Synthetic life

(continuation of „The molecular origins of life" SoSe 2022)

NaturalNews.com

WiSe 2022/23

Zbigniew Pianowski

Overview of the course

artificial ribozymes and aptamers for efficient catalysis and recognition (SELEX, DNAzymes, foldamers);

unnatural base pairing – expansion of the genetic alphabet;

Artificial genetic polymers and oligonucleotide analogues (XNA);

biosynthetic incorporation of **unnatural aminoacids (UAAs)** into proteins;

enzyme engineering – production of enzymes with unknown or unnatural properties, *ab initio* protein design, directed evolution, theozymes;

Artificial lipid vesicles as models for protocell multiplication;

design of artificial organisms

CHAPTER 1

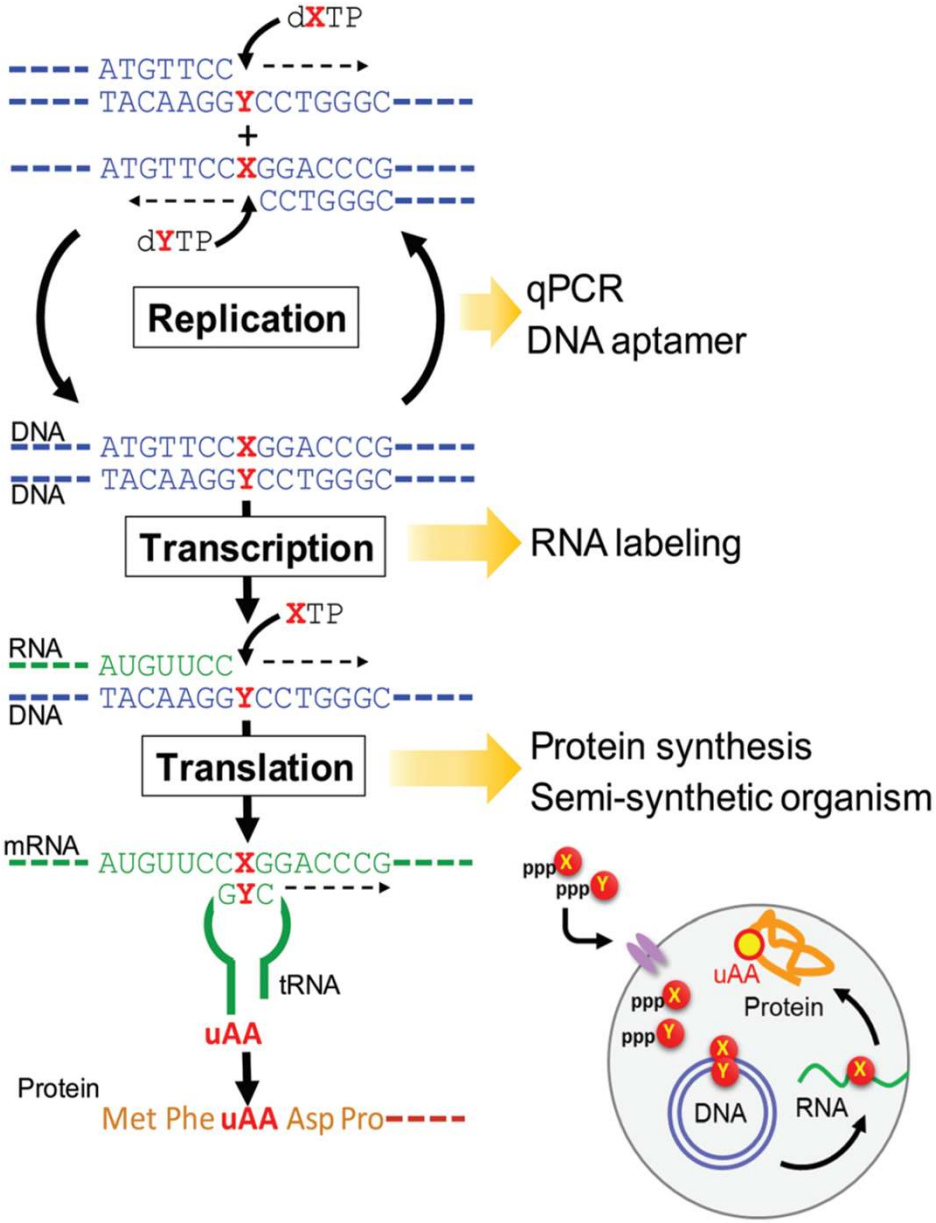
OLIGONUCLEOTIDES

Part 2 – noncanonical nucleobases

Expansion of the genetic alphabet

Expansion of the genetic alphabet and code by creating an unnatural base pair (UBP) as a third pair.

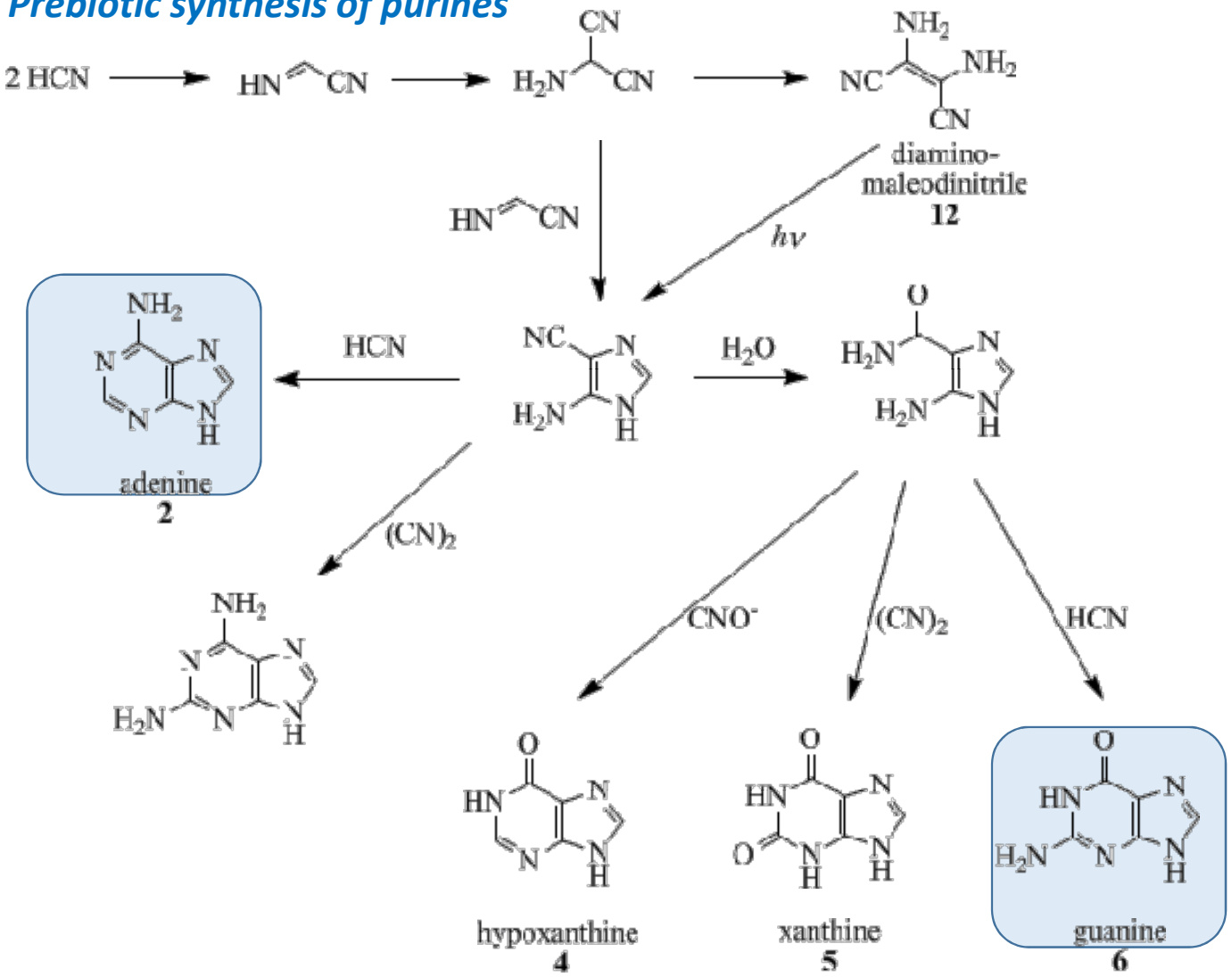
The creation of a UBP (i.e., X–Y) that functions in replication, transcription, and translation as a third base pair with the natural A–T(U) and G–C pairs allows the storage and retrieval of the expanded genetic information in vitro and in vivo, enabling a variety of applications using biopolymers with increased functionalities



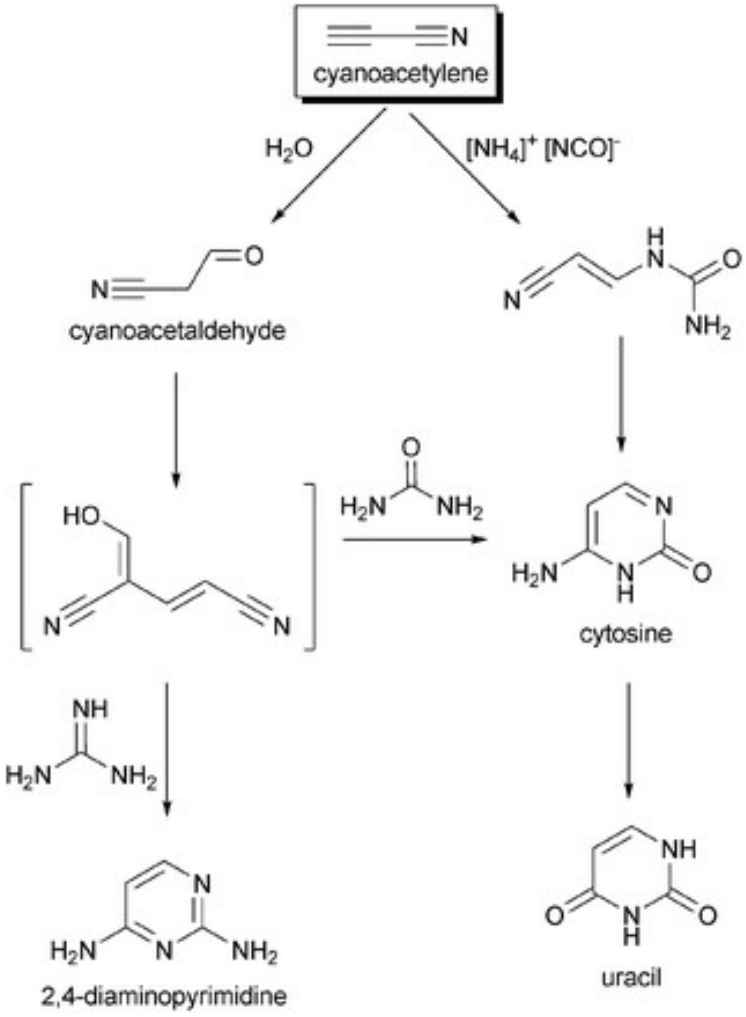
M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Why are A, C, G and T the letters of genetic alphabet.

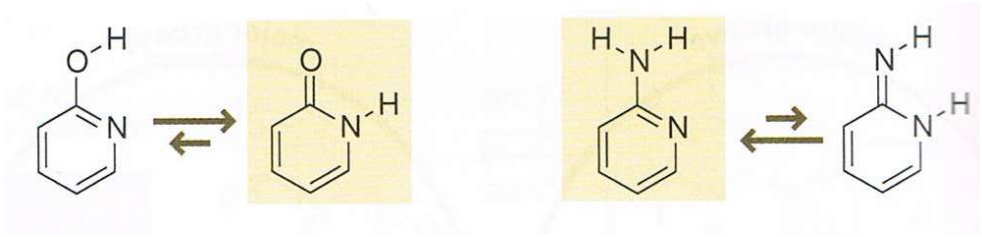
Prebiotic synthesis of purines

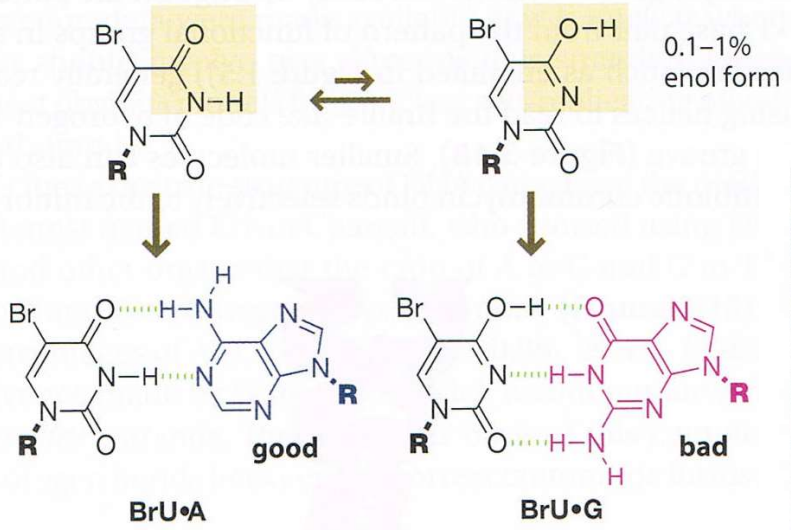


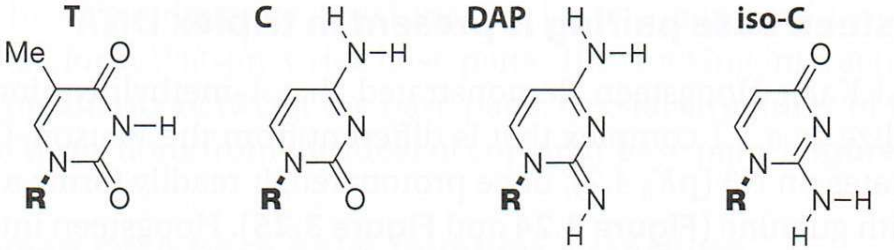
Prebiotic synthesis of pyrimidines



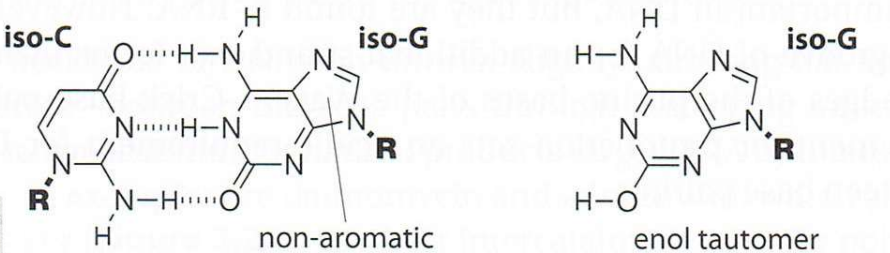
Biological consequences of nucleobase modifications







why not a third type of base pair?



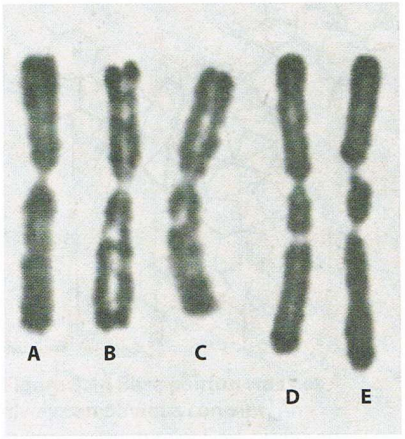
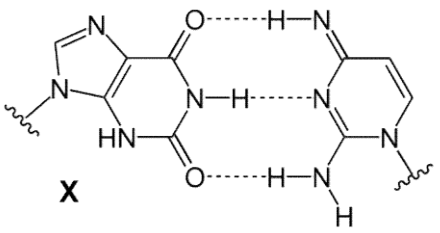
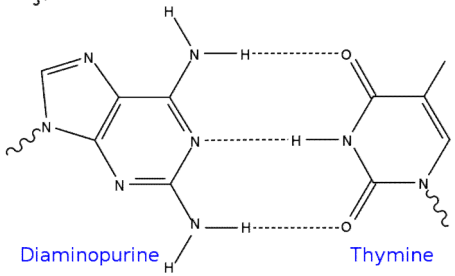


Figure 3.20 Chromosome 1 from hamster cells exposed to bromodeoxyuridine. (A) Normal chromosome. (B–E) Aberrant chromosomes. (From T.C. Hsu and C.E. Somers, *Proc. Natl. Acad. Sci. USA* 47: 396–403, 1961. With permission from the MD Anderson Cancer Center.)

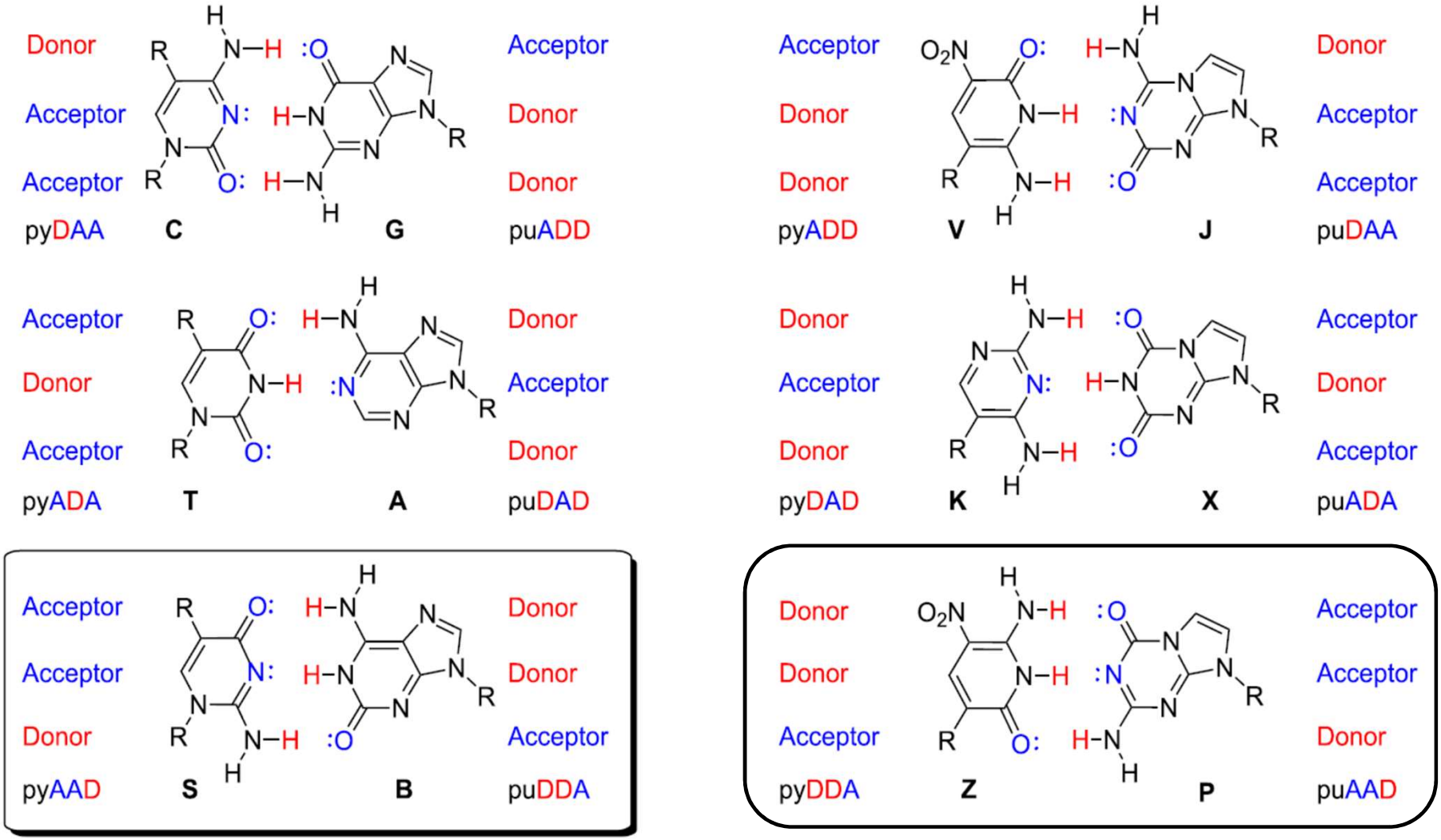
DAP



Cyanophage S-2L:



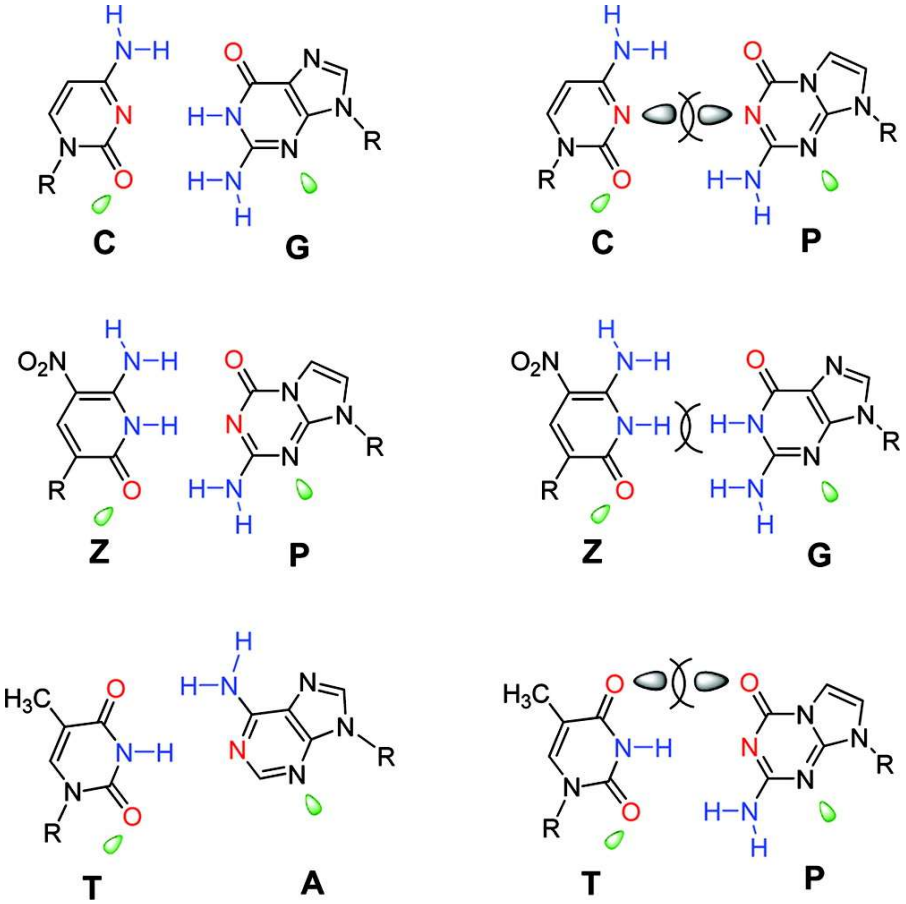
AEGIS – Artificially Expanded Genetic Information System



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

AEGIS – Artificially Expanded Genetic Information System

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



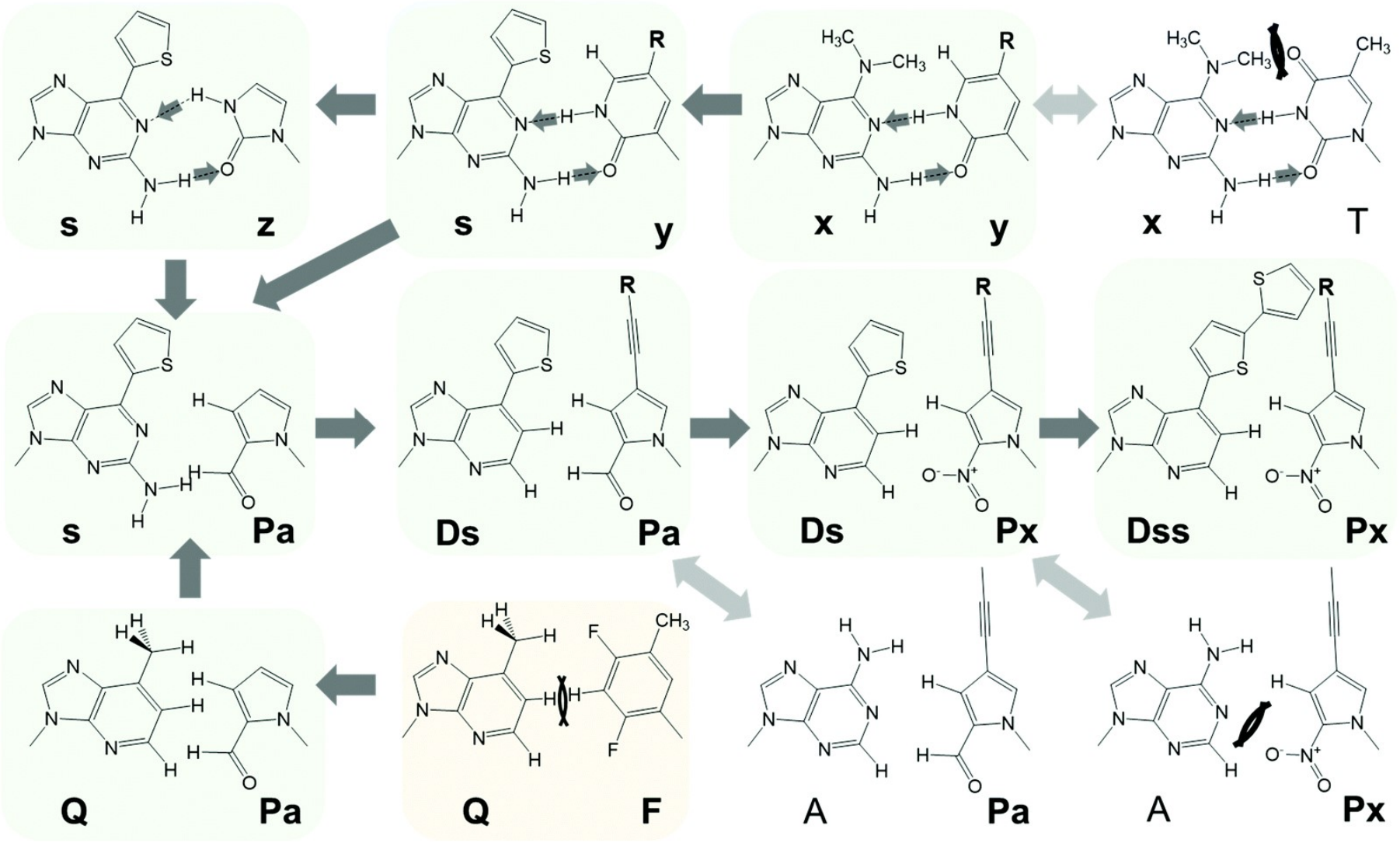
Applications:

- **artificial gene synthesis**
- **Hachimoji DNA/RNA ACGTPZSB genetic system (transcription of spinach RNA aptamer)**
- **AEGIS Cell-SELEX:**
  - **Aptamers against breast cancer cells**
  - **Positive/negative selection cancer/healthy liver cells**
  - **Aptamer + doxorubicin nanotrain**
  - **Aptamers against protein (cancer marker)**

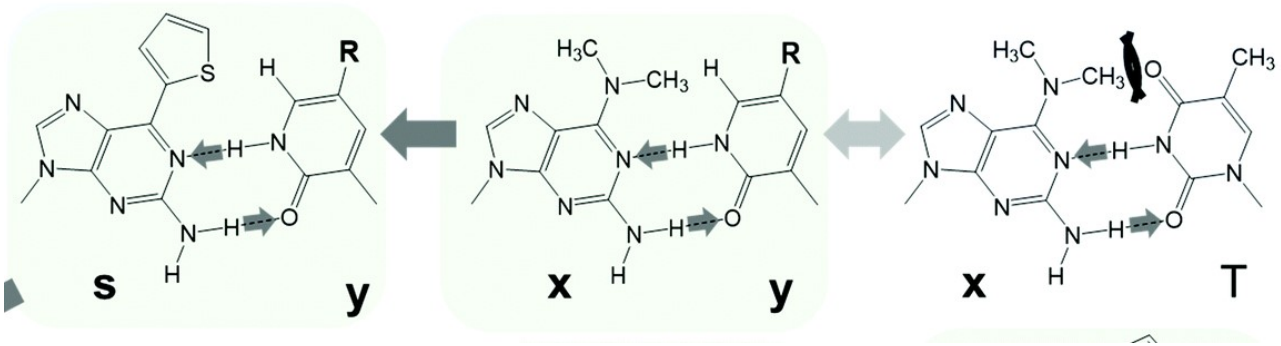
Error rate 0,2% per a PCR cycle – both removal and incorporation of **Z** and **P** ➔ the artificial genetic system capable to evolve.
T7 RNA Polymerase mutant – **ZPSB** transcription

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

Steric exclusion and hydrophobic non-natural base pairs



Steric exclusion and hydrophobic non-natural base pairs



Hirao (2001): the steric hindrance concept to hydrogen-bonded UBPs to exclude the mispairing with natural bases → a series of hydrogen-bonded UBPs: **x–y** and **s–y** pairs

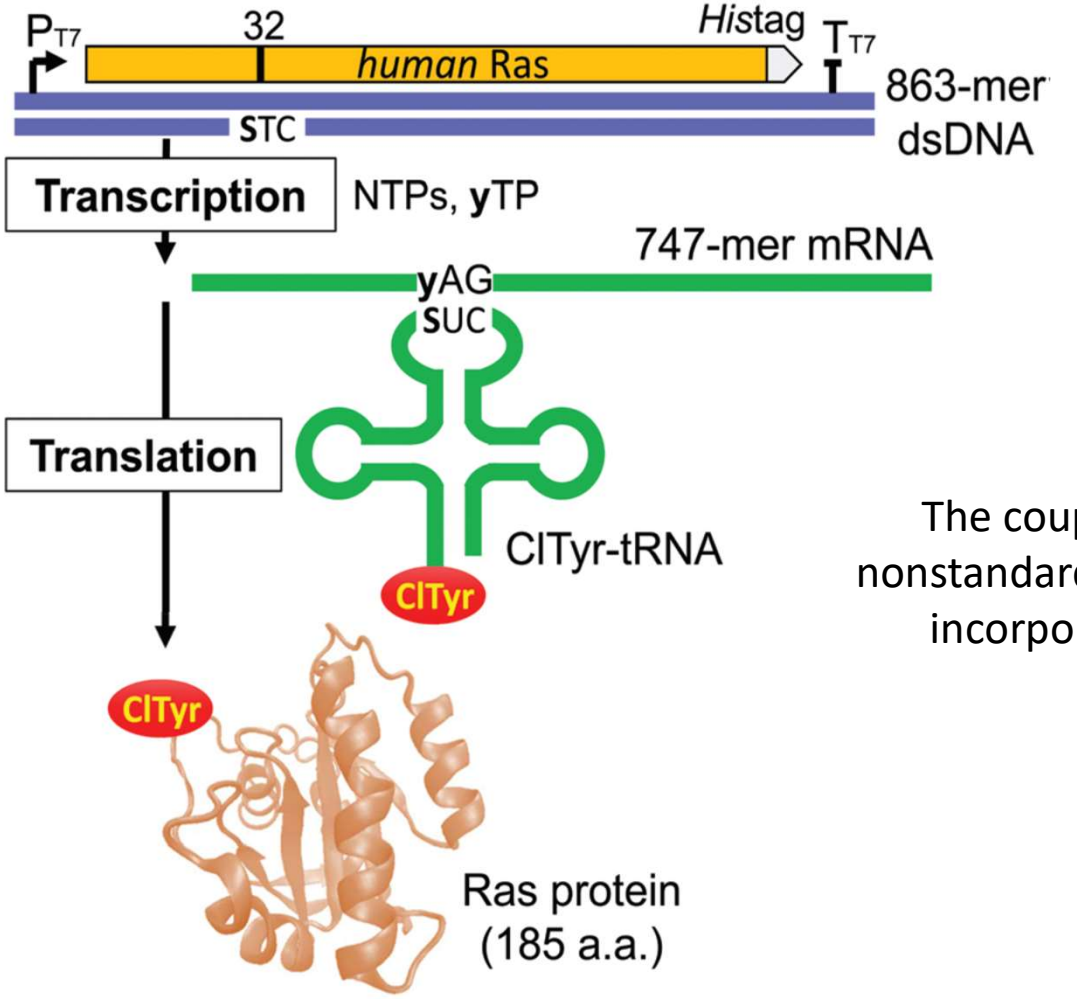
large residues at position 6 of x and s sterically and/or electrostatically clash with the 4-keto group of T, but not with hydrogen of the y base.

x–y and **s–y** pairs function in transcription with T7 RNA polymerase (T7 transcription), and the **y** substrate is incorporated (>96% selectivity **s–y**) site-specifically into RNA transcripts opposite **x** or **s** in DNA templates.

