SUPPLEMENTARY DATA

Table of Contents

Analysis of extra peaks from TCA-treated \textit{S. cerevisiae} extracts - Figures S1-4 ................................. 2
Selection of column material – Figure S5 ........................................................................................................ 5
Development of mobile phase and temperature for HPLC analysis of nucleotides – Figures S6-8........ 5
Extra peaks and final adjustments of HPLC conditions – Figures S9-11 ......................................................... 9
Loading capacity, column dimension, and flow rate – Figure S12 ................................................................. 11
Development of Freon-free TCA extraction – Table S1 .................................................................................. 12
dNTP confirmation assay on different cell extracts – Figure S13 ................................................................. 13
Analysis of cell washes and growth media peaks – Figure S14 ................................................................. 14
HPLC linearity and sensitivity – Figure S15 ................................................................................................. 15
Summary of HPLC chromatograms from different cell extracts – Figure S16 ............................... 15
**Analysis of extra peaks from TCA-treated *S. cerevisiae* extracts - Figures S1-4**

HPLC analyses of samples from *S. cerevisiae* extracted with TCA exhibited three unknown peaks, which were also present in chromatograms from TCA-treated NADPH solutions (Figure S1A). These peaks were denoted Nₓ, Nᵧ, and Nz where N stands for NADPH derivative. The Nₓ peak had a retention time indicating 2´-phospho-ADP-ribose (PADPR), whereas the identities of Nᵧ and Nz remain unknown.

![Figure S1](image-url)

**Figure S1.** NADPH degradation products under acidic conditions. (A) Chromatograms from *S. cerevisiae* extracted with TCA showed three components that were also found in a TCA-treated NADPH solution but were lacking in the NADPH or TCA control solutions. All TCA-containing samples were extracted with chloroform-trioctylamine prior to analysis. (B) The Nₓ peak had a retention time indicating 2´-phospho-ADP-ribose (PADPR). The HPLC analysis was performed with a 150 mm × 2.1 mm ACE C18 column using a mobile phase containing 7% ACN, 1.056 g/l TBA-Br, and 7.36 g/l KPi pH 6. Peak b₁ is an unknown impurity (see Figures S2-3). The column temperature was 40°C, the flow rate was 0.25 ml/min, the detector wavelength was 270 nm, and the loop volume was 5 µl.

Another finding from HPLC analyses of *S. cerevisiae* extracts was an unusually broad peak termed b₁, which disturbed a large region of the chromatogram (Figure S1A-B) and put a restriction on what mobile phase compositions could be used to analyze all four dNTPs. The UV absorption spectrum of this peak was much broader than the nucleotide spectra (Figure S2A). At 310 nm, the absorption of b₁ was still high, whereas it was negligible for all rNTPs. With a wavelength subtraction procedure, it was possible...
to effectively reduce the size of $b_1$ and a second broad peak, $b_2$. Thereby, we could minimize their influence on the analysis of $dGTP$ and $dATP$, respectively (Figure S2B).

![Figure S2](image.png)

**Figure S2.** Development of a subtraction procedure to minimize the $b_1$ and $b_2$ HPLC peaks and thereby improve the baseline in the vicinity of the $dGTP$ and $dATP$ peaks. (A) The HPLC analysis of a *S. cerevisiae* TCA extract measured at different wavelengths shows that the peak heights of rNTPs are negligible above 300 nm. In contrast, the much smaller impurity peak $b_1$ (magnified in the inset) has a broader absorbance spectrum. (B) The two graphs show the HPLC analysis measured at 270 nm at a high magnification to monitor the dNTPs before and after subtraction of 1.8 times the signal recorded at 310 nm to reduce the size of the $b_1$ and $b_2$ peaks. AMP-PNP was added to the sample to determine if it is separated from rNTPs and dNTPs and thereby useful as a loading control (unrelated to the main purpose of this experiment). The HPLC conditions in A were the same as in Figure S1 for optimal separation of the $b_1$ peak from other cellular components, whereas the Fast Protocol was used in B.

