SUPPLEMENTARY FIGURE LEGENDS

Supplementary figure S1: Comparison of t/PK for human, *Tetrahymena* and mouse TERs. t/PK domains of (A) human, (B) *Tetrahymena* and (C) mouse TERs are shown. Human t/PK region sequence changes studied in this work were in the template (pink), template 3'-flanking residues (green, spaced by 3 nt from the underlined template AA bases), P2a.1 bottom strand (orange), template 5'-flanking residues (dark yellow), P3 top and bottom strands (purple and blue respectively). Corresponding regions of *Tetrahymena* and mouse TERs are highlighted using the same color scheme. Proposed alternative conformations of the t/PK are shown on the right: alt-P3 for human and mouse TERs (generated by manual sequence inspection), and stem 3-alt for *Tetrahymena* TER (adapted from (36)).

Supplementary figure S2: Activity of template 3'-flanking region mutants. Comparison of activity of RRL-reconstituted enzyme with mutations in the template 3'-flanking single-stranded region and P2a.1, assayed in the absence or presence of dCTP. Strategy for testing template 5' boundary bypass is shown at the top. Reactions included T_{21}-GTTAGG primer. The blue dot indicates products from template 5' boundary bypass observed only in presence of dCTP, while the red dot indicates products stalled or dissociated prior to complete repeat synthesis observed in absence and presence of dCTP in activity assay reactions. Values of rRAP and rAct were calculated as in Fig. 1, with the same color-coding key duplicated for reference.

Supplementary figure S3: High RAP does not require a specific sequence of the template 3'-flanking single-stranded region. Activity assay of RRL-reconstituted 59-61 substitution mutants carried out with full-length TERT RNP using T_{21}-GTTAGG primer in the presence of all 4 dNTPs. 59-61 WT sequence is in green and substitution nt are in black. Values of rRAP and rAct were calculated as in Fig. 1.

Supplementary figure S4: RAP defects do not derive from reduced product turnover. (A) Schematic of the pulse-chase primer extension assay. RRL-reconstituted telomerase was diluted in activity assay buffer and incubated with 50 nM of 27-nt T_{21}-GTTAGG initial primer at room temperature for 30 min. Primer extension reactions were initiated by adding different dNTPs (spiked with α-^{32}P-dGTP), and allowed to proceed at 30 °C for 10 min. After the initial 10 min pulse, 100-fold excess of the 18-nt T_{12}-GTTAGG chase primer was added, and DNA synthesis was allowed to continue at 30°C while removing aliquots and quenching the reaction at regular time intervals. (B) Pulse-chase assay in reactions with all 4 dNTPs. The 0 min chase lanes are reactions with initial primer elongation for 10 min. (C) First-repeat products from chase primer extension (indicated by a bracket) were divided by the first-repeat products from initial primer extension at 0 min chase, and plotted as a function of increasing chase time. The relative chase for mutants was normalized to that of WT enzyme at each individual time point, and the mean values of relative chase from different times are plotted on the right. Error bars represent the standard deviation from the mean.

Supplementary figure S5: Activity of hTRmin template mutants reconstituted in RRL with full-length TERT or TERT ring RNP. (A) Activity assays for hTRmin enzymes with some or all template adenosines substituted to uridines using either full-length TERT RNP (top) or TERT ring RNP (bottom), with reactions performed in the absence of dCTP. Of note, TERT ring showed a slight gain in RAP for AA49/55UU and Tetra-U mutants compared to WT hTRmin; it was entirely inactive with AA48/54UU mutant for an unknown reason. (B) Activity assay of hTRmin enzymes with all 4 template adenosines substituted to uridines or guanosines, with reactions performed in the presence of all 4 dNTPs. Values of rRAP* and rAct were calculated as in Fig. 3. For TERT ring RNP products, we quantified rRAP with the ≥2 repeat cut-off as in Fig. 1. Similar results were obtained from 2-3 independent experimental replicates.

Supplementary figure S6: Compensatory mutation strategy for rescue of AA48/54GG template. Illustrations show putative alt-P3 and anticipated mature P3 conformations of hTRmin mutants. Color coding and states 'a-g' are the same as in Fig. 4.

Supplementary figure S7: Compensatory mutation strategy for rescue of AA49/55GG template. Illustrations show putative alt-P3 and anticipated mature P3 conformations of hTRmin mutants. Color coding and states 'a-g' are the same as in Fig. 4.

