Supporting Information

Enzyme-Free Copying of 12 Bases of RNA with Dinucleotides

G. Leveau, D. Pfeffer, B. Altaner, E. Kervio, F. Welsch, U. Gerland, C. Richert*
Supporting Information

Contents
1. Materials and methods
2. Synthesis of oligoribonucleotides
3. Protocol for assays
4. MALDI-TOF MS and HPLC analysis
5. Mass spectra from additional copying assays
6. Dissociation constants for complexes of dimers with templates
7. Computational exploration of sequence space
8. References for Supplementary Information
1. Materials and Methods

Reagents and Strands. General chemicals and solvents were purchased from commercial sources and were used without further purification. Dinucleotides were synthesized as described below. Oligoribonucleotides were either synthesized in-house, using TBDMS methodology or purchased from Biomers (Ulm, Germany). Prior to use in assays, cation-exchange to the sodium form was performed. For this, the oligonucleotide was dissolved in an aqueous NaCl solution (1 M, 0.5 mL) and applied to a reversed-phase chromatography cartridge (Waters, Sep-Pak VAC C18 3cc, 1 g). The cartridge was then flushed with a solution of NaCl (1 M, 10 mL), H₂O (15 mL), and the oligonucleotide was then eluted using a mixture (1/1, v/v) of H₂O and acetonitrile. Pure fractions containing the product were combined and lyophilized to dryness.

NMR Spectroscopy. Samples measured in 3 x 100 mm microtubes from Hilgenberg (Malsfeld, Germany). Spectra were recorded on a Bruker Avance III spectrometer with a proton resonance frequency of 700 MHz and a phosphorus resonance frequency of 284 MHz or on a Bruker Avance III HD-NanoBay spectrometer with a proton resonance frequency of 400 MHz and a phosphorus resonance frequency of 162 MHz. Coupling constants are given in Hertz and chemical shifts are reported in δ (ppm), calibrated on the solvent peak or the internal standard.

UV-Vis Spectroscopy. Samples of oligonucleotides were analysed by UV-Vis spectrometer NanoDrop 2000 from PeqLab (Darmstadt, Deutschland). The concentrations of oligonucleotides were determined by UV absorbance, using a 2000c NanoDrop spectrophotometer (Thermo Scientific).

Mass Spectrometry. MALDI-TOF mass spectra were recorded by using a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen, Germany) with an N₂ laser (337 nm). A mixture (2/1, v/v) of matrix (2,4,6-trihydroxyacetophenone solution; 0.2 M in ethanol) and co-matrix (diammonium citrate solution; 0.1 M in H₂O) was used. Spectra were recorded with the Flex Control software in linear negative mode. A mixture of oligonucleotides with known masses was used for external calibration. MALDI-TOF mass spectra were acquired, using a method allowing for quantitative detection. Analysis of mass spectra was done by using the Bruker Flex Analysis software, version 3.4.

Electrospray Mass Spectrometry. All ESI mass spectra were recorded on a microTOFQ mass spectrometer (Bruker Daltonics, Bremen, Germany) in negative mode using the following...
settings: capillary voltage: 4500 V; end plate offset voltage: -500 V; nebulizer gas pressure: 0.3 bar; drying gas velocity: 4.0 L/m; and heater temperature: 200 °C.

2. Synthesis of Oligoribonucleotides

Solid-Phase RNA Synthesis. The synthesis of dinucleotides and some oligoribonucleotides was performed by solid-phase RNA synthesis on an Expedite 8909 Nucleic Acid Synthesis System from PerSeptive Biosystems (Framingham, USA) or an H-2 synthesizer from K&A (Schaafheim, Germany) using the protocol recommended by the manufacturer. Immobilized ribonucleosides on controlled-pore glass (cpg) and 2'-TBDMS-protected phosphoramidites were obtained from Sigma Aldrich (Taufkirchen, Germany) or Glen Research (Sterling, USA). The chemical phosphorylation reagent, (2-cyanoethyl)-2-(2-dimethoxytrityl-ethylsulfonyl)ethyl-(N,N-diisopropyl)-phosphoramidite, was synthesised following a literature known procedure,[S2] and was employed to obtain a phosphorylated 5'-terminus via a conventional extension cycle.

