Semi-automated high-throughput substrate screening assay for nucleoside kinases

Katja F. Hellendahl¹,ǂ, Maryke Fehlau¹,₂,ǂ, Sebastian Hans¹, Peter Neubauer¹,₂, Anke Kurreck¹,²*

¹Technische Universität Berlin, Faculty III Process Sciences, Institute of Biotechnology, Chair of Bioprocess Engineering, Ackerstraße 76, 13355 Berlin, Germany
²BioNukleo GmbH, Ackerstraße 76, 13355 Berlin, Germany

ǂ K.F.H. and M.F. contributed equally to this work.

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Nucleoside kinases (NKs) are key enzymes involved in the in vivo phosphorylation of nucleoside analogues used as drugs to treat cancer or viral infections. Having different specificities, the characterization of NKs is essential for drug design and the production of nucleotide analogues in an in vitro enzymatic process. Therefore, a fast and reliable substrate screening assay for NKs is of great importance. Here, we report the validation of a well-known luciferase-based assay for the detection of NK activity in 96-well plate format. The assay was semi-automated using a liquid handling robot. A good linearity was demonstrated (r² >0.98) in the range of 0 to 500 µM ATP, and it was shown that also alternative phosphate donors like dATP or CTP were accepted by the luciferase. The developed high-throughput assay revealed comparable results to HPLC analysis. The assay was exemplary used for the comparison of the substrate spectra of four nucleoside kinases using 20 (8 natural and 12 modified) substrates. The screening results correlated well with literature data and, additionally, previously unknown substrates were identified for three of the NKs studied. Our results demonstrate that the developed semi-automated high-throughput assay is suitable to identify best performing NKs for a wide range of substrates.
Nucleotides are essential biomolecules involved in all cellular processes as building blocks of nucleic acids, energy storage, cellular communication mediators and cofactors for many enzymatic reactions. Furthermore, nucleotide analogues have a broad range of applications in molecular biology and medicine. As examples, modified nucleotides are used to label nucleic acids in PCR\[1\], for fluorescent in situ hybridization (FISH)\[2\] or microarray\[3\] applications. In addition, they improve nuclease stability of aptamers\[4\] and small interfering RNAs.\[5,6\] The importance of nucleoside 5'-triphosphates (NTPs) is also evident in mRNA vaccines, which have become increasingly important in the current Corona pandemic.\[7\] By incorporating modified NTPs such as N1-methylpseudouridine triphosphates or pseudouridine triphosphates into therapeutic mRNAs significantly less activation of the innate immune response compared to uridine is induced.\[8\] In addition, there is evidence that modified mRNA is translated more efficiently than unmodified mRNA.

Nucleoside analogues are widely used to treat cancer\[9\], viral\[9\] and bacterial\[10\] infections. However, nucleoside analogue drugs are usually active only in the nucleotide form. Therefore, the therapeutic efficacy largely depends on the intracellular phosphorylation of the nucleoside analogue to a 5'-triphosphate. In three consecutive steps nucleoside analogues are phosphorylated by (deoxy)nucleoside kinases ((d)NKs), (deoxy)nucleoside monophosphate kinases and nucleoside diphosphate kinases.\[11\] Thus, to assess the therapeutic potential of new compounds in advance, a high-throughput assay for nucleoside and nucleotide kinases would be of great importance.

Besides the importance in drug design, recombinant nucleoside and nucleotide kinases are valuable biocatalysts to produce modified nucleotides in enzymatic in vitro processes.\[12\] Enzymatic approaches offer an interesting alternative to the chemical synthesis by the use of mild reaction conditions, having a high regio- and stereoselectivity and by avoiding protection and deprotection steps.\[13,14\]

Due to the importance of nucleoside and nucleotide kinases the characterization of their substrate spectra and the identification of the most efficient phosphate donor is highly relevant. Therefore, different methods were developed to determine kinase activity.\[15\] Assays applicable towards a wide range of natural and modified substrates and phosphate donors include radiometric and chromatographic (TLC, HPLC) methods. But these assays are time-consuming and hardly suitable for high throughput applications. In addition, they would have to be optimized for some substrates. Other assays focus on the detection of adenosine 5'-triphosphate (ATP) consumption or production, since ATP is the most commonly used phosphate donor for various kinases.\[16\] Assays based on lactate dehydrogenase, pyruvate oxidase, malachite green, pyruvate kinase, fluorescence polarization, FRET or ELISA use the principle of measuring colorimetric or fluorometric changes.\[17,18\] Although these assays offer the opportunity to be used in high throughput, they are often expensive (especially if an antibody is involved), and require multi-step enzyme cascades. While the latter is not a concern when studying protein kinases, it is challenging for reactions catalyzed by nucleoside or nucleotide kinases because there is a higher risk that the non-natural nucleotides interfere with ATP detection.\[17,18\]

