

Data storage in DNA with fewer synthesis cycles using composite DNA letters

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The density and long-term stability of DNA make it an appealing storage medium, particularly for long-term data archiving. Existing DNA storage technologies involve the synthesis and sequencing of multiple nominally identical molecules in parallel, resulting in information redundancy. We report the development of encoding and decoding methods that exploit this redundancy using composite DNA letters. A composite DNA letter is a representation of a position in a sequence that consists of a mixture of all four DNA nucleotides in a predetermined ratio. Our methods encode data using fewer synthesis cycles. We encode 6.4 MB into composite DNA, with distinguishable composition medians, using 20% fewer synthesis cycles per unit of data, as compared to previous reports. We also simulate encoding with larger composite alphabets, with distinguishable composition deciles, to show that 75% fewer synthesis cycles are potentially sufficient. We describe applicable error-correcting codes and inference methods, and investigate error patterns in the context of composite DNA letters.

DNA-based data storage systems are particularly appealing owing to the high information capacity, in terms of physical volume, of DNA as compared to current state of the art storage media. Storing digital information on DNA involves encoding the information into a sequence over the DNA alphabet (that is, A, C, G and T), producing synthetic DNA molecules with the desired sequence and storing the synthetic biological material. Reading the stored information requires sequencing of the DNA and decoding to obtain the original digital information.

DNA-based storage systems^{1–8} involve several technological and design challenges. Biochemical and technical constraints require the use of custom coding schemes to accommodate possible dropouts and common DNA synthesis and sequencing errors^{4,7,9}. Random access at reduced sequencing overhead requires efficient design of large pools of mutually compatible PCR primers^{5,6,8,10}. Recently, innovative synthesis approaches have been introduced^{11,12}, which may lead to more cost-effective DNA-based data storage. Other molecular biology techniques can also be used for DNA-based storage¹³. DNA synthesis technology, which is based on phosphoramidite chemistry^{14,15}, yields high numbers of molecules for each of the designed DNA sequences¹⁶. Oligonucleotide multiplicity, an important inherent property of current DNA synthesis and sequencing technologies, has not yet been exploited in DNA-storage technologies based on synthesis.

The efficiency of DNA-based storage systems can be evaluated using several quantitative metrics. One is the physical density of the storage medium, as measured by data unit per gram of DNA (for example, gigabytes per gram). A recent study demonstrated a DNA-based storage system with a physical density of 215 PB g⁻¹. This density, when converted to volumetric density, represents roughly six orders of magnitude improvement over current storage media⁷. A second performance metric is the number of synthesis cycles required for a unit of data. This is termed logical density and is the main focus of this current work (as well as of another recent study that was made available during the late stages of this project¹⁷).

We introduce the use of composite DNA letters to increase the logical density of DNA storage above the strict, single-molecule, theoretical limit of 2 bits per synthesis cycle. A composite DNA letter is a representation of a position in a sequence that constitutes a mixture of all four standard DNA nucleotides in a specified predetermined ratio. We use composite DNA letters to form the basis of a DNA synthesis approach that trades sequence multiplicity for increased complexity of the synthesized DNA. This increased complexity effectively extends the available alphabet and therefore allows higher data content per synthesis cycle. We demonstrate an implementation of a complete large-scale, DNA-based storage system using composite DNA letters, develop related methods including error-correction codes and investigate trade-offs and performance metrics.

Results

Composite DNA letters extend the DNA alphabet. A composite DNA letter is a representation of a position in a sequence that constitutes a mixture of all four standard DNA nucleotides in a specified predetermined ratio $\sigma = (\sigma_A, \sigma_C, \sigma_G, \sigma_T)$ where $k = \sigma_A + \sigma_C + \sigma_G + \sigma_T$ is defined as the resolution parameter of the composite letter (Methods). For example, $\sigma = (1, 1, 2, 0)$ represents a position in a composite DNA sequence of resolution $k=4$ in which there is a 25%, 25%, 50% and 0% chance of seeing A, C, G and T, respectively. Writing a composite DNA letter at a given position of a DNA sequence is equivalent to producing (synthesizing) multiple copies (oligonucleotides) of the sequence, so that in this given position the different DNA nucleotides are distributed across the synthesized copies according to the specification of σ . Reading a composite letter requires the sequencing of multiple independent molecules representing the same composite sequence and inferring the original ratio or composition from the observed base frequencies (Fig. 1). Introducing composite letters extends the available alphabet and thus allows the coding of longer messages within a fixed synthesized molecule length. A composite DNA alphabet is a set of composite DNA letters, usually, but not necessarily, sharing a common

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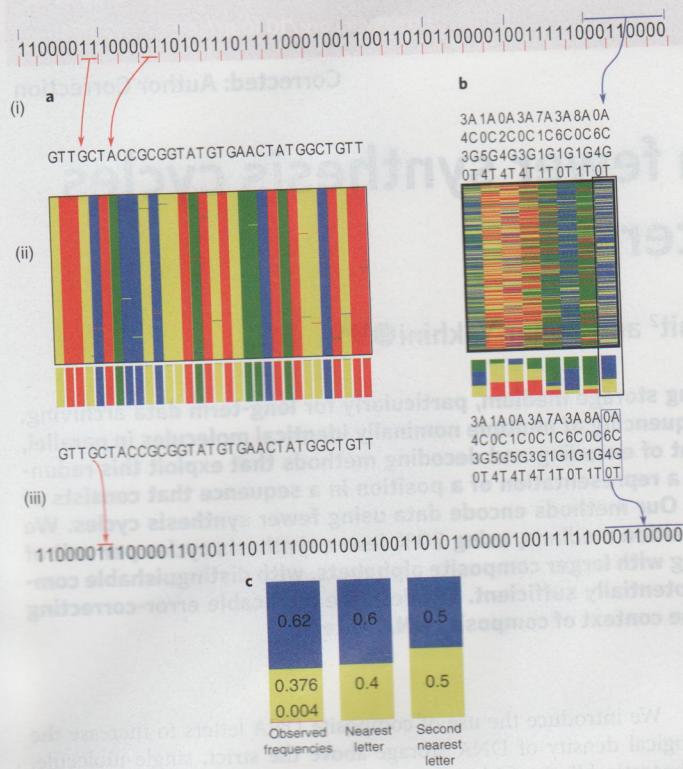


