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Research Article

The monitoring of nucleotide diphosphate kinase activity by blue native polyacrylamide gel electrophoresis

Nucleoside diphosphate kinase (NDPK) has been shown to play a pivotal role in modulating a plethora of cellular processes. In this study, we report on a blue native (BN) PAGE technique which allows the facile assessment of NDPK activity and expression. The in-gel detection of NDPK relies on the precipitation of formazan at the site of immobilized enzyme activity. This is achieved by coupling the formation of ATP, as a consequence of γ-phosphate transfer from NTP to ADP, to hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PDH), oxidized nicotinamide adenine dinucleotide phosphate (NADP), phenazine methosulfate (PMS), and iodonitrotetrazolium chloride (INT). 2-D denaturing gel analysis confirmed that the activity bands corresponded to NDPK as indicated by subunit composition. Furthermore, the sensitivity and specificity of this readily accessible procedure was assessed by monitoring the in-gel activity of NDPK using different concentrations of GTP and CTP as well as deoxynucleoside triphosphates. This electrophoretic technique allows the quick and easy detection of NDPK, a housekeeping enzyme crucial to cell survival.

Keywords:
Activity stain / Blue Native PAGE / NDP kinase DOI 10.1002/elps.200700697

1 Introduction

Cellular systems are reliant on a steady supply of four separate nucleoside triphosphates (NTPs) namely ATP, GTP, CTP, and UTP. Each individual nucleotide is required to drive a variety of cellular processes. As the universal energy currency, ATP drives an enormous number of cellular functions. This is achieved by harnessing the free energy stored within its phosphodiester bonds [1]. For instance, high-energy processes, such as protein synthesis and degradation, DNA replication, and ion transport, would not be possible without ATP. In contrast to ATP, the other nucleotides are involved in some more precise biochemical functions. GTP is strictly utilized by G-proteins for the purpose of signal transduction, vesicular trafficking, and protein translation [2–4]. CTP is generally dedicated to the biosynthesis of phospholipids whereas UTP is required for the production of polysaccharides [5]. Thus, cells must maintain a constant supply of NTP in order to drive numerous cellular processes. The homeostasis of NTP pools is achieved by nucleoside diphosphate kinase (NDPK). This enzyme mediates the transfer of the γ-phosphate from NTP to NDP in order to maintain a proper concentration of these NTP [6]. The importance of this enzyme in biology is illustrated by its conservation throughout various cellular systems [7–9]. Indeed, NDPK plays a pivotal role in numerous cellular processes including DNA replication, signal transduction, and energy metabolism in bacteria, fungi, plants, and mammals. ATP has been described as the principal phosphate donor for this enzymatic reaction [10, 11]. For example, NDPK readily transfers the phosphate from ATP to GDP to provide the necessary GTP molecules for the activation of G proteins [11–13]. However, NDPK is also capable of using GTP, UTP, and CTP as phosphate donors. The *Km* for each respected NTP appears to be dependent on the organism that NDPK has been isolated from [13–16]. Furthermore, deoxynucleotides have been shown to act as phosphate donors in various organisms [16, 17]. The ability of NDPK to use different NTP as well as deoxynucleoside triphosphate (dNTP) in the phosphate transfer reaction clearly demonstrates the pivotal role this enzyme plays in modulating the cellular concentration of nucleotides.

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Abbreviations: BN, blue native; **CFE,** cell-free extract; **dNTP,** deoxynucleoside triphosphate; **G6PDH,** glucose-6-phosphate dehydrogenase; **HK,** hexokinase; **IAA,** iodoacetic acid; **INT,** iodonitrotetrazolium chloride; **NDPK,** nucleoside diphosphate kinase; **PMS,** phenazine methosulfate

NDPK is generally monitored using enzyme-coupled spectrophotometric assays [18]. In these assays, the ATP-generating capabilities of NDPK are coupled to exogenously added hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) allowing the quantification of NADPH production. However, HK and G6PDH from the cell-free extract (CFE) may interfere with the assay. Furthermore, high amounts of sample are required for the spectrophotometric measurements since the sensitivity of these assays are relatively low. In order to compensate for the shortcomings of spectrophotometric assays, techniques employing sensitive technologies such as HPLC or radiolabeled NTP have been developed [15, 18]. Although sensitive, these techniques are quite costly and, in the case of using radiolabeled substrate, extreme care needs to be taken. Thus, the development of a low cost procedure with high sensitivity is crucial for measuring NDPK activity. In previous reports, we described the use of a blue BN-PAGE technique for monitoring the in-gel activity of several enzymes involved in energy metabolism and nicotinamide adenine dinucleotide homeostasis [19–23]. Here, we show that this simple tool can be employed for analyzing the activity of NDPK. The in-gel activity of NDPK was visualized by coupling the production ATP to exogenously added HK and G6PDH. This procedure allows the rapid assessment of NDPK activity and expression.

