Synthetic life

(continuation of „The molecular origins of life" SoSe 2020)

NaturalNews.com

WiSe 2020/21

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;

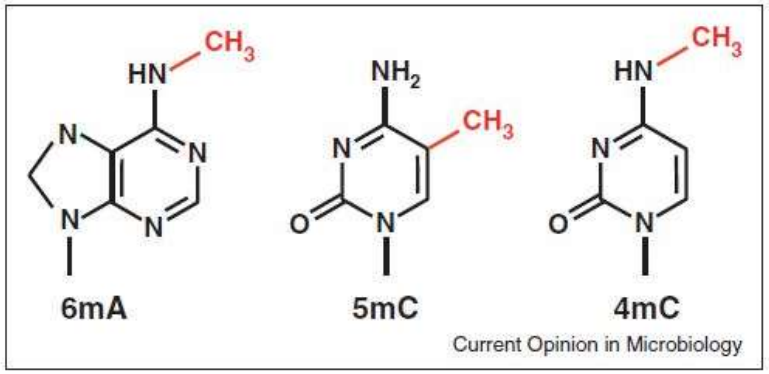
enzyme engineering – production of enzymes with unknown or unnatural properties, *ab initio* protein design, directed evolution, theozymes;

Artificial lipid vesicles as models for protocell multiplication;

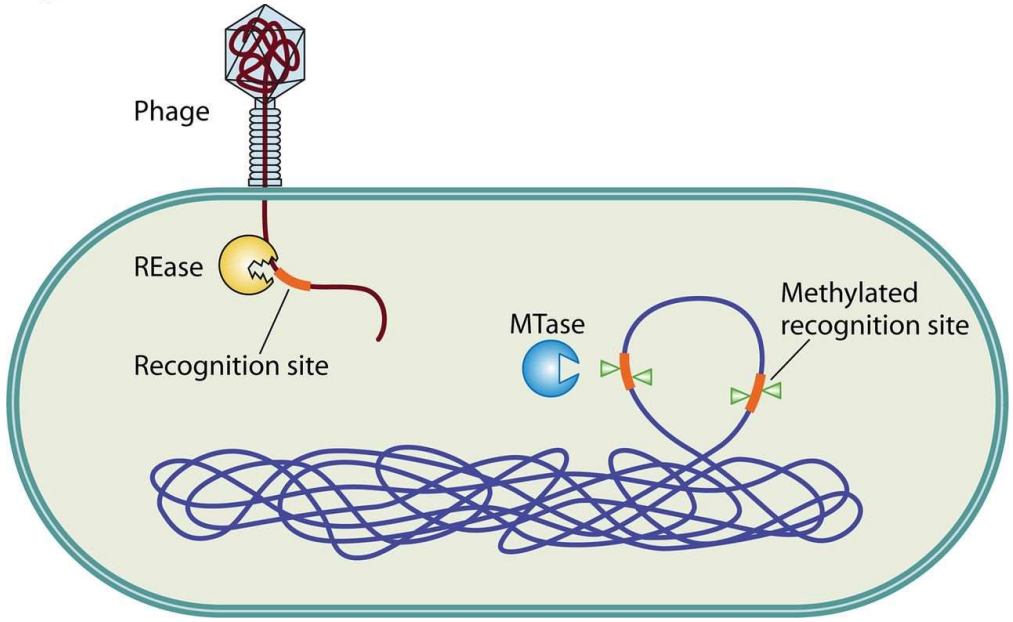
design of artificial organisms

Methylated NB - Restriction modification system

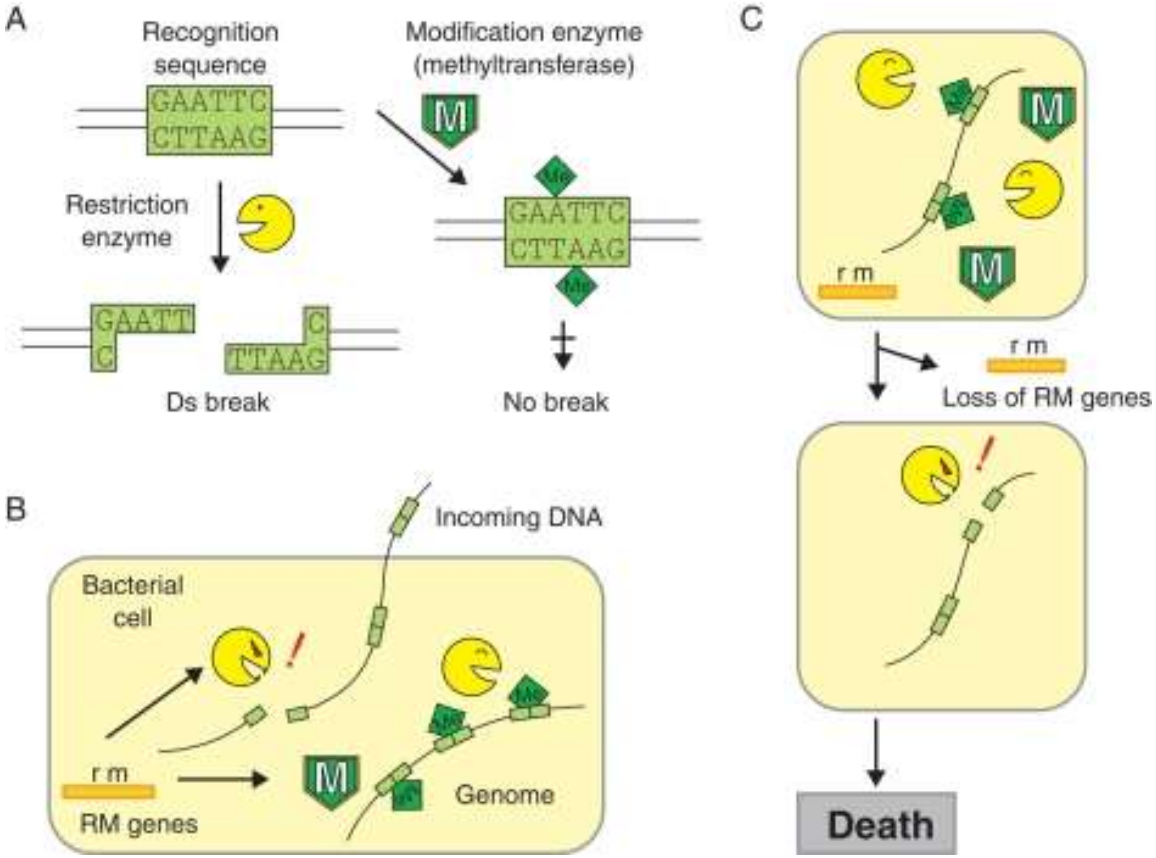
„Immune system" of bacteria and archaea against atacking viruses



Chemical structures of common modified bases generated by DNA methyltransferases.

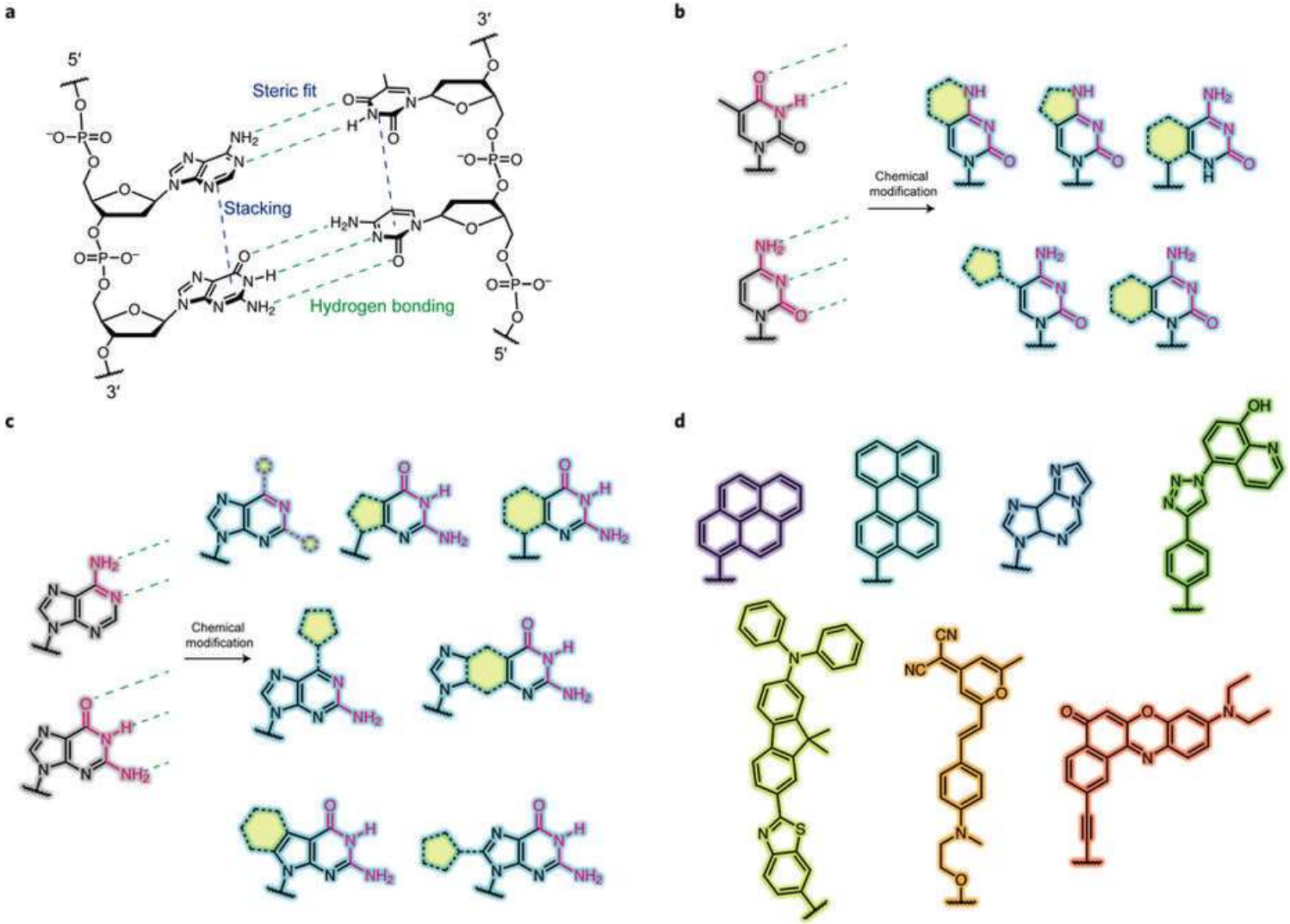


K. Vasu, V. Nagaraja
Microbiol. Mol. Biol. Rev. **2013**, *77(1)*, 53-72



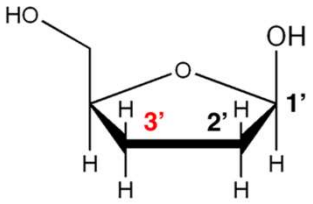
K. Ishikawa *et al. DOI: 10.1093/dnares/dsq027*

Non-canonical fluorescent nucleobases



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

Sanger sequencing



primer

5' 3'

TACGT

ATGCATTAGGGCCTGGCTCTTT

3' 5'

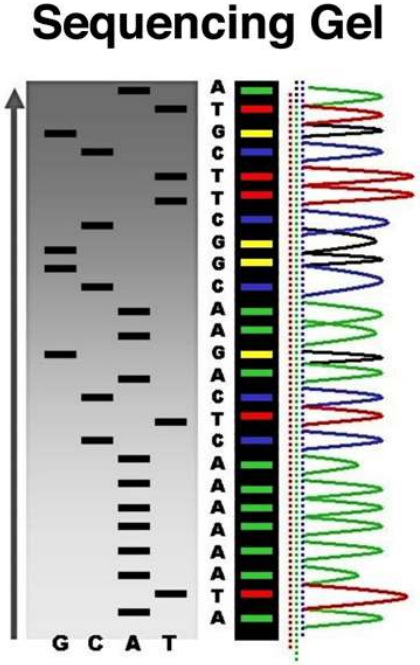
template

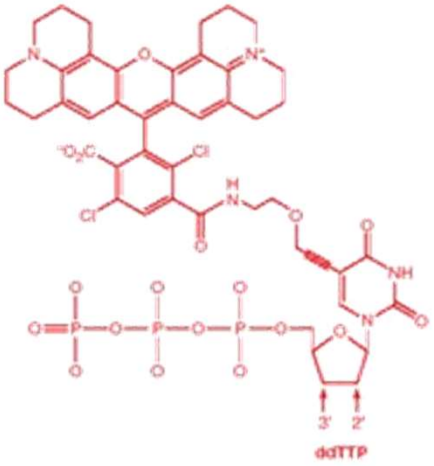
TACGTA
ATGCATTAGGGCCTGGCTCTTT

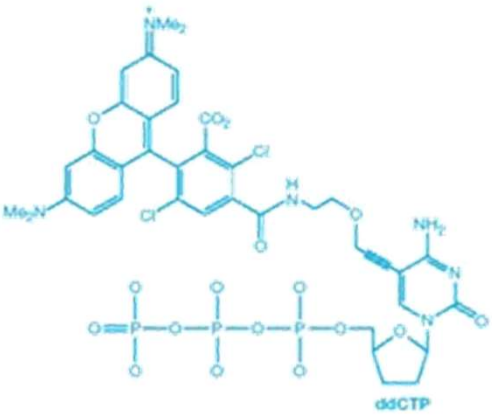
TACGTAA
ATGCATTAGGGCCTGGCTCTTT

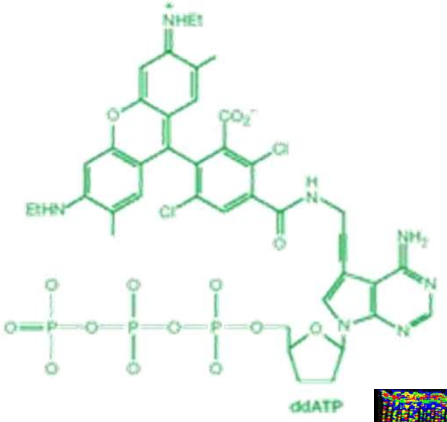
TACGTAATCCCGGA
ATGCATTAGGGCCTGGCTCTTT

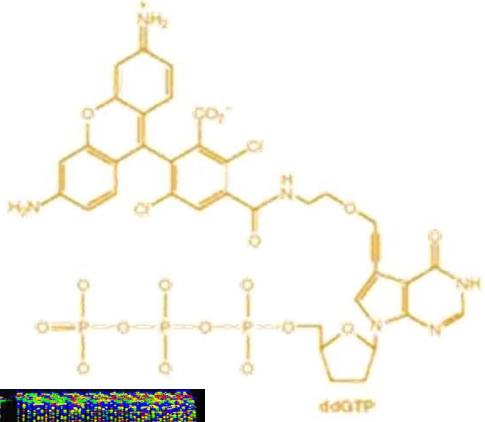
TACGTAATCCCGGACCGA
ATGCATTAGGGCCTGGCTCTTT

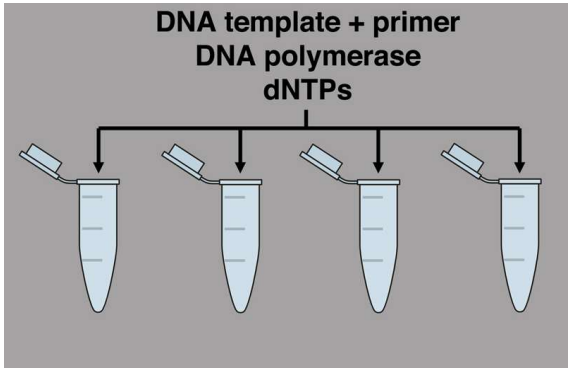


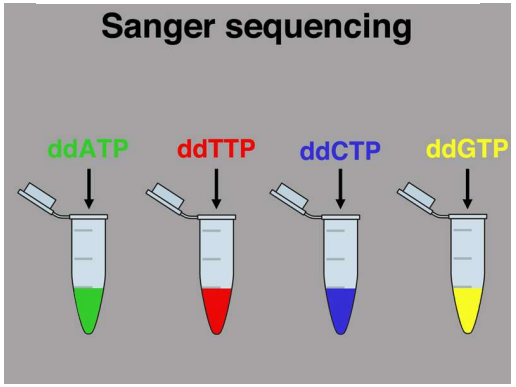


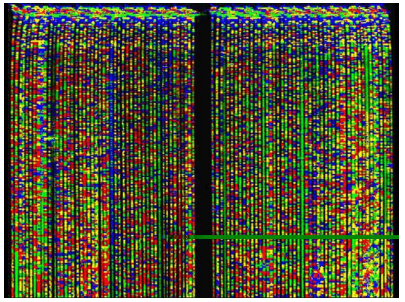




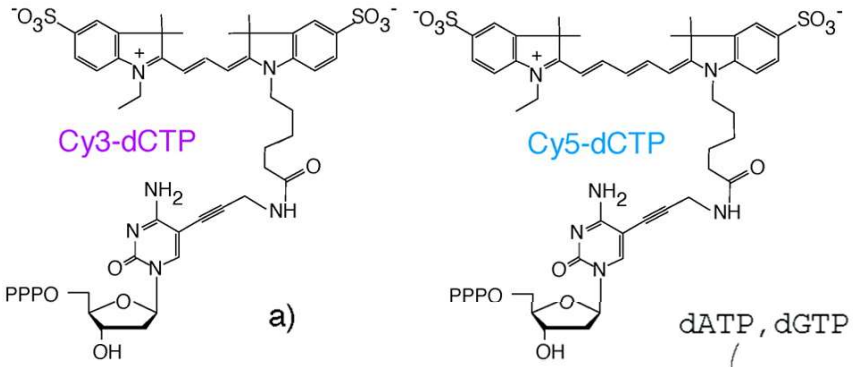


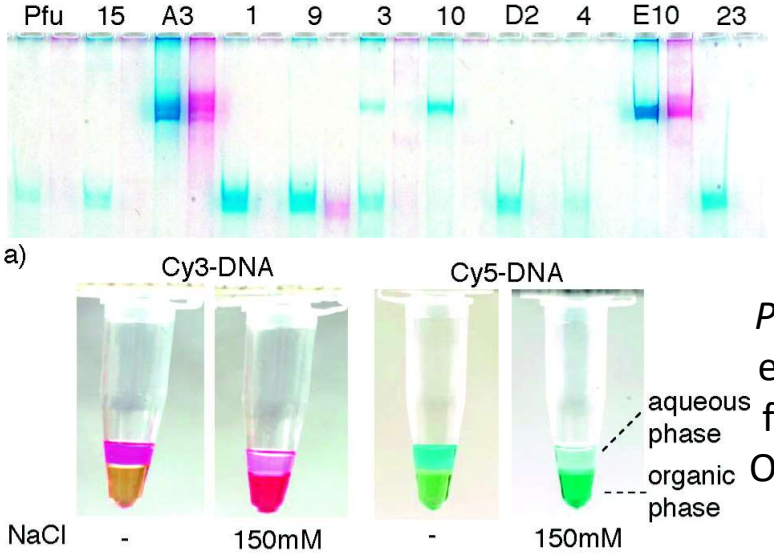




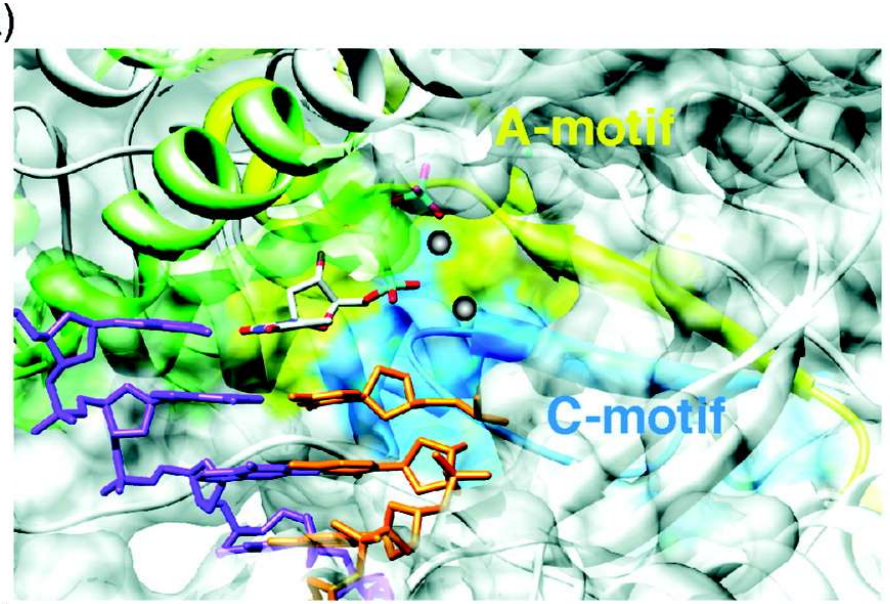


CyDNA – synthesis and replication of highly fluorescently-labelled DNA





Pyrococcus furiosus – an extremophilic *Archaeon* from marine sediments
Optimal life temperature 100^{0}C



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

CHAPTER 1

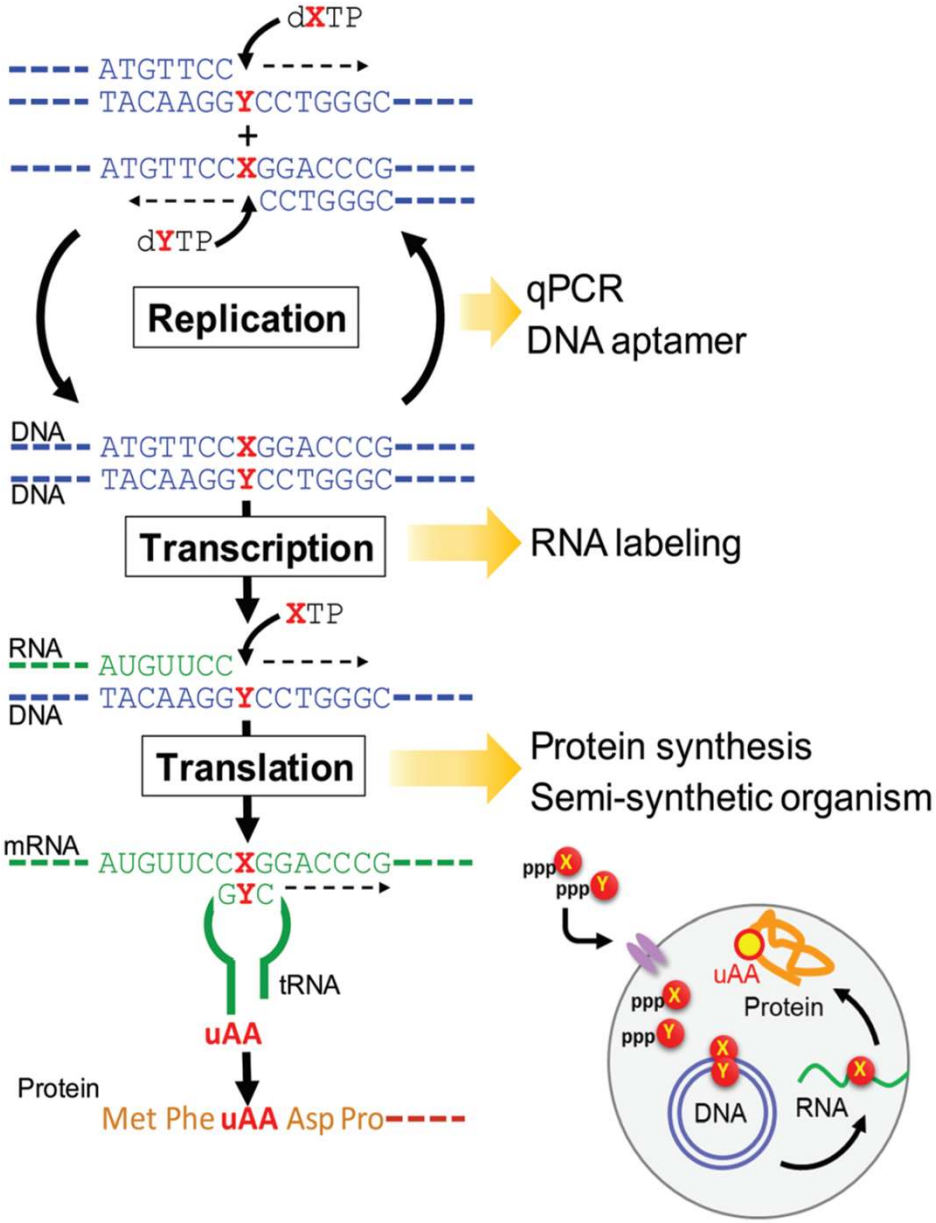
OLIGONUCLEOTIDES

Part 2 – noncanonical nucleobases

Expansion of the genetic alphabet

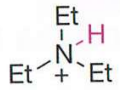
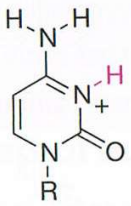
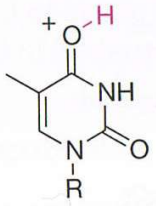
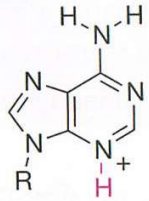
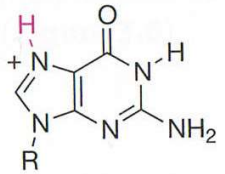
Expansion of the genetic alphabet and code by creating an unnatural base pair (UBP) as a third pair.

