Supplementary Material for:

A comparison of *Dictyostelium discoideum* 3'-5' RNA polymerases reveals a conserved $tRNA^{His}$ guanylyltransferase residue that plays a dual role in catalysis

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Figures S1-S5

Supplementary Figure Legends

Figure S1. Sequence Alignment of Thg1 and TLP enzymes. The sequence alignment was performed in CLUSTAL O(1.2.4) with twelve Thg1/TLP enzymes from all three domains of life: *Dictyostelium discoideum* (Ddi) Thg1, TLP2, TLP3, and TLP4; *Bacillus thuringiensis* TLP (BtTLP): *Acanthamoeba castellanii* (Aca) TLP1 and TLP2; *Homo sapiens* (Hs) Thg1; *Saccharomyces cerevisiae* (Sc) Thg1; *Candida albicans* (Ca) Thg1; *Methanosarcina acetivorans* (Ma) TLP; and *Methanopyrus kandleri* (Mk) TLP (Sievers and Higgins 2014). The shading of conserved residues was performed by Geneious 6.1.8. DdiThg1 numbering is used for important residues. Residues known to be important for both Thg1 and TLP function are indicated in black and residues known to be important for only Thg1 function are indicated in red.

Figure S2. Complete AlphaFold 3 models of complexes with DdiTLP2, DdiThg1, and DdiTLP4.

All models generated with AlphaFold 3 (Abramson et al. 2024) included a protein homotetramer,12 Mg^{2+} ions, 4 ATP, and 4 GTP. Each model was generated with a known substrate tRNA for its relevant activity; DdiTLP2 model includes two copies of *D. discoideum* mt-$tRNA^{His}$. DdiThg1 model includes two copies of *D. discoideum* cyt-$tRNA^{His}$. DdiTLP4 model includes two copies of *S. cerevisiae* $tRNA^{His}$ truncated by one nucleotide at the 5' end and including a G+2-C71 base pair. Protein subunits are colored yellow, magenta, green, and cyan. tRNA has an orange backbone with blue nucleotide bases. Mg^{2+} ions are green. ATP and GTP are green with oxygen colored red and nitrogen colored blue.

Figure S3. SDS PAGE of DdiTLP2 and DdiThg1 mutants tested in this work. (A) 10 µg each of purified DdiTLP2 and DdiThg1 WT and variants (KPND, KPHD, R187D and DCKR refer to DdiTLP2 variants shown in Figure 2) were resolved by 12% SDS-PAGE. Ladder shows the

molecular weight of indicated standards in kD. **(B)** 5 µg each of purified DdiThg1 variants with different alterations at D150 were resolved by 12% SDS-PAGE. Ladder shows molecular weight of indicated standards in kD.

Figure S4. Reaction schemes for single turnover kinetic assays using A+1-U72 mutant tRNA^{His} constructs. (A) Nucleotidyl transfer assay using 5'-[γ^{32}P]-triphosphorylated mt-tRNA^{His}. Use of the triphosphorylated tRNA bypasses the need for 5'-adenylylation, and therefore reports specifically on the nucleotidyl transfer step, as demonstrated previously (Smith and Jackman 2012). Addition of the G-1 nucleotide by DdiTLP2 results in release of labeled pyrophosphate (red P indicates position of labeled phosphate), which can be resolved from the unreacted remaining ppp-tRNA fragment (generated by RNase A cleavage, red arrow). Some hydrolysis of PPi to Pi occurs during reaction processing. Nucleotidyl transfer rates were performed using the same procedure for cyt-tRNA^{His}, which was also altered to contain the terminal A+1-U72 base pair to enable 5'-[γ-^{32}P]-labeling. **(B) Adenylylation assay using 5'-^{32}P- monophosphorylated mt-tRNA^{His}.** Reactions are performed as described in Figure 6, except that the change to the first base pair of the substrate results in generation of a longer oligonucleotide product as the product of G-1 addition to this tRNA.