In contrast, bioluminescence-based assays using a luciferase to detect ATP are fast, high throughput compatible and based on only one enzyme.\[17,19\] In these assays kinase activity is determined in two successive reactions. First, the kinase of interest catalyzes the transfer of a phosphate from a phosphate donor (mainly ATP) to the respective substrate. Afterwards, the residual phosphate donor is converted into a luminescence signal by a luciferase (Scheme 1). Thus, the kinase activity inversely corresponds to the residual phosphate donor concentration. Due to the advantages, bioluminescence-based assays have already been applied successfully for the activity detection as well as for inhibition and kinetic studies of many kinases\[16,20\], including also nucleoside/nucleotide kinases\[19,21–24\]. We recently demonstrated the use of a bioluminescence-based assay for the identification of substrates and kinetics for the human deoxycytidine kinase (HsdCK).\[19\] However, so far this was a manual assay.

Here we adapt this assay to be used on a robotic platform, so it can be applied as a fast, quantitative and reliable semi-automated assay to analyze the phosphorylation of natural and modified nucleosides by
nucleoside kinases using luciferase-based ATP detection. The accuracy of the assay was comparable to HPLC analytics. The advantage of this assay is demonstrated for the characterization of the substrate spectrum of four (d)NKs towards 20 nucleosides in a 96-well plate format. Autoluminescence and luciferase inhibition by the natural and modified nucleosides was not observed.

Results and Discussion

Establishment of the luminescent assay with (deoxy)NTP standards

To develop a high throughput assay for the evaluation of the substrate spectrum of (d)NKs, the commercially available Kinase-Glo Max Luminescent Kinase Assay was chosen because it is described to exhibit high linearity up to an ATP concentration of 500 µM. The assay composition recommended by the manufacturer was slightly adjusted to reach volumes suitable for the 96-well plate format as previously described (for further information see experimental section).[19]

Initially, we were interested in whether the Ultra-Glo luciferase accepts other (deoxy)NTPs next to ATP, since a wide range of phosphate donors are accepted by (d)NKs.[19,25,26] For that, standards of 500 µM ATP, 2’-deoxyadenosine 5’-triphosphate (dATP), cytidine 5’-triphosphate (CTP), 2’-deoxyxytidine 5’-triphosphate (dTTP), uridine 5’-triphosphate (UTP) and thymidine 5’-triphosphate (TTP) were tested as substrates for the recombinant Ultra-Glo luciferase (Figure 1A). Highest luminescence signals were detected for ATP with values of up to 57.892 relative light units (RLU) (Figure 1B). This observation is in good accordance with literature data where a high specificity of luciferases towards ATP was described.[27] However, we also observed the acceptance of additional (deoxy)NTPs as substrates of the Ultra-Glo luciferase. The luminescence signal decreased in the order ATP >> dATP (1128 RLU) > GTP (504 RLU) > UTP (220 RLU) > CTP (161 RLU) > dGTP, dCTP, TTP (< 30 RLU). Except for dGTP Ultra-Glo luciferase showed lower preference for dNTPs and pyrimidine nucleotides compared to NTPs and purine nucleosides, respectively. To study the applicability of ATP, dATP, GTP, CTP and UTP in the luminescent assay in more detail, standard curves in the range of 0 to 500 µM were prepared and revealed high accuracy with r² values > 0.98 (Figure 1C). Hence, except for dGTP, dCTP and TTP all natural (deoxy)NTPs are suitable substrates for the recombinant Ultra-Glo luciferase. This observation widens the applicability of the luminescence-based assay to study nucleoside and nucleotide kinases.
Establishment of the luciferase-based (d)NK activity assay using natural (deoxy)nucleosides as substrates

