations it is critical to have sensitive and accurate methods for measuring intracellular dNTPs.

Previously used methods for nucleotide and deoxynucleotide quantification have included HPLC-UV [7–9], the use of ion-pairing reagents for improved chromatographic separation [10–12] and enzymatic assays [13–15]. HPLC-UV has relatively low sensitivity and therefore often requires a larger sample volume than available for many studies. The use of ion-pairing reagents can require a long run time with additional washes needed to completely remove the reagent from the column, also reducing reproducibility [16]. Additionally, ion-pairing reagents can interfere with ionization of the analytes of interest and result in a reduced signal[17]. Assays utilizing radioactivity and fluorescence are indirect measurements and lack specificity, and are therefore not well suited for measuring several dNTPs concurrently. Finally, while methods exist for quantification of dNTPs from isolated cells [18–20], there are no existing HPLC-MS/MS methods for quantification from tissue. Many published assays use either malignant or replicating cell lines, which have elevated dNTP levels compared to non-replicating cells[14,19]. The presence of connective tissues can act to dilute the sample when compared with isolated cells and therefore demands an even more sensitive assay. Therefore, we developed a robust, sensitive, and specific assay for our analytical needs applicable to tissue and isolated cells.

This study describes a fast and reproducible nucleotide extraction method from both tissue and cells for injection onto a HPLC-MS/MS system with a significantly faster run time than current methods without the use of ion-pairing reagents. The assay successfully separates and quantifies down to the femtomole level for dCTP, dTTP, dATP, and dGTP.

2. Experimental

2.1. Materials

The dNTP standards, 2'-deoxyadenosine 5'-triphosphate (dATP), 2'-deoxyguanosine 5'-triphosphate (dGTP), 2'-deoxycytidine 5'-triphosphate (dCTP), and 2'-deoxythymidine 5'-triphosphate (dTTP), were purchased as a mixture from GenScript (Piscataway, NJ, USA). ATP was purchased as a disodium salt from Sigma-Aldrich, reconstituted in water, and sodium hydroxide was used to adjust the pH to 7 to make it more compatible with our mobile phase. To account for any degradation and confirm the concentration, the concentration was measured again using a DU® 800 spectrophotometer purchased from Beckman Coulter (Fullerton, CA, USA). The internal standard used was C-13 labeled dATP, purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used in mobile phase, sample manager wash, and sample preparation was obtained from a Nanopure™ Analytical Ultrapure Water System produced by Thermo Fisher Scientific (Marietta, OH, USA). Methanol, acetonitrile, and ammonium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium acetate purchased from Avantor (Center Valley, PA, USA). All solvents and buffers were purchased as MS grade or highest purity available.

2.2. Sample preparation and extraction

Tissue or cells were kept frozen at -80°C and stored in liquid nitrogen until immediately prior to use. Tissue samples were removed from liquid nitrogen then ground into a powder using mortar and pestle while kept cold using liquid nitrogen. Depending on the tissue type, 5–30mg of tissue was taken from the powder and massed in a 1.5mL Eppendorf tube. Adjusting to the mass, a minimum of 3µl methanol per mg tissue was added to the sample followed by 1 minute of vortexing. An equal amount of deionized water was then added and the Eppendorf tube was vortexed for an additional 3 minutes. Both the methanol and water were kept at 4°C on ice during use. The sample was sonicated in an ice bath using a Branson B-220 ultrasonic cleaner for a total of 90 seconds. Subsequently, the sample was centrifuged at 4°C for 20 minutes at 13,000×g and the supernatant was stored in a new Eppendorf tube at -20°C until ready for injection on the HPLC-MS/MS system.

This protocol can also be used for isolated cardiomyocytes. Cell isolation was done as described previously [21]. Briefly, hearts were surgically removed, cannulated, and perfused. Cardiomyocytes were dissociated via perfusion with enzymatic digestion buffer and gentle pipetting of the cell suspension. The digestion buffer was washed out and the suspension was briefly centrifuged. Beginning with the pelleted cells, the protocol is identical for isolated cell samples but pulverization was not necessary.