Figure S5. (A) Determination of k_{obs} for nucleotidyl transfer for the non-WC base paired addition of G-1 to cyt-tRNA^{His}. Product formation (as shown on the representative TLC plate in Figure 8A) was plotted as a function of time and fit to eq. 2 to determine k_{obs} for three or four replicates for DdiThg1 WT and D150 variants. k_{obs} determined from each were averaged as reported in Table 2. **(B) Determination of k_{obs} for nucleotidyl transfer for the WC base paired addition of G-1 to mt-tRNA^{His}.** Product formation (as shown on the representative TLC plate in Figure 8A) was plotted as a function of time and fit to eq. 2 to determine k_{obs} for three or four replicates for DdiThg1 WT and D150 variants. k_{obs} determined from each were averaged as

reported in Table 2. **(C) Determination of k_{obs} for adenylylation for the WC base-paired addition of G-1 to mt-tRNA^{His}.** Product formation (as shown on the representative TLC plate in Figure 8C) was plotted as a function of time for three replicates of the DdiThg1 WT and D150 variants. The reaction with D150 variants was fit to eq. 2 to determine k_{obs} . The reaction with DdiThg1 WT was analyzed using the method of linear initial rates (see Methods) using eq. 3, based on the linear fit to the data up to 120 min of reaction. k_{obs} determined form each were averaged as reported in Table 2.