Fig. 1 | Encoding a binary message using standard and composite DNA. A binary message, depicted on top, is encoded into DNA. **a**, Standard DNA-based storage scheme⁷. The binary message is encoded to DNA by mapping every 2 bits (represented by the short red separating lines) to a DNA letter or synthesis cycle (i), the designed DNA sequence will then be synthesized and sequenced by a noisy procedure that introduces some errors (ii). The sequencing output is then used to infer the DNA composition at every position (iii). Decoding of the original message is done assuming the use of an error-correcting code. **b**, The same message is encoded using a composite DNA alphabet of resolution $k=10$ by mapping every 8 bits (represented by the blue separating lines) of the binary message to a single composite DNA position (a single synthesis cycle when using appropriate hardware). Sufficiently deep sequencing allows correct identification of the original composite letters (the right most position, in a black frame, is shown in c) and decoding of the message. The decoding also uses an error-correction mechanism; our implementation uses Reed–Solomon over the appropriate finite field. **c**, An example of the inference step at a single synthesized composite position. The observed frequencies are used to infer the source, $\sigma=(0.6, 4, 0)$, as the closest composite letter, using Kullback–Leibler divergence (Methods). Note that the inference at any fixed position is affected by the sequencing depth obtained there, as well as by sequencing and synthesis errors.

resolution k . The full composite alphabet of resolution k , denoted Φ_k , is the set of all $\sigma = (\sigma_A, \sigma_C, \sigma_G, \sigma_T)$ so that $\sum_{i \in \{A,C,G,T\}} \sigma_i = k$. Note that $|\Phi_k| = \binom{k+3}{3}$, thus the composite alphabet size grows with the resolution parameter and so does the potential logical density, as measured by data units per synthesis cycle (Supplementary Fig. 1).

To read a message coded using composite DNA letters correctly, we must infer, from the observed reads, the original composite letters in sufficiently many positions of the total message. The sequencing readout (that is, the observed sequencing reads) is the product of a complex process, consisting of DNA synthesis, long-term storage^{2,18}, sampling and DNA sequencing. The distribution of counts, for every letter in {A,C,G,T}, resulting from σ at depth

N can be described by a single model in which the readout counts are multinomial:

$$X^{(N)}(\sigma, P_{\text{syn}}, P_{\text{deg}}, P_{\text{seq}}) \sim \text{Multinomial}(N, (p_A(\sigma), p_C(\sigma), p_T(\sigma), p_G(\sigma))) \quad (1)$$

The parameters of the distribution are the designed input letter σ , the sequencing depth N and the errors introduced in the synthesis, storage and sequencing steps of the process, P_{syn} , P_{deg} and P_{seq} (Methods). While each step introduces different errors and biases, the most important parameters that affect the readout are the sampling of molecules to be sequenced and the sequencing depth.

The sequencing readout frequencies will most likely not exactly match any letter from the original alphabet. Inference of the original letter is performed by converting the readout to a vector of base frequencies and comparing it to the base frequencies of the candidate letters in the composite alphabet. The comparison can be done, for example, using the Kullback–Leibler divergence or the L^1 norm. To assess the performance of this inference step, we developed a simulation model and analyzed inference rates for various composite alphabets (Supplementary Fig. 2). The Kullback–Leibler divergence, which corresponds to a maximum-likelihood estimator (Supplementary Note), was found to perform much better and was thus used in the remainder of this study, including the molecular implementation (Methods).

Large-scale composite DNA-based data storage. To show the feasibility of the composite DNA alphabet concept and to demonstrate its potential for improving DNA-based data archiving systems, we performed a large-scale molecular implementation of a storage system based on a six-letter composite alphabet. The system consisted of using our composite letter encoding approach together with an error-correction system, which was based on a combination of Reed–Solomon¹⁹ and fountain^{7,20} schemes (Methods), to produce a composite DNA encoding pipeline (Fig. 2). We first used our system to store and successfully retrieve a 2.12 MB data file from Erlich and Zielinski⁷. Our encoded DNA pool consisted of 58,000 six-letter composite 152-nucleotide oligonucleotides, as compared to 72,000 oligonucleotides of the same length that were required using standard DNA, demonstrating a ~24% increase in logical density, as measured by bits per synthesis cycle (Table 1 and Supplementary Table 4). The six-letter composite alphabet used here was $\Sigma_6 = \{A, C, G, T, M, K\}$, where $M = (1, 1, 0, 0)$ and $K = (0, 0, 1, 1)$. Note that $\Sigma_6 \subset \Phi_2$ ($|\Sigma_6| = 6, |\Phi_2| = 10$). Our error-correcting scheme uses a Reed–Solomon code at the composite DNA level (using the appropriate Galois field) and not at the binary bits level, thereby improving the robustness of the system (Fig. 2; Methods). We further demonstrated the increased logical density of composite DNA by encoding a bilingual interactive version of the Bible, compressed to a 6.42 MB file, using three different composite alphabets. The above six-letter alphabet Σ_6 required 174,000 oligonucleotides, while a five-letter alphabet $\Sigma_5 = \{A, C, G, T, M\} \subset \Phi_2$ required 193,000 oligonucleotides and a standard four-letter alphabet $\Sigma_4 = \Phi_1$ required 217,000 synthetic oligonucleotides, all of the same length of 152 nucleotides (Table 1 and Supplementary Table 4). All the composite DNA oligonucleotides mentioned above were synthesized by Twist Bioscience, using standard DNA-writing hardware and an optimized synthesis process to obtain the desired nucleotide ratios for the letters K and M. We thus demonstrated large-scale composite DNA synthesis. Using the data acquired, we further investigated the characteristics of this approach to composite DNA synthesis.