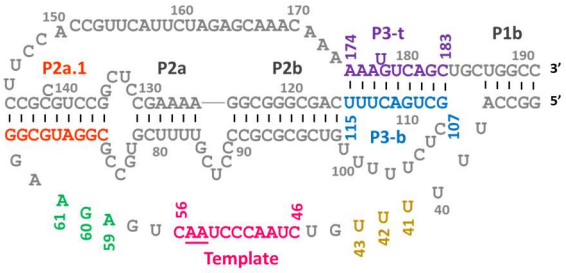
Supplementary figure S8: Compensatory mutation strategy for rescue of C51G template. Illustrations show putative alt-P3 and anticipated mature P3 conformations of hTRmin mutants. Color coding and states 'a-e' are the same as in Fig. 4.

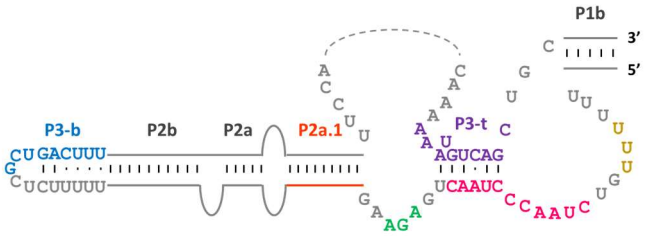
Supplementary figure S9: Formation of P3 duplex but not alt-P3 is essential for RRL reconstitution of active telomerase. (A) Activity assay of hTRmin P3 sequence substitutions reconstituted in RRL, with mutations in P3 top or bottom strand that disrupt P3 duplex base-pairing or compensatory mutations in both strands that restore P3 duplex. (B) Activity assay of hTRmin mutants reconstituted in RRL with complementary mutations in the top and bottom P3 strands that preserve P3 duplex but severely destabilize alt-P3 (schematic in Fig. 6). Assay was carried out with full-length TERT RNP using T_{21}-GTTAGG primer in the presence of all 4 dNTPs. Control reaction without hTRmin is shown at right. (C) Activity assay of U57 hTRmin mutants reconstituted in RRL, with their alt-P3 duplexes indicated at the left. The mutated base is shown in green. Values of rRAP* and rAct were calculated as in Fig. 3. Similar results were obtained from 2 independent experimental replicates.