2 Materials and methods

2.1 Cell culture and fractionation

The strain of *Pseudomonas fluorescens* (ATCC 13525) was grown in a high-phosphate mineral medium containing Na_2HPO_4 (6 g), KH_2PO_4 (3 g), $MgSO_4$ (0.2 g), NH_4Cl (0.8 g), and citric acid (4 g) solubilized in 1 L of distilled and deionized H_2O (ddH_2O) (citrate culture). Following the adjustment of the pH to 6.8 with 2 M NaOH, trace elements were added to a final concentration of 1% v/v as described previously [24]. The medium was dispensed in aliquots of 200 mL in 500 mL Erlenmeyer flasks and autoclaved for 22 min. The media was inoculated with 1 mL of stationary-phase cells grown in a citrate culture and aerated on a gyratory water bath shaker (Model 76; New Brunswick Scientific). Cells were isolated following a 24 h growth period in the citrate medium. Cells were pelleted by centrifuging the growth media at $10\,000 \times g$ for 10 min at 4°C. Following a washing with 0.8% w/v NaCl, the cellular pellet was resuspended in cell storage buffer (CSB; 50 mM Tris-HCl pH 7.3, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, and 1 mM DTT). The cells were homogenized on ice by sonication and centrifuged at $3000 \times g$ for 30 min to remove any intact cells. The cell homogenate was then centrifuged at $180\,000 \times g$ for 3 h to afford a soluble and membrane CFE. The purity of each fraction was tested by performing activity stains for G6PDH (soluble fraction) and succinate dehydrogenase (SDH; membrane fraction). The amount of protein associated with each fraction was determined using the Bradford assay [25]. BSA served as the standard.

2.2 BN-PAGE and 2-D electrophoresis

BN-PAGE was performed as described by Schägger and von Jagow [26] with some modifications. Cellular fractions isolated from *Pseudomonas fluorescens* were prepared in a native buffer (50 mM Bis-Tris, 500 mM ε-aminocaproic acid, pH 7.0, 4°C) at a final concentration of 4 mg of protein *per* mL. For the membrane CFE, 1% v/v β-dodecyl-D-maltoside was included in the preparation in order to solubilize the membrane-bound protein. To ensure optimal protein separation, 4–16% linear gradient gels (8×7 cm^2) were cast with the BioRad MiniProtean™ 2 system using 1 mm spacers. The final volume of the resolving gel was 5.8 mL using 2.9 mL of 4% v/v acrylamide solution and 2.9 mL of 16% v/v acrylamide solution *per* gel. Each acrylamide solution consisted of 50 mM Bis-Tris and 500 mM ε-aminocaproic acid (pH 7.0 4°C). The 16% v/v acrylamide solution also contained 10% v/v glycerol to ensure proper gradient distribution and to aid in maintaining protein stability. Linear gradient gels were produced using a gradient former (BioRad) and a peristaltic pump (Fisher). Proteins (40–160 μg) were loaded into each lane and electrophoresed under native conditions. A voltage of 80 V was applied for the stacking gel. The voltage was then increased to 300 V (note: the amperage never exceeded 15 mA) once the running front entered the separating gel. The blue cathode buffer (50 mM Tricine, 15 min Bis-Tris, 0.02% w/v Coomassie G-250 (pH 7) at 4°C) was changed to a colorless cathode buffer (50 mM Tricine, 15 min Bis-Tris, (pH 7) at 4°C) when the running front was half-way through the gel. This aids in removing any Coomassie G-250 during the electrophoresis. Upon completion, the gel slab was removed from the apparatus and equilibrated for 15 min in a reaction buffer (25 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$). Mg was included since this divalent metal is required for 100% transfer of the phosphate from the donor to the acceptor [15]. The in-gel activity of NDPK was detected by coupling the formation of ATP to PMS and iodonitrotetrazolium chloride (INT). Coupling was achieved using HK and G6PDH which produced the necessary NADPH molecules for formazan precipitation at the site of enzyme activity (Fig. 1). The gel slab was placed in a reaction mixture consisting of 0.75 mM ADP, 0.75 mM NADP, 15 mM glucose, 5 units HK, 5 units G6PDH, 0.5 mg/mL INT, 0.3 mg/mL of PMS, and NTP (GTP, UTP, or CTP) or dNTP (dGTP, dCTP, dTTP). The concentration of NTP and dNTP ranged from 0.3 to 1.5 mM. The final volume of the reaction mixture was 1.5 mL/lane. Once the activity bands reached the desired intensity, the reactions were stopped using a destaining solution (10% v/v glacial acetic acid and 40% v/v methanol). The specificity of the activity bands was assessed by performing reactions with 5 mM iodoacetic acid (IAA, potent kinase inhibitor) or in the absence of NTP

