

## Supporting Information

for manuscript entitled

### **Adenosine residues in the template do not block spontaneous replication steps of RNA**

by

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**General information.** Reagents were the best available grade from Acros (Geel, Belgium) or Aldrich/Fluka/Sigma (Deisenhofen, Germany). Dowex 50 WX8-200 cation exchange resin was from Acros (Geel, Belgium). Template **2** was purchased from biomers.net GmbH (Ulm, Germany) and primer **1** from IBA GmbH (Göttingen, Germany), either in HPLC-purified form. Universal Support II was from Glen Research (Sterling, VA, USA),  $\beta$ -cyanoethyl phosphoramidites of 2'-*O*-TBDMS-protected ribonucleosides of all four protected nucleobases (A<sup>tac</sup>, C<sup>tac</sup>, G<sup>tac</sup>, U) and standard reagents for RNA synthesis were from Proligo Biochemie GmbH (Hamburg, Germany). Activators for RNA synthesis were 4,5-dicyanoimidazole (0.25 M in MeCN) or 5-[3,5-bis(trifluoromethyl)phenyl]-1*H*-tetrazole ("Activator 42", 0.25 M in MeCN). Either were obtained from Proligo Biochemie (Hamburg, Germany). The final step of the purification of synthetic oligoribonucleotides was performed on SepPak RP-C18 cartridges from Waters GmbH (Eschborn, Germany). MALDI-TOF MS spectra were acquired on a Bruker REFLEX IV spectrometer in negative, linear mode. The matrix mixture for MALDI-TOF MS was made up from solution of 2,4,6-trihydroxyacetophenone<sup>1</sup> (0.3 M in EtOH) and diammonium citrate (0.1 M in H<sub>2</sub>O) (2:1 v/v).

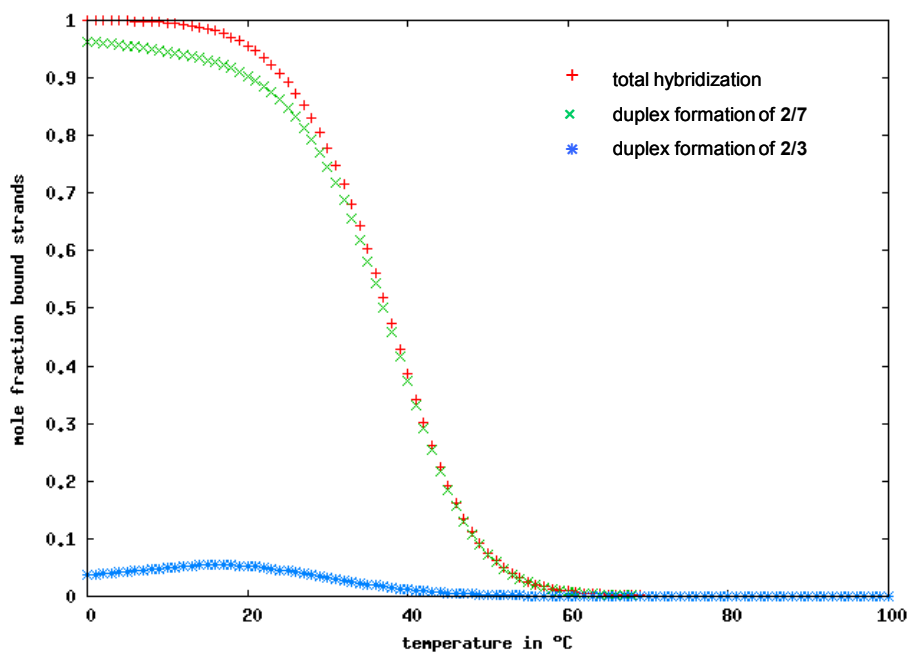
**Abbreviations.** cpg = controlled pore glass; HEPBS = 4-(2-hydroxyethyl)piperazine butanesulfonic acid; HEPES = 4-(2-hydroxyethyl)piperazine ethanesulfonic acid; MeIm = residue of 2-methylimidazole; OAt = residue of hydroxyazabenzotriazole; tac = *tert.*-butylphenoxyacetyl group; TEAA = triethylammonium acetate buffer; THAP = 2,4,6-trihydroxyacetophenone.

