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A Method for Quantification of Nucleotides and Nucleotide Analogues in Thymidine Kinase Assays Utilizing Lanthanum Phosphate Co-Precipitation

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Abstract

Current methodologies for quantifying radiolabeled nucleoside monophosphates and nucleoside analogues result in high retention of unphosphorylated guanosine nucleosides in the case of lanthanum chloride precipitation or inconsistent retention of nucleotides in the case of DEAE cellulose filter papers. This study describes an innovative method for quantifying TK activity that is compatible with both purine and pyrimidine nucleoside analogues by utilizing lanthanum phosphate co-precipitation at a pH of 4. This methodology maintains quantitative precipitation of nucleoside monophosphates and yields minimal background binding from a variety of nucleoside analogues. In addition, use of PCR thermocyclers enhances the temporal precision of TK assays. This method was shown to be useful for assaying TK activity in a broad range of biochemically relevant systems, including purified enzymes, stable cell lines, and virally infected cells. Utilization of this methodology should aid researchers in the evaluation of novel nucleoside analogues and TK enzymes, while decreasing radioactive waste, minimizing assay time, increasing accuracy, and enhancing dynamic range.

Introduction

In mammalian cells, salvage pathway phosphorylation of 2'-deoxythymidine (dThy) is catalyzed by two thymidine kinases (TK), cell-cycle dependent TK1 and constitutively active mitochondrial TK2. These TKs phosphorylate dThy and close structural analogues thereby trapping them within the cell. Normally, these enzymes maintain a sufficient pool of nucleotides for DNA replication and repair. In cancer chemotherapy, human TK, up-regulated in rapidly dividing cells such as malignant cancers, aids in conversion of the chemotherapeutic fluorouracil into its toxic metabolite 5-fluoro-2'-deoxyuridine-5'-monophosphate (5F-dUMP). [1] Viral and insect TKs phosphorylate a wider variety of nucleosides and nucleoside analogues than human TKs.[2–5] Herpes simplex virus-1 thymidine kinase (HSVTK) is one such example

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and is a target for anti-viral pro-drugs such as acyclovir, penciclovir, and their derivatives.[5, 6] HSVTK also has been utilized clinically in the field of suicide gene therapy. Researchers have injected primary tumors with viral vectors encoding HSVTK and then treated with pro-drugs such as ganciclovir to selectively induce apoptosis within the tumor.[7,8] Recently, HSVTK and related mutants have been used as genetically encoded reporters for *in vivo* imaging of viral delivery, promoter activation, protein-protein interactions, and cell migration in living mice through use of radiolabeled nucleoside analogues and positron emission tomography.[9–13] New TK enzymes and substrates with improved specificity and selectivity continue to be developed, [14–17] driving the need for a facile and quantitative *in vitro* method for characterizing these systems.

Current methodologies for quantifying radiolabeled nucleotides result in either high retention of the unphosphorylated nucleoside or unproven retention of nucleotide. For example, methods exist utilizing DEAE cellulose paper for quantifying radiolabeled 2'-deoxythymidine-5'-monophosphate (dTMP), but the samples are subjected to a variety of washing and processing conditions, thus causing variable retention of both nucleosides and nucleotides.[18,19] For tritiated nucleotides, improvements that eliminate β^- particle absorption artifacts by digesting the paper with cellulase have been reported, but this additional step adds time and cost.[20] Methods using aluminum oxide columns for purifying nucleotides have been developed and tested against known standards, but column purification is time consuming and generates significant waste.[21] Another method uses precipitation of dTMP with aggregated lanthanum chloride which is rapid, quantitative, and more sensitive than DEAE cellulose trapping.[22] However, this method has been validated only using dThy as the substrate for HSVTK and applicability to other nucleoside pro-drugs remains to be determined. Here, we describe an innovative method for quantifying TK activity that is compatible with both purine and pyrimidine nucleoside analogues by utilizing lanthanum phosphate co-precipitation.