In a first attempt to establish the NK activity assay the influence of the nucleoside substrates on the luminescence signal was analyzed, since it could not be excluded that certain nucleosides or nucleoside 5'-monophosphates (NMP) would affect the activity of the Ultra-Glo luciferase or show autoluminescence themselves. Therefore, we measured the luminescence of 400 µM ATP alone or together with 400 µM standards of eight natural (deoxy)nucleosides (adenosine (Ado), 2'-deoxyadenosine (dAdo), guanosine (Guo), 2'-deoxyguanosine (dGuo), cytidine (Cyd), 2'-deoxycytidine (dCyd), uridine (Urd), thymidine (Thd)) and 7 natural NMPs (2'-deoxyadenosine 5'-monophosphate (dAMP), guanosine 5'-monophosphate (GMP), 2'-deoxyguanosine 5'-monophosphate (dGMP), cytidine 5'-monophosphate (CMP), 2'-deoxycytidine 5'-monophosphate (dCMP), uridine 5'-monophosphate (UMP), thymidine 5'-monophosphate (TMP)) in NK reaction buffer (Table S1). The impact of adenosine 5'-monophosphate (AMP) was studied in more detail as inhibitory effects on luciferases were described before.\(^{[28]}\) To mimic the conditions of the activity assay, mixtures consisting of different AMP to ATP ratios were analyzed with a total concentration of 400 µM (Figure S1). For all combinations, no significant influence on the luminescence signal was observed (Table S1, Figure S1). Hence, autoluminescence and luciferase inhibition was excluded for all tested (deoxy)nucleosides and (deoxy)NMPs.

To validate the accuracy of the assay, NK reactions were performed with human deoxycytidine kinase (HsdCK), ATP as phosphate donor and eight natural...
(deoxy)nucleosides (Ado, dAdo, Guo, dGuo, Cyd, dCyd, Urd, Thd, Figure 2A) at 37°C for 19 h. Afterwards ATP consumption and NMP formation was analyzed using the luminescence-based assay and data were compared to standard HPLC analytics. Using the luminescence-based assay NMP formation was calculated based on ATP consumption. The determined NMP formation (Figure 2B) and ATP consumption (Figure 2C) were very comparable for both methods used. The deviations between both methods were between 0.49 to 7.53 % (Table S2), with the difference being highest at low (deoxy)NMP formation. For Ado, Guo and Urd there was up to 9 % ATP consumption detected by the luminescent assay while no or only very little NMP formation was observed by HPLC. The susceptibility of the assay to error has been observed previously.\[16\] In order to avoid false positive results at conversions < 10 %, it is therefore advisable to either i) check product formation via HPLC, or ii) to repeat the reaction with increased enzyme concentration to verify the substrate’s acceptance. In general, the assay should be used preferentially to find the best performing NK showing significant substrate conversion > 50%.

In a next step, the substrate spectrum of HsdCK was evaluated and compared to literature data. HsdCK well accepted dAdo, dCyd, dGuo and Cyd with (deoxy)NMP formation being above 80 % (Figure 2, Table S2). While the acceptance of dAdo, dCyd and dGuo has already been described several times in the literature\[19,29\], reports on the acceptance of ribonucleotides are rare. Yet, the use of cytidine as substrates for human T-lymphoblast deoxycytidine kinase has been described before.\[30\]

In this study, we also observed a minor accumulation of TMP (~13 %, HPLC analysis) and AMP (~1 %, HPLC analysis) with HsdCK as biocatalyst (Figure 2).

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**Figure 2.** Validation of the luminescent assay for NK-catalyzed reactions. Natural nucleosides (A) were tested as substrates for HsdCK. HPLC analysis and the luminescent assay were used for the determination of (deoxy)NMP formation (B) and determination of ATP consumption (C). Reactions were performed with 0.33 mM substrate and 0.4 mM ATP in 70 mM Tris-HCl [pH 7.6] supplemented with 5 mM DTT, 10 mM MgCl\(_2\) and 0.0002 U HsdCK at 37°C for 19 h. Mean of the duplicates and standard deviation are indicated. For the luminescent assay, the (deoxy)NMP formation was calculated from the detected ATP consumption.

Ado: adenosine, dAdo: 2'-deoxyadenosine, Guo: guanosine, dGuo: 2'-deoxyguanosine, Cyd: cytidine, dCyd: 2'-deoxycytidine, Urd: uridine, Thd: thymidine
There have been many reports that HsdCK does not accept thymidine, and it is believed that phosphorylation of this nucleoside in vivo is carried out by human thymidine kinase 1 (TK1). However, kinetic studies on wild type and mutant HsdCK revealed that thymidine is accepted with low rate constants, which correlates well with observations made in this study. The acceptance of adenosine as substrate, however, has not been described so far. Although binding of adenosine to HsdCK was shown, AMP forming activity has never been reported. Most probably AMP appears due minor degradation of ATP during the kinase reaction, as low AMP formation was also observed in controls lacking enzymes (data not shown).

