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Cloning and Effective Induction of *Escherichia coli* Nucleoside Diphosphate Kinase by Lactose

Pattama Howhan[a] and Somchai Pornbanlualap[a,b,*]

[a] Interdisciplinary Graduate Program in Genetic Engineering, Graduate School, Kasetsart University, Bangkok, Thailand
[b] Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand
* Corresponding author, E-mail: fsciscpl@ku.ac.th

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ABSTRACT: The complete PCR-derived DNA fragment containing the structural gene for *Escherichia coli* nucleoside diphosphate kinase (*ndk*) gene was cloned into pET-26b, a T_7 promoter-based expression vector. After transforming recombinant pET-*ndk* into the *E. coli* BL21(DE3) pLysS, the expression of *ndk* gene was induced by addition of either IPTG or lactose. Surprisingly, lactose was found to be as effective inducer as IPTG. Both inducers gave almost the same level of expression of *ndk* gene. The rate of induction of nucleoside diphosphate (NDP) kinase by lactose, however, occurred at a much slower rate than by IPTG. Because the pET system had become one of the most powerful and commonly used vector for expressing recombinant proteins, this study to our knowledge is the first to demonstrate that lactose can be substituted for the more costly IPTG as inducer. Furthermore, because the kinetics of induction by lactose occurred at a much slower rate than IPTG, lactose may be the preferred inducer over IPTG in the situation where induction of recombinant protein resulted in formation of insoluble inclusion bodies. The recombinant NDP kinase containing polyhistidine tagged at the C-terminus was purified in a single step by Ni^{2+}-NTA affinity chromatography. The purified enzyme contained two major bands, one that migrated at 16 KDa which corresponding to monomeric form and the other at 32 KDa which corresponding to the dimeric form of enzyme. This purification protocol resulted in a yield of 132 mg of protein per one liter of cell culture. The purified recombinant enzyme that contained polyhistidine tagged at the C-terminus retained its activity, with the specific activity of 2,620 units/mg. This polyhistidine tagged NDP kinase can be reversibly immobilized onto Ni^{2+}-NTA agarose in a column for a more efficient, semi-solid state synthesis of deoxyribonucleoside 5´-triphsophate.

KEYWORDS: Nucleoside diphosphate kinase, *Escherichia coli ndk* gene, mechanism of induction, protein expression, effective inducer, kinetics of induction, lactose.

INTRODUCTION

2´-Deoxyribonucleoside triphosphates (dNTPs) are essential precursors for synthesis of DNA in prokaryotes and eukaryotes.[1] With recent advances in PCR technology, dNTPs have become indispensable reagents used in polymerase chain reactions. Because of their high costs, the ability to synthesize dNTPs and their analogues at a large-scale is an economically important project. Both chemical and enzymatic methods had been developed for synthesis of nucleoside triphosphates. If the nucleoside monophosphate is available, synthesis of the nucleoside triphosphate can be accomplished either by pyrophosphorolysis of nucleoside phosphorimidazolidate[2] or by enzymatic phosphorylation of nucleoside mono- and diphosphate.[3] The enzymatic method was found to be superior to chemical synthesis because conditions that would hydrolyze the nucleotides could be avoided.

Whitesides and coworkers[4] have developed an efficient enzymatic synthesis of ribonucleoside triphosphates (NTPs) on a 10 g scale. Enzymatic conversion of ribonucleoside 5´-monophosphate (NMP) to a ribonucleoside 5´-triphosphate (NTP) requires two kinases: one for NMP and one for NDP. Pyruvate kinase (EC 2.7.1.40) is used to convert all four NDPs (ADP, CDP, GDP, and UDP) to the corresponding NTPs, using phosphoenolpyruvate (PEP) as phosphoryl donor (Scheme 1). Preparation of NDPs from NMPs, however, is more difficult because no single enzyme is known to convert all of the NMPs to NDPs. Thus, conversion of NMP to NDP had been performed separately, using commercially available adenylate kinase (EC 2.7.4.3) to convert AMP to ADP, guanylate kinase (EC 2.7.4.4) to convert GMP to GDP, cytidyl kinase (EC 2.7.4.14) to convert CMP to CDP, and uridyl kinase (EC 2.1.7.48) to convert UMP to