Materials and Methods

Lanthanum Chloride Precipitation Assay

Lanthanum chloride precipitation was performed using previously published methodologies.[22] Briefly, a solution of 100 mM LaCl_3 (Sigma, St. Louis, MO) and 5 mM ethanolamine (Sigma) was prepared. Solution (1 mL) was added to 1.5 mL Eppendorf tubes. Aliquots of radioactive sample (2 to 20 μL) were added to the solution and then vortexed. The tubes were then centrifuged at 14,400 rpm on a table-top centrifuge for 10 min. The pellet was then washed with 1 mL of lanthanum chloride solution, centrifuged, and the supernatant removed. The pellet was re-dissolved in 0.05 M HCl and counted in Ready SafeTM scintillant (Beckman Coulter, Fullerton, CA).

Lanthanum Phosphate Co-Precipitation Assays

A method for assaying nucleotide content was then developed using a lanthanum phosphate/nucleotide co-precipitation strategy. A co-precipitation solution (PS) was prepared with 100 mM sodium acetate (Sigma), 100 mM lanthanum chloride (Sigma), and 0.6 g/L of sodium phosphate mono-basic (Sigma); pH adjusted to 4.0. The PS was well mixed before use, and 500 μL was added to 1.5 mL Eppendorf tubes.

For validating the method with known chemical standards, aliquots of [³H]-thymidine (60–90 Ci/mmol), [³H]-penciclovir (10–30 Ci/mmol), [³H]-guanosine (5–15 Ci/mmol), [³H]-dTMP (25–60 Ci/mmol), [³H]-guanosine-5'-monophosphate (GMP) (5–15 Ci/mmol), and [¹⁴C]-1-(2-deoxy-2-fluoro- β -D-arabinofuranosyl)-5-iodouracil (FIAU) (45–60 mCi/mmol) (Moravsek Biochemicals, Brea, CA) were added to PS. Tubes were vortexed briefly, incubated at room temperature for 30 minutes, and centrifuged at 14,400 rpm on a table top centrifuge for 10 min.

The supernatant was removed using a vacuum flask with a gel loading tip on the tubing end to control flow; this prevented aspiration of the pellet. The pellet was then rinsed once with 500 μ L of PS. After centrifugation, the supernatant was again removed. Pellets were finally re-dissolved in 500 μ L of 0.5 M HCl by stirring with a pipette tip and added to 0.5 mL of Ready Safe™ scintillant in scintillation tubes. Tubes were wiped with Kimwipes™ and equilibrated in the dark before counting on a Beckman scintillation counter.

During an experiment with purified enzymes or cell lysates, an aliquot (1 to 50 μ L) was removed from the TK assay solution and immediately added to tubes containing PS. Afterward, the tubes were vortexed and incubated at room temperature for at least 30 min. The tubes were then centrifuged at 14,400 rpm on a table-top centrifuge for 10 min. The supernatant was removed and rinsed with 0.5 to 1.0 mL of PS, centrifuged, and the supernatant was again removed. The pellets were finally re-dissolved by adding 300 to 500 μ L of 0.5 M HCl and stirred with a pipette tip. The re-dissolved pellets were then transferred to 5 mL of scintillant in scintillation tubes.

HSVTK Enzyme Purification and Assays

For this analysis, wild type HSVTK was purified as previously described with minor modification.[23] A dThy affinity column was prepared from 3-amino-thymidine (Sigma) and a Hi-Trap NHS activated HP column (GE Healthcare, Piscataway, NJ) as per the manufacturer's protocol. TK assay buffer consisted of 5 mM MgCl₂, 5 mM ATP (Sigma), 2 mg/mL BSA (Sigma), and 50 mM Tris (Sigma), pH = 7.5, for comparing HSVTK assays or of 140 mM KCl, 10 mM NaCl (Sigma), 5 mM MgCl₂, 5 mM ATP, 2 mg/mL BSA, 50 mM Tris, pH = 7.5, for quantification of enzyme kinetics. Assay buffer was prepared from 10X stock solutions (stored at -20°C) by diluting with milli-Q water up to the final volume while taking into account the remaining volume of nucleoside to be added. TK assays were performed in thin walled-PCR tubes with reaction volumes ranging from 8 to 100 μ L; the temperature was controlled by a PCR thermocycler with a heated lid. For each assay, a 2 min preparatory hold was performed at 4°C; then at 1.5 minutes into the hold, the nucleoside (e.g., [³H]-dThy (Moravsek); 8 μ L of a 7.3 μ M stock) was added to the reaction and mixed by pipetting the full volume. The thermocycler then rapidly increased the temperature to 37°C to initiate the reaction, and aliquots were manually withdrawn at the required time points. To minimize evaporation, the lid of the thermocycler remained closed between time points.