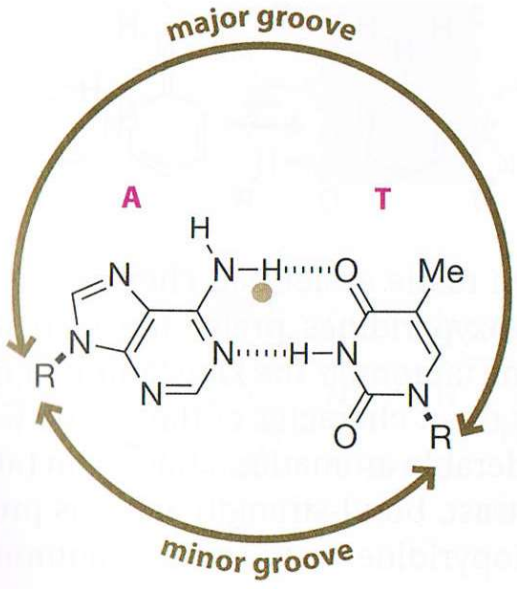
The creation of a UBP (i.e., X–Y) that functions in replication, transcription, and translation as a third base pair with the natural A–T(U) and G–C pairs allows the storage and retrieval of the expanded genetic information in vitro and in vivo, enabling a variety of applications using biopolymers with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

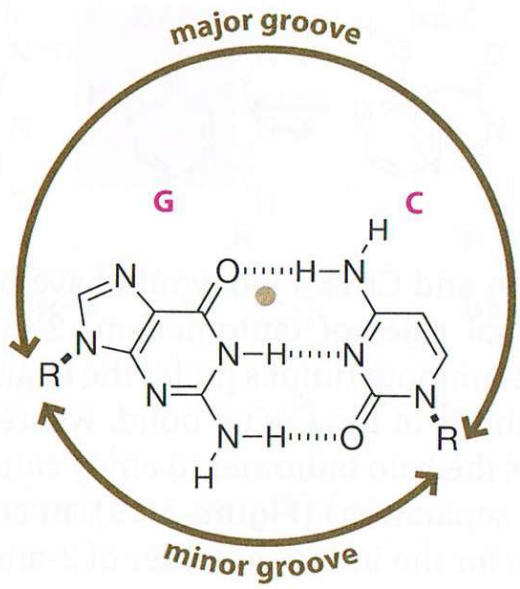


M. Kimoto, I. Hirao *Chem. Soc. Rev.* **2020,** *49,* 7602-7626

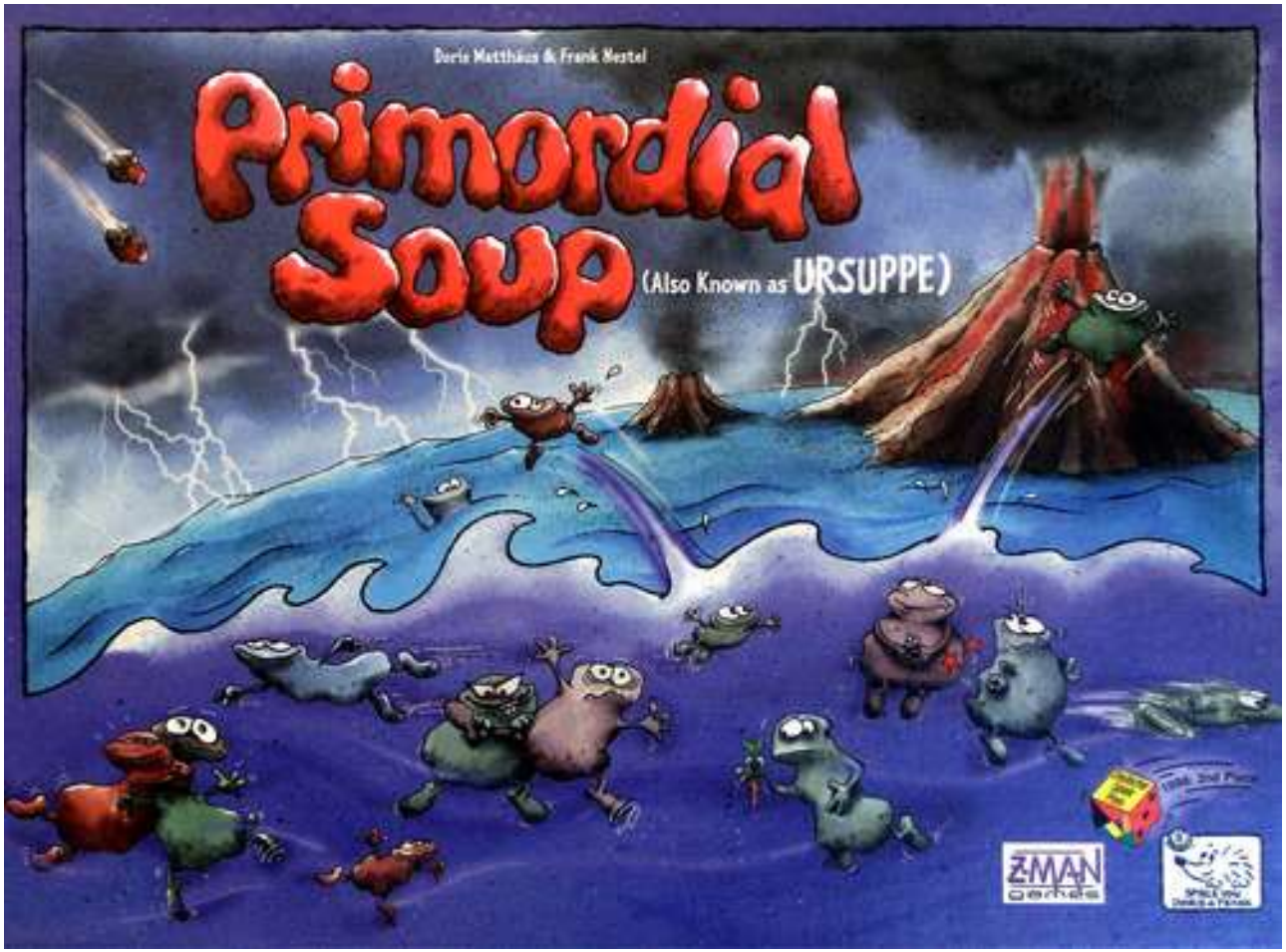
Why are A, C, G and T the letters of genetic alphabet.

	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





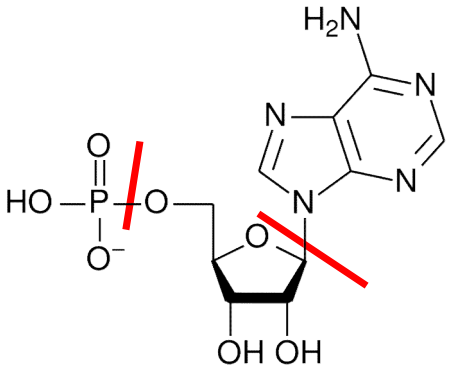
Prebiotic synthesis of nucleotides

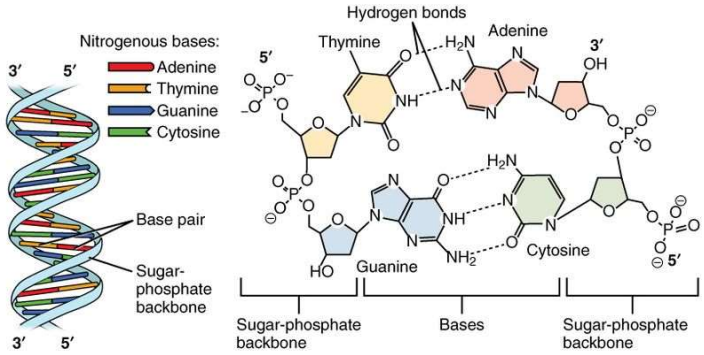


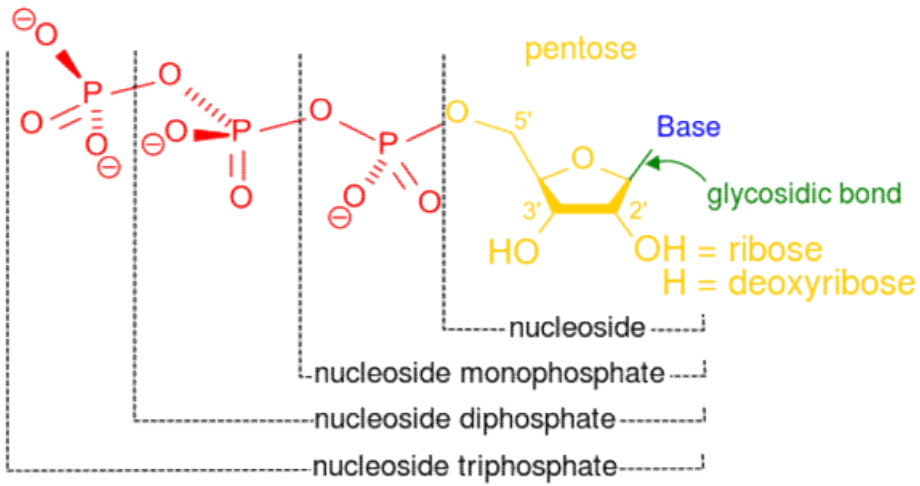
State of the art

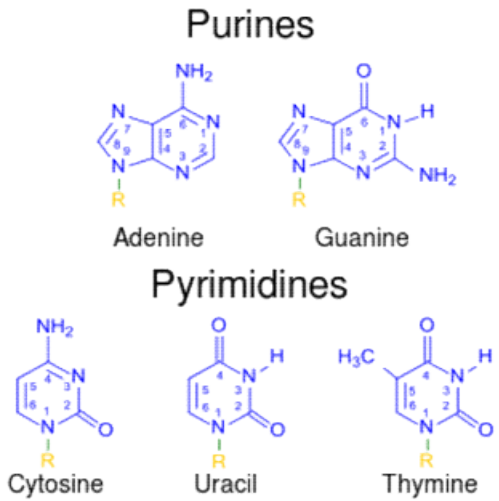
Nucleotides - components

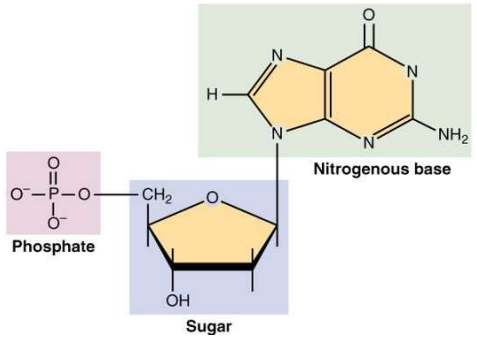
RNA – most likely evolutionarily older („RNA World") than DNA → prebiotic origin of ribose + A, C, G, and U nucleobases





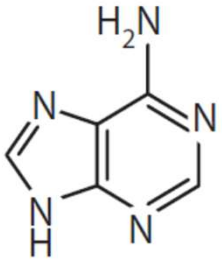




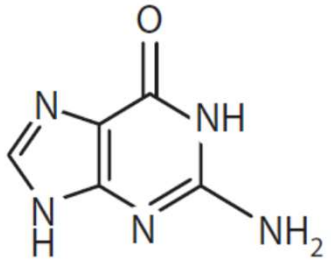


Prebiotic synthesis of nucleobases

Purines

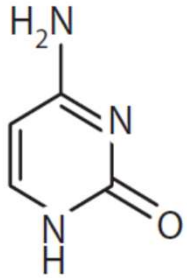


Adenine

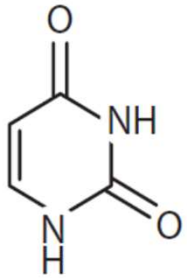


Guanine

Pyrimidines

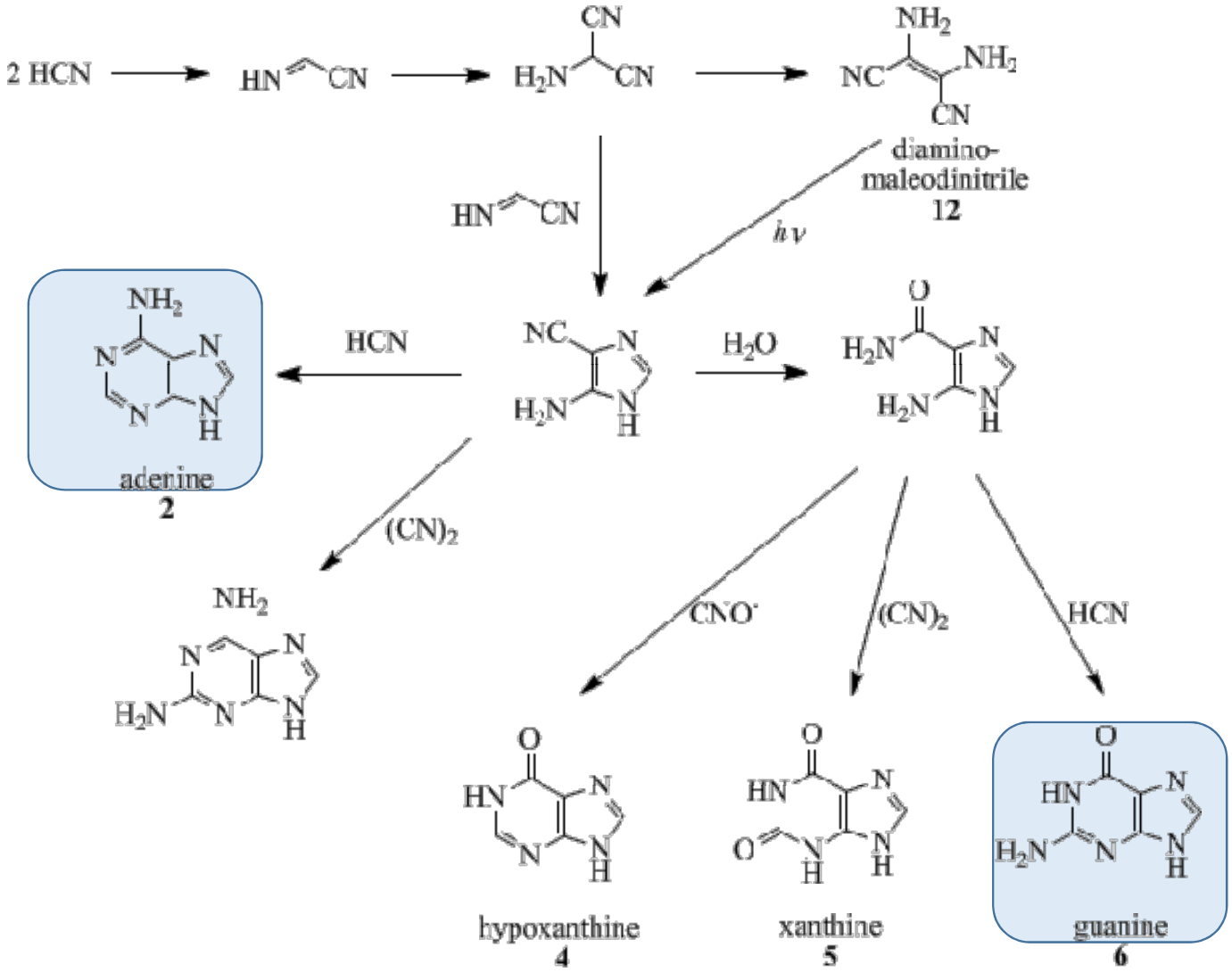


Cytosine

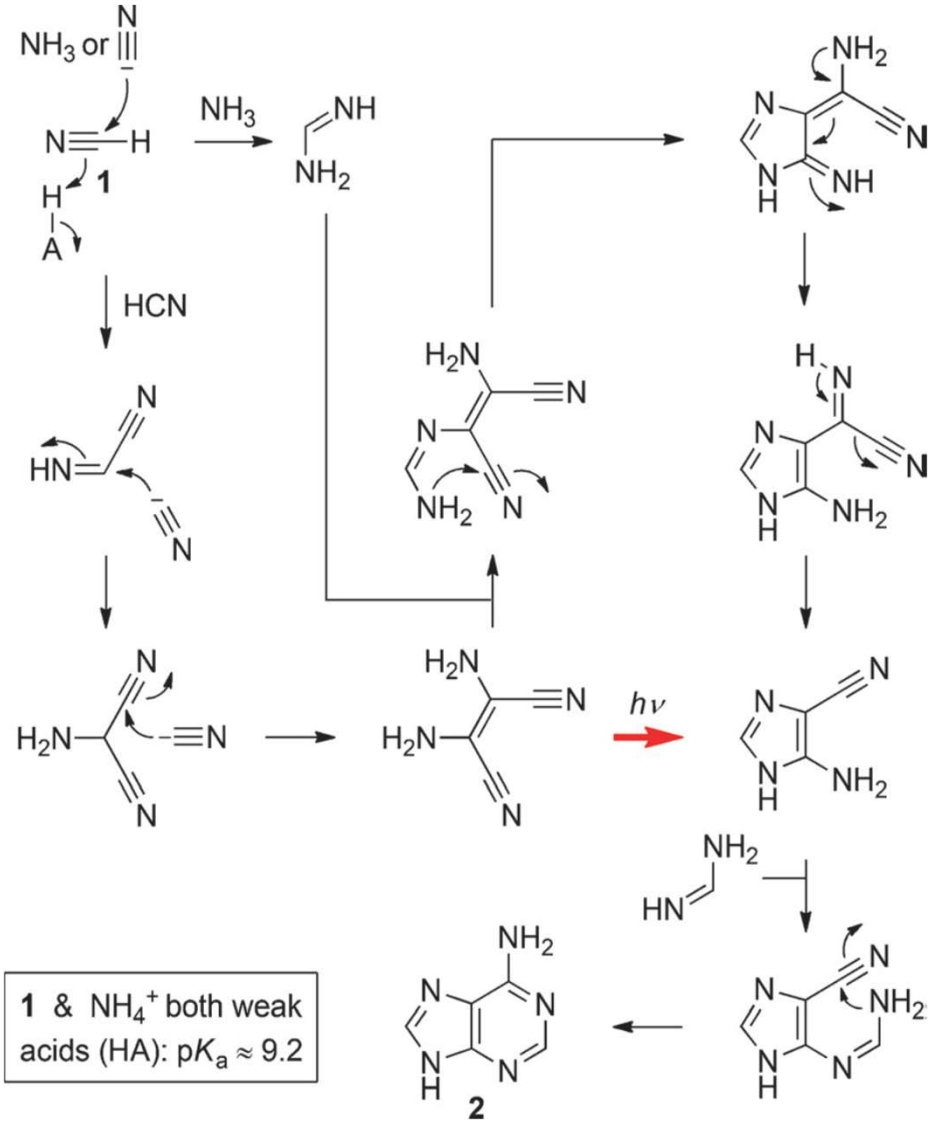


Uracil

Prebiotic synthesis of purines



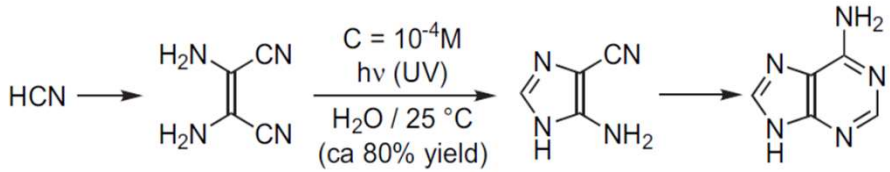
Prebiotic synthesis of adenine



1960 - Oró's synthesis of adenine **2** from hydrogen cyanide **1** and ammonia (general acid–base catalysis, presumed to operate in most steps, is only shown once).
Heating ammonium cyanide at 70^{0}C for a few days
→ 0.5% adenine
Heating HCN with liquid ammonia in a sealed tube → 20% adenine

The photochemical shortcut discovered by Ferris and Orgel is shown by the red arrow.
Optimized yields – up to 20% for adenine, 3% for guanine

Eutectic freezing (-20^{0}C) increases the yield of DAMN formation by concentrating HCN between pure ice crystals