UDP.

$$NMP \xrightarrow{\text{Kinase 1}} NDP \xrightarrow{\text{Kinase 2}} NTP \qquad \text{Scheme 1}$$

The method for synthesis of NTP from NMP described above, however, is not suitable for synthesis of dNTP, because dNDPs are poor substrates for pyruvate kinase. We had recently cloned and over-expressed T_4 deoxyribonucleotide kinase. This bacteriophage enzyme has broad substrate specificity and will phosphorylate dGMP, dTMP 5´-hydroxylmethyldeoxycytidine monophosphate (hmCMP) to their corresponding deoxyribonucleoside diphosphates[5]. The remaining two deoxyribonucleoside diphosphates, dADP and dCDP, can be synthesized by using *Escherichia coli* adenylate kinase[6] and CMP kinase,[7] respectively, since 2´-dexoyribonucleoside monophosphates are also substrates for these enzymes. Once dADP, dTDP, dGDP and dCDP are available, they can be converted to the triphosphate forms, using *E. coli* nucleoside diphosphate (NDP) kinase (EC 2.7.6.4). NDP kinase from *E. coli* has broad specificity and will phosphorylate both the ribose and deoxyribose forms of purines and pyrimidines nucleoside diphosphates, using ATP as phosphate donor.[8] Thus, this paper reports cloning, effective induction and purification of the NDP kinase for enzymatic synthesis of dNTPs. By placing the *ndk* gene under the strong and controllable T_7 promoter-based system such as the pET vectors that had been developed by Studier and co-workers, several milligram quantity of pure protein per liter of bacterial culture can be easily obtained.[9] Furthermore, because the recombinant proteins obtained with this expression vector are polyhistidine-tagged, the enzyme can be reversibly immobilized onto the nickel affinity column for a more efficient, semi-solid state enzymatic synthesis of dNTPs.

Materials and Methods

Strains, Plasmids, and Culture Media

E. coli strains DH5α [*supE*44 Δ*lac*U169 (ϕ80*lac*ZDM15) *hds*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-I *rel*A1], XLBlue [*rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *supE*44 *rel*A1 *lac*(F´*pro*AB *lacI*^{q} ZΔM15 *Tn*10 (Tet^r)^c] and Sϕ3834 (*rps*L Δ*add-uid-man met*B *gua*A *ura*A::Tn10)[10] were grown in LB medium at 37°C. pET-26b (Novagen) was propagated in DH5α in 200 ml LB medium supplemented with 35 μg/ml kanamycin. Strain BL21(DE3) [*hsdS*$_B$ ($r^-_B M^-_B$) F$^-$ *gal*(λts857 *ind*1 *Sam*7 *nin*5 *lac*UV-5-7 gene] pLysS was used for expression of recombinant protein.

Materials

Restriction enzymes *Nde*I, *Xho*I, T4 DNA ligase and deoxyribonucleotides were purchased from New England Biolab (USA). Ni^{2+}-NTA agarose was obtained from Qiagen. Tag DNA polymerase was obtained from Dragon Egg BioLab (Bangkok). Isopropyl-1-thio-β-*D*-galactopyranoside (IPTG), phosphoenolpyruvate, β-NADH, and other chemicals were purchased from Sigma. The designed primers were purchased from KU-vector (Kasetsart University).