Synthesis of Dimers. The following is for 5'-phosphorylated dimer CG and is representative. Starting from cpg loaded with the first nucleoside (70 mg, 2 µmol loading), the protected dimer was synthesized on the synthesizer. After completion of the synthesis, the cpg was treated with ammonium hydroxide for 16 h at 22 °C or with a mixture of aqueous ammonia solution (25%) and methylamine (AMA) (1:1, v/v) at 50 °C for 10 min. The supernatant was collected, and the remaining cpg was washed three times with H2O (3 x 1 mL). The aqueous solutions were combined, and the ammonia was evaporated using a nitrogen stream directed onto the surface of the solution, followed by lyophilization. The resulting solid was treated with triethylamine trihydrofluoride (0.3 mL) and shaken for 5 h at 22 °C. Methoxytrimethylsilane (2.5 mL) was added, and the mixture was shaken for another hour at room temperature. The resulting suspension was centrifuged, the supernatant was removed, and the pellet was dissolved in aqueous triethylammonium acetate (TEAA) solution (0.1 M, 0.5 mL) and then subjected to purification by reversed-phase cartridge chromatography (Sep-Pak VAC C18 3cc, 1 g). The product was eluted with a step-gradient of in acetonitrile in TEAA buffer (0.1 M) in 1% steps, of 5 mL each. Fractions containing the product were combined, lyophilized, and subjected to cation exchange to the sodium form, as described in Chapter 1 under Reagents and Strands.

Table S1. Yields and MS data (ESI-MS) for dimers synthesized.
| Oligonucleotide sequence | Yield [%] | m/z calc.  | m/z found |
|-------------------------|-----------|------------|-----------|
| 5’-pCG-3’               | 40        | 667.092    | 667.092   |
| 5’-pGG-3’               | 46        | 707.098    | 707.098   |
| 5’-pGC-3’               | 53        | 667.092    | 667.091   |
| 5’-pCC-3’               | 49        | 627.085    | 627.084   |
| 5’-pAG-3’               | 23        | 691.103    | 691.102   |
| 5’-pUG-3’               | 27        | 668.076    | 668.075   |
3. Protocol for Assays

The following protocol is for the copying of the sequence containing complementary loci for the four dinucleotides CG, GG, GC, and CC in template 1, downstream of the binding site for primer 2. The protocol is representative. The reaction conditions build on those reported recently for copying with ribodinucleotides. Concentrations in the final reaction volume of 10 µL are given. Aqueous solutions containing primer 2 and template 1 (to give concentration of 45 µM and 60 µM, respectively) were mixed, heated to 80 °C for 5 min, and then allowed to cool to room temperature to allow for annealing. Afterwards, while keeping the combined solution at 4 °C, aliquots of stock solutions of the four dinucleotides (CG, GG, GC, and CC) in water were added to give a concentration of 2 mM, each, in the final solution, followed by adding an aqueous stock solution of the buffer components to give 0.4 M 1-ethylimidazole, 0.08 M MgCl₂, and a freshly prepared solution EDC hydrochloride to give a 0.4 M concentration of the carbodiimide. Water was added to reach a final volume of 10 µL and a pH value of 6.0. The solution was vortexed and kept at 4 °C. The progress of the reaction was monitored by MALDI-TOF analysis, as described below.
4. MALDI-TOF MS and HPLC Analysis

MALDI-TOF Mass Spectra of Assay Solutions. Aliquots of 0.6 µL were taken from assay solutions at stated intervals. A cation exchange was performed by diluting to 10 µL with water and treating the resulting solution with a few beads of Dowex 50 WX8-200 cation exchange resin in the ammonium form for 20 min. A sample of 0.6 µL of the supernatant was spotted and allowed to dry on the MALDI target, followed by adding a solution (0.45 µL) of the mixture of THAP matrix and diammonium citrate co-matrix (see Chapter 1). After acquisition of spectra, the intensity of peaks of the ligation products in the MALDI-TOF mass spectra was measured. From this, the percentage of the corresponding products was determined, using the correction factors determined with authentic samples in the form of synthetic oligoribonucleotides, as described below, following an approach previously published.[53]