Development of a high-throughput semi-automated activity assay for nucleoside kinases

A semi-automated approach using a liquid handling robot was developed to apply the luciferase-based assay in a highly accurate substrate screening assay for nucleoside kinases (Figure 3A). Scripts were written to pipette the kinase reaction by the robot into 96-well PCR plates. Afterwards, the plates were manually transferred to an incubator. After completion of the reaction, plates were manually transferred back to the deck of the robot and the luciferase reaction was pipetted. Subsequently, the assay plate was automatically transported to the plate reader to measure luminescence after 10 min incubation in the dark. Please see Table S3 and the externally hosted Supplementary Information for more detailed information on the approach.

To assess the accuracy of both the kinase reaction and the luminescent assay independently, the pipetting steps performed by the robot were evaluated by using dyes. The dyes were chosen to allow for a differentiation of the influence of the individual components on the overall reaction accuracy (for more information see the experimental section). Robot pipetting accuracy was very high for both the kinase and the luciferase-catalyzed reaction (Figure S2). HsdCK was chosen as biocatalyst to validate the established approach in comparison to reactions which were pipetted manually. Kinase reactions were performed with natural (deoxy)nucleosides that were well accepted by HsdCK before (Figure 2B, dAdo, dGuo, Cyd, dCyd, and Thd). The results of the semi-automated assay were in good accordance to manually pipetted reactions for all substrates tested (Figure 3B).

![Figure 3A](image1)
![Figure 3B](image2)

**Figure 3.** Semi-automated activity assay for nucleoside kinases. (A) The kinase reaction and the luminescent assay automatically prepared by a liquid handling robot. A manual step was implemented to transport the reaction to an incubator and after reaction complementation back to the liquid handling robot (B) HsdCK-catalyzed reactions with ATP as phosphate donor were manually and semi-automated prepared and analyzed using the luminescent assay. Mean of the duplicates and standard deviations are indicated.

dAdo: 2′-deoxyadenosine, dGuo: 2′-deoxyguanosine, Cyd: cytidine, dCyd: 2′-deoxycytidine, Thd: thymidine
Figure 4. Semi-automated substrate screening for DmdNK, HsdCK, HsAK and TK. The substrate spectra of the four (deoxy)nucleoside kinases were tested with 20 nucleosides (A). Activities with purine (B) and pyrimidine (C) nucleoside substrates were determined for the chosen NKS using standard screening conditions. If a discrepancy to literature data was observed, assay conditions were optimized. \[ \text{Semi-automated luminescent assay to compare the substrate spectra of four NKS} \]

With the semi-automated high throughput screening assay in hand, the substrate spectra of three commercially available (d)NKS (HsdCK, human adenosine kinase (HsAK) and a viral thymidine kinase (TK)) were compared to the wide-spectrum DmdNK. In addition to eight natural nucleosides 12 non-natural derivatives were included in the substrate screening
assay. A set of sugar-modified (vidarabine (AraA), gemcitabine (dFdC), 1-(2'-deoxy-2'-fluoro-β-D- arabinofuranosyl) uracil (FanaU), ganciclovir (GCV), acyclovir (ACV)), base-modified (cladribine (CldA), 2-fluoroadenosine (FAdo), 5-fluorocytidine (FCyd), 5-ethynyl-2'-deoxyuridine (EdU)) as well as sugar- and base-modified nucleoside substrates (clofarabine (ClFanaA), fludarabine (FAraA), lamivudine (3TC)) were chosen (Figure 4A). To avoid false-positive results, activities < 10 % were checked with HPLC or the reactions were repeated with higher enzyme concentrations.

In literature, DmdNK and HsdCK were described as wide-spectrum enzymes. Indeed, both enzymes showed a very comparable broad substrate spectrum with the compounds tested. Next to dAdo, dGuo and Thd both enzymes accepted diverse base- and sugar-modified nucleotides like AraA, CldA, ClFanaA, FAraA, FAdo, dFdC, FanaU, FCyd and 3TC (Figure 4B,C). Both DmdNK and HsdCK preferred deoxy- and arabinonucleosides over ribonucleosides. Open ring derivatives GCV and ACV were not accepted by neither DmdNK nor HsdCK. One difference between the two enzymes is the acceptance of uridine and its derivatives. While DmdNK accepted uridine and EdU, this was not the case for HsdCK. Whereas most observations correspond well with literature data available for HsdCK or DmdNK, an activity towards a few substrates was not known before. This concerns the acceptance of FAdo and FCyd by HsdCK and activities towards ClFanaA, FanaU, EdU and 3TC by DmdNK.