Isolation of Chromosomal DNA and Plasmids from *E. coli*

Chromosomal DNA from *E. coli* strains DH5α, XLBlue and Sϕ3834 were isolated by the Triton-Prep method. Briefly, after growing 50 ml of cell culture in LB medium overnight, cells were harvested by centrifugation at 4,000 rpm and resuspended in 9 ml STET buffer [8% sucrose, 5% Triton X-100, 50 mM Tris-HCl (pH 8.0)]. After addition of lysozyme (1 mg) and RNaseA (10 mg), the reaction mixture was boiled for 1 min and centrifuged at 10,000 rpm for 15 min. The supernatant was extracted with equal volume of STET-saturated phenol. To the aqueous layer, 0.1 volume of 4 M lithium chloride was added and placed on ice for 5 min. After centrifugation at 10,000 rpm, equal volume of isopropanol was added to supernatant. Chromosomal DNA was recovered by centrifugation at 10,000 rpm and dissolved in 400 μl TE buffer. A typical yield of DNA obtained was approximately 40 μg. The plasmid pET-26b was isolated from DH5α that contain the plasmid by the standard alkali lysis method and purified as described.[11]

Amplification and Cloning of the *ndk* Gene

PCR amplification reaction was performed using chromosomal DNAs isolated from *E. coli* DH5α, XLBlue, and Sϕ3834 as templates. Based on the nucleotide sequence of the *ndk* gene (Genbank accession D90881), the forward primer (Tong-1: 5´-CCCGGATCCCATATG GCTATTGAACGTACTTTT-3´, where the underline indicated the added *BamH*I and *Nde*I linker) and reverse primer (Tong-2: 5´-GGGCTCGAG ACGGGTGCGCGGGCACAC-3´, where the underline indicated the added *Xho*I linker) were synthesized by KU-vector at Kasetsart University. The optimum PCR conditions were first established for the efficient amplification of the *ndk* gene and then subsequently used as condition for amplification. The reaction solution contained 50 ng of chromosomal DNA, 0.2 μM of forward and reverse primers, 175 μM of dNTP, 0.04 unit of Tag DNA polymerase in 50 mM Tris-HCl, pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, and 1% Triton X-100. The reaction mixture initiated by denaturing at 94°C for 5 min and then subjected to 30 cycles of 1 min denaturation at 94°C, 40 sec of

annealing at 60°C, and 1.40 min of extension at 72°C. After 30 cycles, the reaction mixture was incubated at 72°C for 10 min and then cooled to 4°C. The amplified product was purified with the PCR purification kit (Qiagen) and analyzed on 2% agarose gel. The purified PCR product (4 μg), corresponding to *ndk* gene, was double digested with 12 units of *Nde*I and *Xho*I for 2 h at 37°C. The digested PCR product was again purified with PCR product purification kit. The purified PCR product (100 ng) was ligated into pET-26b (100 ng), which had been previously double digested with *Nde*I and *Xho*I, using T4 DNA ligase (1 U). The ligated DNA product was transformed into competent *E. coli* BL21 (DE3) pLysS cells which had been prepared by the standard calcium chloride procedure. 153 colonies were obtained on LB agar plate contained 30 mg/ml kanamycin. After screening 30 colonies for recombinant plasmid by restriction endonuclease digestion, one colony was found to contain *ndk* gene. This recombinant plasmid was designated as pET-*ndk*.

Assay for NDP Kinase Activity

The activity of NDP kinase was assayed, using dGDP as substrate. The formation of dGTP was measured spectrophotometrically at 340 nm, using pyruvate kinase and lactate dehydrogenase as coupling enzymes.[5] In a typical assay, reaction mixture (1 ml) containing 2 mM phosphoenolpyruvate, 1 mM ATP, 0.4 mM NADH, 0.5 U pyruvate kinase, 0.5 U lactate dehydrogenase, and 1 mM dGDP in buffer A (100 mM Tris-HCl [pH 7.5], 20 mM $MgCl_2$ and 200 mM KCl) was incubated for 1 min at 37°C. The reaction was initiated by addition of NDP kinase. One unit of NDP kinase activity is defined as the amount of protein required to convert 1 nmole of dGDP to dGTP in 1 min at 37°C. Protein concentrations were determined by the Bradford method, using bovine serum albumin as standard.[12]

Comparison of the Kinetic of Induction of the Expression NDP Kinase by IPTG and Lactose

To determine whether IPTG can be substituted by lactose as inducer, *E. coli* BL21 (DE3) pLysS carrying pET-*ndk* was grown at 37°C in two separate flasks containing 50 ml LB supplemented with 30 μg/ml kanamycin to an OD_{600}nm of 0.5. At this time, IPTG at 1 mM or lactose at 5 mM was then added to each flask separately. After 0, 1, 2, 3, 4, and 5 hours of induction, cells were harvested and the level of NDP kinase induced was determined on 15% SDS-PAGE electrophoresis.