Correction Factors for Quantitative Detection of Products. Peak intensities in MALDI-TOF mass spectra depend on the length and sequence of the analyte. To allow for quantitative analysis, 'correction factors' have to be determined for the sequences to be detected. [53] The following oligoribonucleotide sequences were employed in the experiments producing the correction factors:

UAUGCUGGGCG (4)
UAUGCUGGCGGG (6)
UAUGCUGGCGGGCG (8)
UAUGCUGGCGGGCGGCGG (10)
UAUGCUGGCGGGCGGCGGCGG (12)
UAUGCUGGCGGGCGGCGGCGGCGG (23)

In the experimental determination of the correction factors, buffer, template (1 or 22), primer 2, and the authentic RNA sequence (obtained from Biomers, Ulm, Germany) were mixed to obtain the same concentrations as in an assay solution (45 µM copying product and primer, 60 µM template, 0.4 M 1-EtIm, 0.08 M MgCl₂, pH 6, 4 °C). Prior to this, stock solutions of the primer and the ligated primer had been prepared in distilled water at ratios from 0.1 to 3, and the concentrations had been confirmed by UV absorption at room temperature. Aliquots from the buffered solution were treated with Dowex cation exchange resin, followed by MALDI-TOF analysis. Peak ratios between primer and extension product were measured and plotted. A representative calibration plot is shown in Figure S1, below. The correction
factor is defined by the inverse of the slope of the linear regression line obtained by linear fitting of the experimental data.

![Figure S1](image.png)

**Figure S1.** Representative calibration plot for determining MALDI correction factors, compensating for differences in desorption and ionization efficiency for a copying product of assays with dinucleotides and primer 2, as obtained from relative peak intensities in MALDI-TOF mass spectra with strand 12 (UAUGCUGCGGCGGCG). Relative intensities, as determined by absorption spectroscopy, are plotted against the relative peak heights. The black line is the fit from linear regression.

Table S2, below, lists the correction factors for the products of the copying assays with dimers as building blocks. The MALDI spectra were recorded for mixtures containing the strand listed in the first column, together with primer 2 and template 1 (strands 4-12) or template 22 (strand 23), as described above.

| Strand No. | Slope | R²      | Correction factor |
|------------|-------|---------|-------------------|
| 4          | 0.799 | 0.992   | 1.3               |
| 6          | 0.657 | 0.995   | 1.5               |
| 8          | 0.515 | 0.991   | 1.9               |
| 10         | 0.230 | 0.988   | 4.3               |
| 12         | 0.141 | 0.999   | 7.1               |
| 23         | 0.116 | 0.964   | 8.6               |
The relative concentration of the products at the end of the copying assay was calculated for spectra shown in Figure 3 of the main manuscript. The intensity of each peak was adjusted using the correction factor of the corresponding sequence as described above. These values give a more accurate impression of the relative abundance of different products than visual inspection of the spectra, particularly for longer strands, which fly more poorly in the MALDI. For some assays, the sum of percentages is not 100, as peaks for other (side) products exist in the mass range of interest, which were used in the numerical analysis of peak intensities, but were not subjected to the correction procedure.

Table S3. Percentages of products formed, as determined from peak intensities, applying correction factors for the respective sequences.

| Part of Primer | Products |
|---------------|----------|
| Figure 3<sup>a</sup> | 2 | 4 | 6 | 8 | 10 | 12 | 23 |
| A | > 95 | < 1 |
| B | 18 | 25 | 4 | 10 | 25 | 18 |
| C | 16 | 23 | 5 | 9 | 21 | 19 |
| D | 30 | 29 | 5 | 12 | 11 | 12 |
| F | 29 | 28 | 7 | 9 | 5 | 11 | 11 |

<sup>a</sup>See Figure 3 of the main paper for conditions.