### **Synthesis of oligoribonucleotides**

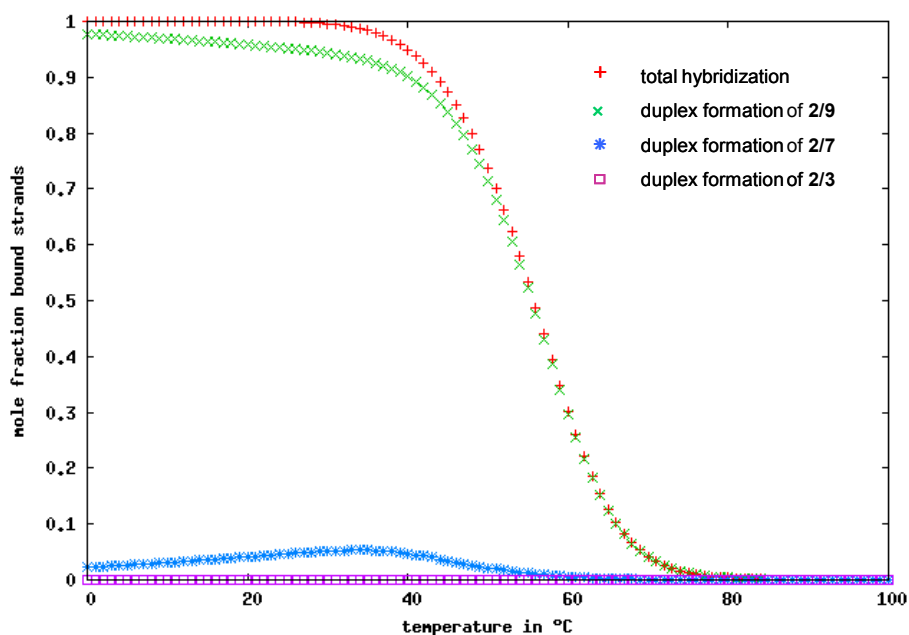
Oligoribonucleotides were synthesized on solid support (Universal Support II) via phosphoramidite chain extension cycles on a Perseptive Biosystem 8909 Expedite DNA synthesizer on 1  $\mu\text{mol}$  scale. The coupling time was extended to 5 min, and 16 equiv. of the respective phosphoramidite were used per coupling step. The last DMT group was left on the 5'-terminal residue. Cleavage from the support and deprotection of all but the 2'-*O*-TBDMS groups was induced by treatment with  $\text{NH}_3$  in MeOH (2M) at room temperature. After 2 h, the supernatant was taken up, and excess ammonia was removed in a gentle stream of air. The remaining methanolic solution was diluted with water and lyophilized to dryness. Desilylation was carried out in  $\text{Et}_3\text{N}\cdot 3\text{HF}$  (100  $\mu\text{L}$ , 1.8  $\mu\text{mol}$  HF) at room temperature for 5 h. Excess HF was quenched with methoxytrimethylsilane (260  $\mu\text{L}$ , 1.9  $\mu\text{mol}$ ). Desilylated oligoribonucleotides were isolated as a pellet that formed upon addition of the quenching reagent. Purification of fully deprotected oligoribonucleotides was performed on SepPak RP-C18 cartridges, using a gradient of  $\text{CH}_3\text{CN}$  in TEAA buffer (0.1 M) at pH 7.0.

### **Simulating micro helper displacement equilibria using ChipCheck<sup>2</sup>**

Hybridization equilibria resulting from the addition of several micro helper strands were simulated with ChipCheck,<sup>2</sup> version 2.0,<sup>3</sup> a program originally developed for simulating binding of oligonucleotides on DNA microarrays. ChipCheck also allows for simulating hybridization events in solution. For the calculations, the appropriate concentrations of template **2** and micro helper RNAs **3**, **7** and **9** (268  $\mu\text{M}$ ) were entered, together with the salt concentrations (0.4 M NaCl, 80 mM  $\text{MgCl}_2$ ). Both situations, displacement of **3** by **7**, and displacement of **3** and **7** by **9** were simulated. The results are shown in Figure S1 and S2, below.



**Figure S1.** Binding versus temperature curves for the displacement of the duplex between template **2** and micro-helper **3** upon the addition of **7** (at 268  $\mu\text{M}$  strand concentration and 0.4 M NaCl, 0.08 M  $\text{MgCl}_2$ ), as obtained using ChipCheck.<sup>2</sup> The duplex between **2** and **7** is the dominant species at room temperature.