Problem: the **y** base cannot exclude the mispairing with A. Therefore, these UBPs cannot be used in replication

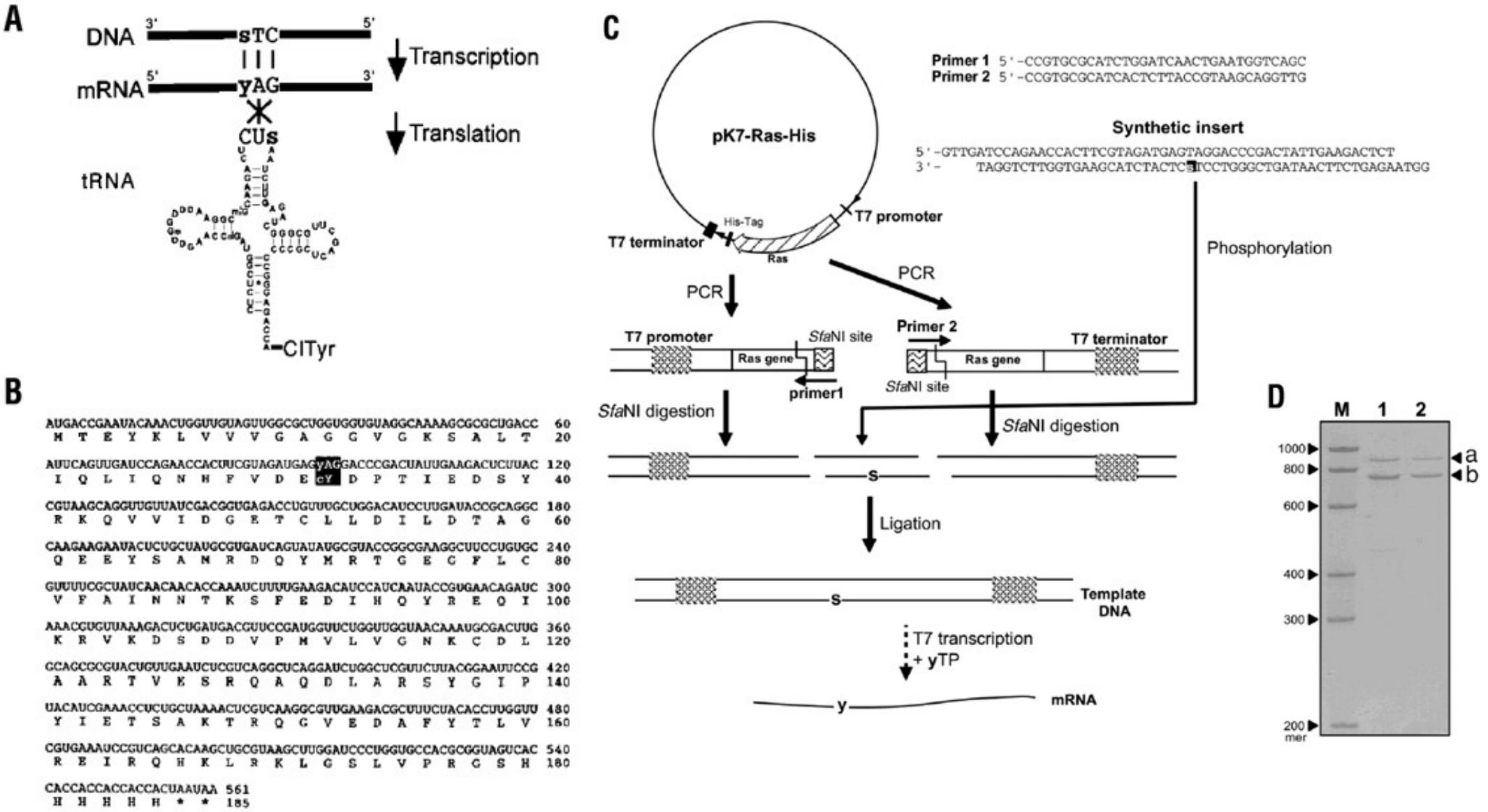
M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Unnatural aminoacid incorporation using a noncanonical base pair



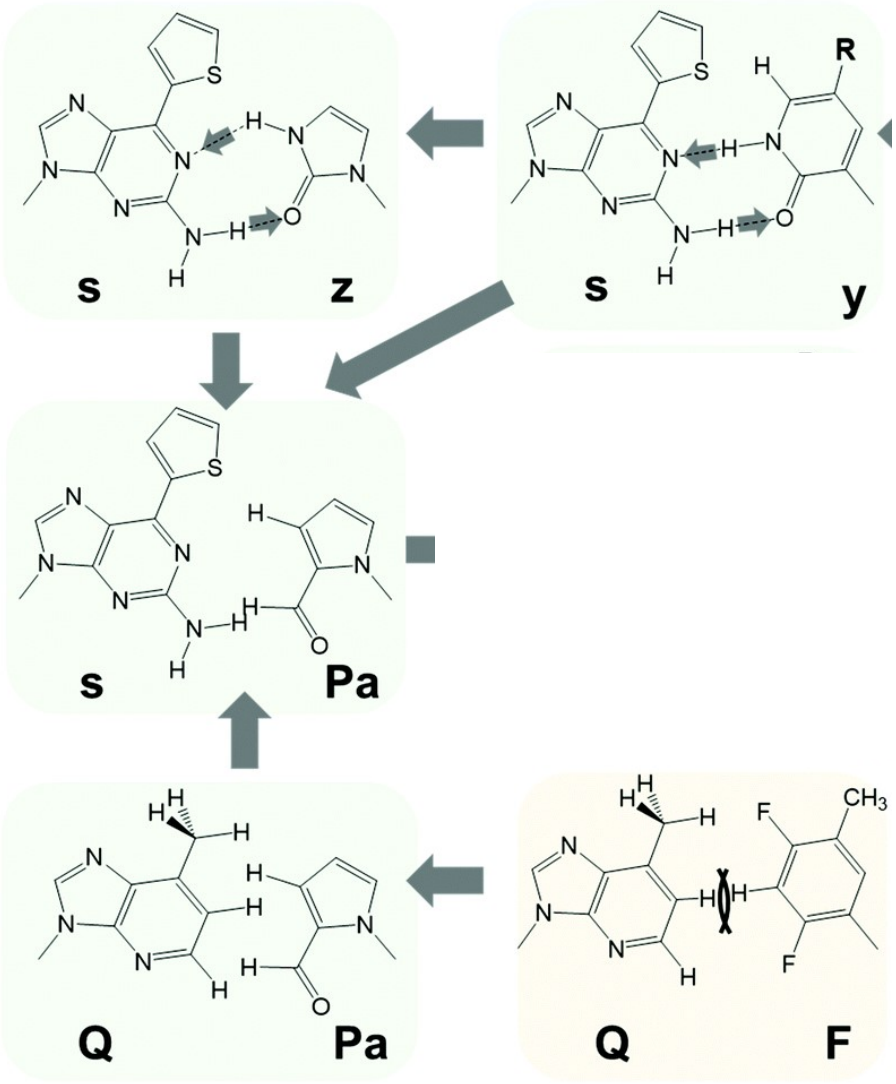
The coupled transcription–translation system using the nonstandard codon–anticodon interaction for the site-specific incorporation of 3-chlorotyrosine into the Ras protein.

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



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

Steric exclusion and hydrophobic non-natural base pairs



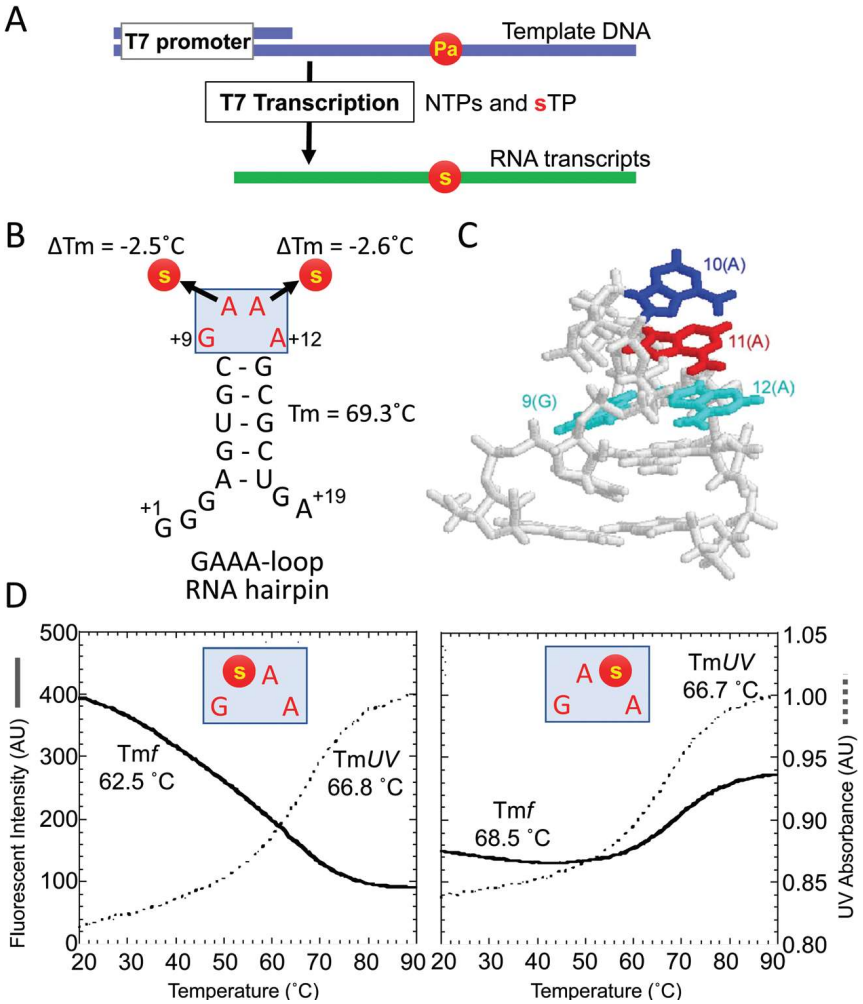
Hirao (2004): The s–z pair increased the s incorporation selectivity opposite z in T7 transcription, as compared to that opposite y in the s–y pair

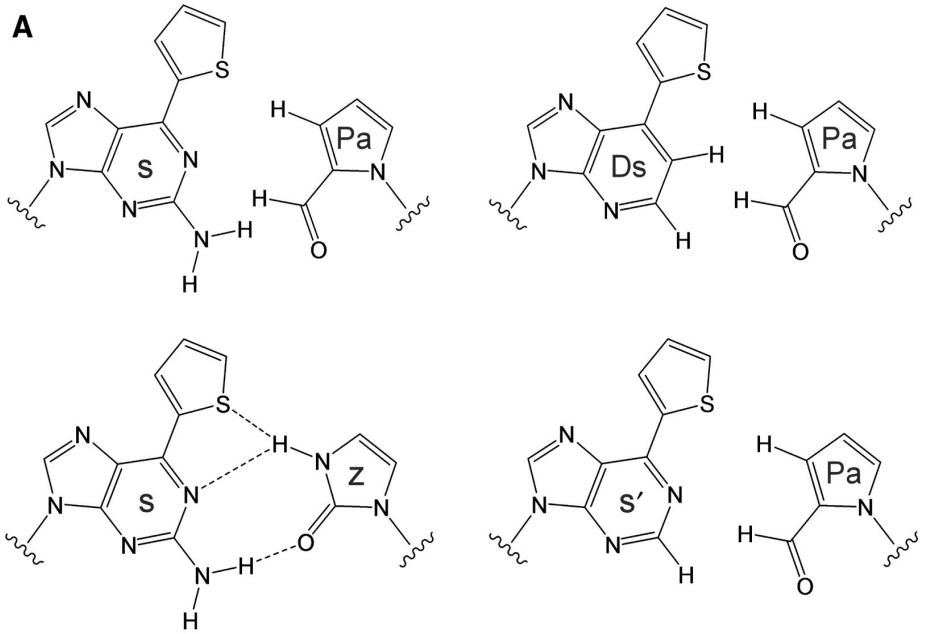
Kool (1999): Q-F pair - shape-complementarity rather than the hydrogen-bond interactions, ➔a new strategy of UBP development using hydrophobic UBs without any hydrogen-bond interactions between the pairing bases.

Hirao (2003): the Q–Pa pair to avoid steric clashes.
Hirao (2007): Pa could be used as a pairing partner of the s base, and the s substrate was incorporated specifically into RNA opposite Pa in templates by T7 transcription.

M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Fluorescent probing for RNA molecules by an unnatural base-pair system



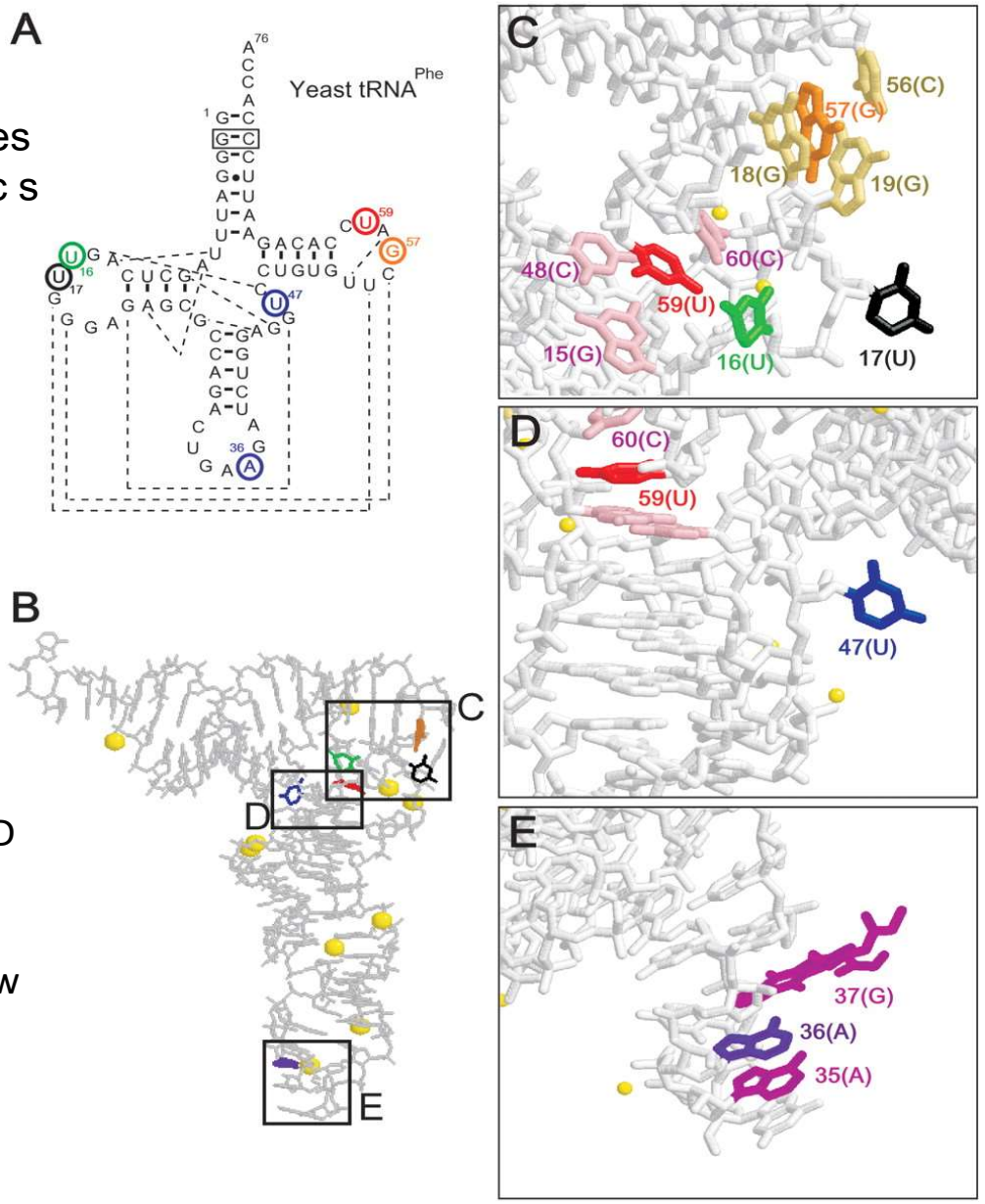


(B) Incorporation of the fluorescent **s** base into GNRA hairpins. (*left*) The secondary structure of the RNA hairpin with a GAAA loop. The second A and third A in the loop are shown in blue and red, respectively. *(right)* The 3D structure of the GAAA-loop hairpin

M. Kimoto, T. Mitsui, Y. Harada, A. Sato, S. Yokoyama and I. Hirao, *Nucleic Acids Res.*, **2007**, *35*, 5360–5369

The „s” incorporation sites in yeast tRNA^{Phe}

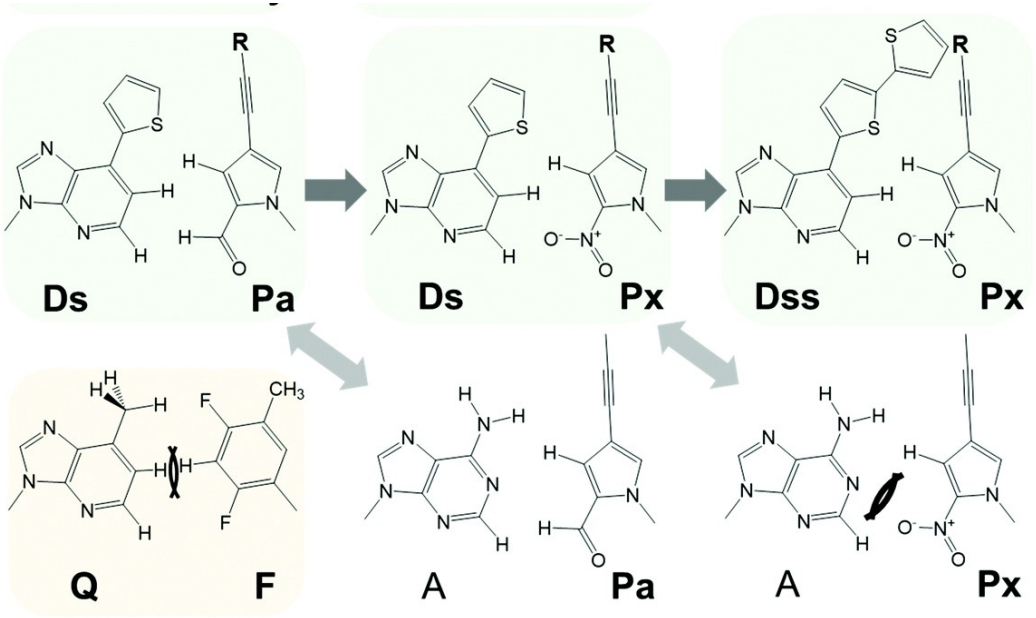
The fluorescent intensity of „s” in RNA molecules changes according to the structural environment. The site-specific s labeling of RNA hairpins and tRNA molecules provided characteristic fluorescent profiles, depending on the labeling sites, temperature and Mg_{2+} concentration.



(**A**) The secondary structure of the original tRNA transcript. The positions substituted with **s** are circled. The broken lines show base–base interactions for the 3D structure.
(**B**–**E**) The deep-colored bases were substituted with **s**, which stacks with the light-colored bases, and the yellow spheres represent Mg^{2+}.

M. Kimoto, T. Mitsui, Y. Harada, A. Sato, S. Yokoyama and I. Hirao, *Nucleic Acids Res.*, **2007**, *35*, 5360–5369

Steric exclusion and hydrophobic non-natural base pairs

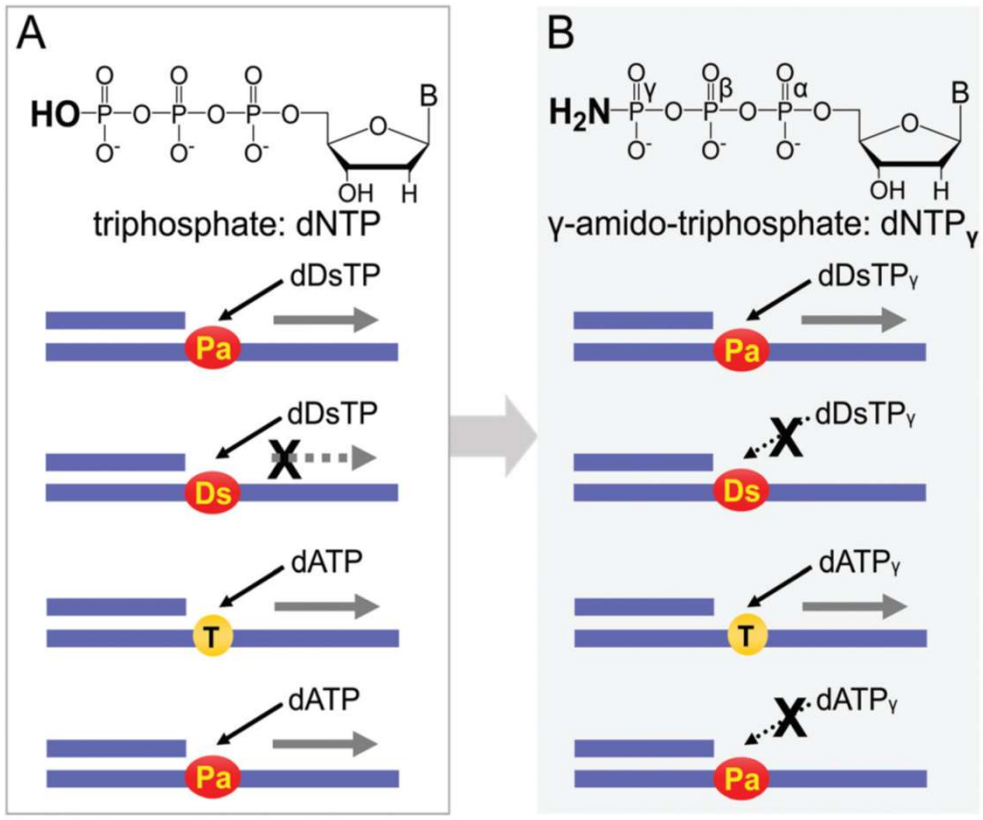


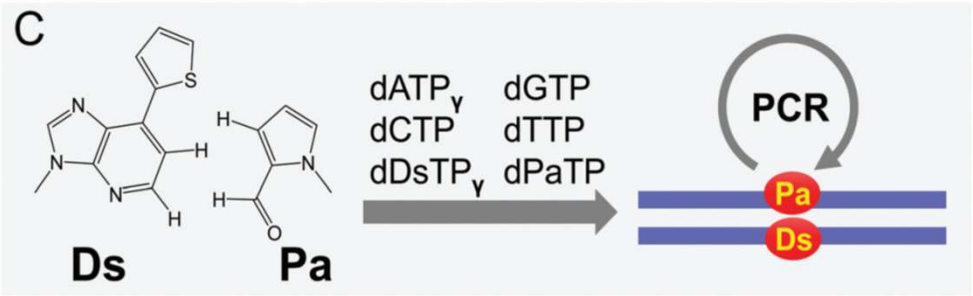
Hirao (2006) the hydrophobic **Ds** base (improved **s**). The **Ds–Pa** pair exhibited high incorporation efficiency in replication. **Problems**: the non-cognate Ds–Ds and A–Pa pairings (solved by using γ-amido-triphosphates of Ds and A, which significantly reduced their mispairing with Ds and Pa - ~99.9% selectivity per cycle in PCR).

Hirao (2007): the **Ds–Px** pair - electrostatic clash with the 1-nitrogen of A (no A–Px pairing), The additional propynyl group reduces the Ds–Ds self-pairing.
The Ds–Px pair exhibits high fidelity and high efficiency in PCR (>99.9% per cycle).

M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Replication of the hydrophobic UBP





The Ds–Pa pair for faithful replication in combination with γ-amidotriphosphates.

(A) The substrates, dDsTP and dATP, are incorporated opposite Pa and T in the templates, respectively. However, dDsTP and dATP are also misincorporated opposite Ds and Pa, respectively. The Ds incorporation opposite Ds inhibits further primer extension.

(B) The γ-amido-triphosphates, dDsTPγ and dATPγ, reduce the Ds–Ds and A–Pa mispairings in replication.

(C) The combination of the usual triphosphates (dG/C/T/PaTP) and the γ-amido-triphosphates (dDs/ATPγ) enables the faithful PCR amplification of 6-letter DNA

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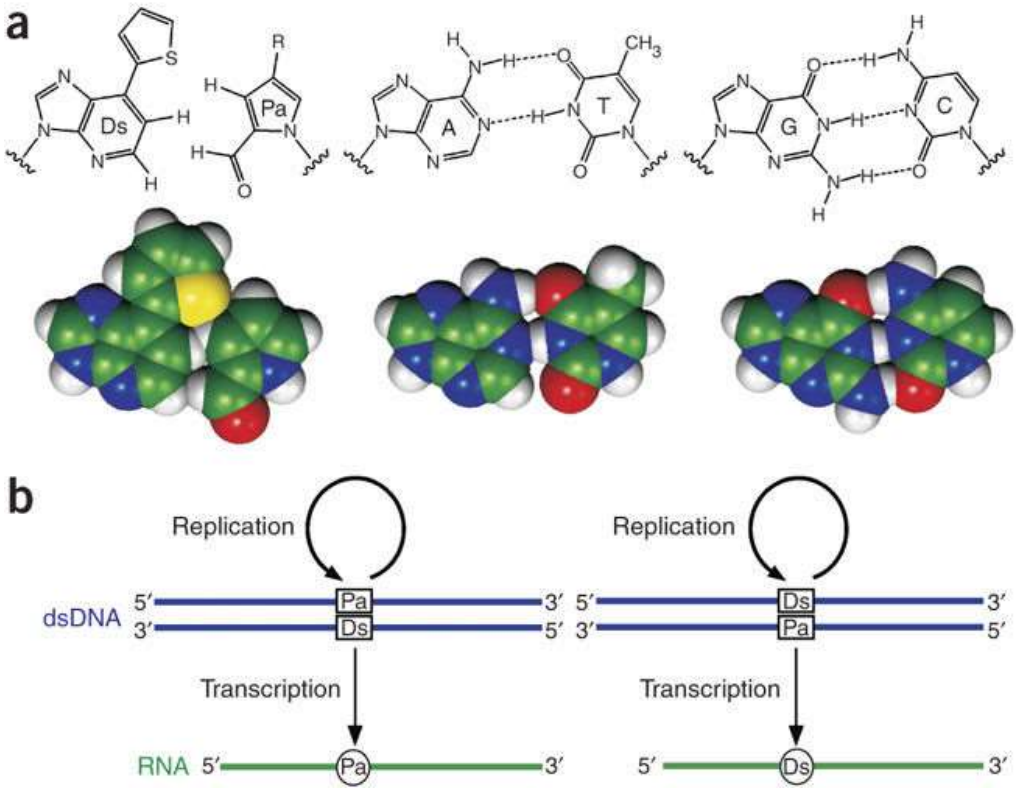
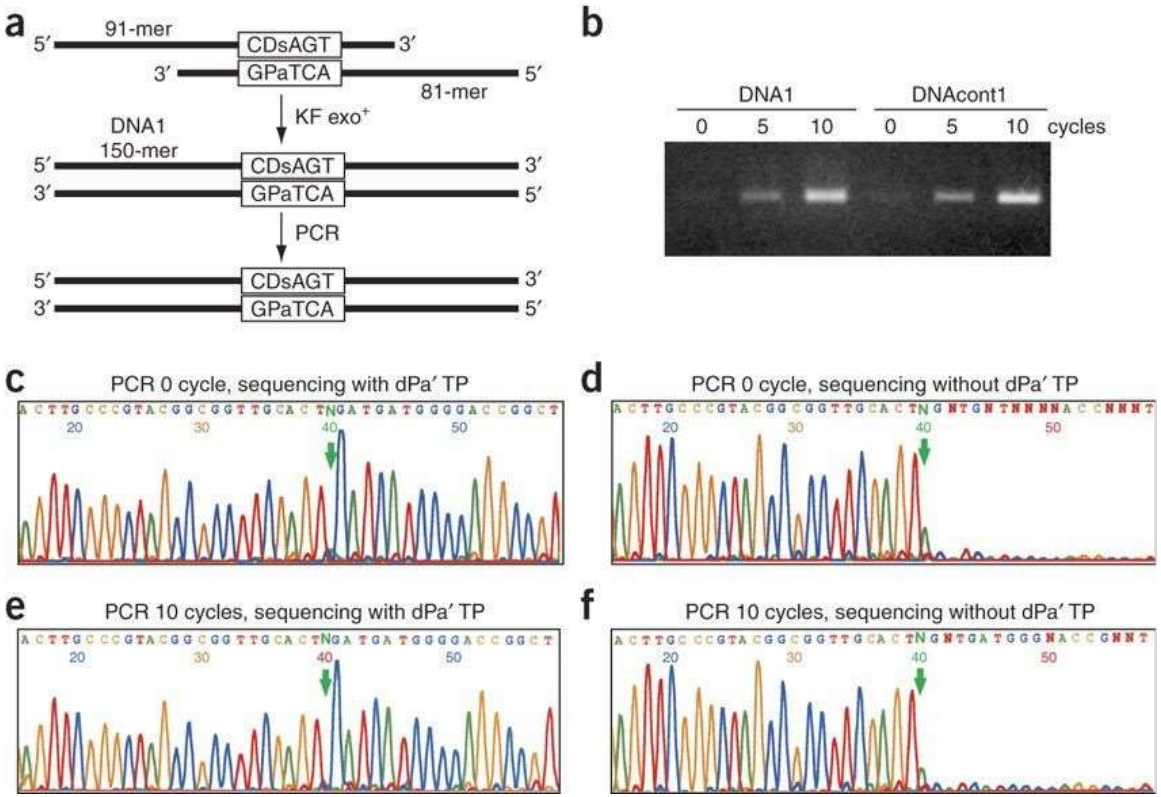
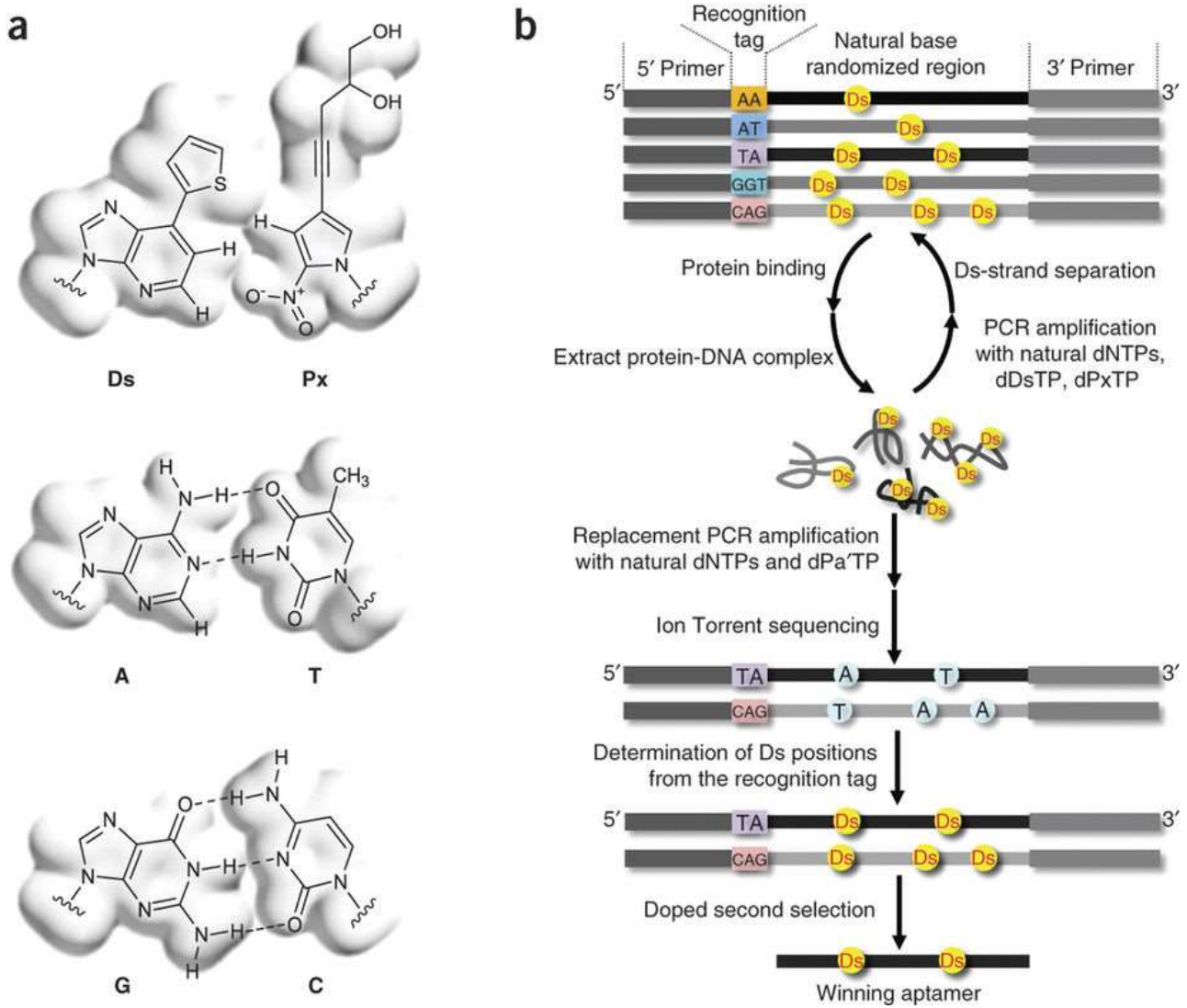
Replication of the hydrophobic UBP