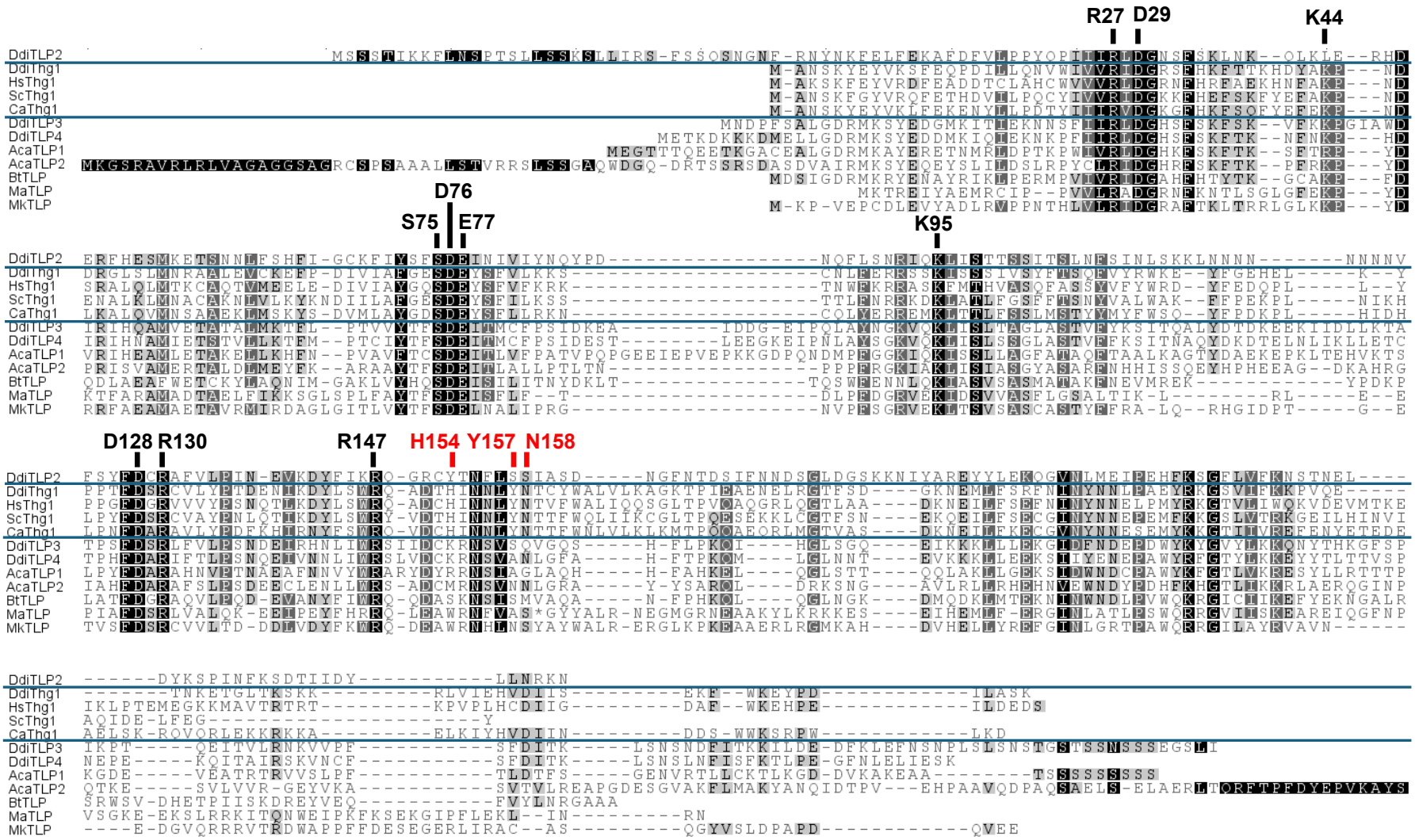
References

Abramson J, Adler J, Dunger J, Evans R, Green T, Pritzel A, Ronneberger O, Willmore L, Ballard AJ, Bambrick J et al. 2024. Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**: 493-500.

Sievers F, Higgins DG. 2014. Clustal Omega, accurate alignment of very large numbers of sequences. *Methods Mol Biol* **1079**: 105-116.

Smith BA, Jackman JE. 2012. Kinetic analysis of 3'-5' nucleotide addition catalyzed by eukaryotic tRNA(His) guanylyltransferase. *Biochemistry* **51**: 453-465.

Figure S1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E77
K95
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H154
Y157
N158
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DdiThg1
HsThg1
ScThg1
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AcaTLP2
BtTLP
MaTLP
MkTLP