Expression and Purification of Recombinant NDP Kinase

For large-scale purification of NDP kinase, *E. coli* BL21 (DE3) pLysS carrying pET-*ndk* was grown at 37°C in 1L of LB containing 30 μg/ml kanamycin to an OD_{600nm} of 0.5. Lactose was then added to the final concentration of 5 mM and cells were allowed to grow for five additional hours. Cells were harvested by centrifugation at 8,000 rpm at 4°C and suspended in lysis buffer (20 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 0.5% Tween 20 and 1 mM PMSF) containing 1 mg/ml lysozyme. After incubation for 15 min at room temperature, the cell-free extract was obtained by centrifugation at 10,000 rpm for 30 min. To cell free extract, ammonium sulfate was added slowly to reach 80% saturation with constant stirring. After additional 30 min of stirring, the precipitated protein was recovered by centrifugation at 10,000 rpm for 30 min at 4°C. The pellet was dissolved in 15 ml of 50 mM potassium phosphate buffer containing 1 mM EDTA and dialyzed two times against 2 liters 50 mM potassium phosphate buffer, pH 7.5 containing 1 mM EDTA. The dialyzed protein was then added onto Ni^{2+}-NTA resin (3 ml bed volume), which had been previously equilibrated with buffer B (20 mM Tris-HCl pH 8.0, 500 mM KCl, and 0.1% Triton X-100). The column was washed several times with the same buffer. The polyhistidine-tagged enzyme was eluted by washing column with buffer B containing 5, 20, 40, 60, and 100 mM imidazole, respectively. The eluted fractions were pooled and concentrated by ultrafiltration, using Amicon pressure cell. Purity of the enzyme was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Construction of the Recombinant pET-*ndk*

The *ndk* gene encoding for nucleoside diphosphate kinase from *E. coli* had been amplified by PCR, using specific primers. Using chromosomal DNA isolated from *E. coli* DH5α, XLBlue, or Sϕ3834 as template, a 451 bp fragment that corresponding to the full-length of *ndk* gene was obtained. This data suggested that the *ndk* gene is presence in all three strains of *E. coli* (Fig 1). The nucleotide sequence of the 451 bp fragment obtained from PCR reaction using *E. coli* DH5α as template was confirmed by DNA sequencing. Determination of the nucleotide sequence of this PCR product revealed that its nucleotide sequence contained an open reading frame (ORF) that is identical to the sequence of the *ndk* gene deposited in GenBank. Thus, this PCR product that contained the *ndk* gene was double digested with *Nde*I and *Xho*I, purified by Qiagen PCR product purification kit, and ligated to the pET-26b, which had been previously double digested with *Nde*I and *Xho*I. Transformation of the ligation mixture into competent BL21(DE3) pLysS resulting in

153 colonies that were able to grow LB agar plate supplemented with 30 μg/ml kanamycin. Plasmids from these clones were isolated by the alkali lysis mini prep. The presence of the *ndk* gene (451 bp) inserted in these plasmids was detected by double digesting the plasmids with *Nde*I and *Xho*I. One of these plasmids, designed as pET-*ndk*, isolated from a colony was found to contain the *ndk* gene.