The wavelength subtraction procedure nearly entirely removes the $b_1$ and $b_2$ peaks from the chromatograms. However, it is only practical with dual-wavelength UV detectors. A less powerful alternative to decrease the size of the two peaks is to use a smaller sample loop volume. Figure S3A shows the comparison between a sample loaded with 100 µl and 20 µl loop volumes. The latter sample was then concentrated five-fold in order to load an equal amount of material in the two cases. Another feature of the $b_1$ and $b_2$ peaks is that they increase in size when the KPi buffer in the mobile phase gets older (compare the top trace in Figure S3A with S3B). The peaks seem therefore to be dependent on both the mobile phase and the type of cell extract, which can be interpreted to mean that there are cell extract components that interact with UV-absorbing impurities in the mobile phase and change their chromatographic behavior. This effect seems to be rather unspecific because traces of the $b_1$ peak (but not $b_2$) could also be observed if mock samples not containing cell extracts were purified by WAX-SPE
Further analyses showed that the solutions used for WAX-SPE were pure, and it was components coming from the resin itself causing this effect. The unspecific causes of the $b_1$ and $b_2$ peaks were in line with our conclusion that the mobile phase itself was the most likely source.

Figure S3. HPLC analyses of *S. cerevisiae* extracts showing the effect of the sample loop volume and the age of the mobile phase KPi solution on the $b_1$ and $b_2$ peaks. (A) Peaks $b_1$ and $b_2$ are more prominent when using a 100 µl sample loop (top trace) than a 20 µl sample loop (bottom trace). The *S. cerevisiae* extract was diluted five-fold before mixing it with loading buffer when using the 100 µl sample loop to give an equal amount of material in both cases. (B) A similar experiment as in A but with a freshly prepared KPi buffer (solution A) and with a loop volume of 100 µl. In A, the KPi buffer was two months old. The HPLC conditions was according to the Fast Protocol.

Finally, we also analyzed how the KPi concentration in the mobile phase affects the relative retention times of the unknown HPLC peaks (Figure S4). The $N_y$ and $N_z$ peaks behaved similarly to the rNTPs shown, whereas the $b_1$ and $b_2$ peaks were more strongly affected by the KPi concentration. The figure shows capacity factors (retention time minus void time) relative to UTP.

Figure S4. Effect of the KPi concentration on the relative capacity factors of $N_y$ (■), $N_z$ ( ), $b_1$ ( ), ATP ( ), $b_2$ ( ), and UTP ( ). The capacity factors (retention time minus void) are relative to UTP as the reference compound (UTP = 1). The HPLC analysis was performed with a 150 mm × 2.1 mm ACE Excel 2 C18 column using a mobile phase containing 7% ACN, 0.7 g/l TBA-Br, and variable concentrations of KPi pH 5.8. The flow rate was 0.3 ml/min and the column temperature was 48°C.
Selection of column material – Figure S5

To develop isocratic conditions for the HPLC analysis of nucleotides, we started out with 5 cm ACE C18, C18-AR, and C18-PFP columns and a mobile phase containing TBA-Br, KPi, and low concentrations of MeOH (Figure S5). The higher polarity phases (C18-AR and C18-PFP) improved the separation of GTP from UTP with the MeOH-based system compared to the regular C18 column (Figure S5). However, the increased retention of GTP is only an advantage under conditions where UTP elutes before GTP, which is not the case when using ACN-based mobile phases (main text, Figure 2). The remaining experiments were therefore performed with regular C18 columns.

Figure S5. HPLC analyses of T. brucei ACN extracts with 2.1 mm × 50 mm ACE Excel 2 C18-PFP, C18-AR, and C18 columns. The mobile phase contained 7% MeOH, 0.7 g/l TBA-Br, and a concentration of KPi adjusted to position the ADP peak between the dCTP and UTP peaks. These concentrations were 7.13 g/l KPi for C18-AR, 4.37 g/l KPi for C18-PFP, and 5.75 g/l KPi for C18. The flow rate was 0.4 ml/min, the detector wavelength was 270 nm, the column temperature was ambient, and the loop volume was 5 µl. dCTP, dGTP and dATP were below the detection limit and the indicated positions were determined by nucleotide standards.