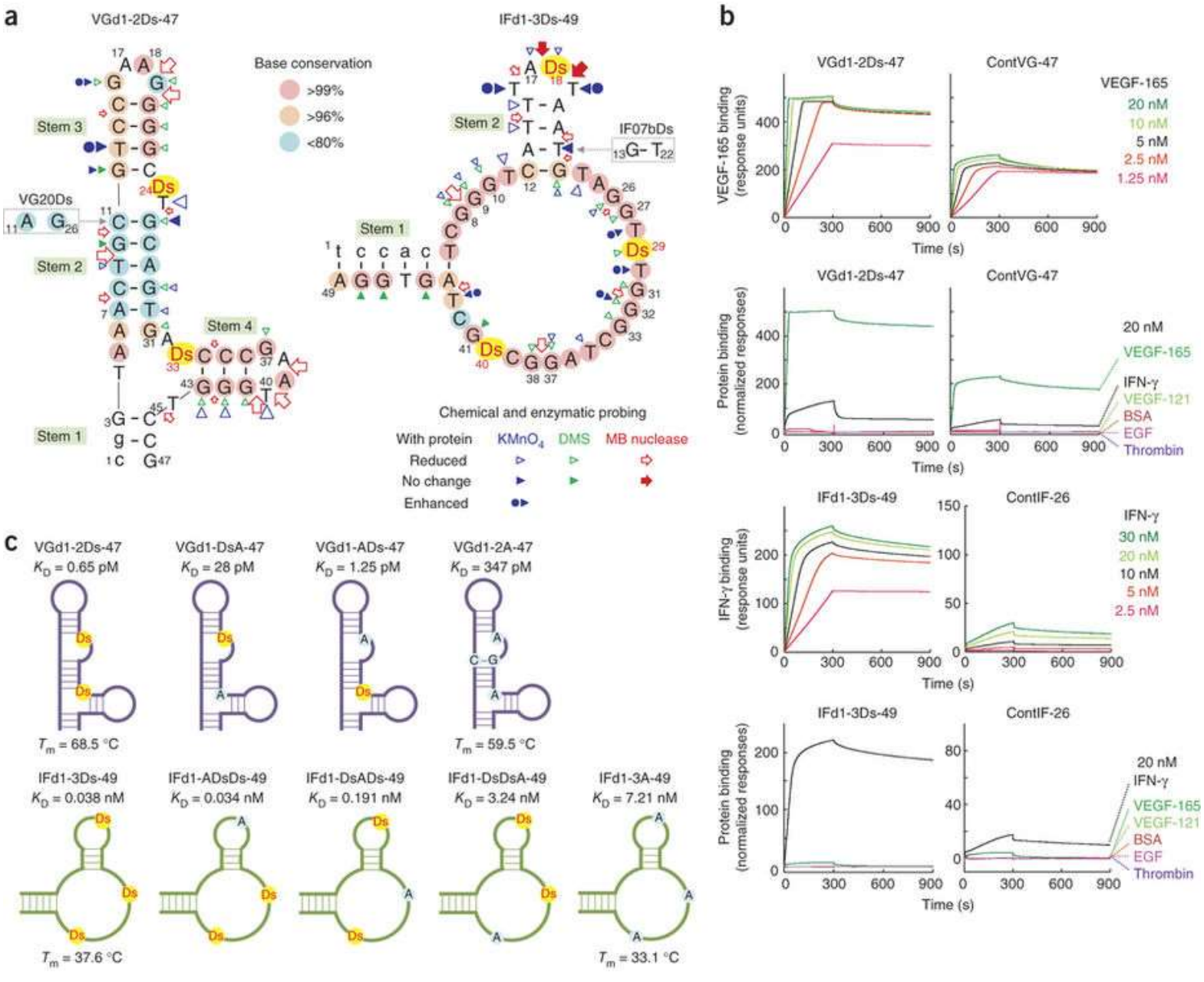
I. Hirao *et al. Nature Methods* **2006**, *3,* 729-735

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



Targets:
VEGF-165
and IFN-γ

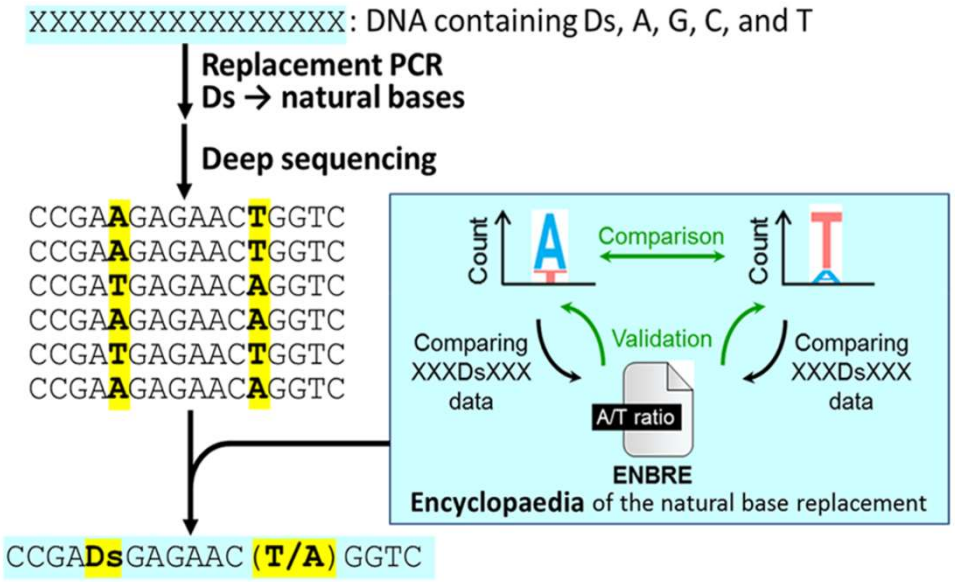
I. Hirao *et al. Nature Biotechnology* **2013**, *31,* 453-458

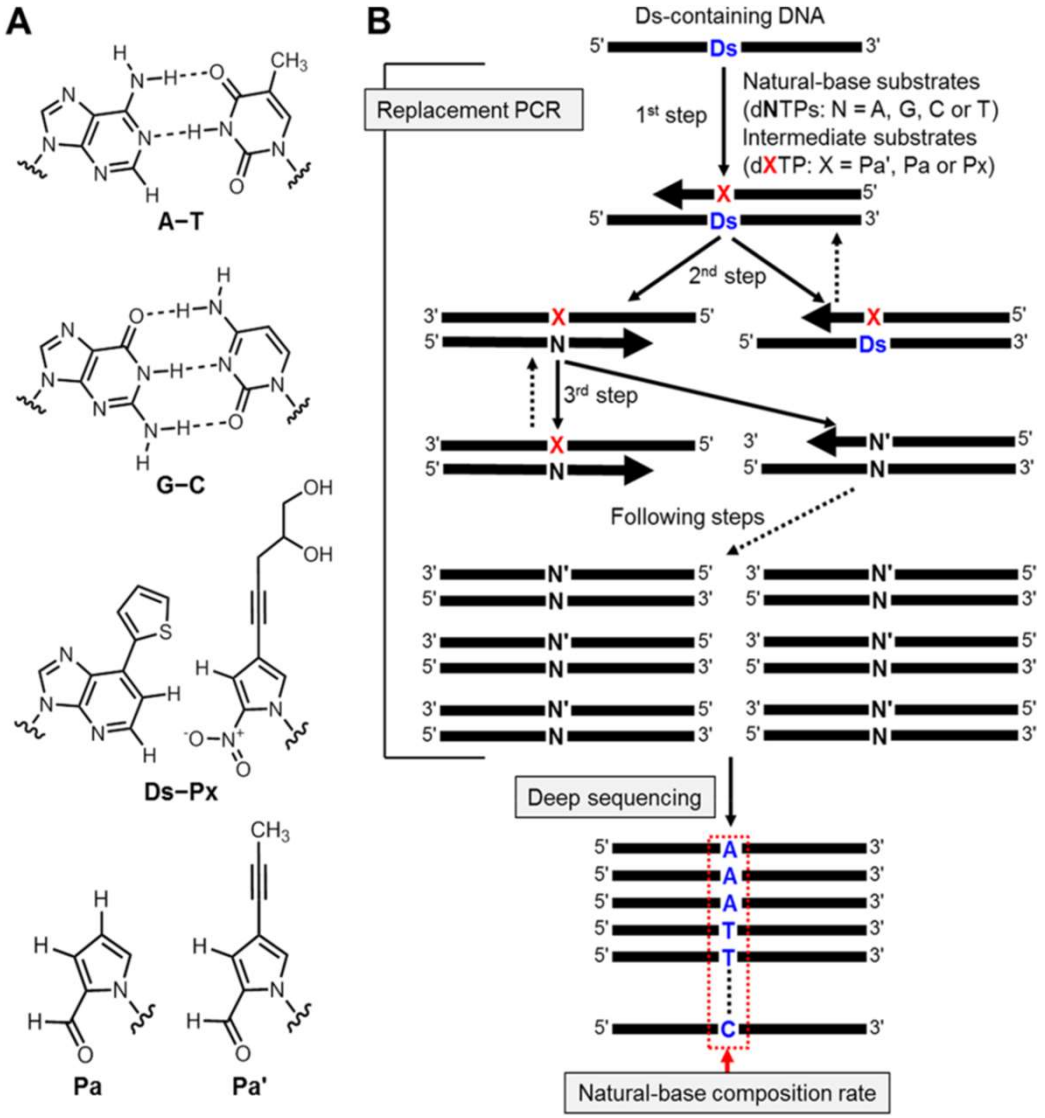


Characterizations and binding affinities of anti–VEGF-165 aptamer (VGd1-2Ds-47) and anti–IFN-γ aptamer (IFd1-3Ds-49)

I. Hirao *et al. Nature Biotechnology* **2013**, *31*, 453-458

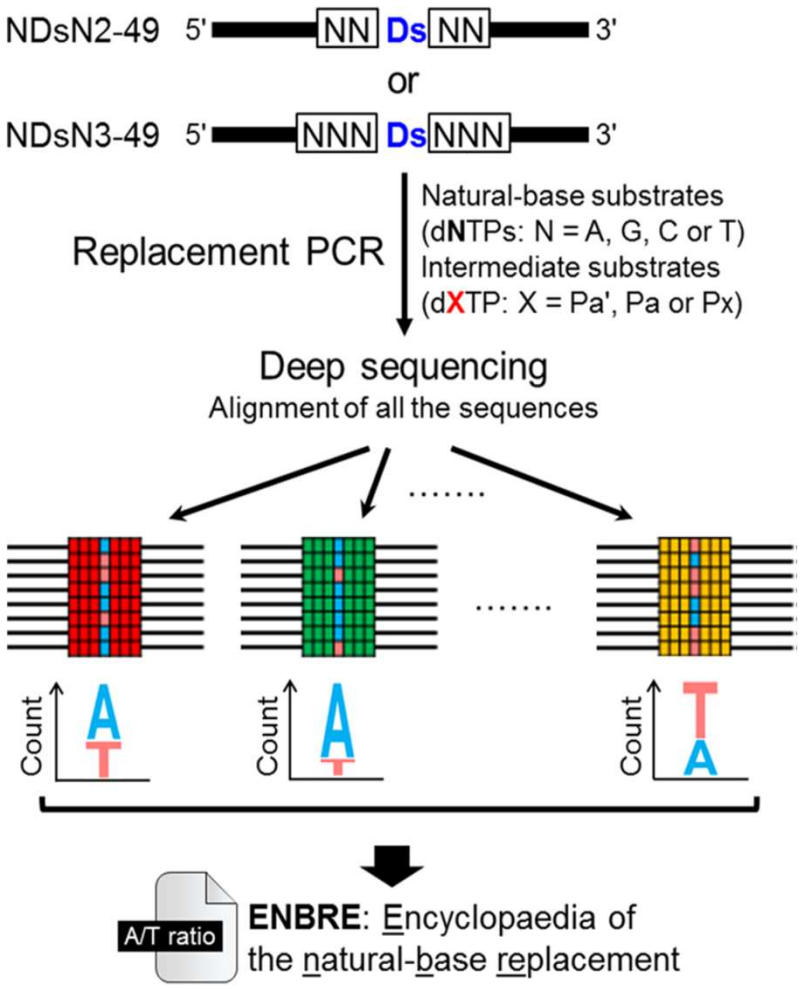
Ds-Pa – need for deep sequencing in the amplification process

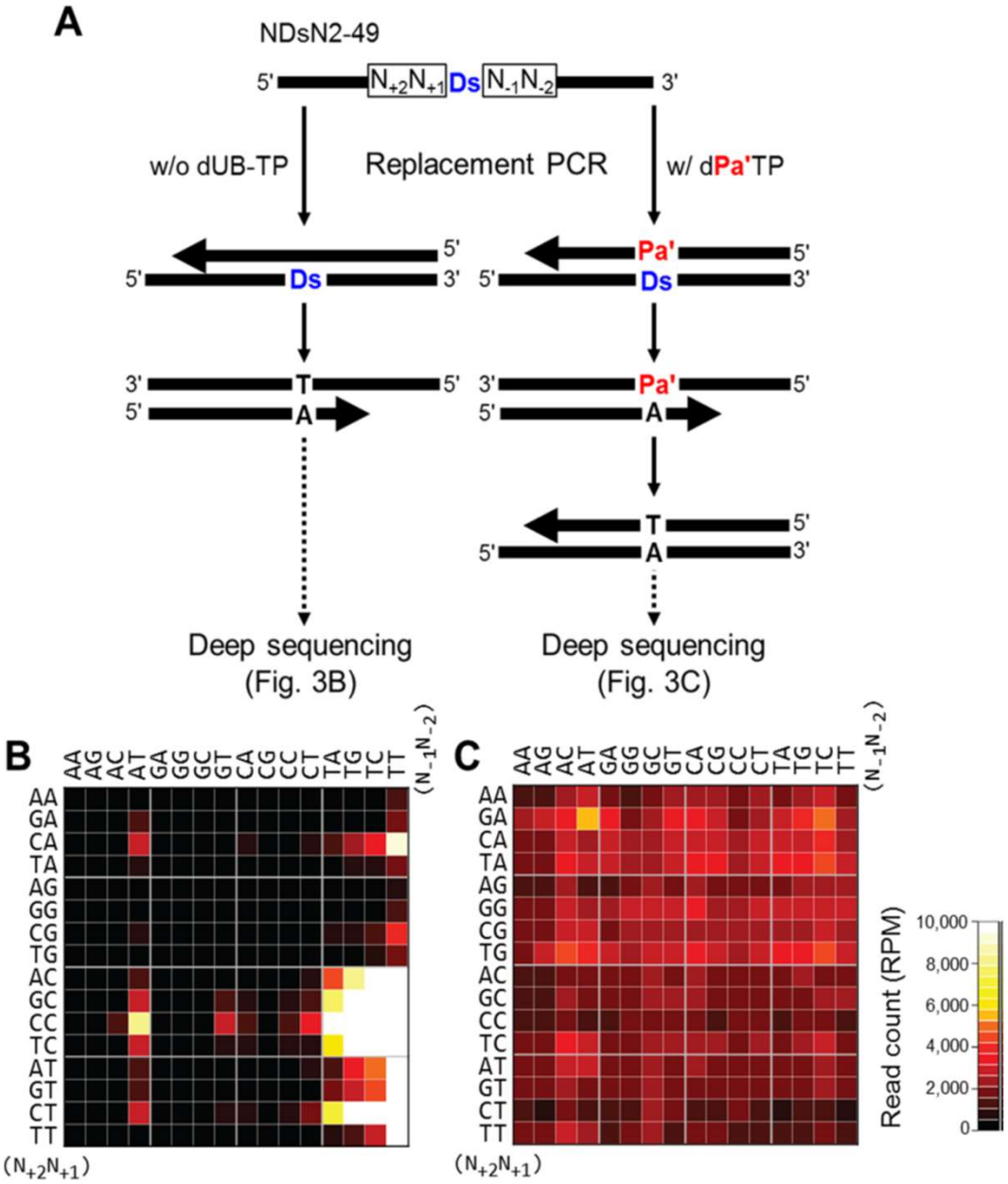




K. Hamashima, I. Hirao *et al. ACS Synth. Biol.* **2019**, *8*, 1401-1410

Ds-Pa – deep sequencing





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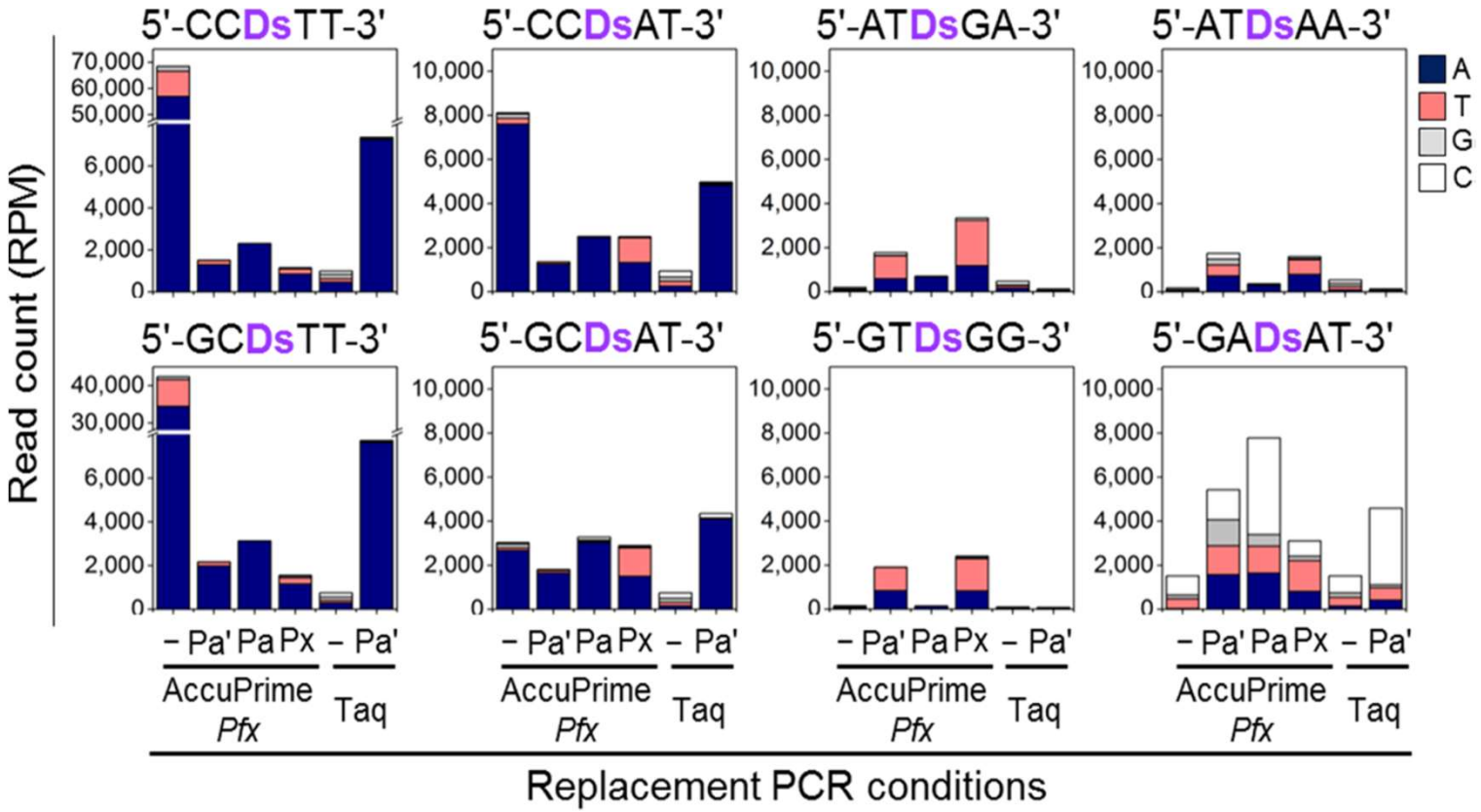
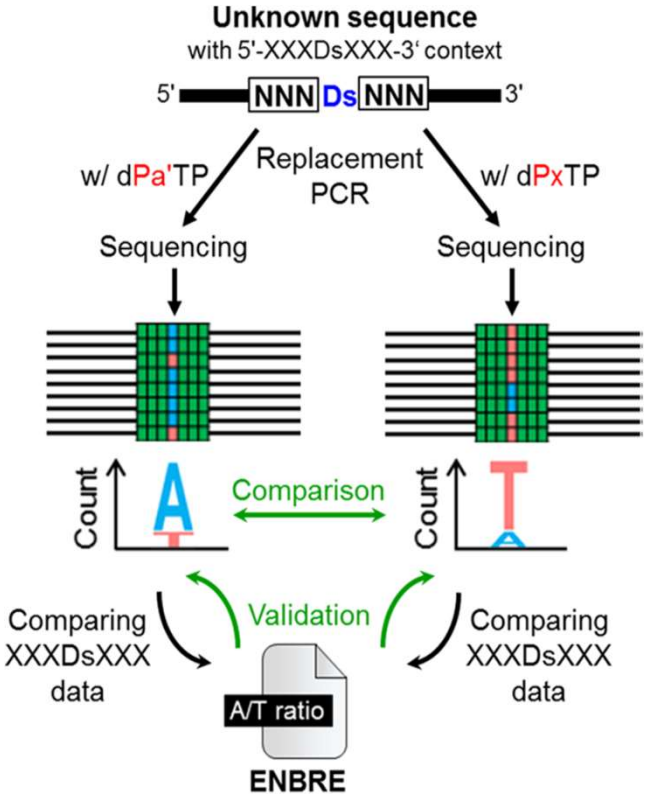
Ds-Pa – deep sequencing





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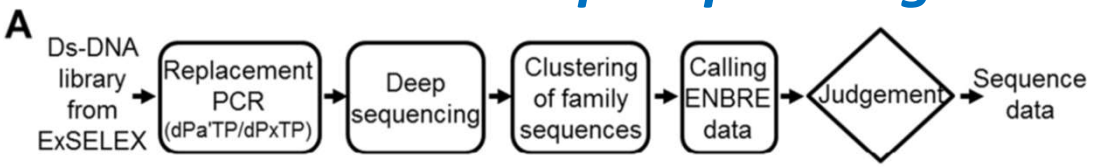
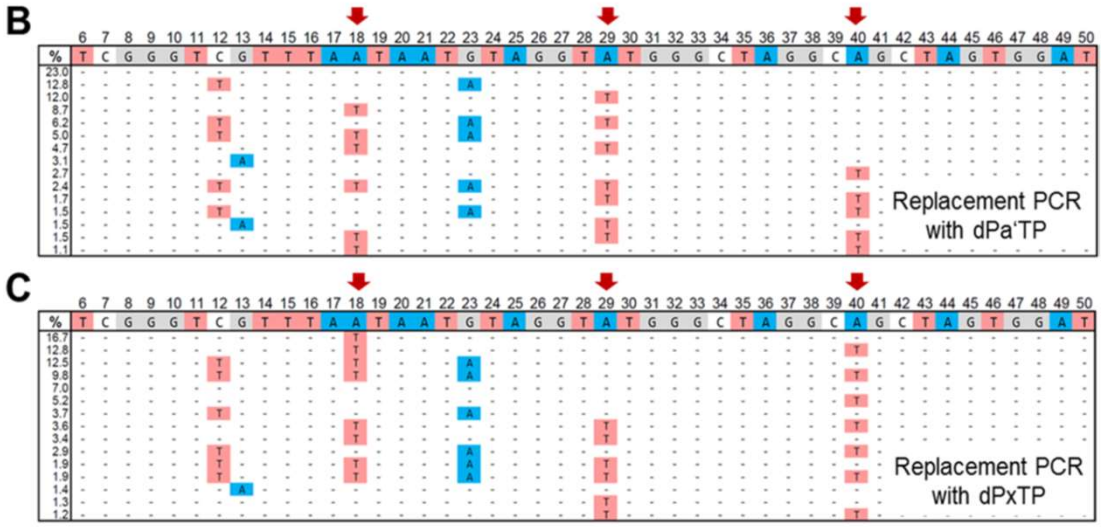
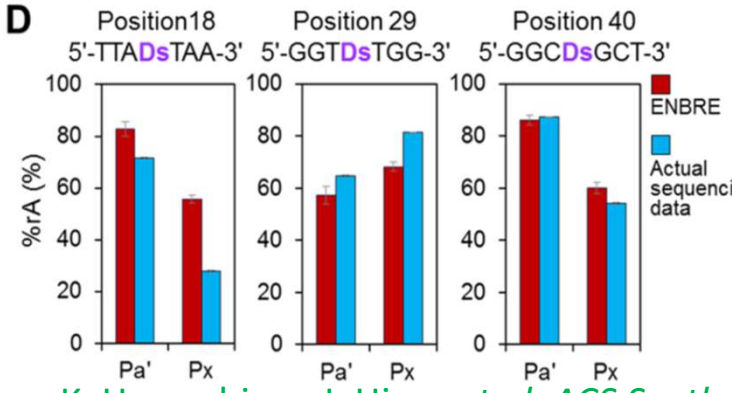
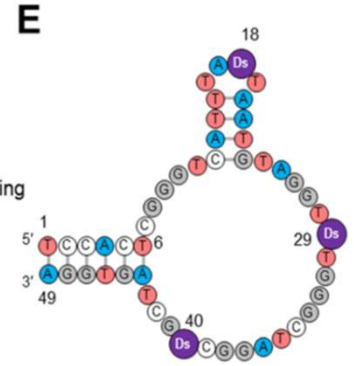
Ds-Pa – deep sequencing



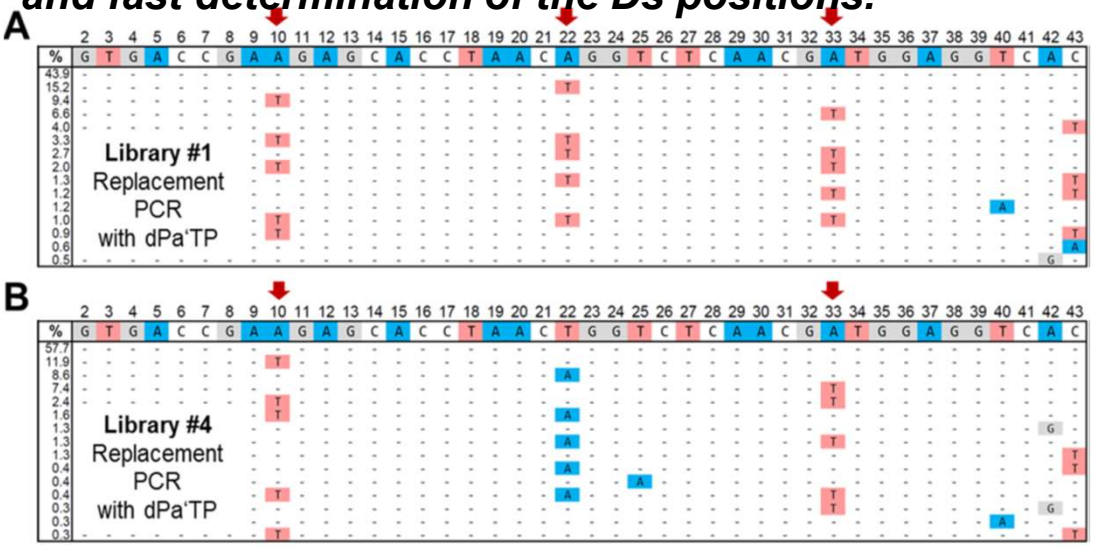


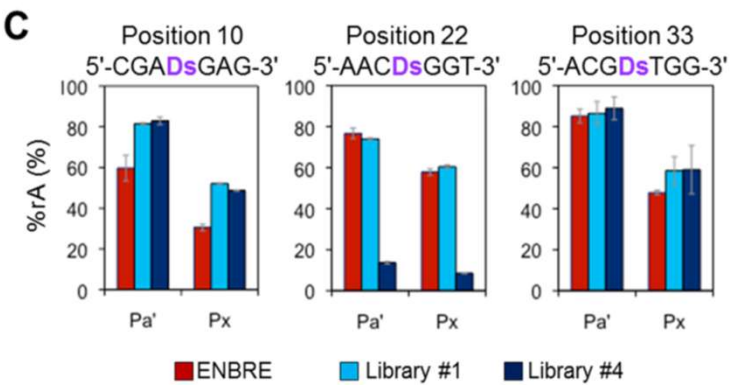


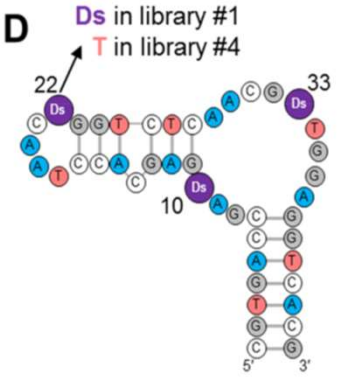


K. Hamashima, I. Hirao *et al. ACS Synth. Biol.* **2019**, *8*, 1401-1410

Referring to the encyclopedia data allows for simple and fast determination of the Ds positions.