The study was designed to compare across like matrices and therefore an internal standard for extraction was not used. When ready to inject, internal standard (labeled C-13 dATP) was added for a final concentration of 1µM C-13 dATP to account for instrument variation.

2.3. HPLC-MS/MS

A Waters Xevo-TQ-S mass spectrometer coupled with a Waters Acuity I-Class HPLC was used for the analysis (Milford, MA, USA). Chromatographic separation was achieved

using a Hypercarb (2.1mm × 50mm, 3μm) column coupled with a Hypercarb guard column (2.1mm × 10mm, 3μm) purchased from Thermo Fisher Scientific. The chromatography was performed at room temperature and 25μl injections were made using a ballistic gradient as follows: 0.1M ammonium acetate in water pH to 9.5 using ammonium hydroxide (solvent A) and 0.1% ammonium hydroxide in acetonitrile (solvent B). The flow rate for analysis was 0.3ml/min. Starting conditions of 100% solvent A were used and held for 1 minute, the % of Solvent B increased to 70% over the next 5 minutes, then further increased to 95% over the next 1 minute. The system was brought back to starting condition in 0.1 minute and allowed to re-equilibrate for 2 minutes for a total run time of 10 minutes. Under these conditions retention times were 3.62, 4.28, 5.10 and 5.14 minutes for dCTP, dTTP, dGTP and dATP, respectively. Analytes were monitored using negative mode via an electrospray ionization source (ESI) acquiring MS-MS ions. Mass Spectrometer parameters were as follows: negative mode, ion spray voltage: 2kV, source temperature: 150°C, desolvation temperature: 400°C, cone gas flow: 150 L/hr, desolvation gas flow: 800 L/hr, and collision gas: 0.15 mL/min, and cone voltage and collision energy were 58V and 24, respectively for all analytes. Multiple reaction monitoring (MRM) transitions were m/z 466.0>158.9, 481.0>158.9, 506.1>158.9, and 490.1>158.9 for dCTP, dTTP, dGTP, and dATP, respectively. Water's MassLynx 4.1 ® was used to acquire data and Water's QuanLynx software was used to process the data.

Initial analysis of biological samples showed a large peak at a retention time very close to that of dGTP. Upon further investigation, it was confirmed the peak was ATP. Optimizations of our chromatographic conditions were able to fully resolve these peaks and ATP was incorporated into our standard solutions to create a calibration curve for ATP at biologically relevant levels.

2.4. Preparation of standard solutions

External standard solutions were prepared from a 10mM stock solution of dNTP mix and a 100mM ATP stock solution, stored at -20°C, by dilution with 1:1 MeOH: H₂O in order to be consistent with the tissue sample preparation. Each calibration standard included all four dNTPs as well as ATP. The calibration standards were adjusted to accommodate the biological samples within their linear range. Calibration standards were prepared at dNTP concentrations of 62.5, 125, 250, 625, and 2500 femtomoles (fmol) while the ATP was in the same solutions at concentrations of 1.25, 2.5, 5, 12.5, and 50 picomoles (pmol). The reason for higher concentration of ATP is to account for the magnitude of difference of dNTP versus ATP concentration in biological samples. Linearity of the calibration curves was assessed and considered acceptable with a regression coefficient >0.95. Separate aliquots of stock solutions were used to prepare the quality control samples (QCs). The QCs were also diluted using 1:1 MeOH: H₂O and were made at concentrations of 250, 625, and 2500 fmol. Internal standard was then added into standards and QCs at a final concentration of 1 pmol of labeled C-13 dATP in all standards.

2.5. Method Validation

Intra and inter-day precision and accuracy were determined by calculating the concentrations using the linear equations obtained from the calibration curves and

comparing those values to the nominal concentrations. Analysis producing concentrations that deviated <25% from nominal for the lower limit of quantification (LLOQ) and <15% for all other points were considered satisfactory for accuracy. Precision was measured using the coefficient of variation (CV(%)) and the acceptance standard was <25% for the lower limit of quantification and <15% for all other points.