**Figure S2.** Binding versus temperature curves for duplex formation between template **2** and micro-helpers **3**, **7**, and **9** at 268  $\mu\text{M}$  strand concentration and 0.4 M NaCl, 0.08 M  $\text{MgCl}_2$ , as obtained using ChipCheck.<sup>2</sup> It can be discerned that the duplex between **2** and **9** far dominates among the three possible duplex species at the temperature of the assays.

### Primer extension assays, general protocol

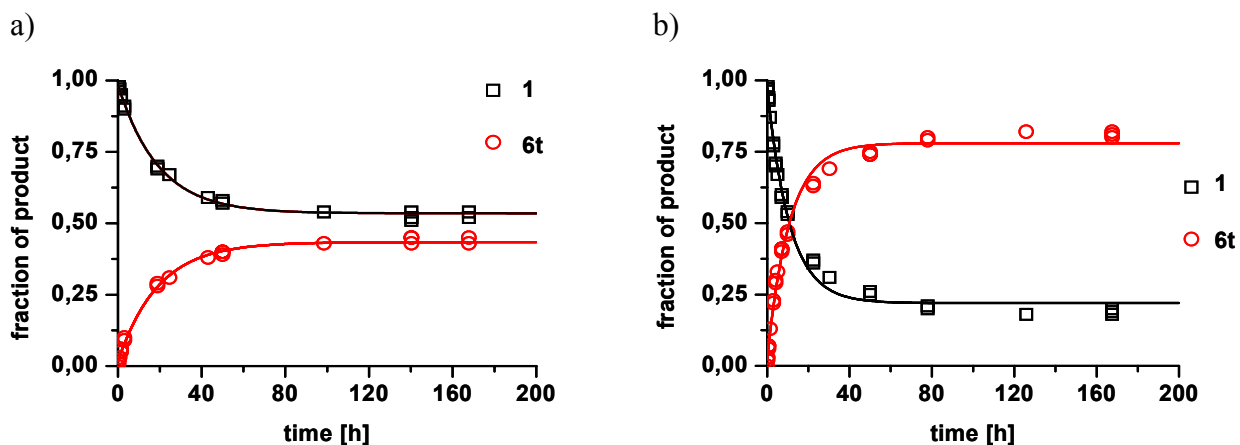
Oligoribonucleotides (template, primer and helper) were dissolved separately in de-ionized water to give stock solutions of defined concentration (1.34 mM). Aliquots (0.5  $\mu\text{L}$  or 1.0  $\mu\text{L}$ ) of the stock solutions were combined in polypropylene reaction vessels to result in a 268  $\mu\text{M}$  concentration of each oligoribonucleotide in the final assay mixture. Separate stock solutions of buffer and activated nucleotides were also prepared. Aliquots of these were combined with the solution of the oligoribonucleotides to give a final total volume of 2.5  $\mu\text{L}$  or 5.0  $\mu\text{L}$ . In case of extension assays with methylimidazolide **5t**, the final reaction mixture contained the following: oligoribonucleotides (268  $\mu\text{M}$ ), **5t** (5 mM or 20 mM), HEPES (0.2 M), NaCl (0.4 M) and  $\text{MgCl}_2$  (80 mM) at pH 7.7. In case of extension experiments with OAt esters **4t** or **4u** the final reaction mixture contained the following: oligoribonucleotides (268  $\mu\text{M}$ ), **4t,u** (1, 2, 5 or 20 mM), HEPBS (0.2 M), NaCl (0.4 M) and  $\text{MgCl}_2$  (80 mM) at pH 8.9. Assays were performed at the temperatures given in the text, between 4  $^\circ\text{C}$  and -20  $^\circ\text{C}$ . At chosen intervals, samples (0.1 - 0.2  $\mu\text{L}$ ) of the assay solution were drawn and diluted 100-fold with de-ionized water. The resulting solution was treated with a few grains of Dowex 50WX8-200 cation exchange resin ( $\text{NH}_4^+$ -form) for ten minutes followed by MALDI-TOF MS analysis.

In the case of assays multiple monomer additions, freshly activated **4t** was added whenever the reaction slowed down detectably. For this, concentrated aqueous stock solutions of **4t** were prepared (0.1 - 0.2 M). Aliquots of these solutions (0.2 - 0.5  $\mu\text{L}$ ) were added to the reaction mixture to generate a final concentration of 9-51 mM of the monomer (depending on the assay and on the addition step).