Cell Culture and Lysate Assays

HeLa cells were cultured in vented flasks at 37°C (Fisher Scientific, Pittsburgh, PA) in a 5% CO₂, 20% O₂, 75% N₂, water-saturated atmosphere. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% v/v heat inactivated fetal bovine serum and 2 mM glutamine. Adenovirus encoding mSR39 HSVTK was prepared and infected as previously published. [24]

Cell lysates were prepared as follows. Lysis buffer consisted of 0.5% Igepal CA-630 (NP-40) (Sigma), 25 mM sodium fluoride (Sigma), and 10 mM Tris, pH=7.0. Cells were cultured in 35 mm dishes and 200 μ L of lysis buffer was added to each dish. Supernatant was removed from the dishes, quantified for protein, and diluted to a final protein concentration of 200 μ g/mL. Fresh beta-mercaptoethanol (Sigma) was then added to a final concentration of 3 mM. Samples were then stored at -20°C for later use, including RT-PCR.

TK assays from HeLa cell lysates were performed similarly to those with purified enzyme. TK assay buffer for cell lysates was prepared fresh with 12 μ M [³H]-penciclovir, 24 mM ATP, 24 mM magnesium acetate (Sigma), 240 mM sodium phosphate pH=7.0. TK assay buffer (3 μ L) was mixed with 5 μ L of protein-normalized cell lysate at 4°C. Samples were processed in a

thermocycler as above with an incubation time of 20 min at 37°C. The entire 8 µL was then processed by lanthanum phosphate co-precipitation as above.

For selected experiments, HeLa cell lysates were prepared as above and then subjected to reverse transcriptase followed by semi-quantitative PCR (RT-PCR). Total RNA was harvested using RNA-WIZ (Ambion, Austin, TX) and quantified by UV-Vis spectroscopy. In addition, samples were run on a denaturing formaldehyde gel and stained with ethidium bromide to confirm concentration and integrity of RNA. RNA (5 µg) was used in a reverse transcription reaction with a Superscript III RT Kit (Invitrogen, Carlsbad, CA). Poly-T primer supplied in the kit was used for first strand synthesis (1h at 50°C), and after stopping by heating to 85°C for 5 min, 2 µL of RT product was used in a 25 µL PCR with Hotmaster Mix (Eppendorf, Westbury, NJ). The primers 5' caatgggcatgccttatgcc (forward) and 5'ctgcagataccgcaccgta (reverse) were used to identify HSVTK. An RT null was included to confirm that no contaminating DNA was present in sample. PCR cycling was performed using an Eppendorf Mastercycler PCR machine and cycling conditions were programmed as follows: 95°C for 2 min, 25 cycles of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C. Samples of each PCR product (10 µL) were separated on a 1% agarose gel, stained with ethidium bromide and photographed. ImageJ was used for quantitative densitometric analysis of bands. Note that no saturated pixels were present in the quantified portion of each image ensuring accurate quantification.

Results and Discussion

Validation with Chemical Standards

First, we tested the conventional lanthanum chloride precipitation methodology with dTMP and several nucleoside analogues. This protocol quantitatively recovered $100 \% \pm 12 \%$ (SD, n=10) of dTMP (Table 1). There also was low non-specific binding of dThy to the pellet ($0.454 \% \pm 0.23 \%$; SD, n=10). These results were in agreement with the published robustness of the lanthanum chloride precipitation assay for recovery of dThy.[22] In contrast, when the method was extended to purine nucleoside analogues such as penciclovir, $3.8 \% \pm 1.3 \%$ (SD, n=24) of the unphosphorylated nucleoside was bound to the pellet. For assaying initial rates of enzyme activity, this would be an unacceptably high background since initial rates must be determined at less than 10 % substrate consumption.