To test the stability of the compounds, the QCs were measured at day 0 then stored at -20°C for 7 days and re-measured. Separately, 10 fold dilutions were made of samples containing 12.5 μmole dNTPs in order to validate dilution as a reliable method to adjust concentrations to be within our linear range if the need arises.

2.6. Method Application

This method was used to quantify dNTP content in mouse heart and skeletal muscle. Furthermore, the method was implemented with isolated cardiomyocytes. Nucleotide extraction occurred one day prior to the run and the samples were stored at -20°C overnight.

3. Results and discussion

3.1. Sample preparation and extraction

The total solvent volume was 150 μL per 15–30mg of tissue, depending on the tissue type. For heart tissue, ~30mg was needed in order to consistently detect levels above our lower limit of quantification (LLOQ). For other matrices that demonstrated higher overall dNTP content, such as skeletal muscle, ~15mg was sufficient for detection in our linear range. The final injection volume was 25 μL . No degradation of chromatographic separation was noticed due to the large injection volume. The relatively small mass of tissue needed allows this assay to be implemented with various tissues from a range of species. Notably, the above-mentioned tissue masses are attainable from mice, an exceedingly common model used in biomedical research.

3.2. HPLC & Mass Spectrometry

Chromatographic separation was achieved with a Thermo Hypercarb column (2.1 $\times$ 50mm, 3 μm) using a mobile phase of 0.1M ammonium acetate in water pH to 9.5 using ammonium hydroxide and 0.1% ammonium hydroxide in acetonitrile. The gradient can be found in the LC-MS/MS in section 2.3. Figure 1 shows a representative chromatogram of the lowest concentration on the calibration curve ([dNTP]=62.5 fmol and [ATP]=1.25 pmol).

Due to the hydrophilic nature of dNTPs, it was necessary that the column used for the liquid chromatography show high selectivity for polar compounds, as well as robustness to maintain peak shape even when equilibrated to 100% aqueous. By increasing the pH of the mobile phase, we were not only able to achieve better separation using NH_4OH , but also increase ion response for analytes in negative mode. Several columns have been used for nucleotide separation, such as HypercarbTM (Thermo Scientific) and SupelcogelTM (Sigma-Aldrich) [19,25]. Of these two columns, Hypercarb column demonstrated excellent separation capabilities of structurally similar compounds and comparatively longer retention times of polar compounds, making it an excellent choice for the chromatography in this

protocol[26]. Commonly used addition for separation and retention of polar analytes are the use of ion pairing reagents such as dimethylhexylamine [11], dibutylamine [12] and tetrabutylammonium[9]. Both effect the intermolecular interaction properties of the mobile and stationary phases and hence the retention of the analytes. By allowing greater interaction with the column packing material, better separation can be achieved between similar analytes species in an analytical run. This however comes at a cost in ionization of analytes when run in negative mode [27]. To avoid these effects we explored the ability to use a graphite column to achieve the desired separation.

It is well known that due to similarity between dNTPs, there is cross talk between the monitoring ion channels [28,29]. During the initial optimization of the chromatography protocol, the standards were prepared as a mixture of the four nucleotides, dATP, dGTP, dCTP, and dTTP, as well as ATP. We observed cross talk, peaks at incorrect retention times in mass channels that were not corresponding to the analyte of interest. This cross talk was most noted by ATP having a peak matching the retention time in the ATP channel however showing up in the dGTP channel as seen in Figure 1. This phenomenon has been reported by other groups [10,30,28]. Subsequently, the dNTPs were run as pure standards with adjustments to the chromatography to provide separation and to minimize cross talk. Once we determined which compounds would interfere with each other, a series of injections of the mix as well as individual standards were made by changing the mobile phase buffers, gradients, and pH of the eluting solvent. We focused on achieved baseline separation of the ATP and dGTP peak, for this proved to be the biggest obstacle due to the drastic dynamic difference in concentration within the biological samples. Using a pH lower than 7.0, minimal separation as well as retention was achieved on the Hypercarb column. By increasing the pH of the aqueous phase greater separation was achieved and we noted a 3x improvement in ion response when 0.1% ammonium hydroxide was added to the organic mobile phase. Ensuring that the nucleotides were not co-eluting allowed us to have minor cross talk between channels without confounding the determination of concentration of each of our analytes of interest. Additional peaks were noted during the analysis, they however did not interfere with any of our peaks of interest and therefore were not further investigated. The applicability of this separation technique is well suited for any LC-MS analysis. The larger packing material size of avoids the need for Ultra-high pressure liquid chromatographic, while the absence of ion pairing reagents allow for drastic improvement in ionization which has in turn provided superior limits of detection over other published methods [10,20].