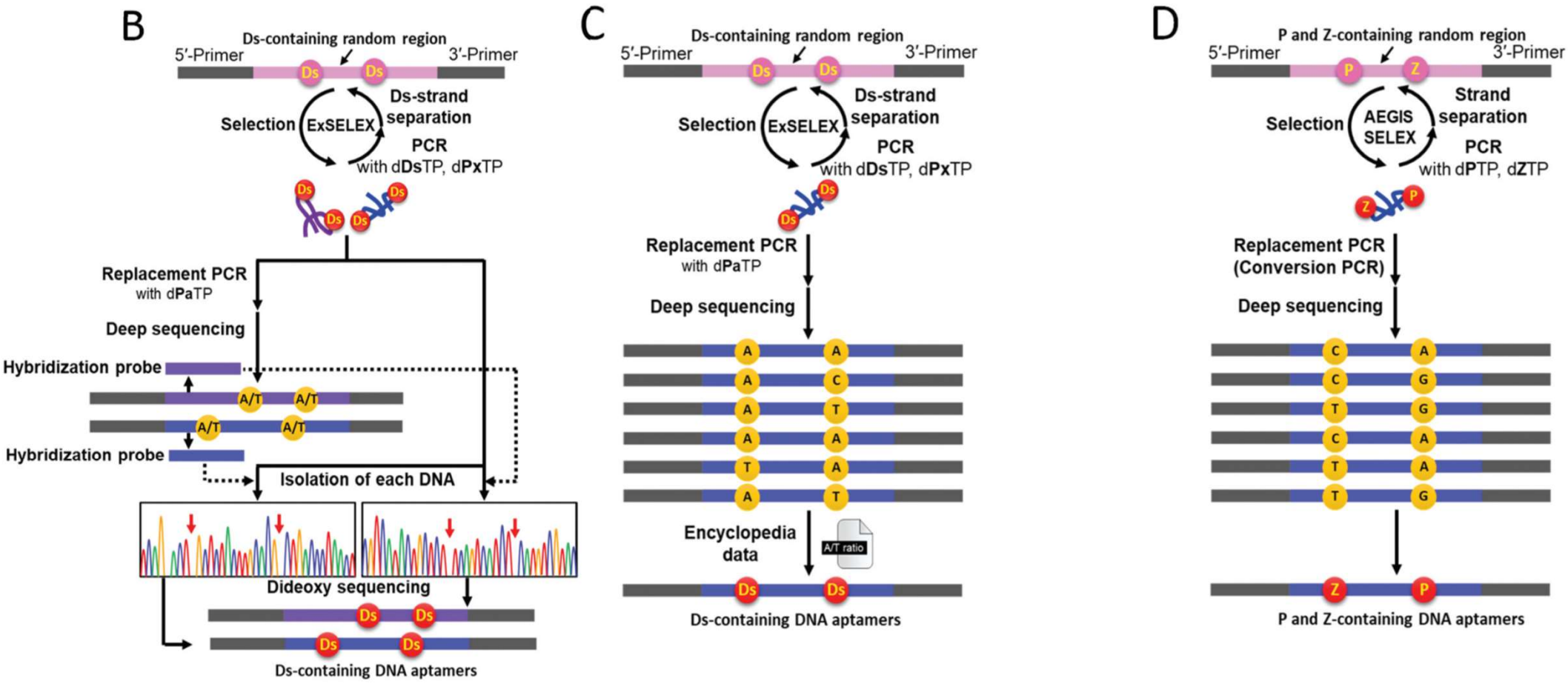






Comparison of the replacement patterns between two conditions enables the Ds positions to be distinguished from other natural-base positions.

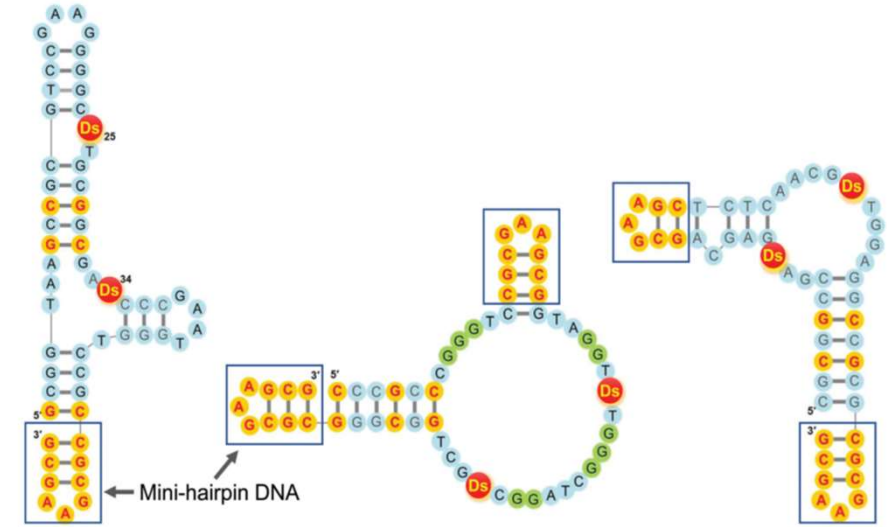
Ds-Pa aptamer selection using deep sequencing



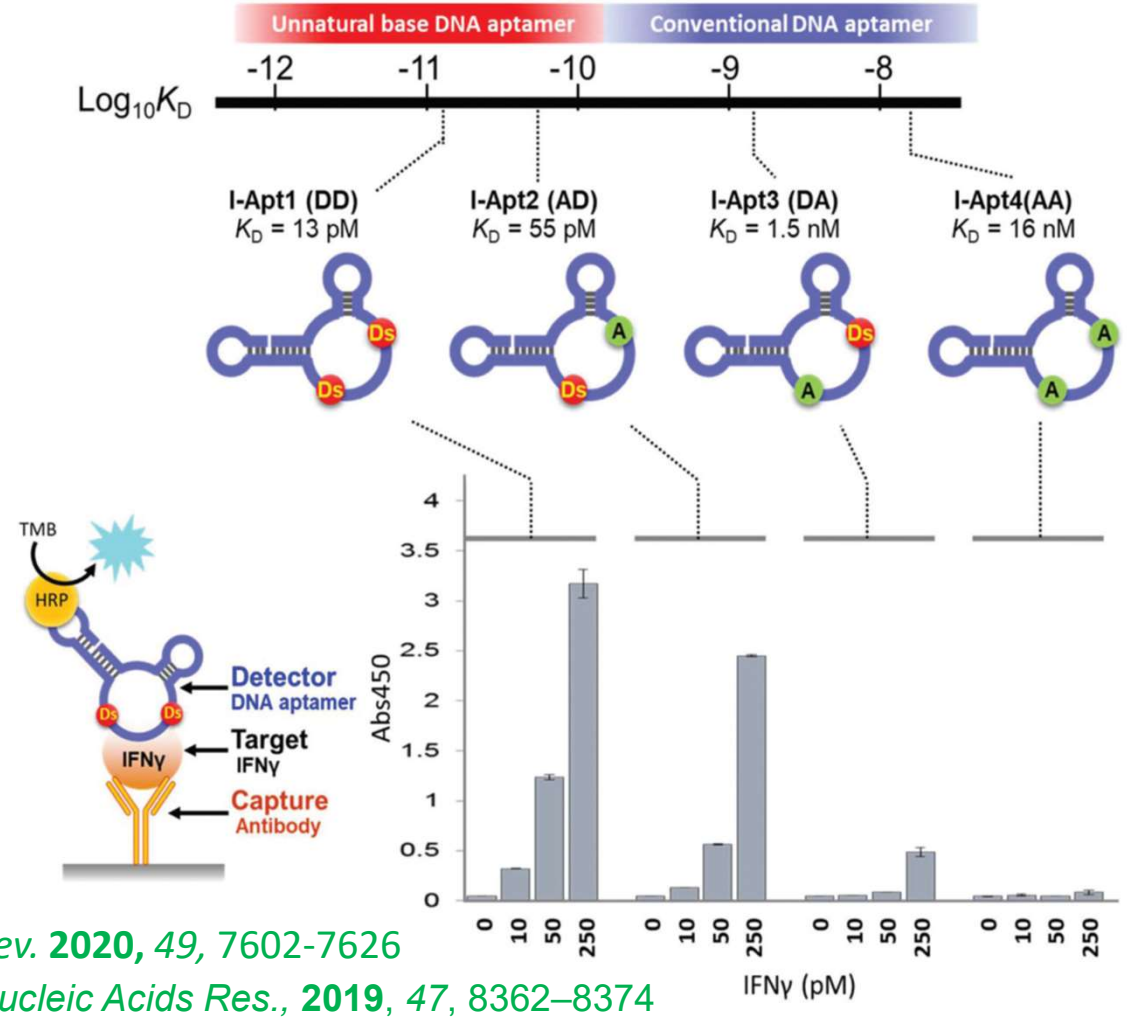
M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Ds-Pa aptamer selection using deep sequencing

Secondary structures of the optimized Ds-DNA aptamers targeting VEGF165, IFNγ, and vWF.



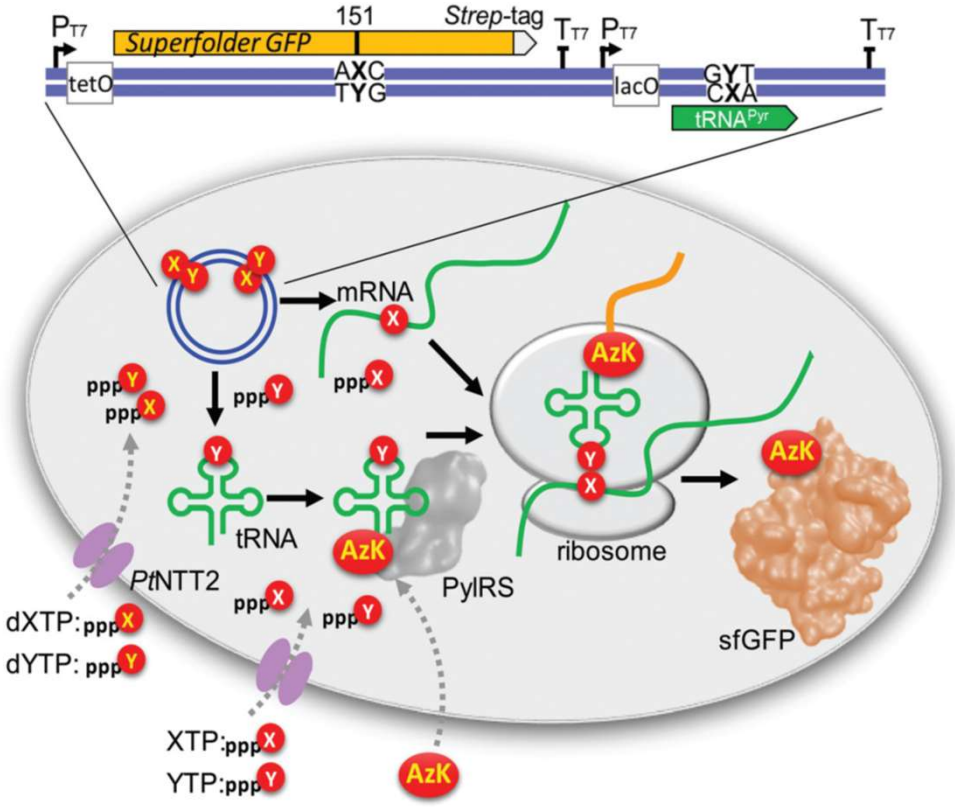
Generation of a molecular affinity ruler using anti-IFNγ Ds-DNA aptamer variants, replacing the Ds base with the natural A base.



M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

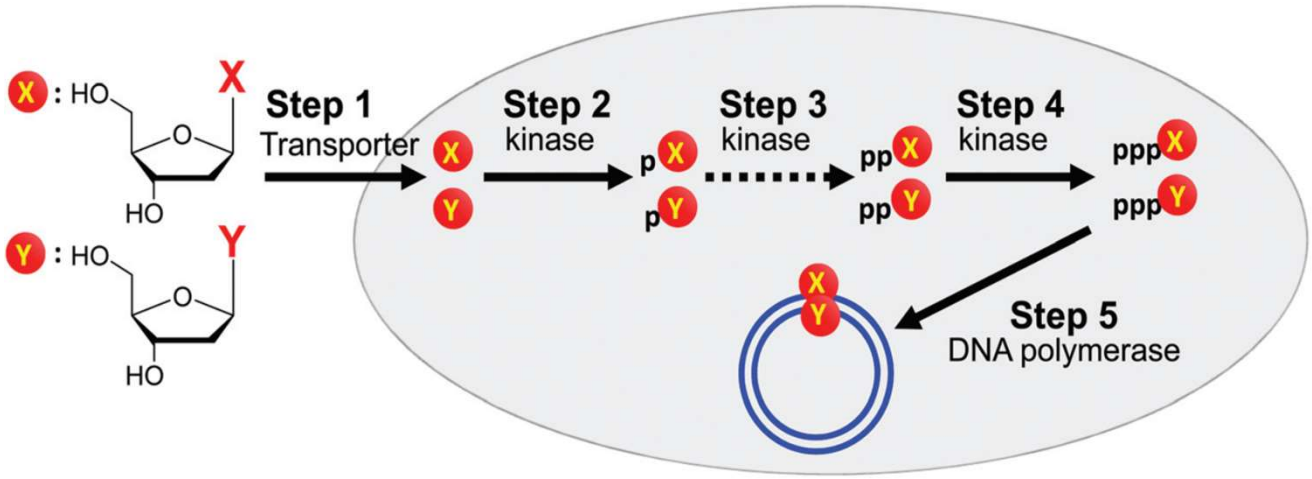
M. Kimoto, Y. W. S. Lim and I. Hirao, *Nucleic Acids Res.*, **2019**, *47*, 8362–8374

A semi-synthetic organism with an expanded genetic alphabet



A semi-synthetic organism with an expanded genetic alphabet

Nucleoside phosphorylation: a biosynthetic pathway to provide unnatural substrates (dX/dYTP) in a cell, using their unnatural nucleosides (dX/dY) as a source.



In Step 2, the nucleoside kinase from D. melanogaster (DmdNK) was explored for its ability to phosphorylate the unnatural nucleosides by three teams in vitro. Benner's team confirmed that the kinase in Step 4 was available for their Z and P bases in vitro. All UB triphosphates are, at least, accepted by the Klenow fragment of E. coli Pol I in vitro

M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

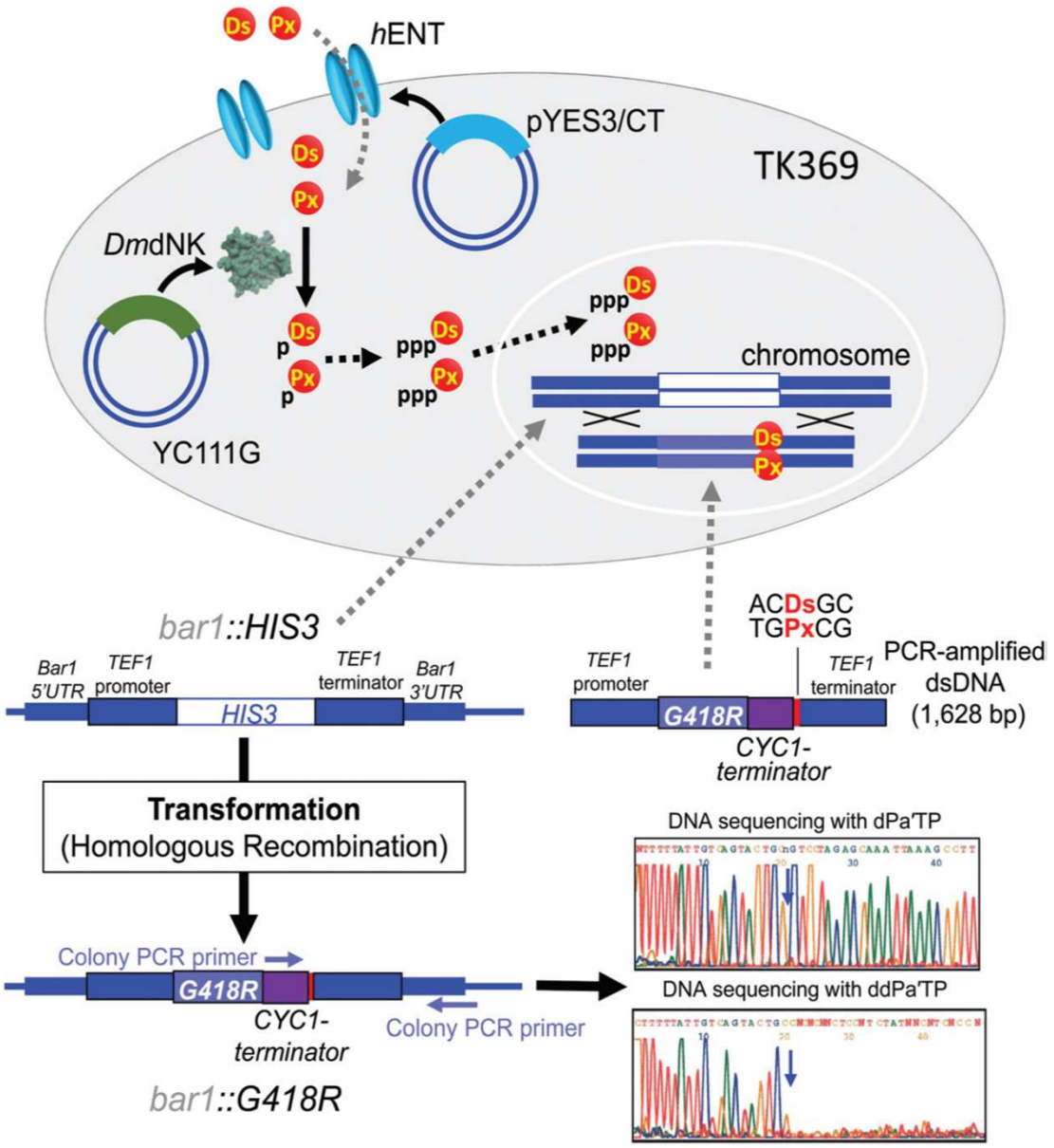
A semi-synthetic organism with an expanded genetic alphabet

Homologous recombination strategy using the yeast strain TK369, to integrate dsDNA with the Ds–Px pair.

DmdNK – nucleoside kinase
hENT - human equilibrium nucleoside transporter

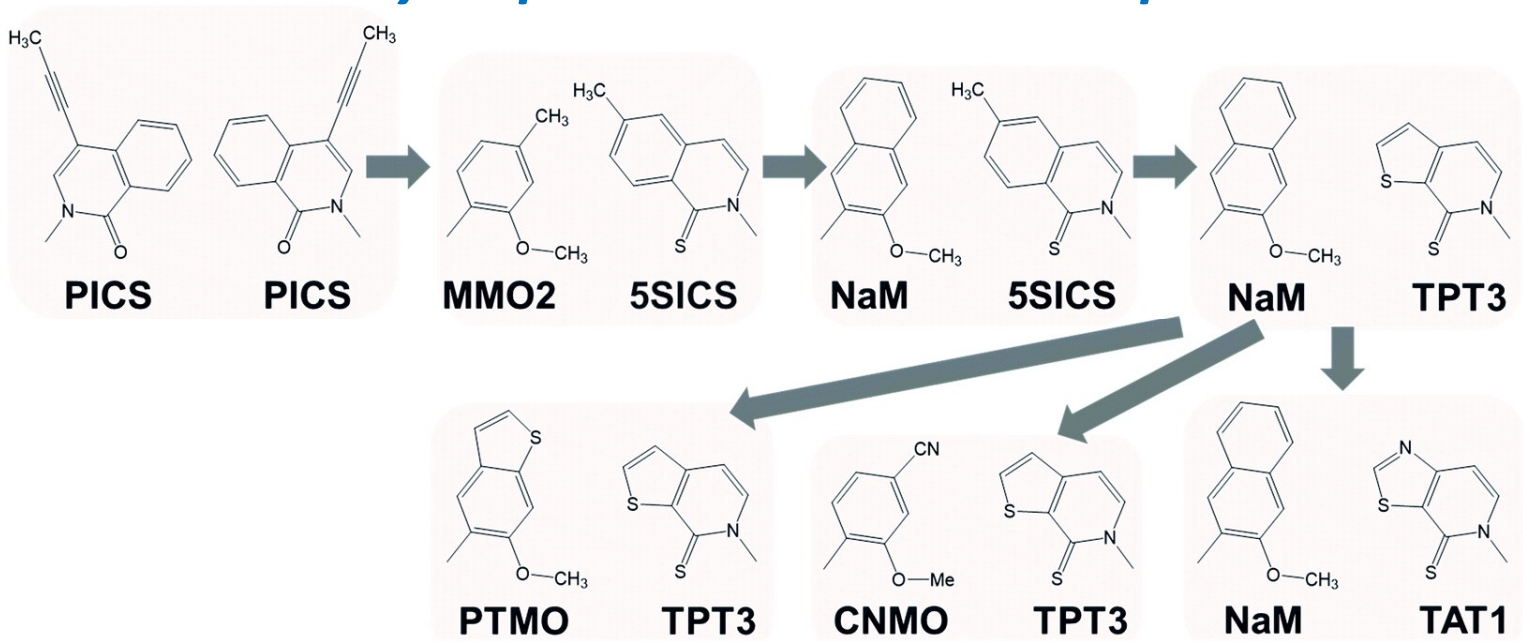
The Ds and Px nucleosides supplied in the media.
The long dsDNA with the Ds–Px pair was prepared by fusion/overlapping PCR,

Analysis: colony PCR with dDsTP and dPxTP, then modified Sanger sequencing.



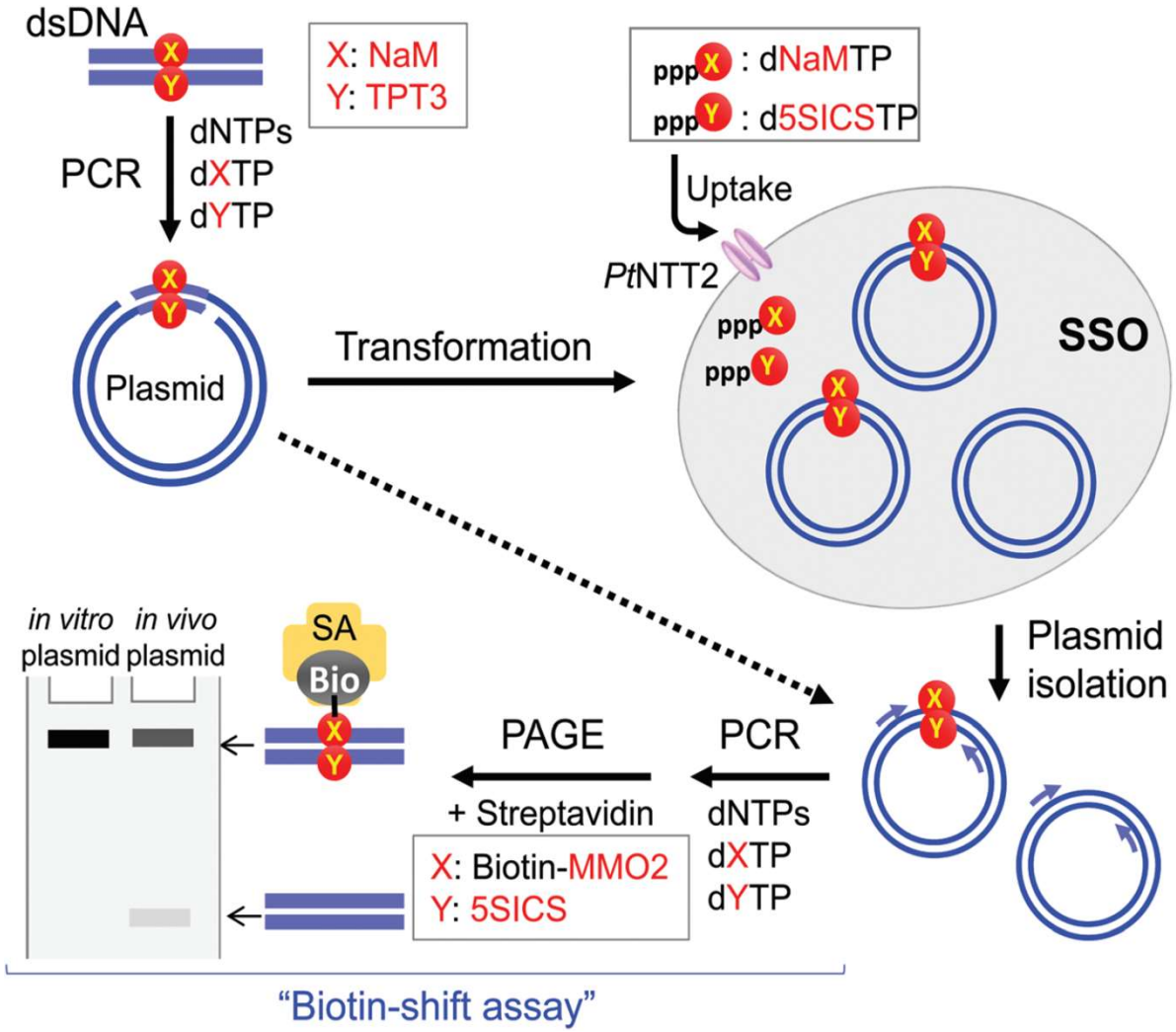
M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

Hydrophobic unnatural base pairs



Romesberg (1999): **PICS–PICS**, but the polymerase reaction is paused at the PICS–PICS pairing position due to self-stacking.
Romesberg (2008): the **MMO2–5SICS** pair - the methyl group of 5SICS prevented the disfavored 5SICS–5SICS self-pairing.
Further optimized into the **NaM–5SICS** pair (>99% fidelity in PCR and transcription).
Romesberg (2014) the **NaM–TPT3** pair (systematic *in vitro* replication screening) ➔ semi-synthetic organism (SSO) of *E. coli* with six-letter DNA.
Optimizations: the **CNMO–TPT3** pair to increase the UBP retention on a plasmid within the SSO,
the **NaM–TAT1** pair for efficient transcription and translation in the SSO.
Using these UBP systems, they reported protein synthesis with the site-specific incorporation of unnatural amino acids into proteins in the SSO.

A semi-synthetic organism with an expanded genetic alphabet



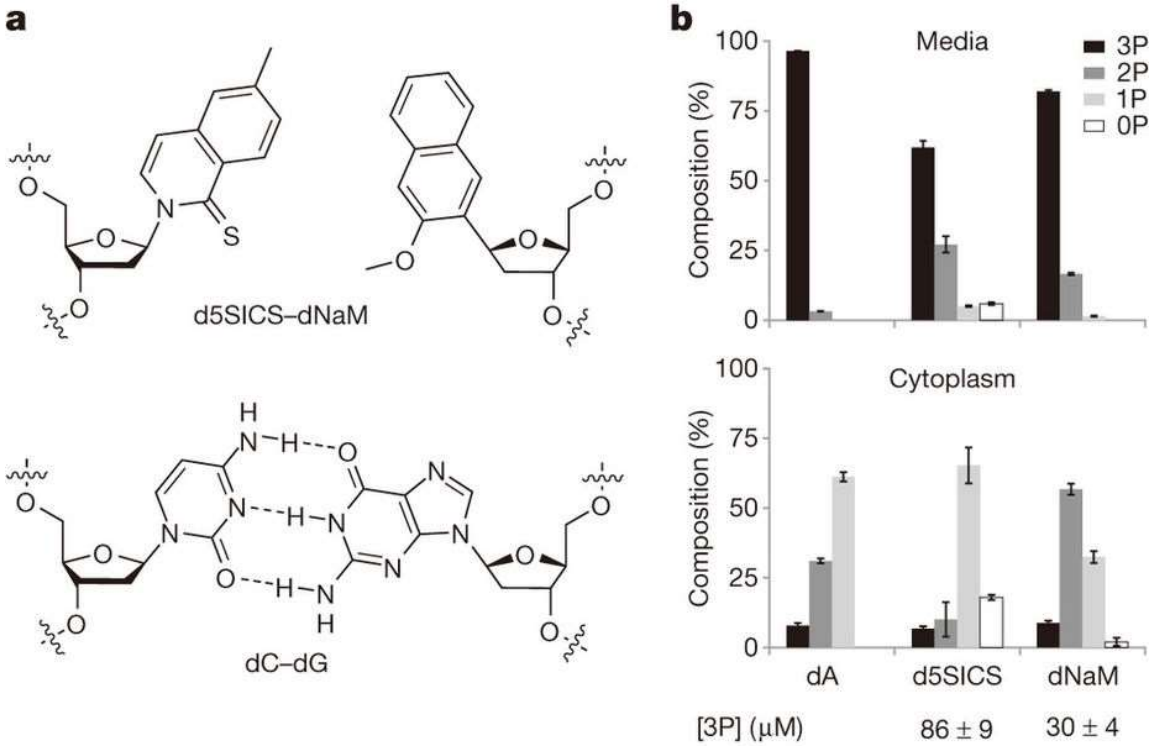
The first creation of an SSO, using the NaM–TPT3 and NaM–5SCIS pairs and E. coli C41(DE3) as the host strain.

The plasmid containing the NaM–TPT3 pair was prepared by PCR and transformed into E. coli C41(DE3) with a PtNTT2 overexpression system. The transformants were cultured in inorganic phosphate-rich growth media in the presence of the NaM and 5SCIS triphosphates.

