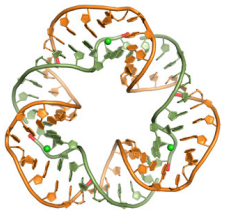
CHAPTER 1

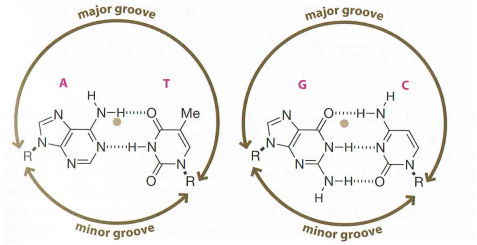


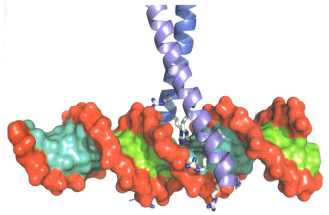
OLIGONUCLEOTIDES

Part 2 – noncanonical nucleobases

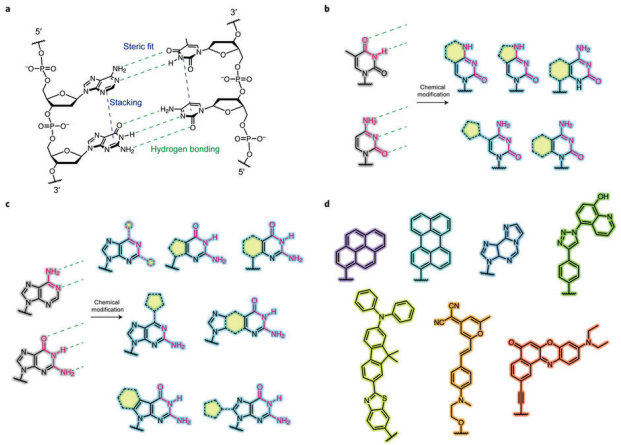
Canonical nucleobase pairing

	common amine base	cytosine	thymine	adenine	guanine
pK_a	10.8	4.2	0.5	4.2	3.3
relative basicity of conj. base	4,000,000	1	0.0002	1	0.1



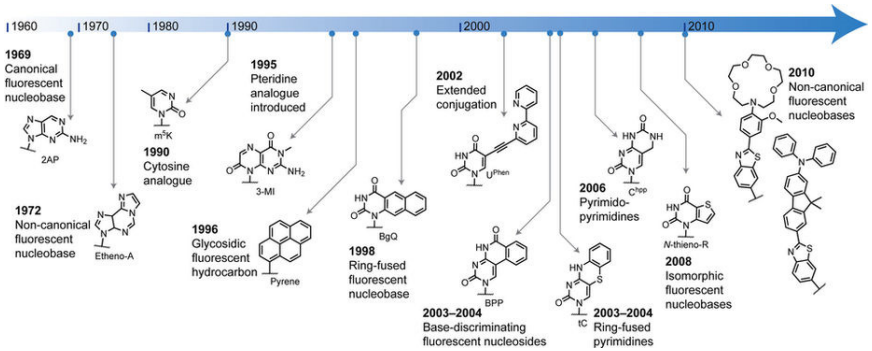


Fluorescent nucleobases for studying DNA and RNA



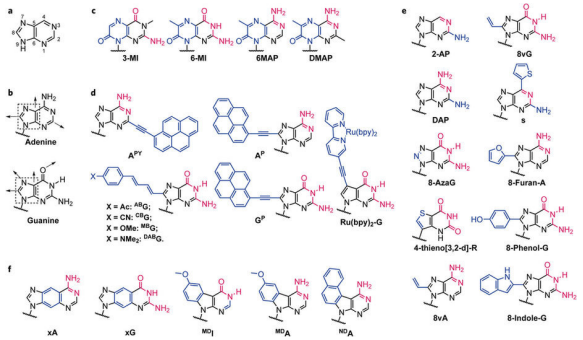
W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9*, 1043-1055

Fluorescent nucleobases for studying DNA and RNA



W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9*, 1043-1055

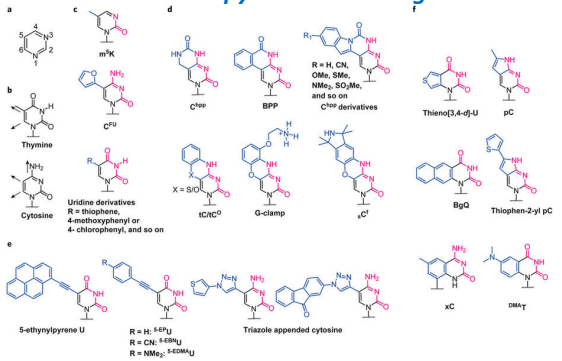
Fluorescent purine analogues



a, Numbering of the purine skeleton. **b**, Potential modification sites of adenine and guanine. Expansion and modification mostly occur on positions 2, 5 and 8 of adenine and positions 5, 6, 7 and 8 of guanine, as indicated by the arrows. **c**, Examples of purine ring fusion modifications. **d**, Examples of extending the purine scaffold through conjugated linkers. **e**, Examples of purine substituent modifications. **f**, Examples of purine ring fusions. Native base-pairing groups are rendered in pink, whereas fluorescence modifications are shown in blue.

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

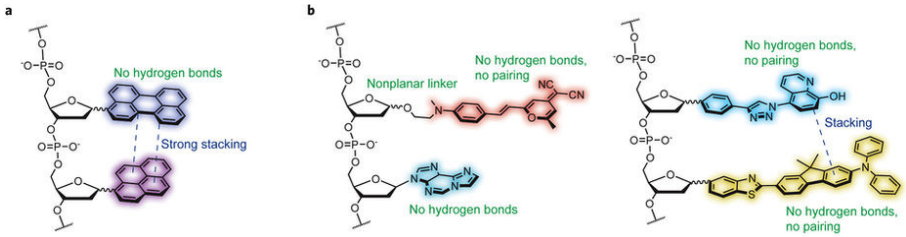
Fluorescent pyrimidine analogues



a, Numbering of the pyrimidine scaffold. **b**, Potential modification sites of thymine and cytosine. Expansion and modification mostly occur on positions 5 and 6 of thymine and positions 4, 5 and 6 of cytosine, as indicated by the arrows. **c**, Modification of the pyrimidine substituents at position 5. This category includes changes at only one especially versatile chemical position. **d**, Modification of the pyrimidine substituents at positions 5 and 6. The extensions form a new six-membered ring containing heteroatoms, which can be further expanded for fluorescent modification. **e**, Extension of pyrimidines via conjugated linkers. **f**, Pyrimidine ring fusion.

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

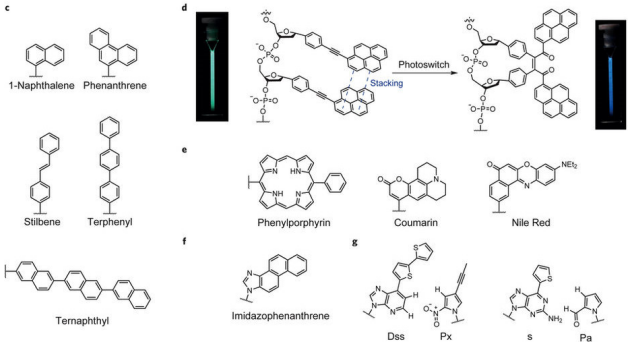
Non-canonical fluorescent nucleobases



a, Nucleobases composed of aromatic hydrocarbons. **b**, Nucleobases composed of planar heterocyclic fluorophores. The lack of hydrogen bonding and weaker π-stacking are compensated by versatile energy states brought from the heteroatoms. These fluorophores contribute to a broader spectrum of emission wavelengths. Functional groups can be added to expand functionality, such as metal binding.