The synthesized DNA was amplified using two different primer pairs as technical repeats. We then sequenced the resulting synthetic DNA sample (100-nucleotide paired-end reads, Illumina HiSeq at the Technion Genome Center). Our library and reaction

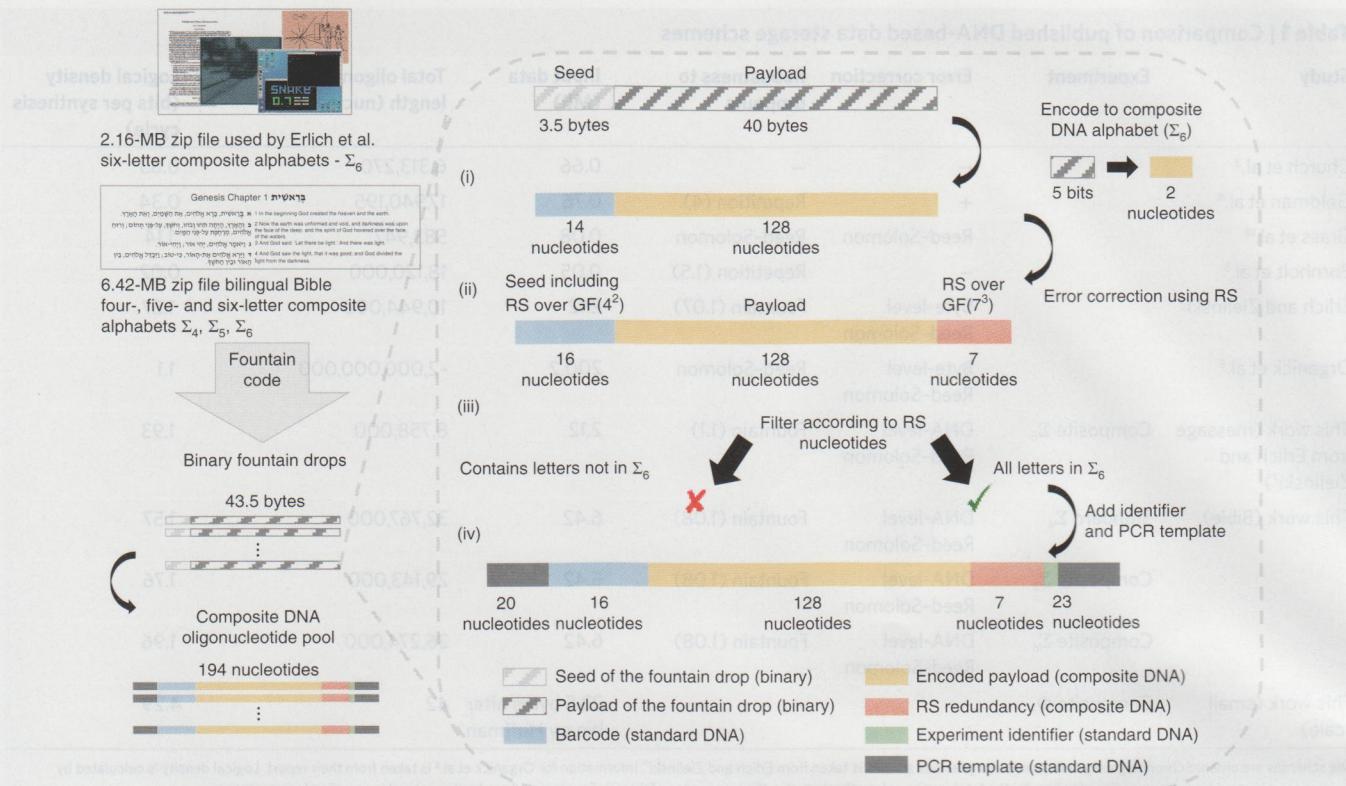


Fig. 2 | Encoding pipeline of a large-scale composite DNA-based data storage. A compressed input file was processed by the fountain code to produce binary droplets. A composite DNA encoding workflow was then applied for each droplet (Methods). (i) The binary message was translated into a composite DNA sequence. The seed sequence was translated to standard DNA sequence, which served as a barcode for the decoding process. The payload was translated to a six-letter composite DNA alphabet (Σ_6) in 5-bit chunks. (ii) Error-correction nucleotides were added to the DNA sequence by using a systematic Reed-Solomon (RS) encoding. The barcode was encoded using Reed-Solomon over $GF(2^4)$ and the payload was padded and encoded using Reed-Solomon over $GF(7^3)$. (iii) Each encoded message was then filtered to verify that the Reed-Solomon redundancy letters are all from Σ_6 (note that the Reed-Solomon code used here is systematic). (iv) Experiment identifier and amplification template sequences were appended to each valid sequence. Similar coding schemes were used for four- and five-letter alphabets (Methods; Supplementary Figs. 3–6).