The m/z transitions to monitor were chosen according to the predominant resulting daughter ion present in the collision activated dissociation (CAD) spectra at collision energy of 35V. Figure 3 shows the CAD for dATP and which fragments correspond to major peaks seen. The largest resulting daughter ion, m/z 158.9, is the transition used in this assay. Similar spectra were used to determine the largest transitions used for the other dNTPs.

3.3. Method validation

The limit of detection (LOD) was 6.25 fmol for dGTP, dCTP, and dTTP, and 3.125 fmol for dATP. The LOD was defined as the lowest concentration at which peaks were detected with a signal to noise ratio >3:1.

Linearity, precision, and accuracy data obtained from the calibration curves is summarized for intra and inter-day in Tables 1 and 2, respectively. All calibration curves demonstrated excellent linearity. The regression coefficient for all dNTPs was >0.99. Calibration curve for each of the nucleotides consisted of five points ranging from 62.5 to 2500 fmol as seen in Figure 4. The range of concentrations were chosen to encompass the range of our biological samples. With this method, excellent linearity was achieved over 2 magnitudes of concentration for all of the dNTPs. By increasing the dynamic range of the curve, we can include larger concentrations of each analyte, however, even with great linearity it decreases our accuracy at the lower end of our analytical standards, and therefore the dynamic range should be evaluated for your analysis to make sure that proper accuracy is achieved.

Intra and inter-day precision, as measured by the coefficient of variation (CV (%)), was <10% for all points and less than 20% for the two lowest points on the curve, which includes the LLOQ. The inter-day accuracy was within 12% of expected concentration for the LLOQ and within 7% for all other points on the calibration curve. The intra-day accuracy was within 22% for the LLOQ and within 11% of all other points on the curve.

Low (250 fmol), medium (625 fmol), and high (2500 fmol) QCs were tested and the accuracy and precision is summarized in Table 3. The low QCs all demonstrated CV values <25% and the medium and high QCs had CV values <20%. All QC values demonstrated accuracy within 12%.

Stability evaluation showed a slight decrease in signal after storing at -20°C for one week, with a more marked decrease in the low and medium (Table 4). The low and medium QC deviated in the range of 20% below nominal while the high QC <15% for all dNTPs. This decrease in dNTP concentration is likely due to degradation and loss of phosphate groups while in storage. The slight decrease seen shows samples can be stored for a short period of time at -20°C prior to analysis without major adverse effects on the results. Analysis of the 10 fold dilutions of the high QC revealed concentrations that were within 0.7% of the expected 10% recovery for all dNTPs, as shown in Table 5. The average accuracy was 98, 98, 94, and 98% for dATP, dGTP, dCTP, and dTTP, respectively. Therefore, the sample integrity was not compromised by dilution and is a valid method for adjusting samples to fall within the linear range of the established calibration curves.