The propagated plasmids were isolated and subjected to the analysis of the NaM–5SCIS pair retention by a biotin-shift assay

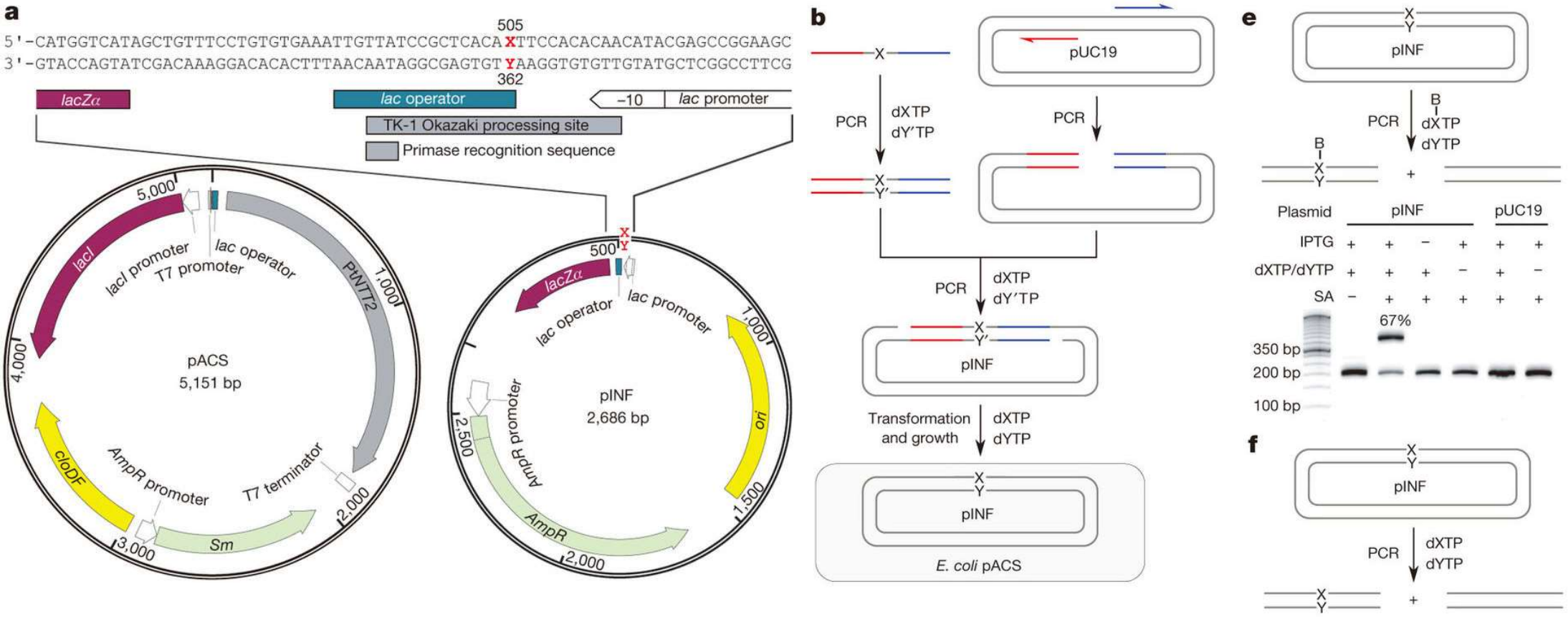
Malyshev, Denis A.; Romesberg, Floyd E. *et al.* *Nature* **2014**, *509,* 385–388

A semi-synthetic organism with an expanded genetic alphabet



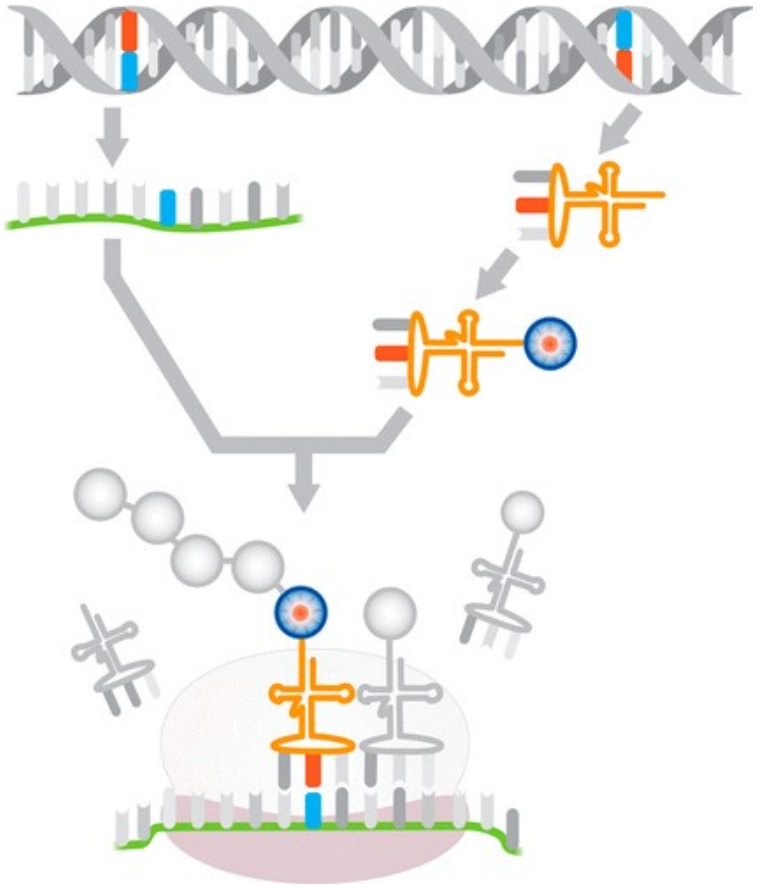
a, Chemical structure of the d5SICS–dNaM UBP compared to the natural dG–dC base pair. **b**, Composition analysis of d5SICS and dNaM in the media (top) and cytoplasmic (bottom) fractions of cells expressing *Pt*NTT2 after 30 min incubation; dA shown for comparison. 3P, 2P, 1P and 0P correspond to triphosphate,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



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

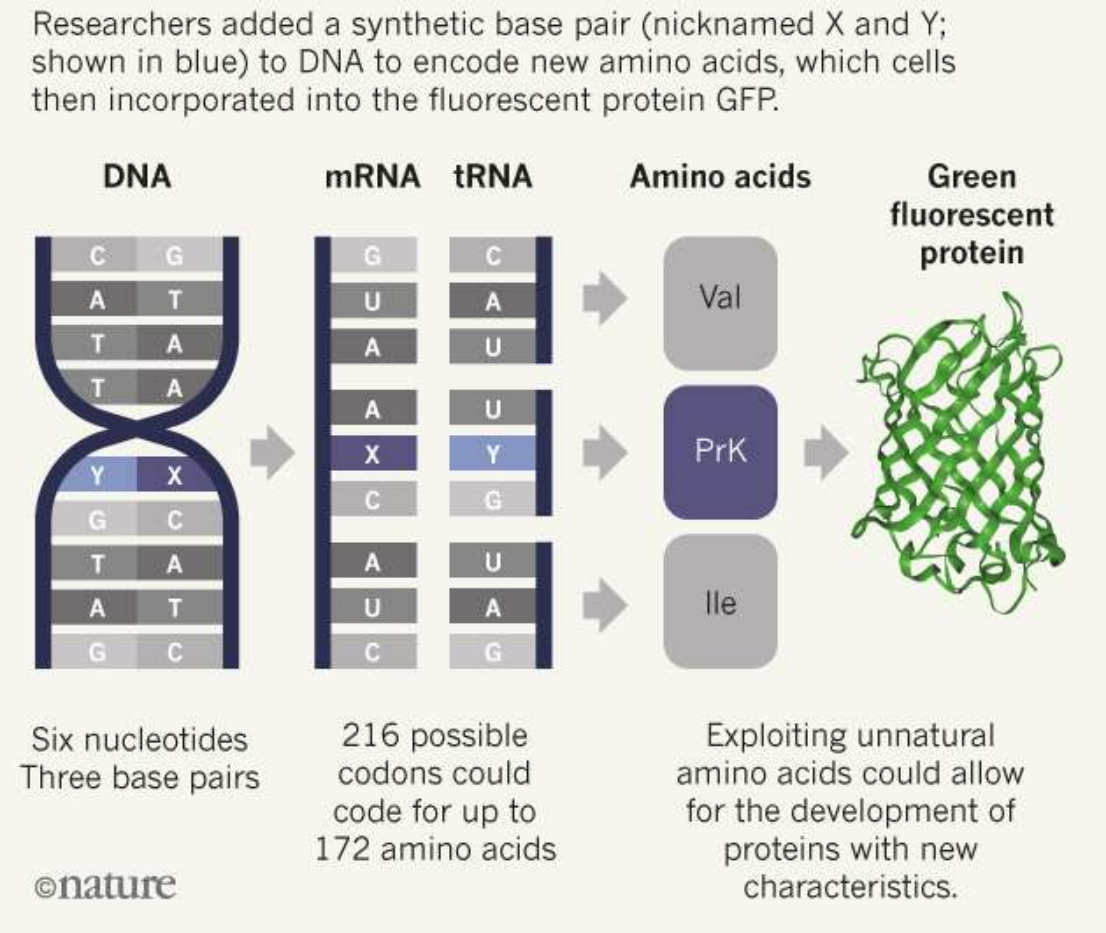
A semi-synthetic organism with an expanded genetic alphabet



The SSO replicates DNA containing the d**NaM**-d**TPT3** UBP (blue and red, respectively), transcribes mRNA and tRNA with complementary codons and anticodons containing **NaM** or **TPT3**, uses an orthogonal synthetase to charge the tRNA with an ncAA, and uses the charged tRNA to translate the mRNA into proteins containing ncAAs

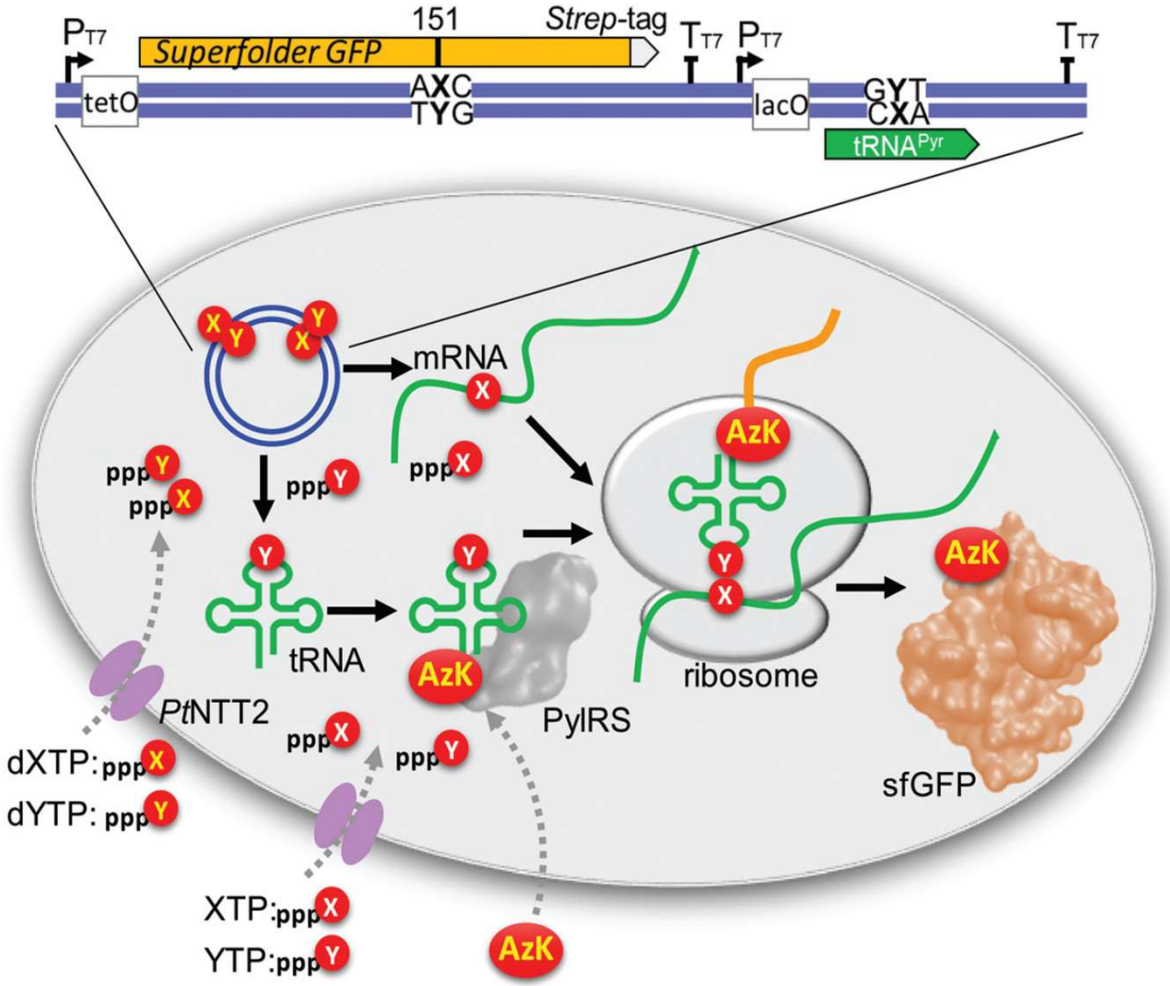
Y. Zhang, F. Romesberg, *Biochemistry* **2018**, *57, 15*, 2177–2178

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

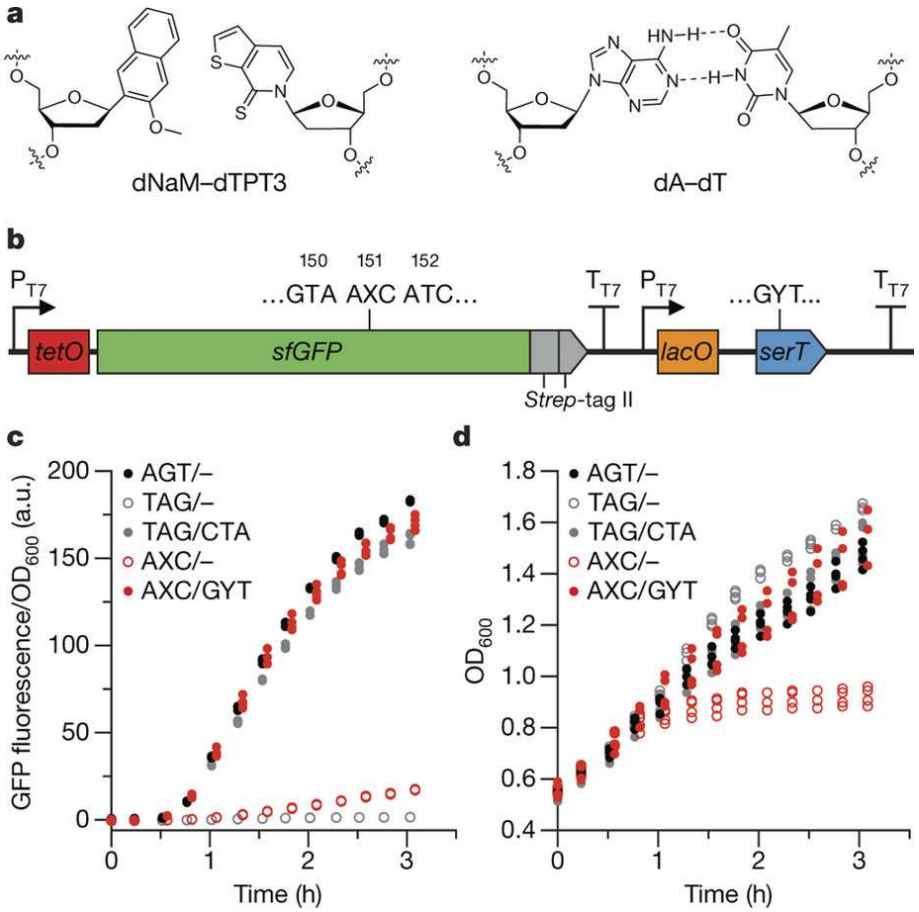


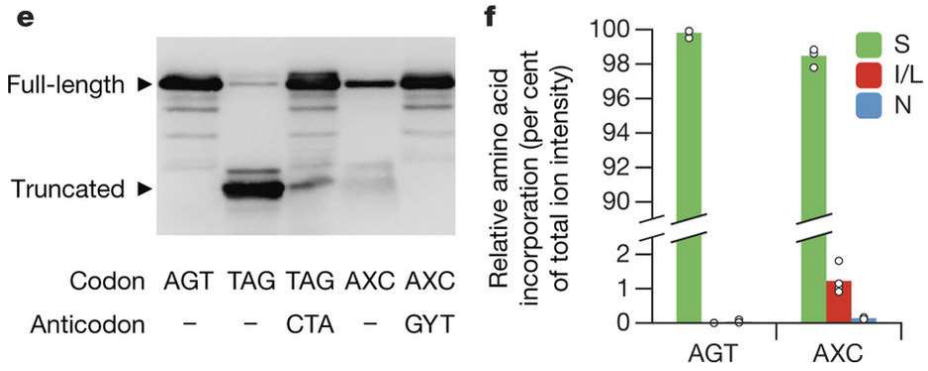
Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551,* 644-647

M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

A semi-synthetic organism with an expanded genetic alphabet

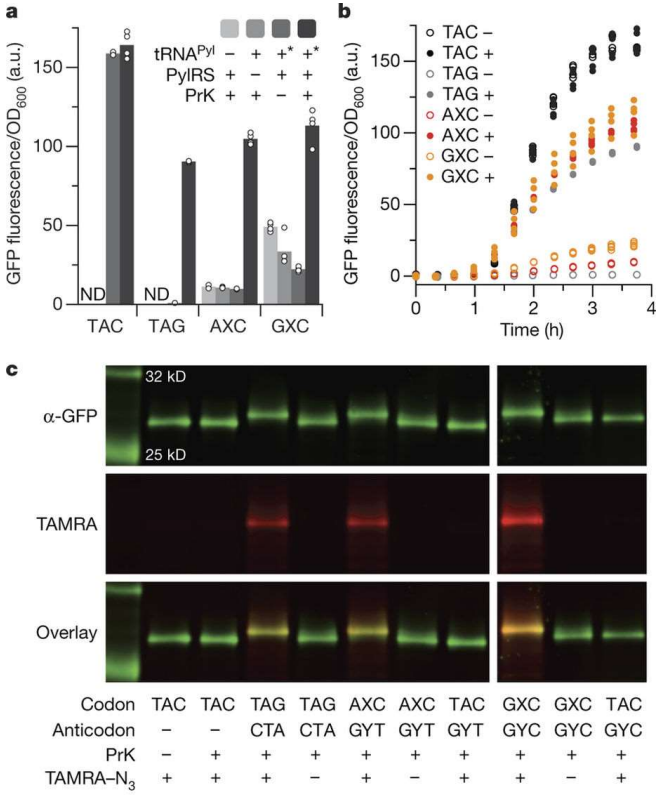
The *in vivo* transcription of DNA containing dNaM and dTPT3 into mRNAs with two different unnatural codons and tRNAs with cognate unnatural anticodons, and their efficient decoding at the ribosome to direct the site-specific incorporation of natural or non-canonical amino acids into superfolder green 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.

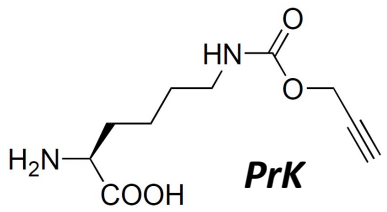




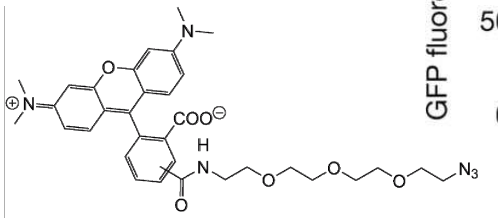
Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551*, 644-647

A semi-synthetic organism with an expanded genetic alphabet

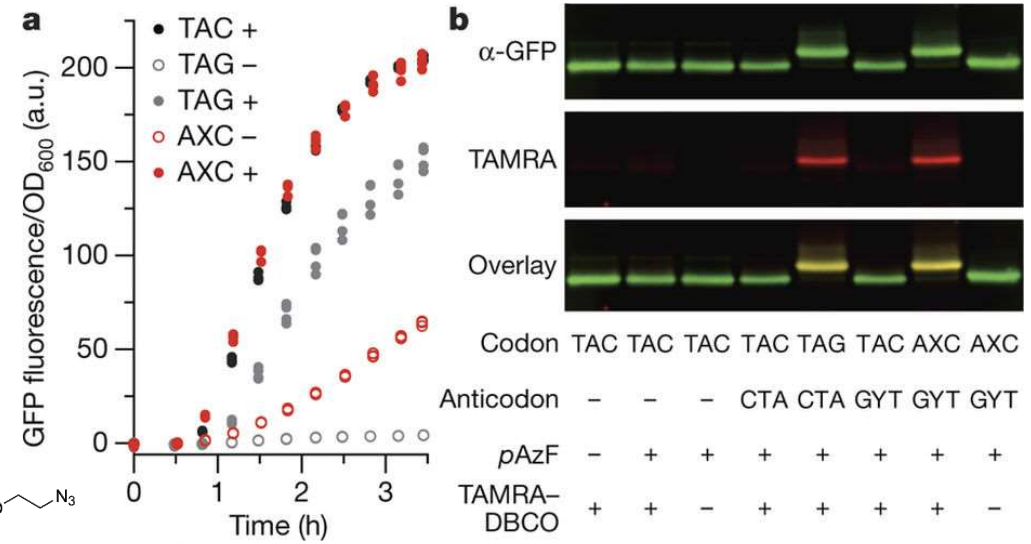


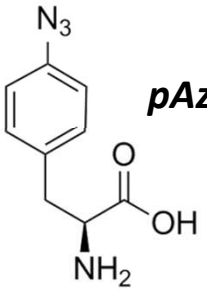


PrK



TAMRA-N_3





pAzF

TAMRA-DBCO

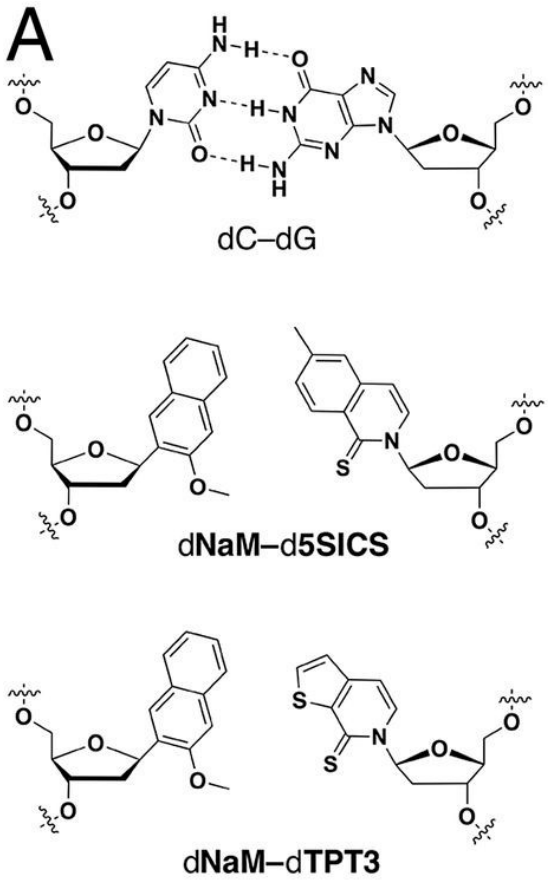
Zhang, Y.; Romesberg, Floyd E. *et al.* *Nature* **2017**, *551*, 644-647

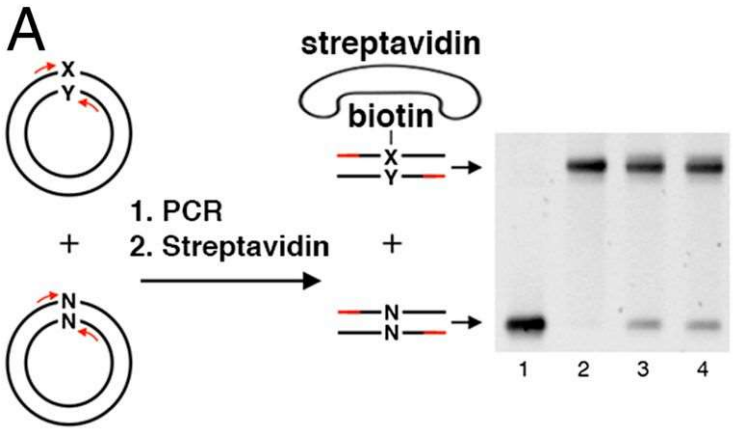
A semi-synthetic organism with an expanded genetic alphabet

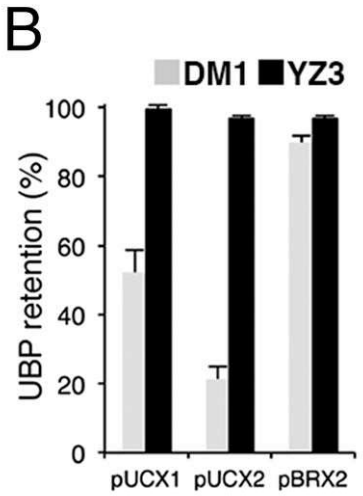
- An unnatural base pair (UBP) would increase the information storage potential of DNA
- and semisynthetic organisms (SSOs) that stably harbor this expanded alphabet would thereby have the potential to store and retrieve increased information,
- *Escherichia coli* grown in the presence of the unnatural 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

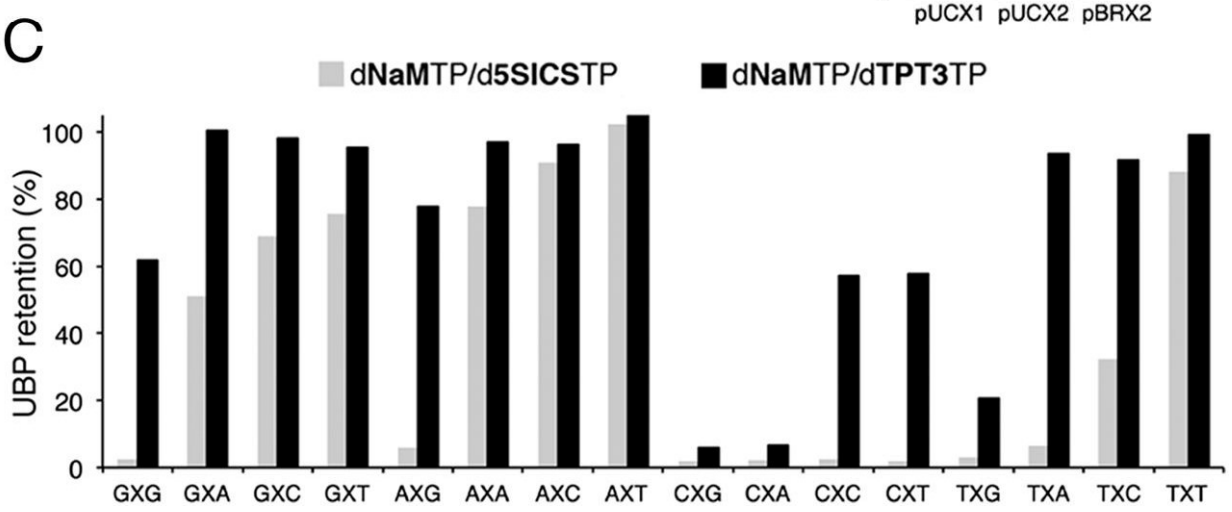
Romesberg, Floyd E. *et al.* *PNAS* **2017**, *114,* 1317-1322

A semi-synthetic organism with an expanded genetic alphabet



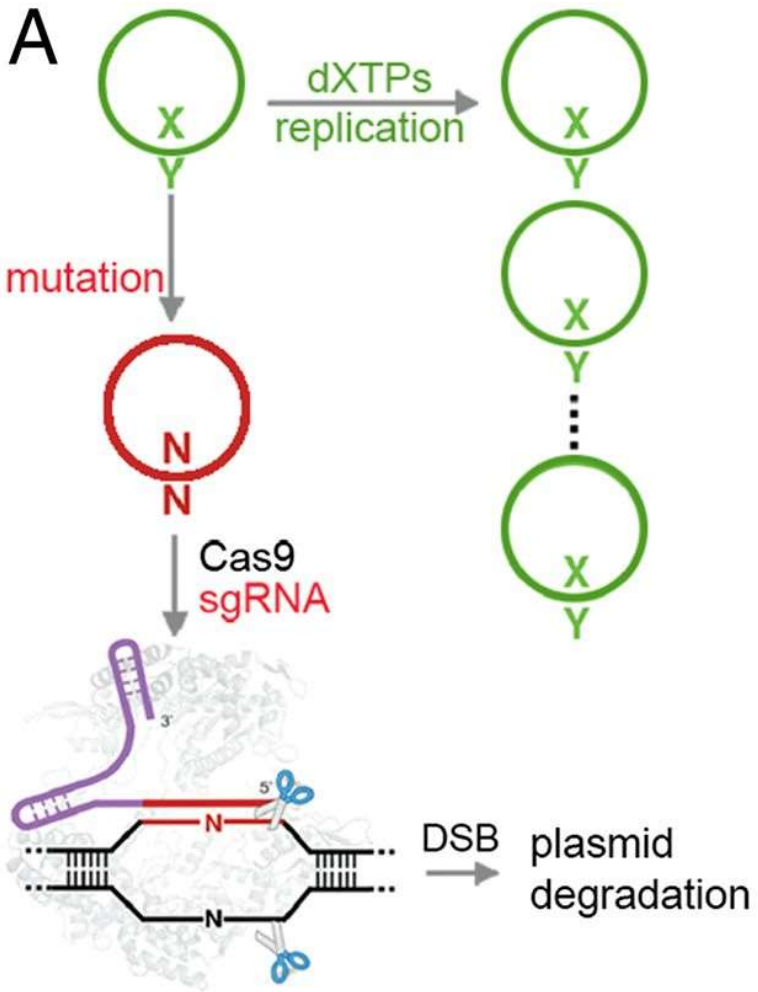


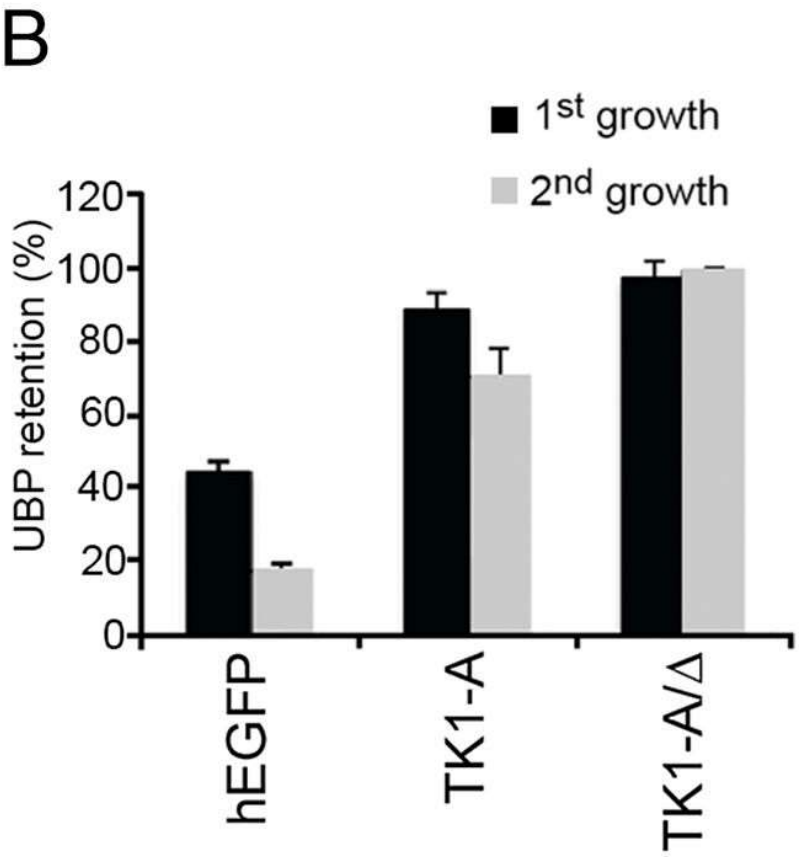




Romesberg, Floyd E. *et al.* *PNAS* **2017**, *114,* 1317-1322

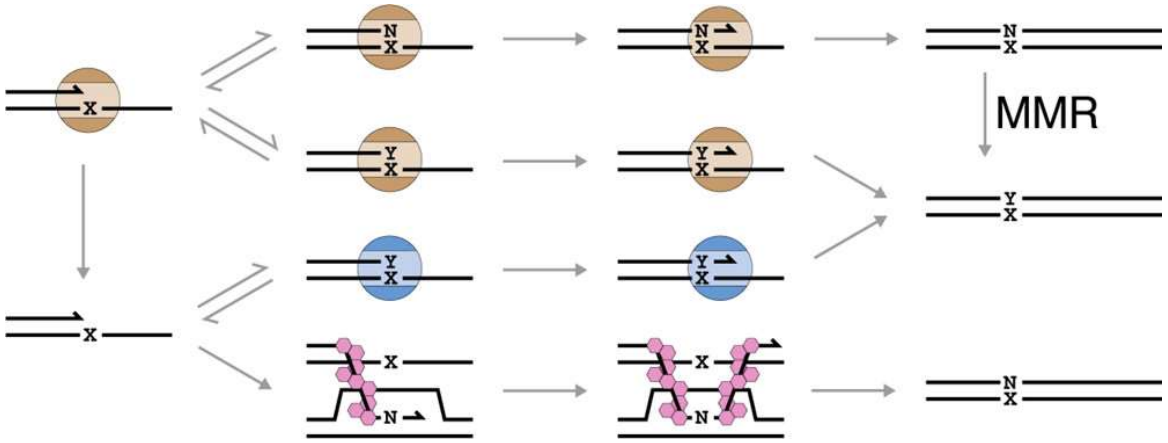
A semi-synthetic organism with an expanded genetic alphabet