design allowed for separately decoding each one of the four test messages, as described above, with each of the primer pairs. In Fig. 3, we describe the process of decoding the results of a sequencing reaction, performed on a synthesized composite DNA library originating from one message (in this case, the 6.4 MB Bible encoded into Σ_6 with a single pair of primers), and of inferring the underlying binary message (Methods). In brief, we first preprocessed the raw reads by assembling paired-end reads, filtering by length and grouping by putative barcode sequence (prefixes). Next, we filtered out prefixes with less than 20 associated reads generating a set of putative barcodes each associated with a group of reads. We inferred the full composite oligonucleotide for each putative barcode using Kullback–Leibler inference. The resulting composite oligonucleotides were Reed–Solomon verified (over $GF(7^3)$ in the case of Σ_6) and the valid oligonucleotides were converted into binary drops. Finally, we applied a binary fountain code decoding to obtain the original message, if successful. We note (Fig. 3c) that the average observed multiplicity, for each one of the inferred putative barcodes was 96 reads. Inference of the full composite oligonucleotide was only done for putative barcodes with more than 20 reads. Figure 3d,e depict frequencies and Kullback–Leibler inference decision boundaries (red dashed lines) for positions that were originally designed as composite. Note that individual synthesized positions were correctly inferred at an error rate of less than 10^{-5} for these filtered putative barcodes. These errors were either corrected by the Reed–Solomon code (over $GF(7^3)$ in the case of Σ_6) or rejected by the same mechanism.

Eventually, sufficiently many accepted drops (172,608 for the Bible encoded into Σ_6) made it to the last decoding step, which used an adaptation of the fountain code mechanism proposed by Erlich and Zielinski⁷ (Fig. 3f).

Dilution and physical density of composite DNA storage. To assess the physical density achieved by our composite DNA-based storage system we performed a dilution experiment. The encoding DNA was sequentially diluted and then amplified, sequenced and processed through our decoding pipeline. Our results include four different encodings in four dilution experiments. In a physical density of 6 PB g^{-1} we managed to successfully decode the Bible message encoded using Σ_6 (See Supplementary Table 1 for physical density calculations). We recovered 167,093 of the 174,000 original composite oligonucleotides (Fig. 3b). Further dilution of the DNA yielded only partial recovery of the composite oligonucleotides, below the redundancy level that is recoverable by the fountain code. From the message from Erlich and Zielinski⁷, encoded using Σ_6 and representing a 30 PB g^{-1} density, we successfully recovered 92% of the original oligonucleotides. This was slightly lower than required by the fountain code to decode the message. We observed that even in the standard DNA (Σ_4) experiment we achieved a lower physical density than that reported by Erlich and Ziellinski. This could be due to the modified synthesis process or to the larger scale of the experiment. From the complete set of dilution results, we estimated that using a six-letter composite alphabet we can achieve a physical density of $20\text{--}30 \text{ PB g}^{-1}$.

Table 1 | Comparison of published DNA-based data storage schemes

Study	Experiment	Error correction	Robustness to dropouts	Input data (MB)	Total oligonucleotide library length (nucleotides)	Logical density (bits per synthesis cycle)
Church et al. ³		—	—	0.66	6,313,270	0.83
Goldman et al. ⁴		+	Repetition (4)	0.76	17,940,195	0.34
Grass et al. ¹⁸		Reed-Solomon	Reed-Solomon	0.08	583,947	1.14
Bornholt et al. ⁵		—	Repetition (1.5)	0.05	18,120,000	0.02
Erlich and Zielinski ⁷		Byte-level Reed-Solomon	Fountain (1.07)	2.12	10,944,000	1.57
Organick et al. ⁸		Byte-level Reed-Solomon	Reed-Solomon	200.2	~2,000,000,000	1.1
This work (message from Erlich and Zielinski ⁷)	Composite Σ_6	DNA-level Reed-Solomon	Fountain (1.1)	2.12	8,758,000	1.93
This work (Bible)	Standard Σ_4	DNA-level Reed-Solomon	Fountain (1.08)	6.42	32,767,000	1.57
	Composite Σ_5	DNA-level Reed-Solomon	Fountain (1.08)	6.42	29,143,000	1.76
	Composite Σ_6	DNA-level Reed-Solomon	Fountain (1.08)	6.42	26,274,000	1.96
This work (small scale)	Composite Φ_3	—	—	22.5 bytes after binary Huffman	42	4.29

The schemes are ordered chronologically. Information for previous studies is taken from Erlich and Zielinski⁷. Information for Organick et al.⁸ is taken from their report. Logical density is calculated by dividing total binary input data, measured in bits, by the total number of synthesis cycles (Supplementary Table 2). Fountain code redundancy level is specified in parentheses.

Our dilution experiment evidently involved PCR amplification of the diluted material. By investigating the distribution of the K and M compositions in the different dilution steps, we therefore also examined the potential composition biases introduced by PCR together with those originating from the dilution itself. The analysis is presented in Supplementary Fig. 7. We concluded that the distribution of base frequencies had higher variance but there was only a minimal shift in the mean frequencies (0.3% for K and 0.05% for M after three additional cycles of PCR).

Higher resolutions, compositions and sequencing depth. As $\Phi_2 \subset \Phi_k$ for every even value of k , we can use the two composite letters from Φ_2 (that is, K and M) to calculate, on the basis of our experimental data, correct inference rates for these two letters in the context of larger composite alphabets. In Fig. 3d,e we further indicate the decision boundaries that would have been used under Φ_4 to distinguish, for example, $K = (0,0,2,2)$ from $\sigma = (0,0,3,1)$. Using these decision boundaries we would have had up to 7% of the positions designed as K (or M) potentially leaked to be interpreted as one of the two neighboring composite letters in Φ_4 (at the current sequencing depth). We further analyzed the effect of sequencing depth and the implication of extending the composite alphabet in Fig. 4. We observed that the mean base frequency of the composite letters K and M was slightly shifted toward G and C, respectively (Fig. 4b and Supplementary Fig. 8). As an immediate result, the leakage into neighboring letters was mainly toward G and C when considering Φ_2 decision boundaries. As expected, the leakage rate was anticorrelated with sequencing depth.