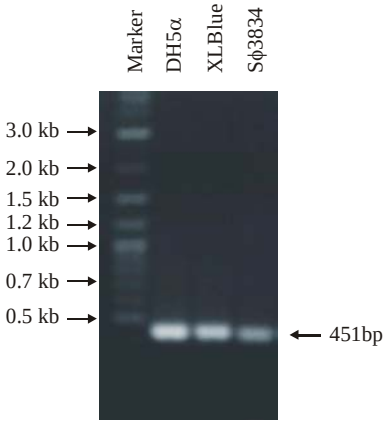

Fig 1. PCR amplification of *ndk* gene from *E. coli* chromosomal DNA. *Lane 1*, molecular weight markers; lanes 2-4 are PCR reactions performed by using 50-100 ng of chromosomal DNA isolated from strain of *E. coli* DH5α, XLBlue, and Sφ3834 as templates, respectively.

Comparison of the Level Expression of NDP Kinase Induced by IPTG and Lactose

IPTG is the most commonly used inducer to induce the expression of the desired gene placed under the control of the strong T_7, *lac* or *tac* promoter. The advantage of using this synthetic inducer is that it cannot be hydrolyzed by β-galactosidase.[13] However, the use of IPTG for the large-scale production of human therapeutic proteins is undesirable because of its toxicity and high cost.[14-15] For these reasons, experiment was performed to determine whether IPTG could be substituted by lactose as inducer. After allowing cells carrying pET-*ndk* to grow and reach OD_{600nm} of 0.5 in two separated flasks, expression of recombinant NDP kinase was induced by addition of either IPTG at 1 mM or lactose at 5 mM. The kinetics of induction of the expression of NDP kinase by IPTG and lactose were followed at different hour after induction. Our results showed that kinetic of induction of the expression of NDP kinase by IPTG occurred rapidly (Fige 2A). After one hour of induction, significant amount of NDP kinase was observed (Fig 2A, *lane* 3). The amount of NDP kinase continued to increase and reached plateau at approximately 3 hours after induction. The kinetic of induction of NDP kinase by lactose, however, occurred at a much slower rate (Fig 2B). Even after 2 hours of induction, only small amount of the enzyme was observed (Fig 2B, *lane* 4). However, after 5 hours of induction, the amount of NDP kinase reached almost the same level (~25% of total protein consisted of NDP kinase) as that induced with IPTG. The ability of lactose to effectively induce the expression of NDP kinase is not limited to pET-*ndk* as reported in this study. Lactose at 3 to 5 mM was found to be as effective inducer for the expression of other genes such as genes coding for *E. coli* CMP kinase, adenylate kinase and Tag DNA polymerase that placed under the control of T_7 or *tac* promoter as well (Howhan, Poopanitpan and Pornbanlualap, manu-script in preparation). Thus, these studies strongly suggest that lactose can be used as substituted inducer for IPTG for expression of genes placed under the control of T_7, *tac* or *lac* promoter.

The slower rate of induction of NDP kinase by lactose perhaps can be explained as the following. When platted on LB agar containing IPTG and X-Gal, BL21 (DE3) pLysS colonies turn blue, indicating that BL21 (DE3) contains *lac*Z gene (Data not shown). Thus, when lactose was added to medium, some of these molecules were converted to allolactose (6-O-β-*D*-galactopyranosyl-*D*-glucose) by β-galactosidase existed within cells. Allolactose but not lactose is known to be the natural inducer of the *lac* operon[16]. In addition to its ability to catalyze cleavage of lactose and certain analogs of it, β-galactosidase also catalyzes the transglycosylation that converted lactose to allolactose.[17] Thus, the slower rate of induction of NDP kinase by lactose may be due to a slower rate of accumulation of allolactose. Allolactose and IPTG had been reported to bind to *lac* repressor with same affinity.[18] Equilibrium binding data showed that the association rate of IPTG to *lac* repressor protein is 1.2×10^6 M^{-1} where as that for allolactose is 1.7×10^6 M^{-1}. Because of its slower rate of induction, lactose may be the preferred inducer over IPTG in cases where induction of proteins resulted in accumulation of proteins as insoluble inclusion bodies. In spite of its many merits and wide usage, the T_7-base expression systems may pose considerable problems such as formation of insoluble inclusion bodies and inconsistency in level of expression. Currently, the parameters that contribute to formation of inclusion bodies remain unclear.[15] The problem with formation of inclusion bodies presumably resulted from expressing protein from these relatively high-copy number T_7 vectors.[20] To solve the insolubility problem, various conditions to reduce the level of expression, including expression for shorter time and at 30 °C, reducing the level of IPTG, and using the low-copy number T_7 expression vector had been recommended.[20] Because of its slower rate of induction, lactose may be the preferred inducer over IPTG in