It was previously reported that lanthanum binds guanidine residues in DNA at the N7 position on the guanine ring.[25] Because the N7 position has a pK_a between 3 and 4,[25,26] non-specific binding of guanine nucleoside analogues could result from interaction of the base with lanthanum ions at the N7 position. Therefore, we reasoned that non-specific binding should be reduced by lowering the pH of the co-precipitation solution. When the published lanthanum chloride precipitation solution was shifted to an acid pH, no visible precipitate was formed and no radioactive counts above background were detected when utilized in a TK assay (data not shown). Thus, it appeared that acid pH solubilized lanthanum chloride precipitates. A suitable co-precipitant for the phosphorylated nucleosides should be highly insoluble in the presence of lanthanum at acidic pH, be inexpensive and available in laboratories, readily precipitate phosphorylated nucleosides, and ideally, re-dissolve for transfer to scintillation vials. Sodium phosphate was then chosen as the co-precipitant because, unlike the published methodology, lanthanum phosphate is completely insoluble at a pH of 1 ($pK_s = 26.15$), and thus, it should be a suitable co-precipitant for nucleotides at acid pH.[27] A pH of 4 was selected as the solution pH because both non-specific binding of the nucleoside and acid catalyzed hydrolysis of the nucleotide should be minimized.[28] Sodium phosphate was also a readily available and inexpensive laboratory reagent which should allow quick adoption by any laboratory. Finally, the lanthanum phosphate pellet and corresponding bound nucleotides were readily re-dissolved in 0.5 M HCl for further processing.

Shifting to acid pH and utilizing phosphate as the co-precipitant permitted the quantitative extraction of dTMP while providing advantageously low background binding of the corresponding nucleosides. Furthermore, the lanthanum phosphate co-precipitation method was successful in retaining quantitative extraction of phosphorylated purine nucleosides while decreasing non-specific binding of purine nucleoside analogues. When different co-precipitation times (1, 10, 30, and 120 min) were experimentally tested with [^3H]-GMP, there was quantitative co-precipitation and recovery of [^3H]-GMP at all time points ($107 \pm 5\%$; SD; $n = 3$ each). Thus, the system rapidly reached an equilibrium allowing for flexibility in sample processing. As evidence for the overall increase in sensitivity, lanthanum phosphate co-precipitation yielded a 5-fold increase in the signal to noise ratio for recovery of [^3H]-dTMP (Table 1). Importantly, the background binding of penciclovir was suppressed to 0.2 %, a level that should readily allow quantification of initial rates of enzyme activity. Since the lanthanum phosphate co-precipitant met all of the requirements for a suitable co-precipitant, no others were tested. Thus, by shifting the solution to pH 4.0, and changing the co-precipitant from an aggregate of lanthanum, chloride, and ethanolamine to lanthanum phosphate, we now retained quantitative trapping of both phosphorylated purine and pyrimidines while minimizing non-specific binding of a variety of nucleosides and nucleoside analogues.

Lanthanum phosphate co-precipitation conditions are further advantageous because the method immediately stops TK activity upon addition of samples to PS. To experimentally test this, 2 μL of a 5 mM ATP solution, the same amount of ATP as in TK assay buffer, was added to PS. ATP visually precipitated out of solution as fast as it could be added. Since the solubility constant for lanthanum phosphate is on the order of 10^{-26} , the TK reaction would be terminated immediately due to loss of the critical substrate ATP. Secondly, the acid pH and low temperature of PS should inhibit any residual enzyme activity.

When the lanthanum phosphate co-precipitation assay was compared with the DEAE cellulose methodology, two major advantages became apparent. First, both a purine and a pyrimidine monophosphate were precipitated and retained quantitatively, which had not been demonstrated for these techniques.[18,19] In earlier publications utilizing the DEAE cellulose method, the percent of a nucleoside retained after washing was calculated from quantitative standards.[29] As reported, the best washing method (ethanol washes $\times 2$), showed that 71% of dTMP was retained vs. 0.76% of dThy. This yields a specific to non-specific ratio of 93; the signal to noise could not be compared as no standard deviations were reported. The more common method of washing, i.e., extensive ammonium formate followed by water and ethanol, was first described by Breitman.[30] dTMP was retained quantitatively on filters while 2% of dThy was retained, yielding a specific to non-specific ratio of 50.[30] However, no raw data or methods indicating how these controls were performed was presented, so the signal to noise ratio could not be calculated. Overall, the lanthanum phosphate co-precipitation methodology reported herein yielded a 10- to 20-fold increase in specific to non-specific ratios for thymidine over DEAE cellulose methodologies (Table 1). The second major advantage of this technique was the significant reduction in radioactive waste and time. For the DEAE cellulose treatments as well as the column chromatography treatments, between 20 mL and 100 mL of liquid radioactive waste are generated per sample. Utilizing the lanthanum phosphate co-precipitation, only ~ 1 mL of liquid radioactive waste was generated, a 100-fold reduction. Furthermore, because the pellet could be re-solubilized in HCl, there were no β^- particle absorption artifacts. This eliminated a time consuming (hours) digestion step at 37°C from the DEAE cellulose trapping methodology in order to maintain accurate quantification of tritiated compounds.