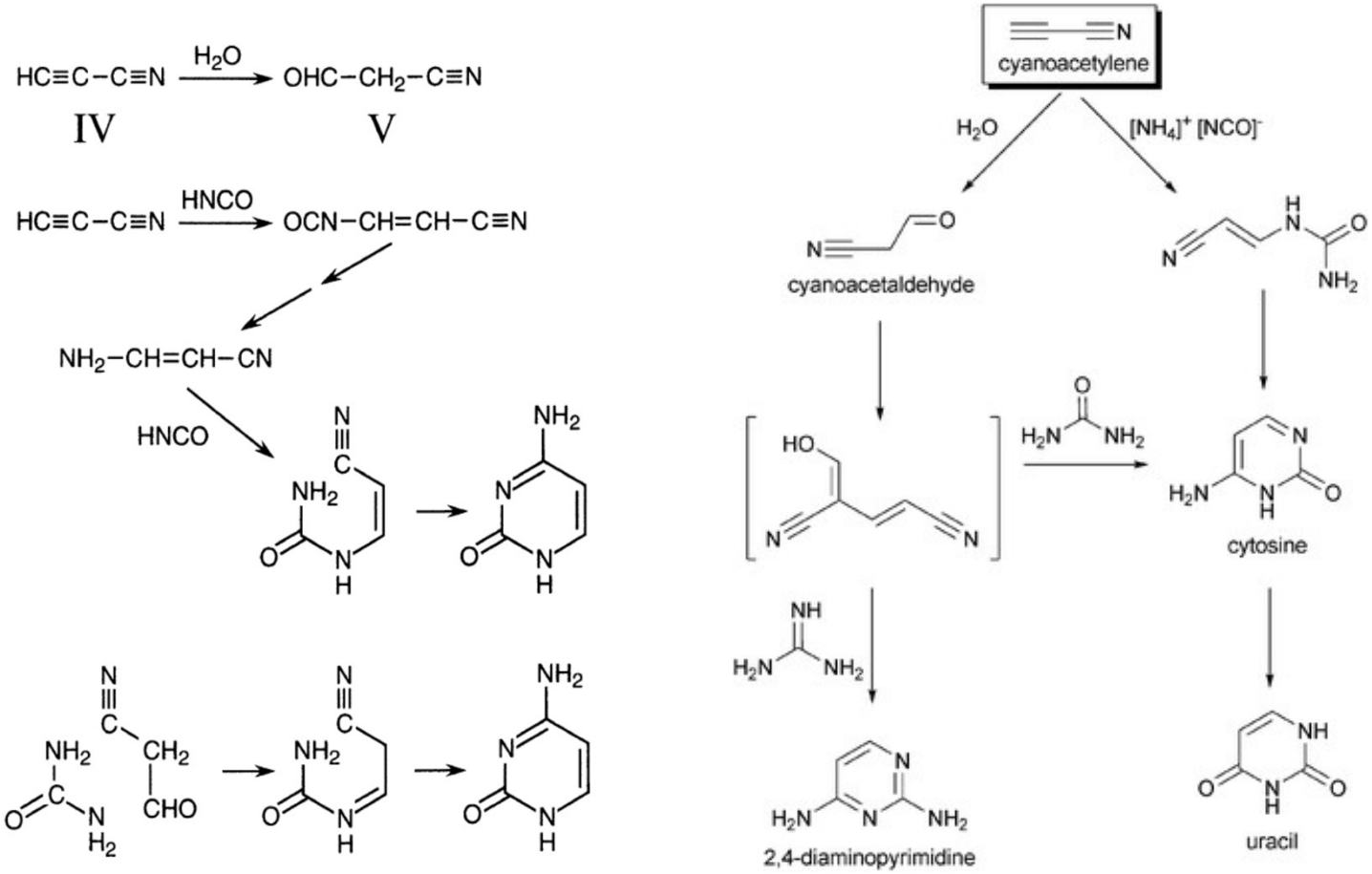


J. Oro *Biochem. Biophys. Res. Commun.* **1960**, *2*, 407.

J. P. Ferris, L. E. Orgel, *J. Am. Chem. Soc.* **1966**, *88*, 1074

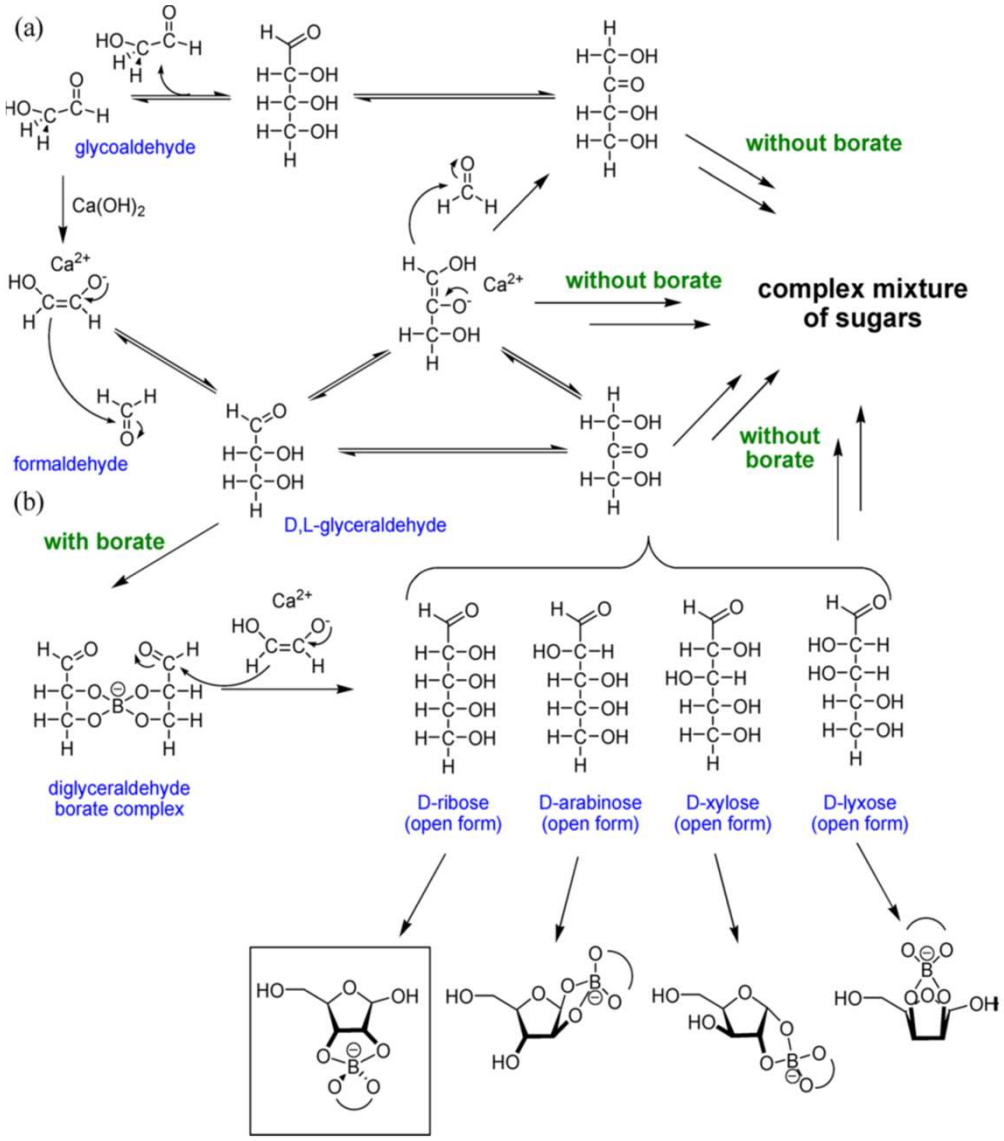
Prebiotic synthesis of pyrimidines

Cyanoacetylene is a major product of electric discharges in the mixture of nitrogen and methane



Cyanoacetylene incubated with saturated solution of urea yields up to 50% cytosine.
Other methods typically yield up to 5% cytosine.It is further converted to uracil by hydrolysis.

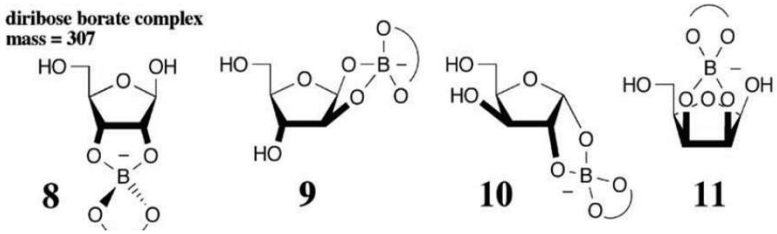
Formose reaction in presence of borates



Pentose formation in the presence of borate

With borate (left)
Without borate (right)
Colemanite (background)

By NMR, the ribose borate complex **8** has the structure shown; cyclic structures for other pentoses are speculative.

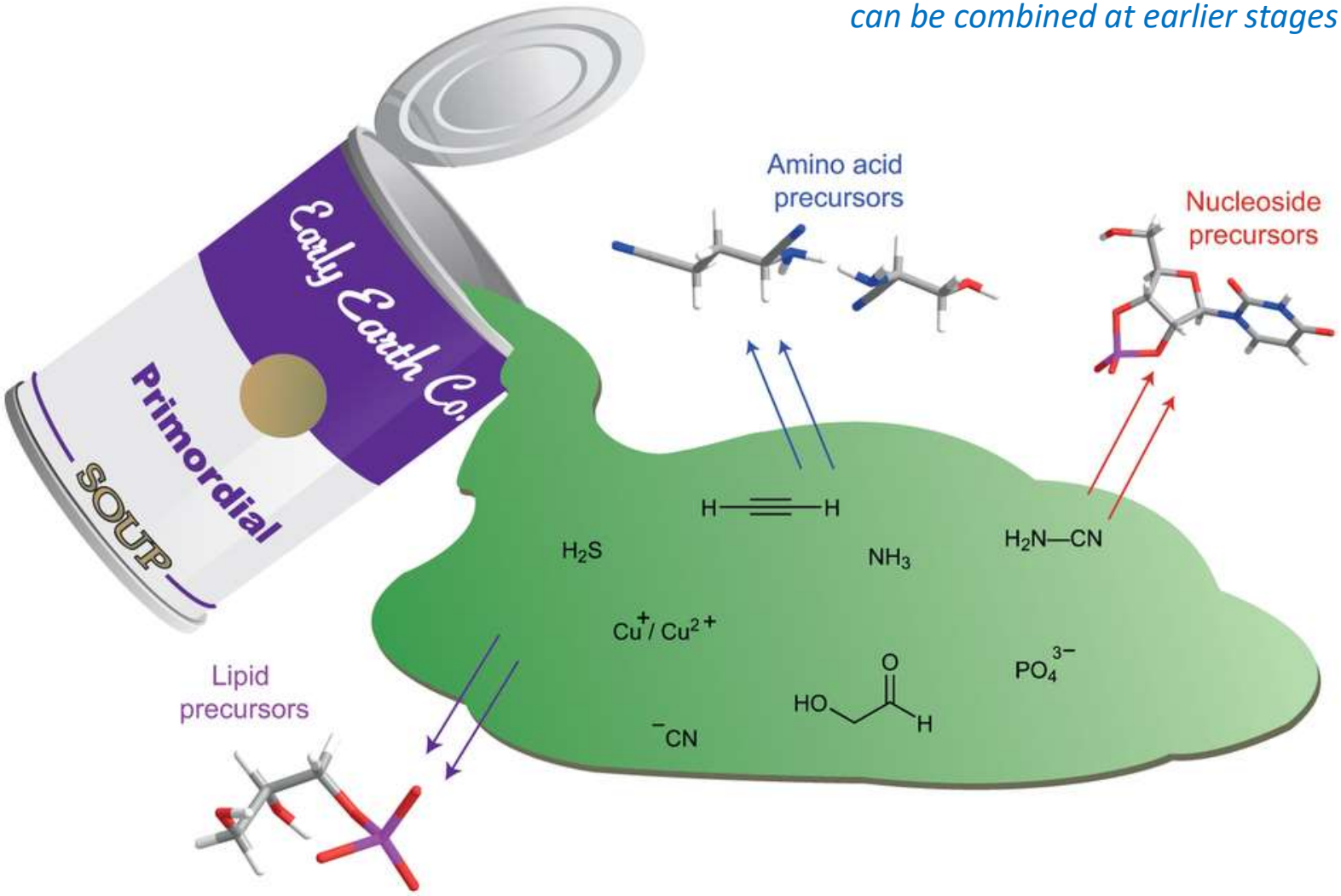


A. Ricardo, M. A. Carrigan, A. N. Olcott, S. A. Benner
Science **2004**, *303*, 196

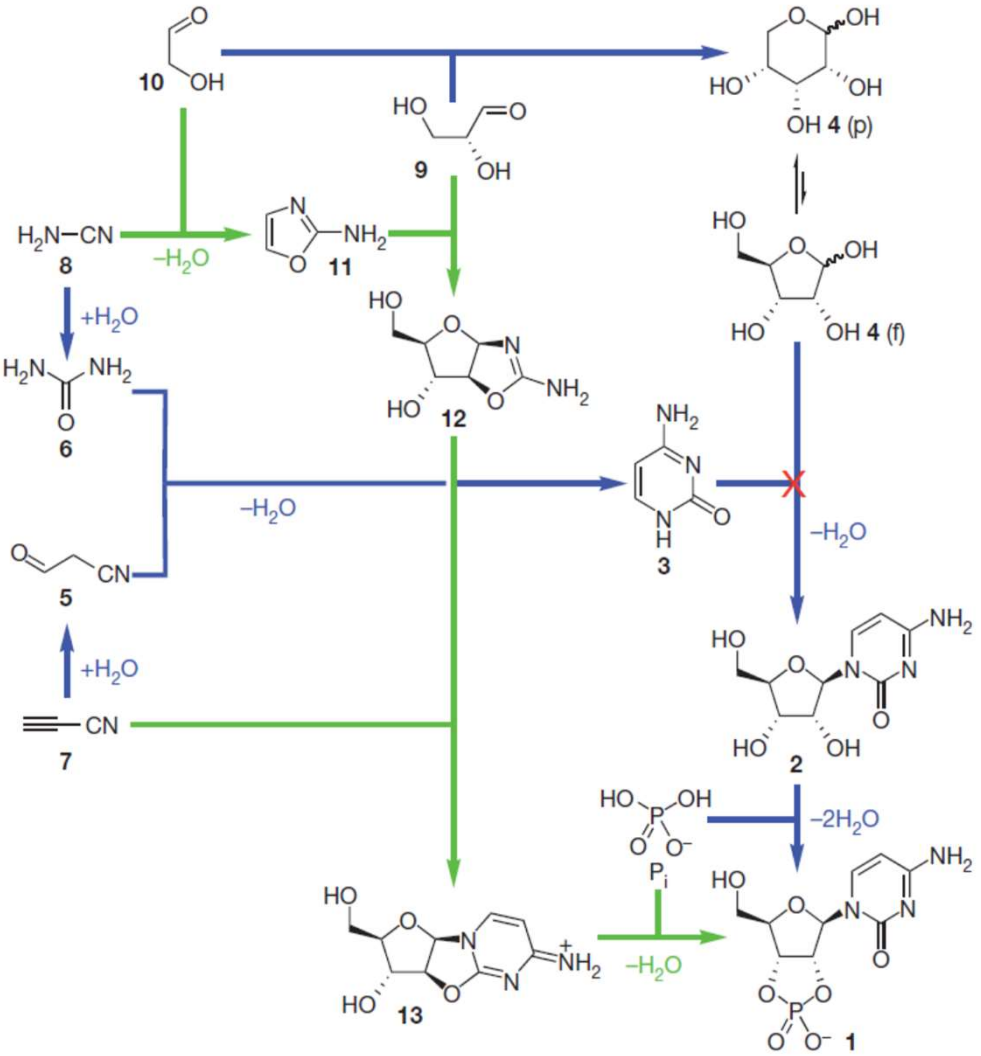
Escosura

Cyanosulfidic chemistry

The aldol chemistry of sugars and cyanide chemistry of nucleobases can be combined at earlier stages than glycosylation.

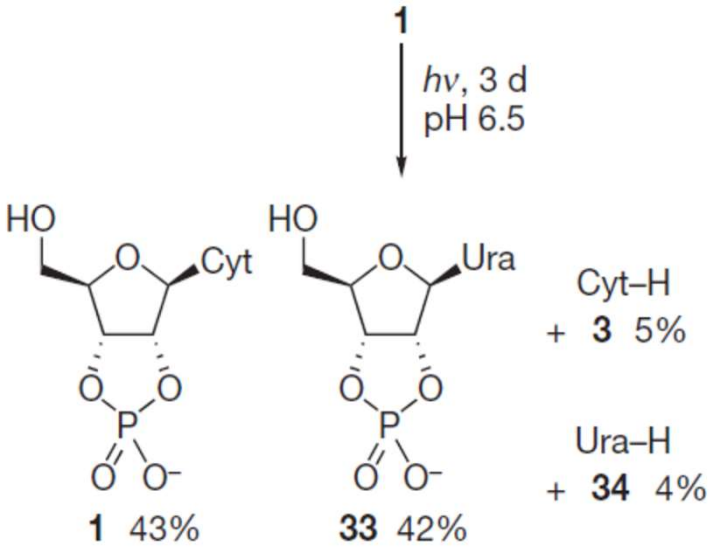


Cyanosulfidic chemistry



M. W. Powner, B. Gerland, J. D. Sutherland, *Nature* **2009**, *459*, 239–242

Cyanosulfidic chemistry



Photochemistry of *beta*-ribocytidine-2',3'-cyclic phosphate **1**. Under conditions of irradiation that destroy most other pyrimidine nucleosides and nucleotides, **1** undergoes partial hydrolysis and slight nucleobase loss. Ura, N1-linked uracil; Cyt–H, cytosine; Ura–H, uracil.

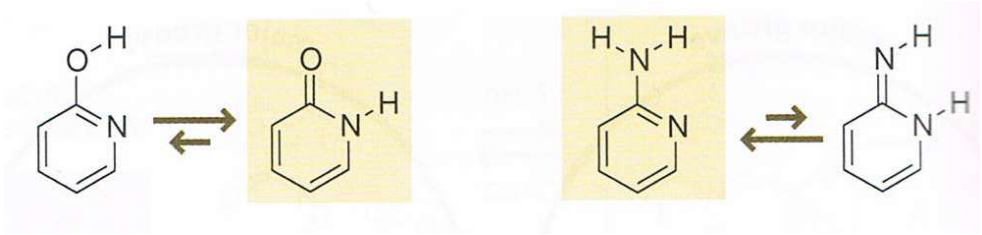
M. W. Powner, B. Gerland, J. D. Sutherland, *Nature* **2009**, *459*, 239–242

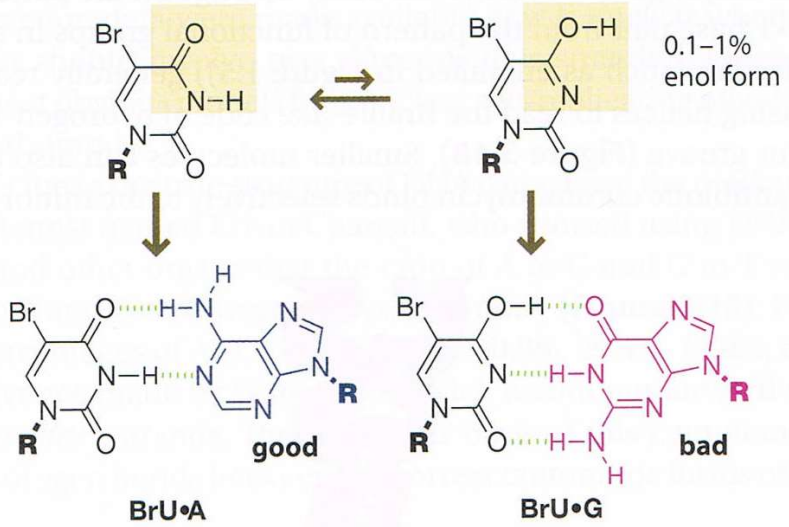
J. D. Sutherland, *Angew. Chem. Int. Ed.* **2016**, *55*, 104-121.

B. H. Patel, C. Percivalle, D. J. Ritson, C. D. Duffy, J. D. Sutherland, *Nat. Chem*. **2015**, *7*, 301–307.

J. D. Sutherland, *et al. Nat. Chem*. **2013**, *5*, 383–389.

Biological consequences of nucleobase modifications





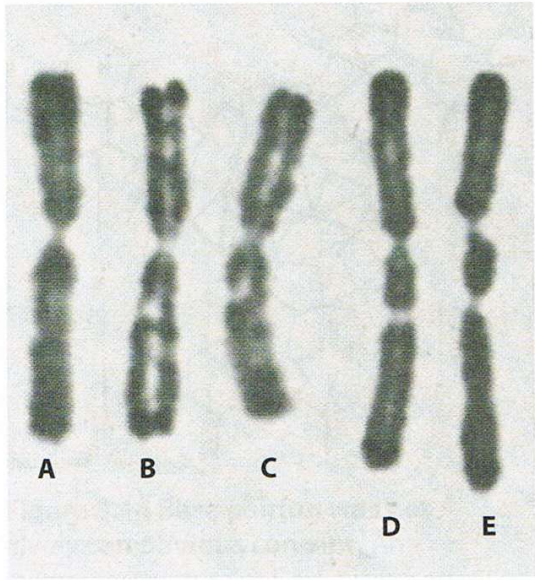
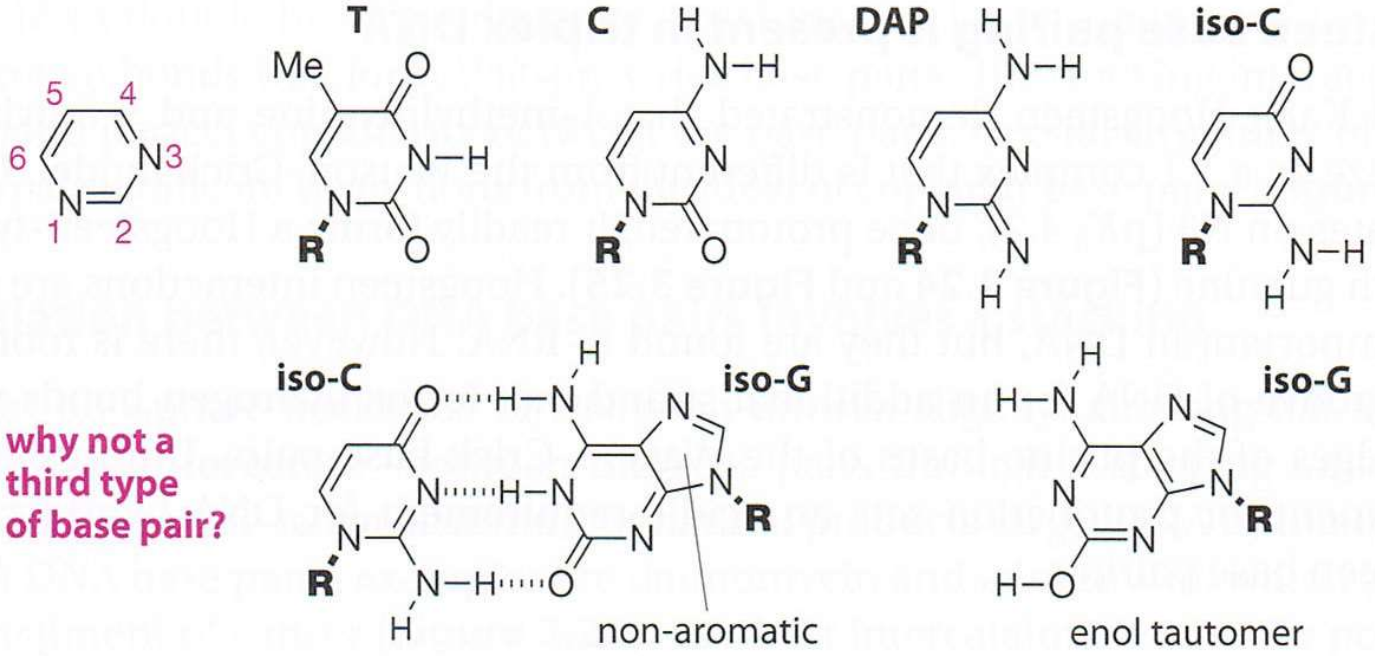
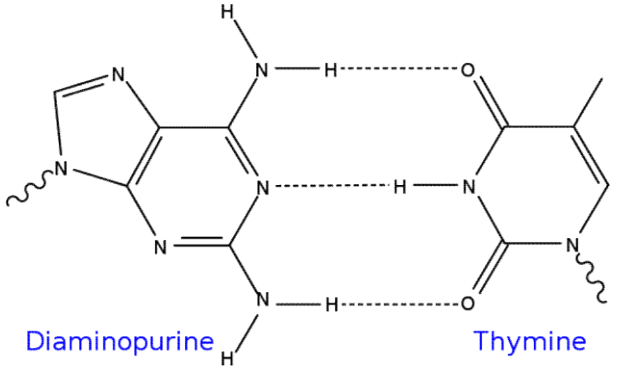


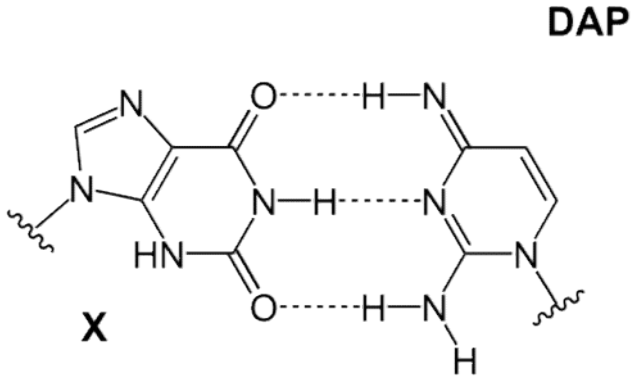
Figure 3.20 Chromosome 1 from hamster cells exposed to bromodeoxyuridine. (A) Normal chromosome. (B–E) Aberrant chromosomes. (From T.C. Hsu and C.E. Somers, *Proc. Natl. Acad. Sci. USA* 47: 396–403, 1961. With permission from the MD Anderson Cancer Center.)

