Supplementary Material for:

Thg1 family 3'-5' RNA polymerases as tools for targeted RNA synthesis

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Supplementary Table 1: DNA duplexes used to generate RNA templates by *in vitro* transcription.

Name	DNA duplex sequences: top: T7 promoter oligonucleotide bottom: templating oligonucleotide[a]
19 bp	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CTTTGGCAACACCAGAGGGCGCGCGCG* 5′
13 bp	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CAACACCAGAGGGCGCGCGCG* 5′
10 bp	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CACCAGAGGGCGCGCGCG* 5′
8 bp	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CCAGAGGGCGCGCGCG* 5′
7 bp	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CAGAGGGCGCGCGCG* 5′
AU	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CAGAGGGTATATATA* 5′
Mixed	5′ TAATACGACTCACTATA 3′ 3′ ATTATGCTGAGTGATAT*CAGAGGGAGGTTCAG* 5′

[a] underlined sequence corresponds to reverse complement of template RNA

Supplementary Figure 1: Optimization of the 5'-targeted extension assay to achieve single nucleotide resolution.

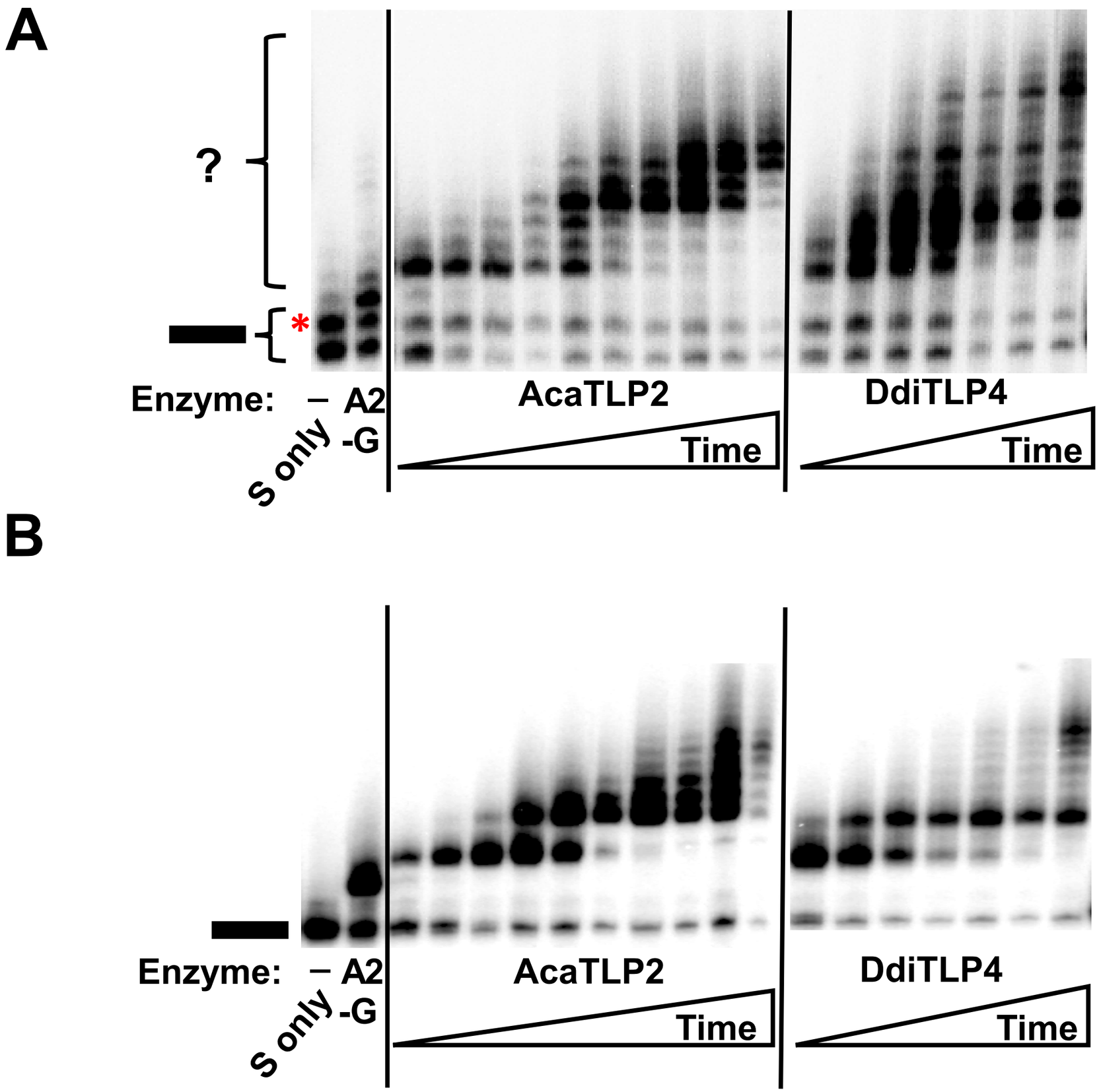
3'-5' polymerase activity was assayed with 8 bp RNA duplexes as described in methods with AcaTLP2 and DdiTLP4 (15 μM) under single turnover conditions over a 0-30 minute time course using either **(A)** 26-mer RNA substrate derived from run-off transcription, or **(B)** 26-mer RNA substrate derived from transcription with a 3'-HDV ribozyme sequence to generate a homogeneous 3'-end. The "S only" lanes are control reactions containing labeled substrate RNA, in which the heterogeneity of the run-off transcript is clearly visible in panel A (marked with red star), whereas the labeled substrate migrates as a nearly homogeneous (~95%) single band in panel B. Lane A2-G corresponds to the control reaction where only CTP was added, and thus marks the migration of the single C-addition product, which again corresponds to a single band in the HDV-derived transcript (panel B) vs. multiple bands in the original run-off transcript (panel A). Thus, use of the HDV-derived transcript was required to definitively identify products of 3'-5' polymerase activity with the RNA duplexes tested here.

Supplementary Figure 2: MsTLP and BtTLP bind RNA duplexes with relatively lower affinities compared to AcaTLP2, DdiTLP4 and ScThg1.

Binding affinity of MsTLP and BtTLP for each RNA duplex was measured using a double filter-binding assay as described in the main text and compared to affinity for $tRNA^{His}$. The fraction of bound RNA product was plotted vs. the concentration added in each reaction (0-10 μM), with linear fits representing non-specific binding (K_D > 10μM) for enzymes that exhibited detectable RNA binding in the assays. Average fraction bound was determined from triplicate assays

performed with each enzyme. The data were fit to Eq. 2 to determine the apparent K_D for each enzyme binding to the tested RNAs, which are indicated in Table 1.

Supplementary Figure 1



Supplementary Figure 2