Validation with Purified Enzymes and Mammalian Cell Lysates

Purified HSVTK enzyme activity was readily detectable utilizing the lanthanum phosphate co-precipitation method. Assays for purified HSVTK activity were conducted over a wide range of enzyme or substrate concentration. The lanthanum phosphate co-precipitation methodology yielded a K_m and k_{cat} for dThy with HSVTK of 0.30 ± 0.14 (SEM) μM and 0.50 ± 0.08 (SEM) 1/sec, respectively. The K_m was within the range of reported values (0.2 to 0.9 μM). [23,31–34] Meanwhile, k_{cat} was slightly higher than reported values in the literature (0.46 1/sec) as would be expected since quantitative recovery of dTMP now has been demonstrated with the present technique. The assay was tested with other substrates as well. As an example, the linearity of penciclovir phosphorylation was tested with regard to time and concentration of HSVTK (Figure 1). Purified HSVTK was diluted to the indicated concentrations and incubated in TK assay buffer (50 μL) supplemented with 61 μM [3H]-penciclovir for 5 sec before precipitating aliquots (45 μL) in PS. Time and penciclovir concentration were selected such that less than 10% of the substrate was converted for all concentrations of enzyme tested. Linearity ($R^2 > 0.90$) was clearly maintained over time and a significant range of HSVTK concentrations (Figure 1). The assay could be readily extended to lower concentrations of enzyme by increasing assay time from 5 seconds to minutes (data not shown).

The methodologies developed herein were also validated for quantifying HSVTK activity in cell lysates. A HeLa cell line that stably expresses mSR39 HSVTK (a mutant HSVTK) was previously generated and characterized.[13] This cell line has proven useful for evaluating radiolabeled nucleosides for imaging applications. For HSVTK assays using [3H]-penciclovir, lysates from this cell line as well as a parental HeLa cell line were prepared. These extracts were not further purified, and therefore contained other endogenous proteins, including native human TK1 and TK2, nucleosides and nucleotides that could potentially interfere with quantification of HSVTK. Nonetheless, the assay yielded a signal to noise ratio of 63,000 and target to non-target ratio of 1,900 indicating the robustness and sensitivity of the methodology for detection of HSVTK activity in complex cell lysates (Figure 2A). Note that reducing incubation times such that less than 10% of the penciclovir would be converted to penciclovir-monophosphate should increase the linearity of the assay with regard to HSVTK enzyme concentration, but would decrease the sensitivity of the assay.

HSVTK has proven utility as a marker for imaging gene therapy both *in vitro* and *in vivo*. To demonstrate use of the assay for studying these vectors, HeLa cells were infected with an adenovirus that encodes for mSR39 HSVTK, a reporter strategy that has been utilized for imaging lung infection *in vivo*. [24] Cell lysates were prepared from both uninfected and infected HeLa cells. Samples from each cell lysate were analyzed by the TK assay using the lanthanum phosphate co-precipitation methodology and also subjected to reverse transcriptase followed by semi-quantitative PCR. The data were quantified and a strong correlation was found between the TK activity assay and TK mRNA; $r = 0.998$; $p = 0.0025$ (Figure 2B). Despite the multiple layers of experimentation involved in this assay, the lanthanum phosphate co-precipitation methodology showed a clear linear relationship between TK activity and mRNA post viral infection in living cells. This technique also enabled quantification of TK activity *ex vivo* post lung infection with adenovirus in living animals (data not shown).