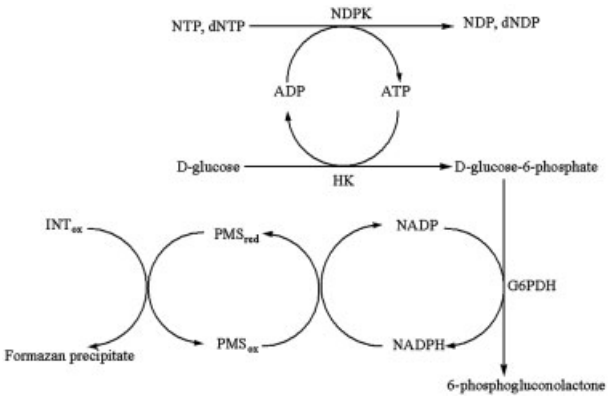

Figure 1. Schematic representation of the reactions involved in the in-gel detection of NDPK activity.

or dNTP [27]. Proper protein loading was assured by staining for total protein with Coomassie R-250. BSA served as the molecular weight standard. Bands were quantified using SCION imaging for windows (SCION, Frederick, MD).

The analysis of protein expression levels and subunit composition was performed using 2-D electrophoresis [28, 29]. To quantitate protein expression, the activity bands were precision cut and loaded into a BN gel (2-D BN-PAGE). Electrophoresis was performed as described above. Protein subunit analysis was carried out using a modified 2-D SDS PAGE technique originally described by Laemmli [30]. The activity bands were excised and incubated for 15 min in the electrophoresis buffer (24 mM Tris, 191 mM glycine, 1% w/v SDS) at room temperature. The bands were then loaded into the wells and electrophoresed at 80 V. 2-D BN-PAGE and 2-D SDS-PAGE analyses were performed on activity bands isolated from separate experiments. Upon completion the gel slabs were fixed in destaining solution. Proteins were detected with silver stain.

3 Results and discussion

The maintenance of NTP levels is important in sustaining cellular systems. By adjusting the relative concentration of different NTP, NDPK can aid in directing the cell to perform specific functions. The importance of NDPK in biological systems prompted us to identify a facile method for measuring the activity of this enzyme. By using the BN-PAGE technique, we were able to evaluate the activity and expression of NDPK. BN-PAGE is an electrophoretic technique that maintains the native form of proteins and enzymes as they migrate through the gel. This procedure allows the colorimetric visualization of enzyme activity in the gel matrix. The negative charge provided by the Coomassie G-250 and the maintenance of protein stability by ε-aminocaproic acid aid in optimizing the sensitivity of this technique. The ability of NDPK to produce ATP *via* the transfer of a γ-phosphate from an NTP to ADP was exploited to assess the in-gel activity of this enzyme. With the aid of exogenously added HK and G6PDH, the NADPH produced donates its electrons to INT to yield a purple precipitate at the site of immobilized enzyme activity. The activity of NDPK was measured in the soluble and membrane fractions isolated from *P. fluorescens* grown for 24 h in a high phosphate citrate-containing medium. As shown in Fig. 2 (panel 1), activity bands were observed only when GTP was present in the reaction mixture. No activity bands were present when GTP was removed. Furthermore, the inclusion of 5 mM IAA, a known