Alternative base pairs – synthetic biology



Cyanophage S-2L uses diaminopurine instead of adenine (3 H-bonds!)



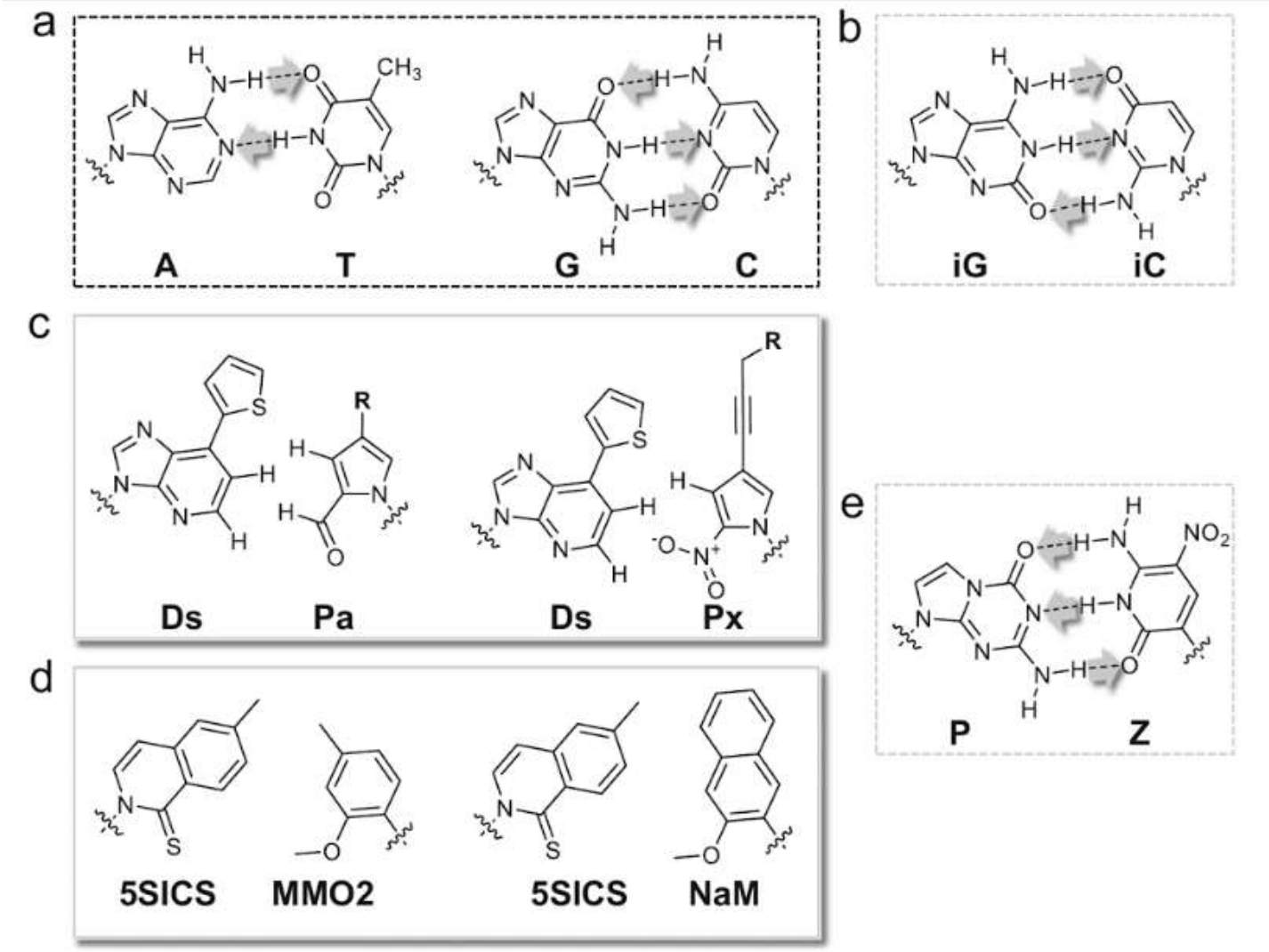


DAP – one tautomer forms a base pair with guanine

iso-C/iso-G

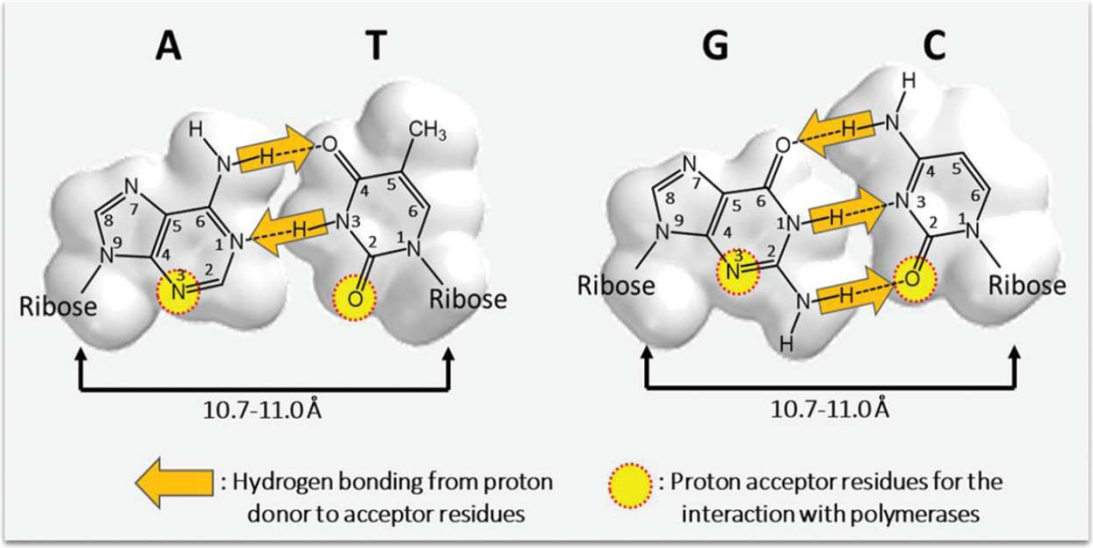
- specificity (the enol tautomer of iso-G, stabilized by aromatization, complementary to thymine)
- the 2-amino group of iso-C hydrolyses easily to uracil

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



Unnatural base pair (UBP) design rules:

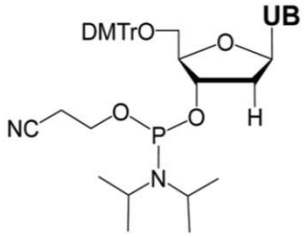
Design



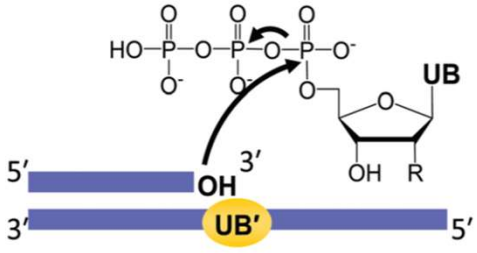
- Distance of 10.7-11.0 Å between the glycosidic bonds of the pair
- no mispairing with natural bases
- Reasonable hydrophobicity
- Chemical stability (phosphoramidite chemistry)
- Recognition by DNA and RNA polymerases (fidelity, efficiency)

Chemical synthesis

Phosphoramidite reagents
for DNA chemical synthesis

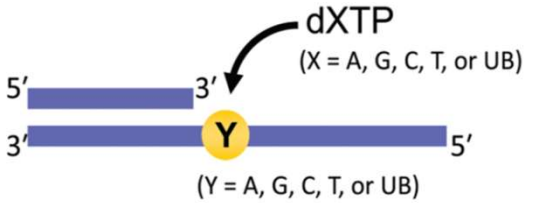


Nucleoside triphosphates
(R = H: d(UB)TP, R = OH: (UB)TP)

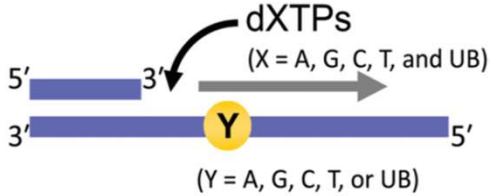


Polymerase reaction tests

Single-nucleotide insertion

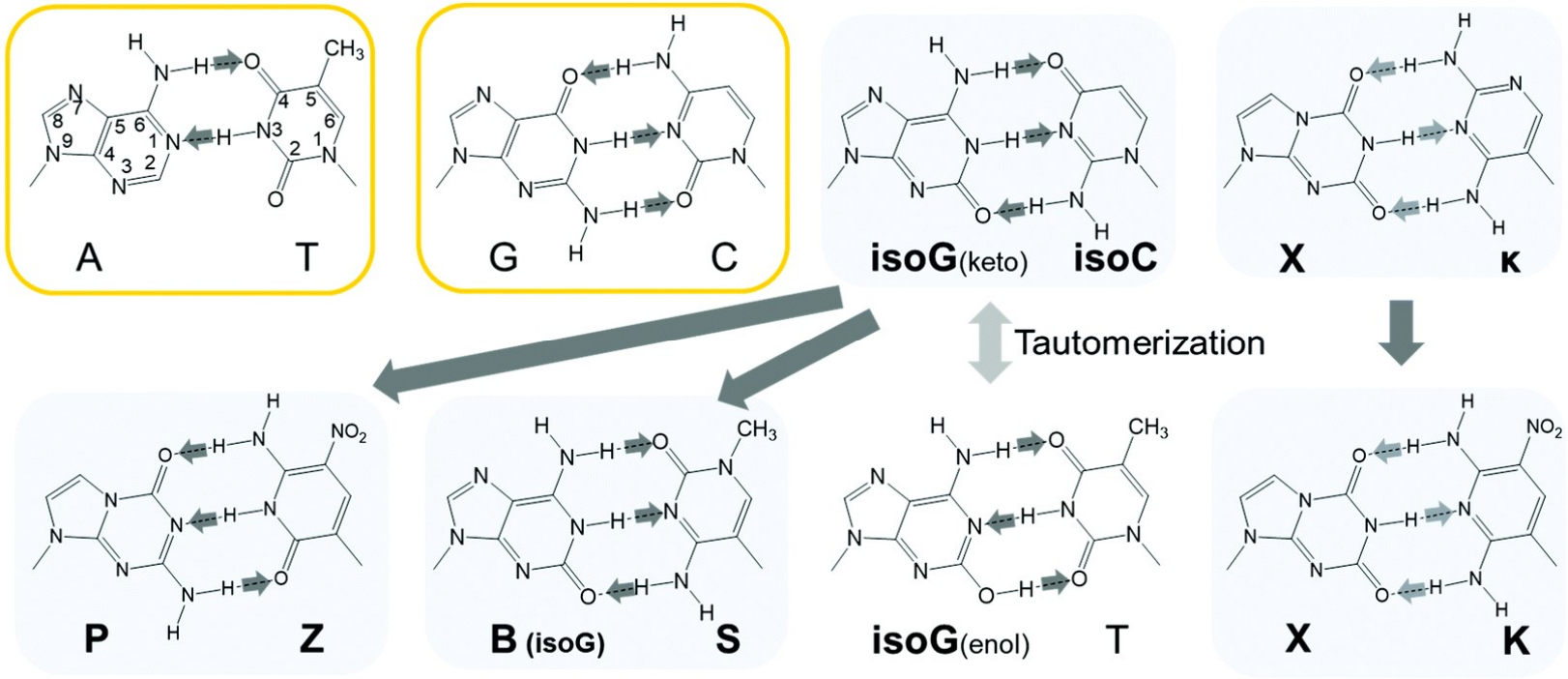


Primer extension



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

Non-natural nucleobases compatible with polymerases



Alexander Rich (1962): isoG–isoC pair

Steven Benner (1989-95): the artificially expanded genetic information system (AEGIS) including the isoG–isoC and X–κ pairs → *in vitro* replication, transcription, and translation systems.

Benner and Prudent (2004): new quantitative PCR (qPCR) methods, such as Plexor, using the isoG–isoC pair

Benner (2007): Z-P pair - the P base by removing the hydrogen at position 1 of G to exclude the keto–enol tautomerism, by introducing the nitro group into Z, the chemical stability of the nucleoside was improved;

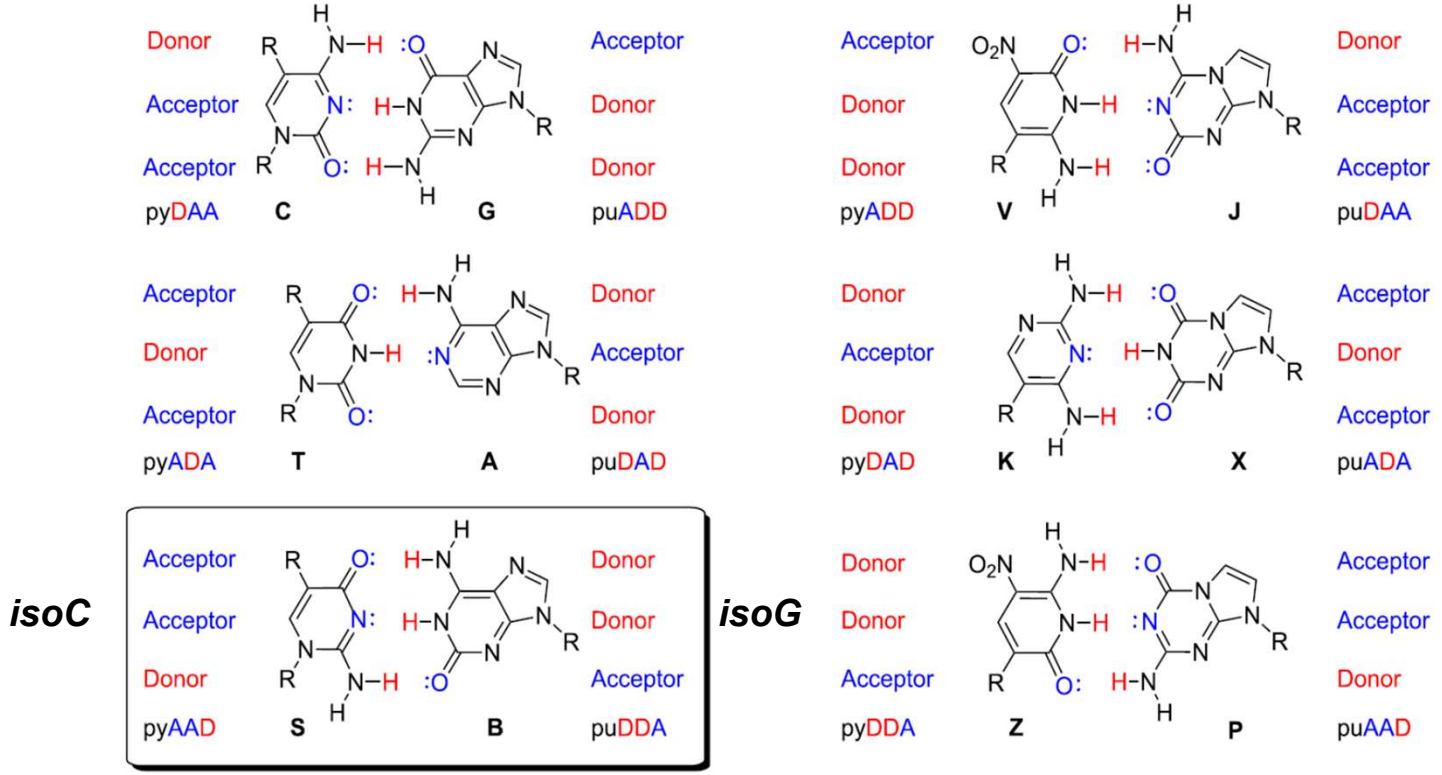
With the same strategy, they also developed the B–S and X–K pairs from isoG–isoC and X–κ pairs, respectively

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

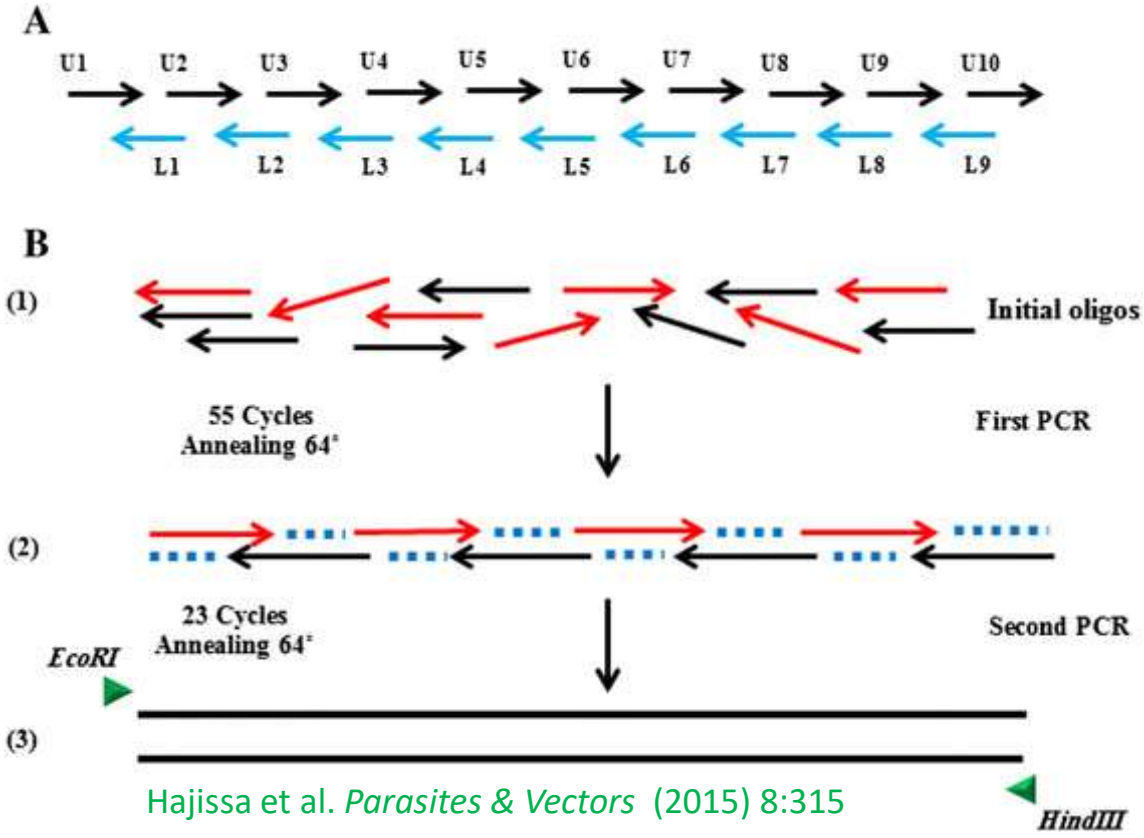
Artificial Gene Synthesis

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:

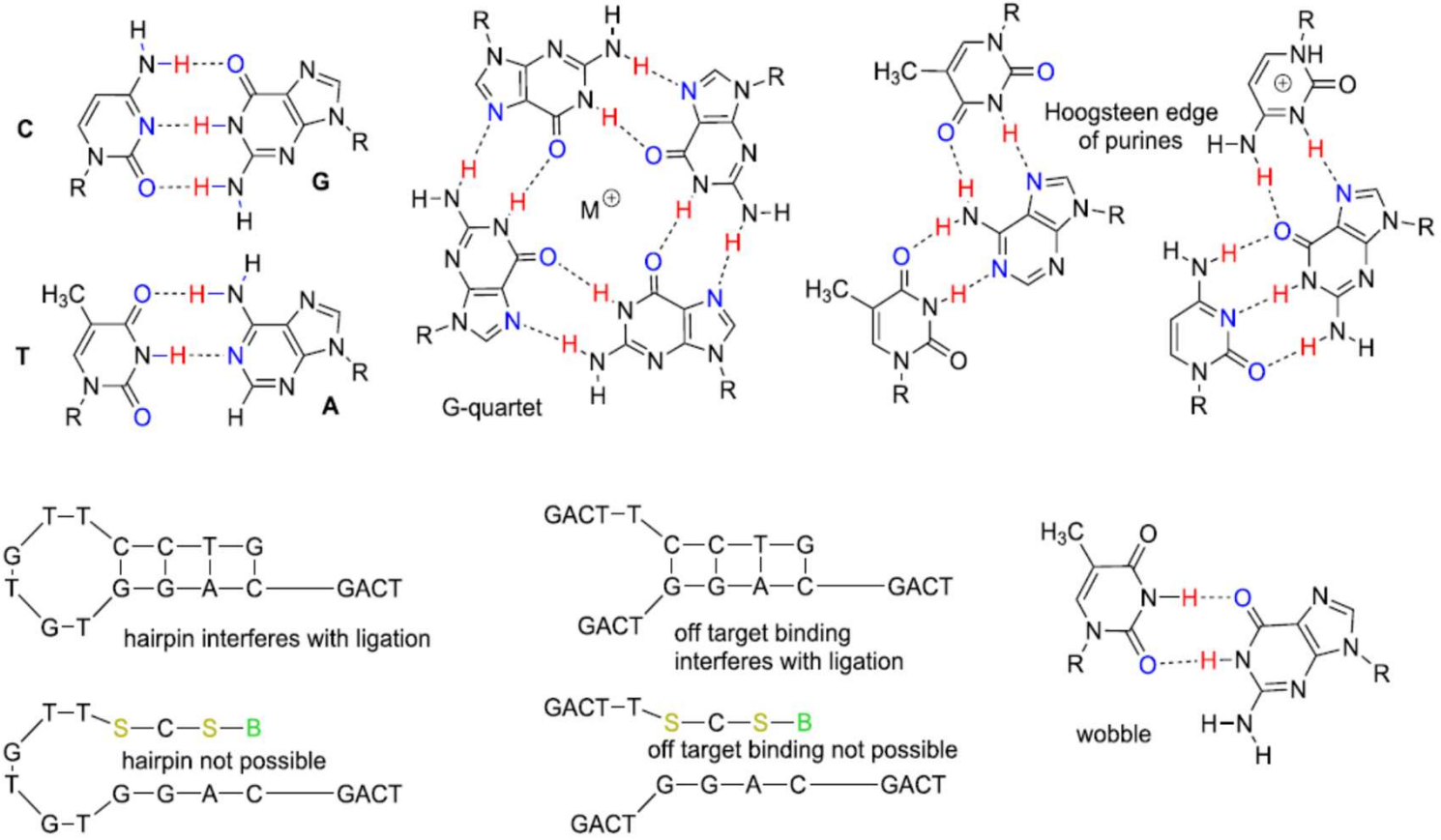
- recombinant DNA technology including heterologous gene expression, vaccine development, gene therapy and molecular engineering.
- The synthesis of nucleic acid sequences can be more economical than classical cloning and mutagenesis procedures
- the ability to safely obtain genes for vaccine research 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