Conclusion

We have demonstrated an extension of the lanthanum chloride precipitation method for detecting TK activity to now include assaying a broad spectrum of nucleoside analogues by utilizing lanthanum phosphate co-precipitation at a pH of 4. This methodology maintained quantitative co-precipitation of both purine and pyrimidine mono-phosphates and yielded minimal background binding from a variety of nucleoside analogues. In addition, use of PCR thermocyclers enhanced the temporal precision of TK assays. This method was shown to be

useful for assaying TK activity in a broad range of biochemically relevant systems, including purified enzymes, stable cell lines, and virally infected cells. Adoption of lanthanum phosphate co-precipitation for TK assays will aid researchers in the evaluation of novel nucleoside analogues and TK enzymes, while decreasing radioactive waste, minimizing assay time, increasing accuracy, and enhancing dynamic range.

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References

1. Pinedo HM, Peters GF. Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988;6:1653–64. [PubMed: 3049954]
2. Johansson M, van Rompay AR, Degreve B, Balzarini J, Karlsson A. Cloning and characterization of the multisubstrate deoxyribonucleoside kinase of *Drosophila melanogaster*. *J Biol Chem* 1999;274:23814–9. [PubMed: 10446143]
3. Vogt J, Perozzo R, Pautsch A, Protá A, Schelling P, Pilger B, Folkers G, Scapozza L, Schulz GE. Nucleoside binding site of herpes simplex type 1 thymidine kinase analyzed by X-ray crystallography. *Proteins* 2000;41:545–53. [PubMed: 11056041]
4. Balzarini J, Liekens S, Solaroli N, El Omari K, Stammers DK, Karlsson A. Engineering of a single conserved amino acid residue of herpes simplex virus type 1 thymidine kinase allows a predominant shift from pyrimidine to purine nucleoside phosphorylation. *J Biol Chem* 2006;281:19273–9. [PubMed: 16702226]
5. Black M, Newcomb T, Wilson H, Loeb M. Creation of drug-specific herpes simplex virus type 1 thymidine kinase mutants for gene therapy. *Proc Natl Acad Sci USA* 1996;93:3525–3529. [PubMed: 8622970]
6. Gupta R, Wald A. Genital herpes: antiviral therapy for symptom relief and prevention of transmission. *Expert Opin Pharmacother* 2006;7:665–75. [PubMed: 16556084]
7. Stermán DH, Recio A, Vachani A, Sun J, Cheung L, DeLong P, Amin KM, Litzky LA, Wilson JM, Kaiser LR, Albelda SM. Long-term follow-up of patients with malignant pleural mesothelioma receiving high-dose adenovirus herpes simplex thymidine kinase/ganciclovir suicide gene therapy. *Clin Cancer Res* 2005;11:7444–53. [PubMed: 16243818]
8. Hinata N, Shirakawa T, Terao S, Goda K, Tanaka K, Yamada Y, Hara I, Kamidono S, Fujisawa M, Gotoh A. Progress report on phase I/II clinical trial of Ad-OC-TK plus VAL therapy for metastatic or locally recurrent prostate cancer: Initial experience at Kobe University. *Int J Urol* 2006;13:834–7. [PubMed: 16834676]
9. Ponomarev V, Doubrovin M, Lyddane C, Beresten T, Balatoni J, Bornman W, Finn R, Akhurst T, Larson S, Blasberg R, Sadelain M, Tjuvajev J. Imaging TCR-dependent NFAT-mediated T-cell activation with positron emission tomography in vivo. *Neoplasia* 2001;3:480–488. [PubMed: 11774030]
10. Richard JC, Zhou Z, Chen DL, Mintun MA, Piwnica-Worms D, Factor P, Ponde DE, Schuster DP. Quantitation of pulmonary transgene expression with PET imaging. *J Nucl Med* 2004;45:644–654. [PubMed: 15073262]
11. Le LQ, Kabarowski JHS, Wong S, Nguyen K, Gambhir SS, Witte ON. Positron emission tomography imaging analysis of G2A as a negative modifier of lymphoid leukemogenesis initiated by the BCR-ABL oncogene. *Cancer Cell* 2002;1:381–391. [PubMed: 12086852]
12. Gross S, Piwnica-Worms D. Spying on cancer: molecular imaging in vivo with genetically encoded reporters. *Cancer Cell* 2005;7:5–15. [PubMed: 15652745]
13. Luker G, Sharma V, Pica C, Dahlheimer J, Li W, Ochlesky J, Ryan C, Piwnica-Worms H, Piwnica-Worms D. Noninvasive imaging of protein-protein interactions in living animals. *Proc Natl Acad Sci USA* 2002;99:6961–6966. [PubMed: 11997447]