t/PK
Mature P3

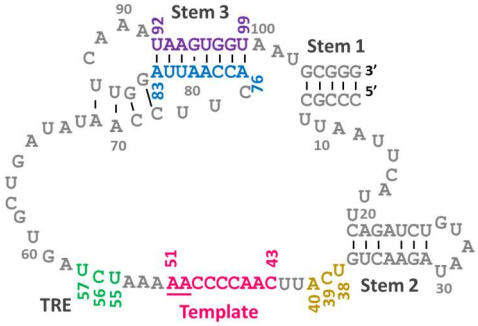
t/PK
Alt-P3

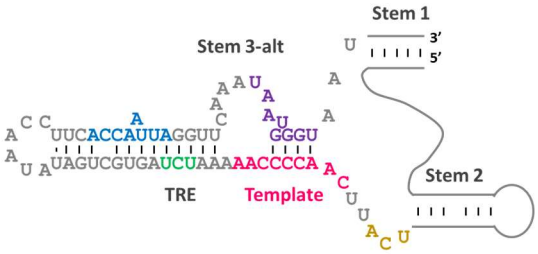
A Human



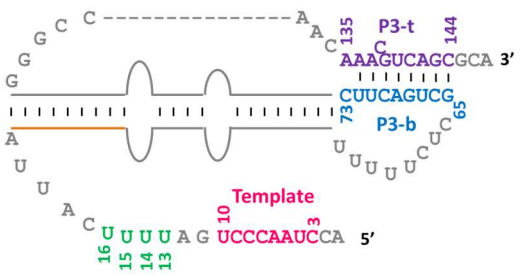


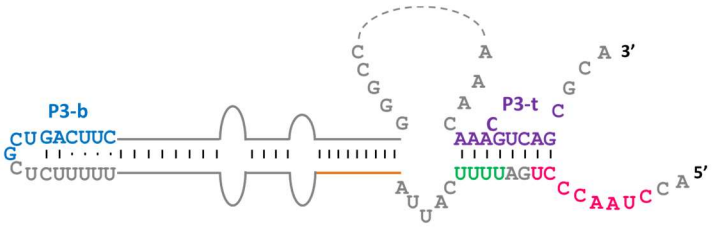
B *Tetrahymena*



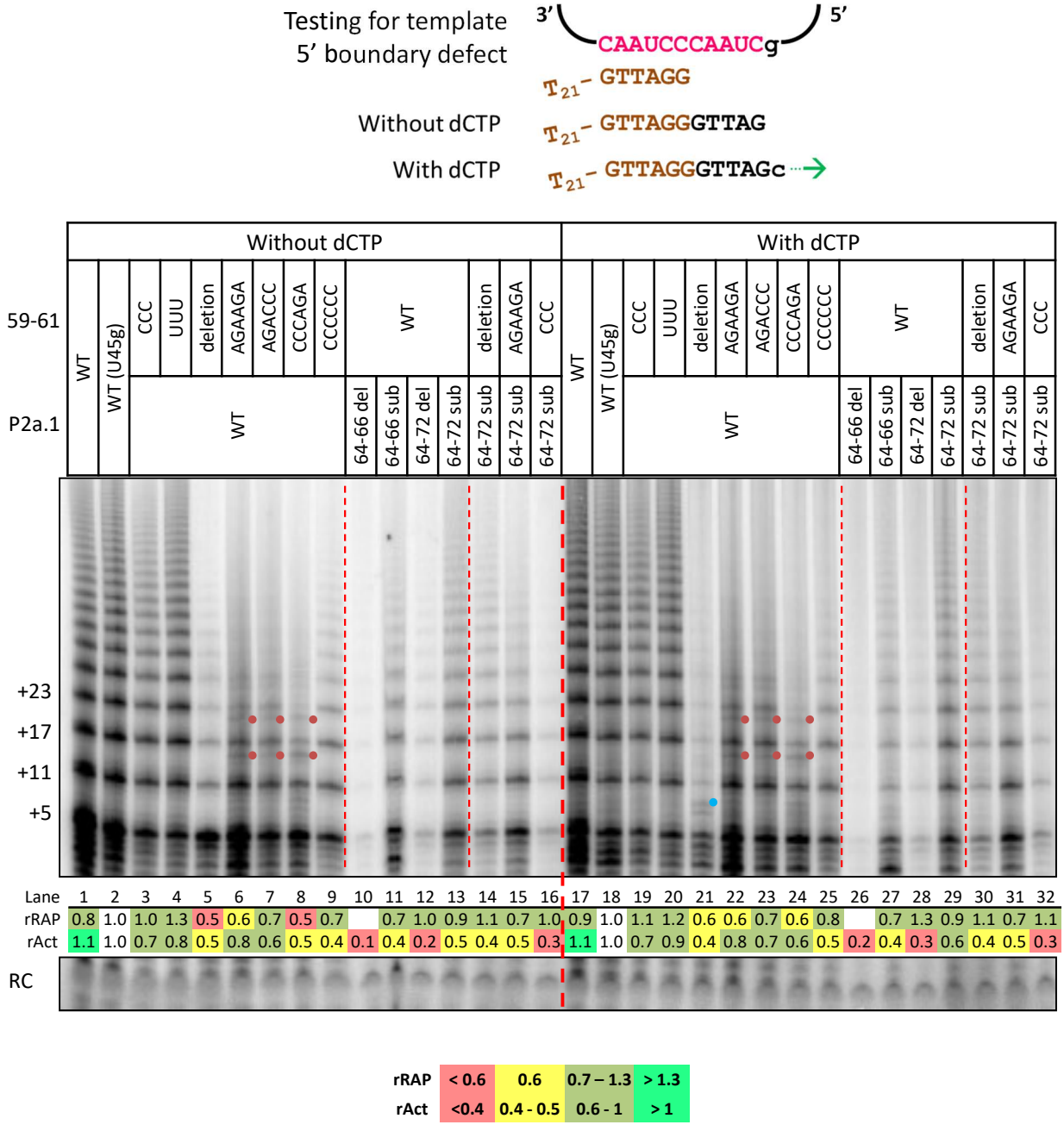