Development of mobile phase and temperature for HPLC analysis of nucleotides – Figures S6-8

The conditions of our final two HPLC methods (High-Resolution Protocol and Fast Protocol) are based on an analysis of how the mobile phase composition and column temperature affect the separation of the nucleotides. For optimizing the HPLC mobile phase composition, we found it convenient to use a ternary mixture of three aqueous solutions A, B, and C, where solution A contained pH-adjusted 23 g/l potassium phosphate (KPi) in 7% (v/v) ACN, whereas solution B only contained 7% ACN and solution C contained 3.5 g/l TBA-Br in 7% ACN. This ternary mixture simplified the testing of different mobile phase compositions. In Figure S6, we have provided the final concentrations of the components in the mobile phase and indicated the A-B-C composition in footnotes. The indicated pH value in each experiment represents the pH in solution A, which is also very close to the final pH after mixing the solutions (generally within 0.05 units).

When testing out the separation of nucleotides, we found a general effect where increased pH, KPi concentration, ACN concentration, and temperature gave decreased retention times of the nucleotides.
This can be explained by higher ACN concentration and temperature reducing the hydrophobic interaction with the column material, whereas the phosphate ions, which become more ionized at higher pH ($pK_a = 6.7$), compete with the nucleotides for binding to TBA. The increased ionization of the phosphate ions at the higher pH increases their capacity to elute the triphosphates at the higher pH, whereas the diphosphates were less affected by the pH (Figure S6). This can be explained by their $pK_a$ values ($pK_a \sim 6.8$ for nucleoside diphosphates, and $\sim 7.7$ for the triphosphates), where the increased elution power of the phosphate ions is partially compensated for by stronger binding of the diphosphates at the higher pH (charge transition from $–2$ to $–3$). In contrast, the triphosphates do not have a $pK_a$ close to the working pH range.

**Figure S6.** The effect of mobile phase composition and temperature on the retention times of nucleotides on a 150 mm $\times$ 4.6 mm Sunshell C18-WP column. (A) Effect of pH on nucleotide retention times. The mobile phase contained 9.2 g/l KP$_i$, 7% ACN, and 0.7 g/l TBA-Br*. (B) The effect of KP$_i$ concentration on nucleotide retention times. The mobile phase contained 4.6–9.2 g/l KP$_i$, pH 6.3, 7% ACN, and 0.7 g/l TBA**. (C) The effect of ACN concentration on nucleotide retention times. The mobile phase contained 6.21 g/l KP$_i$, pH 6.3, 5.94–10.71% ACN, and 0.7 g/l TBA-Br***. (D) The effect of temperature on nucleotide retention times. The mobile phase contained 6.21 g/l KP$_i$, pH 6.3, 7% ACN, and 0.7 g/l TBA****. The symbols indicate: dATP (□), ATP (■), dTTP (◊), PADPR (×), dUTP (△), dGTP (▽), UTP (▲), GTP (▼), dCTP (○), CTP (●), UDP-GlcA (*, green line), and dADP-ADP-dTDP (blue-colored □, ○, ◊). dUTP is highlighted with a dashed line. The selection of the symbols is categorized into rNTPs (filled symbols), dNTPs (corresponding open symbols), rNDPs/dNDPs (blue symbols), and nucleotide-conjugated sugars (crosses: * and ×). The flow rate was 0.8 ml/min, the column temperature was ambient unless indicated, the detector wavelength was 270 nm, and the loop volume was 50 µl. The ordinates have logarithmic-based distances between the numbers.