Subsampling of reads. We performed a subsampling experiment in which we repeatedly sampled different portions of the reads and assessed the read subsets using our decoding pipeline. For the message from Erlich and Zielinski⁷, we show that using as little as 29 reads per oligonucleotide on average (30% sampling) was still sufficient to successfully decode the message with ~97% of the oligonucleotides successfully recovered (Fig. 4a).

Subsampling of the reads resulted in a wider distribution of base frequencies (Fig. 4b), while examining only oligonucleotides with higher coverage generated a narrow distribution (Fig. 4c and Supplementary Fig. 9). In particular, inference of both K and M under hypothetical use of Φ_2 or of Φ_4 was perfect, even at 160 reads per barcode (Fig. 4d). When considering Φ_6 and Φ_8 , we obtained reasonable performance at the higher depths. It is important to note that some errors in inference can be tolerated as we use a Reed-Solomon error correction on the complete composite oligonucleotide at the composite alphabet level.

Composite alphabets increase logical density. To assess and establish the potential of large composite alphabets we combined simulations of large-scale composite DNA systems and a smaller-scale experimental proof of concept.

First, we calculated the potential logical density of storage systems based on large composite alphabets (Supplementary Table 2). A system using Φ_{10} , which consists of 286 letters, potentially achieves logical density of 6.4 bits per synthesis cycle, which is a fourfold increase over the standard DNA system (Supplementary Table 2). For Φ_{10} we further performed a full simulation study, working with experimentally motivated error rates, to understand the potential under non-perfect conditions. Planted errors include deletions, mismatches and insertions as derived from our data (Methods). We encoded the message from Erlich and Zielinski⁷ using 17,585 composite oligonucleotides and simulated the synthesis and sequencing using different error rates and sequencing depths (Supplementary Tables 5 and 6). At an average sequencing depth of 2,000 reads and an overall error rate of 1:500 bases or less we correctly infer more than 99.95% of the composite oligonucleotides allowing for correct decoding of the message (Fig. 5a). Using Φ_5 , an alphabet with 56 letters, we achieve a logical density of 4.5 bits per synthesis cycle (a 2.8-fold increase) and encoded the same message using 24,848 composite oligonucleotides (Supplementary Tables 5 and 6). With an average sequencing depth of 2,000 reads we successfully decoded the message even with an error rate of 1:50 bases while with

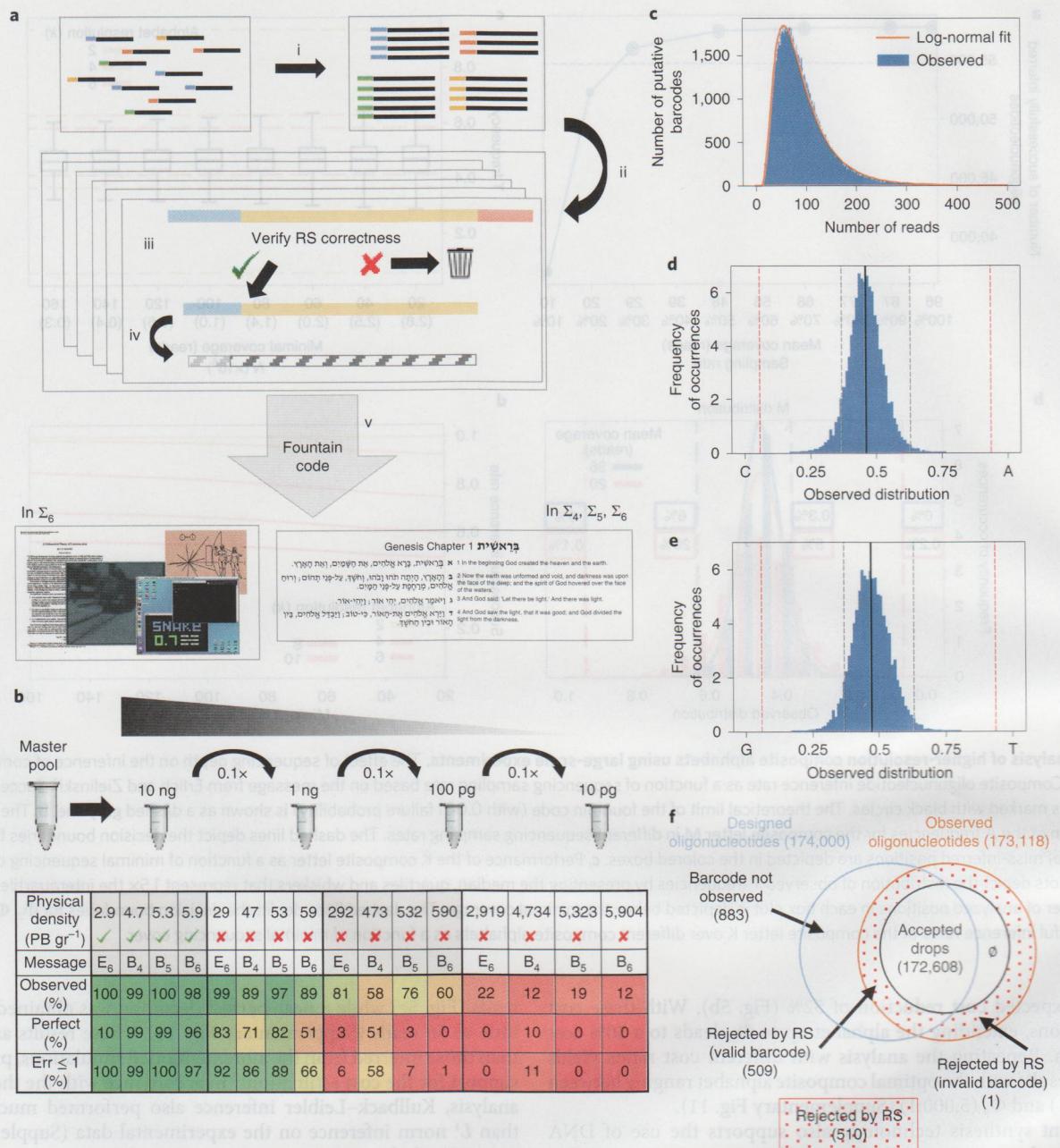


Fig. 3 | Performance of a large-scale composite DNA-based storage system. Decoding a composite library to infer the original encoded message.