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

Non-canonical fluorescent nucleobases



c, Examples of nucleobases based on hydrocarbons. **d**, Photoreaction of adjacent phenethynylpyrene nucleobases yields a colour change in emission. The left image shows phenylalkynylpyrene excimer emission whereas the right image shows pyrene monomer emission, both excited at 360 nm. **e**, C-glycosidic nucleobases based on known fluorophores. **f**, Simple heterocyclic nucleobases used in the detection of DNA repair activity. **g**, Nucleobase pairs based on shape complementarity. Although they lack hydrogen bonding, the conformation of these bases counterpart each other, thus forming unnatural base pairing.

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

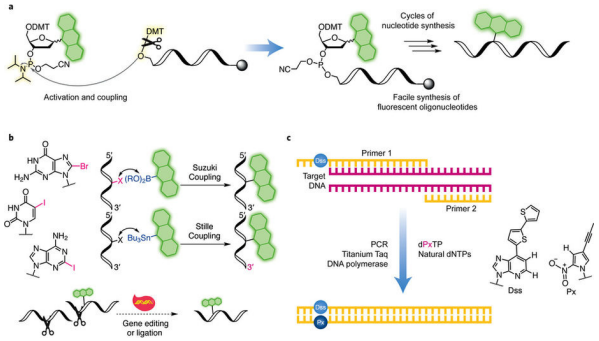
Strategies for incorporating fluorescent nucleobases

Table 1 | Comparison of three labelling methods for incorporating fluorescent nucleobases into DNA or RNA.

Labelling methods	Advantages	Disadvantages
Direct chemical synthesis	Site-specific incorporation at any position Little or no constraint on fluorophore structure	High cost on preparative synthesis scales Requires access to DNA synthesizer ~100 nt or less in length
Post-synthesis modification	Site-specific incorporation at any position Less expensive than direct synthesis	Limited structural diversity available May require challenging purification
Enzymatic incorporation	Low cost Access to labelled DNAs/RNAs ~100–1,000 nt in length	Some constraints on positional labelling Fluorophore structure limited by enzyme constraints Base-pair choices limited

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

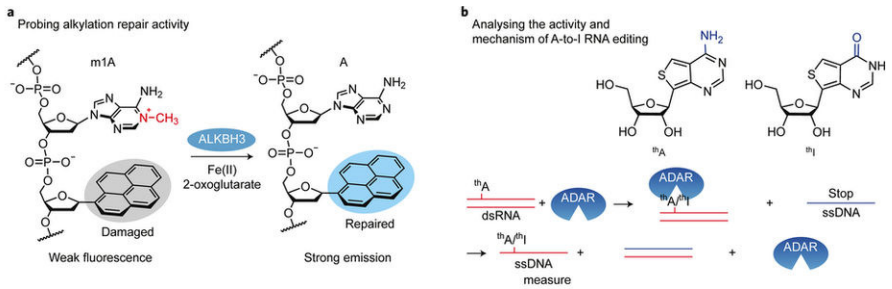
Strategies for incorporating fluorescent nucleobases



a, Direct oligonucleotide synthesis via synthesizer and phosphoramidite chemistry. **b**, Post-synthesis modification using mild coupling methods or gene-editing methods. Gene-editing and ligation methods enzymatically join smaller labelled strands to make longer ones. **c**, Direct enzymatic incorporation using fluorescent nucleoside triphosphate derivatives. When the fluorescent nucleobases are labelled in the primers or supplied as free nucleobases in the pool, polymerases that recognize them can incorporate the fluorescent nucleobases into DNA sequences.

W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

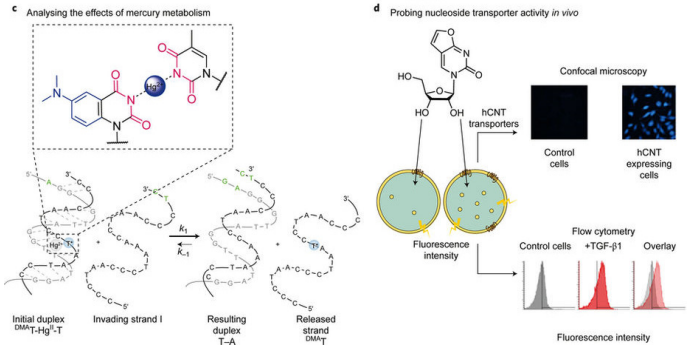
Fluorescent nucleobases for studying DNA and RNA



a, Fluorogenic sensing of a demethylation enzyme, ALKBH3. The emission of pyrene is initially quenched by the positive charge of 1-methylated adenine (m1A). When ALKBH3 demethylates m1A, the quenching effect is removed and a signal is generated.
b, Fluorogenic analysis of adenine-to-inosine RNA editing enzyme. The emission maxima of the thiolated adenine (thA) and inosine (thI) are different. Hence by measuring the intensity of thA and thI at their respective maximal wavelengths, the activity of the A-to-I enzyme can also be measured.

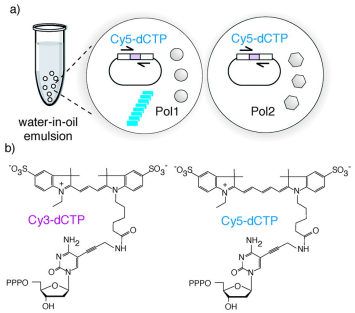
W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

Fluorescent nucleobases for studying DNA and RNA



c, Kinetic and thermodynamic investigation of the effects of mercury on DNA metabolism. The fluorescent thymine can chelate mercury with another thymine ring and link DNA strands. This can be used to probe mercury metabolism in vivo and to study the effects of mercury on DNA status.
d, Visualization and analysis of human concentrative nucleoside transporters (hCNTs). The fluorescent nucleoside can enter the plasma membrane through the transporters, thus allowing the measurement of the transport activity. TGF-β1, transforming growth factor β1. W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

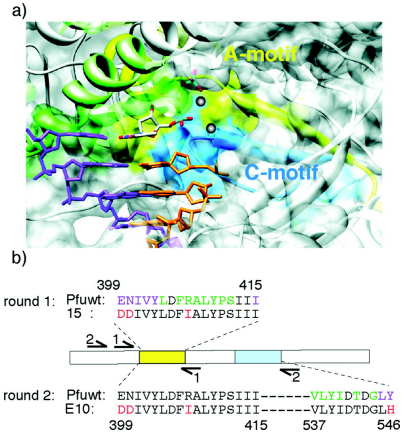


DNA Polymerase is evolutionarily optimized by mutagenesis to accept fluorescent Cy3 or Cy5 nucleotides instead of simple dCTP. Here, the polymerase replicates a short segment of its own encoding gene. Water/oil compartmentalization allows testing many independent mutations in paralell

Polymerases (Pol1 (left compartment)) that are capable of utilizing Cy5-dCTP are able to replicate, i.e., produce "offspring", while polymerases like Pol2 (right compartment) that are unable to utilize it disappear from the gene pool.

P. Holliger *et al. J. Am. Chem. Soc.* **2010**, *132*, 5096-5104

CyDNA – synthesis and replication of highly fluorescently-labelled DNA



(a) Structural model of the active site of a polB-family polymerase (RB69, PDB: 1IG9). The polypeptide chain is shown as a ribbon diagram overlaid with a transparent surface model. Primer and template strands are shown in orange and purple, incoming nucleotide triphosphate in elemental colors, and gray spheres represent the two catalytic Mg^{2+} ions. Regions corresponding to the A-motif are colored yellow, and those for the C-motif cyan. Also shown is the conserved B-motif (green).