Romesberg, Floyd E. *et al.* *PNAS* **2017**, *114,* 1317-1322

Reprograming the replisome of a semi-synthetic organism

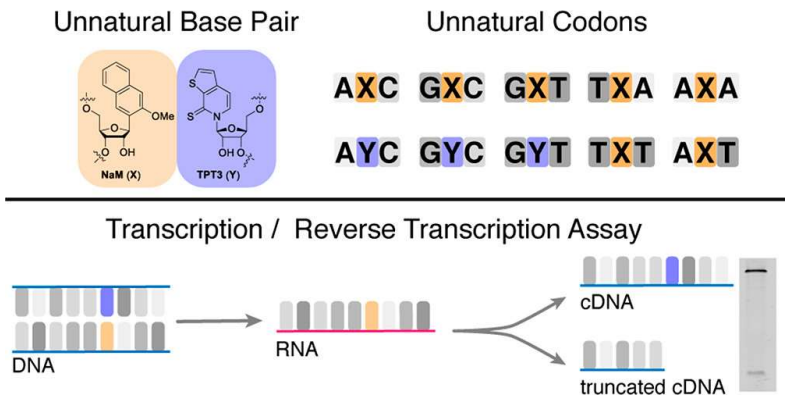


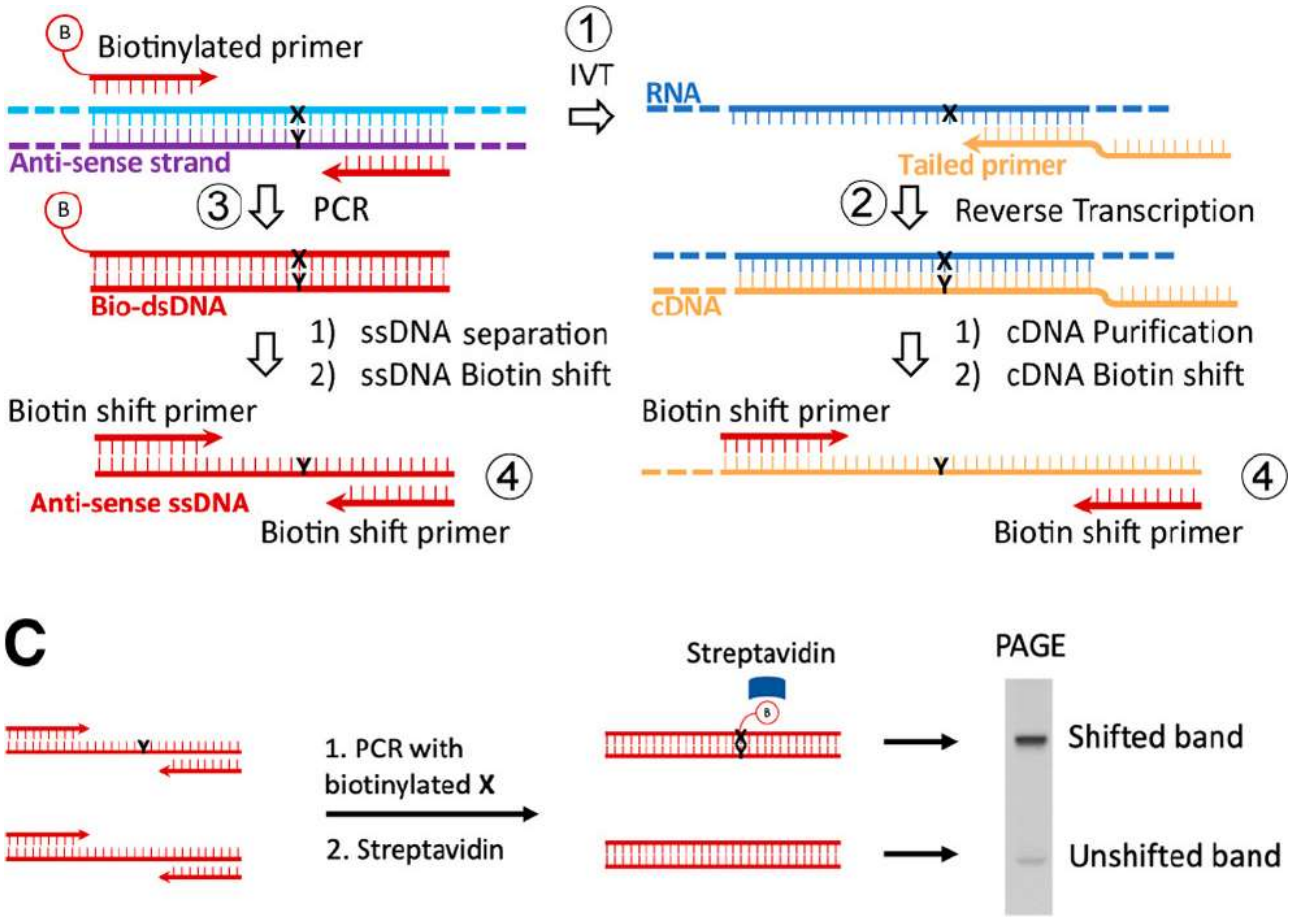
Proposed mechanism of UBP replication. *UBP retention is mediated by the activities of Pol III (tan), Pol II (blue), and MMR. UBP loss is mediated by polymerase replication errors or RecA (pink)-mediated RER.*

UBPs are not well retained in all sequences, limiting the information that can be encoded, and are invariably lost upon extended growth. The contributions of the *E. coli* DNA replication and repair machinery to the propagation of DNA containing d**NaM**-d**TPT3** have been explored and showed that replication by DNA polymerase III, supplemented with the activity of polymerase II and methyl-directed mismatch repair contribute to retention of the UBP and that recombinational repair of stalled forks is responsible for the majority of its loss.

M. P. Ledbetter, R. J. Karadeema, and F. E. Romesberg *J. Am. Chem. Soc.* **2018**, *140, 2,* 758-765

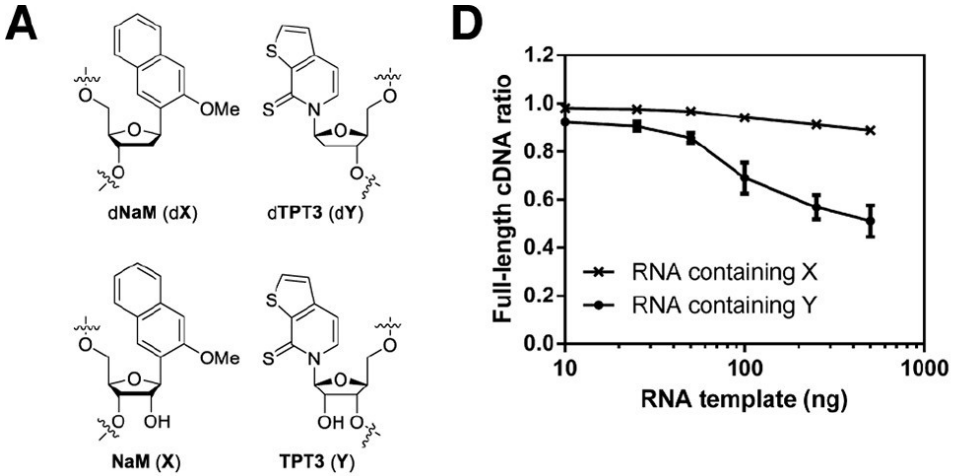
Transcription and reverse transcription in vitro and in SSO

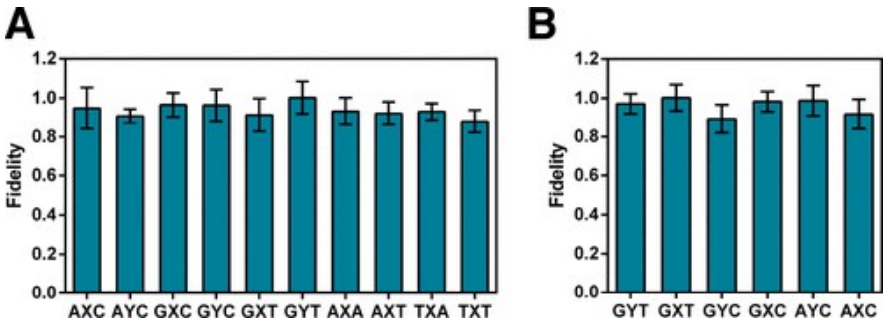




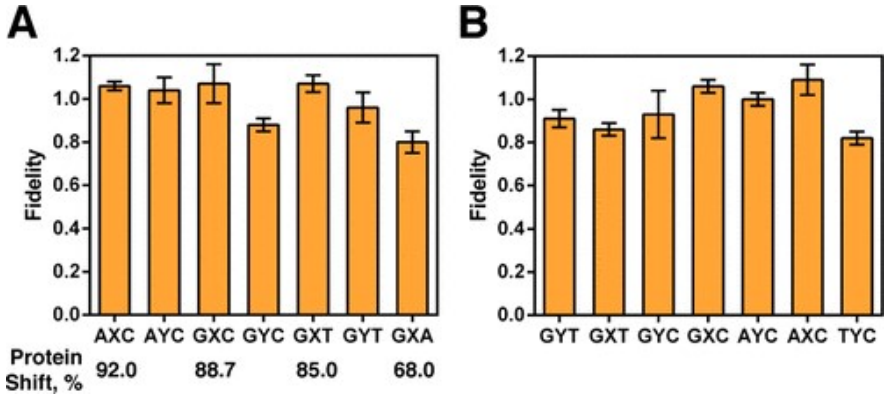
A. X.-Z. Zhou, X. Dong, F. Romesberg,
J. Am. Chem. Soc., **2020**, *142*, 19029-19032

Transcription and reverse transcription in vitro and in SSO





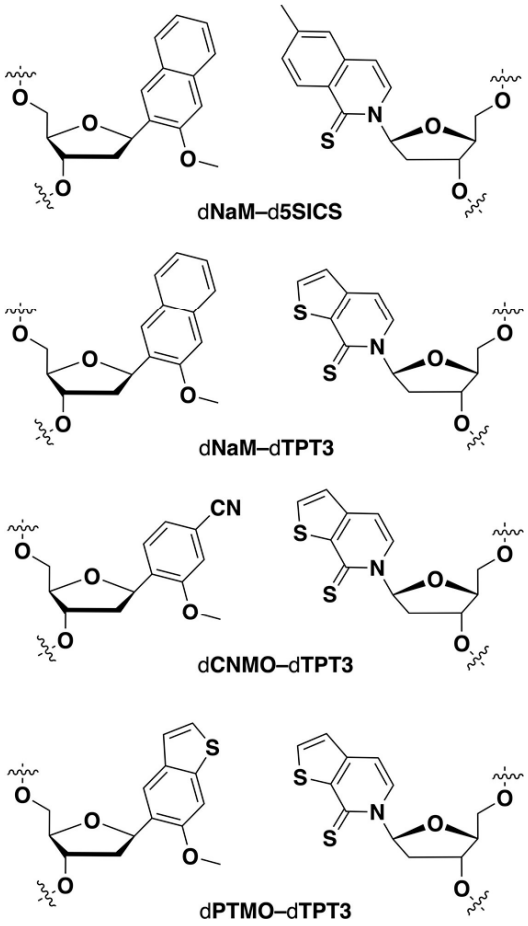
Transcription fidelity measured by T-RT assay of (A) mRNA and (B) tRNA made by in vitro transcription using T7 RNA polymerase.

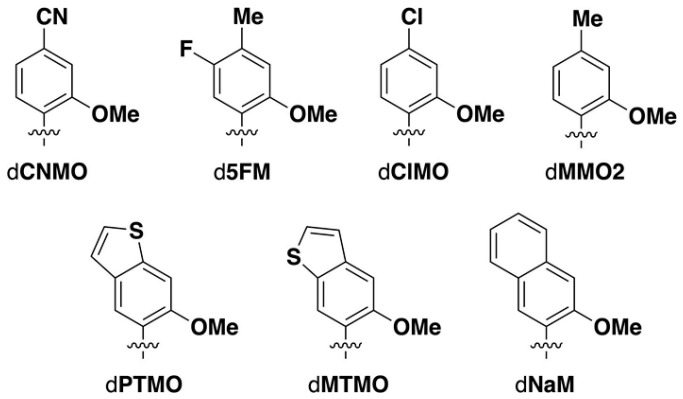


Transcription fidelity measured by T-RT assay of (A) mRNA and (B) tRNA extracted from SSO in vivo translation experiments

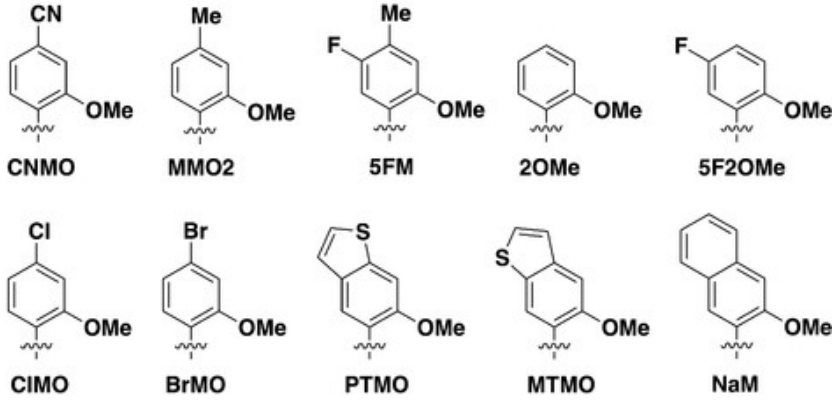
A. X.-Z. Zhou, X. Dong, F. Romesberg,
J. Am. Chem. Soc., **2020**, *142*, 19029-19032

Expanded genetic alphabet - 2019





dXTP analogues

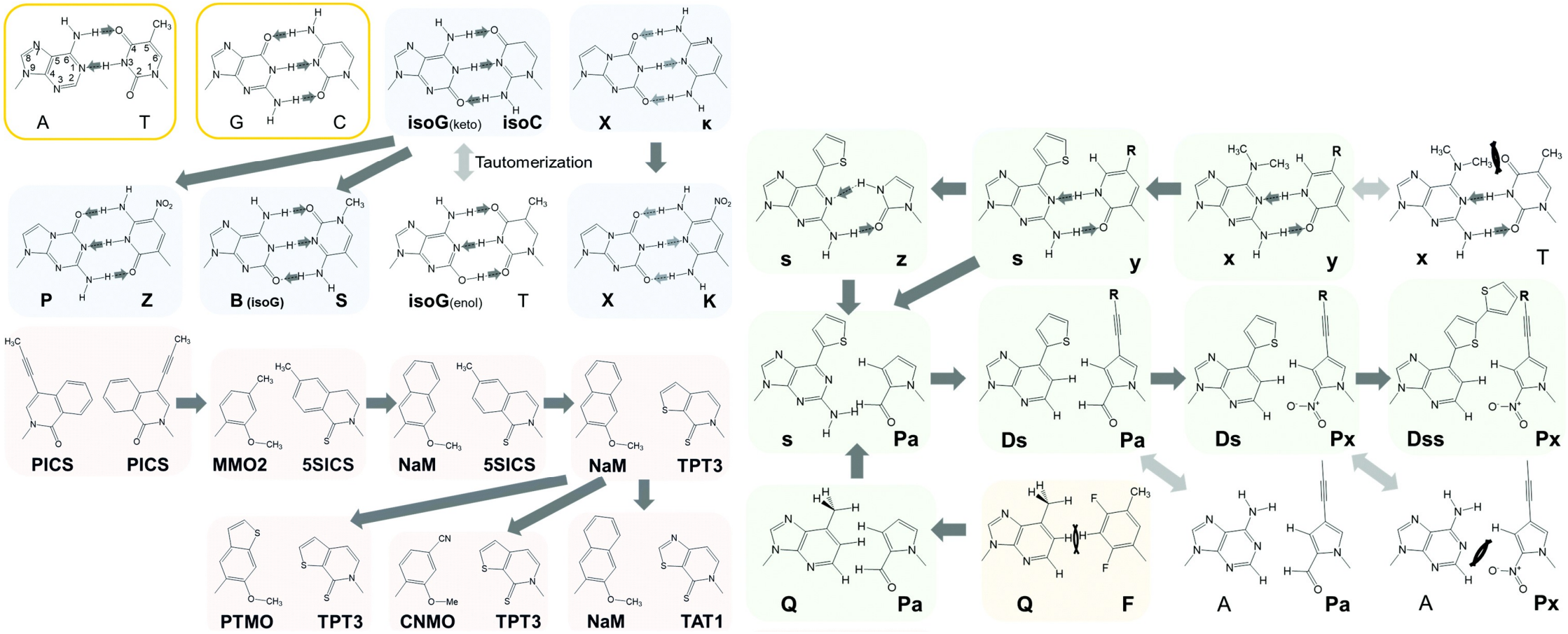


XTP analogues

YTP analogues

Romesberg, Floyd E. *et al.*
J. Am. Chem. Soc. **2019**, *141, 27,* 10644–10653

Unnatural nucleobases - overview



CHAPTER 1

OLIGONUCLEOTIDES

Part 3 – noncanonical backbone

Overview of the course

artificial ribozymes and aptamers for efficient catalysis and recognition (SELEX, DNAzymes, foldamers);

unnatural base pairing – expansion of the genetic alphabet;

Artificial genetic polymers and oligonucleotide analogues (XNA);

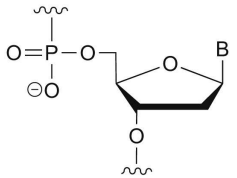
biosynthetic incorporation of **unnatural aminoacids (UAAs)** into proteins;

enzyme engineering – production of enzymes with unknown or unnatural properties, *ab initio* protein design, directed evolution, theozymes;

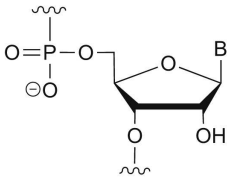
Artificial lipid vesicles as models for protocell multiplication;

design of artificial organisms

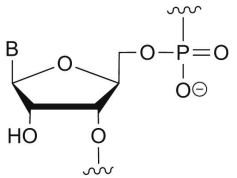
Artificial genetic polymers



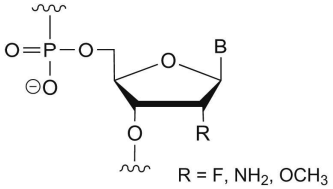
DNA



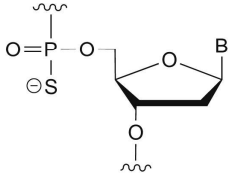
D-RNA
(natural)



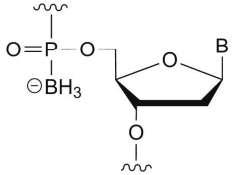
L-RNA
(unnatural)



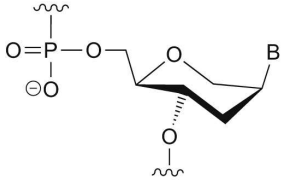
2'-modified RNA



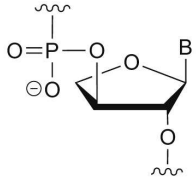
Phosphorothioate



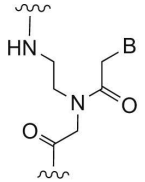
Boranophosphate



Hexitol Nucleic Acid
(HNA)



Threose Nucleic Acid
(TNA)

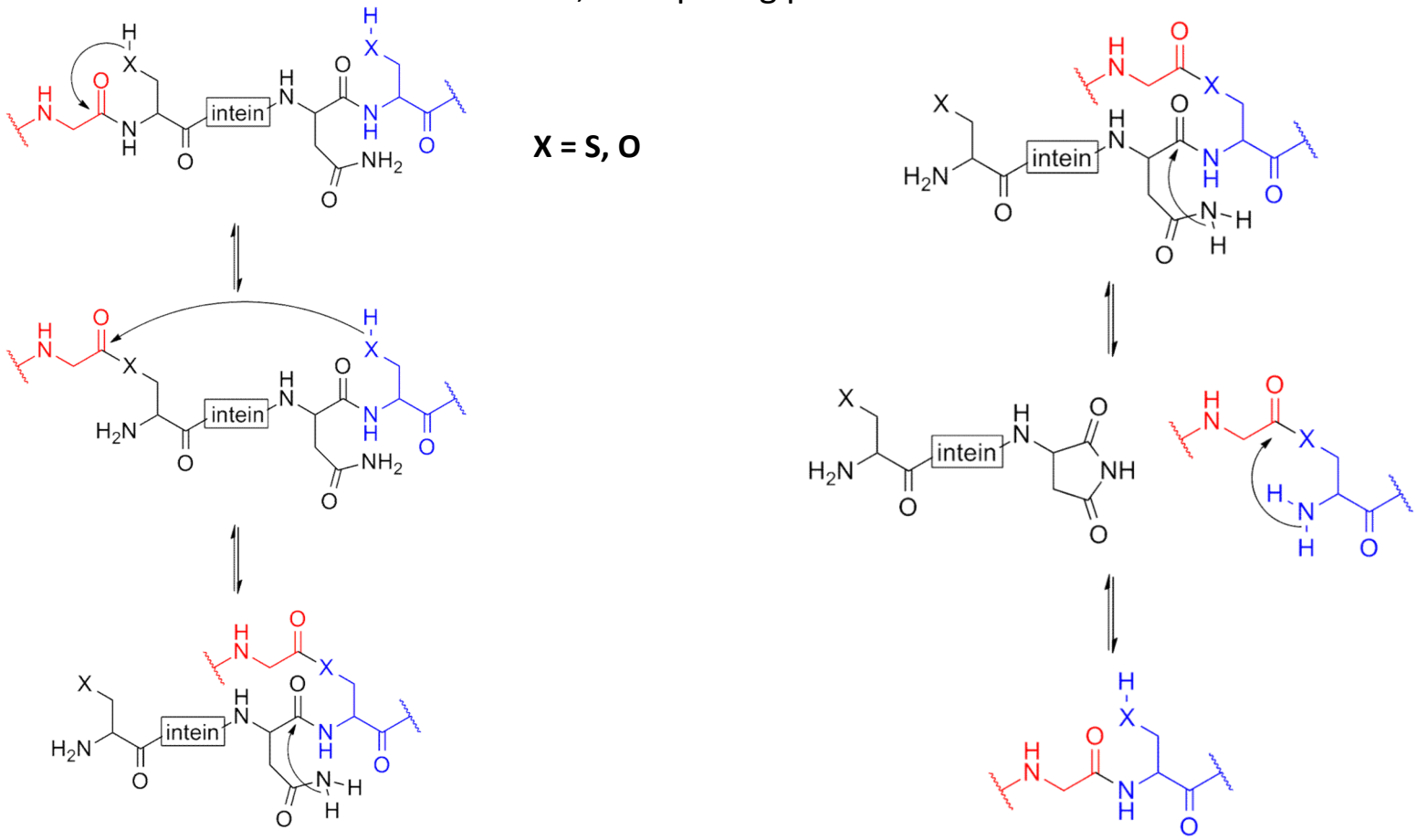


Peptide Nucleic Acid
(PNA)

Intein splicing

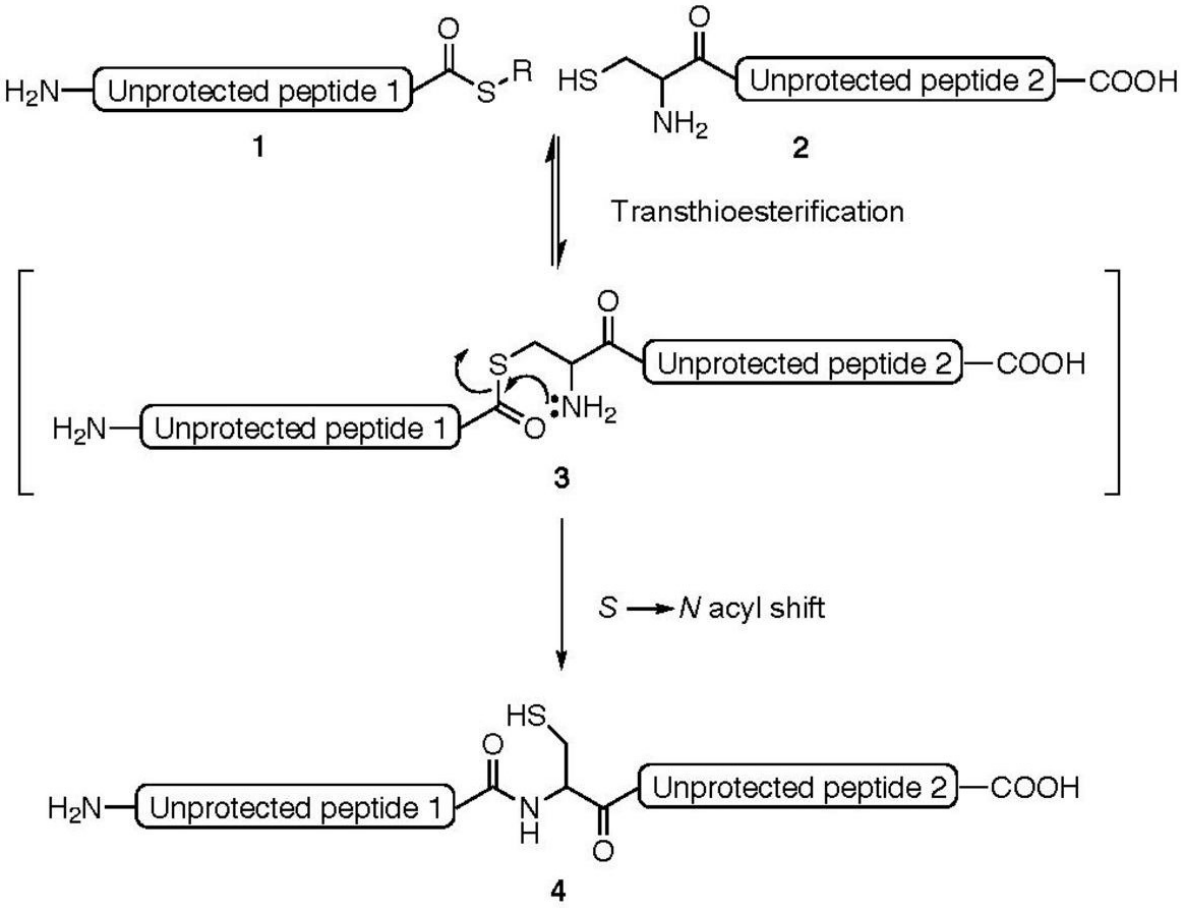
An **intein** is a segment of a protein that is able to excise itself and join the remaining portions (the **exteins**) with a peptide bond in a process termed protein splicing. Inteins have also been called "protein introns".

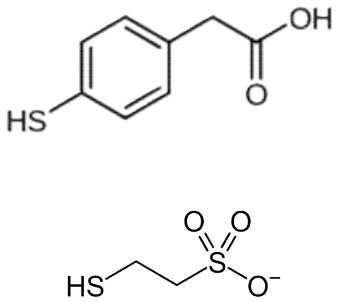
Intein-mediated protein splicing occurs after the intein-containing mRNA has been translated into a protein. This precursor protein contains three segments—an **N-extein** followed by the intein followed by a **C-extein**. After splicing has taken place, the resulting protein contains the N-extein linked to the C-extein; this splicing product is also termed an extein.



Native chemical ligation

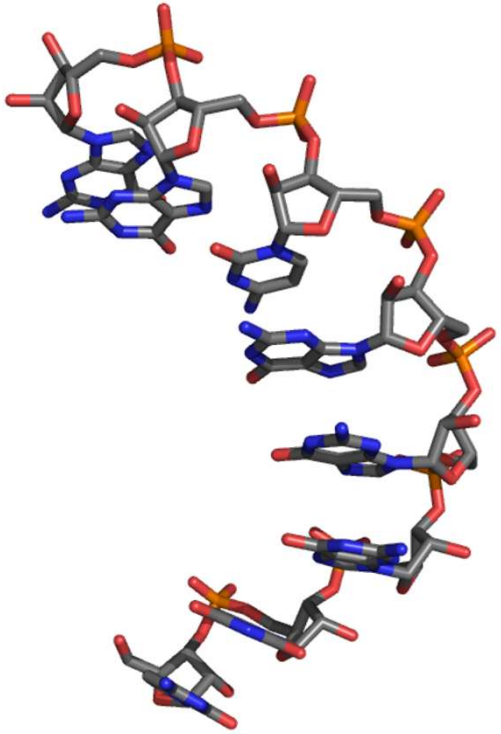
Native chemical ligation or ***NCL*** is an important extension of the chemical ligation field, a concept for constructing a large polypeptide formed by the assembling of two or more unprotected peptides segments. Especially, NCL is the most powerful ligation method for synthesizing proteins (native or modified) of moderate size (i.e., small proteins< 200 AA).





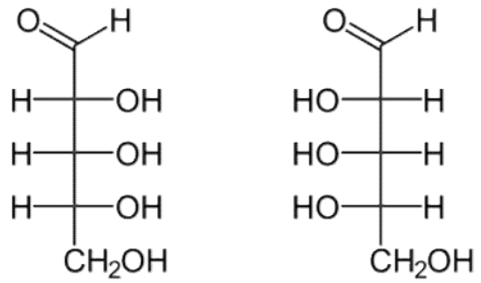
Spiegelmers: L-RNA

Aptamers (from the Latin aptus – fit, and Greek meros – part) are oligonucleotide or peptide molecules that ***bind to a specific target molecule***. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist in riboswitches.