Although viral thymidine kinases are known to accept a broad spectrum of nucleoside analogs, the tested TK displayed a narrower substrate spectrum compared to DmdNK or HsdCK (Figure 4B,C). The screening mainly revealed activities towards deoxy- or arabinonucleoside substrates. Not only pyrimidines but also some purine nucleoside analogues were converted by TK, although to a much lower extent than by DmdNK and HsdCK. Interestingly, unlike DmdNK and HsdCK, TK phosphorylates the open-ring guanosine analogs GCV and ACV, which is in good agreement with literature data on viral thymidine kinases. The ability of a viral TK to phosphorylate CldA and FanaU was shown for the first time in this study.

Compared to the other three analyzed enzymes HsAK appears to be the most specific kinase. Here, this enzyme only showed an activity for the natural purine nucleoside Ado. However, HsAK seems to be more promiscuous. Previous studies by Yamada et al. showed phosphorylation of dAdo and AraA with AK from human liver, and we showed the conversion of FAdo. Both studies relied on optimized conditions to reach significant conversions, e.g. the addition of potassium chloride (KCl) was described to be beneficial for HsAK activity, something which could be analyzed with the here described assay.

Application of the high-throughput semi-automated assay to optimize reaction conditions for HsAK

To validate the impact of optimized reaction conditions on the activity of HsAK, the semi-automated assay was applied. Reactions with potassium addition and/or an increased enzyme concentration were analyzed and compared to the standard conditions. While the addition of 50 mM KCl did not improve NMP formation, a 20-fold increase in HsAK lead to an increased substrate conversion of dAdo, AraA and FAdo (Figure 5). A combination of KCl addition and increased enzyme concentration further improved NMP formation for dAdo and FAdo. In accordance with literature data, no significant conversion of AraA was observed, which confirms the results by Yamada et al., who showed HsAK activity towards AraA to be max. 7 % compared to Ado under optimized conditions.

Higher enzyme concentrations might be applied in future screenings to avoid overlooking small activities towards single nucleoside analogues. In this context, the example of HsAK revealed that variations in reaction conditions might be necessary to ensure optimal conditions for each enzyme.
Figure 5. HsAK activity for Ado, dAdo, AraA and FAdo. Standard screening conditions are compared to optimized conditions (KCl addition and/or 20-fold increase of enzyme concentration). Reactions were performed with 0.33 mM substrate and 0.4 mM ATP in 70 mM Tris-HCl [pH 7.6] supplemented with 5 mM DTT, 10 mM MgCl$_2$, 0/50 mM KCl and 0.0002/0.004 U HsAK at 37°C for 19 h. Mean of the duplicates and standard deviations are indicated. The NMP formation was calculated from the detected ATP consumption. Ado: adenosine, dAdo: 2’-deoxyadenosine, AraA: vidarabine, FAdo: 2-fluoroadenosine.

Concluding remarks

The luciferase-based assay is very suitable to screen the activity of (d)NKs towards a wide range of nucleoside substrates. Interference of the nucleoside substrates or the NMP products with the luminescent assay was not observed. The results obtained in this study correlated very well with literature data. In addition, further previously unknown substrates were identified for HsdCK, DmdNK and the viral TK. By automation, a non-radiometric, fast, inexpensive, reliable and simple method was established which shows comparable accuracy as standard HPLC analytics. The automated luciferase-based assay has great potential to be used to screen the substrate spectrum of other kinases involved in nucleotide metabolism like NMP and NDP kinases or in inhibition/kinetic studies of nucleoside kinase catalyzed reactions. Furthermore, the impact of varying phosphate donors on the activity of nucleoside or nucleotide kinases can be evaluated as the Ultra-Glo luciferase also accepts dATP, GTP, CTP and UTP next to ATP.

