nature portfolio

Peer Review File

A universal polyphosphate kinase powers in vitro transcription

Corresponding Author: Professor Tomoaki Matsuura

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Reviewer's comments for
A universal polyphosphate kinase powers in vitro transcription
Matsumoto et al.

In their manuscript, Matsumoto et al describe a polyphosphate kinase (PPK) with broad activity towards nucleotide monophosphates (NMPs) and nucleotide diphosphates (NDPs). NTP recycling is definitely a bottleneck in biocatalysis and cell-free biology applications, and broad-specificity PPKs have not been reported yet, which makes the findings of Matsumoto et al. interesting and novel. In general, the manuscript is well-written and provides the field with an interesting new tool. I am very supportive of the work, but a couple of points need to be considered before potential publication.

My biggest concern is the fact that the enzyme shows some broad specificity, but also still has clear preferences, which makes applications not completely straight forward. The authors coupled their systems to an in vitro translation system, however, they cannot run NTP recycling continuously, which would be the real bottleneck to overcome. Thus, the proof-of principle is currently less convincing. I would have appreciated, if the authors has really managed to establish a continuous recycling system (i.e., by parallel operation of two different PPKs (one ATP/GTP-specific plus the broad specificity enzyme that covers UTP/CTP) or by changing the NTP concentrations towards more favorable ratios (e.g., running high CMP and low AMP) to achieve balanced regeneration.

Secondly, the authors report that the enzyme creates multiple and long-chain phosphorylated NTPs. I am wondering, how much this causes troubles when operating the enzyme in a continuous recycling system, because after multiple turnovers/recycling rounds, all NTPs should stochastically end up trapped in long-chain states (or could the authors show reversibility?). Along the same lines: The authors claimed that they can control the level of long-chain phosphorylation, but this pomise is actually kept very vague. I think they would need to become more concrete and provide examples (e.g., at PP-10, the level is below X%, etc.), so that the community can really start using the system.

Without such proof of principles for the two main concerns, the authors need to be very cautious about the general claims of broad specificity and applicability.

Additional (minor) concerns:

Can the authors make any statements on the selectivity of PPKs for NMPs/NDPs over dNMPs/dNDPs?

What is the shortest polyphosphate MAN can use to produce NTPs? Would pyrophosphate suffice?

Does MAN have any phosphate-transfer activity between NTPs and NDPs/ NMPs?

Editorial comments:

The color scheme, particularly that of the bar plots in Figures 2 and 3 and that of the graphs in Figure 5, relies on the reader's ability to distinguish red from green. The authors should rework the figures to be more accessible to color-blind

readers.
In Figure 1, the authors show the mode of binding for A and G of CHU. Here, the (predicted) network of binding for U and C would be a sensible addition. Even though it is not necessary for the explanation of why the N138 family was chosen for deeper analysis, it would be a nice visual aid for the reader to understand the active site, and why purine binding is more favored compared to pyrimidines. This could also be done for MAN, where kinetic data is provided to support such analysis of the binding site.

The choice of plot for the showcasing of percent conversion to NTP (such as Figure 2B) was very unintuitive. I would suggest that these plots are replaced, e.g. by line plots. In particular, the yellow lines are hard to make out.

In Figure S5, it is unclear to me whether the data points shown are the mean of replicates or individual measurements. It also isn't indicated how many replicates were used to generate the curves

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
In this manuscript, the authors report the identification, characterization, and engineering of a universal polyphosphate kinase (designated MAN) capable of synthesizing all four nucleoside triphosphates (NTPs). Through gene screening and protein engineering, the authors optimize the catalytic activity of MAN and demonstrate its application in driving in vitro transcription reactions. The work is generally well executed and the manuscript is clearly written. However, several points should be addressed to strengthen the overall impact and clarity of the study.

Major comments:

1. The current title may be somewhat misleading. The majority of the manuscript (six out of seven Results subsections) focuses on the screening, characterization, and engineering of MAN, while only the final section explores its application in in vitro transcription. As such, in vitro transcription is not the core emphasis of the study. The title should be revised to better reflect the main content and contribution of the work.

2. In the in vitro transcription experiments (Figure 5B), both transcription reactions appear to plateau at around 4 hours. The authors should clarify the reason for this termination. Whether it results from substrate (NTP) depletion, T7 RNA polymerase inactivation, or other factors. In additionally, quantification of NTP concentrations remaining after the 4-hour reaction would be informative. The final yields of all four NTPs from the MAN-catalyzed one-pot one/two-step reactions should be reported as well.

3. The methodology for using 0.125 mM of each NTP in the control reaction is clear, but it remains ambiguous how the "0.125 mM NTP mixture" from the MAN-generated sample was determined. HPLC quantification should provide precise concentrations of each NTP, and it would be more appropriate to match these concentrations when comparing to the control. Alternatively, if fixed concentrations (e.g., 0.125 mM) are used, their actual composition should be justified. It would also be useful to assess whether the reaction profiles of the MAN-synthesized and commercial NTPs are equivalent under matched conditions (i.e, the same concentration of each NTP).

4. The manuscript would benefit from a standalone Discussion section, where the implications, limitations, and future directions of this work can be addressed more comprehensively. Currently, such discussion is embedded within the Conclusion, limiting its visibility and depth.

5. The authors use analytical ultracentrifugation to determine whether truncated proteins form monomers or dimers. While valid, this method is relatively specialized. For accessibility and reproducibility, a more commonly used technique such as size exclusion chromatography (SEC) should be included as supportive validation.

6. The practical utility of the MAN platform is not fully demonstrated. The authors are encouraged to explore additional applications of MAN-synthesized NTPs. For example:

6.1. Can the in vitro transcribed RNA be used to produce functional proteins (e.g., therapeutic or fluorescent proteins) in a cell-free protein synthesis system?

6.2. What is the maximum length of RNA that can be synthesized using the MAN-generated NTPs?

6.3. Given the enzyme's promiscuity, can it be applied to synthesize non-canonical NTP analogs with biotechnological or

therapeutic relevance?

Minor comments:

7. In Figure 4A/D, please label residue position numbers for clarity.

8. Consider including a basic cost comparison between the reported method and commercially available NTPs.

9. Although the manuscript mentions the use of SDS-PAGE for protein analysis in Methods, no gel images are provided. Please include representative SDS-PAGE results in the supplementary materials.