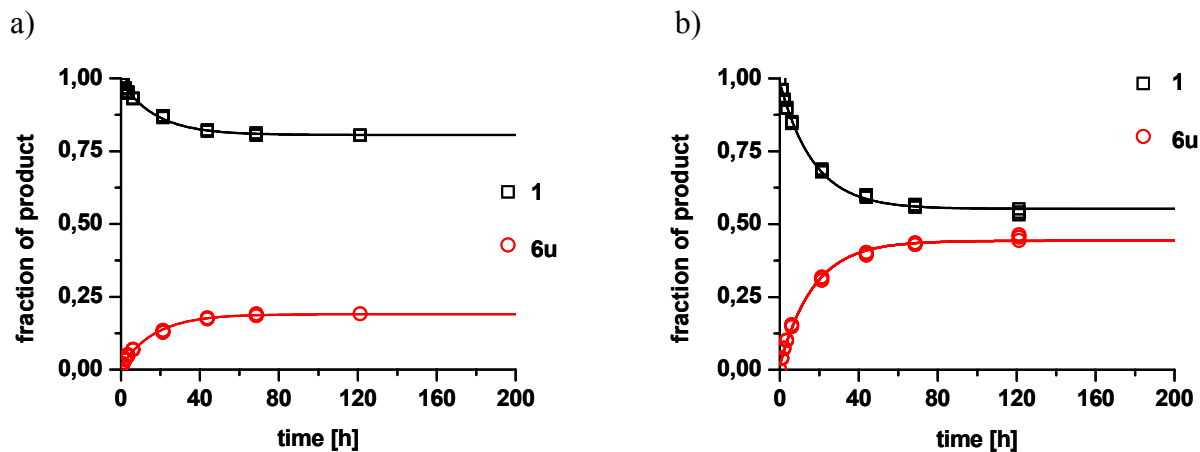
### **MALDI-TOF analysis and kinetic model**

The methodology used for acquisition of MALDI-TOF mass spectra, and the kinetic model applied to determine rate constants was similar to that described in earlier publications.<sup>4,5,6</sup> Briefly, aliquots of diluted samples were spotted on a stainless steel MALDI target plate and dried at 0.1 Torr. To these, 0.5  $\mu\text{L}$  of matrix solution (0.3 M THAP in EtOH and 0.1 M diammonium citrate in  $\text{H}_2\text{O}$ , 2:1, v/v) were added and allowed to crystallize. After insertion of the target plate into the mass spectrometer three spectra with more than 150 shots were acquired of each spot. Relative peak intensities of primer (**1** or **6u**) and extension products (**6t,u**, **8t,u**, or **10t,u**) were determined based on peak heights. The resulting data was subjected to fitting in the programs SlideWrite or Origin. To allow for incomplete conversion of primer, the following functions were used:  $f(t) = a_2 + a_1 \cdot \exp(-a_0 \cdot t)$  for the primer concentration, with starting values of  $a_2 = 0$ ,  $a_1 = 1$  and  $a_0 = 0.1$  for non-linear curve fitting. For the concentration of extension products the function  $f(t) = a_2 - a_1 \cdot \exp(-a_0 \cdot t)$  was used with starting values for non-linear curve fitting of  $a_2 = 1$ ,  $a_1 = 1$  and  $a_0 = 0.1$ .

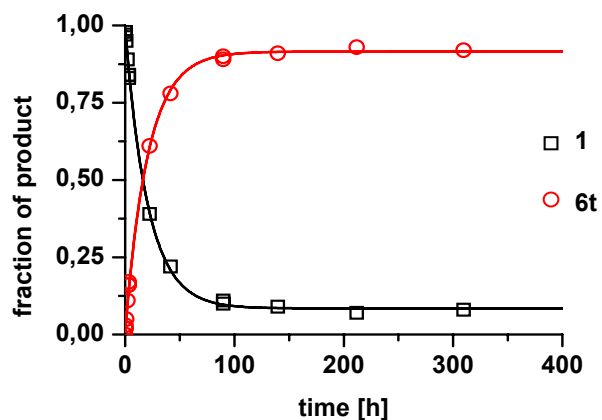
### Representative kinetics for extension of primer 1 by one nucleotide



**Figure S3.** Representative kinetics of chemical extension of primer 1 with 20 mM OAt-ester **4t** at 4 °C a) without micro helper RNA and b) in the presence of helper **3** (conditions: 268  $\mu$ M oligos, 200 mM HEPBS, 0.4 M NaCl, 80 mM MgCl<sub>2</sub>, pH 8.9).