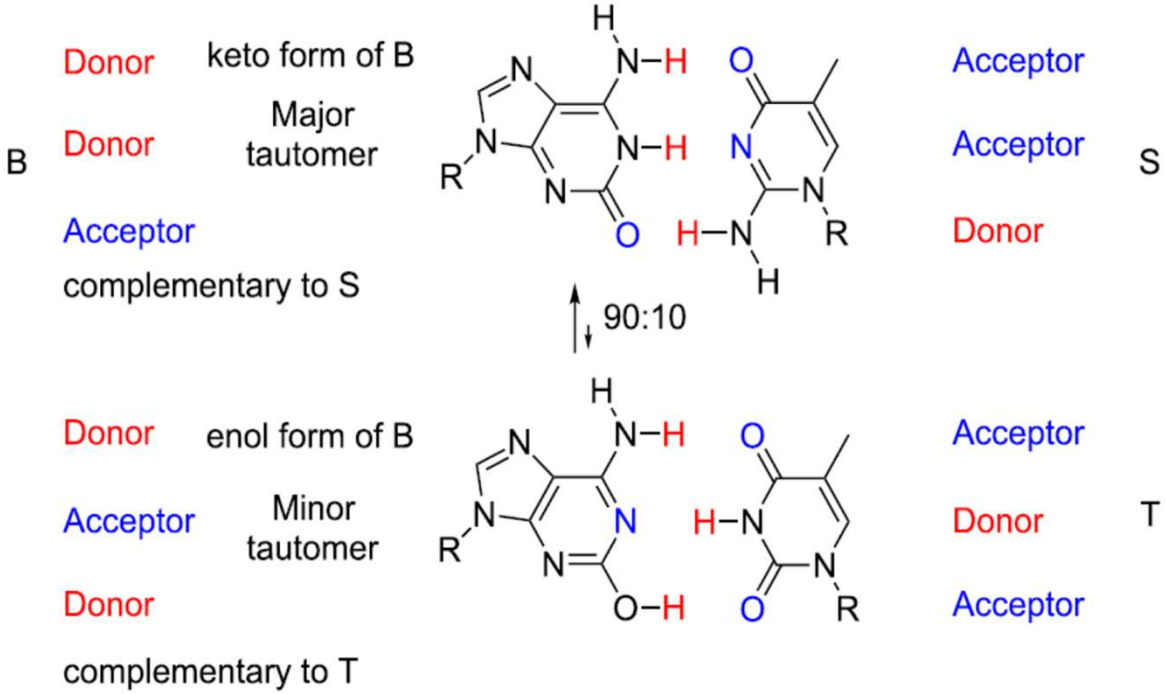


S. Benner *et al., Beilstein J. Org. Chem.* **2014,** *10,* 2348–2360. doi:10.3762/bjoc.10.245

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

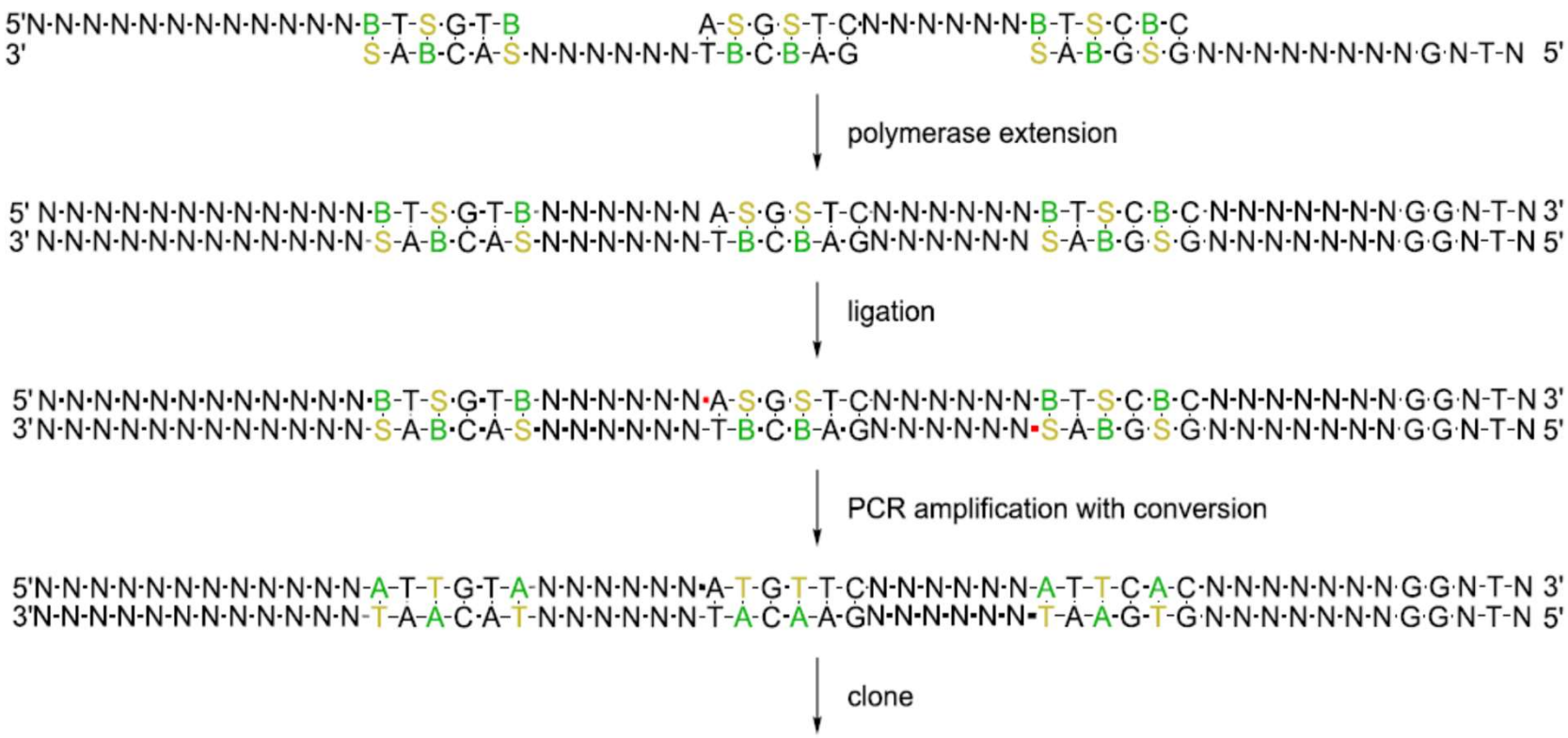
Conversion occurs when polymerases are forced to mismatch a standard nucleotide opposite an AEGIS nucleotide by
(a) not being provided the complementary AEGIS triphosphate and
(b) exploiting a chemical feature of the AEGIS nucleotide that directs a specific mismatch.



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).

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

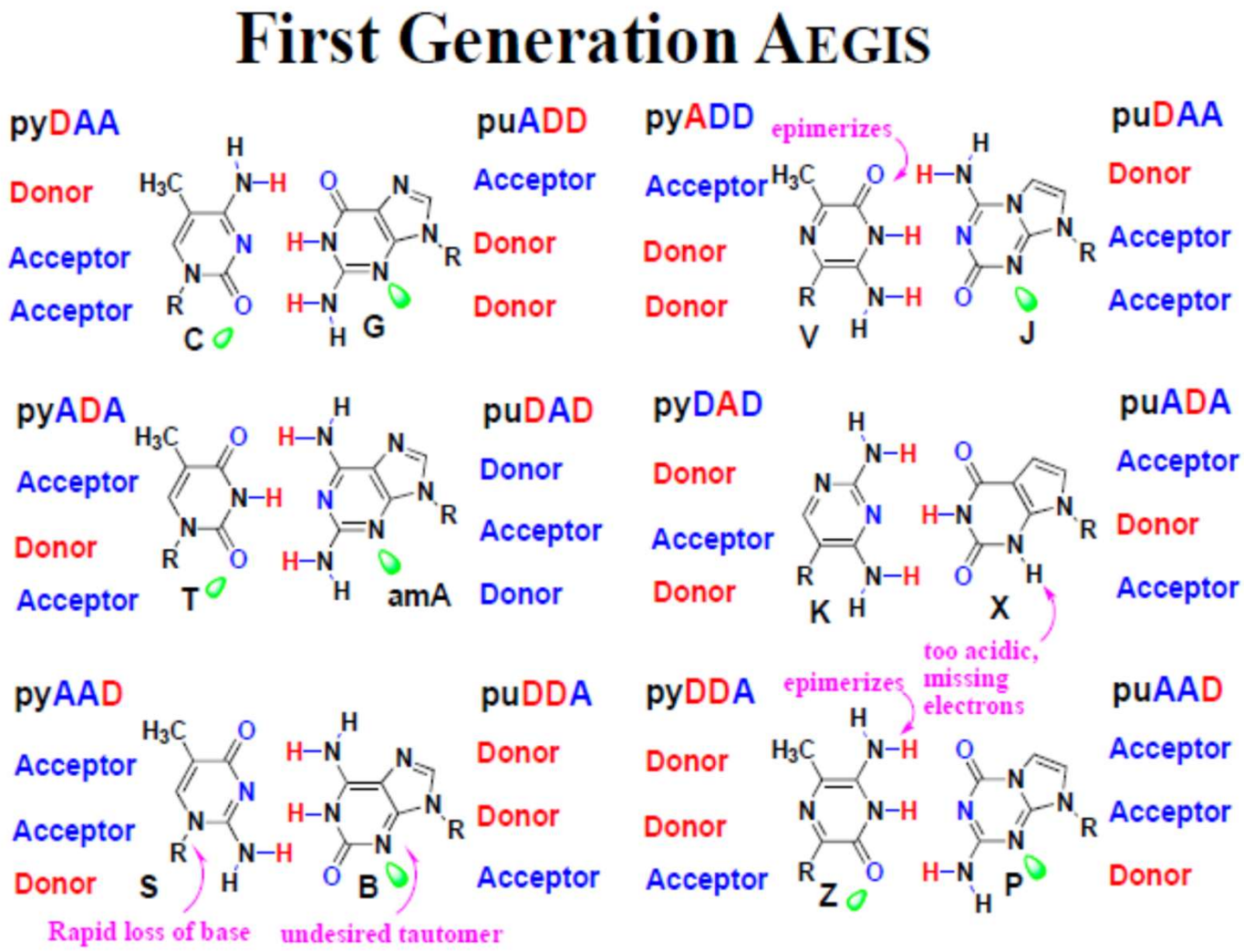
Self-assembly of whole genes and DNA nanostructures



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.

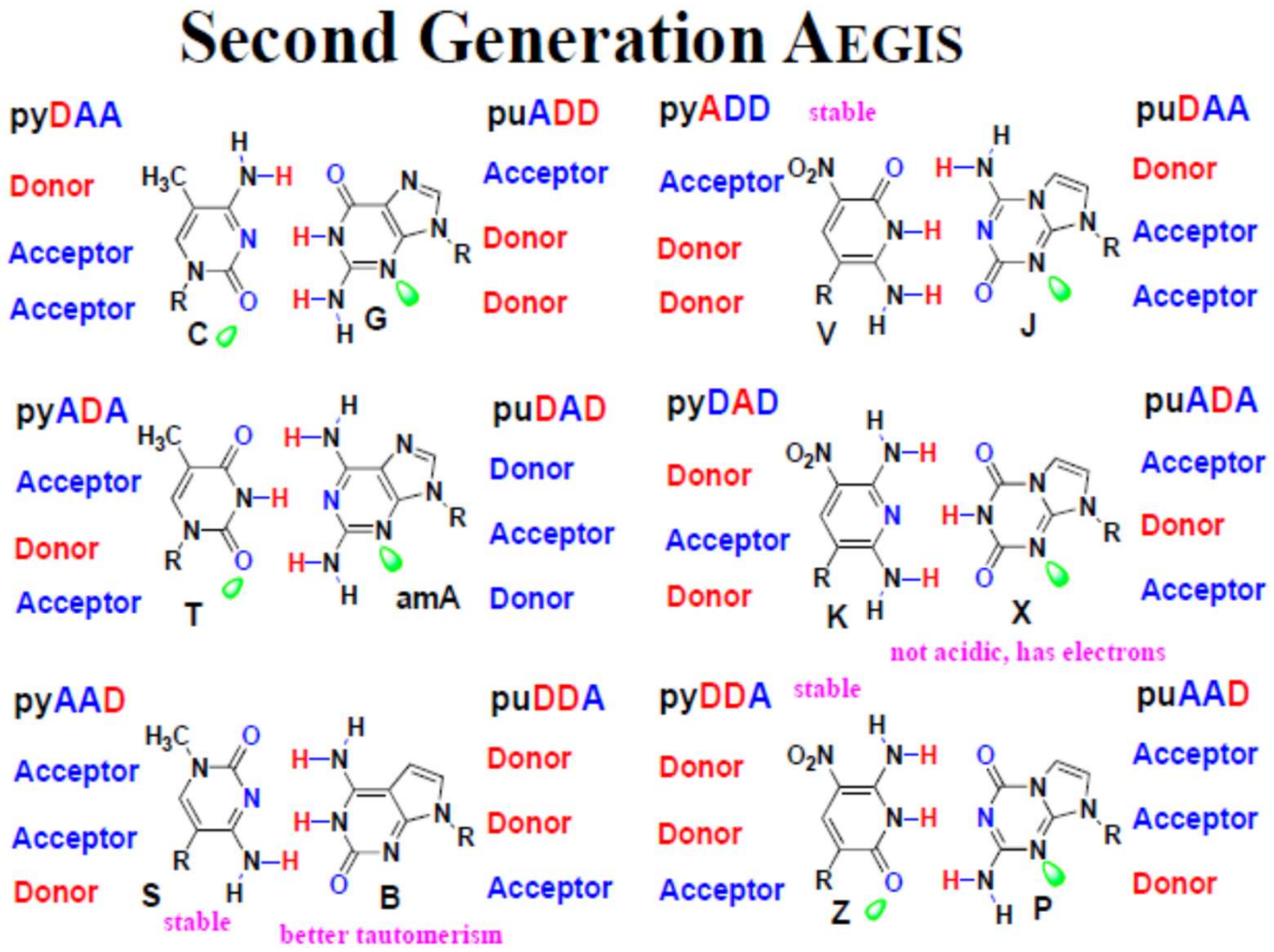
S. Benner *et al., Beilstein J. Org. Chem.* **2014,** *10,* 2348–2360. doi:10.3762/bjoc.10.245

AEGIS – Artificially Expanded Genetic Information System



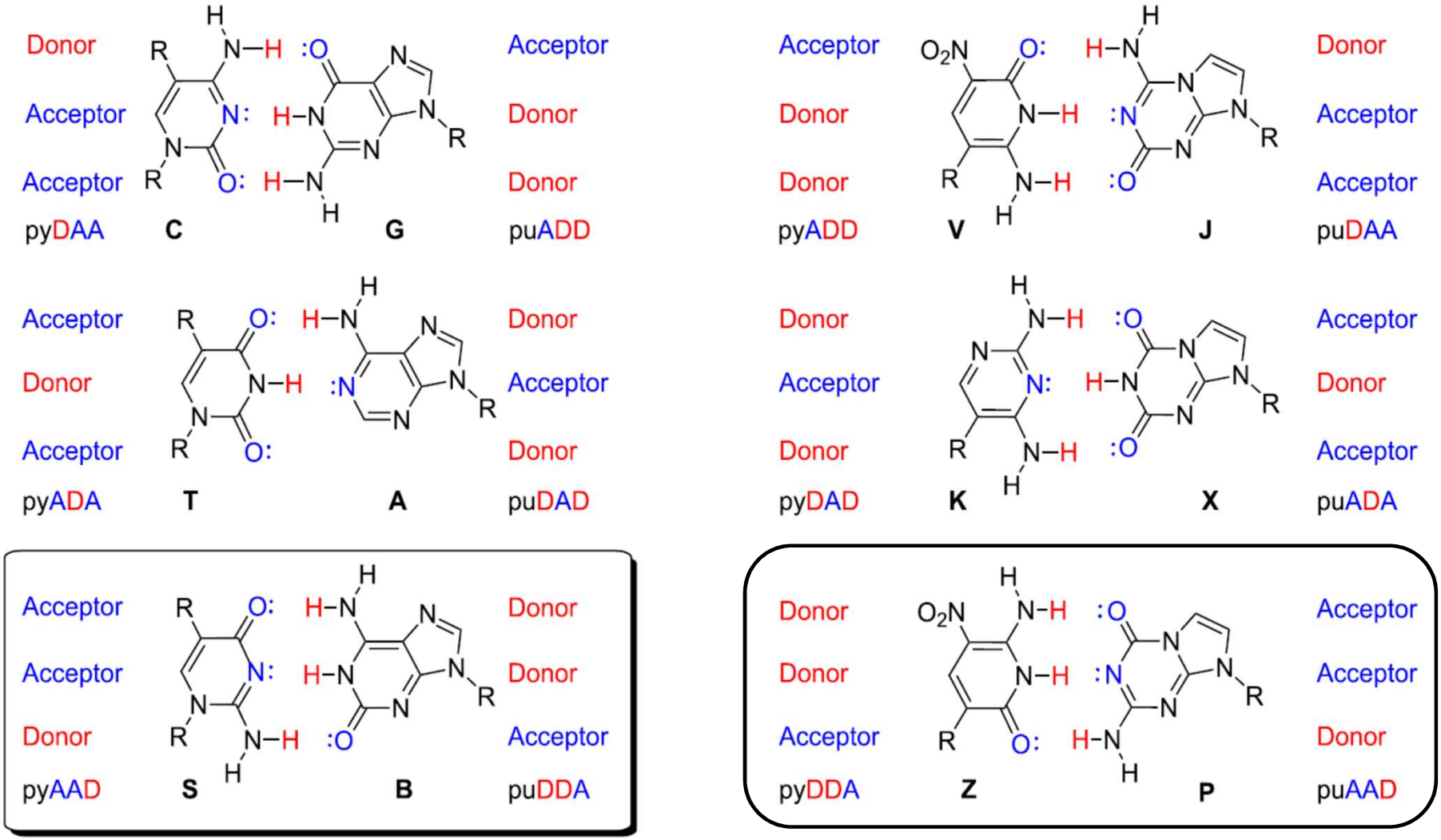
E. Biondi, S. Benner *Biomedicines* **2018**, *6*, 53; *doi:10.3390/biomedicines6020053*

AEGIS – Artificially Expanded Genetic Information System



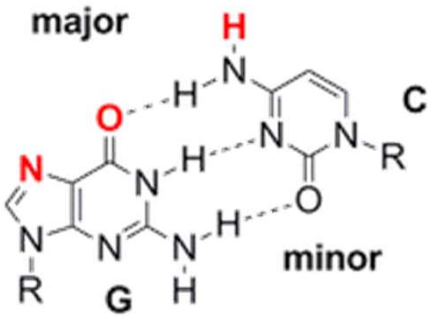
E. Biondi, S. Benner *Biomedicines* **2018**, *6*, 53; *doi:10.3390/biomedicines6020053*

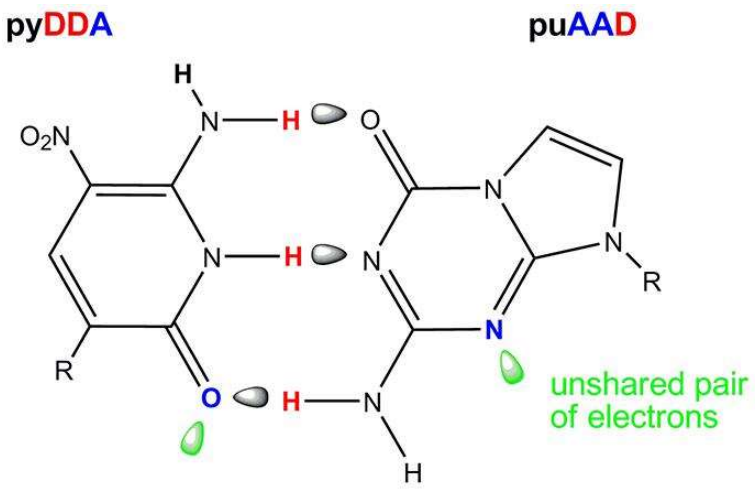
AEGIS – Artificially Expanded Genetic Information System