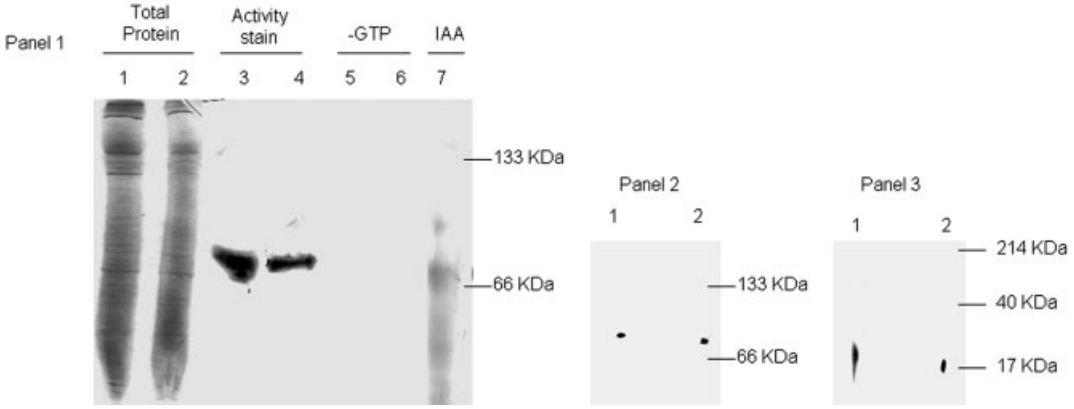

Figure 2. In-gel activity stain for NDPK. Following a 24 h growth period cells were isolated and the membrane fractions were tested for NDP kinase activity using GTP and ADP. Activity stains were performed in duplicate. An 80 μg equivalent to membrane fraction was utilized. Panel 1: in-gel detection of NDPK. Lane 1 and lane 2: total protein stain using CBB R-250. Lane 3 and lane 4: in-gel activity stain for NDP kinase. Lane 5 and lane 6: band specificity was assured by excluding GTP (negative control). Lane 7: reaction performed in the presence of 5 mM IAA, a known kinase inhibitor. Note: these reactions were also performed in soluble fractions however no bands were detected. Panel 2: 2-D BN-PAGE analysis of the molecular weight of the activity bands from panel 1 (lanes 3 and 4). Bands were excised and loaded into the gel. Protein levels were determined by silver stain. Panel 3: 2-D SDS-PAGE analysis of the subunit composition of the activity bands. The experiment in panel 1 was repeated and the resulting activity bands were excised and loaded into a 15% denaturing gel. Protein levels were determined by silver stain.

kinase inhibitor, prevented the precipitation of the formazan. The soluble fraction from *P. fluorescens* was also tested for NDPK, however, no bands were observed (data not shown). This is consistent with the observations made by Kapatral *et al.* using *Pseudomonas aureginosa*. Indeed, NDPK activity and expression was only observed in the membrane fraction of cells isolated at the stationary growth phase [31]. To evaluate the nature of the activity bands further, 2-D BN-PAGE was performed. The molecular mass of the native protein from the excised activity band appeared to be approximately 70 kDa (Fig. 2, panel 2). This is consistent with previous data which have shown that the native NDPK has a molecular mass ranging from 60 to 120 kDa [16, 32–34]. To further confirm that the observed activity band was indeed NDPK, we analyzed the subunit composition. 2-D SDS-PAGE of the excised activity bands revealed low molecular mass subunits of approximately 15 kDa (Fig. 2, panel 3). It is worthy to note that the 2-D BN-PAGE and 2-D SDS-PAGE were performed on activity bands from separate experiments. NDPK has been previously reported to be a multimeric protein comprised of several 15–17 kDa subunits [9]. Thus, the subunits from the activity bands corresponded to the molecular weight of the subunits from NDPK.

