## **Supporting Information**

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MEC(U)

RNA template DNA non-template DNA AAUA AUCGAGAGGG TCGGTAGCTCTCCCGGT AGCCATCGAGAGGGGCCA

MEC(A)

RNA template DNA non-template DNA

AAUA AUCGAGAGGGG<sup>A</sup> GTCGGTAGCTCTCCCCG CAGCCATCGAGAGGGGGC GCTTATCGGT CGAATAGCCA

EC1

AUCGAGAGGGACA RNA template DNA ATGTCGGTAGCTCTCCCTGTGCCGCTTATCGGT TACAGCCATCGAGAGGGACACGGCGAATAGCCA non-template DNA

FC2

AAUA AUCGAGAGG RNA template DNA CGGTAGCTCTCCACGTGCC GCCATCGAGAGGTGCACGG GCTTATCGGT CGAATAGCCA non-template DNA

AAUA AUCGAGAGGG

EC3

RNA non-template DNA

template DNA

EC4

CGTAGCTCTCCCTGTGC

AAUA AUCGAGAGAGG RNA template DNA GTAGCTCTCCCTCTGC non-template DNA

EC5

AAUA AUCGAGAGAGG RNA template DNA GGTAGCTCTCCC CCATCGAGAGGG non-template DNA

EC6

AAUA AUCGAGAGAGG RNA template DNA non-template DNA GTAGCTCTCCCGATGCC CATCGAGAGGGCTACGG

Fig. S1. Elongation complexes used in the study. Shown are the sequences of nucleic acid scaffolds used to assemble elongation complexes.



Fig. S2. Kinetics of phosphodiester bond hydrolysis in MEC(U) and MEC(A) by WT,  $\Delta$ TL, Q1242A, R1239A, and H1242A RNAPs. Quantification of the data is shown in Fig. 1 *B* and *C*, respectively. Gels and quantification of WT and  $\Delta$ TL cleavage reactions are shown in Fig 1 *B* and *C*.



**Fig. S3.** Template misalignment does not increase rate of misincorporation. (*A*) Cartoon in the top of the panel shows formation of elongation complexes  $MEC(G)^n$  backtracked by 1 bp [from elongation complex EC4 (Fig. S1)] and  $MEC(GG)^n$  backtracked by 2 bp [from elongation complex EC3 (Fig. S1)] Below: Kinetics of misincorporation of 1 mM GTP in EC3 and EC4 and hydrolysis of the second and the third phosphodiester bonds in  $MEC(GG)^n$  and  $MEC(GG)^n$ , respectively, by WT RNAP. (*B*) Quantification of the misincorporation kinetics of GTP in EC3 and EC4 by *T. aquaticus* WT RNAP (data from A), UTP in EC5 and EC6 by *T. aquaticus* WT RNAP (data from Fig. 4A), and by *E. coli ECWT* RNAP (data from Fig. 4C).



Fig. 54. pH profiles of second phosphodiester bond hydrolysis in MEC(U) (*Red*) and MEC(A) (*Blue*) complexes (colors correspond to reactions shown schematically above the plots) by ΔTL RNAP.

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