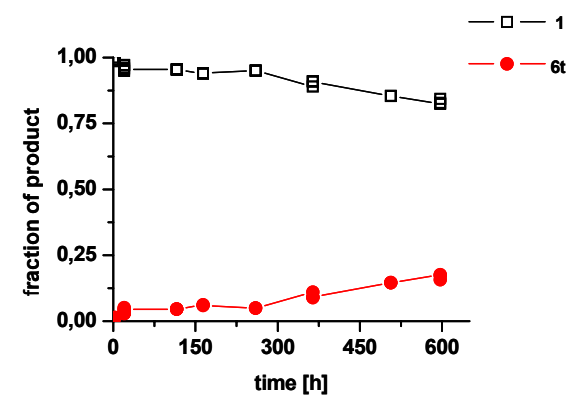


**Figure S4.** Representative kinetics of chemical extension of primer 1 with 5 mM OAt-ester **4u** at 4 °C a) without micro helper RNA and b) in the presence of helper **3** (conditions: 268  $\mu$ M oligos, 200 mM HEPBS, 0.4 M NaCl, 80 mM MgCl<sub>2</sub>, pH 8.9).

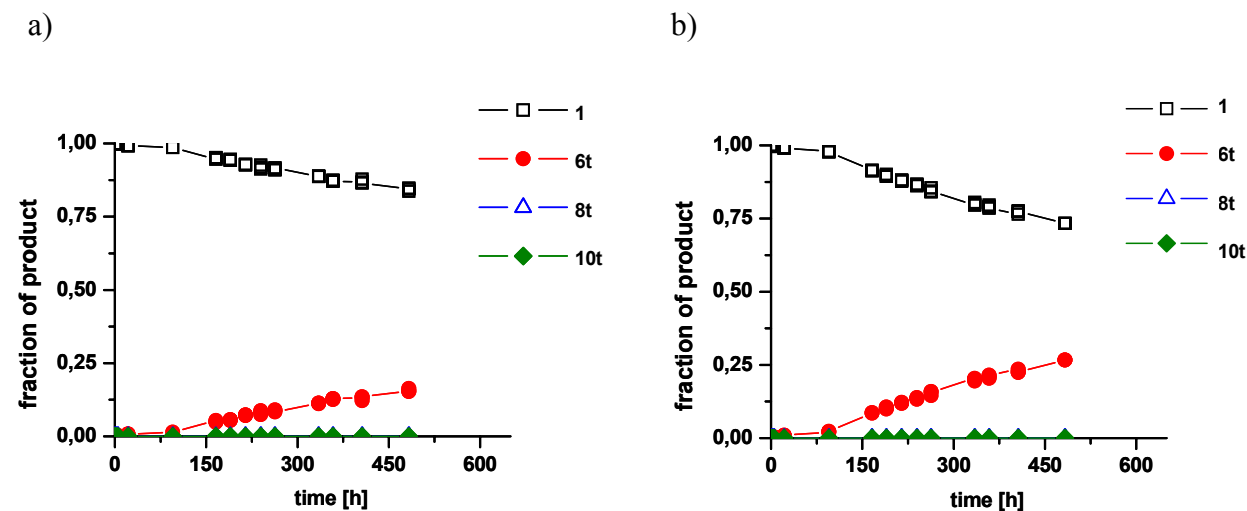


**Figure S5.** Representative kinetics of chemical extension of primer 1 with 5 mM OAt-ester **4t** at -20 °C with micro helper RNA **3** (conditions: 268  $\mu$ M oligos, 200 mM HEPBS, 0.4 M NaCl, 80 mM MgCl<sub>2</sub>, pH 8.9).

## Kinetics and product distribution for primer extension with methylimidazolide **5t**



**Figure S6.** Representative plot of product formation during extension of **1** under conditions known from the literature<sup>7</sup> for multiple extensions. The assay was performed with methylimidazolide **5t** (50 mM) at 0 °C in the absence of a helper oligonucleotide. Note that no multiple extension products were observed. This data is the basis of the corresponding entry in Table 1.



**Figure S7.** Representative plots of product distribution during extension of primer **1** with methylimidazolide **5t**, templated by **2** at -20 °C. The assays were started with 5 mM of **5t**. After 100 h, the concentration of activated nucleotide **5t** was increased to 20 mM. Resulting rate constants can be found in Table 1.

a) Assay without micro helper **3**.