a, The steps of the decoding process (Methods): (i) Preprocessing and grouping by prefix sequences; (ii) generation of a set of putative oligonucleotides; (iii) inference of composite oligonucleotides using Kullback-Leibler inference and Reed-Solomon error correction; (iv) conversion into binary drops; and (v) binary fountain code decoding to obtain the original message. **b**, A dilution experiment testing the physical density achieved by the composite DNA approach. DNA was sequentially diluted, amplified, sequenced and tested for decoding. For each dilution, physical density is presented for all four encodings. (The message from Erlich and Zielinski⁷ is marked E6 and the Bible is marked B₆). The percentage of observed barcodes is presented together with the composite oligonucleotide inference rates and the rate of composite oligonucleotides inferred with up to one error (Err ≤ 1). **c–f**, Descriptive statistics related to the decoding process. Numbers indicated are for the 6.4-MB Bible message encoded into Σ_6 composite DNA. **c**, The number of reads associated to each 16-nucleotide prefix (putative barcode). The distribution follows a log-normal shape with a median of 81 reads and a mean of 96 reads. **d,e**, The distribution of base frequencies per synthesized position. For this counting we consider the positions that were designed to be composite—either K or M. Kullback-Leibler decision boundaries are also depicted. **f**, Acceptance statistics for the designed composite oligonucleotides.

500 reads we decoded the message with an error rate of 1:500 bases or less (Supplementary Fig. 10).

Using composite DNA has the potential to reduce the costs of DNA-based storage. This reduction is due to the increased logical density leading to reduced DNA synthesis cost, which is related to the total number of synthesis cycles. We analyzed the effect of

using a large composite alphabet on the overall cost of a DNA-based storage system, taking into account the reduction in synthesis cost together with the increase in sequencing costs (Methods). We performed the analysis using different assumptions on the synthesis cost to sequencing cost ratio ($C_{syn}:C_{seq}$). With a moderate cost ratio of 1,000:1 we observe that using Φ_5 (56 letters) is optimal, with an

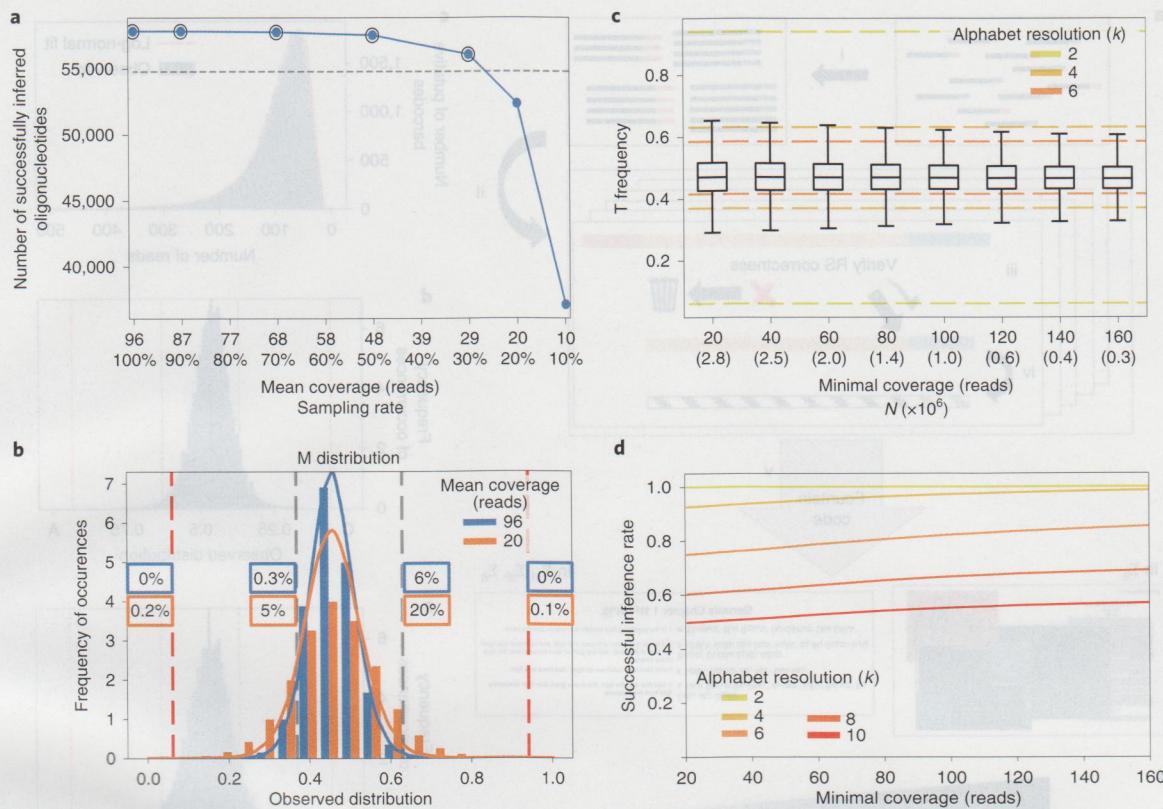


Fig. 4 | Analysis of higher-resolution composite alphabets using large-scale experiments. The effect of sequencing depth on the inference of composite letters. **a**, Composite oligonucleotide inference rate as a function of sequencing sampling rate based on the message from Erlich and Zielinski⁷. Successful decoding is marked with black circles. The theoretical limit of the fountain code (with 0.001 failure probability) is shown as a dashed gray line. **b**, The distribution of the A frequencies for the composite letter M in different sequencing sampling rates. The dashed lines depict the decision boundaries for Φ_2 and Φ_4 . Rates of miss-inferred positions are depicted in the colored boxes. **c**, Performance of the K composite letter as a function of minimal sequencing coverage. The box plots depict the distribution of observed T frequencies by presenting the median, quartiles and whiskers that represent 1.5x the interquartile range. The number of analyzed positions in each box plot is depicted below the minimal coverage. The dashed lines depict the decision boundaries for Φ_2 , Φ_4 and Φ_6 . **d**, Successful inference rates of the composite letter K over different composite alphabets as a function of minimal sequencing cover.