14. Solaroli N, Johansson M, Balzarini J, Karlsson A. Substrate specificity of three viral thymidine kinases (TK): vaccinia virus TK, feline herpesvirus TK, and canine herpesvirus TK. *Nucleosides Nucleotides Nucleic Acids* 2006;25:1189–92. [PubMed: 17065088]
15. Solaroli N, Johansson M, Balzarini J, Karlsson A. Enhanced toxicity of purine nucleoside analogs in cells expressing *Drosophila melanogaster* nucleoside kinase mutants. *Gene Ther* 2007;14:86–92. [PubMed: 16885999]
16. Balatoni JA, Doubrovin M, Ageyeva L, Pillarsetty N, Finn RD, Gelovani JG, Blasberg RG. Imaging herpes viral thymidine kinase-1 reporter gene expression with a new 18F-labeled probe: 2'-fluoro-2'-deoxy-5-[18F]fluoroethyl-1-beta-d-arabinofuranosyl uracil. *Nucl Med Biol* 2005;32:811–9. [PubMed: 16253805]
17. Willmon CL, Krabbenhoft E, Black ME. A guanylate kinase/HSV-1 thymidine kinase fusion protein enhances prodrug-mediated cell killing. *Gene Ther* 2006;13:1309–12. [PubMed: 16810197]
18. Litteria M, Popoff CG. An efficient washing procedures for thymidine kinase assay. *Neurochem Res* 1981;6:931–4. [PubMed: 7312102]
19. Munch-Petersen B, Cloos L, Tyrsted G, Eriksson S. Diverging substrate specificity of pure human thymidine kinases 1 and 2 against antiviral dideoxynucleosides. *J Biol Chem* 1991;266:9032–8. [PubMed: 2026611]
20. Gerber S, Folkers G. A new method for quantitative determination of tritium-labeled nucleoside kinase products adsorbed on DEAE-cellulose. *Biochem Biophys Res Comm* 1996;225:263–267. [PubMed: 8769128]
21. el Kouni MH, Cha S. A simple radioisotopic assay for nucleoside kinases employing alumina for separation of nucleosides and nucleotides. *Anal Biochem* 1981;111:67–71. [PubMed: 6263130]
22. Wolcott R, Colacino J. Detection of thymidine kinase activity using an assay based on precipitation of nucleoside monophosphates with lanthanum chloride. *Anal Biochem* 1989;178:38–40. [PubMed: 2729577]
23. Kokoris MS, Black ME. Characterization of Herpes Simplex Virus type 1 thymidine kinase mutants engineered for improved ganciclovir or acyclovir activity. *Protein Science* 2002;11:2267–2272. [PubMed: 12192082]
24. Richard JC, Zhou Z, Ponde DE, Dence CS, Factor P, Reynolds PN, Luker GD, Sharma V, Ferkol T, Piwnica-Worms D, Schuster DP. Imaging pulmonary gene expression with positron emission tomography. *Am J Respir Crit Care Med* 2003;167:1257–63. [PubMed: 12505860]
25. Tajmir-Riahi HA, Ahmad R, Naoui M. Interaction of calf-thymus DNA with trivalent lanthanum, europium, and terbium ions. Metal ion binding, DNA condensation and structural features. *J Biomolec Structure Dynamics* 1993;10:865–877.
26. Rogstad KN, Jang YH, Sowers LC, Goddard WA 3rd. First principles calculations of the pKa values and tautomers of isoguanine and xanthine. *Chem Res Toxicol* 2003;16:1455–62. [PubMed: 14615972]
27. Firsching FH, Brune SN. Solubility products of the trivalent rare-earth phosphates. *J Chem Engineering Data* 1991;36:93–95.
28. Oivanen M, Schnell R, Pfeleiderer W, Lonnberg H. Interconversion and hydrolysis of monomethyl and monoisopropyl esters of adenosine 2'-monophosphates and 3'-monophosphates - kinetics and mechanisms. *J Organic Chem* 1991;56:3623–3628.
29. Furlong NB. A rapid assay for nucleotide kinases using C14-or H3-labeled nucleotides. *Anal Biochem* 1963;5:515–22. [PubMed: 13945869]
30. Breitman TR. The feedback inhibition of thymidine kinase. *Biochim Biophys Acta* 1963;67:153–5. [PubMed: 14015204]
31. Wurth C, Thomas RM, Folkers G, Scapozza L. Folding and self-assembly of Herpes Simplex Virus Type 1 thymidine kinase. *J Molec Biol* 2001;313:657–670. [PubMed: 11676546]
32. Balzarini J, Liekens S, Esnouf R, De Clercq E. The A167Y mutation converts the Herpes Simplex virus type 1 thymidine kinase into a guanosine analogue kinase. *Biochemistry* 2002;41:6517–6524. [PubMed: 12009916]
33. Kokoris M, Sabo P, Adman E, Black M. Enhancement of tumor ablation by a selected HSV-1 thymidine kinase mutant. *Gene Therapy* 1999;6:1415–1426. [PubMed: 10467366]