* Mixture of 40% A, 40% B, and 20% C using A solutions with different pH values.
** 20–40% A pH 6.3, 20% C, and the remaining percentage B.
*** 27% A pH 6.3, 53% B, and 20% C using variable concentration of ACN of 5–14% in solution B.
**** 27% A pH 6.3, 53% B, and 20% C.
The graphs shown in Figure S6 give information about general trends, but in order to get to know the movement of different nucleotides relative to each other, the graphs were replotted where the capacity factor (retention time minus void time) of each nucleotide was divided by that of UTP in the same experiment (Figures S7-8). The numbers shown just above the top trace in each panel indicate the retention time for the last eluting peak to give information about the time each experiment takes. Figure S7A shows the relative capacity data replotted from the pH experiment in Figure S6A. The increased pH had a much stronger effect on the diphosphates (indicated in blue) than the triphosphates. A similar trend was also observed when increasing the KP concentration (Figure S7B). UDP-GlcA was also strongly affected by the pH and KP concentration but with the opposite pH trend compared to the diphosphates. A challenge during optimization was to find conditions where no other peaks overlapped with dCTP (○). At pH 6 UDP-GlcA interfered with dCTP analysis, whereas good nucleotide separations could be obtained on both sides of this value. However, when using the higher pH values, it was important to have a compensatory reduction of the KP concentration in order to decrease the extensive overlap between diphosphates and triphosphates (Figure S7B). This shifted all rNDPs, dNDPs, and UDP-GlcA ahead of the rNTPs and dNTPs. However, a consequence was that the total elution time of the nucleotides became longer. Another trend was that the gap between each dNTP and its corresponding rNTP was smaller at the higher pH values. This effect is relatively small and is clearer in plots with capacity factors of each rNTP divided by that of the corresponding dNTP (Figure S7A-inset).

**Figure S7.** The effect of pH (A) and KP, concentration (B) on the relative capacity factors of different nucleotides compared to UTP. The experimental conditions are the same as in Figure S6A-B but replotted with relative capacity factors instead of retention times on the ordinate. rNDPs and dNDPs are highlighted in blue and UDP-GlcA in green. In the inset of A, the data are re-plotted as $k_{\text{CTP}}/k_{\text{UTP}}$ (●), $k_{\text{UTP}}/k_{\text{dCTP}}$ (▲), $k_{\text{GTP}}/k_{\text{dGTP}}$ (▼), and $k_{\text{dATP}}/k_{\text{ATP}}$ (■). The data points for pH 6.5 in the inset are from an experiment with a lower KP concentration (7.36 g/l instead of 9.2 g/l) to avoid insufficient retention times. In the main figures, the symbols indicate: dATP (○), ATP (●), dTTP (◊), PADPR (×), dUTP (△), dGTP (▼), UTP (▲), GTP (▼), dCTP (○), CTP (●), UDP-GlcA (●, green line), and dADP-dADP-dTDP (blue-colored ○, ▲, ◊) using the same categorization of symbols as in Figure S6 with rNTPs being filled symbols, dNTPs open symbols, and nucleotide-conjugated sugars crosses. dUTP is highlighted with a dashed line.
Figure S8 shows the effects of the mobile phase ACN concentration and column temperature on the relative capacity factors of the nucleotides. Both parameters had similar effects on nucleotide separation with a relative decrease in capacity factors of purines over pyrimidines (especially dATP, ATP, dGTP, dADP, and ADP, whereas the effect on GTP is less prominent), and a relative increase in the capacity factors of the two included nucleotide-conjugated sugars UDP-GlcA and PADPR. Our original goal to separate dUTP and dGTP could be achieved at both low and high ACN concentrations, but at the higher concentration dGTP merged with UTP instead (Figure S8A). Another factor of importance was the $k_{\text{CTP}/\text{dCTP}}$ factor, which came closer to 1 (meaning no separation) at higher ACN concentration but was much less affected by temperature (insets in Figure S8A-B). Based on the studies in Figures S6-9, we selected the ACN concentration to be 5.8% at pH 5.6, whereas the KPi concentration and column temperature were fine-tuned to optimize peak separation with both standard nucleotides and biological extracts as described in the next section.