**HPLC Analysis.**

To study product distribution by an independent method, HPLC analysis was performed for a representative assay employing template 1, primer 2 and CG and GG as dimers, analogous to the one whose result is shown in Figure 3B.

First, the HPLC method was established using a mixture of synthetic compounds of the same sequences as those of the extension products. The HPLC runs were performed on a Dionex UltiMate 3000 (Thermo Scientific), using an XBridge C18 RP column (3.0 x 150 mm, 3.5 µm, Waters, Milford (MA), USA) at 75 °C and a flow rate of 0.6 mL/min, with a gradient of acetonitrile in 0.1 M triethylammonium acetate buffer (TEAA). Typically, 10 µL of the crude mixture was injected and eluted using a gradient of CH$_3$CN from 2% for 5 min and to 15% in 30 min. Peaks were detected via UV absorption at 260 nm. Fractions were analyzed by
MALDI-TOF mass spectrometry. The sequences of the strands are shown in Figure S2 again for clarity. Figure S3 shows the chromatogram for an equimolar mixture of strands, and Figure S4 shows the MALDI-TOF mass spectra for the fractions corresponding to the peaks labeled in Figure S3.

Figure S2. Sequences of strands used in validation run. This is an excerpt of Figure 2 of the manuscript.

**template (1)**

\[
3' \text{--CACCCACCACAUCAGACCCGCCCAGGC--5'} \\
5' \text{--UAUGCUUG (2)} \\
5' \text{--UAUGCGGCG (4)} \\
5' \text{--UAUGUGCGG (6)} \\
5' \text{--UAUGCGUGCG (8)} \\
5' \text{--UAUGUGGGCGG (10)} \\
5' \text{--UAUGCGUGCGGCGG (12)}
\]

Figure S3. Expansion of the relevant part of the HPLC chromatogram of the mixture of authentic strands injected at a concentration of 10 µM each.

Figure S4. Overlay of MALDI-TOF mass spectra of HPLC fractions for peaks labeled in Figure S3, above. The time values given on the right-hand side of each spectrum is the retention time for the peak analyzed. See the listing below for the massed measured for the main peak of each spectrum.
MALDI-TOF data for the spectra shown in Figure S4.
UAUGCUGG (2), \( t_R = 10.1 \) min. MALDI MS calcd for \([M - H]\) 2525.6, found 2525.8.
UAUGCUGGCGG (4), \( t_R = 12.3 \) min. MALDI MS calcd for \([M - H]\) 3175.9, found 3176.4.
UAUGCUGGCGGG (6), \( t_R = 13.6 \) min. MALDI MS calcd for \([M - H]\) 3866.4, found 3865.6.
UAUGCUGGCGGGCGG (8), \( t_R = 14.3 \) min. MALDI MS calcd for \([M - H]\) 4516.7, found 4515.9.
UAUGCUGGCGGCGGG (10), \( t_R = 15.3 \) min. MALDI MS calcd for \([M - H]\) 5207.2, found 5205.3.
UAUGCUGGCGGCGGGCGG (12), \( t_R = 16.1 \) min. MALDI MS calcd for \([M - H]\) 5857.6, found 5855.0.

After validating the method, it was applied to analyze the product mixture at the end of the assay with dimers CG and GG. The results are shown below. The retention times and the appearance of the chromatogram differ slightly from that of the validation runs due to a more complex mixture of compounds in the assay.

**Figure S5.** Expansion of the relevant part of the HPLC chromatogram of assay mixture from the assay with dimers CG and GG on template 1 with primer 2. The assay was run analogously to the one that produced the spectrum in Figure 3B. See Figure S6, below, for mass spectra of the fractions labeled.

**Figure S6.** Overlay of MALDI-TOF spectra for peaks labeled in Figure S5, above. The time points are the retention times for each peak.
MALDI-TOF data for the spectra shown in Figure S6.