cases where induction of proteins resulted in accumulation of proteins as insoluble inclusion bodies. A slower rate of induction by lactose may allow sufficient time for proper protein folding.

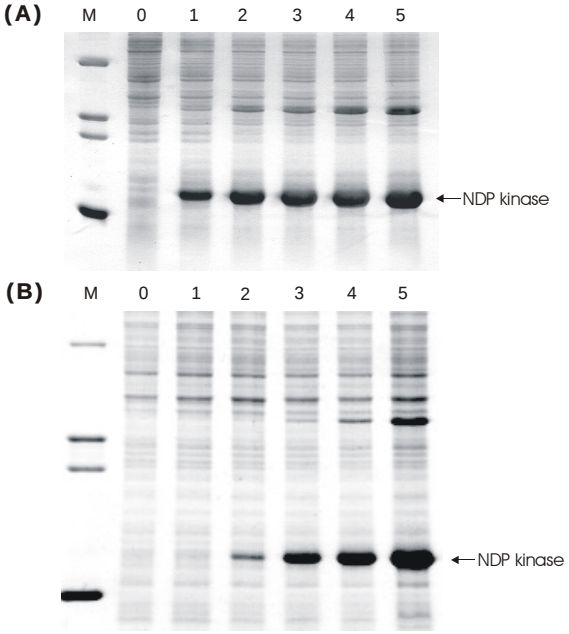

Fig 2. Comparison of effectiveness of induction of the expression of the *ndk* gene by IPTG and lactose. **A-** *Lane 1,* molecular markers (66, 30, 25, and 14.9 kDa); *lanes* 2-7 are cell-free extract at 0, 1, 2, 3, 4, and 5 hours of induction with 1 mM IPTG. **B-** Lane 1, *Lane 1,* molecular markers (66, 30, 25, and 14.9 kDa); *lanes* 2-7 are cell-free extract at 0, 1, 2, 3, 4, and 5 hours of induction with 5 mM lactose.

Expression and Purification of the Recombinant NDP Kinase

The recombinant NDP kinase was purified from one-liter cell culture. After induced with 5 mM lactose, NDP kinase consisted of approximately 25% of total protein in cell-free extract. After 0-80% ammonium sulfate fractionation, the total unit of enzyme activity increased from 100 to 182% (Table 1). This data suggested that ammonium sulfate fractionation and dialysis removed some of endogenous inhibitors that may have existed in cell-free extract. After the dialyzed enzyme was loaded onto Ni^{2+}-NTA affinity column, the column was washed with five volumes of buffer B. NDP kinase was subsequently eluted with buffer B containing 20, 40, 60 and 100 mM imidazole, respectively. The enzymes began to elute at 40 mM imidazole and consisted of two major bands, one that migrated at 16 KDa and the other at 32 KDa (Fig 3). The 32 KDa is dimeric form of NDP kinase observed after prolong storage of enzyme at –20°C that is not entirely dissociated under denaturing conditions on SDS-PAGE. Similar observation had been reported with recombinant FlgM protein.[19]

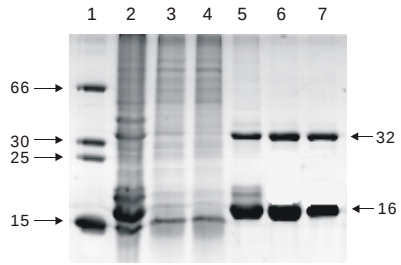

Fig 3. SDS-electrophoresis analysis of the recombinant His_6-tagged nucleoside diphosphate kinase in each step of purification. Proteins from each step of purification were analysed on 15% polyacrylamide gel. *Lane* 1, the molecular mass marker (66, 30, 25 and 14.9 KDa); *lane* 2, cell-free extract; *lane* 3, flow through fractions; *lane* 4, fraction eluted with buffer A containing 20 mM imidazole; *lane* 5, fraction eluted with buffer B containing 40 mM imidazole; *lane* 6, fraction eluted with buffer containing 60 mM imidazole; *lane* 7, fraction eluted with buffer containing 100mM imidazole.