**Figure S8.** Effect of ACN concentration (A) and temperature (B) on the relative capacity factors of different nucleotides compared to UTP. The experimental conditions are the same as in Figure S6C-D but replotted with relative capacity factors instead of retention times on the ordinate. rNDPs and dNDPs are highlighted in blue and UDP-GlcA in green. In the insets, the data is replotted as $k_{\text{CTP}/\text{dCTP}}$ (●), $k_{\text{UTP}/\text{dUTP}}$ (▲), $k_{\text{GTP}/\text{dGTP}}$ (▼), and $k_{\text{ATP}/\text{dATP}}$ (■). In the main figures, the solid symbols indicate rNTPs, open symbols dNTPs, blue symbols rNDPs-dNDP, and different types of crosses nucleotide-conjugated sugars: dATP (○), ATP (■), dTTP (◇), PADPR (×), dUTP (◇), dGTP (▼), UTP (▲), GTP (▼), dCTP (○), CTP (●), UDP-glucuronic acid (⋆), and dADP-ADP-dTDP (grey-colored ○, □, ◇). dUTP is highlighted with a dashed line and was not included in the standard for the 19°C data because it was inseparable from dGTP under these conditions.
From the analysis of different biological extracts, we found three additional peaks to the ones described in Figure S1-8. Two of them, denoted c and g, were observed in HPLC analyses of mouse embryonal extracts (Figure S9A), and the third one named t was an impurity in the TCA solution used for extraction of cells. As shown in Figure S9A, the c-peak was partially overlapping with dCTP and illustrates the need for the final optimization of the HPLC conditions. Figure S9B shows that the retention times of the c, g, and t-peaks are less dependent on the KPi concentrations than the surrounding rNTPs and dNTPs as evident by the shallower slopes of the curves. At the lowest KPi concentration tested, the c-to-dCTP, g-to-dGTP, and t-to-dTTP separations were all improved. Figure S9C shows the effect of column temperature on the retention times. Although higher temperatures helped to reduce the complexity around the dCTP peak, the separation between dTTP and ATP was not sufficient at 50°C and the dGTP and dUTP peaks merged when using mid-range temperatures.

**Figure S9.** The effect of the HPLC mobile phase and temperature on the separation of nucleotides and the three unknown extract components named c, g, and t. (A) HPLC analysis of a sample prepared from mouse embryos extracted with 50% ACN revealed two unknown components named c and g. The column temperature was ambient, and the mobile phase consisted of 5.8% ACN, 0.7 g/l TBA-Br, and 9.6 g/l KPi pH 5.6. (B) Effect of HPLC mobile phase KPi on retention times. The HPLC analyses were performed with mobile phases containing 5.8% ACN, 0.7 g/l TBA-Br, and variable concentrations of KPi pH 5.6. To simplify the testing of several KPi concentrations, this mixture was created from three solutions named A, B and C, which were similar to the ones in Figure 6 but with an ACN concentration of 5.8% instead of 7%*. (C) Effect of column temperature on retention times. The HPLC analyses were performed with varying temperatures and a mobile phase consisting of 5.8% ACN, 0.7 g/l TBA-Br, and 7.36 g/l KPi pH 5.6*. The c, g, and t-components and nucleoside diphosphates are highlighted with green and blue curves, respectively. The remaining components are rNTPs and dNTPs using the following symbols: dATP (○), ATP (■), dTTP (△), t (), dUTP (▽), dGTP (▽), g (□), UTP (▲), GTP (▼), dCTP (○), CTP (●), c (Θ), dADP (blue-colored ○), and ADP (blue-colored □). GTP, UTP, and dADP are not included in A.

*In B, the mixture was 32-55% A with a pH of 5.6, 20% C, and the remaining percentage B. In C, the mixture was 32% A, 48% B, and 20% C.
Optimizing the KP<sub>i</sub> concentration for speed and separation under ambient conditions resulted in the High-Resolution Protocol, a method that was able to separate ADP, rNTPs, and all dNTPs including dUTP from the other peaks in the chromatograms (Figure S10A and Figure 3). A similar optimization with a column temperature of 30°C gave the best separation at higher KP<sub>i</sub> concentration and consequently a shortened analysis time (the Fast Protocol), but with the drawback of not being able to discriminate between dUTP and dGTP. The relative positions of most peaks were the same with the two methods with the notable exception of the t-peak, which elutes just after dTTP using the Fast Protocol (Figure S10B). For the studies of this peak, we used a 20× t-standard prepared by extracting TCA with chloroform-triocylamine. Because this peak was present in TCA preparations extracted with chloroform-triocylamine but not in similar experiments with TFA, we concluded that the TCA solution itself was the source of the impurity (Figure S10B).