Reviewer #4

(Remarks to the Author)
The authors demonstrated that the PPK from Mangrovibacterium marinum (MAN) was able to phosphorylate all four common nucleotide monophosphates (AMP, GMP, CMP, UMP) and diphosphates (ADP, GDP, CDP, UDP) to their corresponding triphosphates (ATP, GTP, CTP, UTP). This uPPK2 has the potential for application in nucleotide phosphorylation and synthesis.
There are the comments:
1. The author demonstrated the one-pot nucleotide synthesis and in vitro transcription reaction, and emphasized that this work will reduce the cost of DNA and RNA production. Before this statement, the authors should conduct in-depth comparisons and cost analyses. Otherwise, such a statement would be completely unfounded.
2. The author should define uPPK2.
3. There were some typos in the manuscript, such as "…diphosphates (ADP, GDP, CDP, UDP) to their corresponding triphosphate (ATP, GTP, CTP, UTP)…" on page 3 should be "triphosphates".
4. It is already known that Class III PPK2 can accept both adenine and guanine, so it is not surprising that MAN could phosphorylate different ribonucleotides.
5. The author should give a brief introduction about the Class III PPK2 in the Introduction Part, including the structural information, sequence length, and the mechanism study (such as CHU).
5. "N138 contributes to utilization of all nucleotides". Does it mean that, except for MAN, other wild-type PPK2 can also utilize all nucleotides, or through simple protein engineering? The author should give some supplementary studies and a discussion.

Reviewer #5

(Remarks to the Author)
The manuscript introduces and characterizes MAN, a Class III PPK2 enzyme described as the first "universal" polyphosphate kinase capable of efficiently phosphorylating all eight canonical ribonucleotides, including both monophosphates and diphosphates. While MAN indeed displays a broad substrate range and may hold practical potential in nucleotide regeneration systems, I find that the central claims regarding catalytic efficiency and universality are significantly overstated. This, combined with superficial mechanistic analysis and a key citation error, limits the manuscript's overall scientific strength and its suitability for Nature Communications.

1. The core issue concerns the interpretation of catalytic performance. The claim that MAN efficiently phosphorylates all eight ribonucleotides is directly contradicted by the kinetic data presented in Table 1. Specifically, the catalytic efficiencies for CMP and CDP are 170–270 times lower than that for AMP. UMP and UDP also exhibit significantly lower values than purines. Throughout the manuscript, the authors rely on endpoint conversion after 60 minutes of incubation as a measure of efficiency, but this approach masks differences in reaction rate and does not reflect intrinsic catalytic capacity. The kinetic analysis in Supplementary Figure S5 lacks necessary details, and the fitting curves for several substrates appear irregular and have low resolution. Without reporting Vmax values, replicate experimental data, or fitting parameters, it is impossible to judge the reliability of the kinetic constants in Table 1.

2. Although the authors attempt to explore the molecular basis for substrate generality, including through mutation of residue N138, truncation of the C-terminal helix, and domain-swapping constructs, the analysis remains largely descriptive. For example, N138 mutations lead to a non-uniform drop in NTP synthesis across substrates, with AMP being largely unaffected. In my opinion, this suggests that the N138 mutation alone cannot explain the substrate breadth of the enzyme. Likewise, truncation of the C-terminal helix improves purine activity at the cost of pyrimidine conversion. The authors attribute this to changes in oligomerization or conformational dynamics, but without structural or biophysical data, this remains speculative. The chimeric constructs suggest a complex functional landscape but do not lead to predictive insights. Overall, while the term "rugged functional landscape" is used to describe MAN's substrate behavior, the underlying molecular factors remain insufficiently resolved.

3. The application of MAN in in vitro transcription is an interesting demonstration, but the setup reveals limitations. The need for a two-step substrate addition protocol (pyrimidines followed by purines). The choice to use polyP-10 due to cost considerations, despite its lower catalytic performance compared to longer polyP chains, further limits the enzyme's demonstrated potential. While these are understandable compromises in an experimental context, they reduce the strength of the claim that MAN is broadly and practically applicable. For an enzyme being proposed as a universal catalyst for

nucleotide regeneration, its performance should be evaluated under ideal rather than constrained conditions.

4. There is a critical citation error in the manuscript that undermines confidence in the structural analysis. In Figure 1C, the authors cite Kuge et al. (2025) as a reference for structural insights into Class III PPK2 enzymes like CHU, but that reference in fact describes a Class II enzyme. This misattribution is especially problematic given the absence of any experimentally determined structure for MAN or its homologs.

5. While the enzyme itself may be of interest in applied settings, its discovery is empirical rather than conceptual. The study does not advance fundamental principles in enzymology, protein evolution, or mechanistic biochemistry. The narrative linking MAN to themes such as enzymatic promiscuity and origin-of-life chemistry is speculative and not supported by novel insight. As it stands, the work offers an example of a broadly active enzyme but does not contribute new mechanistic understanding or a framework for rational design. This limits its relevance for a high-impact journal like Nature Communications.

6. Several technical issues related to candidate enzyme selection and data quality that deserve attention. The authors state that PPK2 sequences were filtered using three criteria. While the first criterion is understandable, the latter two raise questions. If the goal is to discover a broadly active enzyme with possible biotechnological utility, then excluding thermophilic homologs from the start appears contradictory, especially given that thermostable enzymes often have broader utility, and yet the phylogenetic tree in Figure 1A includes many thermophilic sequences. The logic behind including them in the tree but excluding them from screening is never explained. Likewise, restricting the search to genomes with only one PPK2 gene in order to avoid “division of labor” is speculative. This assumption may discard functional variants that have evolved broad specificity or versatility precisely because of coexistence with other paralogs. Overall, the criteria appear designed more to narrow to a preselected outcome than to support an unbiased screen. Also, there are issues in the analytical methods. In Figure 5B, the HPLC chromatograms show significant co-elution of AMP and UTP, whose retention times appear to overlap. This is a critical technical flaw in a study where relative nucleotide conversion is a central output. If AMP and UTP cannot be resolved under the chosen conditions, then it is difficult to assess conversion efficiency accurately, particularly for AMP-containing reactions or where UTP is being produced from UMP. Better chromatographic resolution, such as using ion-pairing or alternative detection methods (e.g., MS), is essential to support the conclusions.

For these reasons, I do not believe the manuscript meets the threshold for publication in Nature Communications. The authors may consider substantially revising the claims and performing deeper mechanistic analysis before resubmitting to a more application-focused journal.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all remaining points.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
The authors addressed most of my previous comments. However, regarding my query about the 0.125 mM NTP mixtures generated via MAN catalysis, I remain unconvinced by the explanation that “the concentration of the NTP mixture was calculated assuming 100% NMP to NTP conversion for all nucleotides,” based on the presented data.

The HPLC analysis of authentic nucleotides in Figure S2 was helpful, but after comparing Figure S2 and Figure 5B, I believe further clarification is needed on the following points:

1. The retention time of ATP in Figure S2 is before 4 min, whereas in Figure 5B, it appears after 5 min. Could the authors explain this discrepancy?

2. For accurate comparison of nucleotide retention times, the X-axis (retention time) in Figure 5B should be presented using the same scale as in Figure S2.