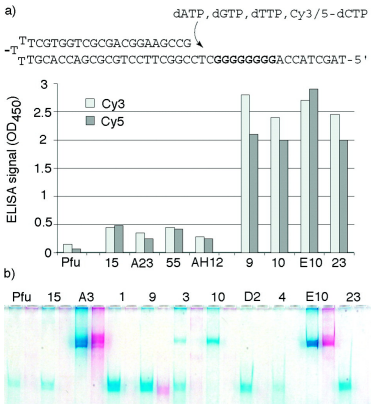
(b) For round 1 selection, diversity was focused on the A-motif and vicinity (399–415) comprising random mutation spike at the core of the A-motif (lime) as well as phylogenetic diversity in the adjacent sequences (purple). For round 2, successful clones from round 1 (e.g., 15) were diversified in the C-motif region (cyan), and selected for replication of A- and C-motif (399–546) yielding polymerase E10 (selected mutations in red)

P. Holliger *et al. J. Am. Chem. Soc.* **2010**, *132*, 5096-5104

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

380 selected variants from round 1 were screened by Polymerase-ELISA and ranked for their ability to incorporate 4 consecutive Cy5-dCTPs or Cy3-dCTPs. Polymerase-ELISA identified 4 mutant polymerases with significantly enhanced ability to incorporate either Cy3-dCTPs or Cy5-dCTPs compared with wild-type Pfuexo-:
- A23 (N400D, I401L, R407I),
- AH12 (E399D, N400G, I401L, V402A, R407I, Q572H),
- 55 (N400G, R407I), and in particular
- 15 (V337I, E399D, N400G, R407I).

a) activities of round 1 clones (15, A23, 55, AH12) and round 2 clones (9, 10, E10, 23). Clones were chosen i.a. on the their ability to incorporate both Cy3- and Cy5-dCTPs with comparable efficiency.
b) PAGE gel of a 0.4kb PCR amplification (70% GC) with complete replacement of dCTP with either Cy5- or Cy3-dCTP comparing selected clones to wild-type Pfuexo- (Pfu). Only E10 (and to a lesser extent A3) are able to perform PCR amplification with both dyes.



P. Holliger *et al. J. Am. Chem. Soc.* **2010**, *132*, 5096-5104

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

The polymerase fidelity in the selected mutant „E10" was not significantly compromised as compared to the starting Pfu DNA polymerase (*Pyrococcus furiosus*)

Fulvio314

Pyrococcus furiosus – an extremophilic *Archaeon* from marine sediments
Optimal life temperature 100°C

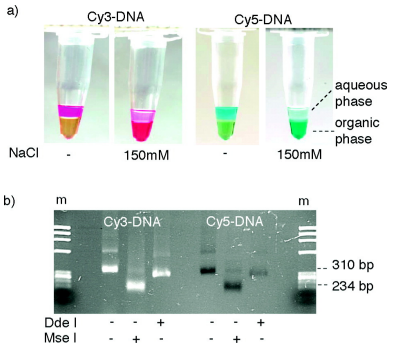
Table 1. Polymerase Fidelity

polymerase	PCR substrates	mutations/kb	mutation rate/bp/doubling[a]
Pfuexo-	dNTPs	1.1	4.4×10^{-5} (4.7×10^{-5})[b]
E10	dNTPs	0.4 (2.6 without additives[c])	1.6×10^{-5} (1.04×10^{-4})[c]
Pfuexo-	dNTPs[b]	2.7	6.0×10^{-5}
E10	dATP, dGTP, dTTP, Cy3-dCTP	4.3	9.6×10^{-5}
E10	dATP, dGTP, dTTP, Cy5-dCTP	4.9	1.1×10^{-4}

[a] Corrected for the number of doublings (PCR cycles). [b] As determined by a lacZ reversion assay.[44] [c] In the absence of additives (1% formamide, 10% glycerol, 10 μg/mL RNase, 1 mM DTT).

P. Holliger *et al. J. Am. Chem. Soc.* **2010**, *132*, 5096-5104

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

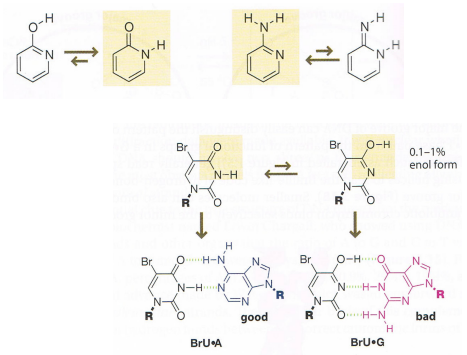


(a) Organic phase partitioning of CyDNA is shown for Cy3-DNA (left) and Cy5-DNA (right). Essentially 100% partitoning occurs in the presence of 150 mM NaCl (the yellow color of the phenol phase is due to addition of 8-hydroxyquinoline to prevent oxidation).

(b) Agarose gel electrophoresis of CyDNA restriction digests. Restriction endonucleases are sensitive probes of noncanonical DNA conformations such as those which occur under torsional strain. Both Cy3- and Cy5-DNA are resistant to cleavage by the restriction endonuclease DdeI (C'TNAG) but are cut by MseI (T'TAA). This indicates that at least the local regions of AT-sequence in Cy-DNA adopt a canonical B-form conformation.

P. Holliger *et al. J. Am. Chem. Soc.* **2010**, *132*, 5096-5104

Nucleobase modifications for biosynthetic



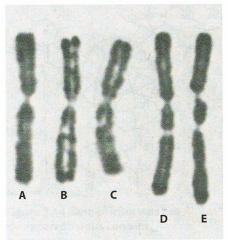
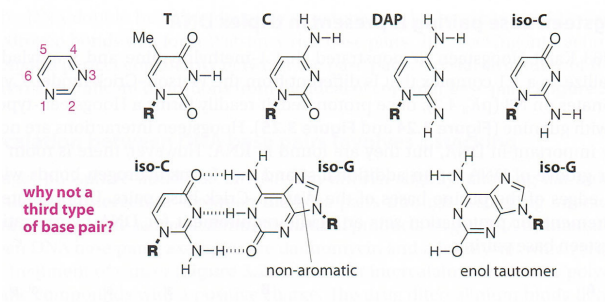


Figure 3.20 Chromosome 1 from hamster cells exposed to bromodeoxyuridine. (A) Normal chromosome. (B–E) Aberrant chromosomes. (From T.C. Hsu and C.E. Somers, *Proc. Natl. Acad. Sci. USA* 47: 396–403, 1961. With permission from the MD Anderson Cancer Center.)

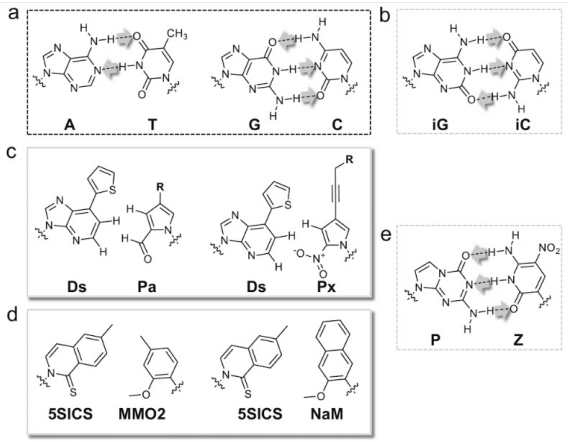
Alternative base pairs – synthetic biology



DAP – one tautomer forms a base pair with guanine

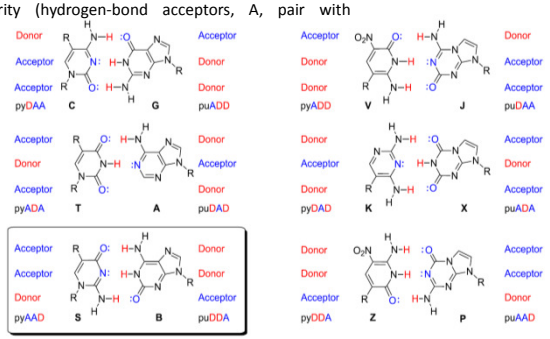
iso-C/iso-G
- specificity (the enol tautomer of iso-G, stabilized by aromatization, complementary to thymine)
- the 2-amino group of iso-C hydrolyses easily to uracil

Natural and non-natural base pairs that function in polymerase reactions



AEGIS – Artificially Expanded Genetic Information System

Watson–Crick pairing rules:
(a) size complementarity - large purines pair with small pyrimidines
(b) hydrogen-bonding complementarity (hydrogen-bond acceptors, A, pair with hydrogen-bond donors, D).



Rearranging donor and acceptor groups on the nucleobases, while not changing the geometry of the Watson–Crick pair, creates an artificially expanded genetic information system (AEGIS). AEGIS components add information density to DNA strands built from them.