34. Hinds TA, Compadre C, Hurlburt BK, Drake RR. Conservative mutations of glutamine-125 in Herpes Simplex virus type 1 thymidine kinase result in a ganciclovir kinase with minimal deoxypyrimidine kinase activities. *Biochemistry* 2000;19:4105–4111. [PubMed: 10747801]

Figure 1.

A) Linearity of enzyme reaction as a function of time as determined with 0.01 μM HSVTK and 1 μM [³H]-penciclovir, $R^2 > 0.99$. B) Linearity of penciclovir phosphorylation rate as a function of enzyme concentration using 61 μM penciclovir. Best fit to data (GraphPad Prism, GraphPad Software Inc., San Diego, CA) is represented as solid line, $R^2 > 0.90$. Concentration of phosphorylated penciclovir was calculated by subtracting empirically determined non-specific binding of nucleoside for each assay and normalizing to specific activity.

Figure 2.

A) Cell lysates were prepared from parental HeLa cells, HeLa cells stably expressing mSR39 HSVTK (mTK HeLa), HeLa cells infected with 10^8 pfu adenovirus (HeLa + nullAd), or HeLa cells infected with 10^8 pfu adenovirus encoding for mSR39 HSVTK (HeLa + mSR39TKAd). Lysates were analyzed for TK activity with [3 H]-penciclovir and then processed by lanthanum phosphate co-precipitation. Data are displayed as CPM. Error bars represent standard deviations ($n = 3$). B) HeLa cells were infected with mSR39TKAd at virus titers: 0, 10^8 , 10^9 , or 10^{10} pfu. Cell lysates were prepared and subjected to semi-quantitative RT-PCR or the lanthanum phosphate co-precipitation TK assay as in A. TK assay results correlated with

mSR39TK mRNA; $r = 0.998$ ($p = 0.0025$). The best linear fit was found to be: $Y = (26.95 \times 10^4) X - 0.156 \times 10^4$; $R^2 = 0.995$.

Table 1

Comparing recovered nucleotides and nucleosides with lanthanum phosphate co-precipitation and lanthanum chloride precipitation methodologies. Signal to noise ratio was calculated as % bound phospho-nucleoside over SD of bound nucleoside or nucleoside analogue; SD refers to standard deviation. Specific to non-specific ratio was calculated as % bound phospho-nucleoside over % bound corresponding nucleoside or analogue. ND; not determined.

Quantitative Comparison of Precipitation Methods for Recovery of Nucleosides and Nucleotides

	% Bound (SD)	Specific/Non-Specific	Signal/Noise
Lanthanum Phosphate Co-Precipitation			
<i>Phosphorylated Nucleosides</i>			
2'-deoxy-thymidine 5'-monophosphate	100 (3.80)		
guanosine 5'-monophosphate	105 (15.0)		
<i>Nucleosides and Analogues</i>			
thymidine	0.105 (0.048)	952	2085
FIAU	0.166 (0.100)	602	998
guanosine	0.200 (0.030)	525	3540
penciclovir	0.227 (0.013)	462	7997
Lanthanum Chloride Precipitation			
<i>Phosphorylated Nucleosides</i>			
2'-deoxy-thymidine 5'-monophosphate	100 (12.0)		
<i>Nucleosides and Analogues</i>			
thymidine	0.454 (0.230)	220	432
penciclovir	3.80 (1.30)	ND	ND