3. In Figure 5B (top), the CMP peak should be clearly indicated with an arrow. Does the first peak correspond to CMP? If so, the first (major) peak in Figure 5B (bottom) would also represent CMP. This observation makes it difficult to support the assumption of “100% NMP to NTP conversion for all nucleotides,” as notable peaks are still present in the HPLC traces.

4. Given the appearance of multiple peaks in Figure 5B, I recommend the authors clearly label all relevant peaks and provide a more detailed retention time scale (1 min, 2 min, 3 min, 4 min and 5 min) on the X-axis to facilitate interpretation.

Reviewer #4

(Remarks to the Author)
After review and detailed revisions, the revised manuscript has made significant improvements and has met the requirements of Nature Communications.

Reviewer #5

(Remarks to the Author)
The authors have made commendable progress in addressing the previous round of critiques, and I can see the clear improvements in the revised manuscript. However, from my perspective, a few critical issues still need to be resolved to ensure the study is as robust and clear as it can be.

1. I still have concerns about the kinetic data. While the presentation is better, the standard errors for GMP and GDP are high enough that I find it difficult to draw firm conclusions about their precise catalytic efficiency. I strongly believe the manuscript should explicitly acknowledge this limitation in the text or figure legend to prevent readers from over-interpreting these particular values. Furthermore, I am genuinely curious about what might be causing this variability. Is it a pH shift caused by the addition of high concentrations of GMP or GDP? Or is it a sign of nonideal kinetics of guanine nucleotides, such as substrate inhibition? Even a brief speculative discussion would add significant depth and rigor to this section.

2. My second point revolves around the enzyme discovery narrative. I feel the presentation of the mining strategy could be more transparent. The decision to focus on mesophiles is a valid strategic choice, but I think it should be framed clearly as such, acknowledging that this specific filter might have excluded other interesting candidates. More importantly, the error in the gene count criterion is a significant issue that isn't fully resolved by just removing it from the report. The simple fact that two of the four successfully characterized enzymes come from genomes with multiple PPK2 genes directly challenges the initial "division of labor" idea. For the story to be fully credible, I think authors need to openly discuss this outcome, conceding that the evolutionary story is more complex and that broad specificity can indeed coexist with multiple paralogs in a genome.

3. For the mechanistic insights, the link between the C-terminal truncation, conformational dynamics, and substrate specificity remains quite speculative. The data shows a clear change in oligomerization, but the rest is a hypothesis. I believe even a relatively short MD simulation and DCCM analysis to compare the dynamics of the full-length and truncated proteins would provide invaluable preliminary support and greatly strengthen this part of the report.

4. My rigorous critique stems entirely from a desire to see this foundational work built on the most solid methodological footing, preventing any potential misunderstandings that could lead other researchers astray. If the authors can address these points thoroughly, I would be very pleased to support the publication of this manuscript in Nature Communications. Personally, having studied PPK enzymes for many years, I find it deeply gratifying to see the field progress through work like this. The discovery of MAN is a meaningful step. As Reviewer 1 (R1.6) points out, the ability to harness pyrophosphate remains a holy grail for our PPK field, as the accumulation of unusable short-chain polyphosphates poses numerous problems. While MAN doesn't achieve this, its broad specificity provides a fantastic foundation for future engineering toward that ultimate goal. I will be following the continued investigation of the N138 position with great interest.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
I have no further comments.

Reviewer #5

(Remarks to the Author)
I have no other questions.

A point-by-point response to Reviewers for *A universal polyphosphate kinase powers in vitro transcription* by Matsumoto *et al*.

Reviewer comments are in black text. Author responses are in blue text.

Reviewer #1 (Remarks to the Author)

R1.1 In their manuscript, Matsumoto et al describe a polyphosphate kinase (PPK) with broad activity towards nucleotide monophosphates (NMPs) and nucleotide diphosphates (NDPs). NTP recycling is definitely a bottleneck in biocatalysis and cell-free biology applications, and broad-specificity PPKs have not been reported yet, which makes the findings of Matsumoto et al. interesting and novel. In general, the manuscript is well-written and provides the field with an interesting new tool. I am very supportive of the work, but a couple of points need to be considered before potential publication.

We thank the Reviewer for their enthusiasm for our work. We have addressed all of the points raised by the Reviewer and we believe that the manuscript has improved significantly as a result of these additions.

R1.2 My biggest concern is the fact that the enzyme shows some broad specificity, but also still has clear preferences, which makes applications not completely straight forward. The authors coupled their systems to an in vitro translation system, however, they cannot run NTP recycling continuously, which would be the real bottleneck to overcome. Thus, the proof-of principle is currently less convincing. I would have appreciated, if the authors has really managed to establish a continuous recycling system (i.e., by parallel operation of two different PPKs (one ATP/GTP-specific plus the broad specificity enzyme that covers UTP/CTP) or by changing the NTP concentrations towards more favorable ratios (e.g., running high CMP and low AMP) to achieve balanced regeneration.

As the Reviewer notes, *in vitro* transcription (IVT) is not a recycling system because the NTPs that are produced are directly incorporated into the growing mRNA molecule and cannot be used again. We are not aware of a reaction system, short of a living cell, that would require *continuous and simultaneous recycling* of all four nucleotides. However, we now demonstrate continuous ATP and GTP recycling from AMP/ADP and GDP, respectively, by MAN using a modified version of PUREfrex 2.0, a reconstituted *in vitro* translation system. These data are presented in the newly added **Figure S15**. As anticipated, MAN is able to recycle ATP and GTP with sufficient efficiency so as to allow for the production of the fluorescent protein GFP. We have updated the main text with the newly added section, *Continuous purine nucleotide regeneration by MAN*, and expanded the Discussion section to better explain and contextualize the points raised by the Reviewer.

R1.3 Secondly, the authors report that the enzyme creates multiple and long-chain phosphorylated NTPs. I am wondering, how much this causes troubles when operating the enzyme in a continuous recycling system, because after multiple turnovers/recycling rounds, all

NTPs should stochastically end up trapped in long-chain states (or could the authors show reversibility?).

The Reviewer raises an insightful point. As *in vitro* transcription is not a recycling system, the impact of APn accumulation in this way is not a major concern. To address this question, we instead used the modified PURExfrex 2.0 system as described above. We find that MAN can recycle ATP and GTP to synthesize GFP, and that neither APn nor GPn were accumulated in significant amounts, even after 2 hours of continuous recycling. These data are presented in the newly added **Figure S15** and in the newly added section, *Continuous purine nucleotide regeneration by MAN*.

R1.4 Along the same lines: The authors claimed that they can control the level of long-chain phosphorylation, but this pomise is actually kept very vague. I think they would need to become more concrete and provide examples (e.g., at PP-10, the level is below X%, etc.), so that the community can really start using the system.