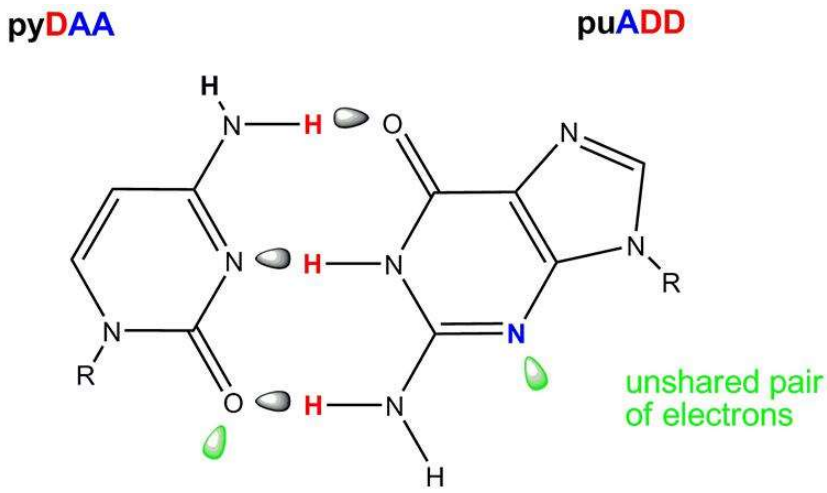


S. Benner *et al., Beilstein J. Org. Chem.* **2014,** *10,* 2348–2360. doi:10.3762/bjoc.10.245

AEGIS – Artificially Expanded Genetic Information System



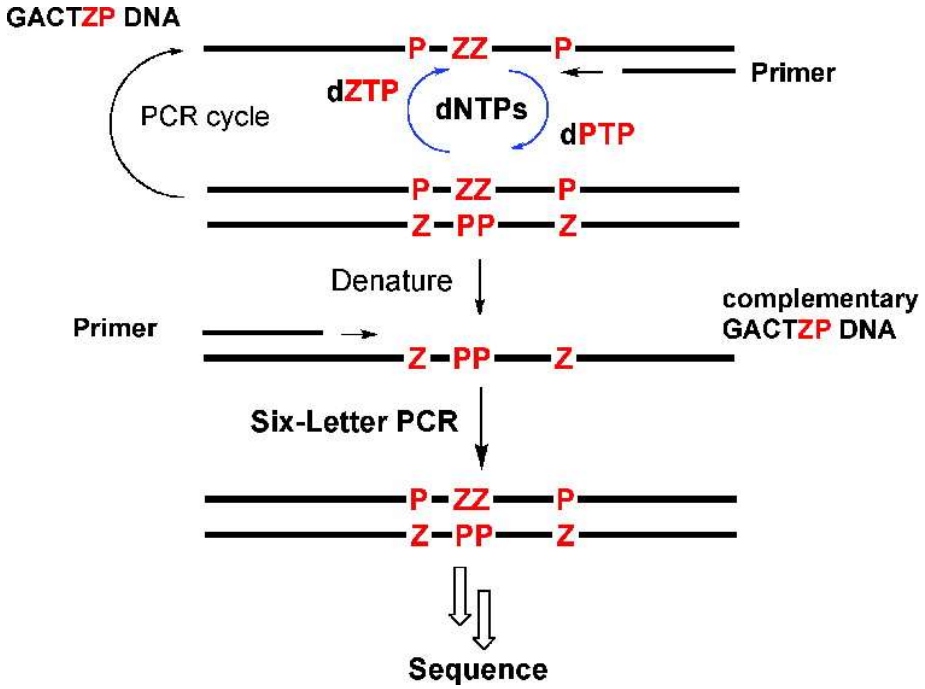




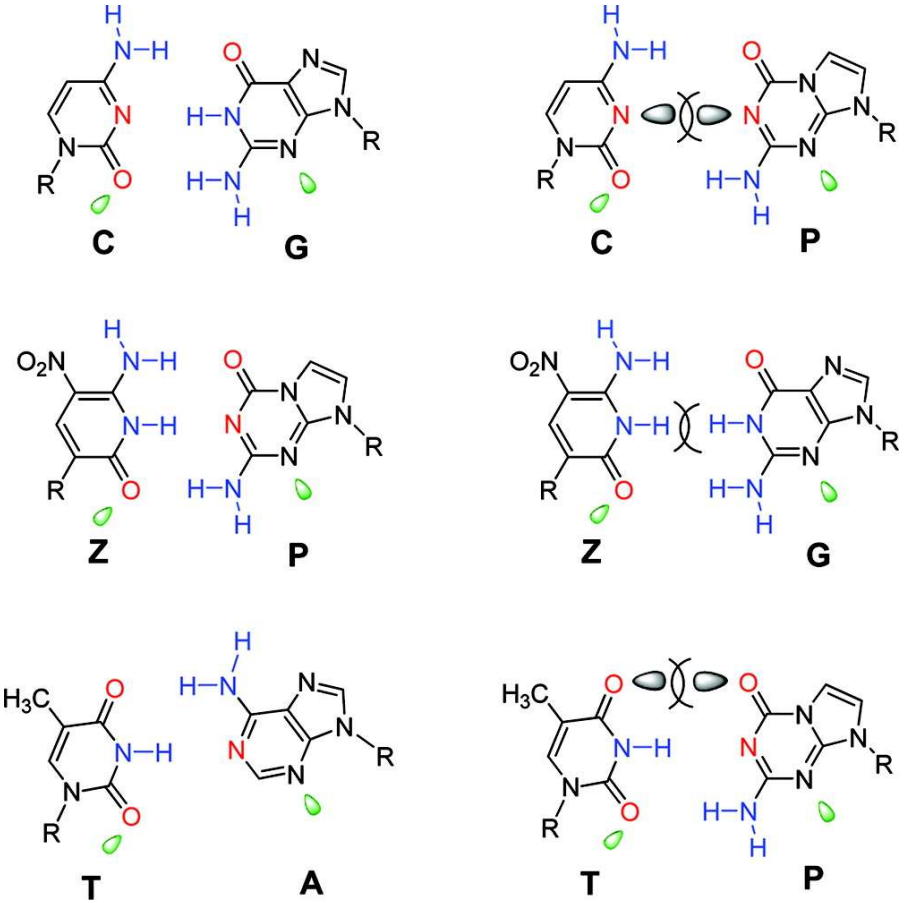
Z **P** **C** **G**

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

AEGIS – Permanent orthogonal nucleobases surviving PCR



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

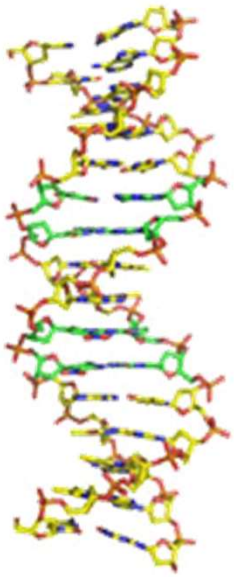


Error rate 0,2% per a PCR cycle – both removal and incorporation of **Z** and **P** ➔ the artificial genetic system capable to evolve.

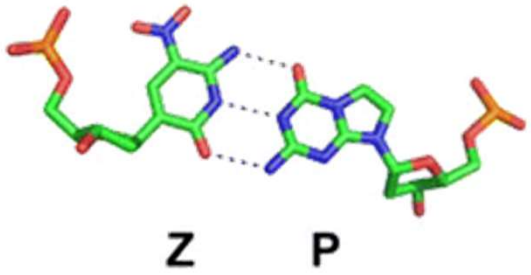
Pol: Deep Vent – 2 Z/P, Taq/Phu – 3-4 Z/P
dZTP (*deprotonated*) at higher pH pairs slightly with G
→ loss of some **Z**, but also gain of some new **Z** mutants.

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

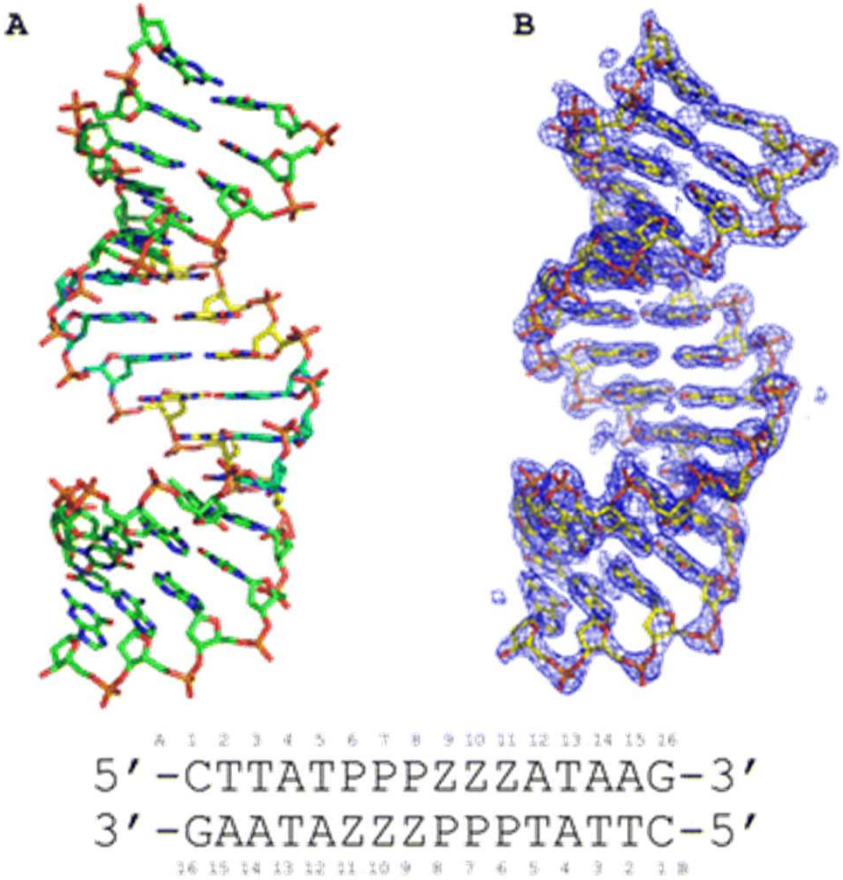
ACGTZP-DNA crystal structures



B-DNA



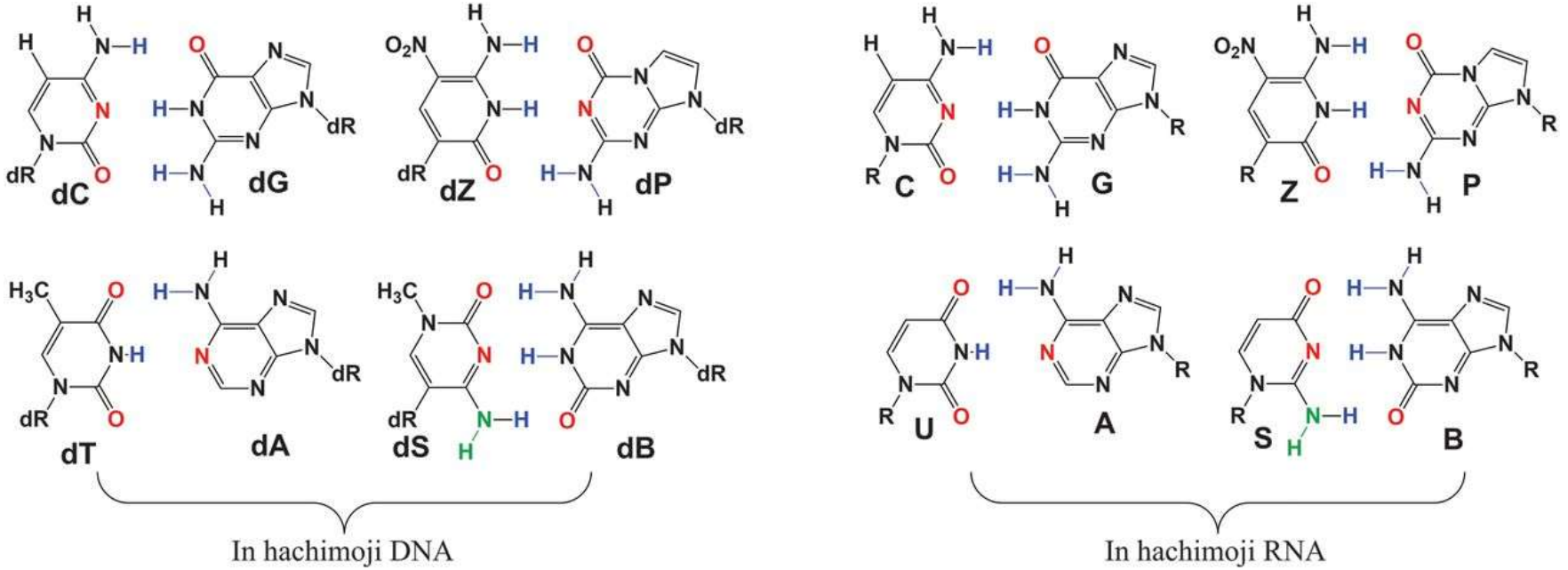
A-DNA

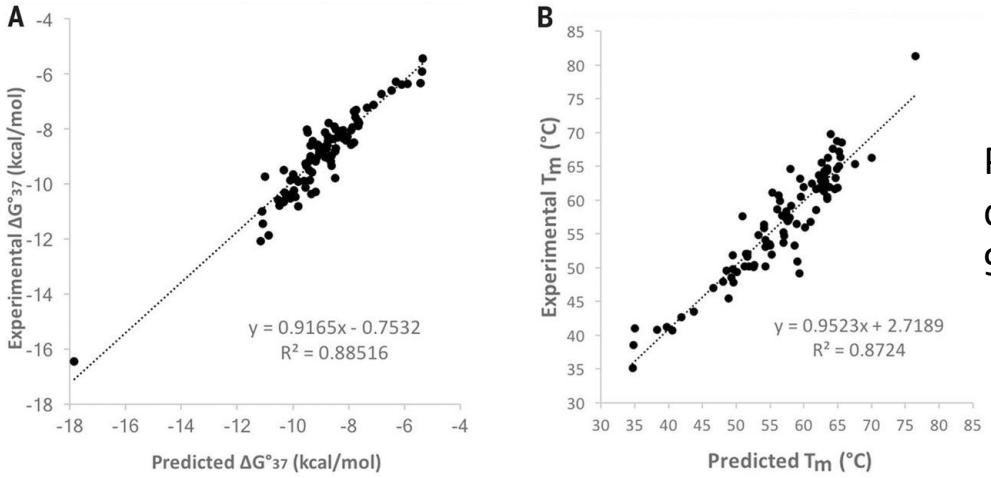


*18-mers: 2+2 **Z:P** pairs → B-DNA*
*6 consecutive **Z:P** → A-DNA*
*0,1 nm wider, but otherwise alike **G:C** pairs*

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

Hachimoji DNA and RNA – a genetic system with eight (Jap.- Hachi) letters

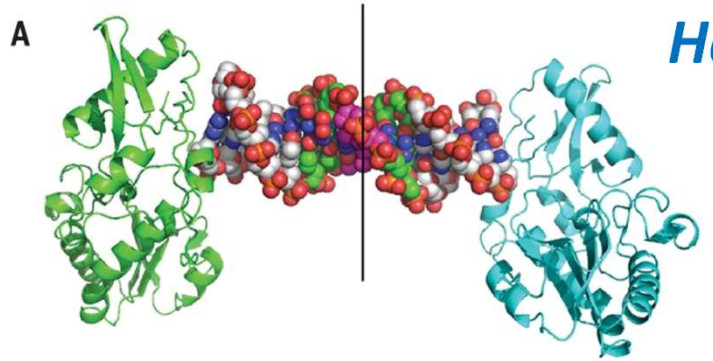




Plots of experimental versus predicted **(A)** free energy changes (ΔG°_{37}) and **(B)** melting temperatures T_m for 94 SBZP-containing hachimoji DNA duplexes.

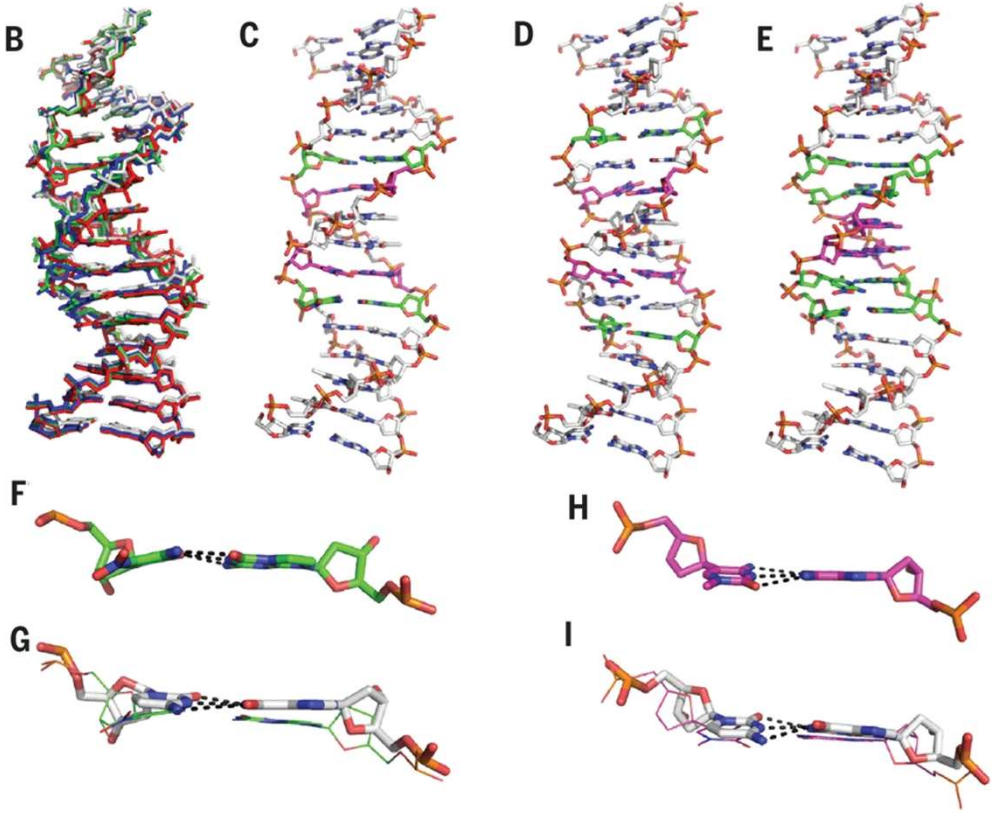
S. Hoshika *et al., Science* **2019**, *363,* 884-887

Hachimoji DNA and RNA



Crystal structures of hachimoji DNA.

(**A**) The host-guest complex with two N-terminal fragments from Moloney murine leukemia virus reverse transcriptase bound to a 16-mer PP hachimoji DNA; Z:P pairs are green and S:B pairs are magenta.



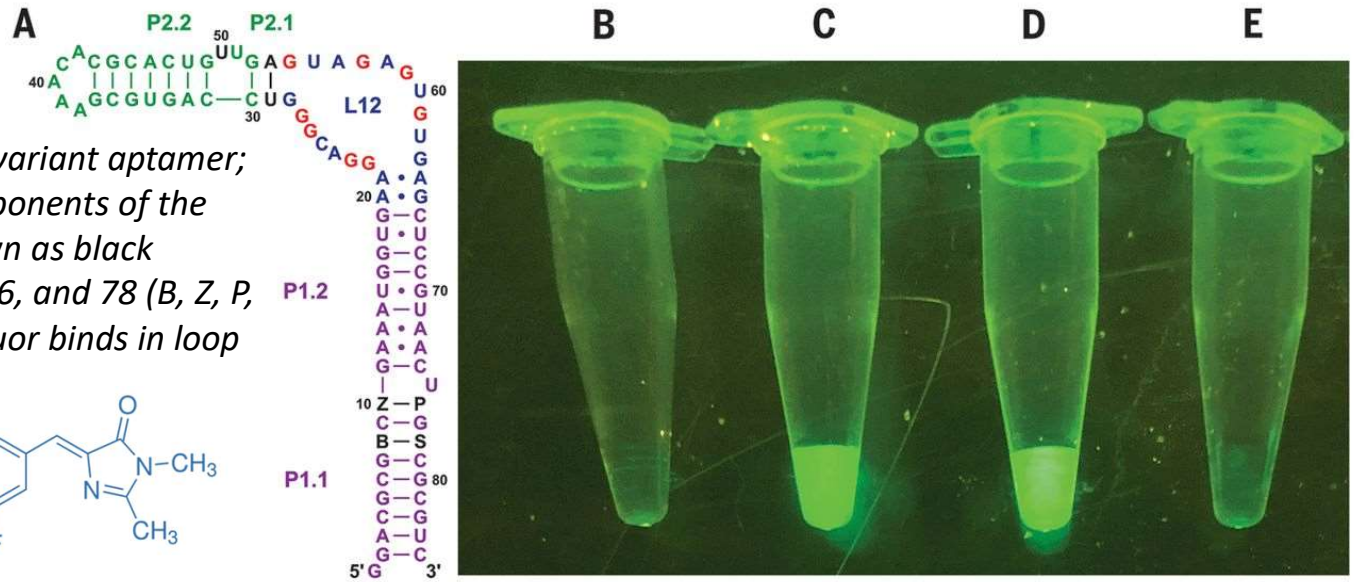
(**B**) Hachimoji DNA structures PB (green), PC (red), and PP (blue) are superimposed with GC DNA (gray).
(**C**) Structure of hachimoji DNA with self-complementary duplex 5′-CTTATPBTASZATAAG (“PB”).
(**D**) Structure of hachimoji DNA with self-complementary duplex 5′-CTTAPCBTASGZTAAG (“PC”).
(**E**) Structure of hachimoji DNA with self-complementary duplex with six consecutive nonstandard 5′-CTTATPPSBZZATAAG (PP) components.

(**F** to **I**) Examples of largest differences in detailed structures. The Z:P pair from the PB structure (F) is more buckled than the corresponding G:C pair (G). The S:B pair from the PB structure (H) exhibits a propeller angle similar to that in the corresponding G:C pair (I).