**Figure S10.** Final HPLC protocols and characterization of the t-peak. (A) HPLC analysis with the High-Resolution Protocol on a nucleotide standard consisting of 1 µM ADP and rNTPs, 0.5 µM dNTPs, 0.1 µM 2'-phospho-ADP ribose (APS), 0.2 µM of dTDP, dADP, UDP-GlcA, and 20× t-standard (mock TCA extract diluted 1:20 in water). (B) Zoomed-in chromatogram using the Fast Protocol on a nucleotide mixture in TCA-MgCl<sub>2</sub>, extracted with Freon-trioctylamine or chloroform-triocylamine, and purified by WAX-SPE. The t-peak was only present when using TCA but not in corresponding experiments with TFA. The position of the t-peak came after dTTP when using the Fast Protocol.

The mobile phase itself can also be a source of extra peaks. In our case, there was a negative peak (m<sup>−</sup>) just before ADP and a positive peak (m<sup>+</sup>) just before CTP (Figure S11A). The negative peak only appeared when using a straight flow cell (ST cell) and was not visible using the default tapered flow cell (TP cell) having a tapered shape designed to minimize refractive index changes in the UV detector (Figure S11B). None of the peaks interfered significantly with nucleotide analysis in cell extracts because ADP and CTP are generally much more abundant than dNTPs. In common with the c, g, and t-peaks, the positions of the m<sup>+</sup> and m<sup>−</sup> peaks were less dependent of the KP<sub>i</sub> concentration than the positions of the nucleotides (Figure S11B).
Figure S11. HPLC peaks coming from the mobile phase. (A) Loading an empty sample (Mock) gave one negative peak, m−, coming just before ADP and one positive peak, m+, coming just before CTP using the Fast Protocol. Both peaks shifted to the right if the temperature was decreased to room temperature (Mock: RT). The last chromatogram shows the peaks using the High-Resolution Protocol (Mock: 8.7 KPi, RT). (B) Peak m+ (arrow) was much less affected by the mobile phase KPi concentration (given in g/l) than the surrounding nucleotides. The same is true for m− (compare Mock: RT and Mock: 8.7KPi, RT in A).

Loading capacity, column dimension, and flow rate – Figure S12

When analyzing cellular extracts, the loading capacity is an important factor because the rNTP pools are several orders of magnitude higher than the dNTPs and overloading results in broad rNTP peaks and low theoretical plates per meter (Figure S12A). The loading capacity is a known limitation in ion-pair chromatography, most likely because the repulsion between molecules of the same charge puts a limit on the coverage of the ion-pairing agent on the column (1). To avoid overloading, it was advantageous to use 4.6 mm ID columns because columns with narrower inner diameters require shorter detector flow cells and therefore more sample per gram of chromatography material to achieve the same sensitivity. Another feature of the ion-pairing chromatography was that nucleotide separation required lower flow rates for optimal separation than non-charged compounds (Figure S12B).

Figure S12. Effect of nucleotide amount (A) and flow rate (B) on theoretical plates. The experiment was performed using the Fast Protocol with variable flow rates in B and 1.2 ml/min in A. The compounds tested were ATP in A and dGTP (△), dCTP (○), and 2-fluorodeoxyadenosine (★, dotted line) in B. 2-fluorodeoxyadenosine is not a nucleotide and was used for comparison.
Development of Freon-free TCA extraction – Table S1

Table S1 shows the recovery of nucleotides when mixed with TCA or TFA and subjected to Freon-trioctylamine or chloroform-trioctylamine extraction for the removal of the acid. The recovery of nucleotides was nearly as good with chloroform substituting for Freon in the extraction step and was equally efficient with TFA and TCA.