Figure S2

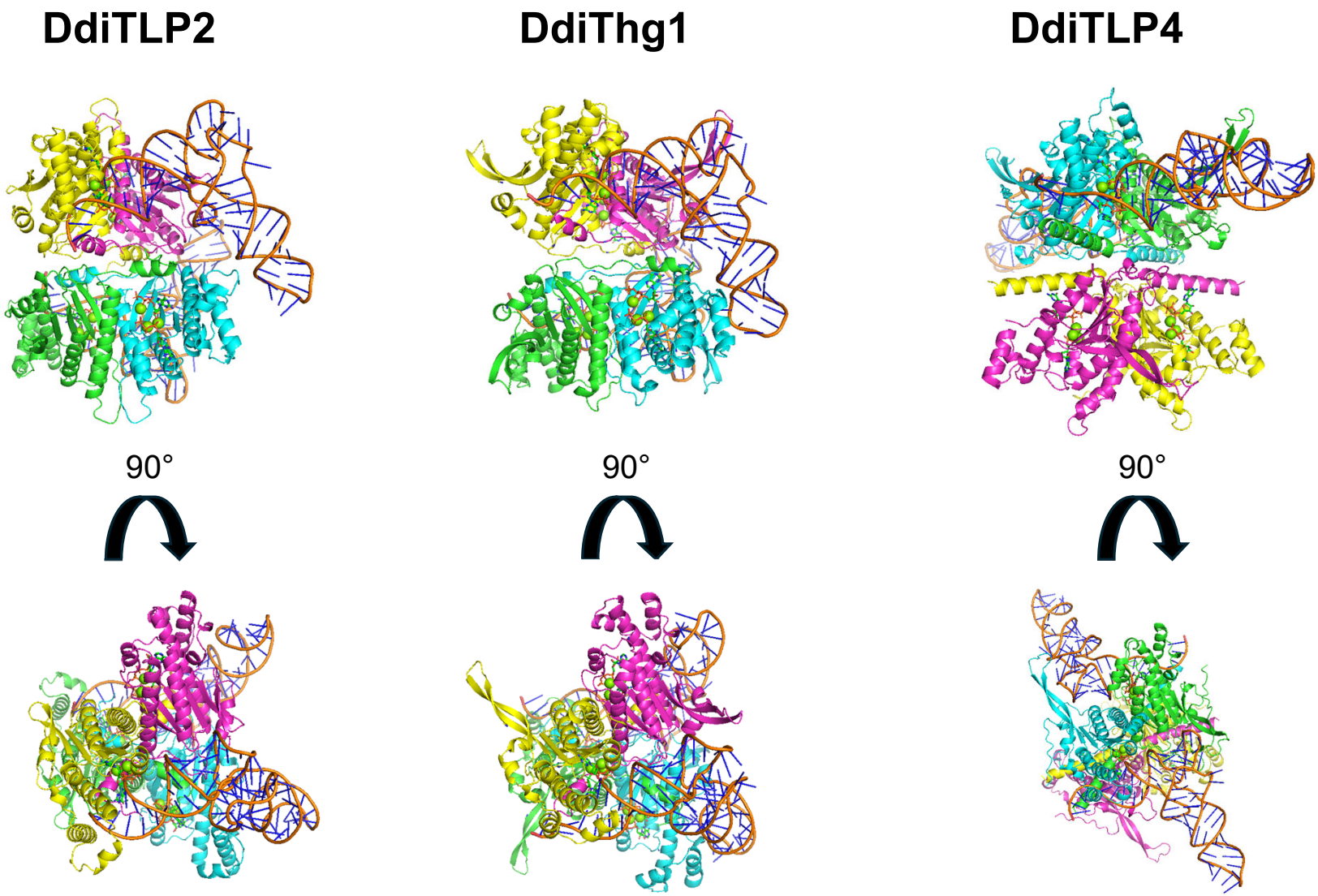
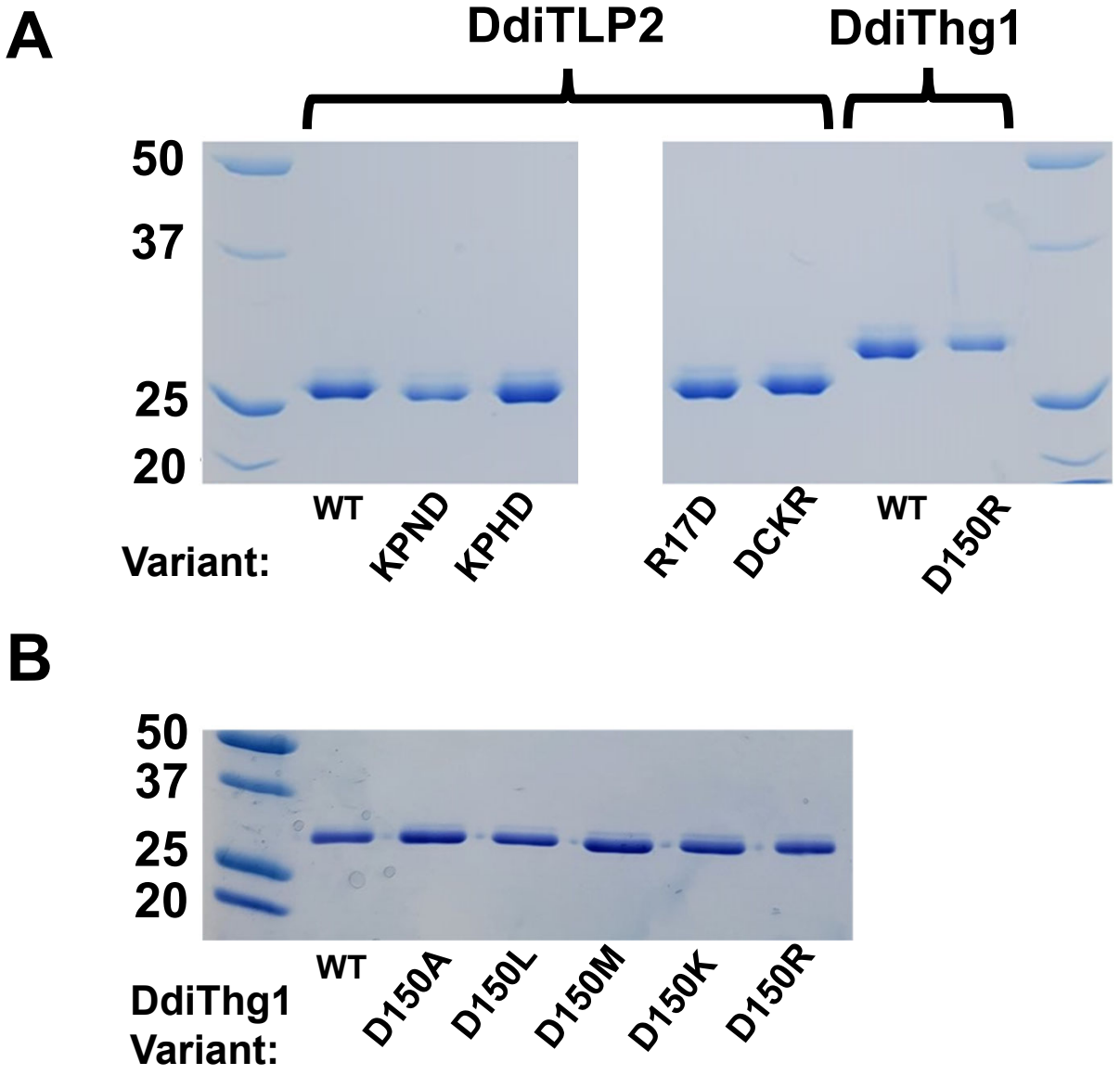