C Mouse





Supplementary figure S1



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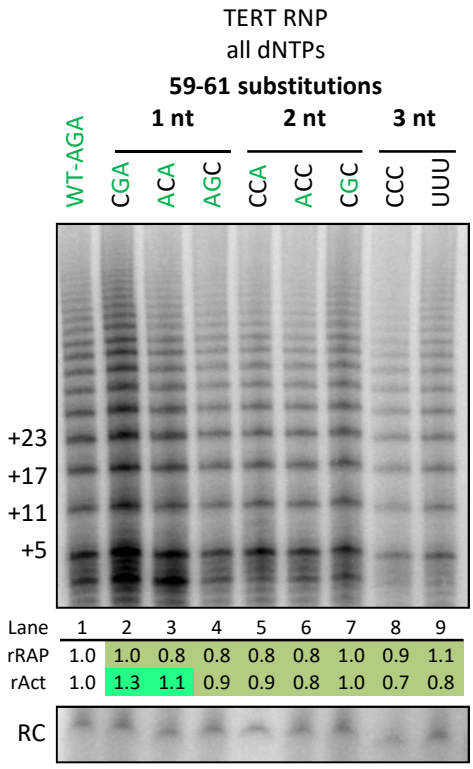

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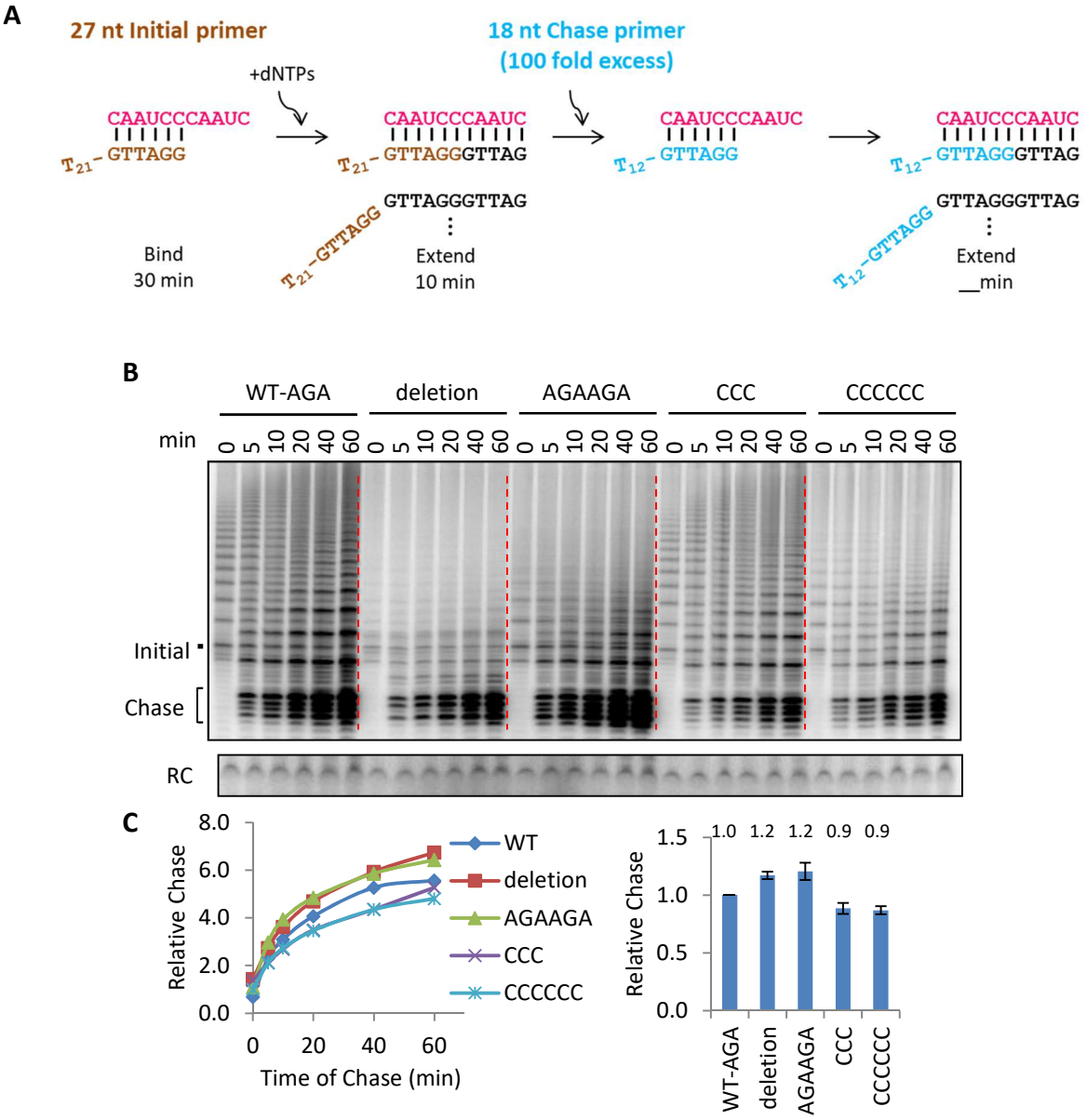