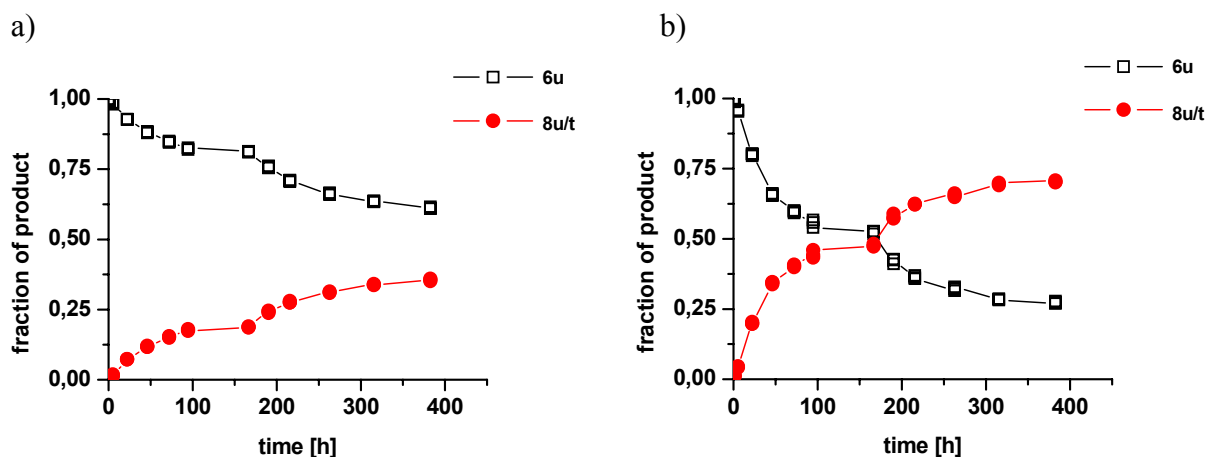
b) Assay in the presence of **3**.

Note that less than 10 % primer extension occurs at 5 mM **5t** [first phase in b)], even in the presence of **3**.

### Probing the second elongation step: Data for assays involving extension of 6u

As mentioned in the text, parallel assays were performed under identical conditions in terms of monomer concentration, temperature and sequence of addition of monomers that only differed in the presence or absence of primer 6u during the first phase of the assay. This led to the presence of hydrolyzed monomer when the solutions of the "delayed" assay were treated with 6u. Assays were run under two different sets of conditions: at 20 mM starting concentration of 4t and 4 ° or 5 mM starting concentration of 4t and -20 °C. The results indicate that the presence of hydrolyzed monomers slows down the reaction and makes it less efficient in terms of conversion.

#### Primer in the solution from the very beginning



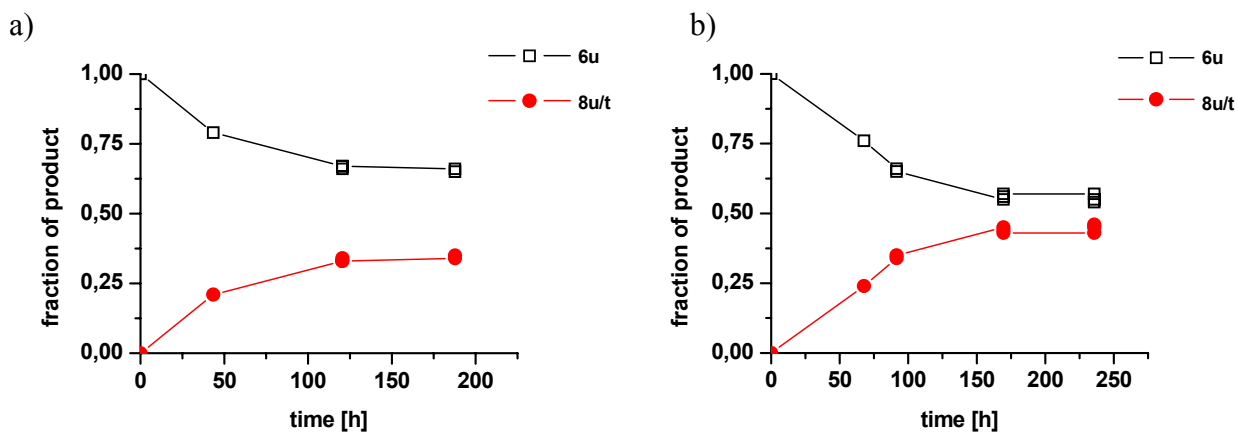
**Figure S8.** Representative plots of product distribution during the templated reaction of synthetic primer 6u with OAt-ester 4t (5 mM starting concentration) at -20 °C (see also legend to Table 2). After 170 h further, activated nucleotide 4t (80 mM) was added to give a second reaction phase to extension product 8u/t.

a) Assay without micro helper 7.

b) Assay in the presence of 7.