overall expected cost reduction of 52% (Fig. 5b). With these cost assumptions, extending the alphabet up to Φ_{10} leads to a 30% cost reduction. Repeating the analysis with different cost ratios yields similar results with the optimal composite alphabet ranging between Φ_4 (500:1) and Φ_9 (5,000:1) (Supplementary Fig. 11).

Current synthesis technology also supports the use of DNA mixtures that represent higher-resolution composite alphabets, albeit on a small scale. To further explore the properties of large alphabets we encoded a short message (38 bytes in ASCII, 22.5 bytes after a binary Huffman compression) using composite alphabets of four different types, resulting in logical density of up to 4.29 bits per synthesis cycle (Table 1 and Supplementary Table 2; Methods).

The four different alphabets used are the standard DNA alphabet Φ_1 , the full composite alphabets Φ_2 and Φ_3 , and an alphabet containing the 15 IUPAC letters. The input English phrase, ‘DNA STORAGE ROCKS!’, was encoded to each of these alphabets using Huffman coding with the appropriate alphabet. The four resulting composite oligonucleotides were synthesized by IDT and sequenced by the Technion Genome Center (Methods; Supplementary Fig. 12 and Supplementary Table 3).

First, we examined the minimal sequencing depth required to decode the message correctly for each of the four composite alphabets. As expected, extending the alphabet by using higher resolutions requires deeper sequencing. In all four alphabets that were tested, a fully successful decoding was observed in as little as 100

reads (Fig. 5c) while a near-perfect decoding was obtained with as little as 50 reads (Supplementary Fig. 13). These results are better than those inferred from the aforementioned simulations, providing support for the cost estimations. In accordance with the theoretical analysis, Kullback–Leibler inference also performed much better than L^1 norm inference on the experimental data (Supplementary Figs. 14 and 15).

As predicted by the statistical model, some composite letters are harder to identify than others (Fig. 5d). However, contrary to the model prediction, when examining different letters from the same composite archetype (that is, letters that are different permutations of the same probability vector) we observe significant differences ($P < 10^{-10}$; Z test for proportion difference for the letters GGA and GGC) (Fig. 5d). These higher-resolution results also suggest that the position of the letter in the synthesized oligonucleotide affects the identification rate. To further explore the differences between different composite letters, we designed another synthetic DNA oligonucleotide containing all the equimolar letters (represented by the 15-letter IUPAC alphabet), with multiple copies of each composite letter distributed along the designed sequence (Methods; Supplementary Fig. 16). We examined the inference rate at a depth of 15 reads and reported the results as a function of the letter and the position in the oligonucleotide (Supplementary Fig. 17). We observed a small but persistent decrease in inference rates as a function of the position on the synthesized oligonucleotide, starting from the 5' end.