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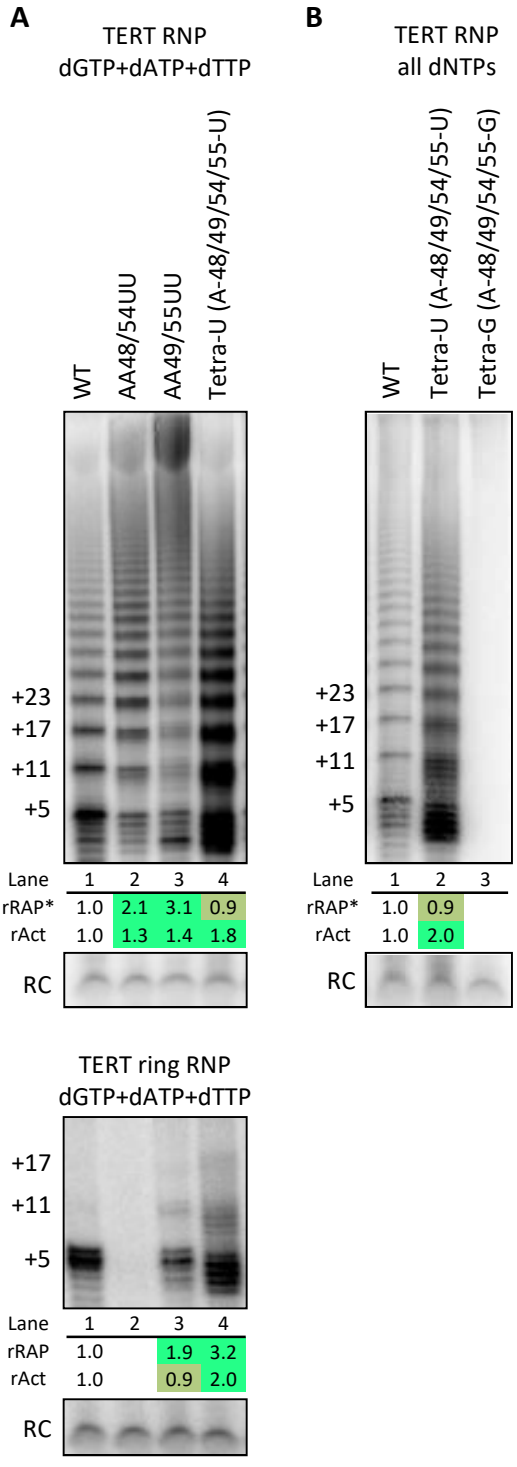