UAUGCUGG (2), $t_R = 11.0$ min. MALDI MS calcd for $[M - H]^-$ 2525.6, found 2524.7.
UAUGCUGGCGG (4), $t_R = 12.1$ min. MALDI MS calcd for $[M - H]^-$ 3175.9, found 3175.5.
UAUGCUGGCGGCGG (6), $t_R = 13.5$ min. MALDI MS calcd for $[M - H]^-$ 3866.4, found 3866.9.
UAUGCUGGCGGCGGG (8), $t_R = 14.3$ min. MALDI MS calcd for $[M - H]^-$ 4516.7, found 4518.0.
UAUGCUGGCGGCGGGCGG (10), $t_R = 15.2$ min. MALDI MS calcd for $[M - H]^-$ 5207.2, found 5205.7.
UAUGCUGGCGGCGGGCGGG (12), $t_R = 15.7$ min. MALDI MS calcd for $[M - H]^-$ 5857.6, found 5857.7.

The product distribution calculated from peaks in the HPLC trace (Figure S5, above) and the molar extinction coefficients for the different strands is shown in Table S4. Given that these are from separate assays that there is significant noise in both MALDI spectra and HPLC trace, and that incomplete denaturation under HPLC conditions cannot be ruled out, there is acceptable agreement between both methods, confirming that more than 10% of the full length copying product was formed in the assays.

| Table S4. Product distribution (%), as obtained from the HPLC trace of Figure S5. |
|---------------------------------|--|--|--|--|--|--|
| Quantity | Strand |
|         | 2 | 4 | 6 | 8 | 10 | 12 |
| peak area | 3.2 | 5.3 | 0.7 | 6.0 | 3.6 | 5.4 |
| molar fraction (%) | 20 | 28 | 3 | 22 | 12 | 16 |
5. Mass spectra from additional copying assays

5.1 Assays with a higher concentration of monomers

Assay with two monomers (C and G)

Assay with four monomers (A/C/G/U).

Figure S7a. MALDI-TOF mass spectrum of copying reaction using primer 2 and template 1 with 20 mM monomers CMP and GMP each after 13 d reaction time.

Figure S7b. MALDI-TOF mass spectrum of copying reaction using primer 2 and template 13 at 20 mM monomers AMP, CMP, UMP and GMP (5 mM each) after 13 d reaction time.

5.2 Assays with fewer equivalents of dimer and lower overall concentration

Two assays were performed to explore the effect of a smaller excess of dimer building blocks and a lower overall concentration. To shed light on these less favorable conditions for copying with dimers, we also used a less favorable template, where a single dimer can bind to the terminal region of a template section. For this, instead of a primer-template duplex, hairpin 24
was used, which contains a templating and primer region linked by a hexaethylene glycol (HEG) linker, as similarly used in the work of Chapter 6, below. The first assay was performed with two equivalents over hairpin 24 (2 mM). The reaction conditions were the same as those of other assays: 0.4 M 1-ethylimidazole, 0.08 M MgCl₂, 0.4 M EDC. The second assay was performed in 10-fold more dilute solution, with final concentrations of 0.04 M 1-ethylimidazole, 0.008 M MgCl₂, 0.04 M EDC, 0.2 mM hairpin (24) and 0.4 mM CG. The temperature and the pH value were the same in both cases: 4°C and a pH of 6.0. The MALDI-TOF spectra below show the expected decrease in conversion when using fewer equivalents and a more dilute solution are used (Figure S8).

Figure S8. Effect of fewer equivalents and dilution: MALDI-TOF mass spectra of ligation reactions using hairpin 24 and dimer CG. a) Assay with two equivalents building block CG at 2 mM hairpin 24, 0.4 M 1-ethylimidazole, 0.08 M MgCl₂, 0.4 M EDC. b) Assay with ten-fold dilution: 0.2 mM hairpin (24), 0.4 mM CG, 0.04 M 1-ethylimidazole, 0.008 M MgCl₂, 0.04 M EDC. Because this was exploratory work, the time points are not exactly the same. However, the much decreased conversion expected upon dilution can clearly be discerned. When correcting for differences in desorption and ionization, the assay of part a) gave 33% conversion after a reaction time of 138 h, which is approx. half of the reaction time of the assays of Figures 3A-D. In the dilute solution, there was approx. 3% conversion after 120 h.
6. Dissociation Constants for Complexes of Dimers with Templates