S. Benner *et al., Beilstein J. Org. Chem.* **2014,** *10,* 2348–2360. doi:10.3762/bjoc.10.245

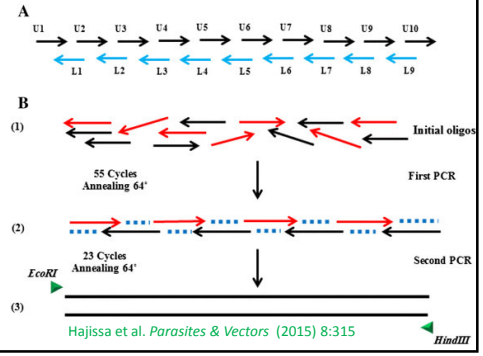
Artificial Gene Synthesis

Artificial gene synthesis (DNA printing) - method in synthetic biology to create artificial genes in the laboratory:

- *currently based on solid-phase DNA synthesis,*
- *the user does not have to begin with preexisting DNA sequences.*
- *Therefore, it is possible to make a completely synthetic double-stranded DNA molecule with no apparent limits on either nucleotide sequence or size.*

Applications:

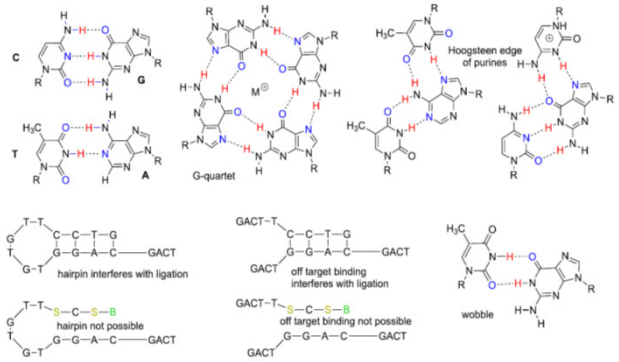
- recombinant DNA technology including heterologous gene expression, vaccine development, gene therapy and molecular engineering.
- The synthesis of nucleic acid sequences can be more economical than classical cloning and mutagenesis procedures
- the ability to safely obtain genes for vaccine research without the need to grow the full pathogens.
- to optimize protein expression in a particular host, or to remove non-functional DNA segments
- For DNA digital data storage and computing
- For synthetic biological circuits



Hajissa et al. *Parasites & Vectors* (2015) 8:315

Self-assembly of whole genes and DNA nanostructures

Limitations of DNA puzzle assembly: unequal A:T vs. G:C strength, insufficient ACGT information density, higher-order structures

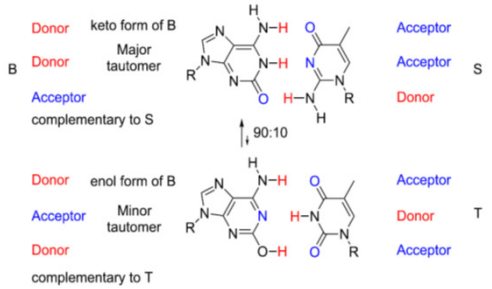


S. Benner *et al., Beilstein J. Org. Chem.* **2014,** *10,* 2348–2360. doi:10.3762/bjoc.10.245

Self-assembly of whole genes and DNA nanostructures

Solution: an orthogonal pair from the AEGIS system, that can be removed from the product, yielding native DNA structures

Conversion occurs when polymerases are forced to mismatch a standard nucleotide opposite an AEGIS nucleotide by
(a) not being provided the complementary AEGIS triphosphate and
(b) exploiting a chemical feature of the AEGIS nucleotide that directs a specific mismatch.



B in its major tautomeric form pairs with **S**; in its minor tautomeric form, **B** pairs with standard **T**. Asembly of the target gene/DNA nanostructure is followed by conversion of the **S:B** pairs to **T:A** pairs after two cycles of PCR:
B → A via an intermediate **B:T** mispairing, **S → T** (intermediate **S:B** followed by a second **B:T** mispairing).

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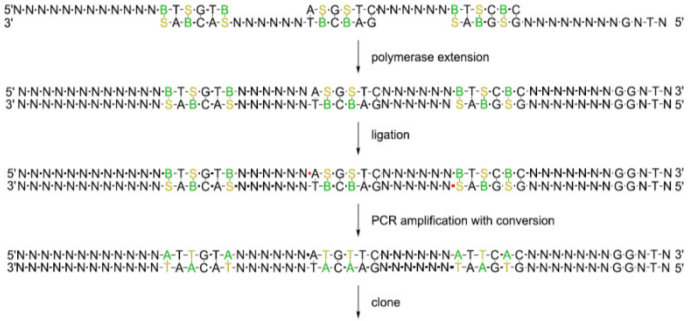
Self-assembly of whole genes and DNA nanostructures



The technology tested by assembly of the kanamycin-resistance gene and growing the bacteria in the environment containing kanamycin after assembly and conversion of that gene.

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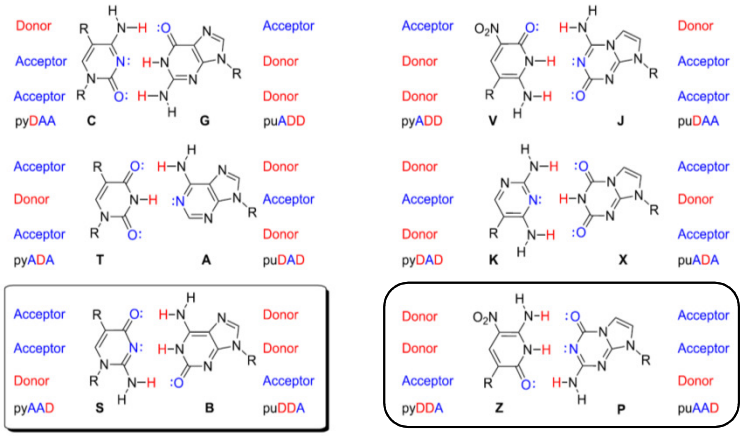
AEGIS – Artificially Expanded Genetic Information System



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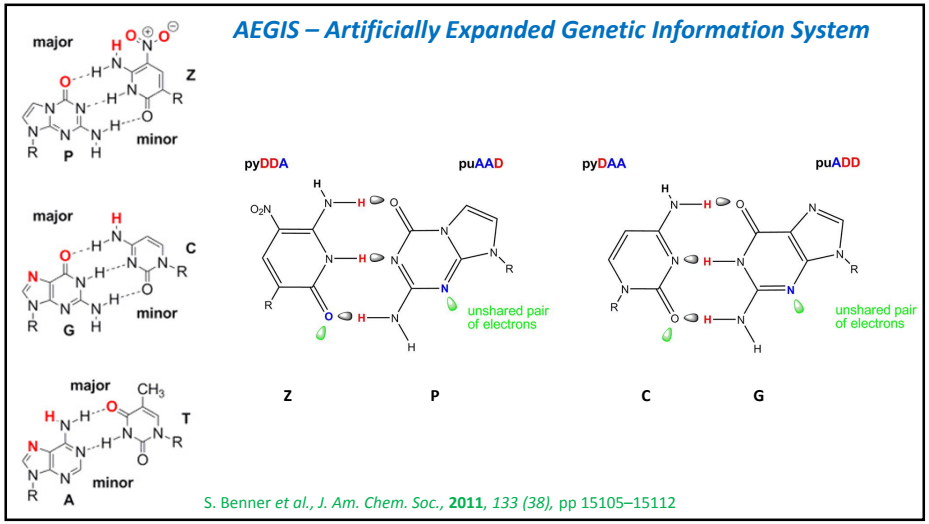
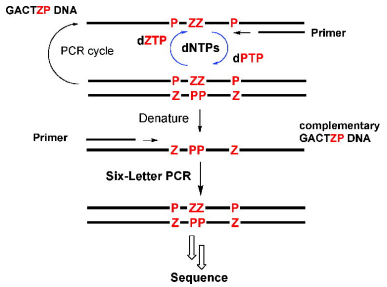
AEGIS – Artificially Expanded Genetic Information System



S. Benner *et al., J. Am. Chem. Soc.,* **2011**, *133 (38),* pp 15105–15112

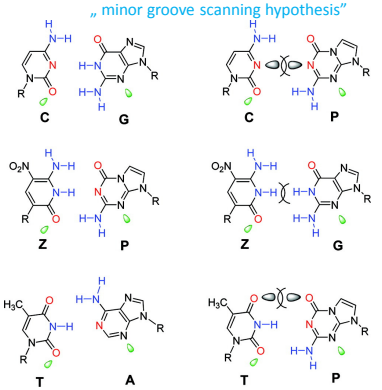
AEGIS – Permanent orthogonal nucleobases surviving PCR



Error rate 0,2% per a PCR cycle – both removal and incorporation of **Z** and **P** → the artificial genetic system capable to evolve.