3.4. Method application

Table 6 summarizes the dNTP content in two different mouse tissues using this extraction procedure. The concentration from each sample was calculated using a calibration curve run on the same day. Total amount of dNTP was then determined by adjusting for the total volume of solvent used to prepare the sample compared to the amount injected into the HPLC-MS/MS and then normalized using the mass of the tissue prior to extraction. dNTP content is reported in pmol/mg tissue. Our results show dNTP content in mouse

gastrocnemius muscle ranges from 0.179–0.340 pmol/mg tissue, comparable to values measured by Ylikallio et al., at a range of 0.121–0.253 pmol/mg tissue, using an enzymatic assay. Comparison of standard deviations from the dNTP measurements to those found by Ylikallio et al. show similar variability between samples with the exception of dATP where our assay demonstrates a variability that is greater than 10 fold lower. The magnitude of difference in variation is likely due to the non-specific nature of enzymatic assays for quantification of similar compounds. Figures 2A and 2B show a representative chromatogram from a mouse gastrocnemius muscle sample and adult mouse isolated cardiomyocytes, respectively. Our method for extraction and analysis can be applied to tissue for different matrices as well as isolated cells. We expect this method to be used to study changes in dNTP levels during development, and disease conditions such as cancer and mitochondrial disease. Our group has a unique interest in measuring dNTP as we have a novel method for treating cardiac dysfunction by altering dNTP and in particular dATP levels in cardiomyocytes. We have previously shown that small increases in dATP levels increases cardiac force and rate of force development by altering myosin cycling [31,32]. Our novel gene therapy method increases cardiac intracellular dATP levels by cardiac-specific overexpression of ribonucleotide reductase, the rate-limiting step in the de novo dNTP synthesis pathway, and is shown to increase contractility in rodents and pigs with cardiac dysfunction[32,33].

4. Conclusion

This assay implements a highly sensitive HPLC-MS/MS method to quantify endogenous dNTP content in various tissues. By implementing chromatographic buffers that exclude ion pairing reagents and the use of ammonium hydroxide in the organic solvent our LLOQ in this study was 62.5 fmols for all four dNTPs. Our reported LLOQ is at least 4 fold more sensitive than the lowest published value of 0.25 pmol [20]. Other reported published LLOQ range from 0.3 to 10 pmol [9,19,29], which is significantly less sensitive than our method reported here. This increase in sensitivity and detection will have profound impact on our ability to measure low level concentrations in tissue samples where analysis was previously impossible. The method uses a relatively small injection volume of 25 μ l, and has a much shorter run time of only 10 minutes compared to existing assays that range from 26 minutes to 160 minutes [7,10,34]. We have demonstrated the samples can be successfully diluted, which can be useful in measuring samples with higher concentration than the upper limit of our calibration curve. Additionally, this method was able to chromatographically separate ATP and dGTP, whereas previous methods were unable to achieve separation [10,19,28,35], had to use run times of up to 1 hour and switch ion modes [20], or utilize ion-pairing reagents [36] for successful separation. In summary, the assay is a fast, reproducible, and robust method that efficiently extracts nucleotide from small tissue samples and quantifies four intracellular deoxynucleosides triphosphates simultaneously. As demonstrated, this is well suited for our analytical needs.

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Highlights

- HPLC-MS/MS method for dNTP quantification from mouse tissue and isolated cells.
- Avoids ion-pairing reagents and achieves shorter run time.
- Successful chromatographic separation of dGTP from ATP.
- Improved sensitivity allows measurement at femtomole level

Figure 1. Representative chromatogram of dNTP levels at the LLOQ

Ion chromatograms showing relative abundance for dGTP, ATP, dATP, labeled C-13 dATP, dTTP, and dCTP upon injection of 62.5 fmol dNTPs, 1,250 fmol ATP, and 2.5 pmol of labeled C-13 dATP.

Figure 2. Representative chromatogram of dNTP levels in mouse skeletal muscle and isolated cardiomyocytes demonstrating ability to detect all four dNTPs in different matrices
 A. Ion chromatograms showing relative abundance over time for dGTP, ATP, dATP, labeled C-13 dATP, dTTP, and dCTP upon injection of nucleotides extracted from mouse gastrocnemius muscle as described in section 2.2. *Sample preparation and extraction*. B. Ion chromatograms showing relative abundance over time for dGTP, ATP, dATP, labeled C-13 dATP, dTTP, and dCTP upon injection of nucleotides extracted from mouse isolated cardiomyocytes as described in section 2.2. *Sample preparation and extraction*.