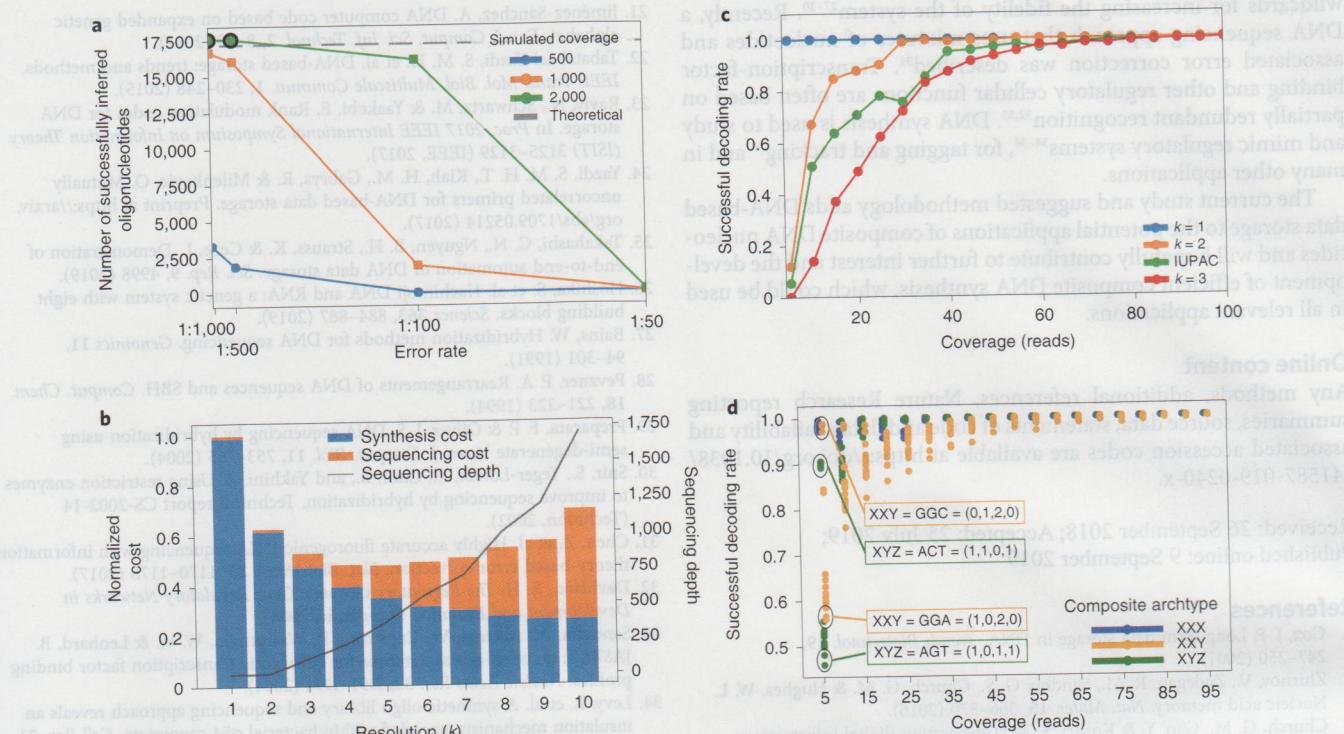


Fig. 5 | Data storage systems based on large composite alphabets. **a**, Successful inference of a Φ_{10} composite storage system storing the message from Erlich and Zielinski⁷ on the basis of simulations ($N=5$). The number of correctly inferred oligonucleotides is shown as a function of the simulated error rates for three sequencing depths (Methods). The theoretical limit of the fountain code (with 0.001 failure probability) is shown as a dashed gray line. Instances for which a successful decoding was achieved are marked with a black circle. **b**, Cost analysis for a composite DNA-based storage system using different alphabets. Cost components are normalized to the total cost of a standard DNA-based system obtained in the molecular implementation using Kullback-Leibler inference as a function of sequencing depth for the four composite DNA alphabets. **c**, Inference rates for letters in Φ_3 (multiple occurrences along the oligonucleotide) as a function of sequencing depth. The letters are colored by their composite archetype.

Discussion

We applied composite DNA letters to enable DNA-based data storage using fewer DNA synthesis cycles. Composite DNA schemes could be combined with other approaches such as orthogonal base pair systems²¹, efficient coding^{6,7,9,22} and random access approaches^{5,6,10,23,24} to increase capacity and fidelity of DNA-based storage systems. However, the logical density advantage of using composite DNA is traded off with several performance metrics as discussed below.

Incorporating composite DNA into future DNA-based storage systems will require further investment in several directions. First and foremost, any large-scale implementation will require scaling up the currently limited commercial hardware for synthesis of composite DNA. The current implementation of a six-letter alphabet did not increase the cost per synthesized oligonucleotide. Progress to even higher resolutions will require slight modifications in the design of synthesis hardware or the adaptation of other synthesis approaches. In a recent study, the authors describe the construction of a flexible laboratory-size synthesis system²⁵. This system can be configured to accommodate higher-resolution alphabets. Second, using highly multiplexed composite DNA sequences will require better understanding of the effect of composite DNA on different chemical processes involved in DNA manipulation. Previous studies dealt with the chemical limitations of these processes either by employing strict encoding schemes^{3–6,9} or by using coding methodologies like DNA fountains to handle sequence dropout⁷. Employing composite DNA inherently generates balanced DNA molecules, resulting from the combinatorial space associated with

every designed composite sequence. While unwanted sequences will unavoidably be part of the ensemble of synthesized molecules, the inherent independence of the different positions renders them negligible, representing an extra benefit of the composite DNA approach. Third, the design principles for composite DNA sequences, or of related coding approaches, as well as the decoding pipeline, can be further tuned for optimal results. Mixed composite alphabets can be generated to minimize inference errors without compromising the alphabet size, by only selecting subsets of the full alphabets Φ_k . Technical calibration of the actual base frequencies, on the basis of further experimental investigation thereof (such as in Fig. 4b), can be added to the decoding pipeline allowing the correction of systematic synthesis biases.

The use of composite DNA affects required sequencing depth and physical density, as our data show. Using current technologies, synthesis cost per position is approximately four orders of magnitude larger than sequencing cost per base, yielding a potential overall reduction in cost when using composite DNA. This holds despite the increase in sequencing costs entailed by the required depth. We further analyzed the effect of both factors on the overall cost, as described in the text and in Fig. 5b and Supplementary Fig. 11. The physical density reported herein is about a single order of magnitude less than the best previously reported.

It is important to note, in relation to the work presented here, that increased alphabet size for data storage can also be achieved, to a limited extent, by introducing synthetic orthogonal nucleotide pairs^{21,26}. In the early days of DNA sequencing by hybridization, degenerate and semidegenerate bases were proposed as

wildcards for increasing the fidelity of the system^{27–30}. Recently, a DNA sequencing approach that uses mixtures of nucleotides and associated error correction was described³¹. Transcription-factor binding and other regulatory cellular functions are often based on partially redundant recognition^{32,33}. DNA synthesis is used to study and mimic regulatory systems^{34–36}, for tagging and tracking³⁷ and in many other applications.

The current study and suggested methodology adds DNA-based data storage to the potential applications of composite DNA nucleotides and will hopefully contribute to further interest and the development of efficient composite DNA synthesis, which could be used in all relevant applications.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of code and data availability and associated accession codes are available at <https://doi.org/10.1038/s41587-019-0240-x>.

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Author contributions

L.A. and Z.Y. initiated and designed the coding and algorithmic approach. L.A. developed the software and performed data analysis. I.V. and O.A. performed the experiments. L.A., R.A. and Z.Y. wrote the manuscript. R.A. and Z.Y. supervised the study.

Competing interests

L.A., Z.Y. and R.A. are the inventors of a patent application for the method described in this article. The initial filing was assigned United States Provisional Patent Application No. 62/674,114. The remaining authors declare no competing financial interests.

Additional information

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