Without such proof of principles for the two main concerns, the authors need to be very cautious about the general claims of broad specificity and applicability.

We note that the data requested by the Reviewer has been provided in the initial manuscript. As per **Figure 3**, the fraction of APn that is produced is given for a range of conditions – including at different temperatures, in the presence of different metal ions, and at different concentrations and lengths of PolyP. For example, APn production is described in **Figure 3C** (left-hand side) for 6 different PolyP-10 concentrations. We now provide **Table S2,** which describes in full the reaction conditions and product distribution for each condition tested in **Figures 2C**, **3**, and **S8**.

In addition, we believe that one important aspect of APn production was not presented: Time. We now provide time course data in the newly added **Figure S6** showing the formation of APn at 55 ºC in the presence of PolyP-60 (65 mM phosphate units). Consistent with our view that the level of long-chain phosphorylation can be controlled, we observe significant ATP production within the first 10 min of the time course. At 30 min, APn production becomes detectable. By 45 min, APn is the dominant species in solution.

We conclude that temperature, Poly-P length and concentration, metal ions, and time are all viable levers with which to control the production of PolyP. In **Figure 3**, we present clear conditions that both favor and suppress APn production.

Additional (minor) concerns:
R1.5 Can the authors make any statements on the selectivity of PPKs for NMPs/NDPs over dNMPs/dNDPs?

We have already collected these data and, indeed, MAN can phosphorylate dNMPs as well. However, we are currently developing applications using this functionality to be reported in a separate manuscript.

R1.6 What is the shortest polyphosphate MAN can use to produce NTPs? Would pyrophosphate suffice?

The Reviewer raises an interesting question. We now provide data exploring the limits of PolyP length utilization in the newly added **Figure S8**. We find that while pyrophosphate cannot be used, triphosphate and tetraphosphate are modestly reactive, with 12-20% conversion to ATP. The circular trimetaphosphate, on the other hand, is largely non-reactive, with only a small amount of NDP production, perhaps due to contaminating linear triphosphates. Finally, we show that hexametaphosphate – which is the most commonly used polyphosphate in the food industry and cosmetics, and often contains a mixture of polyphosphate forms, and thus is among the cheapest polyphosphates available in the market – can support 61% conversion to ATP.

R1.7 Does MAN have any phosphate-transfer activity between NTPs and NDPs/ NMPs?

The reviewer raises another important point. MAN does not efficiently catalyze phosphoryl transfer between NTPs and NMPs of the same class These data are presented in the newly added **Figure S5**.

Reviewer #2 (Remarks to the Author)

R2.1 I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We are glad to have participated in this initiative.

Reviewer #3 (Remarks to the Author)

R3.1 In this manuscript, the authors report the identification, characterization, and engineering of a universal polyphosphate kinase (designated MAN) capable of synthesizing all four nucleoside triphosphates (NTPs). Through gene screening and protein engineering, the authors optimize the catalytic activity of MAN and demonstrate its application in driving in vitro transcription reactions. The work is generally well executed and the manuscript is clearly written. However, several points should be addressed to strengthen the overall impact and clarity of the study.

We thank the Reviewer for their enthusiasm for our work. We believe we have addressed all of the points raised by the Reviewer.

Major comments:
R3.2 The current title may be somewhat misleading. The majority of the manuscript (six out of seven Results subsections) focuses on the screening, characterization, and engineering of MAN,

while only the final section explores its application in in vitro transcription. As such, in vitro transcription is not the core emphasis of the study. The title should be revised to better reflect the main content and contribution of the work.

We respectfully disagree. In our view, the value of a universal PPK2 enzyme must be clearly communicated in the title. It is not sufficient to say that we have discovered such an enzyme, it is also important to communicate how this enzyme or enzymes like it can benefit biotechnology. Since the focus of the paper is to show the application of a universal polyphosphate kinase, and we now provide additional data to support its application in *in vitro* RNA and protein production (**Figures S15**, **S16**, **S17**), we would like to keep the title as it is.

R3.3 In the in vitro transcription experiments (Figure 5B), both transcription reactions appear to plateau at around 4 hours. The authors should clarify the reason for this termination. Whether it results from substrate (NTP) depletion, T7 RNA polymerase inactivation, or other factors. In additionally, quantification of NTP concentrations remaining after the 4-hour reaction would be informative. The final yields of all four NTPs from the MAN-catalyzed one-pot one/two-step reactions should be reported as well.

As the termination of the reaction occurs with authentic nucleotides as well as MAN-synthesized nucleotides at about the same time, this question relates to the properties of the T7 polymerase kit (TOYOBO Code No. TLR-201) more than to the properties of MAN-catalyzed NTP synthesis.

In the newly added **Figure S16**, we show that significant amounts of NTPs remain after 4 h of incubation for both authentic and MAN-synthesized NTPs. This result suggests that a loss T7 RNA polymerase activity is the causal factor and thus outside the scope of the present study.

R3.4 The methodology for using 0.125 mM of each NTP in the control reaction is clear, but it remains ambiguous how the “0.125 mM NTP mixture” from the MAN-generated sample was determined. HPLC quantification should provide precise concentrations of each NTP, and it would be more appropriate to match these concentrations when comparing to the control. Alternatively, if fixed concentrations (e.g., 0.125 mM) are used, their actual composition should be justified. It would also be useful to assess whether the reaction profiles of the MAN-synthesized and commercial NTPs are equivalent under matched conditions (i.e, the same concentration of each NTP).

The concentration of the NTP mixture was calculated assuming 100% NMP to NTP conversion for all nucleotides. This approach emphasizes differences between the MAN-synthesized NTPs and authentic NTP mixtures because if MAN-mediated NTP production is limited, it will directly result in less RNA production. We have updated the text to make sure this point is clearly communicated.

R3.5 The manuscript would benefit from a standalone Discussion section, where the implications, limitations, and future directions of this work can be addressed more comprehensively. Currently, such discussion is embedded within the Conclusion, limiting its visibility and depth.

We agree and have added a Discussion section to the manuscript.

R3.6 The authors use analytical ultracentrifugation to determine whether truncated proteins form monomers or dimers. While valid, this method is relatively specialized. For accessibility and reproducibility, a more commonly used technique such as size exclusion chromatography (SEC) should be included as supportive validation.

AUC is the gold standard for mass determination, and it is generally considered more accurate than SEC. Interactions between a protein and the matrix can result in an apparently lower molecular weight. Elongated molecules, on the other hand, can have apparently larger molecular weights. AUC, on the other hand, involves no potential interactions with the matrix and requires no assumptions of globularity (in fact, AUC can provide information about the shape of the protein or protein complex). These strengths stem from the fact that AUC measures sedimentation and diffusion, and these properties are both related to the molecular weight of a molecule (as well as its degree of hydration and overall shape). We refer the Reviewer to PMIDs: 28479353, 33351266 as well as the textbook *Modern Analytical Ultracentrifugation*, edited by Schuster and Laue, which may be useful resources, and clearly explain the benefits of AUC over SEC for macromolecular weight determination.