Author Information

Corresponding Author

*Dr. Anke Kurreck, anke.wagner@tu-berlin.de, orcid.org/0000-0001-6919-725X

Other Authors

Katja F. Hellendahl, orcid.org/0000-0002-5408-3679
Maryke Fehlau, orcid.org/0000-0002-9365-6716
Dr. Sebastian Hans, orcid.org/0000-0002-5346-869X
Dr. Peter Neubauer, orcid.org/0000-0002-1214-9713

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Conflict of Interest

A.K. is CEO, M.F. and P.N. are board members of the biotech company BioNukleo GmbH. The authors declare no competing interest.

Experimental

General remarks

All chemicals and solvents were of analytical grade or higher and purchased, if not stated otherwise, from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), Fluka Chemie GmbH (Buchs, Switzerland), PanReac AppliChem (Darmstadt, Germany) or VWR (Darmstadt, Germany). Nucleosides and nucleotides were acquired from Alfa Aesar (Kandel, Germany), Carl Roth (Karlsruhe, Germany), Sigma-Aldrich (Steinheim, Germany), Carbosynth Limited (Berkshire, United Kingdom) and TCI (Eschborn, Germany). Stock solutions with concentrations of 100, 10 or 1 mM were prepared in deionized water and aliquots were stored at -20°C.

The human adenosine kinase (HsAK, NK14), human deoxycytidine kinase (HsdCK, NK13) and viral thymidine kinase (TK, NK16) were obtained from BioNukleo GmbH (Berlin, Germany) and stored at -20°C.
The *Drosophila melanogaster* deoxynucleoside kinase (*DmdNK*) plasmid was kindly provided by Prof. Birgitte Munch-Petersen (Roskilde University).

Expression of *DmdNK*

*DmdNK* was produced as described before. Briefly, it was expressed as a GST-fusion protein in *Escherichia coli* BL21 and purified using glutathione sepharose. The GST tag was cleaved off using thrombin and the enzyme was stored in aliquots at -20°C including 50% glycerol, 1% Triton X-100 and 1 mM dithiothreitol.

The luminescent assay

The Kinase-Glo Max assay (Promega, Madison/Wisconsin, USA) was used to detect (deoxy)NTPs in 96-well white assay plates (Costar 655094, Greiner Bio-One, Kremsmünster, Austria) as previously described. Briefly, the assay was performed with 10 µL sample/standard and 10 µL Kinase-Glo reagent following the 1:1 ratio suggested by the manufacturer. The total assay volume was adapted for the use in 96-well plate format to 100 µL by adding 80 µL deionized water. Luminescence was measured in a Synergy® Mx Multi-Mode Microplate Reader (Bio Tek Instruments, Winooski/Vermont, USA) for 1s and extended dynamic range after 10 min incubation in the dark.

Unless otherwise stated, each standard was tested twice and each sample three-times.

Detection of (deoxy)NTP and mixed ATP standards

The acceptance of (deoxy)NTPs as substrates for the Ultra-Glo luciferase were analyzed by the preparation of standards ranging from 0 to 500 µM of ATP, UTP, GTP, CTP, dATP, TTP, dGTP and dCTP in NK reaction buffer (70 mM Tris [pH 7.6], 10 mM MgCl₂, 5 mM DTT). Autoluminescence and possible luciferase inhibition, were tested with 400 µM ATP alone or together with 400 µM of Ado, dAdo, Guo, dGuo, Cyd, dCyd, Urd, or Thd in NK reaction buffer, respectively.

To analyze the impact of AMP, standards in NK buffer with 0.4 mM ATP, 0.3 mM ATP/0.1 mM AMP, 0.2 mM ATP/0.2mM AMP, 0.1 mM ATP/0.3 mM AMP and 0.4 mM AMP were prepared.

Nucleoside kinase reactions

The NK reactions were performed in a total volume of 150 µL consisting of 0.4 mM ATP, 0.33 mM nucleoside and 0.0002 U enzyme in NK reaction buffer (70 mM Tris [pH 7.6], 10 mM MgCl₂, 5 mM DTT). Reaction mixtures were incubated at 37°C for 19 h and stopped by a heat step at 75°C for 10 min.

Optimized reaction conditions included 6x (0.0012 U for TK) or 20x (0.004 U for *DmdNK* and *HsAK*) increased enzyme concentration and/or the addition of 50 mM KCl (*HsAK*).

The following controls were prepared on the same assay plate as the reactions to ensure same incubation conditions: (a) negative control without enzyme, (b) substrate control without ATP and (c) basal activity control without substrate.