Pol: Deep Vent – 2 Z/P, Taq/Phu – 3-4 Z/P
dZTP (*deprotonated*) at higher pH pairs slightly with G
→ loss of some **Z**, but also gain of some new **Z** mutants.

Electron density presented to the minor groove
→ recognition site by polymerases
„ minor groove scanning hypothesis"



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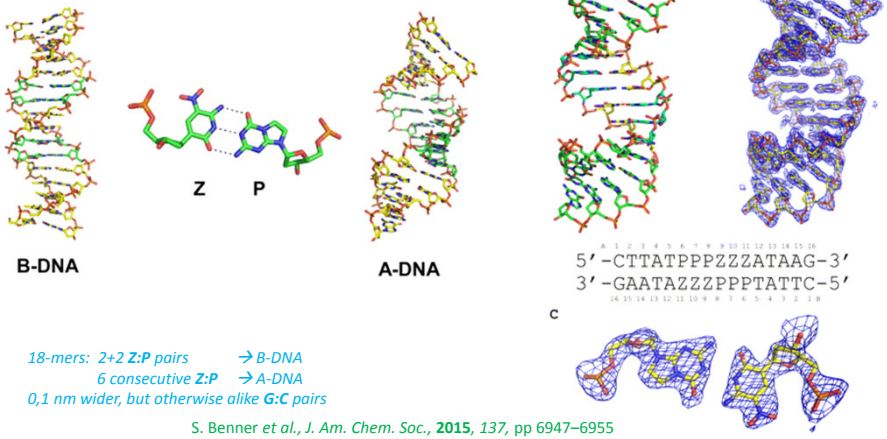
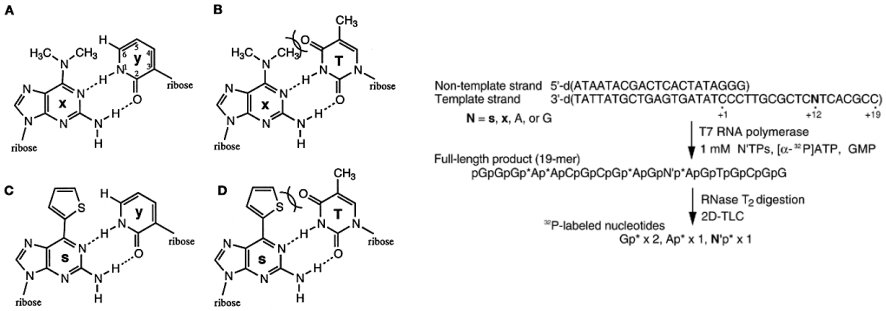
ACGTZP-DNA crystal structures



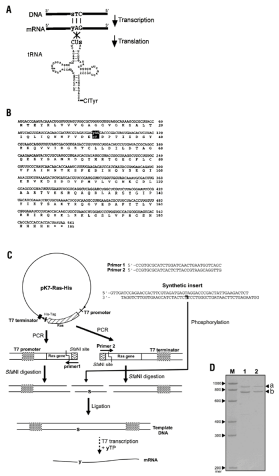
*18-mers: 2+2 **Z:P** pairs → B-DNA*
*6 consecutive **Z:P** → A-DNA*
*0,1 nm wider, but otherwise alike **G:C** pairs*

S. Benner *et al., J. Am. Chem. Soc.*, **2015**, *137*, pp 6947–6955

Unnatural aminoacid incorporation using a noncanonical base pair



I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)



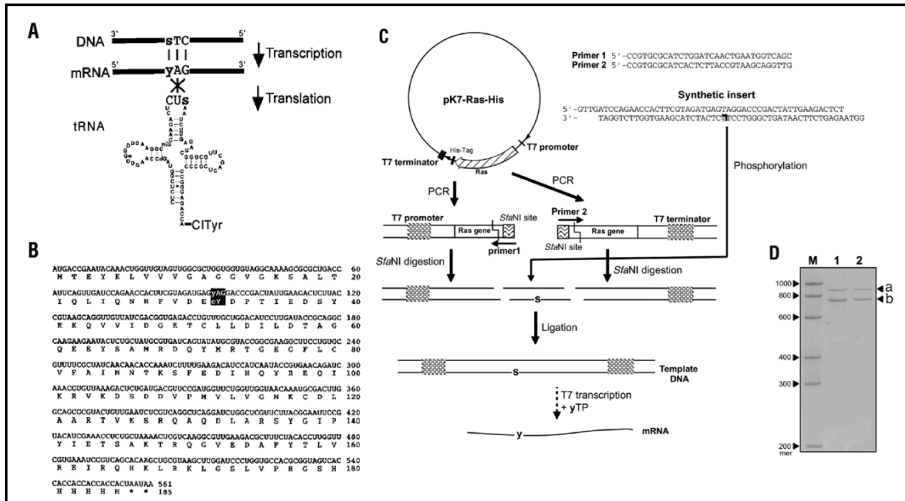
(A) The coupled transcription–translation system using the nonstandard codon–anticodon interaction for the site-specific incorporation of 3-chlorotyrosine into the Ras protein.

(B) The mRNA and amino acid sequences of human c-Ha-Ras replaced with the **y**AG codon for 3-chlorotyrosine at position 32.

(C) Construction of the DNA template containing the **s**TC sequence.

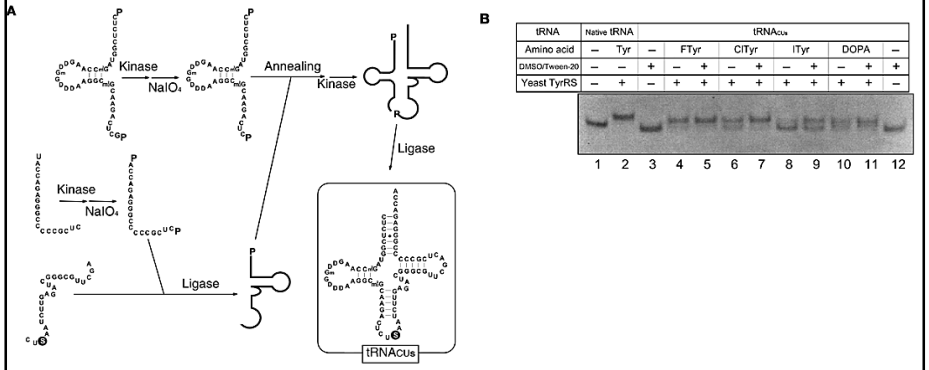
(D) Gel electrophoresis of the T7 transcripts. Lane 1 shows the transcripts from the DNA template containing the **s**TC sequence, and lane 2 shows those from the DNA template without **s** for the wild-type Ras protein. Bands a and b show the transcript terminated at the end of the template DNA (863-mer) and at the T7 terminator (747-mer), respectively.