To assess the sensitivity of the in-gel detection of NDPK, reactions were performed over a period of 80 min. A faint band was recorded following a 10 min reaction however, the intensity increased exponentially when reactions were left for 20 to 60 min (Fig. 3). When reactions were left for longer than 60 min the band intensity remained constant. Figure 4 depicts the dependence of the activity bands on protein concentration. A faint band was observed when 40 μg of protein was reacted for 30 min. The intensity of the activity bands increased dramatically when 80, 120, and 160 μg were used. Thus, as little as 40–60 μg of protein is required to detect NDPK in contrast to the higher amounts required for spectrophotometric assays. To evaluate the ability of NDPK to use other NTP, we tested whether CTP and UTP can also yield activity bands. Indeed, activity bands were produced when CTP and UTP were used as phosphate donors. However, the reactions took significantly longer since the precipitate was only observed following a 120 min reaction with CTP and a 60 min reaction with UTP (Fig. 5). This is in contrast to the

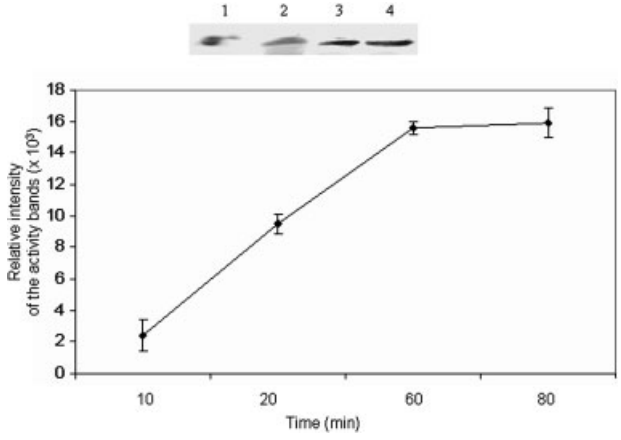

Figure 3. In-gel detection of NDPK over a period of 80 min. Membrane CFE from *P. fluorescens* grown in a high-phosphate citrate medium was isolated and probed for the presence of NDPK. An 80 μg equivalent to membrane fraction was utilized. Reactions were performed and stopped at various time intervals. Lane 1: 10 min, lane 2: 20 min, lane 3: 60 min, lane 4: 80 min. The relative intensity of each activity band was quantified using SCION imaging for Windows. Reactions were performed in triplicate. $n = 3$, $p \leq 0.05$, mean ± SD.

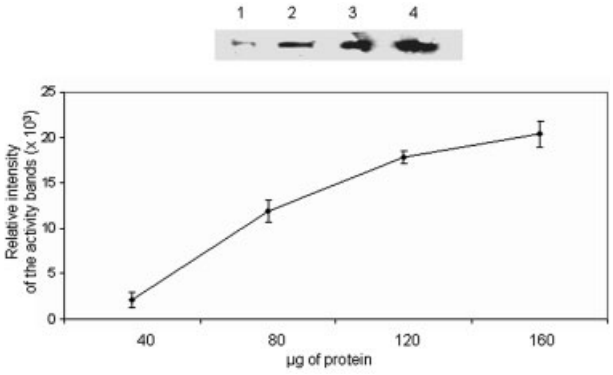

Figure 4. Impact of protein concentration on the in-gel detection of NDP kinase activity. Following a 24 h growth period in a high-phosphate citrate medium cells were isolated and the membrane fractions were tested for NDP kinase activity using GTP and ADP. Lanes 1, 2, 3, and 4 correspond to 40, 80, 120, and 160 μg of membrane protein, respectively. Bands were quantified using SCION imaging for Windows. Reactions were performed for 30 min. Reactions were performed in triplicate. $n = 3$, $p \leq 0.05$, mean ± SD.

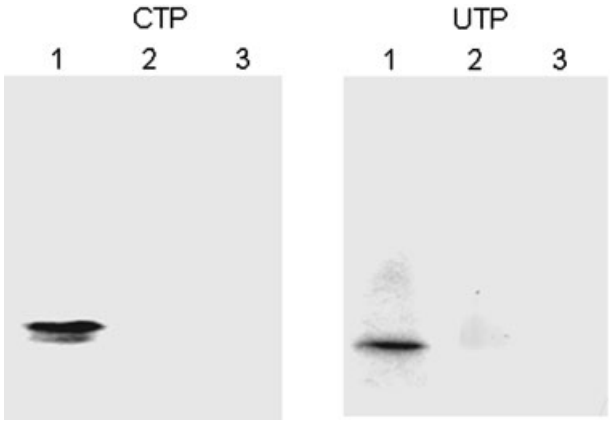

Figure 5. In-gel detection of NDPK using CTP and UTP as phosphate donors. Membrane fraction from *P. fluorescens* grown in a high-phosphate citrate medium was isolated for BN PAGE analysis. An 80 μg equivalent to membrane fraction was utilized. NDPK was detected using a reaction mixture consisting of Lane 1: 1.5 mM CTP or UTP, lane 2: 1.5 mM CTP or UTP + 5 mM iodoacetate, and lane 3: reactions devoid of CTP or UTP. Reactions were performed for 120 min (CTP) and 60 min (UTP).