Nevertheless, we have collected SEC data (updated **Table S3**). We find that SEC mass estimates are likely artefactual, indicating trimers or hexamers, which is inconsistent with crystallographic evidence and the previously collected AUC data. Crucially, however, the SEC data confirm a change in the oligomerization state upon truncation of the C-terminal α-helix.

R3.7 The practical utility of the MAN platform is not fully demonstrated. The authors are encouraged to explore additional applications of MAN-synthesized NTPs. For example:

Can the in vitro transcribed RNA be used to produce functional proteins (e.g., therapeutic or fluorescent proteins) in a cell-free protein synthesis system?

Yes! We now show that MAN-powered IVT can produce mRNA that can be translated into functional proteins (in this case, GFP) by PURExpress 1.0 *in vitro* translation system. We find that MAN-derived mRNA and authentic nucleotide-derived mRNA showed nearly identical GFP synthesis (**Figure S17**). This result demonstrates that MAN-produced NTPs and any carryover reagents from the NTP-production reaction do not introduce errors into the translation step. These data are presented in the newly added **Figure S17** and the main text has been updated accordingly.

R3.8 What is the maximum length of RNA that can be synthesized using the MAN-generated NTPs?

We now demonstrate RNA production using both Pepper RNA (188 bp) and GFP (971 bp) in the newly added **Figure S17**.

R3.9 Given the enzyme's promiscuity, can it be applied to synthesize non-canonical NTP analogs with biotechnological or therapeutic relevance?

We believe so, and screening of diverse non-canonical nucleotides is planned. To address this question in the meantime, we now provide phosphorylation data for inosine monophosphate (IMP). We observe the phosphorylation of IMP to ITP, but also find that inosine nucleotides are far more prone to over-phosphorylation than either adenine or guanine, despite their structural similarity. These data are presented in the newly added **Figure S4**.

Minor comments:

R3.10 7. In Figure 4A/D, please label residue position numbers for clarity.

We have updated the figure accordingly.

R3.11 8. Consider including a basic cost comparison between the reported method and commercially available NTPs.

We now provide a cost comparison analysis between our approach and the standard approach in the newly added Discussion section. This section references both the traditional enzymatic reaction schemes that produce NTPs (**Figure S18**) and some key reagent costs (**Table S4**). We reproduce this new analysis here:

> Reagent costs and synthesis pipelines for the large-scale production of nucleotides and nucleic acids are considered proprietary information and are not publicly disclosed. However, a price comparison between NTPs ($1095/mmol, sum of 1 mmol of ATP, GTP, UTP and CTP) and NMPs ($78/mmol, sum of 1 mmol of AMP, GMP, UMP and CMP) confirms that the phosphorylation step is a key contributor to NTP production costs (**Table S4**). This price difference is likely because NMPs can be readily produced, for example, by simply hydrolyzing biologically derived RNA[50]. Subsequent NMP phosphorylation, if performed enzymatically, will be different if using cell-pathway inspired reaction schemes (according to KEGG database[51]) or a uPPK2 (**Figure S18, Table S4**). With cellular reactions, the synthesis of one mol unit of NTPs from NMPs needs 6 enzymes and 8 mol unit of creatine phosphates ($296/8 mmol). On the other hand, uPPK2-mediated synthesis of one mol unit of NTP requires one enzyme and 8 mol unit of polyphosphate ($0.128/8 mmol). Moreover, while the cellular-inspired reaction process requires at least 6 pots, the uPPK2-based process requires only one pot, further reducing production costs. In summary, NMP to NTP synthesis by MAN is far cheaper than that by cell-pathway inspired reaction.

R3.12 9. Although the manuscript mentions the use of SDS-PAGE for protein analysis in Methods, no gel images are provided. Please include representative SDS-PAGE results in the supplementary materials.

We now include an SDS-PAGE gel demonstrating the purity of MAN, CHU, LEE, ARE, truncated CHU, truncated LEE, and truncated ARE (**Figure S18**).

Reviewer #4 (Remarks to the Author)

R4.1 The authors demonstrated that the PPK from Mangrovibacterium marinum (MAN) was able to phosphorylate all four common nucleotide monophosphates (AMP, GMP, CMP, UMP) and diphosphates (ADP, GDP, CDP, UDP) to their corresponding triphosphates (ATP, GTP, CTP, UTP). This uPPK2 has the potential for application in nucleotide phosphorylation and synthesis. There are the comments:

The author demonstrated the one-pot nucleotide synthesis and in vitro transcription reaction, and emphasized that this work will reduce the cost of DNA and RNA production. Before this statement, the authors should conduct in-depth comparisons and cost analyses. Otherwise, such a statement would be completely unfounded.

We now provide a cost analysis in the Discussion section. See also response **R3.11**.

R4.2 The author should define uPPK2.

We have reworded the introduction for improved clarity.

R4.3 There were some typos in the manuscript, such as "…diphosphates (ADP, GDP, CDP, UDP) to their corresponding triphosphate (ATP, GTP, CTP, UTP)…" on page 3 should be "triphosphates".

We have corrected this typographical error and carefully checked the text.

R4.4 It is already known that Class III PPK2 can accept both adenine and guanine, so it is not surprising that MAN could phosphorylate different ribonucleotides.

Presumably the Reviewer is referring to the CHU enzyme (*ACS Catal.* **8**, 10746–10760 (2018).). As we have shown in **Figure 1C**, CHU is less efficient against cytosine and significantly over-phosphorylates guanine nucleotides. Because of these reasons, CHU is not able to power IVT (**Figure 5B**). To date, no class III PPK2 has been shown to phosphorylate all common ribonucleotides with nearly similar efficiency. We note that both adenine and guanine are purines (double ring structure), whereas uracil and cytosine are pyrimidines (single ring structures). The presumption that both classes of nucleotides should be substrates for PPK2 enzymes is incorrect, as demonstrated by **Figure 2**. Moreover, many enzymes with broader substrate profiles (high promiscuity) often have poor conversion efficiency (*Annu. Rev. Biochem.* **79**, 471–505 (2010)), greatly limiting their potential applications.

R4.5 The author should give a brief introduction about the Class III PPK2 in the Introduction Part, including the structural information, sequence length, and the mechanism study (such as CHU).

We have updated the manuscript to better introduce Class III PPK2 enzymes.

R4.6 "N138 contributes to utilization of all nucleotides". Does it mean that, except for MAN, other wild-type PPK2 can also utilize all nucleotides, or through simple protein engineering? The author should give some supplementary studies and a discussion.