I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)



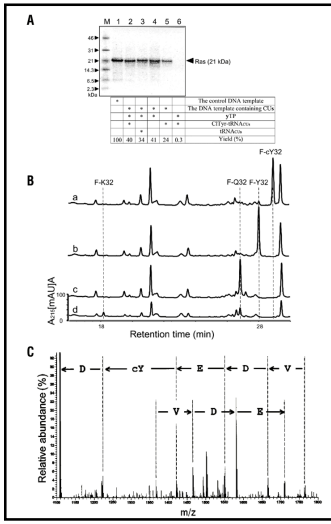
I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)

Unnatural aminoacid incorporation using a noncanonical base pair



(A) Construction of $tRNA_{CUs}$. (B) Acidic-gel electrophoresis of the products after aminoacylation of *S. cerevisiae* tRNA and $tRNA_{CUs}$ with tyrosine and the 3′-substituted analogs, in the absence or presence of 20% DMSO and 0.25% Tween-20. The upper bands are the aminoacylated tRNAs, and the lower bands are the noncharged tRNAs.

I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)



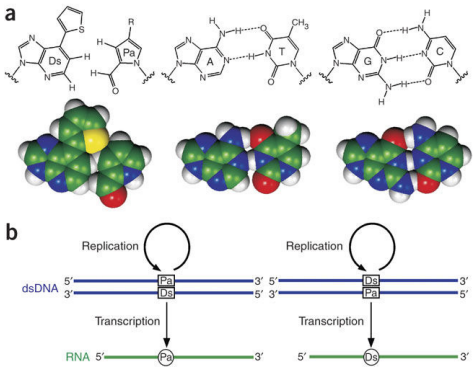
A) Autoradiogram of *in vitro* transcription–translation products labeled with l-[^{14}C]leucine. The conditions are noted at the bottom of each lane.

B) LC patterns of the products digested by Lys-C. Chart a shows the products obtained from the reaction in the presence of the DNA template containing the **CTs** sequence, **y**TP, and ClTyr-$tRNA_{CUs}$; chart b shows those obtained in the presence of the standard DNA template and Tyr; chart c shows those obtained in the presence of the DNA template containing **CTs** but in the absence of **y**TP and $tRNA_{CUs}$; and chart d shows those obtained in the presence of the DNA template containing **CTs** and **y**TP but in the absence of $tRNA_{CUs}$.

C) Tandem mass spectrum of the F-cY32 fragment. The partial sequence, VDE**cY**D, of F-cY32 was confirmed from the ion series

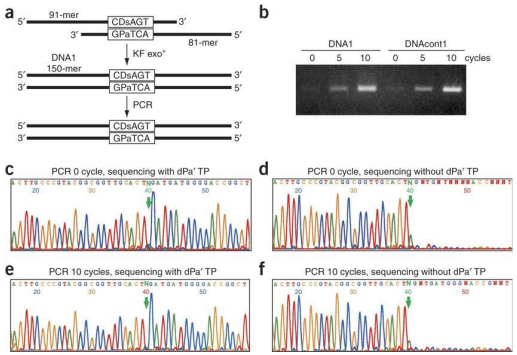
I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)

Unnatural aminoacid incorporation using a noncanonical base pair



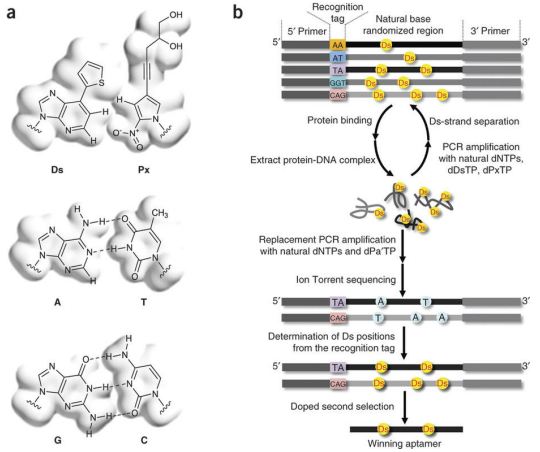
(**a**) The unnatural Ds-Pa and natural base pairs (Pa: R = H, Pa': R = C≡C–CH_3). (**b**) The unnatural base pair system that functions in PCR amplification, primer extension, DNA sequencing and T7 transcription. Original DNA templates were prepared by chemical synthesis and ligation, and were amplified by PCR with unmodified dNTPs (dPaTP, dGTP, dCTP and dTTP) and modified $dNTP_N$s (γ-amidotriphosphates, $dDsTP_N$ and $dATP_N$). RNA molecules containing Pa or modified Pa bases at specific positions were transcribed from DNA templates containing Ds in the template strands, by T7 RNA polymerase with PaTP (or modified PaTP) and the natural NTPs (left). RNA molecules containing Ds were transcribed from DNA templates containing Pa in the template strands, with DsTP and the natural NTPs (right).

I. Hirao *et al. Nature Methods* **2006**, *3*, 729-735



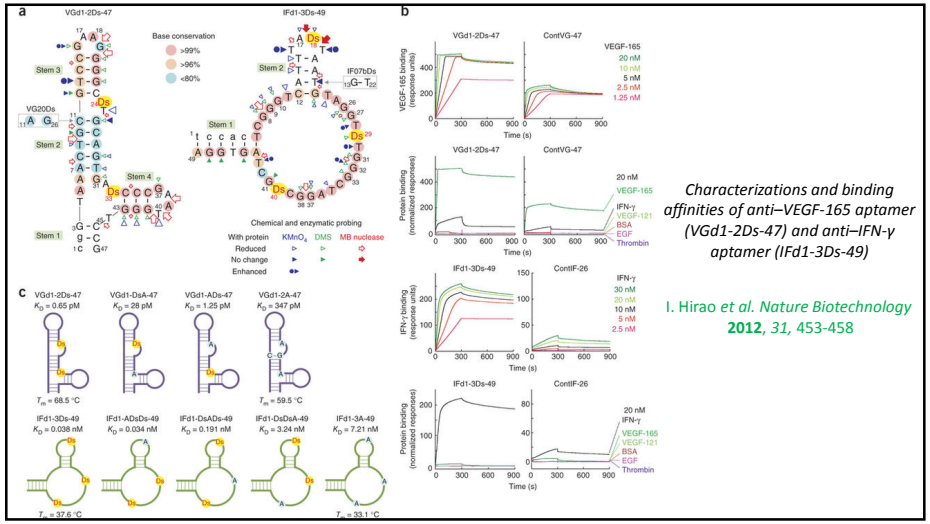
(**a**) The double stranded DNA fragment (150-mer, DNA1) was prepared by primer extension using chemically synthesized DNA fragments (91-mer and 81-mer) containing Ds and Pa. (**b**) Agarose-gel analysis of original DNA fragments (0 cycle) and PCR products after 5 and 10 cycles of amplification. For DNA1, PCR was performed with 0.04 unit/μl Vent DNA polymerase and the following cycle conditions: 0.5 min at 94 °C, 0.5 min at 45 °C and 4 min at 65 °C. For DNAcont1 consisting only of the natural bases, PCR was performed with 0.01 unit/μl Vent DNA polymerase with the following cycle conditions: 0.5 min at 94 °C, 0.5 min at 45 °C and 1 min at 72 °C. (**c**–**f**) DNA sequencing, in the presence (**c**,**e**) or absence (**d**,**f**) of dPa′TP, of the original DNA1 (**c**,**d**) and PCR-amplified DNA1 after 10 cycles using the unnatural base pair system (**e**,**f**).