The purified recombinant NDP kinase that contained polyhistidine tagged at the C terminus retained its activity, with specificity activity of 2,620 units/mg (Table 1). The recombinant NDP kinase contained eight additional amino acids (Lue-Glu-His-His-His-His-His-His) tagged at the C-terminus. The polyhistidine tagged NDP kinase along with T_7 deoxyribonucleotide kinase, which had been

Table 1. Summary of the Purification of Nucleoside Diphosphate Kinase from *E. coli.*

Step	Total Protein (mg)	Specific activity (unit/mg)	Fold purification	Recovery (%)	Total unit (unit)
Cell-free extract	767	332	1.0	100	255,000
0-80% NH_3SO_4	305	1520	4.6	182	464,000
Ni-NTA affinity	132	2620	7.9	136	346,000

previously cloned and expressed in our laboratory, can be easily immobilized onto Ni^{2+}-NTA-agarose column for semi-solid state synthesis of dTTP and dGTP from dTMP and dGMP, respectively. Unlike conventional immobilization of enzyme, the process of immobilizing polyhistidine tagged enzymes onto Ni^{2+}-NTA-agarose is reversible. Thus, once these enzymes lose their activities over time, they can be easily eluted out from the Ni^{2+}-NTA agarose with imidazole. New batch of active enzymes can be immobilized again onto Ni^{2+}-agarose for further synthesis of dNTP. However, because reactions catalyzed by NDP kinase and T_4 deoxyribonucleotide kinase are reversible, this resulted in yield of ~40-60% conversion of dNMP to dNTP (Data not shown). The conversion of dNMP to dNTP can be driven to completion by addition of pyruvate kinase and phosphoenolpyruvate to reaction mixture[3]. Thus, cloning and over-expression of polyhistidine tagged pyruvate kinase is currently underway in our laboratory. Once polyhistidine tagged pyruvate kinase was obtained, enzymatic synthesis of dNTP can be accomplished by immobilizing NDP kinase, T_4 deoxyribonucleotide kinase and pyruvate kinase onto Ni^{2+}-NTA agarose in a column as shown in Fig 4.

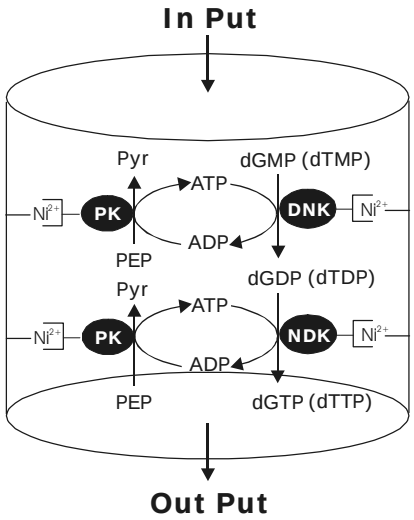

Fig 4. Diagram of the strategy of enzymatic synthesis of deoxyribonucleoside triphosphate by immobilization of polyhistidine tagged enzymes on Ni^{2+}-NTA agarose. The abbreviations used are DNK for T_4 deoxyribonucleotide kinase, NDK for *E. coli* nucleoside diphosphate kinase, PK for pyruvate kinase, PEP for phosphoenolpyruvate, and Pyr for pyruvate. In this diagram, all three polyhistidine tagged enzymes are immobilized onto the Ni^{2+}-NTA agarose in a column.

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