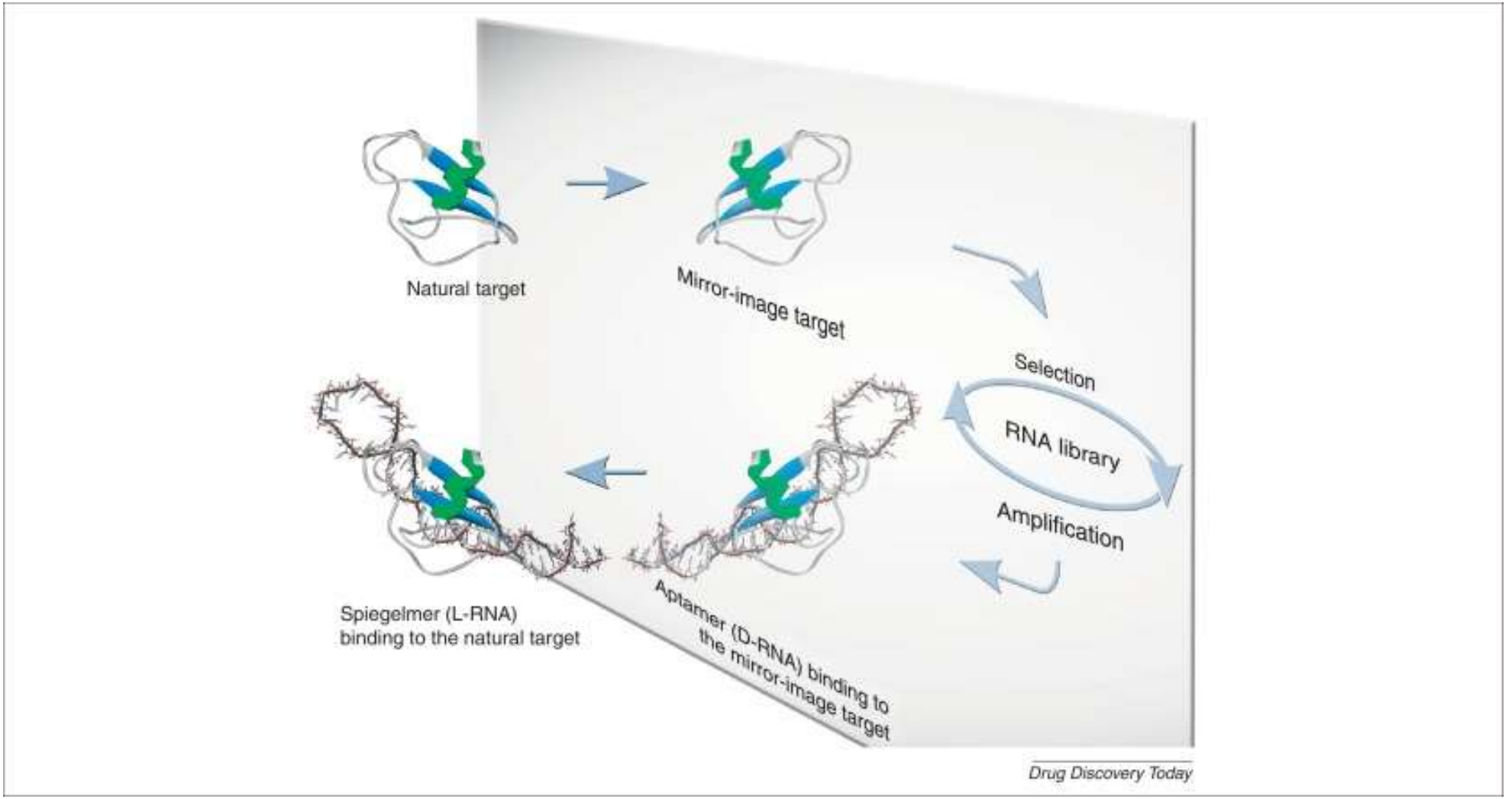
An ***L-ribonucleic acid aptamer*** (L-RNA aptamer, trade name ***Spiegelmer*** – from German Spiegel "mirror" – by Noxxon Pharma) is an RNA-like molecule built from L-ribose units.
It is an artificial oligonucleotide named for being a mirror image of natural oligonucleotides.

L-RNA aptamers are a form of aptamers. Due to their L-nucleotides, they are highly resistant to degradation by nucleases. ***Spiegelmers*** are considered potential drugs and are currently being tested in clinical trials.



A. Vater, S. Klussmann, *Drug Discovery Today* **2015**, *20,* 147-155

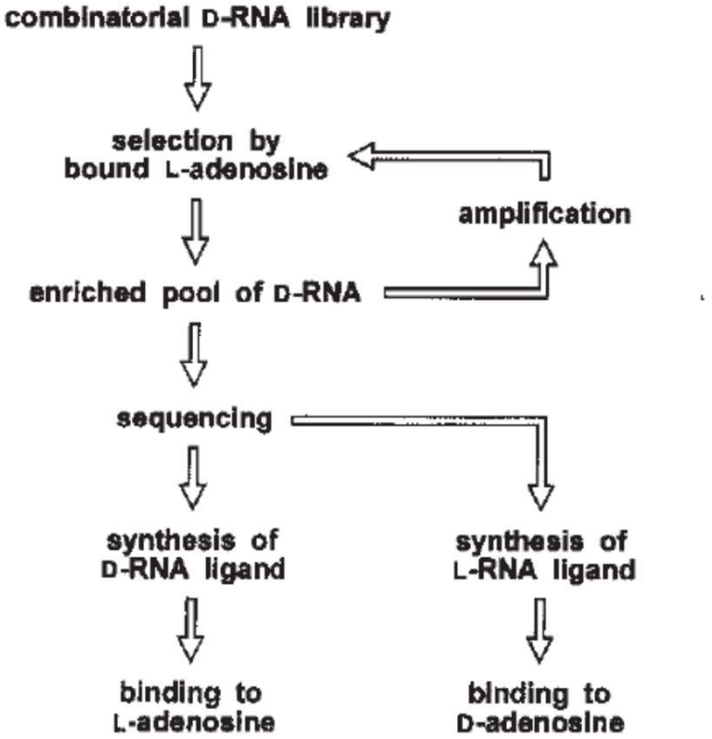
Spiegelmers: L-RNA

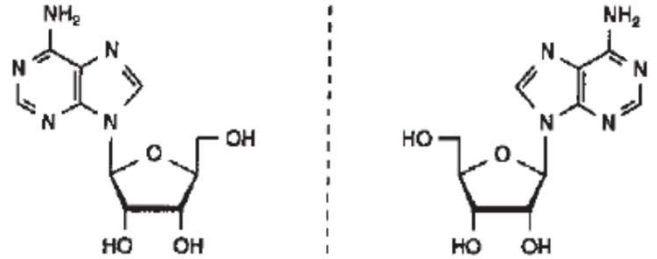


A. Vater, S. Klussmann, *Drug Discovery Today* **2015**, *20,* 147-155

B.E. Young, N. Kundu, J.T. Szczepanski, *Chem. Eur.J.* ***2019***, *25*, 7981 –7990

Mirror-image RNA that binds D-Adenosine





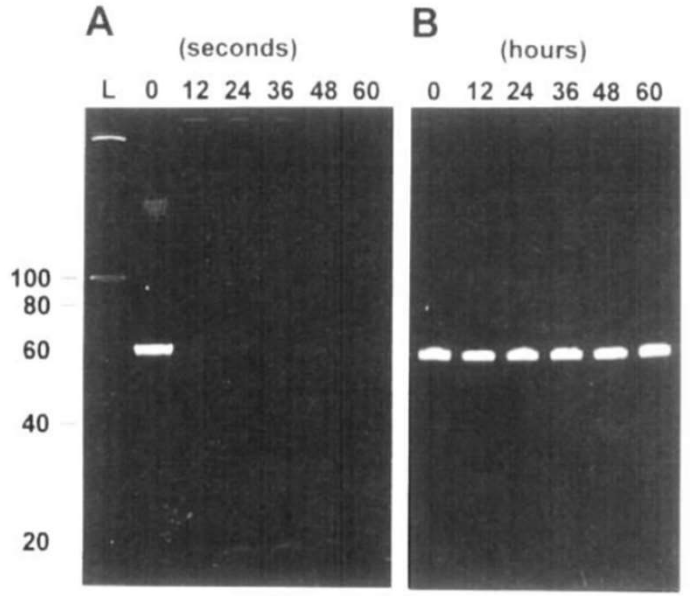


Figure 5. Stability of the 58-mer RNA ligands in human serum. (A) Aptamer D-A42d and (B) spiegelmer L-A42d. Aliquots were taken at the indicated times. L marks the size standard (10 bp DNA ladder). The results were reproduced in an independent experiment.

S. Klussmann, J. Furste, et al. *Nature Biotech.* **1996**, *14,* 1112-1115

Spiegelmers: L-RNA

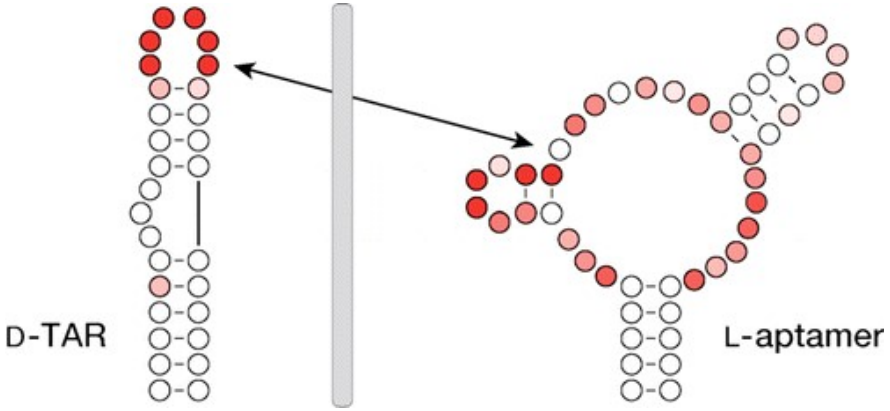
Selected pharmaceutically relevant targets of spiegelmers

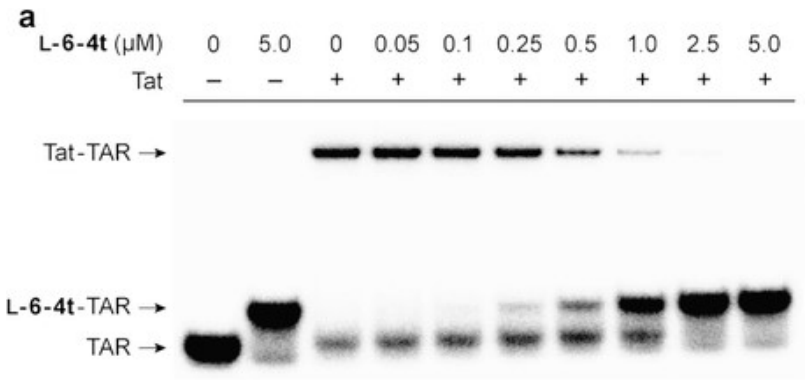
Monocyte chemoattractant peptide MCP-1/CCL2 NOX-E36/ emapticap pegol	Placebo-controlled Phase I, SAD and four-week MAD completed,PD-effect:reduction of $CCR2_+$ monocytes in peripheral blood. Double-blind, placebo-controlled Phase IIa trial in diabetic patients with albuminuria (three months treatment,three months follow-up): reductions in albuminuria and improved glycemic control(HbA1c); renoprotective effect maintained in absence of drug (three months)
Stromal-cell-derived factor-1 (SDF-1/CXCL12) NOX-A12/ olaptesed pegol	Hematopoietic stem cell/WBC mobilization in mice and/or monkeys; Phase I, SAD: hematopoietic stem cell and WBC mobilization Phase IIa for chemosensitization in MM (with VD) and CLL (with BR) ongoing
Hepcidin NOX-H94/ lexaptepid pegol	Phase I:dose-dependent increases in serum iron in healthy Volunteers; PK/PD study: inhibition of LPS-induced serum iron decrease Phase IIa in anemia of cancer ongoing

A. Vater, S. Klussmann, *Drug Discovery Today* **2015**, *20,* 147-155

Spiegelmers: L-RNA

Aptamer selection against RNA with natural D-chirality





An *L*-RNA aptamer was developed that binds the natural d-form of the HIV-1 trans-activation responsive (TAR) RNA (K_d = 100 nM). The aptamer initially was obtained as a *D*-aptamer against *L*-TAR RNA through *in vitro* selection.

It binds *D*-TAR exclusively at the six-nucleotide distal loop, but does so through tertiary interactions rather than simple Watson–Crick pairing.

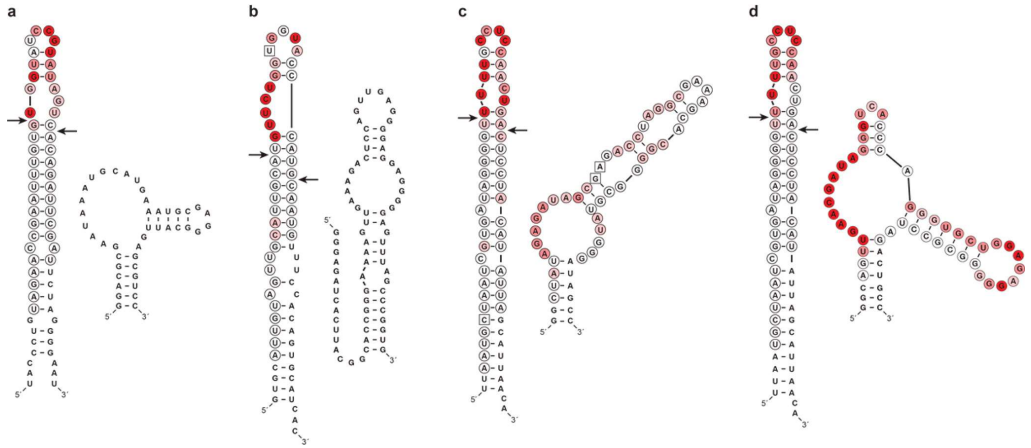
This complex is the first example of two nucleic acids molecules of opposing chirality that interact through a mode of binding other than primary structure.

Binding of the *L*-aptamer to *D*-TAR RNA inhibits formation of the Tat-TAR ribonucleoprotein complex that is essential for TAR function ➔ therapeutic interest

J.T. Szczepanski, G.F. Joyce, *J. Am. Chem. Soc.* **2013**, *135, 36,* 13290–13293

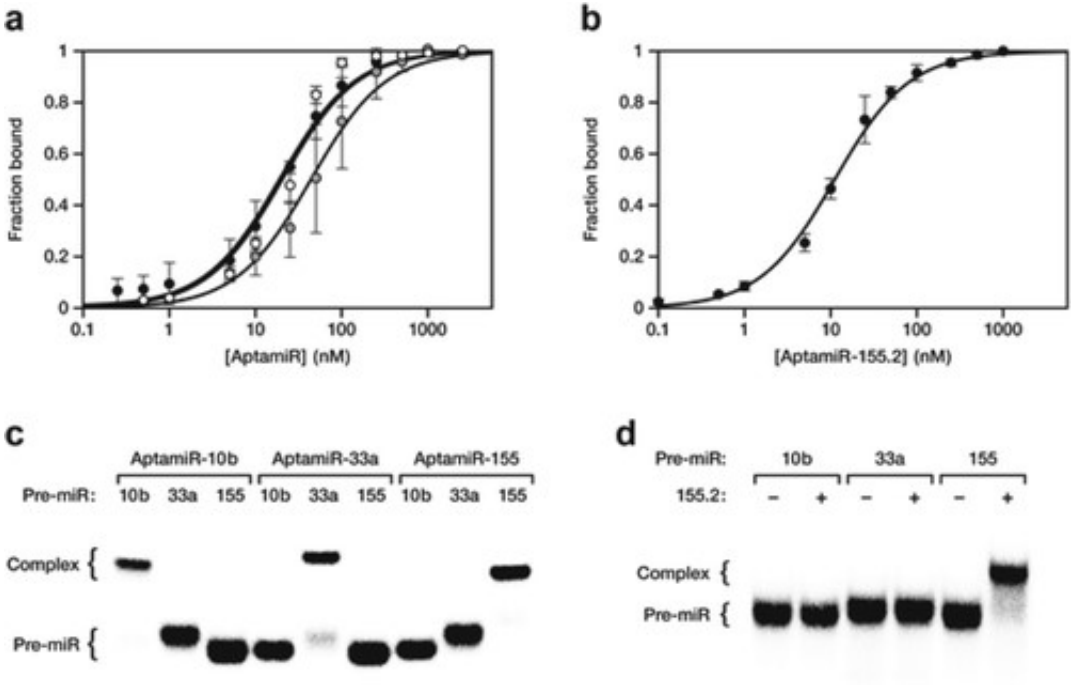
Spiegelmers: L-RNA

Spiegelmers/"AptamiRs" - a new class of miR inhibitors.



In vitro selection was used to obtain *L*-RNA aptamers that bind the distal stem-loop of various precursor microRNAs (pre-miRs). These *L*-aptamers, termed "aptamiRs", bind their corresponding pre-miR target through highly specific tertiary interactions rather than Watson–Crick pairing. Formation of a pre-miR–aptamiR complex inhibits Dicer-mediated processing of the pre-miR, which is required to form the mature functional microRNA. One of the aptamiRs, which was selected to bind oncogenic pre-miR-155, inhibits Dicer processing under simulated physiological conditions, with an IC_{50} of 87 nM

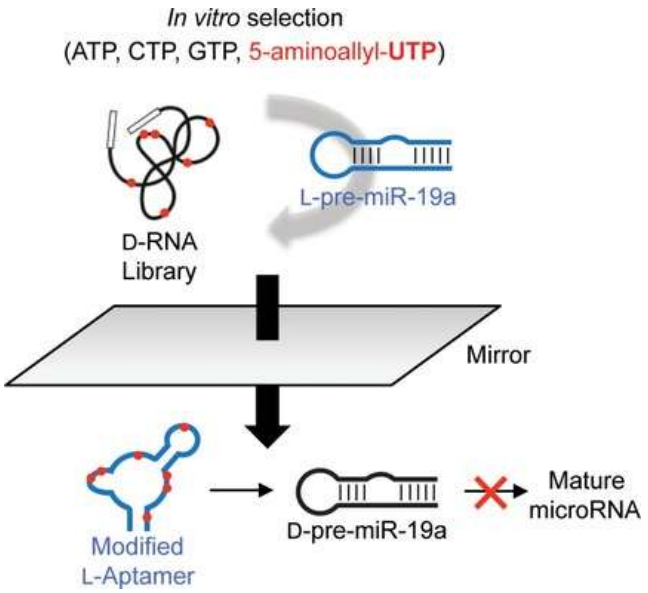
MicroRNAs (miRs) are small, noncoding RNAs that act as post-transcriptional regulators of gene expression, involved in development, differentiation, and apoptosis. Alterations in their expression patterns can contribute to the pathogenesis of human disease.



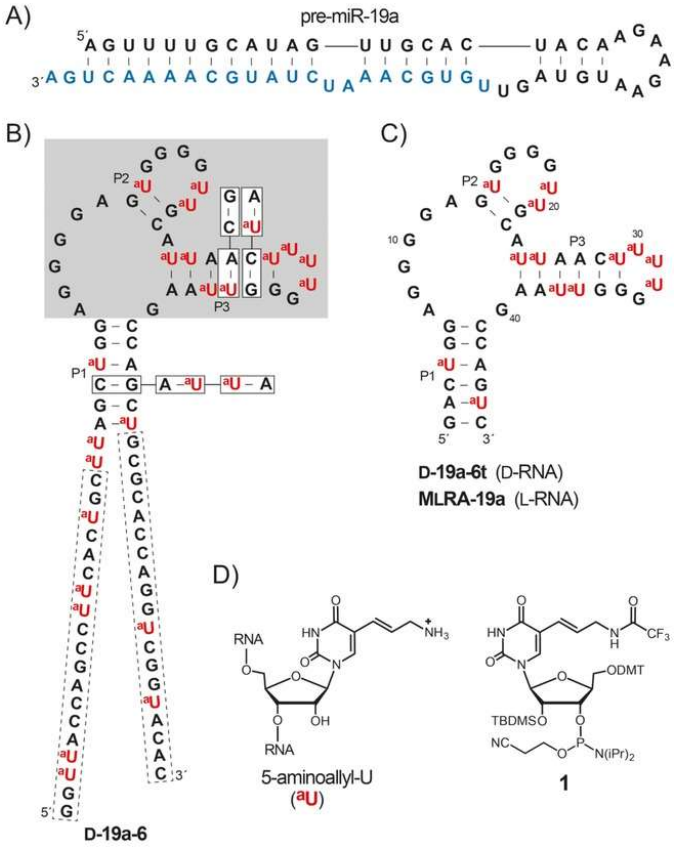
J.T. Szczepanski, G.F. Joyce, *J. Am. Chem. Soc.* **2015**, *137, 51,* 16032–16037

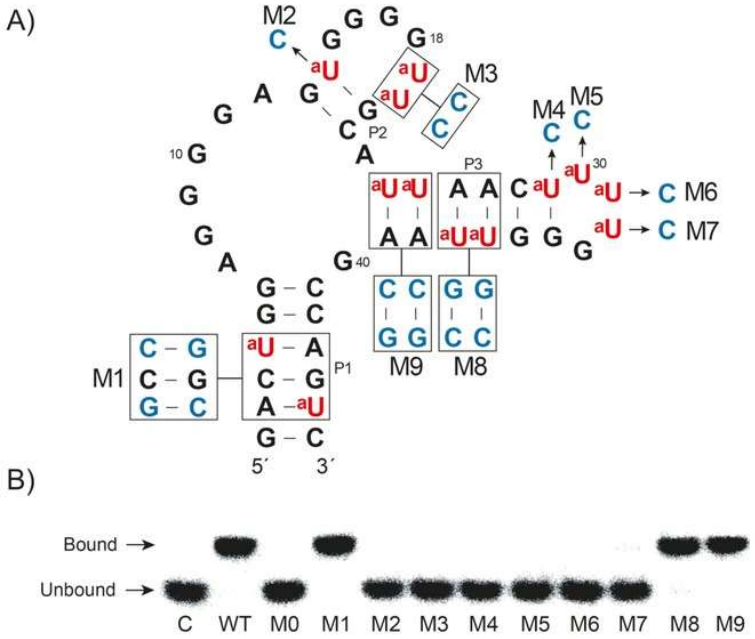
Spiegelmers: L-RNA

Spiegelmers with modified nucleotides



By employing 5-aminoallyl-UTP during mirror image in vitro selection, a modified *L*-RNA aptamer was isolated with exceptional affinity (IC_{50}= 4nM) towards oncogenic precursor microRNA-19a. These studies demonstrate that expanding the chemical functionality of *L*-aptamers enables development of robust RNA-binding reagents.

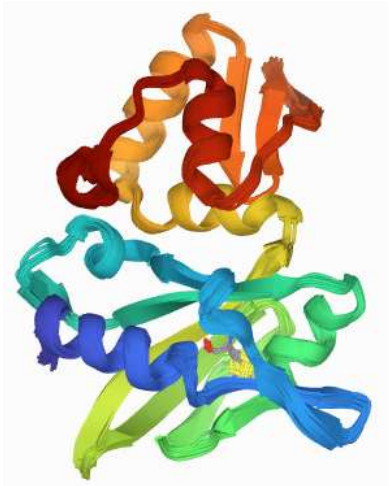




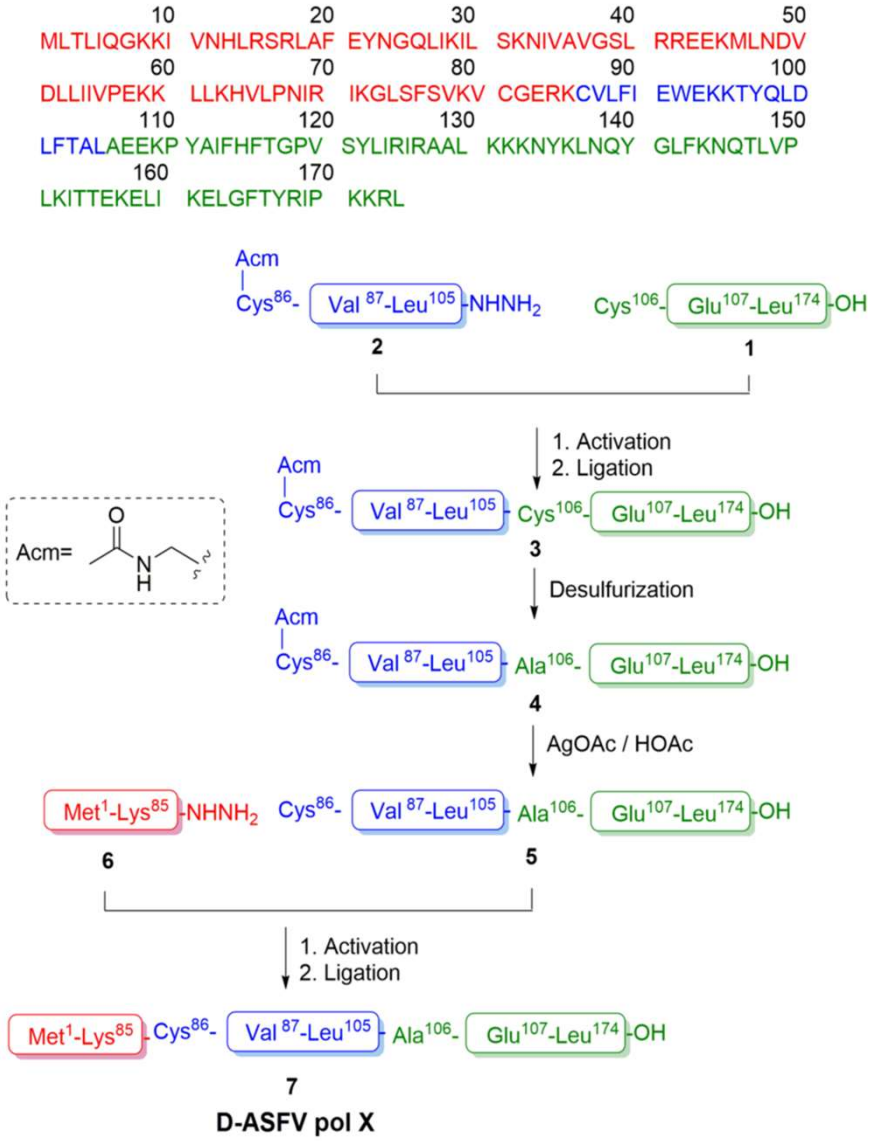
A.M. Kabza, J.T. Szczepanski,
ChemBioChem. **2017**, *18,* 1824-1827

Processing of the mirrored genetic information

Synthesis of a mirrored 832-residue Taq polymerase or the 604-residue Klenow Fragment from *D*-aminoacids is still beyond reach with the current methods. However, progress in long peptide synthesis enabled solid-support synthesis followed by fragment ligation (NCL) of the mirror image configuration of polymerase X from African swine fever virus (**ASFV**), the shortest known polymerase (174 amino acids).



PDB: 1JQR



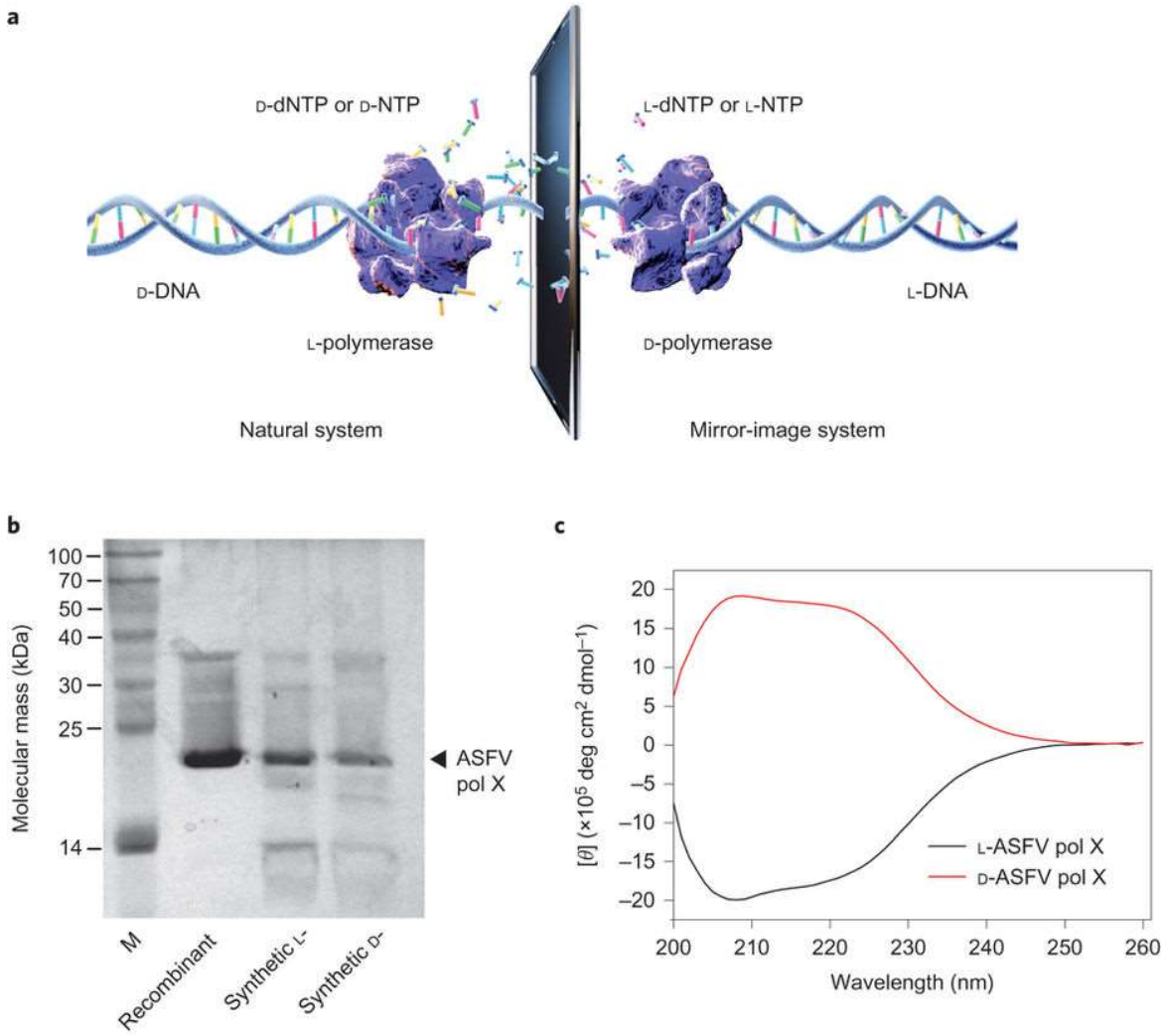
Showalter A.K. et al. *Nat Struct Biol* **2001**, *8,* 942-946

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

L-DNA polymerase

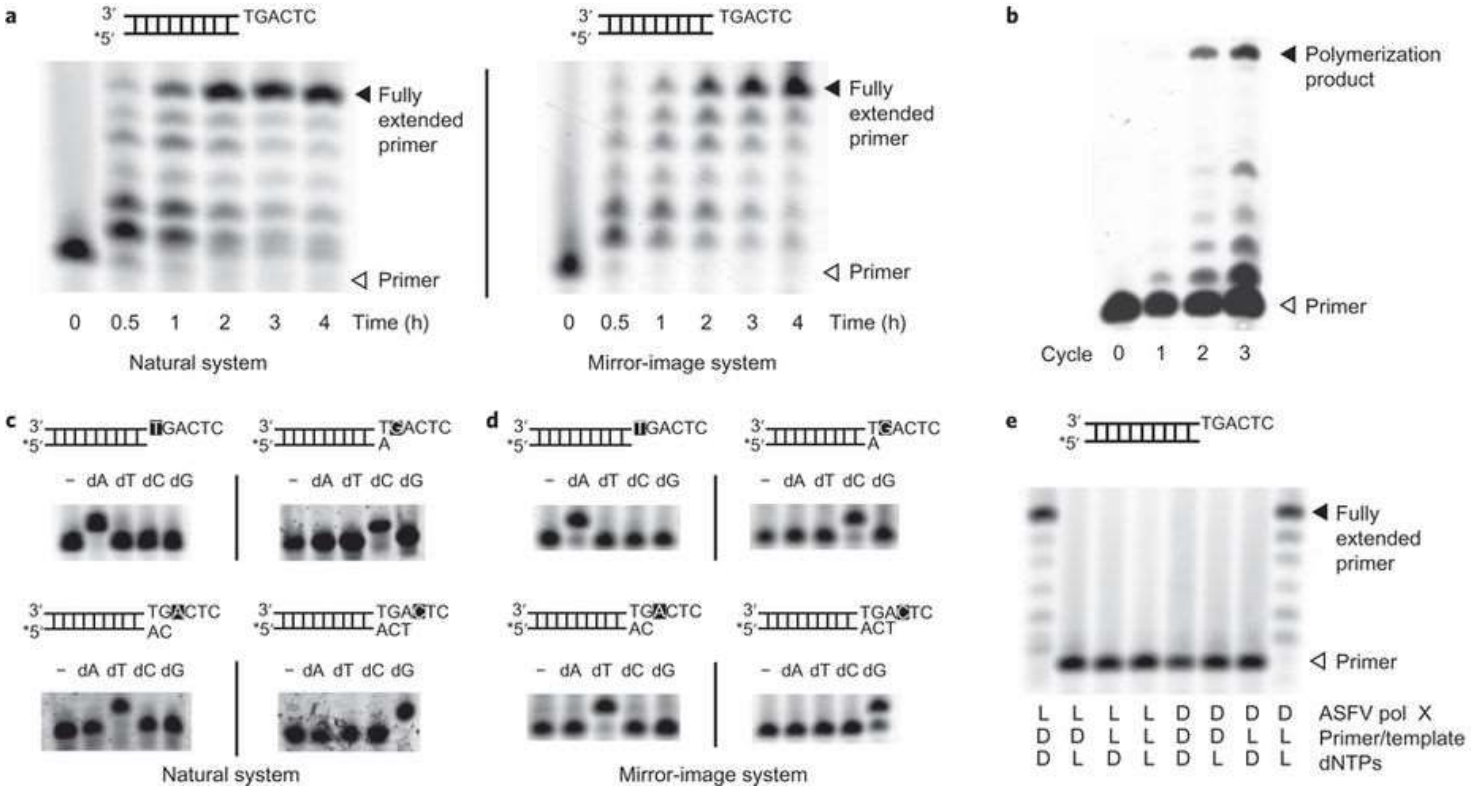
D-ASFV Pol X elongated an *L*-DNA primer with *L*-dNTPs - a functional 56-mer *L*-DNAzyme was made within 36 hours.