To calculate the consumed ATP in the kinase reaction the following equation (1) was used, whereby R is the average luminescence signal of the kinase reaction, B is the difference of the negative control and the basal activity, S is the substrate control and N is the average luminescence signal of the negative control:

\[
\text{Consumed ATP}_{\text{consumed}} \% = 100 - \left( \frac{100 \times R + B + S}{N + S} \right)
\]

Due to the molar ratio of ATP and substrate of 1.2 : 1 the (deoxy)NMP product formation was calculated by multiplying the consumed ATP with the factor 1.2 (equation (2)).

\[
\text{Product}_{\text{consumed}} \% = \text{Consumed ATP}_{\text{consumed}} \times 1.2
\]

Values were restricted to 0-100%.

Semi-automated assay to determine the activity of nucleoside kinases

Pipetting steps were performed by the liquid handling robot Microlab Star (Hamilton Bonaduz AG, Bonaduz, Switzerland). The reaction mixture was incubated for 19 h at 37°C in 96-well PCR plates (Greiner Bio-One 652290, Kremsmünster, Austria). The PCR plates were closed with EASYseal™ sealing film (Greiner Bio-One A5596) and arched auto-sealing lids (Bio-Rad, Hercules/California, USA) preheated to 50°C. The reaction was stopped by heating to 75°C for 10 min with lid heat set to 85°C in a Primus 96 plus (MWG Biotech Inc, Ebersberg, Germany) PCR cycler. All kinases
reactions were performed in duplicates and the luciferase assay was performed in triplicates.

Accuracy of the assay
The accuracy of the assay was analyzed with colored-solutions. Therefore, the kinase reaction PCR plate and the assay plate were prepared independently by the liquid-handling station. Then, the absorbance between 300 and 700 nm were recorded and the differences in the spectra were evaluated. For the kinase reaction plate, the dyes blue (water), pink (buffer), green (substrate) and black (enzyme) were used. From the PCR plate, 100 µL were manually transferred to an assay plate for analysis. For the assay plate, blue (water), pink (kinase reaction) and black (Kinase-Glo reagent) were applied.

The Z’-value for each plate was calculated according to equation (3)[16], whereby SD\text{negative} and SD\text{substrate} are the standard deviations and Average\text{negative} and Average\text{substrate} are the means for the negative and the substrate controls, respectively.

\[
Z'\text{value} = 1 - \frac{3 \cdot SD\text{negative} + 3 \cdot SD\text{substrate}}{\text{Average}\text{negative} - \text{Average}\text{substrate}}
\]

High performance liquid chromatography (HPLC)
For HPLC analysis, 100 µL of the kinase reaction was mixed with 100 µL MeOH and centrifuged at 4°C and 21,500xg for 20 min. 75 µL of the supernatant were added to 25 µL deionized water and the mixture was transferred to HPLC vial with inlet. Samples were analyzed by HPLC-DAD (Agilent 1200 series, Santa Clara/California, USA) at 260 nm using a Kinetex Evo column (C18, 100 A, 250x4.6 mm, Phenomenex, Aschaffenburg, Germany) as described previously.[12]

Briefly, a flow rate of 1 ml min\(^{-1}\), a separation temperature of 34 °C and a gradient of A (KH\(_2\)PO\(_4)/K\(_2\)HPO\(_4\); 0.1 M, tetrabutylammonium bisulfate: 8 mM, pH 5.4) and B (70 % A, 30 % methanol) was applied: 0 min – 80 % A, 4 min – 80 % A, 14 min – 40 % A, 35 min – 36.5 % A, 35.5 min – 80 % A and 38 min – 80 % A.

Typical retention times [min] were for AMP – 7.3, ADP – 14 and ATP – 20.8. The retention times of the (deoxy)nucleosides and (deoxy)nucleoside monophosphates are given in the Supplementary Material (Table S5).

The consumed ATP and formed product in the kinase reaction mix was calculated using the following equations (4) and (5), whereby \(A_X\) is the peak area of ATP, \(A_{total}\) is the sum of all adenosine nucleotides (ATP, ADP, AMP), \(P_X\) is the peak area of the produced NMP and \(P_{total}\) is the sum of the substrate and product(s):

\[
\text{Consumed ATP}\% = \left( 1 - \frac{A_X}{A_{total}} \right) \times 100 \tag{4}
\]

\[
\text{Product}\% = \frac{P_X}{P_{total}} \times 100 \tag{5}
\]

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