Figure S3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DdiTLP2
DdiThg1
50
37
25
20
WT
KPND
KPHD
R17D
DCKR
WT
D150R
Variant:
B
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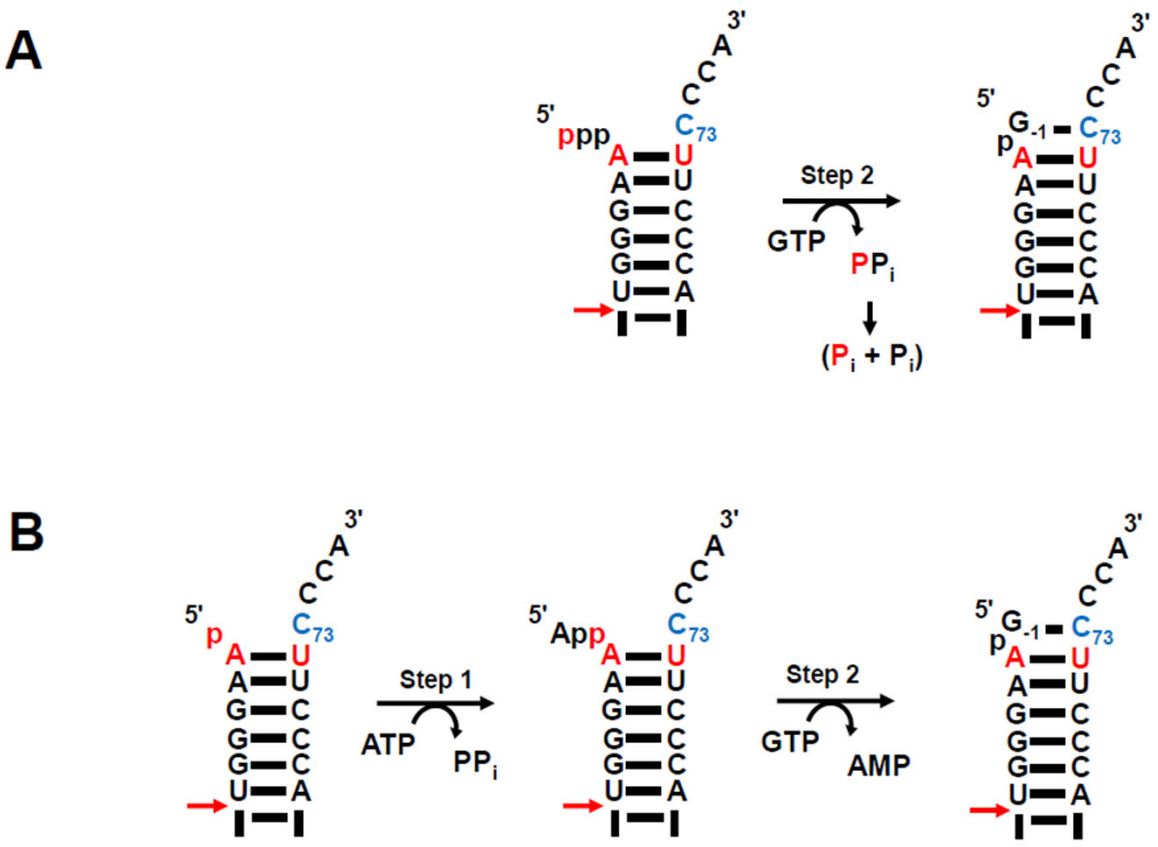
37
25
20
WT
D150A
D150L
D150M
D150K
D150R
DdiThg1
Variant:

Figure S4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G
G
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C73
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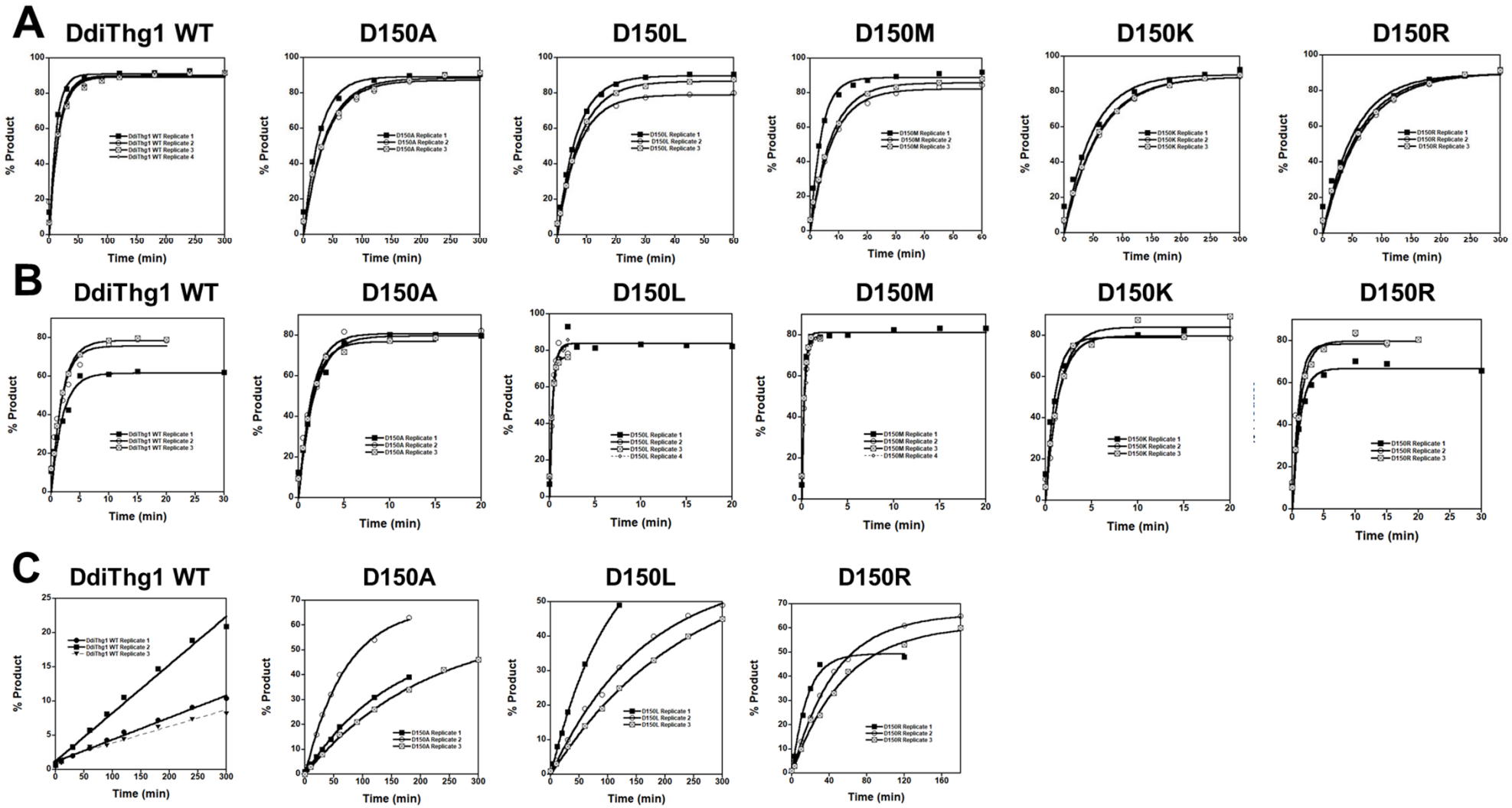
U
C
C
C
A
3'
C
C
A
Step 2
GTP
PPi
(Pi + Pi)
5'
pG-1
pA
C73
3'
B
5'
p
ATP
Step 1
PPi
5'
Appp
GTP
Step 2
AMP

Figure S5


A
DdiThg1 WT
D150A
D150L
D150M
D150K
D150R
B
DdiThg1 WT
D150A
D150L
D150M
D150K
D150R
C
DdiThg1 WT
D150A
D150L
D150R
% Product
Time (min)