Table S1. Recovery of a nucleotide standard mixed with a 0.6 M TCA/TFA-acidified solution containing 15 mM MgCl₂ and subsequently extracted with Freon-trioctylamine (FTOA) or chloroform-trioctylamine (CTOA). As indicated in the table, different concentrations were used for dNTPs, ADP, ATP, and other rNTPs to better reflect physiological ratios.

| Nucleotide | Conc. (µM) | TCA-FTOA Recovery (%) | TCA-CTOA Recovery (%) | TFA-CTOA Recovery (%) |
|------------|-----------|------------------------|------------------------|------------------------|
| ADP        | 0.75      | 97.3 ± 1.0             | 91.0 ± 2.5             | 90.7 ± 3.0             |
| CTP        | 5         | 95.7 ± 4.6             | 90.5 ± 1.8             | 91.6 ± 2.8             |
| dCTP       | 0.5       | 96.6 ± 4.7             | 91.5 ± 2.0             | 92.9 ± 3.7             |
| GTP        | 5         | 94.2 ± 5.1             | 88.8 ± 0.8             | 90.8 ± 1.5             |
| UTP        | 5         | 96.7 ± 3.8             | 90.7 ± 0.5             | 92.0 ± 1.1             |
| dGTP       | 0.5       | 92.5 ± 4.5             | 85.5 ± 2.2             | 87.1 ± 4.5             |
| AMP-PNP    | 0.5       | 90.3 ± 2.6             | 84.4 ± 2.9             | 86.5 ± 3.1             |
| dTTP       | 0.5       | 99.1 ± 4.3             | 89.7 ± 3.2             | 94.3 ± 2.7             |
| ATP        | 20        | 97.9 ± 4.5             | 91.1 ± 0.5             | 94.0 ± 1.9             |
| dATP       | 0.5       | 98.1 ± 4.8             | 90.6 ± 0.5             | 93.2 ± 3.1             |
dNTP confirmation assay on different cell extracts – Figure S13

A dNTP confirmation assay showed that there were no co-eluting peaks disturbing the HPLC analyses of cell extracts from Balb/3T3 fibroblasts, *T. brucei*, or *S. cerevisiae* (Figure S13A-C). Each figure includes a pairwise comparison of two samples from the same extract, where the first sample was mock-treated and the second was incubated with DNA-polymerization agents. AMP-PNP added prior to treatment served as a loading control to ensure that the dilution effect during the post-incubation filtering step was roughly equal in the two samples. As shown for *S. cerevisiae* (Figure S13D), the rNTPs can also serve as an internal indicator of the amount loaded. The only advantage of adding AMP-PNP is for visualization purposes because it can be shown with the same scaling as the dNTPs and does not require a separate panel.

**Figure S13.** HPLC analyses of cellular extracts from mouse Balb/3T3 fibroblasts (A), *T. brucei* (B), and *S. cerevisiae* (C) treated with the DNA polymerase-dependent dNTP confirmation assay. (D) The same chromatogram as in C but zoomed out to show ADP and rNTPs. The top graphs show control samples in which the incubation was without DNA polymerase, primers, and M13 DNA, while the bottom graphs highlight the specific removal of dNTPs in the treated samples. Peaks a, b1, g, and t are from non-identified components, and AMP-PNP (Ctrl-load) was added as a loading control in assays A and B. The Fast Protocol was used for the HPLC analyses in A-B and the High-Resolution Protocol was used in C-D.
Analysis of cell washes and growth media peaks – Figure S14

Growth medium components are potential sources of extra peaks in HPLC analyses, and our initial studies of *S. cerevisiae* grown in YPD showed that the cell extracts often give a whole range of undefined peaks over the entire chromatogram. Figure S14 shows chromatograms from samples prepared by mixing different growth media with TCA and extracting them with chloroform-trioctylamine. Tissue culture media used for the growth of mammalian cells and *T. brucei* (DMEM and HMI-9) were free from extra peaks except for the t-peak coming from the TCA solution (Figure S14A). In contrast, YPD medium samples gave plenty of unresolved peaks, which could only partially be removed by the WAX-SPE procedure (Figure S14B). YPD is a broth-type of medium containing yeast extract and peptone, and both components gave HPLC peaks (Figure S14C), with the yeast extract being the main contributor.