![Diagram](image)

**Figure S9.** Measuring binding of dimers to hairpins. a) Equilibrium studied, with hairpin and corresponding dimer; HEG = hexaethyleneglycol linker; b) and c) hairpin sequences used for the ¹H-NMR titration with dimer GG and dimer CC, respectively.

The procedure employed for determining dissociation constants of duplexes of dimers with the complementary template-primer complex via NMR titration is literature known and used RNA hairpins with an overhang as models for the primer-template duplexes.[⁵⁴,⁵⁵] The following is a brief description of the titration involving dinucleotide GG and RNA hairpin 5'-CCCAG-HEG-CUG-3' as binding partner (Figure S9), where HEG stands for the hexaethyleneglycol linker bridging the hairpin strands, and where the templating sequence CC given in boldface. The protocol is representative. The hairpin (100 nmol) was dissolved in deuterated buffer (0.2 mL volume; 0.2 M phosphate buffer, 0.4 M NaCl, 0.5 mM TPS internal standard, pH 7.0, uncorrected for deuterium effect), resulting in a concentration of 0.5 mM. Spectra were recorded at 25 °C on a Bruker Avance 700 spectrometer with a helium-cooled probe. Peak assignment was based on 2D-NMR spectra (NOESY, TOCSY, and COSY). After acquiring the spectra of the hairpin by itself, aliquots of a stock solution containing the dinucleotide (140 mM) and the hairpin (0.5 mM) in the same buffer were added, and a one-dimensional ¹H-NMR spectrum was acquired after each addition. One-dimensional spectra were measured using a pulse sequence with a D1-time of 2 seconds. Chemical shifts were determined in the ¹H channel, using Topspin 4.0.8 software. In the case of the sequences named above, the chemical shift of the resonance of proton H5 of residue U7 was analyzed in detail. Its chemical shift was plotted against the concentration of the dinucleotide and the data was fitted using Origin version 9.1 (*OriginLab Corporation*, Northampton, MA, USA). Determination of the $K_d$ value...
followed the literature-known protocol, based on the following equation, where $K_d$ is the dissociation constant.$^{[S4]}$

$$\Delta \delta_{\text{obs}} = \Delta \delta_{\text{D,PT}} \cdot \frac{[D]}{K_d \left( 1 + \frac{[D]}{K_d} \right)}$$

The same protocol was employed to determine the dissociation constant for the complex of GG with the corresponding hairpin representing the primer-template complex. Analogous titrations with the dinucleotides CG and GC were inconclusive because of self-pairing of the hairpins containing self-complementary single-stranded overhangs. Here, the $K_d$ value was estimated, using the arithmetic average of the values measured for CC and GG.

**Figure S10.** Representative data used for determining dissociation constants via $^1$H-NMR titration. In each case, the relevant parts of the NMR spectra are shown on the left, and the plot of the chemical shift displacement versus concentration of the dimer is shown on the right. a) Overlay of $^1$H-NMR spectra of hairpin CC at increasing concentrations of dinucleotide GG; b) Spectra and plot for hairpin CC and dimer CC. Lines in the plots are fits using the equation given above and squares are experimental data points.
**Attempted Kd determination for hairpin CG and dimer pGC**

**Figure S11.** Overlay of $^1$H-NMR spectra of hairpin CG at increasing concentrations of dinucleotide GC, showing that several transitions take place simultaneously, most probably including self-pairing of hairpins, rendering the determination of a dissociation for the complex between dimer and hairpin impractical. Here, an estimated K$_d$ value was used for the computational work.

|        | CC  | GG  | CG  | GC  |
|--------|-----|-----|-----|-----|
| K$_d$ (mM) | 0.2 | 1.0 | (0.6)$^a$ | (0.6)$^a$ |