Ds-Px noncanonical base pair – High-affinity DNA aptamers



Targets:
VEGF-165
and IFN-γ

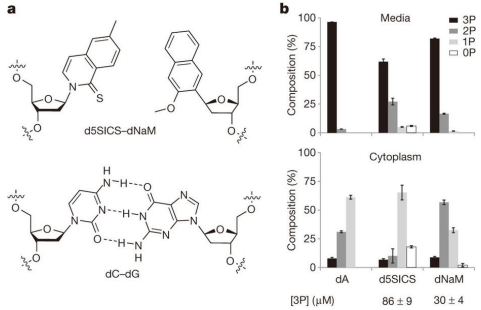
I. Hirao *et al. Nature Biotechnology* **2012**, *31*, 453-458



Characterizations and binding affinities of anti–VEGF-165 aptamer (VGd1-2Ds-47) and anti–IFN-γ aptamer (IFd1-3Ds-49)

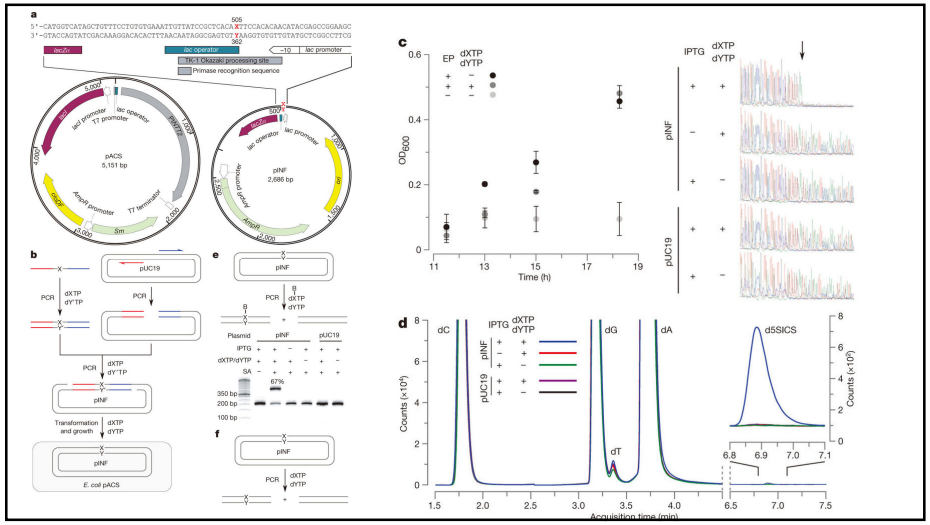
I. Hirao *et al. Nature Biotechnology*
2012, *31*, 453-458

A semi-synthetic organism with an expanded genetic alphabet

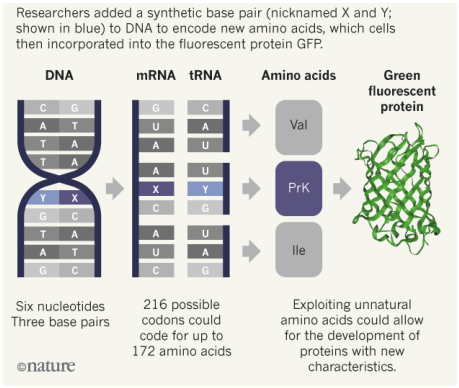


a, Chemical structure of the d5SICS–dNaM UBP compared to the natural dG–dC base pair. **b**, Composition analysis of d5SICS and dNaM in the media (top) and cytoplasmic (bottom) fractions of cells expressing *Pt*NTT2 after 30 min incubation; dA shown for comparison. 3P, 2P, 1P and 0P correspond to triphosphate, diphosphate, monophosphate and nucleoside, respectively; [3P] is the intracellular concentration of triphosphate.

Malyshev, Denis A.; Romesberg, Floyd E. *et al. Nature* **2014**, *509*, 385–388



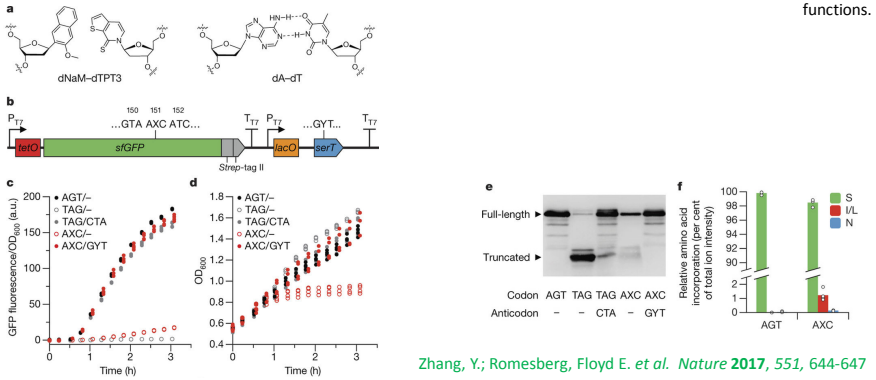
A semi-synthetic organism with an expanded genetic alphabet



Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551,* 644-647

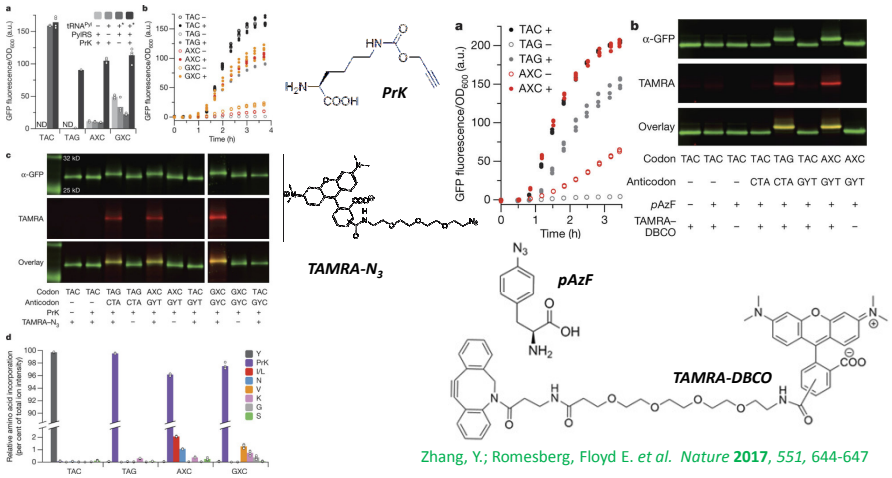
A semi-synthetic organism with an expanded genetic alphabet

The *in vivo* transcription of DNA containing dNaM and dTPT3 into mRNAs with two different unnatural codons and tRNAs with cognate unnatural anticodons, and their efficient decoding at the ribosome to direct the site-specific incorporation of natural or non-canonical amino acids into superfolder green fluorescent protein. The resulting semi-synthetic organism both encodes and retrieves increased information and should serve as a platform for the creation of new life forms and functions.



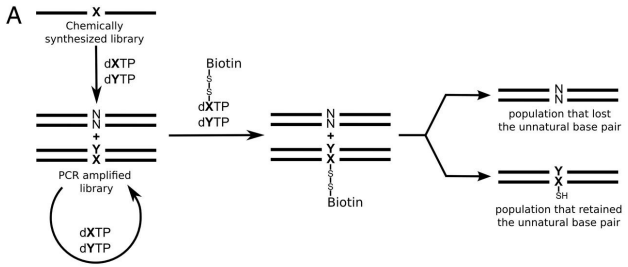
Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551,* 644-647

A semi-synthetic organism with an expanded genetic alphabet



Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551,* 644-647

A semi-synthetic organism with an expanded genetic alphabet



(A) PCR selection scheme. X = NaM (or when biotinylated, its analog MMO2; see Fig. S5) and Y = 5SICS. (B) Library design. The regions proximal to the unnatural base pair that were analyzed for biases are shown in red, and the distal regions used as a control are shown in green. Sublibrary-specific two-nucleotide barcodes that indicate the position of the unnatural base pair flank the randomized regions and are shown in italics. Primer binding regions are denoted as PBR

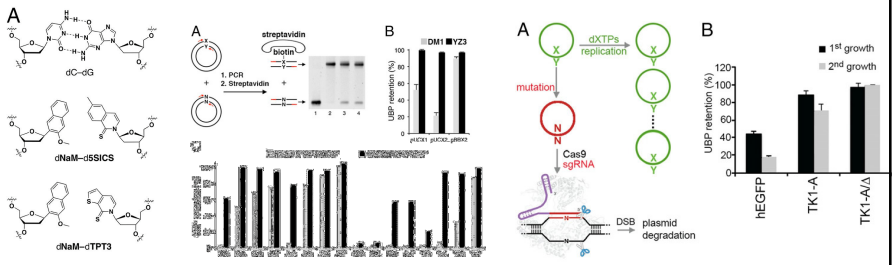
Malyshev, Denis A.; Romesberg, Floyd E. *et al.* *PNAS* **2012**, *109 (30),* 12005-12010