reactions with GTP which yield intense activity bands following a 30 min reaction (Fig. 4). These data indicate that other NTP can be used to ascertain the activity of NDPK. However, UTP and CTP were not as effective as GTP in generating activity bands. It has been previously reported that NDPK preferentially utilizes GTP [18]. To evaluate the concept that NDPK has a higher affinity for GTP than CTP we performed reactions with concentrations of GTP and CTP ranging from 0.3 to 1.5 mM. Activity bands were readily observed when membrane fractions were reacted with 0.3 mM GTP for 120 min (Fig. 6). In contrast, CTP generated activity bands only at concentrations of 1 and 1.5 mM. Thus, GTP is preferentially used in the phosphotransferase reaction. These data are consistent with the previously observed Km values for NDPK isolated from *Solanum chacoense* [18]. The NDPK in this case had a calculated *Km* value of 75 μM for GTP in comparison to a value of 1030 μM for CTP.

The in-gel activity of NDPK was probed further using dNTP. Activity bands were generated with dGTP, dTTP, and dCTP indicating that these deoxynucleotides participate in the phosphorylation reactions (Fig. 7). However, dATP did not appear to be a substrate for NDPK activity, even when reactions were performed with high concentrations of dATP. It appears that NTP are better substrates for the kinase in *P. fluorescens* than their deoxy counterparts, a finding that is consistent with previous observations [35]. Indeed, when gels were incubated in low concentrations of dNTP, activity bands were only observed for gels incubated in 0.5 mM dTTP or dCTP (data not shown). However, the activity bands were generated only after a lengthly incubation period. A readily accessible pool of dNTP is required to maintain the high fidelity of DNA replication and cell division. Interestingly, it has been proposed by a recent study that high levels of dNTP prevent cell division and cell cycle progression [36]. It is quite possible that NDPK may also function to maintain the pool of dNTP within a narrow range in order to promote cell division and preserve the energy status of the cell. When the levels of dNTP are increased, NDPK may rectify this high concentration by donating the γ-phosphate to an NDP mole

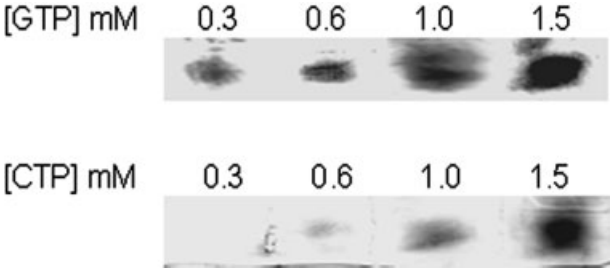

Figure 6. In-gel detection of NDPK activity exposed to different concentrations of GTP and CTP. Membrane fraction from *P. fluorescens* grown in a high phosphate citrate medium was isolated for BN PAGE analysis. An 80 μg equivalent to membrane fraction was utilized. Reactions were performed for 120 min and then stopped using destaining solution.

Figure 7. Detection of NDPK using deoxynucleotides. Membrane fraction from *P. fluorescens* grown in a high-phosphate citrate containing medium was isolated for BN-PAGE analysis. NDPK activity was detected using a reaction mixture consisting of lane 1: 1.5 mM deoxynucleotide, lane 2: 1.5 mM deoxynucleotide + 5 mM iodoacetate, lane 3: 0 mM deoxynucleotide. Reactions were performed for a minimum of 60 min to provide maximal band intensity.

cule. Thus, this BN-PAGE technique allowed the detection of NDPK activity using a range of NTP and dNTP as phosphate donors.

In conclusion, BN-PAGE is an invaluable tool for the evaluation of NDPK activity and expression. This technique enables the facile monitoring of the status of NDPK and provides critical information on cellular nucleotide homeostasis.

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