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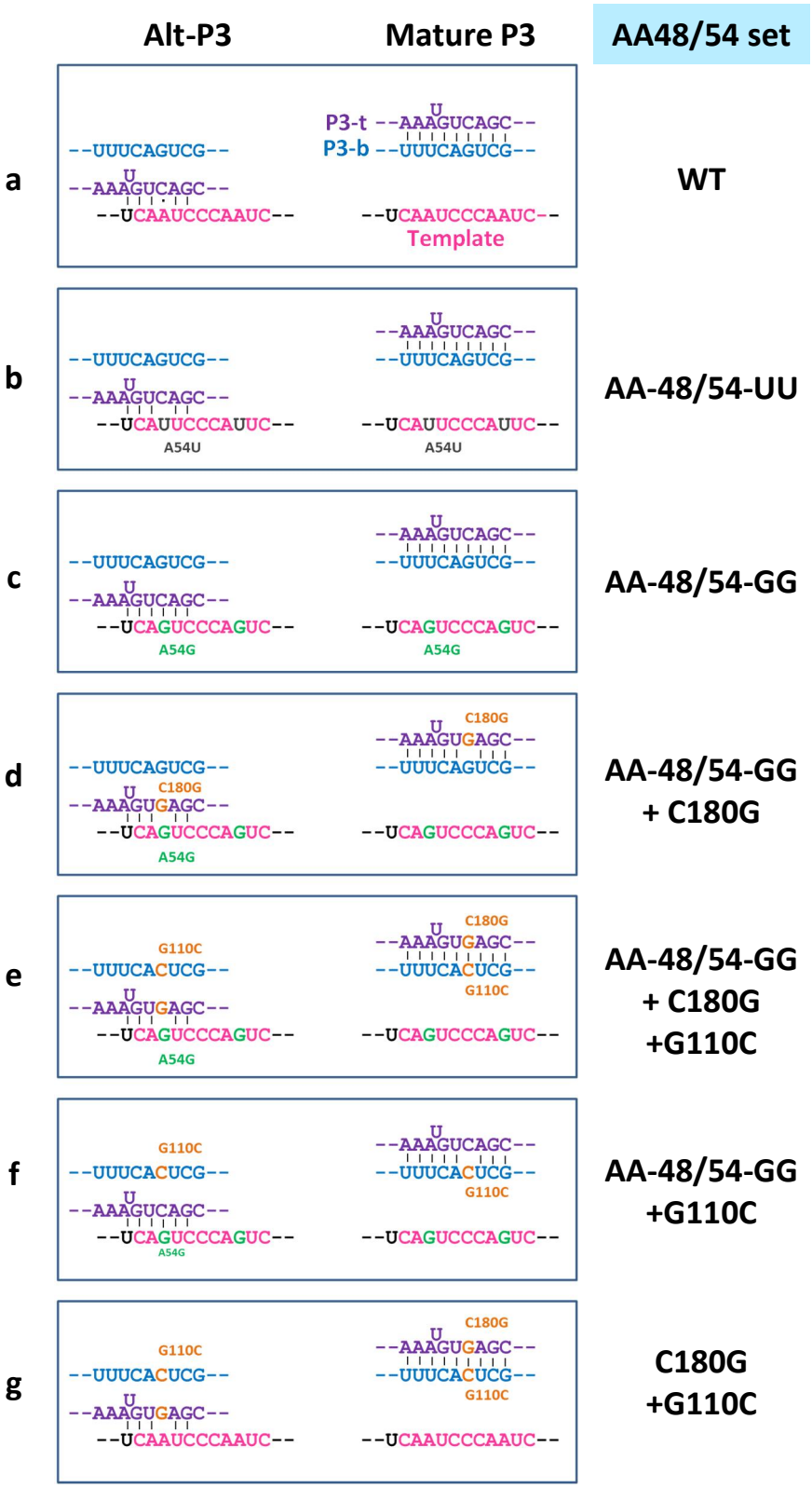