Figure 3. Daughter scan of dATP

Collision activated spectra following injection of 25 pmole dATP at collision energy of 35 V. Insert shows the molecular structure of dATP with structure and corresponding molecular weight associated with the major fragments.

Figure 4. Standard curve for all four dNTPs

Peak area ratio is plotted against dNTP concentration (A–D). Each point represents mean and standard deviation from the interday data. Linear regression is plotted with corresponding R^2 value for each curve.

Table 1

Accuracy and precision, and linearity of inter-day calibration standards (n=3)

Calibration standards (fmol)		62.5	125	250	625	2500	slope	r ²	y-intercept
dATP									
	Mean ± SD	65.9 ± 11.9	117.1 ± 21.7	254.0 ± 9.4	625.7 ± 29.7	2499.7 ± 7.0	6.54E-04 ± 1.12E-04	1.0000 ± 0.0001	-0.0018 ± 0.0093
	CV (%)	18.1%	19.6%	4.7%	5.7%	0.3%			
dGTP	Accuracy (%)	105%	93%	101%	100%	100%			
	Mean ± SD	70.3 ± 12.3	126.8 ± 8.2	248.1 ± 14.4	614.9 ± 23.4	2502.4 ± 4.3	2.47E-04 ± 4.18E-05	1.0000 ± 0.0002	-0.0001 ± 0.0068
	CV (%)	17.5%	6.4%	5.8%	3.8%	0.2%			
	Accuracy (%)	112%	101%	99%	98%	100%			
dCTP									
	Mean ± SD	62.5 ± 8.8	119.9 ± 11.9	248.8 ± 6.7	632.8 ± 32.2	2498.4 ± 6.7	5.37E-04 ± 1.32E-04	1.0000 ± 0.0002	-0.0042 ± 0.0122
	CV (%)	14.1%	9.9%	2.7%	5.1%	0.3%			
	Accuracy (%)	100%	96%	100%	101%	100%			
dTTP									
	Mean ± SD	67.1 ± 13.2	122.9 ± 9.6	242.5 ± 10.5	630.8 ± 33.4	2499.3 ± 6.9	5.59E-04 ± 1.11E-04	1.0000 ± 0.0003	-0.0052 ± 0.0116
	CV (%)	19.7%	7.8%	4.3%	5.3%	0.3%			
	Accuracy (%)	107%	98%	97%	101%	100%			

CV: Coefficient of Variance

Table 2

Accuracy, precision, and linearity of intra-day calibration standards (n=3)

Calibration standards (fmol)		62.5	125	250	625	2500	slope	r ²	y-intercept
dATP									
	Mean ± SD	67.2 ± 13.3	126.0 ± 25.6	259.3 ± 7.5	606.5 ± 49.8	2503.5 ± 10.7	5.46E-04 ± 1.32E-05	0.9999 ± 0.0009	-0.0085 ± 0.009
	CV (%)	19.8%	20.3%	2.9%	8.2%	0.4%			
dGTP	Accuracy (%)	107%	101%	104%	97%	100%			
	Mean ± SD	60.8 ± 15.0	133.6 ± 6.5	239.6 ± 22.1	628.8 ± 36.4	2499.7 ± 6.8	1.91E-04 ± 2.19E-05	1.0000 ± 0.0002	-0.0033 ± 0.0123
	CV (%)	24.7%	4.8%	9.2%	5.8%	0.3%			
	Accuracy (%)	97%	107%	96%	101%	100%			
dCTP									
	Mean ± SD	62.3 ± 11.7	105.8 ± 5.7	258.9 ± 24.2	638.9 ± 11.6	2496.6 ± 1.0	4.31E-04 ± 2.71E-05	1.0000 ± 0.0002	-0.0022 ± 0.0122
	CV (%)	18.8%	5.3%	9.3%	1.8%	0.1%			
	Accuracy (%)	111%	89%	103%	100%	96%			
dTTP									
	Mean ± SD	76.4 ± 1.4	123.4 ± 21.4	272.2 ± 17.4	582.3 ± 35.7	2508.2 ± 7.0	4.75E-04 ± 2.80E-05	0.9999 ± 0.0009	-0.0113 ± 0.0047
	CV (%)	1.9%	17.3%	6.4%	6.1%	0.3%			
	Accuracy (%)	122%	99%	109%	93%	100%			