A semi-synthetic organism with an expanded genetic alphabet

- An unnatural base pair (UBP) would increase the information storage potential of DNA
- and semisynthetic organisms (SSOs) that stably harbor this expanded alphabet would thereby have the potential to store and retrieve increased information,
- *Escherichia coli* grown in the presence of the unnatural nucleoside triphosphates dNaMTP and d5SICSTP, and provided with the means to import them via expression of a plasmid-borne nucleoside triphosphate transporter, replicates DNA containing a single dNaM-d5SICS UBP,
- to fortify and vivify the nascent SSO, a more chemically optimized UBP dTPT3 was used, and the power of the bacterial immune response was harnessed by using Cas9 to eliminate DNA that had lost the UBP.
- The optimized SSO grows robustly, constitutively imports the unnatural triphosphates, and is able to indefinitely retain multiple UBPs in virtually any sequence context. This SSO is thus a form of life that can stably store genetic information using a six-letter, three-base-pair alphabet

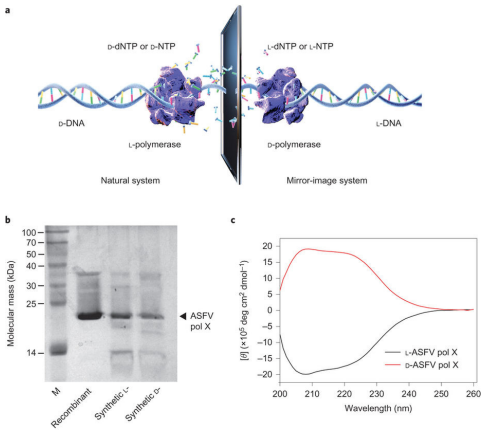
Malyshev, Denis A.; Romesberg, Floyd E. *et al.* *PNAS* **2017**, *114,* 1317-1322

A semi-synthetic organism with an expanded genetic alphabet



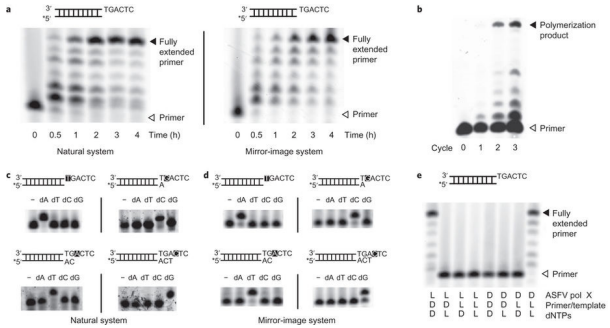
Malyshev, Denis A.; Romesberg, Floyd E. *et al.* *PNAS* **2017**, *114,* 1317-1322

Spiegelmers: L-DNA + D-aminoacids



Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8,* 698-704

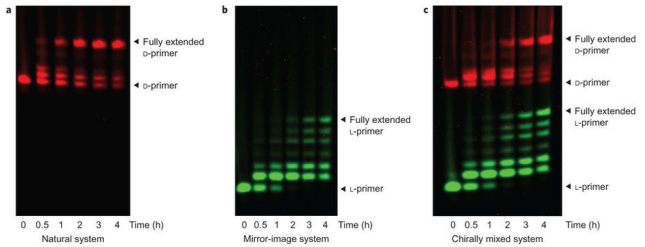
Spiegelmers: L-DNA + D-aminoacids



a, Template-directed primer extension by synthetic L-ASFV pol X (natural system) and D-ASFV pol X (mirror-image system) with the corresponding D- and L-DNA primers, templates and dNTPs. **b**, Repeated cycles of polymerization by D-ASFV pol X: **c**,**d**, The nucleotide substrate specificities of synthetic L- and D-ASFV pol X. **e**, Chiral specificity assay with different chiral combinations of polymerases, primer/template pairs and dNTPs.

Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8,* 698-704

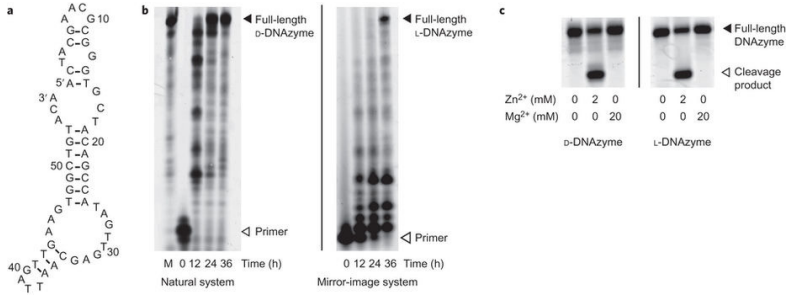
Spiegelmers: L-DNA + D-aminoacids



a,b, Primer extension by synthetic L- and D-ASFV pol X with the corresponding D-DNA primer (5′-Cy5 labelled) and L-DNA primer (5′-FAM labelled), templates and dNTPs. **c**, The above two polymerization reactions were carried out in a racemic mixture under the same conditions as described above, with the L- and D-ASFV pol X, D- and L-primers, D- and L-templates and D- and L-dNTPs added, incubated for up to 4 h at 37 °C.

Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8,* 698-704

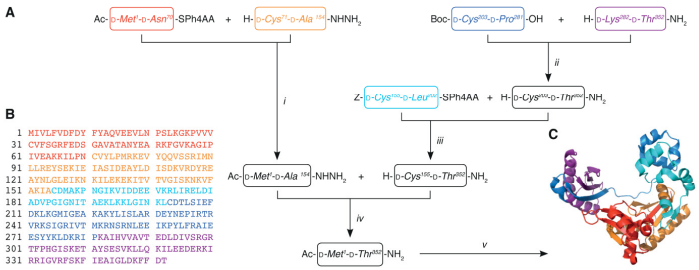
Spiegelmers: L-DNA + D-aminoacids



a, Sequence and predicted secondary structure of the previously reported Zn^{2+}-dependent self-cleaving DNAzyme[29]. **b**, Primer extension on a 66 nt template to produce the Zn^{2+}-dependent self-cleaving DNAzyme. **c**, Self-cleavage of the enzymatically polymerized Zn^{2+}-dependent D- and L-DNAzymes.

Z. Wang, W. Xu, L. Liu, T. F. Zhu *Nature Chem.* **2016**, *8,* 698-704

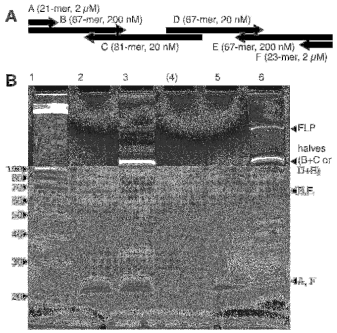
Spiegelmers: A thermostable D-polymerase



Synthesis strategy for d-Dpo4-3C. (**A**) five fragments were synthesized and assembled as follows: (i) native chemical ligation (NCL) of fragments 1 and 2. Isolated yield ≈ 18%. (ii) Segment condensation of fully protected fragments 4 and 5 followed by deprotection. Isolated yield ≈ 15%. (iii) NCL of fragments 3 and 4•5 followed by Z-deprotection. Isolated yield ≈ 25%. (iv) Thioester-conversion of fragment 1•2 and NCL with fragment 3•4•5. Isolated yield: 10%. (v) Folding. (**B**) sequence of d-Dpo4-3C; coloring as in panel A. (**C**) folded d-Dpo4-3C (artist impression based on PDB 3PR4 (31)).

S. Klussmann *Nucl. Acid Res.* **2017**, *45,* 3997-4005

Spiegelmers: A thermostable D-polymerase



Assembly of a mirror-image gene. (**A**) schematic of the oligonucleotide setup. (**B**) lane 1, 3 µl of 10 bp DNA ladder. Lane 2, mirror-image no-enzyme control. Lane 3, mirror-image gene assembly. Lane 4, empty. Lane 5, natural handedness no enzyme control. Lane 6, natural handedness gene assembly.

S. Klussmann *Nucl. Acid Res.* **2017**, *45,* 3997-4005