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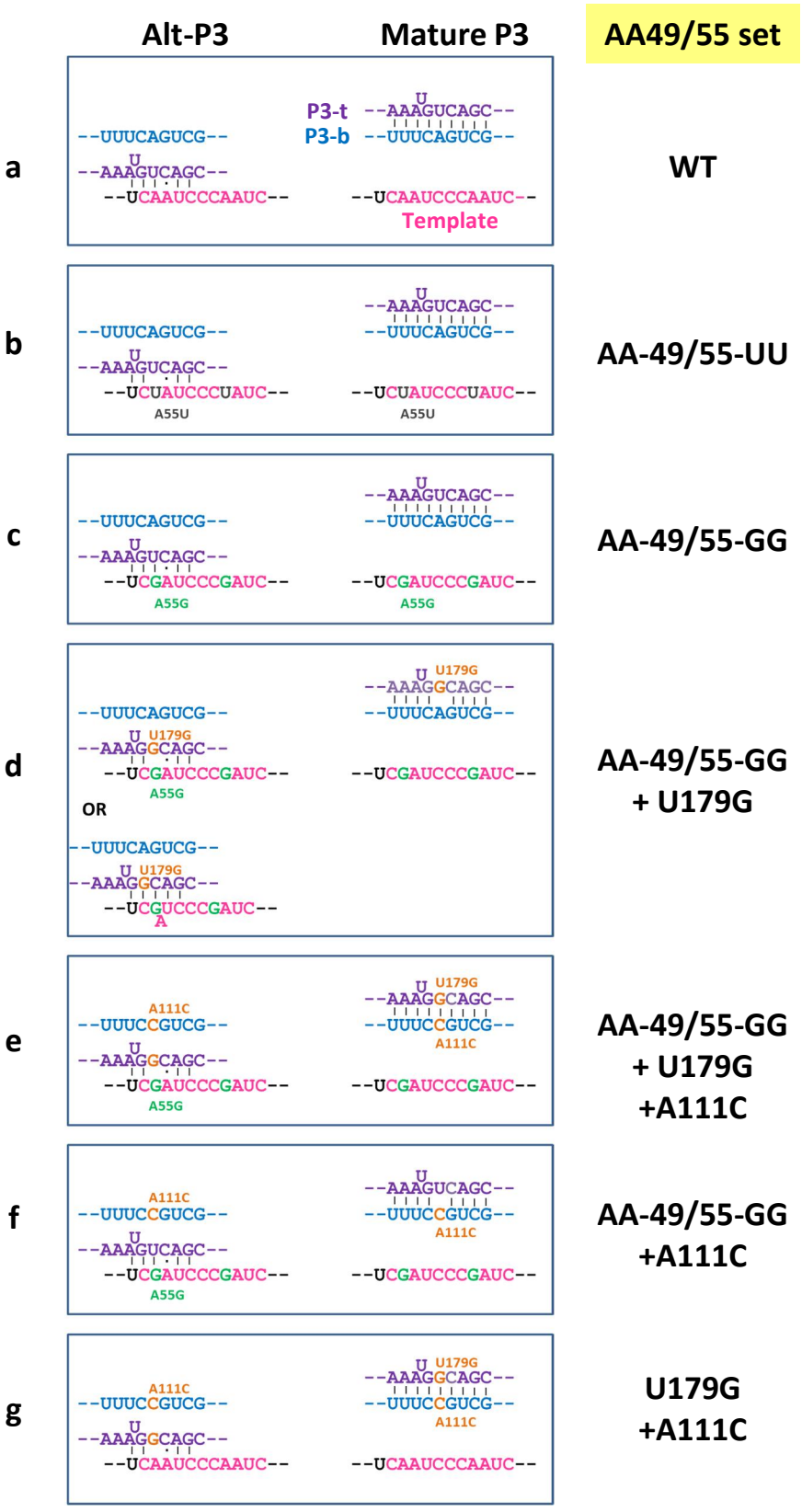

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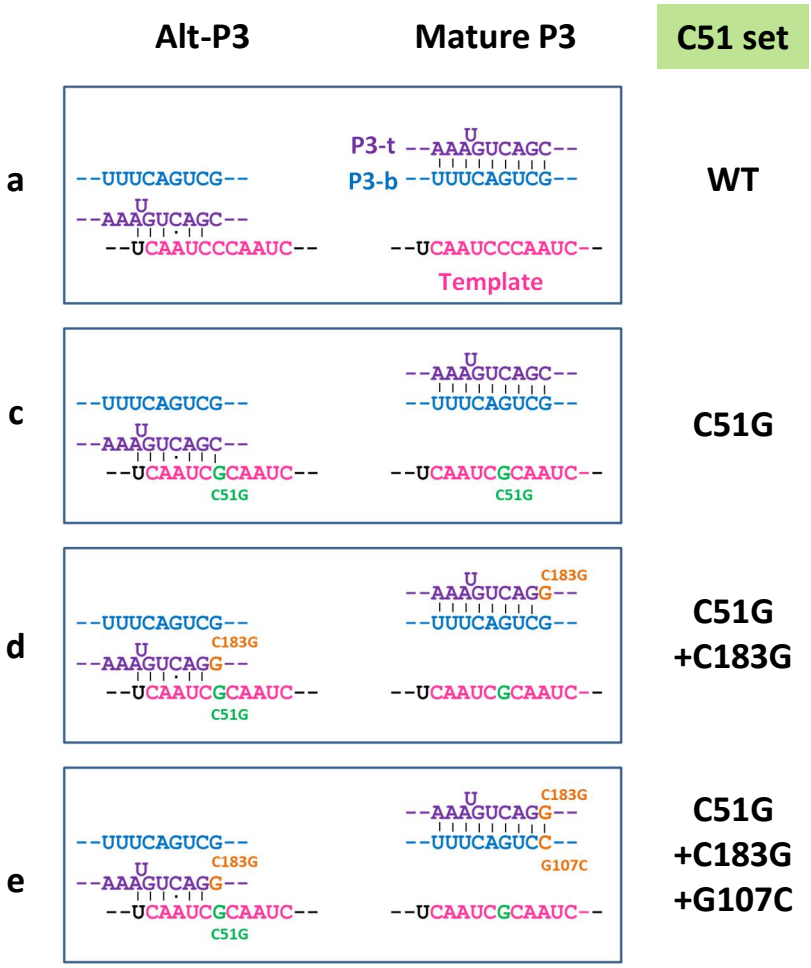