$^a$ Estimated
7. Computational exploration of sequence space

As a basis to computationally explore the sequence dependence of the copying times, we first performed separate single-step primer extension experiments in presence of only one of the four dimers CG, GG, GC, or CC. These experiments followed the same protocol as described in Section 3 and 4 above, but with the product fractions (yield) quantified at closely spaced time points during the initial phase during which the reaction is far from complete. The time-dependent yield for each of the four dimers is shown in Figure S12, where the experimental data points (filled circles) are connected by lines as a guide to the eye. The initial data points (displayed in orange in Figure S12) were fitted by linear regression (black lines). Because in these experiments templates were provided in excess of primers, and dimers in large excess of both templates and primers, the rate constants obtained from the linear regression of the initial time-dependent yield of extended primers can be interpreted as effective kinetic rate constants for primer extension at the experimental dimer concentration (2 mM). The obtained best fit values for these effective rate constants $k_0(D)$, with $D \in \{CC, CG, GC, GG\}$, are provided in Figure S12.

![Figure S12](image-url)

**Figure S12.** Kinetics of primer extension for individual dimers. The four panels show time-dependent product fractions (yield) data for the integration of each of the four dimers GG, CC, CG, and GC (data points represented by filled circles, connected by lines to guide the eye). The effective rate constant $k_0$ for each dimer is obtained by linear regression (black lines) of the initial time points (orange filled circles). The values obtained are indicated in each panel.
We then devised a simple model for the kinetics of enzyme-free copying with dimers, based on the extracted rate constants $k_0(D)$. Because the multi-step copying assays in the presence of all four dimers were performed at the same conditions and dimer concentrations (2 mM for each dimer) as the single-dimer primer extensions described above, the rate constants $k_0(D)$ can be applied for an approximate description of the copying kinetics. Our simple model neglects cooperative and competitive binding effects of multiple dimers on the template strand, and estimates the total copying time for a given template sequence as the sum of the extension times of the corresponding complementary dimers. This procedure also neglects the dependence of the rate constants $k_0(D)$ on the identity of the 3′-terminal nucleotide of the primer, which we expect to be a reasonable approximation, given that for the case of DNA this dependence was found to be modest,\cite{S6} whereas a strong dependence on the bases of the incoming dimer (including the second base) was observed.\cite{S3} Since the average time for a single extension with dimer $D$ is the inverse of the extension rate, $\tau(D) = k_0(D)^{-1}$, the copying time for a given template sequence within our simple model is the sum over the $\tau(D)$ for the corresponding complementary dimers. Using this model, we inferred the times that would be required to copy each of the 1024 possible template sequences of ten bases consisting only of C/G nucleotides, as presented in Figure 4 of the main text.
8. References for Supporting Information

S1. D. Sarracino, C. Richert, Quantitative MALDI-TOF spectrometry of oligonucleotides and a nuclease assay. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 2543–2548.

S2. T. Horn, M. S. Urdea, A chemical 5′-phosphorylation of oligodeoxyribonucleotides that can be monitored by trityl cation release. *Tetrahedron Lett.* **1986**, *27*, 4705-4708.

S3. M. Sosson, D. Pfeffer, C. Richert, Enzyme-free ligation of dimers and trimers to RNA primers. *Nucleic Acids Res.* **2019**, *47*, 3836-3845.

S4. E. Kervio, B. Claasen, U.E. Steiner, C. Richert, The strength of the template effect attracting nucleotides to naked DNA. *Nucleic Acids Res.* **2014**, *42*, 7409-7420.

S5. E. Kervio, M. Sosson, C. Richert, The effect of leaving groups on binding and reactivity in enzyme-free copying of DNA and RNA. *Nucleic Acids Res.* **2016**, *44*, 5504-5514.

S6. E. Kervio, A. Hochgesand, U. Steiner, C. Richert, Templating efficiency of naked DNA. *Proc. Natl. Acad. Sci. USA*, **2010**, *107*, 12074-12079.