S. Hoshika *et al., Science* **2019**, *363,* 884-887

Hachimoji RNA aptamer

T7 RNA polymerase incorporates ZTP, PTP, and BTP, but not STP opposite to dP, dZ, dS, and dB, respectively. A mutant of T7 RNA Pol (Y639F H784A P266L, "**FAL**") incorporated also STP – full DNA→RNA conversion possible



the full hachimoji spinach variant aptamer; additional nucleotide components of the hachimoji system are shown as black letters at positions 8, 10, 76, and 78 (B, Z, P, and S, respectively). The fluor binds in loop L12

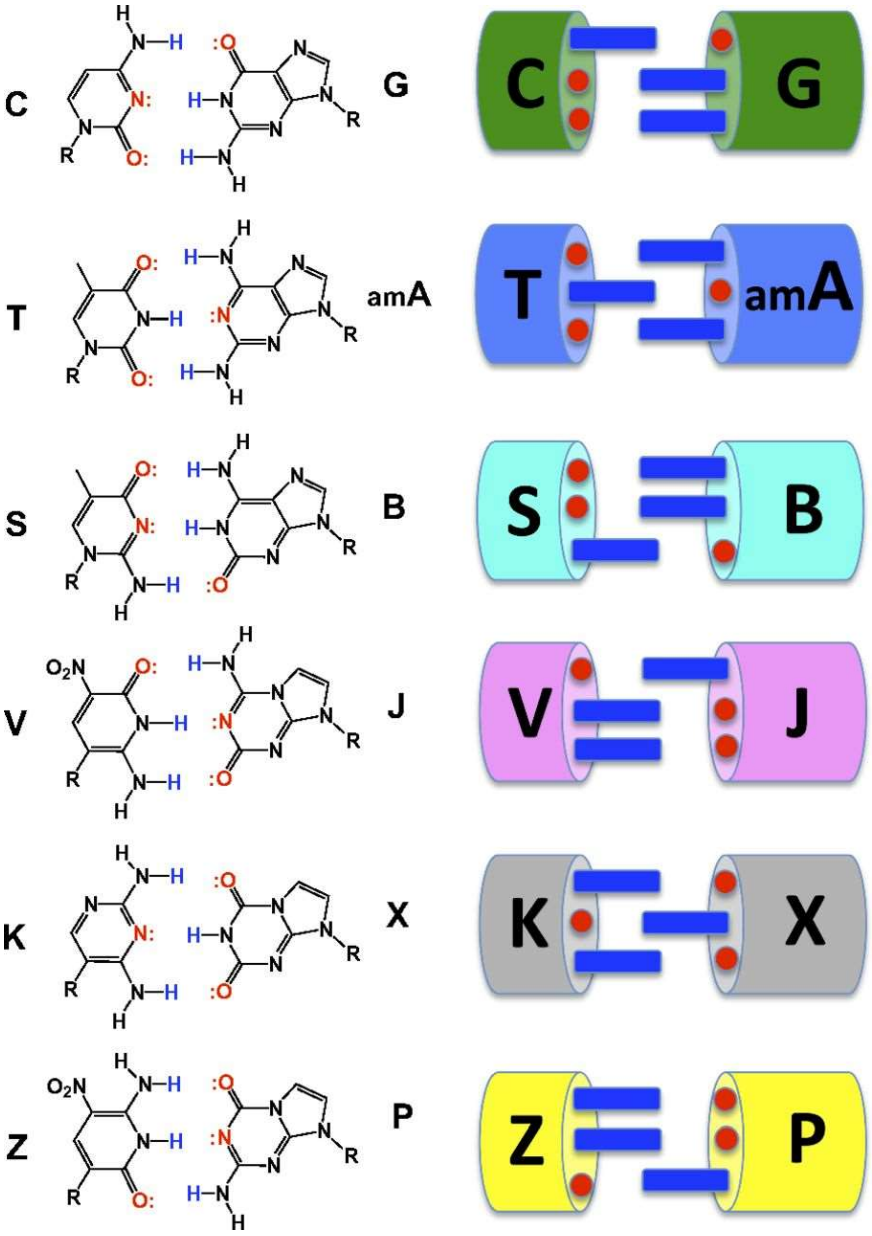
The hachimoji variant of the spinach fluorescent RNA aptamer. In its standard form, spinach folds and binds 3,5-difluoro-4-hydroxybenzylidene imidazolinone, which fluoresces green when bound.

(B) Control with fluor only, lacking RNA.
(C) Hachimoji spinach with the sequence shown in (A).
(D) Native spinach aptamer with fluor.
(E) Fluor and spinach aptamer containing Z at position 50, replacing the A:U pair at positions 53:29 with G:C to restore the triple observed in the crystal structure. This places the quenching Z chromophore near the fluor;

S. Hoshika *et al., Science* **2019**, *363,* 884-887

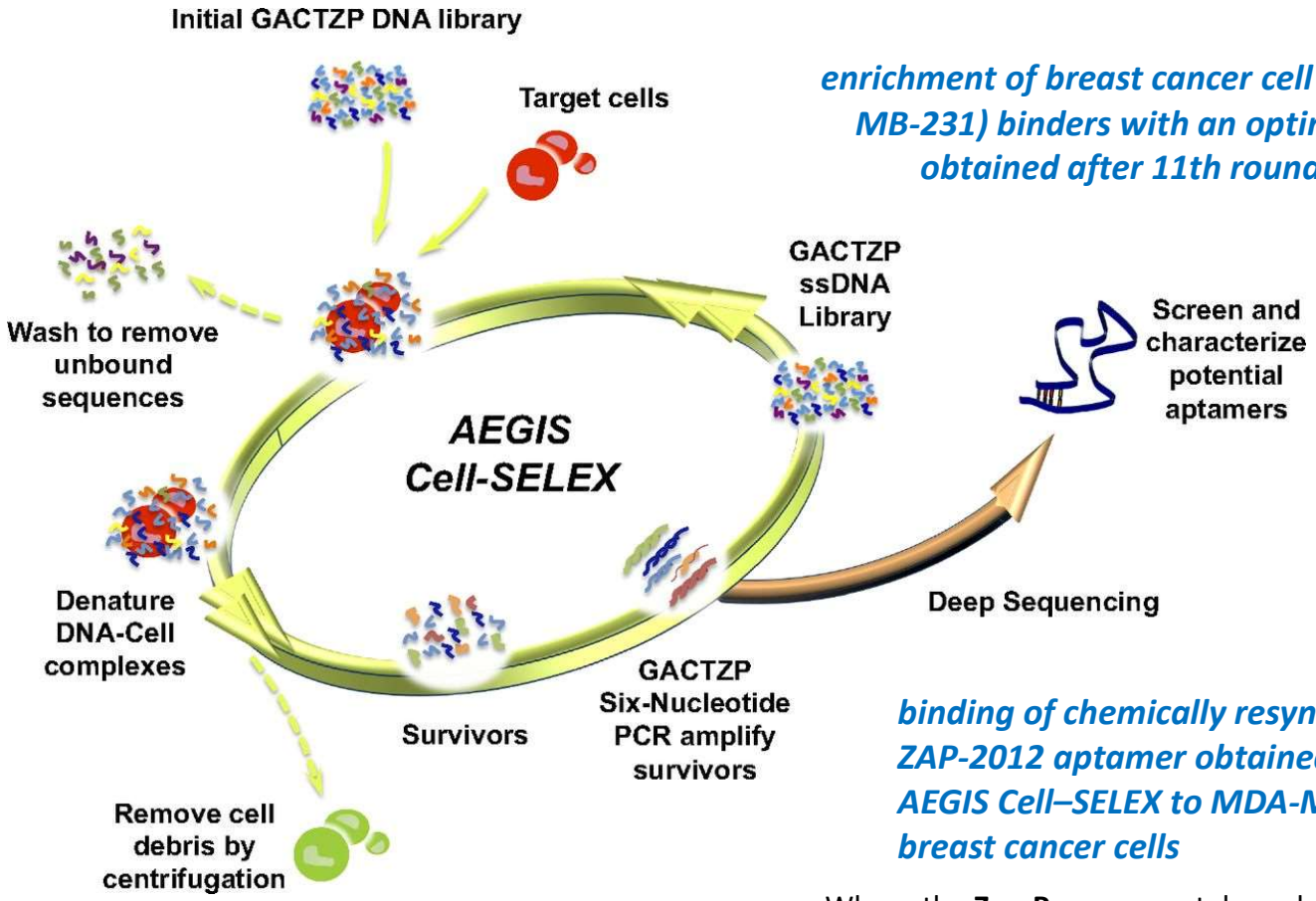
AEGIS – Artificially Expanded Genetic Information System

*An **xNA** biopolymer having functionalized AEGIS components may allow SELEX to yield protein-like aptamers better than the standard DNA and RNA biopolymers.*

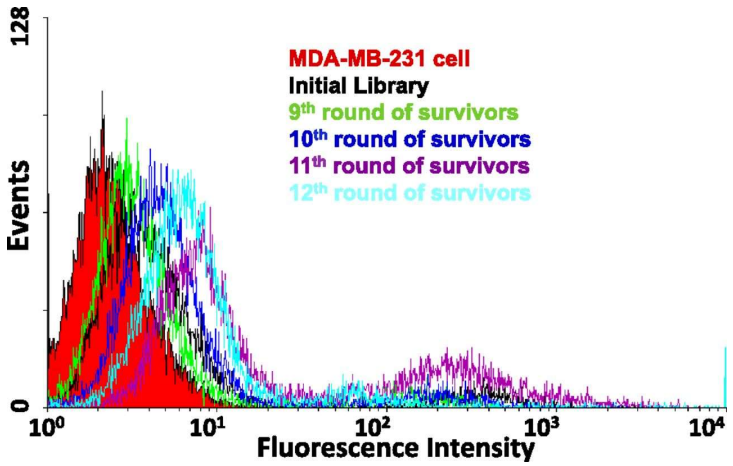


K. Sefah *et al., Proc. Natl. Acad. Sci. USA* **2014,** *111 (4),* 1449-1454.

ACGTZP-aptamers

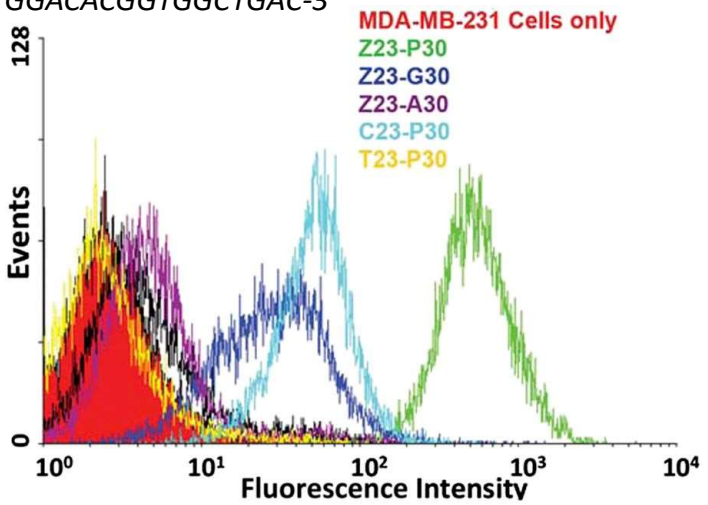


enrichment of breast cancer cell (MDA-MB-231) binders with an optimum obtained after 11th round



ZAP-2012 (Z23-P30) K_d = 30 nM

5′-biotin-TCCCGAGTGACGCAGC-
CCCCGGZGGGATTPATCGGT-
GGACACGGTGGCTGAC-3′

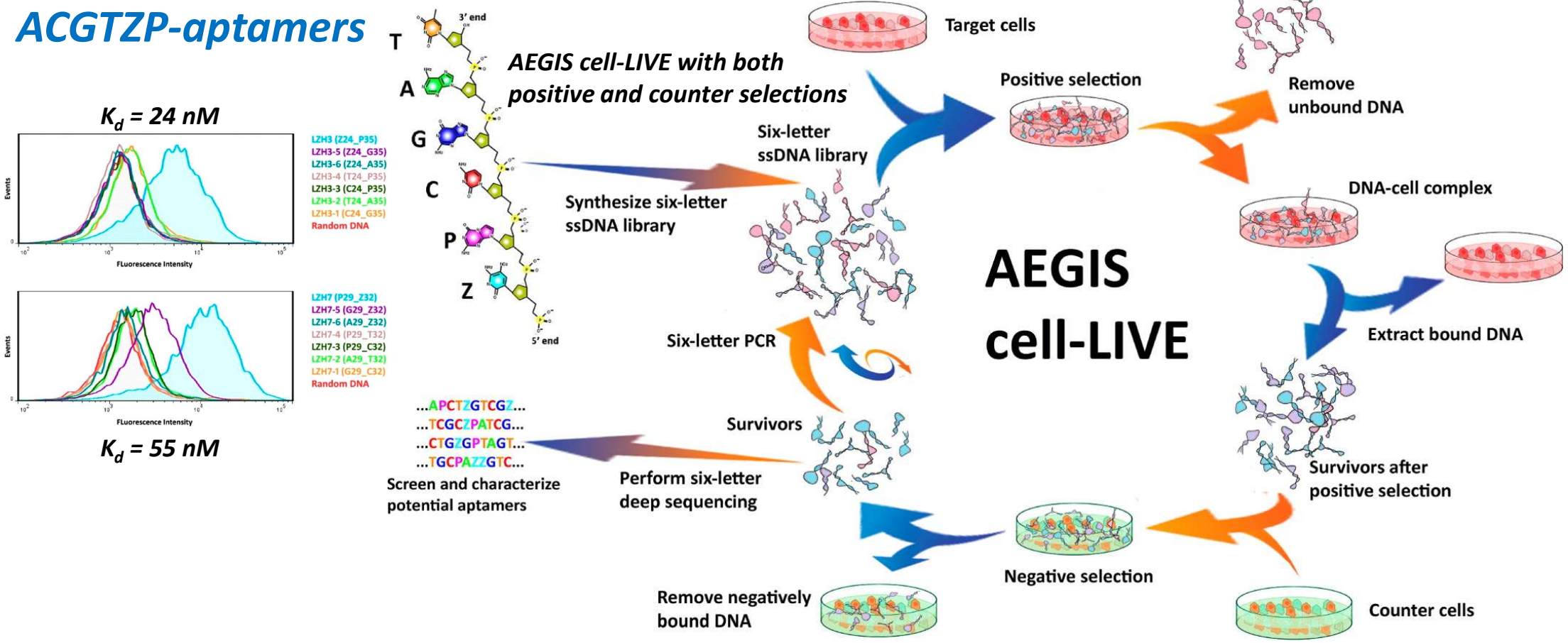


binding of chemically resynthesized ZAP-2012 aptamer obtained using AEGIS Cell–SELEX to MDA-MB-231 breast cancer cells

Where the **Z** or **P** are separately replaced with standard nucleotides, binding affinity is reduced. Where the **Z** and **P** are both replaced with standard nucleotides, binding affinity is lost (not shown).

K. Sefah *et al., Proc. Natl. Acad. Sci. USA* **2014,** *111 (4),* 1449-1454.

ACGTZP-aptamers



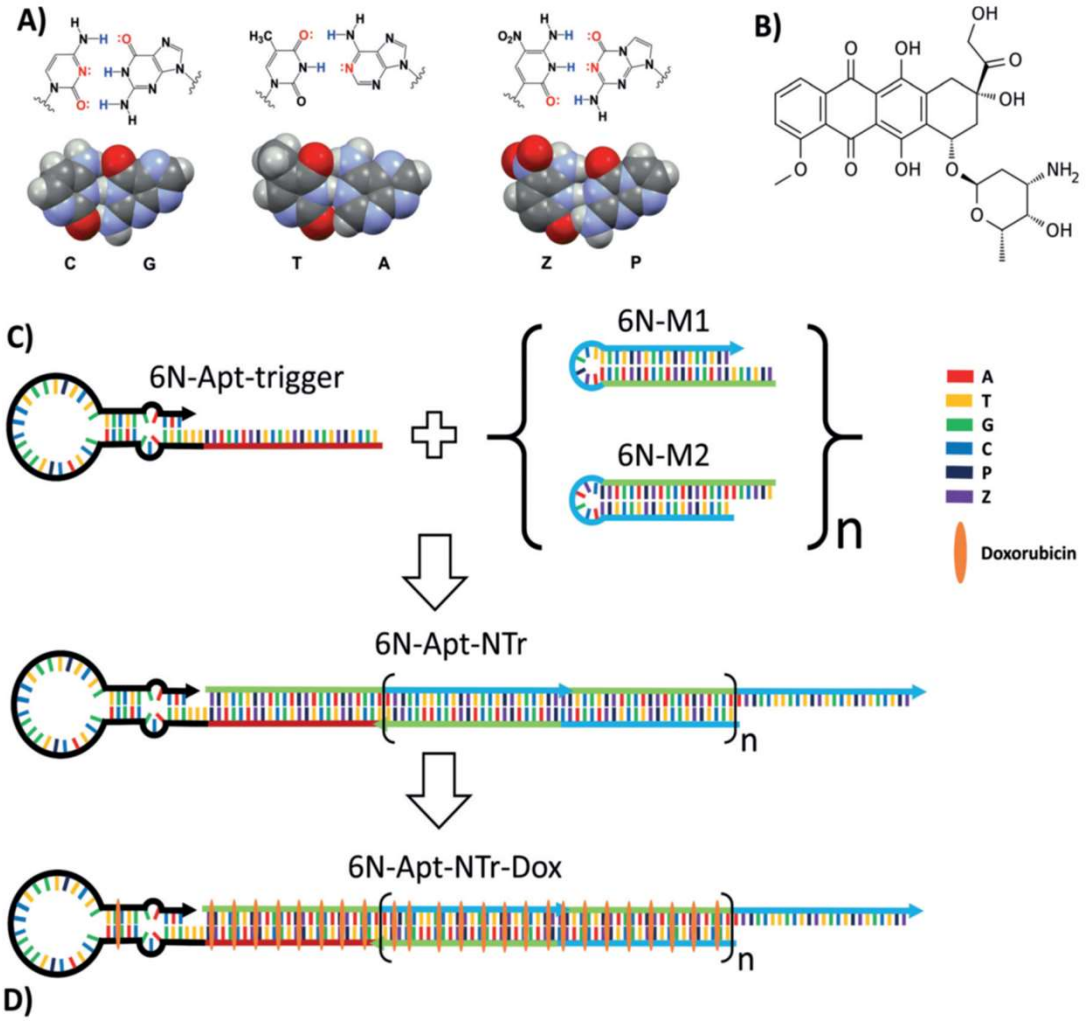
Nucleotides **Z** and **P** were added to a library of oligonucleotides used in a laboratory *in vitro* evolution (LIVE) experiment; the GACTZP library was challenged to deliver molecules that bind selectively to **liver cancer cells**, but not to untransformed **liver cells**. Unlike in classical *in vitro* selection, low levels of mutation allow this system to evolve to create binding molecules not necessarily present in the original library. Over a dozen binding species were recovered. The best had multiple Z and/or P in their sequences.

S. Benner *et al., J. Am. Chem. Soc.*, **2015**, *137,* pp 6734-6737

Aptamer-Nanotrain

The aptamer-nanotrain assembly, charged with doxorubicin, selectively kills liver cancer cells in culture, as the selectivity of the aptamer binding directs doxorubicin into the aptamer-targeted cells. The assembly does not kill untransformed cells that the aptamer does not bind.

The previously selected 6-letter aptamer which binds liver cancer cells

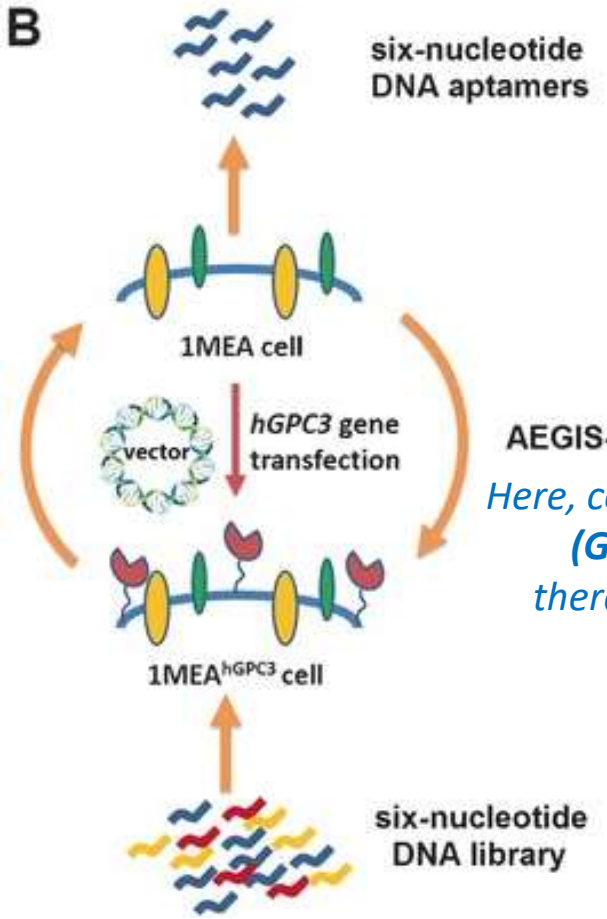


6N-Apt-trigger	5′-TGC TPZ TGC TPZ TGC TPZ ACG AZP TTT GTG ACG CAG CAG CTA CPT GGG CCC TGG TPT CTG TGC TGG ACA C-3′
6N-M1	5′-ZPT CGT PZA GCA PZA GCA PZA GCA AZP GCT TGC TPZ TGC TPZ TGC TPZ-3′
6N-M2	5′-TGC TPZ TGC TPZ TGC TPZ ACG AZP PZA GCA PZA GCA PZA GCA AGC ZPT-3′

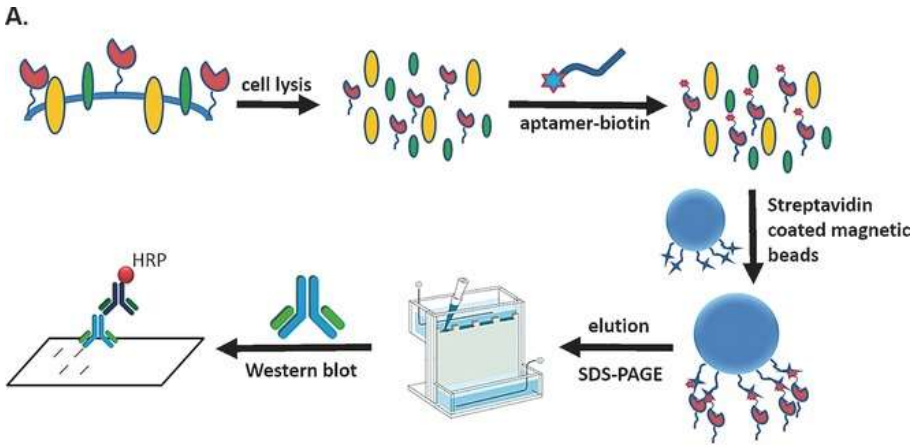
This architecture, built with an expanded genetic alphabet, is reminiscent of antibodies conjugated to drugs, which presumably act by this mechanism as well, but with the antibody replaced by an aptamer.

L. Zhang *et al., Angew. Chem., Int.Ed.*, **2020**, *59*, 663-668

ACGTZP-aptamers against a specific protein

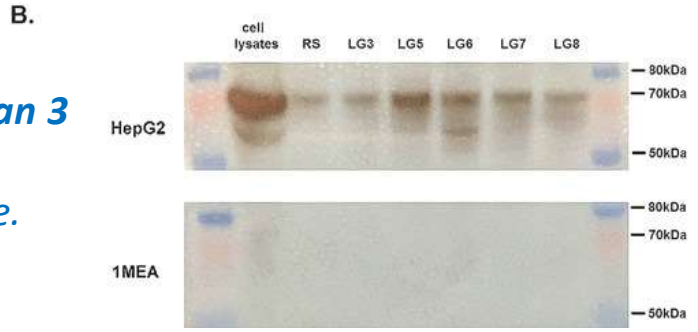


Laboratory in vitro evolution (LIVE) might deliver DNA aptamers that bind proteins expressed on the surface of cells.