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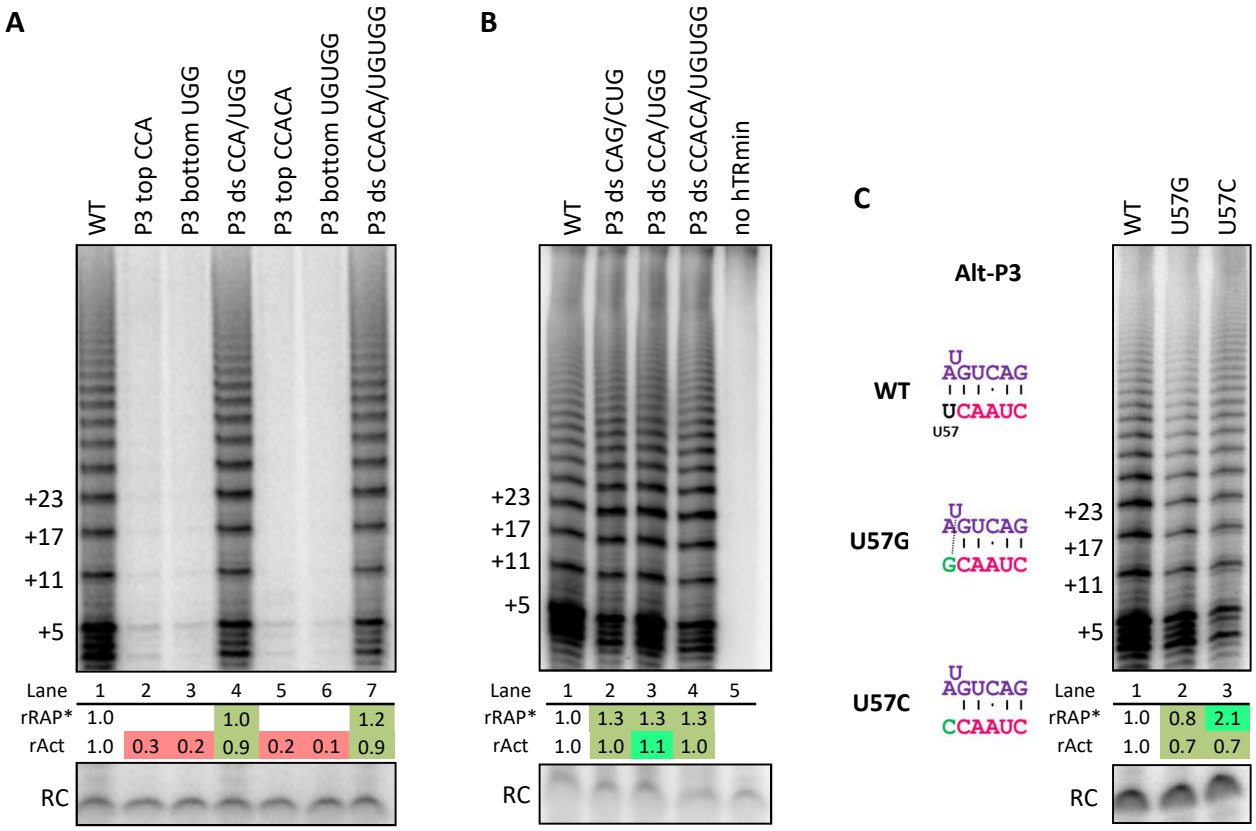

Supplementary figure S9