This poses an important proof of concept, however, polymerase X is a thermo-labile repair enzyme and its catalytic activity does not meet the requirements for a standard PCR



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

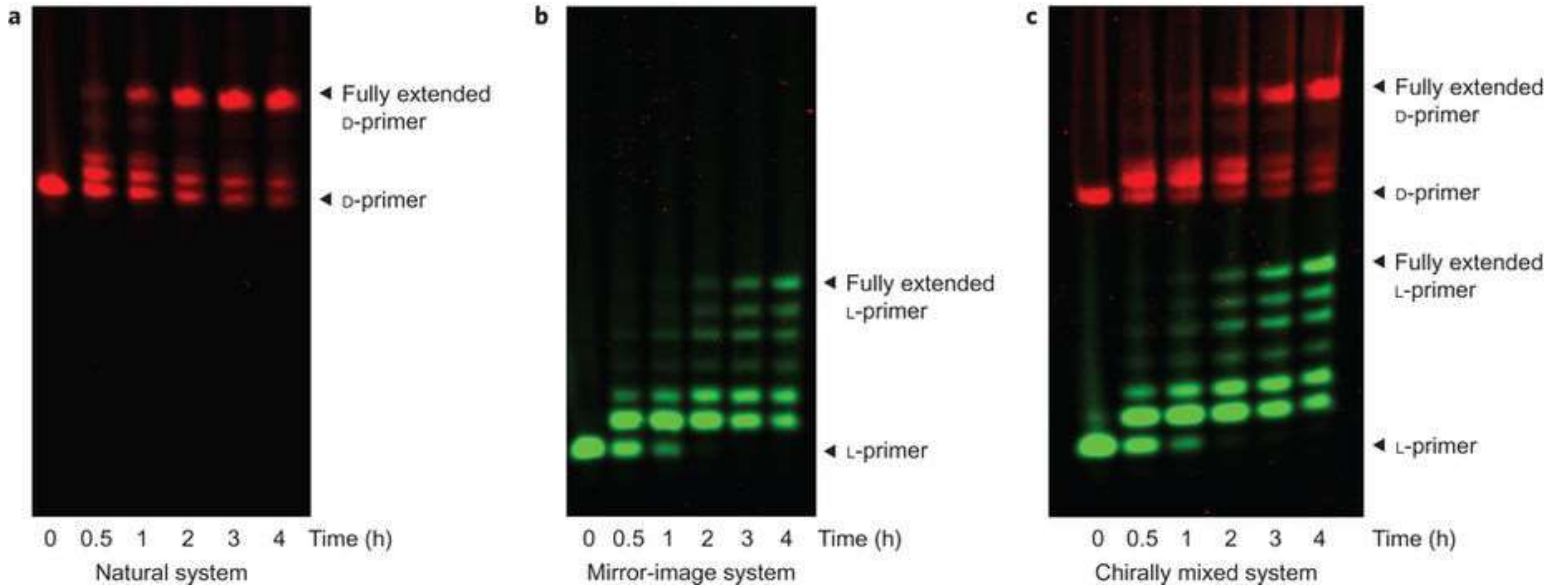
L-DNA polymerase



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

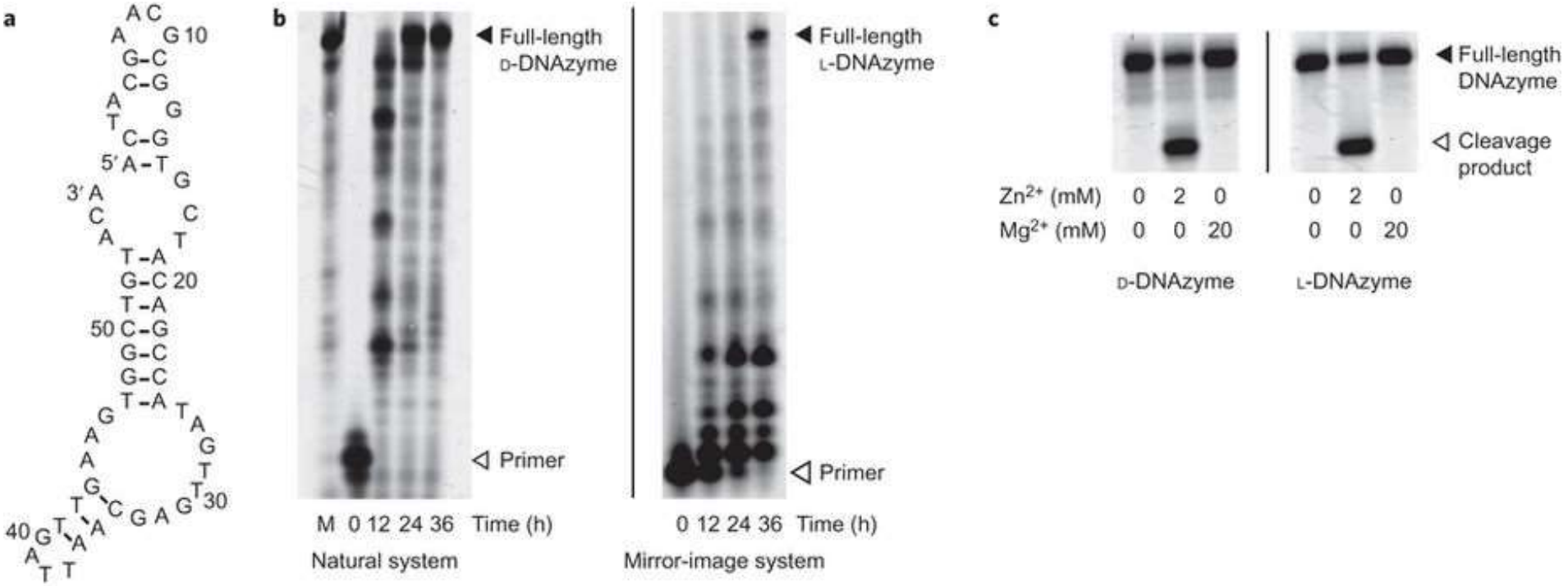
L-DNA polymerase



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

L-DNAzyme

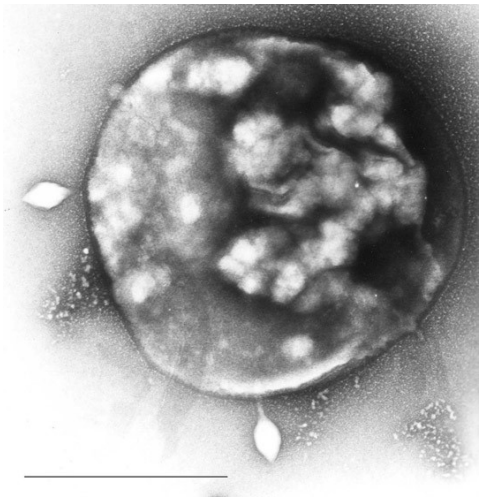


a, Sequence and predicted secondary structure of the previously reported Zn^{2+}-dependent self-cleaving DNAzyme. **b**, Primer extension on a 66 nt template to produce the Zn^{2+}-dependent self-cleaving DNAzyme. **c**, Self-cleavage of the enzymatically polymerized Zn^{2+}-dependent D- and L-DNAzymes.

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

A thermostable L-DNA-polymerase

A thermostable mirror-image polymerase ***D*-Dpo4-3C** has been produced, that is able to amplify L-DNA in a classical PCR reaction and can even be used to assemble an L-DNA gene from L-DNA oligonucleotides. This artificial enzyme is a mutant of DNA polymerase IV from *Sulfolobus solfataricus*, a Y-family polymerase consisting of 352 amino acids, the longest protein made by chemical synthesis thus far.

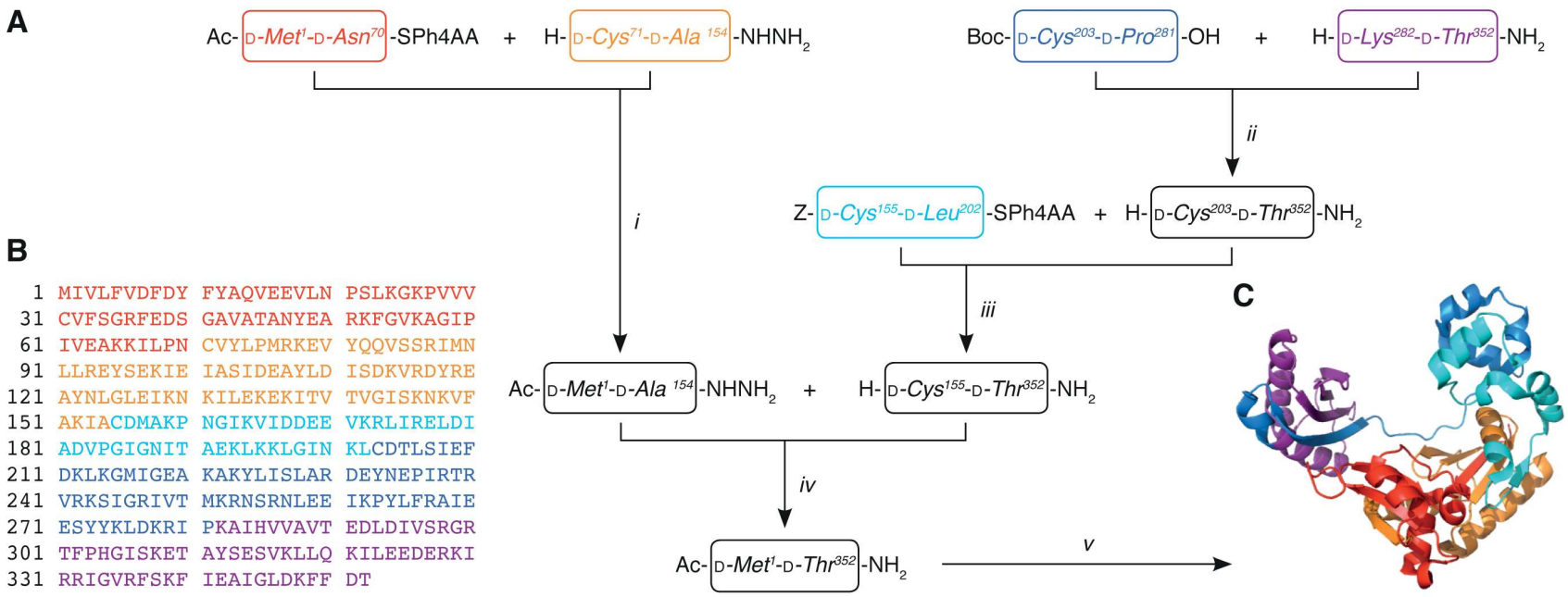


Cell of *Sulfolobus* infected by virus STSV1 observed under microscopy. Two spindle-shaped viruses were being released from the host cell.

Furthermore, with an additional single point mutation (Tyr12Ala or Tyr12Ser), this DNA polymerase can be tuned to accept also ribonucleotides as substrates with reasonable efficiency. Thus, this enzyme may be hijacked to act as a DNA-dependent RNA polymerase to prepare longer stretches of *L*-RNA

A. Pech, S. Klussmann *et al. Nucl. Acid Res.* **2017**, *45,* 3997-4005

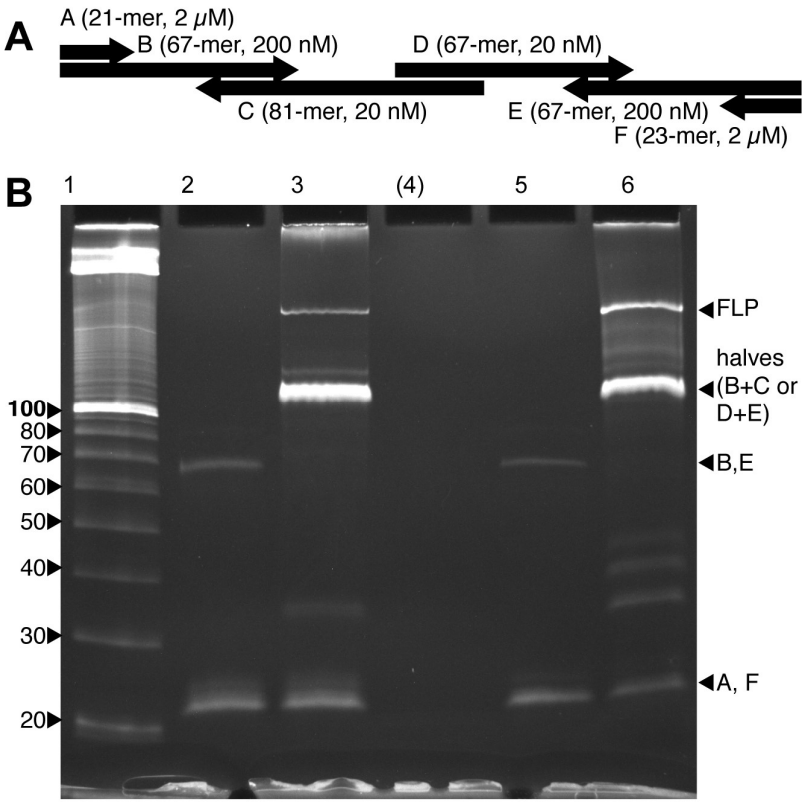
A thermostable L-DNA-polymerase



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)).

A. Pech, S. Klussmann *et al. Nucl. Acid Res.* **2017**, *45*, 3997-4005

A thermostable L-DNA-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.

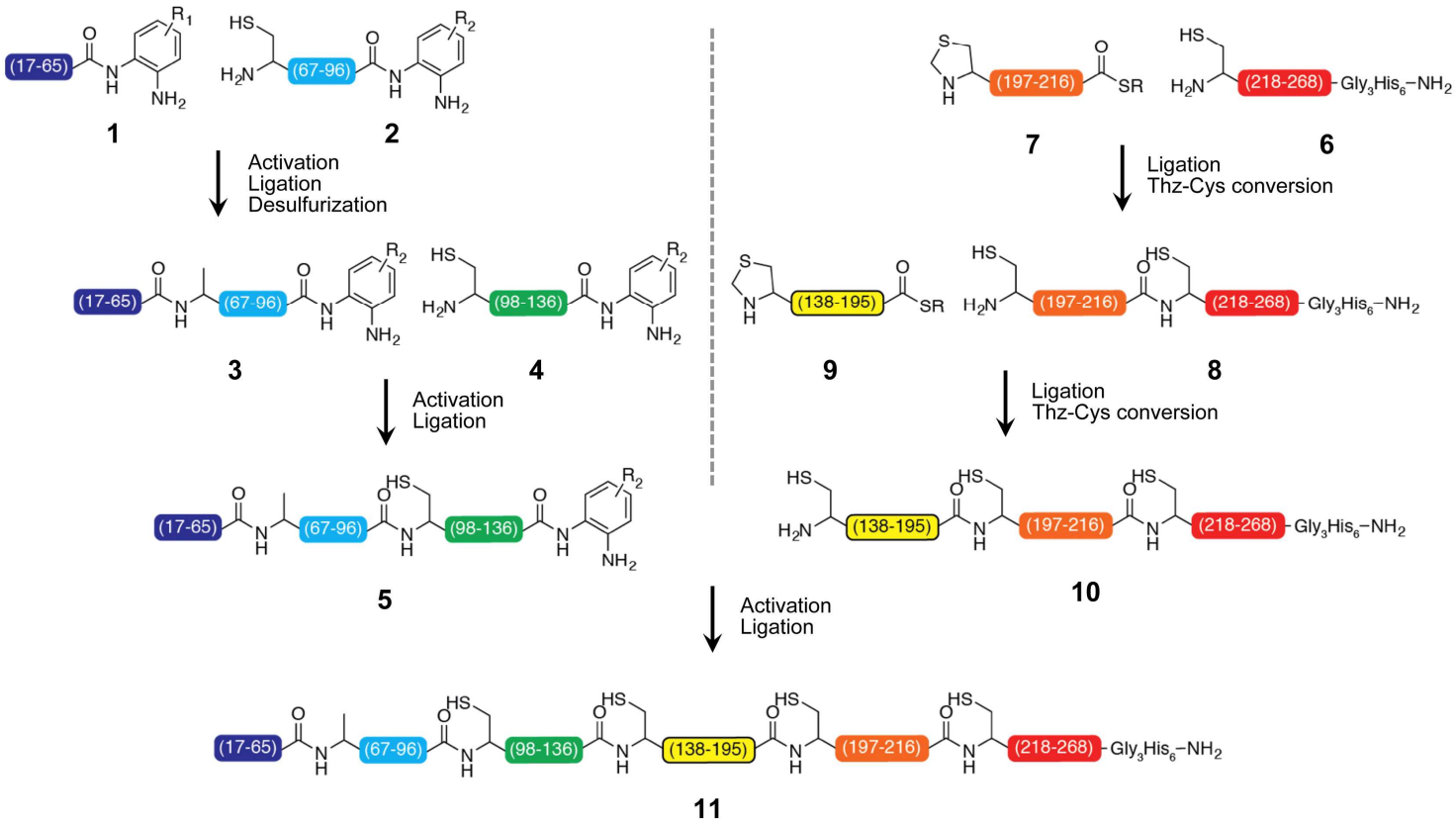
A. Pech, S. Klussmann *et al. Nucl. Acid Res.* **2017**, *45,* 3997-4005

Mirror-image DNA ligase

A

MANSDLMLLHTYNNQPIEGWVMSEKLDGVRGYWNGKQLLTRQGQRLSPPAYFIKDFPPFAIDGELFSERNHFEEISTITKCFKGDGW
EKLKLYVFDVPDAEGNLFERLAKLKAHLLEHPTCYIEIIEQIPVKDKTHLYQFLAQVENLQGEGVVVRNPNAPYERKRSSQILKLKT
ARGEQCTVIAHHKGKGQFENVMGALTCKNHRGEFKIGSGFNLNERENPPPIGSVITYKYRGITNSGKPRFATYWREKKGGGHHHHHH

B



A functional DNA-ligase in the D-enantiomeric conformation has been synthesized. It exhibited DNA ligation activity on chiraly inverted nucleic acids in L-conformation, but not acting on natural substrates and with natural co-factors. The ligase was based on the known structure of the *Paramecium bursaria* chlorella virus DNA-ligase and the homologous but shorter DNA-ligase of *Haemophilus influenza*. The structure and the activity of the mirror-image ligase were characterized, documenting its enantiospecific functionality.

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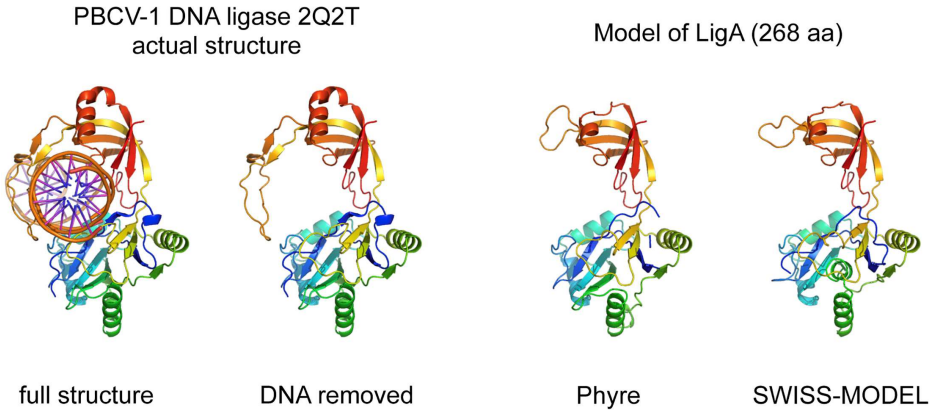
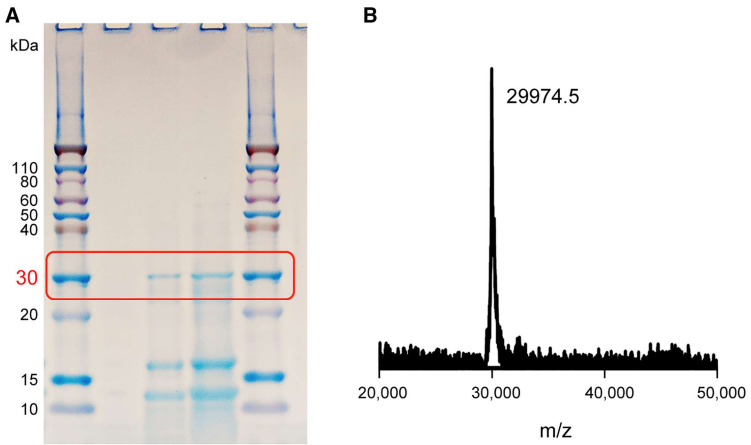
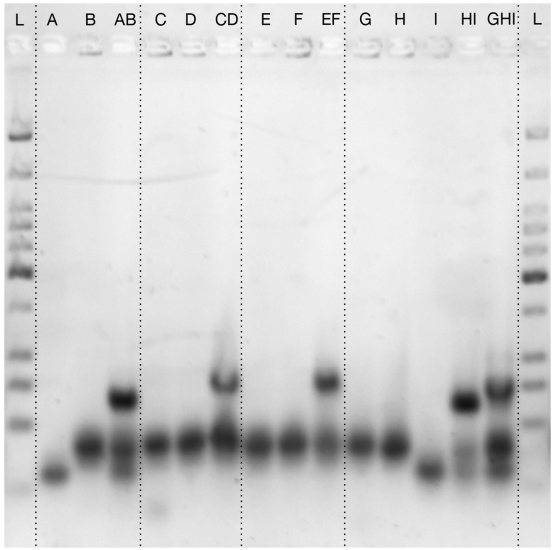
Mirror-image DNA ligase



Prediction of the LigA structure



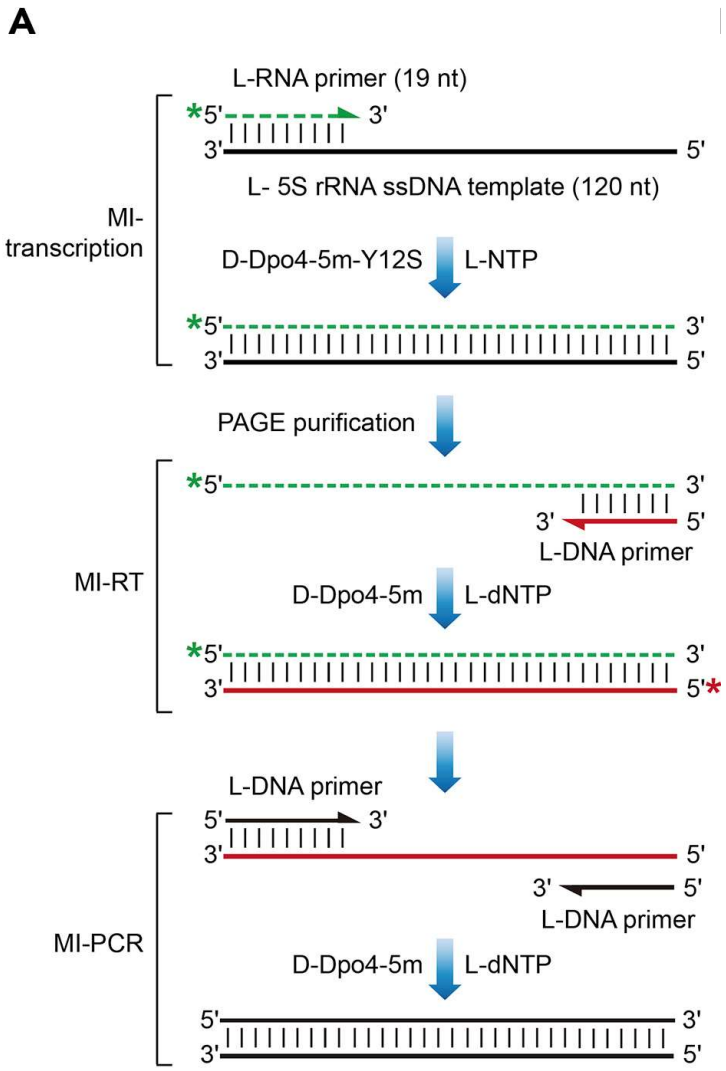
D-Protein LigA: (A) The product of the final chemical ligation after 12 h. The two peptides **5** and **10** and the final, full-length protein of about 30 kDa can be seen. (B) MALDI-TOF mass spectrum of LigA. Measured: 29,974.5 Da theoretical: 29,966.1 Da.



Enzymatic Ligation of Gene Fragments Made of Synthetic L-DNA

J. Weidmann *et al.*
Cell Chemical Biology ***2019*** *26(5), 645-651.e3*

Mirror-image transcription, reverse transcription and amplification

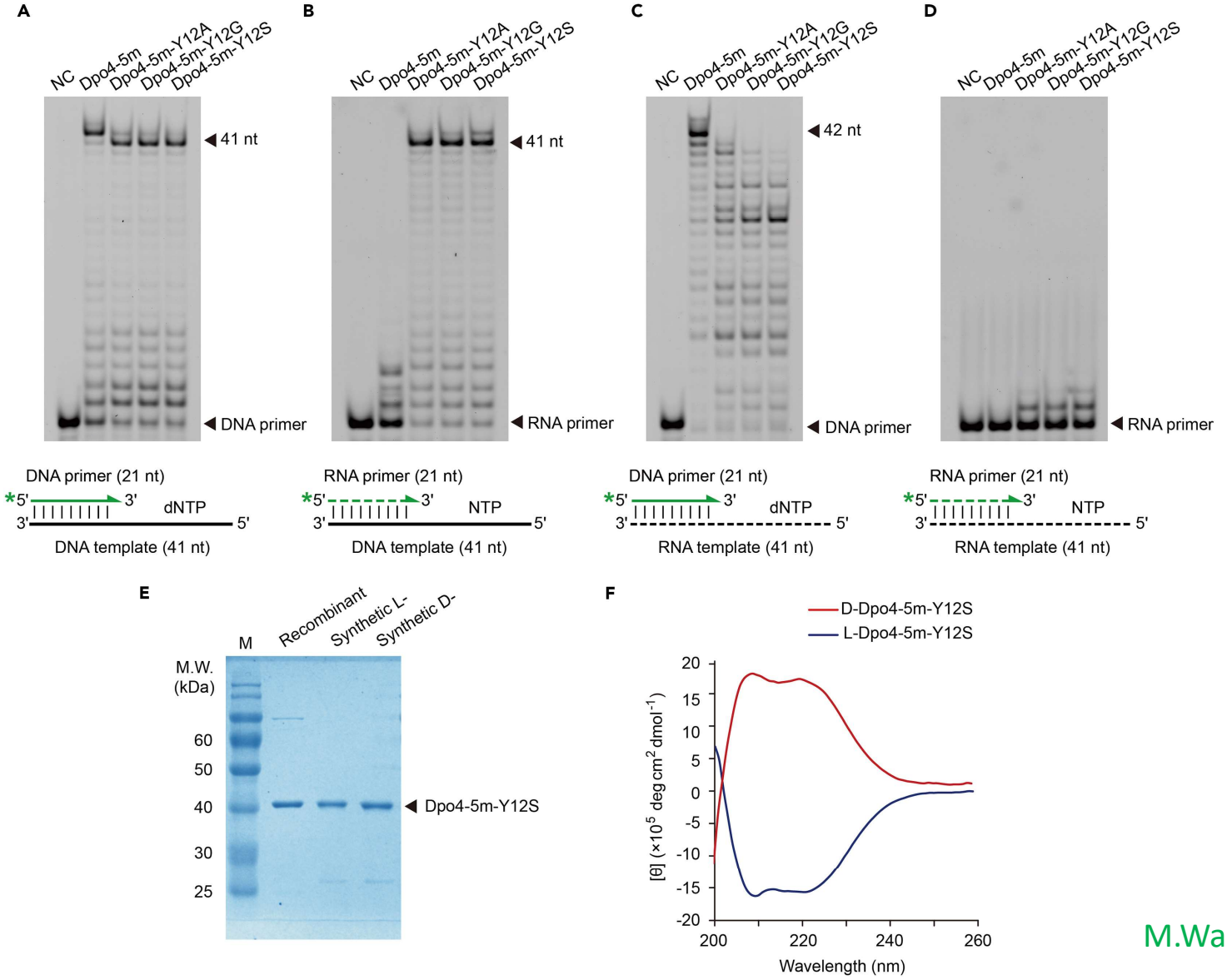


The transcription of a mirror-image gene into L-RNA, as well as reverse transcription of *L*-RNA into *L*-DNA by synthetic D-polymerases, based on designed mutants of Dpo4, have been demonstrated. The efficient mirror-image transcription system may enable enzymatic preparation of *L*-RNA molecules to further enable clinical applications of nuclease-resistant aptamer biosensors and drugs or studies on mirror-image or cross-chiral ribozymes and aptamers. The enzymatically transcribed ***L*-5S rRNA** shown in this study could be used as one component in a future effort to assemble a mirror-image ribosome, a step toward the realization of mirror-image life

***Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4)**

M.Wang *et al. Chem*. **2019**, *5 (4)*, 848-857

Mirror-image transcription, reverse transcription and amplification



Sulfolobus solfataricus P2
DNA polymerase IV (Dpo4)

M.Wang *et al. Chem*. **2019**, *5 (4)*, 848-857