We believe that this statement has been misunderstood. We intended to describe the role of N138 in MAN. Substitution of N138 in MAN results in a loss of broad specificity. PED, ARE, LEE, and CHU have N138, while they do not show as broad specificity as MAN. These data suggest that N138 in MAN *contributes* to the utilization of all nucleotides in MAN, but is clearly not the sole factor that determines the broad specificity. The nature of the contribution (be it dynamical, related to protein stability, or through direct interactions) cannot be determined as of yet.

Reviewer #5 (Remarks to the Author)

R5.1 The manuscript introduces and characterizes MAN, a Class III PPK2 enzyme described as the first "universal" polyphosphate kinase capable of efficiently phosphorylating all eight canonical ribonucleotides, including both monophosphates and diphosphates. While MAN indeed displays a broad substrate range and may hold practical potential in nucleotide regeneration systems, I find that the central claims regarding catalytic efficiency and universality are significantly overstated. This, combined with superficial mechanistic analysis and a key citation error, limits the manuscript's overall scientific strength and its suitability for Nature Communications.

We provide a detailed response to each of the Reviewer's comments below.

R5.2 The core issue concerns the interpretation of catalytic performance. The claim that MAN efficiently phosphorylates all eight ribonucleotides is directly contradicted by the kinetic data presented in Table 1. Specifically, the catalytic efficiencies for CMP and CDP are 170–270 times lower than that for AMP. UMP and UDP also exhibit significantly lower values than purines.

We agree that MAN does have clear substrate preferences. Despite these preferences, however, all four common ribonucleotide NTPs can be produced with sufficient yield on a short timescale (90 min) to drive *in vitro* transcription. Moreover, endpoint product distributions after just 60 minutes show ~70% conversion of NMPs and NDPs to NTPs. Other reported PPK2 enzymes (*e.g.*, CHU) do not have these key properties. We certainly agree that engineered forms of MAN with even flatter substrate preferences would be of biotechnological value.

R5.3 Throughout the manuscript, the authors rely on endpoint conversion after 60 minutes of incubation as a measure of efficiency, but this approach masks differences in reaction rate and does not reflect intrinsic catalytic capacity.

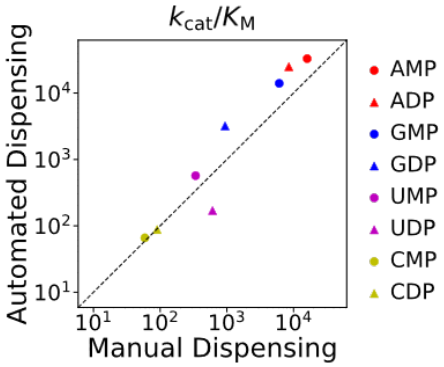
MAN kinetic data (k_{cat}, K_M, k_{cat}/K_M) for each of the 8 nucleotide substrates (purines and pyrimides, monophosphates and diphosphates) are provided in **Table 1**.

R5.4 The kinetic analysis in Supplementary Figure S5 lacks necessary details, and the fitting curves for several substrates appear irregular and have low resolution. Without reporting Vmax values, replicate experimental data, or fitting parameters, it is impossible to judge the reliability of the kinetic constants in Table 1.

We have re-collected the data in **Table 1** and **Figure S9** (previously Figure S5), now with three replicates, because the previous data was performed with only a single replicate. The Methods section has been updated to clearly describe the data collection and fitting procedure. In addition, for each fit in Figure S9 (formerly Figure S5), fitting parameters are provided as insets.

Previously, pipetting was done by hand (Manual Dispensing). The initial velocity errors were estimated from the linear regression of product distribution data from HPLC (N=1) collected at various timepoints. Note that the standard errors of the initial velocities were not shown on the previous version of **Figure S9** but are now shown. Fitting to the Michaelis-Menten equation was then performed using the initial velocities and their standard errors. The standard errors associated with V_{max} and K_M were then obtained from the covariance matrix of the fit, and incorporated the individual velocity errors as weights.

We now follow essentially the same procedure, except that each timepoint used in the calculation of the initial velocity is collected in triplicate (N=3) and pipetting was performed using the Echo 525 liquid handling system (Beckman; Automated Dispensing). The results from these two approaches(manual and automated dispensing) are in good agreement (see plot below). The manuscript has been updated to include only the automated dispensing results.



R5.5 Although the authors attempt to explore the molecular basis for substrate generality, including through mutation of residue N138, truncation of the C-terminal helix, and domain-swapping constructs, the analysis remains largely descriptive. For example, N138 mutations lead to a non-uniform drop in NTP synthesis across substrates, with AMP being largely unaffected. In my opinion, this suggests that the N138 mutation alone cannot explain the substrate breadth of the enzyme.

We thank the Reviewer for acknowledging the diverse types of constructs that we characterized, including point mutations, truncations, and chimeric constructs. We fully agree that an Asn at position 138 cannot explain the substrate breadth of the enzyme on its own. We claim only that this position can *contribute to* broad specificity, not that it is the sole causative factor. Please see also response **R4.6**.

R5.6 Likewise, truncation of the C-terminal helix improves purine activity at the cost of pyrimidine conversion. The authors attribute this to changes in oligomerization or conformational dynamics, but without structural or biophysical data, this remains speculative.

We previously provided analytical ultracentrifugation data (**Figure S13**) and now provide size exclusion chromatography data (updated **Table S3**), both of which demonstrate that full-length and truncated PPK2 enzymes adopt different oligomerization states. How these changes in oligomerization may affect the enzymatic properties of MAN is an intriguing question, and we are currently pursuing crystallization studies to this end.

R5.7 The chimeric constructs suggest a complex functional landscape but do not lead to predictive insights. Overall, while the term "rugged functional landscape" is used to describe MAN's substrate behavior, the underlying molecular factors remain insufficiently resolved.

We share the Reviewer's frustration. Rugged functional landscapes, by their nature, result in unpredictable behavior that makes evolutionary exploration (and laboratory characterization!) difficult. To quote the discussion section:

> However, our analysis suggests that PPK2 enzymes in general (and MAN in particular) have a relatively rugged functional landscape, particularly for non-adenine bases. This conclusion follows from the diverse properties of N138 Clade III PPK2 enzymes (**Figure 2**) and the idiosyncratic, non-uniform changes in substrate preference upon mutation (**Figures 4B** and **Figure S11**) and chimerization (**Figure 4C** and **E**). The general sensitivity of these enzymes to reaction conditions (**Figure 3**) may also be an indication of a rugged functional landscape.