Table 3

Accuracy and precision of QC samples (n=3)

Nucleotide Content (fmol)				
		250	625	2500
dATP				
	Mean ± SD	236.5 ± 53.5	573.2 ± 27.1	2376.4 ± 138.6
	CV (%)	23.0%	5.0%	6.0%
	Accuracy (%)	95%	92%	95%
dGTP				
	Mean ± SD	247.1 ± 59.3	530.4 ± 34.4	2596.4 ± 338.8
	CV (%)	24.0%	6.0%	13.0%
	Accuracy (%)	99%	85%	104%
dCTP				
	Mean ± SD	219.9 ± 60.5	530.4 ± 96.4	2465.4 ± 124.4
	CV (%)	28.0%	15.0%	5.0%
	Accuracy (%)	88%	104%	99%
dTTP				
	Mean ± SD	230.6 ± 54.2	571.5 ± 115.7	2466.5 ± 116.4
	CV (%)	23.0%	20.0%	5.0%
	Accuracy (%)	92%	91%	99%

Table 4Stability evaluation of samples stored at -20°C for one week (n=3)

Nucleotide Content (pmol)				
		250	625	2500
dATP				
	Mean $\pm$ SD	190.8 $\pm$ 23.6	466.5 $\pm$ 65.2	2154.4 $\pm$ 252.8
	CV (%)	12.4%	14.0%	12.0%
	Accuracy (%)	76%	74.6%	86.2%
dGTP				
	Mean $\pm$ SD	199.5 $\pm$ 28.0	440.5 $\pm$ 31.4	2045.4 $\pm$ 288.8
	CV (%)	14.0%	7.0%	14.0%
	Accuracy (%)	79%	70.5%	81.8%
dCTP				
	Mean $\pm$ SD	199.6 $\pm$ 28.2	448.0 $\pm$ 103.6	2168.0 $\pm$ 198.1
	CV (%)	14.1%	23.0%	9.0%
	Accuracy (%)	80%	71.7%	86.7%
dTTP				
	Mean $\pm$ SD	219.7 $\pm$ 17.6	457.6 $\pm$ 39.7	2156.9 $\pm$ 307.3
	CV (%)	8.0%	9.0%	14.0%
	Accuracy (%)	88%	73.2%	86.3%

Table 5

Dilution (10-fold) Evaluations (n=4)

dATP				
	Recovery (%)	9.8%	±	0.00
	Accuracy (%)	97.7%		
dGTP				
	Recovery (%)	9.9%	±	0.01
	Accuracy (%)	98.5%		
dCTP				
	Recovery (%)	9.3%	±	0.01
	Accuracy (%)	94.4%		
dTTP				
	Recovery (%)	10.0%	±	0.00
	Accuracy (%)	98.4%		

Table 6

dNTP content in mouse tissue (n=3)

Tissue		Mean $\pm$ standard deviation (pmol/mg tissue)
Gastrocnemius muscle		
	dATP	0.178 $\pm$ 0.025
	dGTP	0.260 $\pm$ 0.036
	dCTP	0.228 $\pm$ 0.067
	dTTP	0.344 $\pm$ 0.026
Heart		
	dATP	0.021 $\pm$ 0.007
	dGTP	1.019 $\pm$ 0.366
	dCTP	0.128 $\pm$ 0.065
	dTTP	0.198 $\pm$ 0.049