Note that primer extension templated by the second A of the AAA block is quite efficient, if started with fresh activated monomer.

**Primer added to the solution after initial phase without primer**



**Figure S9.** Representative plots of product distribution during extension of primer **6u** with OAt ester **4t** in the presence of hydrolyzed monomer building block.

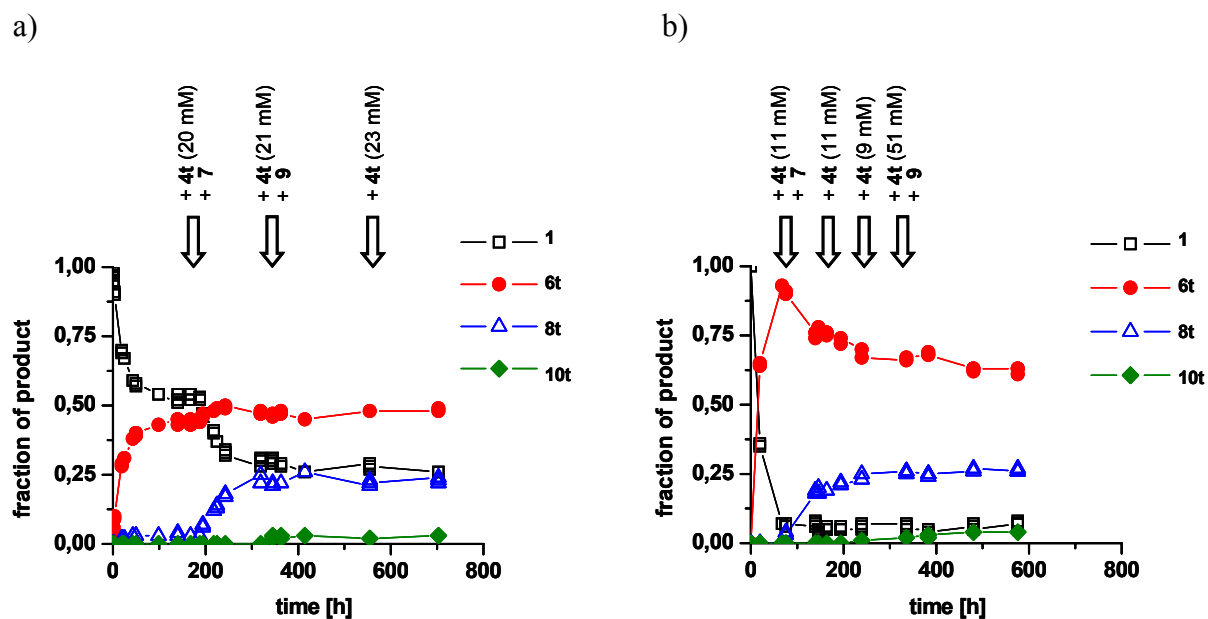
a) Assay at 4 °C with 20 mM starting concentration of **4t** and addition of **6u** after 170 h;

b) Assay at -20 °C with 5 mM starting concentration of **4t** and addition of **6u** after 74 h.

The time scales given at the x-axis reflect the time *after* addition of the primer, not the entire time of the assay. Note that the reactions are slower and give less conversion than those discernible in the first phase of the plots shown in Figure S8. Their rate is comparable to those of the second phase of the reactions shown in Figure S8.

## Representative plots of kinetic data for multiple extension reactions

Results shown in Figure S10, below, were obtained under optimized conditions. The spectrum shown in Figure 2b of the main manuscript was acquired during the assay whose results are shown in Figure S10b.



**Figure S10.** Representative plots of product distribution during templated reactions of primer **1** with OAt-ester **4t** a) at 4 °C with 20 mM starting concentration of **4t**, b) at -20 °C with 5 mM starting concentration of **4t**. Arrows atop the graphs indicate the time points when compounds were added to the reaction mixture. Note that the lower temperature gives higher yielding conversions.

## References for Supporting Information

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