R5.8 The application of MAN in in vitro transcription is an interesting demonstration, but the setup reveals limitations. The need for a two-step substrate addition protocol (pyrimidines followed by purines). The choice to use polyP-10 due to cost considerations, despite its lower catalytic performance compared to longer polyP chains, further limits the enzyme's demonstrated potential. While these are understandable compromises in an experimental context, they reduce the strength of the claim that MAN is broadly and practically applicable. For an enzyme being proposed as a universal catalyst for nucleotide regeneration, its performance should be evaluated under ideal rather than constrained conditions.

We agree. We now present the effect of PolyP length and concentration on MAN-powered IVT in the newly added **Figure S19**. We found that PolyP-10 at 30 mM was the best among the tested conditions, though PolyP-60 and PolyP-700 could also be used. The two-step addition setup may be overcome by optimizing the reaction conditions or engineering MAN.

R5.9 There is a critical citation error in the manuscript that undermines confidence in the structural analysis. In Figure 1C, the authors cite Kuge et al. (2025) as a reference for structural insights into Class III PPK2 enzymes like CHU, but that reference in fact describes a Class II enzyme. This misattribution is especially problematic given the absence of any experimentally determined structure for MAN or its homologs.

We thank the reviewer for calling our attention to this citation mistake. However, we would like to note that (a) the correct citation was also present, (b) the main text discussion does not include the incorrect citation, (c) the indicated PDB structure codes and AF3 models are correct and refer to the appropriate enzymes.

R5.10 While the enzyme itself may be of interest in applied settings, its discovery is empirical rather than conceptual. The study does not advance fundamental principles in enzymology, protein evolution, or mechanistic biochemistry. The narrative linking MAN to themes such as enzymatic promiscuity and origin-of-life chemistry is speculative and not supported by novel insight. As it stands, the work offers an example of a broadly active enzyme but does not contribute new mechanistic understanding or a framework for rational design. This limits its relevance for a high-impact journal like Nature Communications.

We believe that this work provides the most detailed characterization of a PPK2 enzyme substrate preference to date, reports the broadest specificity PPK2 to date, and shows the first application of a PPK2 enzymes to key *in vitro* reaction system.

As protein scientists with a deep interest in primitive biology and synthetic biology, we find the connection between the two fields inspiring. Indeed, primitive biology has been a throughline of the PPK2 field since its inception because polyphosphate is a potential primitive store of phosphorylating power and because PPK2 enzymes are members of one of the most ancient and ubiquitous enzyme families, P-Loop NTPases. We have updated the Discussion to better communicate the connection between our work and primitive biology research.

R5.11 Several technical issues related to candidate enzyme selection and data quality that deserve attention. The authors state that PPK2 sequences were filtered using three criteria. While the first criterion is understandable, the latter two raise questions. If the goal is to discover a broadly active enzyme with possible biotechnological utility, then excluding thermophilic homologs from the start appears contradictory, especially given that thermostable enzymes often have broader utility, and yet the phylogenetic tree in Figure 1A includes many thermophilic sequences.

Whether thermophilic enzymes have generally broader substrate profiles is unclear. The Andreas Wagner group, for example, has recently demonstrated that “high temperature delays and low temperature accelerates” the evolution of new phenotypes (Zheng et al., 2024, *Nature Communications*). At high temperatures, enzymes can be more sensitive to destabilizing mutations, blocking further functional exploration. Older work, for example, from the Dan Tawfik

Group, has noted the importance of chaperones for related reasons (Tokuriki and Tawfik, *Nature,* 2009). Brian Shoichet and others have noted that protein stability and function can trade-off (Beadle and Shoichet *J Mol Biol* 2002), suggesting the possibility that broad functional profiles may come at the expense of thermostability.

While we certainly appreciate the intuition put forth by the Reviewer – likely stemming from the fact that (a) many researchers believe the LUCA was a thermophile and (b) primitive cells are thought to have required generalist enzymes to run their metabolism – the connection between habitat and enzyme promiscuity has not been well established, and the evidence noted above supports an alternative view.

R5.12 The logic behind including them [thermophilic sequences] in the tree but excluding them from screening is never explained.

There are key methodological reasons to include genes from thermophilic organisms when calculating a gene tree, even if one does not plan to characterize those genes experimentally. In short, excluding genes of a given type can lead to an unnecessary loss of evolutionary context. Genes from thermophiles, for example, may form deep-branching clades on the gene tree, which can help clarify the topology of the resulting tree. Instead, it is preferable to cluster genes based on sequence similarity and choose representatives – for example, by using the program CD-HIT, as we do here – to calculate the phylogenetic tree. This approach will achieve a more even sampling of sequence space and a more accurate phylogenetic tree.

R5.13 Likewise, restricting the search to genomes with only one PPK2 gene in order to avoid "division of labor" is speculative. This assumption may discard functional variants that have evolved broad specificity or versatility precisely because of coexistence with other paralogs.

Upon re-analysis of our code, we found a mistake related to this criterion. While the parent organisms of MAN and LEE have just a single PPK2 gene, the parent organisms of PED and ARE have more than one PPK2 gene. This mistake occurred because contigs, not assembled genomes, were downloaded from GenBank in error. (Note that our previous study of PPK2 gene distribution across prokaryotes, PMID 39691974, used the GTDB database and was not subject to this error.) We apologize for the mistake and we have removed this criterion from the manuscript.

R5.14 Overall, the criteria appear designed more to narrow to a preselected outcome than to support an unbiased screen.

We stand by our criteria and are disheartened that the Reviewer believes we have intentionally misrepresented the selection process. We also apologize again for the error regarding the PPK2 gene count criteria.

R5.15 Also, there are issues in the analytical methods. In Figure 5B, the HPLC chromatograms show significant co-elution of AMP and UTP, whose retention times appear to overlap. This is a

critical technical flaw in a study where relative nucleotide conversion is a central output. If AMP and UTP cannot be resolved under the chosen conditions, then it is difficult to assess conversion efficiency accurately, particularly for AMP-containing reactions or where UTP is being produced from UMP.

We note that only **Figure 5** is affected by peak ambiguity between nucleotides, as all other experiments involved only the mono-, di-, and tri-nucleotide forms with the same base. Therefore, the phosphorylation efficiencies of the 8 common nucleotides were quantified properly.

In the case of **Figure 5**, there are actually *two* probes of NTP formation: The first is HPLC and the second is RNA aptamer formation. Regarding the chromatogram, we can infer the production of CTP from the disappearance of CMP and CDP. And, given that Pepper RNA is ultimately produced, we can be confident that all four NTPs were present.

To improve the clarity of this figure, we have updated our HPLC analysis for mixed-nucleotide samples so that all triphosphate nucleotides can be unambiguously assigned. The results are in full agreement with our previous interpretation that all four NTPs are present. We have updated **Figure 5** accordingly.

R5.16 Better chromatographic resolution, such as using ion-pairing or alternative detection methods (e.g., MS), is essential to support the conclusions.