**Figure S14.** HPLC analyses of different cell culture media. (A) Both DMEM and HMI-9 were clean from peaks interfering with rNTP and dNTP analysis. The culture media were mixed 1:5 with TCA and extracted in a similar way as the cell extract from Balb/3T3 fibroblasts, which is included for comparison, but with the WAX-SPE purification step omitted. Additional peaks include the t-peak coming from the TCA solution, UDP-GlcA, NADPH degradation products (PADPR, Ny, and Nz), and the unknown peak a. (B) TCA-extracted YPD gives many peaks, which can only partially be removed by the WAX-SPE step. (C) Analysis of yeast extract and peptone components of YPD shows the presence of additional peaks in both of them, but more prominently in the yeast extract. Both components were prepared at the same concentration as in YPD medium using the same purification as in B but with the WAX-SPE step included. The Fast Protocol was used for the HPLC analyses.
**HPLC linearity and sensitivity – Figure S15**

Figure S15 compares the linearity of peak height and area measurements for ADP and rNTPs over a wide range of concentration units using a logarithmic scale on the abscissa and relative units on the ordinate (i.e. per pmol). The peak heights deviate from linearity when the column starts to reach saturation at around 500 pmol, whereas the areas are linear in the entire interval studied. Note that a broken ordinate is introduced in B to avoid overlap between the symbols. This will also emphasize the variation between the data points, and it is only at nucleotide amounts below 10 pmol that peak heights vary less than areas.

![Figure S15](image)

**Summary of HPLC chromatograms from different cell extracts – Figure S16**

Figure S16 shows HPLC chromatograms of nucleotides from mammalian fibroblasts (Balb/3T3), *T. brucei*, and *S. cerevisiae* extracted with different techniques. The chromatograms have logarithmic axes to visualize both rNTPs and dNTPs in the same plot. However, this type of plot should not be used for assessments of quantity or peak resolution as illustrated in the Figure S16A inset that shows the corresponding linear plot of the TCA experiment from the main figure. Note that the logarithmic plot underestimates the ATP-ADP ratio and emphasizes the overlap between the GTP and UTP peaks.

Figure S16 illustrates the following:

- Figure S16A. With TCA as the extracting solution, the HPLC chromatograms contain additional peaks compared to solutions based on ACN or MeOH. These extra peaks include the t-peak, which is an impurity from TCA, as well as the three NADPH degradation products PADPR, N\(\gamma\), and N\(\delta\). The t-peak and the NADPH degradation products are described in Figures S10 and S1, respectively.
- Figure S16B-C. The broad peaks b\(_1\) and b\(_2\) are present in HPLC chromatograms from *S. cerevisiae* and *T. brucei*. If necessary, these peaks can be eliminated by a UV-subtraction procedure as illustrated in Figure S2.
- Additional peaks of minor importance are also present in some chromatograms such as the m\(^+\) peak from the mobile phase, the a-peak from the Balb/3T3 extract, and the g-peak from *T. brucei*.
- Two HPLC methods were developed, the High-Resolution Protocol in Figure S16D and the Fast Protocol in Figure S16A-C. The High-Resolution Protocol is able to separate dUTP from dGTP and in addition gives better separation of dTTP from a TCA impurity (the t-peak).
Figure S16. HPLC peaks in different types of extracts. (A) Chromatograms from Balb/3T3 fibroblasts extracted with 80% MeOH (grey chromatogram) and TCA (white chromatogram in the background). The Fast Protocol was used for the HPLC analysis. Note that PADPR, t, Ny, and Nz are only present in the TCA extract. The inset shows the chromatogram from TCA-extracted cells with linear axes for comparison. (B-C) Chromatograms from TCA-extracted T. brucei (B) and S. cerevisiae (C) analyzed by the Fast Protocol. The T. brucei analysis also shows a peak labeled Ctrl-load, coming from added AMP-PNP, which is a useful standard for measuring recovery (see example in Fig. S13). (D) TCA-extracted S. cerevisiae cells analyzed by the High-Resolution Protocol. The dUTP pool is below the detection limit.

References

1. Dai, J., Carr, P.W. and McCalley, D.V. (2009) A new approach to the determination of column overload characteristics in reversed-phase liquid chromatography. J Chromatogr A, 1216, 2474-2482.