AEGIS-LIVE

*Here, cell engineering was used to place **glypican 3 (GPC3),** a possible marker for liver cancer theranostics, on the surface of a liver cell line.*



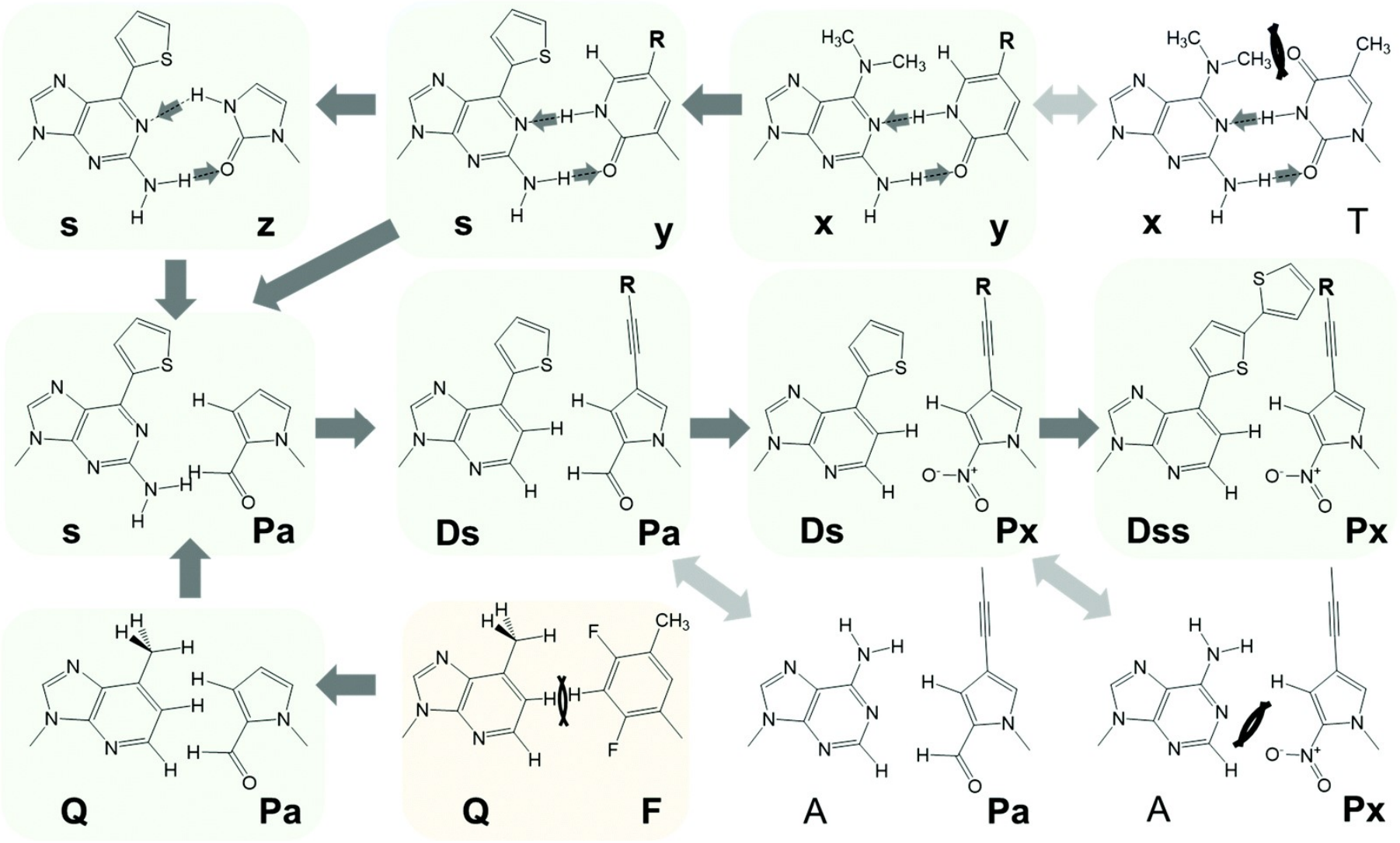
Libraries were then built from a six-letter genetic alphabet. With counterselection against non-engineered cells, eight AEGIS-containing aptamers were recovered. Five bound selectively to GPC3-overexpressing cells.

Name	Sequence	Percentage
LG1	~PGGTGGGCGGAGGTCTZGCTACAPGPTTTGGPGGC~	11.37%
LG2	~PGCCCGGGPTAPPGTGPTGGGTGTTCGCTATCCAG~	7.98%
LG3	~GGTAACTAGTAGTTGACCCTGPAGTGZTGTPTCTG~	6.01%
LG4	~GGCGGGGTZGPGTAAGGGGTCTAAGGCATTGGGTC~	4.48%
LG5	~GGAGGAAGTGGTCCTTGCTTTGCZTCGTATCTGGG~	2.57%
LG6	~GGTZGATTATTPGGTTCAATAACACPTCCTGGTGG~	1.96%
LG7	~PGCACAGTGTGZZCCATAGGTTGTAATGACPTZTG~	1.04%
LG8	~GGCAGCZCCTGPAGTPGAGTGTPATGGCTTATTCG~	0.91%

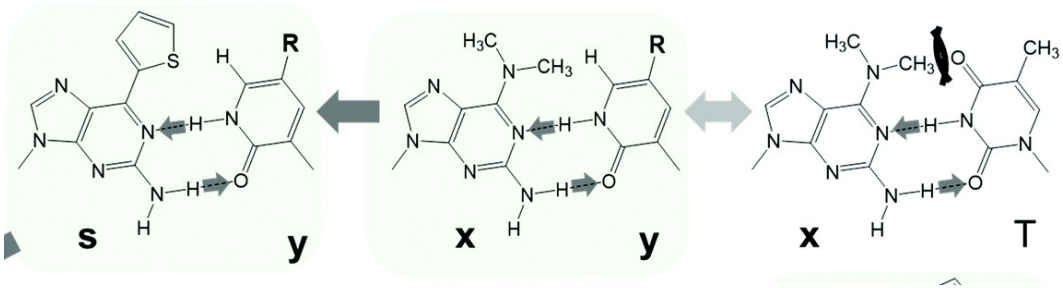
LG5 K_d = 6 nm (without Z – no binding)

L. Zhang *et al., Angew. Chem., Int.Ed.,* **2016**, *55,* 12372-12375

Steric exclusion and hydrophobic non-natural base pairs



Steric exclusion and hydrophobic non-natural base pairs



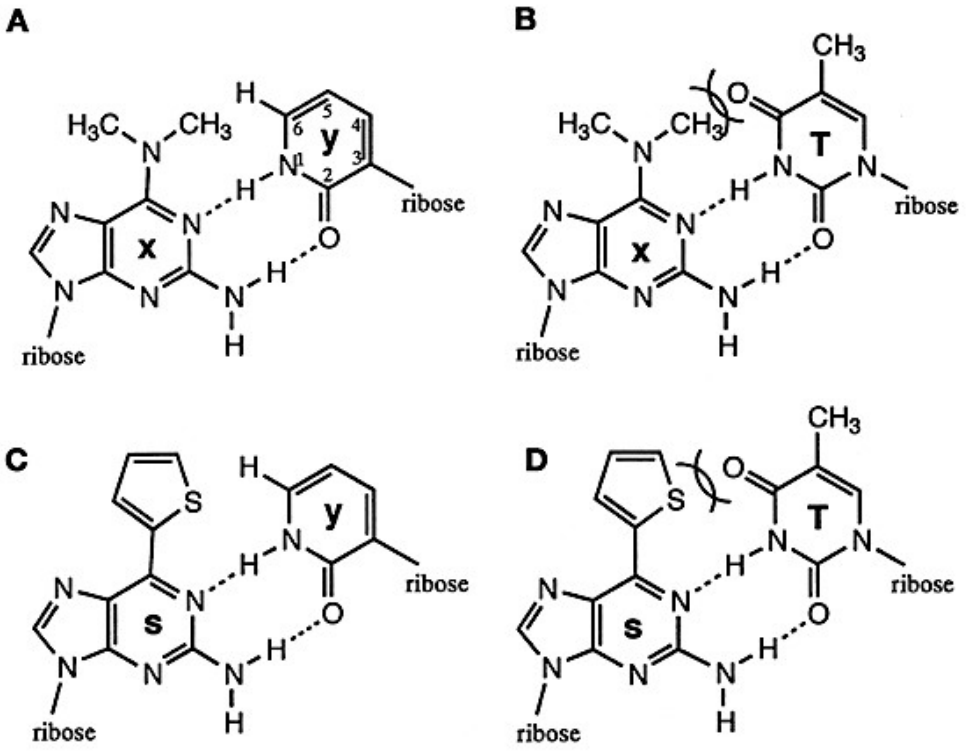
Hirao (2001): the steric hindrance concept to hydrogen-bonded UBPs to exclude the mispairing with natural bases → a series of hydrogen-bonded UBPs: **x–y** and **s–y** pairs

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

Unnatural aminoacid incorporation using a noncanonical base pair



Non-template strand 5'-d(ATAATACGACTCACTATAGGG)
Template strand 3'-d(TATTATGCTGAGTGATATCCCTTGCGCTCNTCACGCC)
+1 +12 +19
N = **s**, **x**, A, or G

↓ T7 RNA polymerase
1 mM N'TPs, [α-^{32}P]ATP, GMP

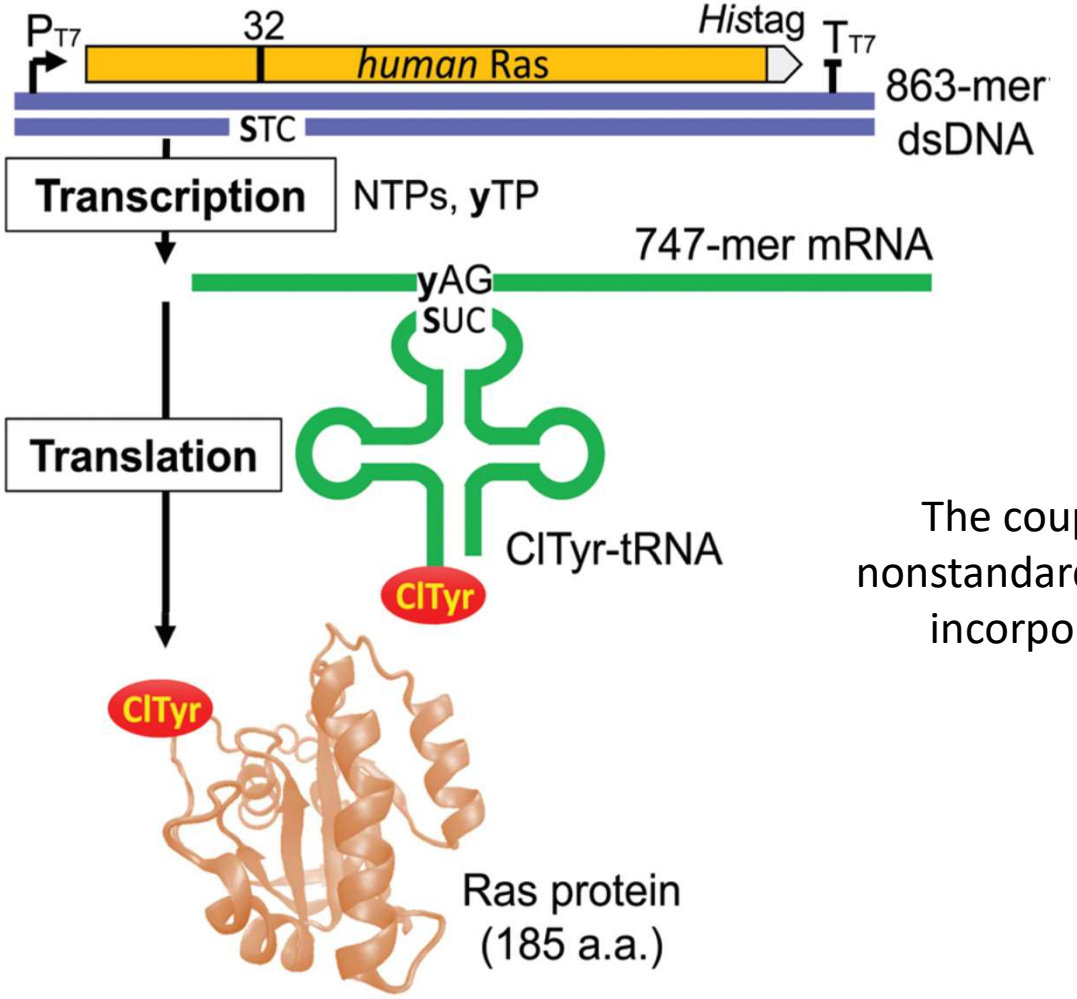
Full-length product (19-mer)
pGpGpGp*Ap*ApCpGpCpGp*ApGpN'p*ApGpTpGpCpGpG

↓ RNase T_2 digestion
2D-TLC

^{32}P-labeled nucleotides
Gp* x 2, Ap* x 1, **N'**p* x 1

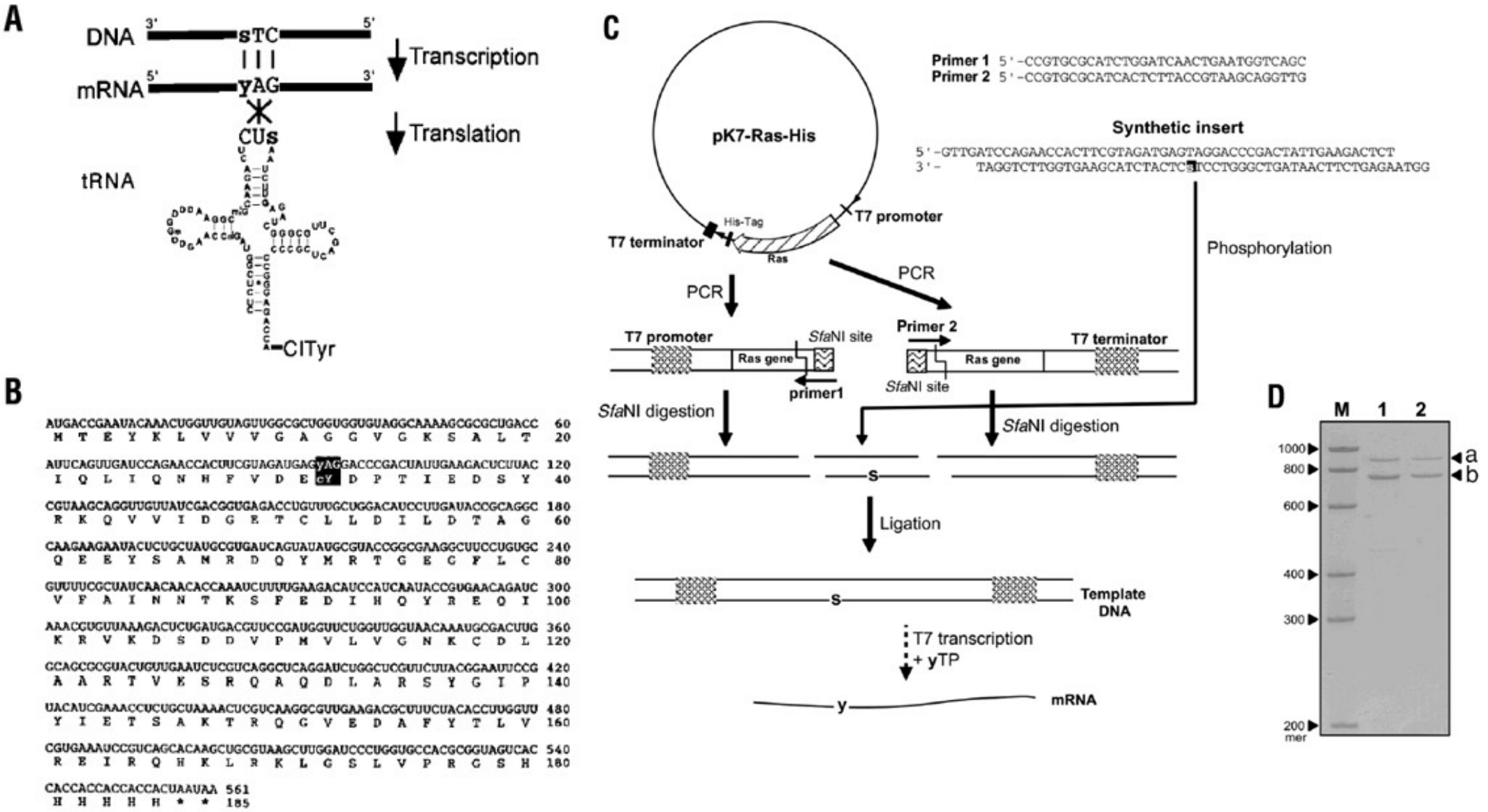
I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)

Unnatural aminoacid incorporation using a noncanonical base pair



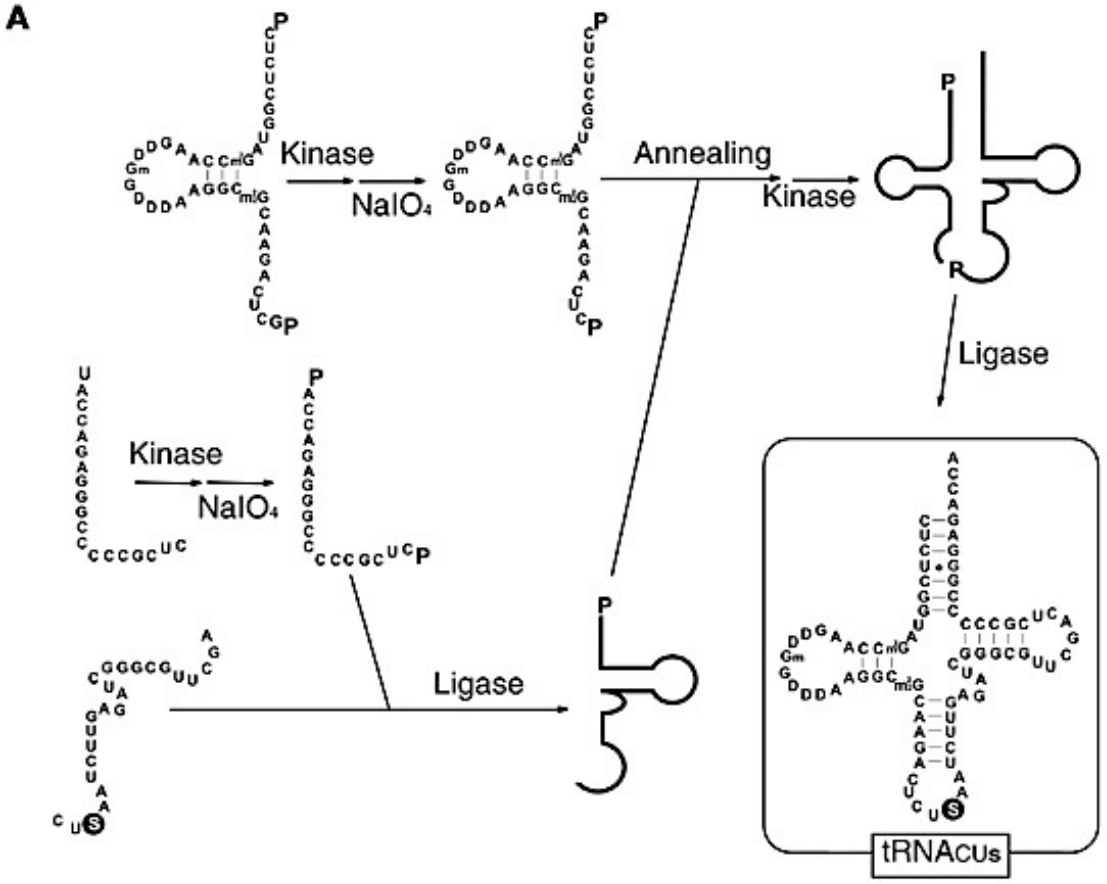
The coupled transcription–translation system using the nonstandard codon–anticodon interaction for the site-specific incorporation of 3-chlorotyrosine into the Ras protein.

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



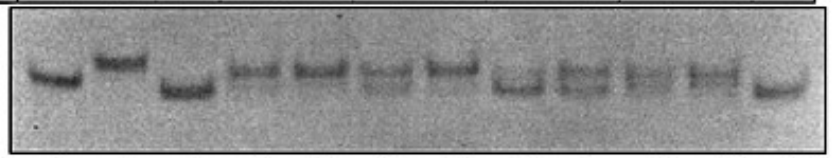
I. Hirao *et al. Nature Biotechnology* **20**, 177–182 (2002)

Unnatural aminoacid incorporation using a noncanonical base pair



B

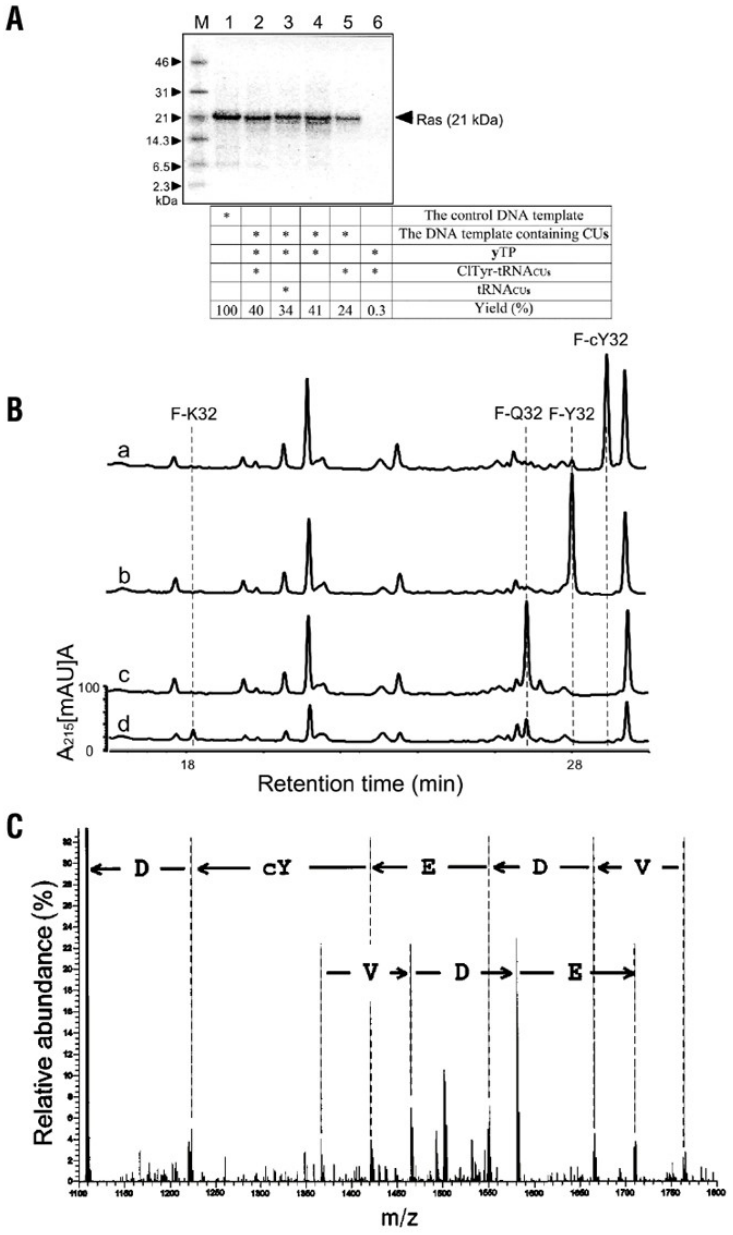
tRNA	Native tRNA		tRNA$_{CUs}$									
Amino acid	–	Tyr	–	FTyr		ClTyr		ITyr		DOPA		–
DMSO/Tween-20	–	–	+	–	+	–	+	–	+	–	+	+
Yeast TyrRS	–	+	–	+	+	+	+	+	+	+	+	–



1 2 3 4 5 6 7 8 9 10 11 12

(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 CT**s** 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 CT**s** but in the absence of **y**TP and tRNA$_{CUs}$; and chart d shows those obtained in the presence of the DNA template containing CT**s** 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 nucleobases - overview