We are currently using triethylammonium acetate as an ion pairing agent. We have updated the Methods section to clearly state the role of triethylammonium acetate.

A point-by-point response to Reviewers for *A universal polyphosphate kinase powers in vitro transcription* by Matsumoto et al.

Reviewer comments are in black text. Author responses are in blue text.

Reviewer #3 (Remarks to the Author)

R3.1 The authors addressed most of my previous comments. However, regarding my query about the 0.125 mM NTP mixtures generated via MAN catalysis, I remain unconvinced by the explanation that "the concentration of the NTP mixture was calculated assuming 100% NMP to NTP conversion for all nucleotides," based on the presented data.

We believe that there has been a misunderstanding about what we mean when we say that we assume "100% conversion". We respond to this comment in detail below.

The HPLC analysis of authentic nucleotides in Figure S2 was helpful, but after comparing Figure S2 and Figure 5B, I believe further clarification is needed on the following points:
1. The retention time of ATP in Figure S2 is before 4 min, whereas in Figure 5B, it appears after 5 min. Could the authors explain this discrepancy?
2. For accurate comparison of nucleotide retention times, the X-axis (retention time) in Figure 5B should be presented using the same scale as in Figure S2.

The Reviewer is correct – the retention times in **Figure S2** and **Figure 5B** are different. Previously, Reviewer 5 raised concerns about the resolution of the HPLC chromatogram in **Figure 5B** (see R5.15 in the previous point-by-point reply). We updated the protocol from one column to two columns in tandem to improve the resolution of samples that contain nucleotides of all four bases. This change is only relevant to **Figure 5B** and is detailed in the **Methods** section, but was not noted clearly in the main text.

We have updated the figure caption for improved clarity. In addition, we now provide a chromatogram of authentic samples using the updated ("enhanced") protocol as part of **Figure 2**.

3. In Figure 5B (top), the CMP peak should be clearly indicated with an arrow. Does the first peak correspond to CMP? If so, the first (major) peak in Figure 5B (bottom) would also represent CMP. This observation makes it difficult to support the assumption of "100% NMP to NTP conversion for all nucleotides," as notable peaks are still present in the HPLC traces.

The positive control contained 0.125 mM of each NTP, whereas test samples contained 0.125 mM nucleotide mixtures derived from a one-pot reaction with PPK2, assuming complete conversion of NMPs to NTPs. In reality, it is a mixture of NXPs. We thus have reworded the main text to be clearer on this point as below.

"The test samples contained 0.125 mM of each nucleotide type (AXP, GXP, CXP, and UXP) derived from a one-pot reaction with PPK2 using NMPs as the initial substrates. Only if the PPK2 phosphorylation reaction is efficient will the nucleotides be predominantly triphosphates."

4. Given the appearance of multiple peaks in Figure 5B, I recommend the authors clearly label all relevant peaks and provide a more detailed retention time scale (1 min, 2 min, 3 min, 4 min and 5 min) on the X-axis to facilitate interpretation.

We have updated the figure for clarity.

Reviewer #5 (Remarks to the Author)

R5.1 The authors have made commendable progress in addressing the previous round of critiques, and I can see the clear improvements in the revised manuscript.

We thank the Reviewer for their positive remarks.

However, from my perspective, a few critical issues still need to be resolved to ensure the study is as robust and clear as it can be.

1. I still have concerns about the kinetic data. While the presentation is better, the standard errors for GMP and GDP are high enough that I find it difficult to draw firm conclusions about their precise catalytic efficiency. I strongly believe the manuscript should explicitly acknowledge this limitation in the text or figure legend to prevent readers from over-interpreting these particular values.

We have updated the manuscript to better highlight the fact that the coefficient of variation (CV) values for the k_{cat} and K_M of GDP and GTP are larger than for other substrates (ranging from 0.37 - 0.92 for GMP and GDP versus 0.09-0.24 for all other nucleotides).

Furthermore, I am genuinely curious about what might be causing this variability. Is it a pH shift caused by the addition of high concentrations of GMP or GDP? Or is it a sign of nonideal kinetics of guanine nucleotides, such as substrate inhibition? Even a brief speculative discussion would add significant depth and rigor to this section.

The pH of the nucleotide stock solutions were adjusted; thus, we think it is unlikely that pH differences caused the variability. Guanine nucleotides are well known to assemble in solution, and we have encountered this property in other experimental contexts. We have updated the text to include this as a possible explanation for the greater error for GMP and GDP kinetic constants.

2. My second point revolves around the enzyme discovery narrative. I feel the presentation of the mining strategy could be more transparent. The decision to focus on

mesophiles is a valid strategic choice, but I think it should be framed clearly as such, acknowledging that this specific filter might have excluded other interesting candidates.

We agree, and have updated the narrative to include the insightful discussion that happened in the Reviews/Response to Reviews.

More importantly, the error in the gene count criterion is a significant issue that isn't fully resolved by just removing it from the report. The simple fact that two of the four successfully characterized enzymes come from genomes with multiple PPK2 genes directly challenges the initial "division of labor" idea. For the story to be fully credible, I think authors need to openly discuss this outcome, conceding that the evolutionary story is more complex and that broad specificity can indeed coexist with multiple paralogs in a genome.

We removed all references to this criterion in our text because it no longer applies. Thus, the “division of labor” idea does not appear in the main text of the current version. However, we agree it is an interesting point, and we have updated the discussion.

3. For the mechanistic insights, the link between the C-terminal truncation, conformational dynamics, and substrate specificity remains quite speculative. The data shows a clear change in oligomerization, but the rest is a hypothesis. I believe even a relatively short MD simulation and DCCM analysis to compare the dynamics of the full-length and truncated proteins would provide invaluable preliminary support and greatly strengthen this part of the report.

We had previously reached out to an expert in molecular dynamics and were strongly encouraged to get a crystal structure before proceeding. We were strongly encouraged to start simulations with a crystal structure if possible. We are aggressively pursuing crystallization, and recent results are highly encouraging.

4. My rigorous critique stems entirely from a desire to see this foundational work built on the most solid methodological footing, preventing any potential misunderstandings that could lead other researchers astray. If the authors can address these points thoroughly, I would be very pleased to support the publication of this manuscript in Nature Communications. Personally, having studied PPK enzymes for many years, I find it deeply gratifying to see the field progress through work like this. The discovery of MAN is a meaningful step. As Reviewer 1 (R1.6) points out, the ability to harness pyrophosphate remains a holy grail for our PPK field, as the accumulation of unusable short-chain polyphosphates poses numerous problems. While MAN doesn't achieve this, its broad specificity provides a fantastic foundation for future engineering toward that ultimate goal. I will be following the continued investigation of the N138 position with great interest.

We thank the Reviewer for their positive remarks. We agree that the efficient utilization of very short-chain polyphosphates will be an exciting frontier.