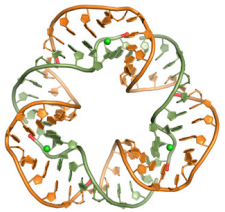
Synthetic life SL2

WiSe 2018/19

Zbigniew Pianowski

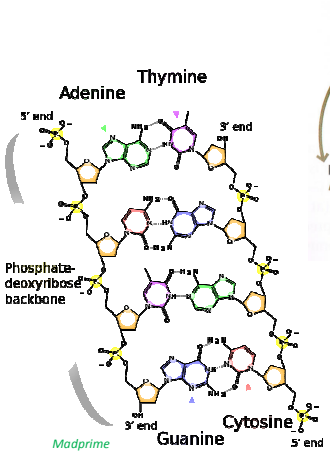
CHAPTER 1

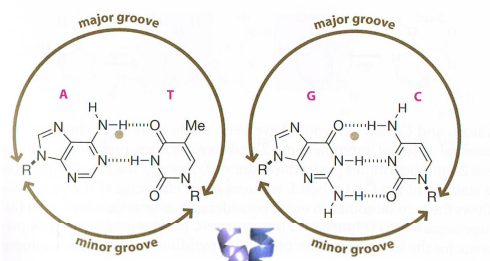


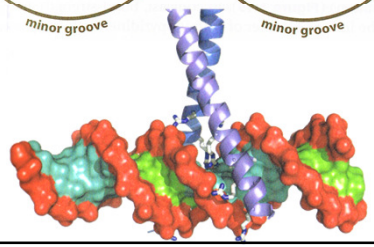
OLIGONUCLEOTIDES

Part 1 – modified canonical nucleobases

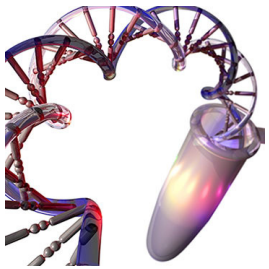
Canonical nucleobases and Watson-Crick pairing in DNA

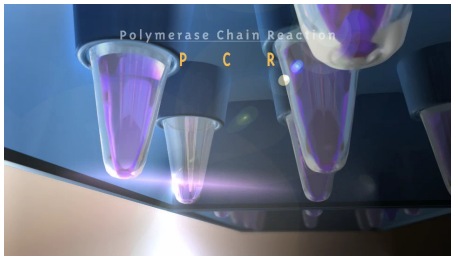






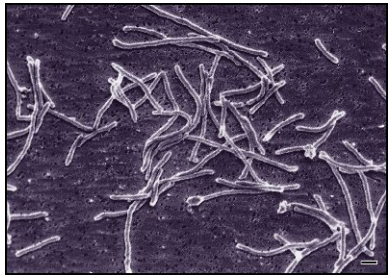
PCR – Polymerase Chain Reaction





National Library of Medicine, National Institutes of Health

PCR – Polymerase Chain Reaction

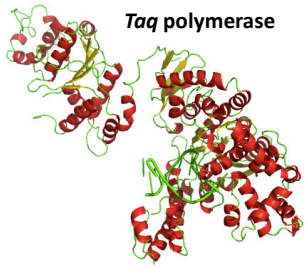


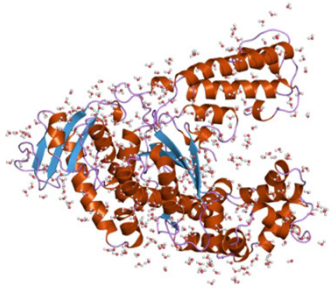
Brian W. Schaller, Yellowstone Park

Thermus aquaticus is a thermophilic bacteria from hot springs in Yellowstone Park
70^{0}C – optimum, living range: 50-80^{0}C

It is a source of thermostable enzymes

PCR – Polymerase Chain Reaction





Taq polymerase withstands denaturing conditions (hot temperatures) detrimental for most enzymes. *Activity optimum: 75-80^{0}C, half-life at 95^{0}C > 2.5 h*

1990 – Kary Mullis optimized the PCR technique with *Taq* polymerase (1993 Nobel Prize)

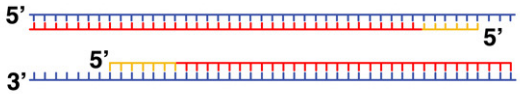
PCR – Polymerase Chain Reaction

We begin with a single molecule of DNA.

We can melt the DNA (break the hydrogen bonds holding the helix together) by heating it to 98 degrees.

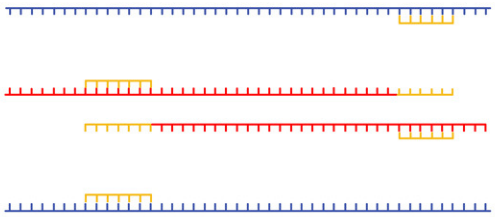
PCR – Polymerase Chain Reaction

Two DNA primers (18-22 bp, T_m: 50-60^{0}C) are designed to anneal to a known sequence. The primers are separated in the sequence that we are targeting by a few hundred base pairs. Cooling the reaction from 98^{0}C to a more moderate temperature allows annealing to take place.



Now we have two primed templates. With dNTPs and DNA polymerase in the reaction mixture, new DNA is synthesized.

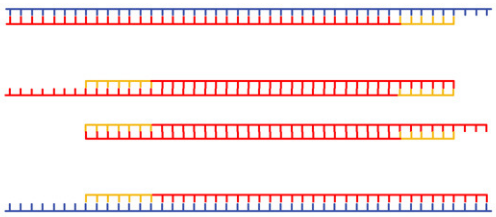
PCR – Polymerase Chain Reaction



The DNA is molten for another cycle.
Because there is a vast molar excess of primers, when we cool the mixture, we again anneal primers

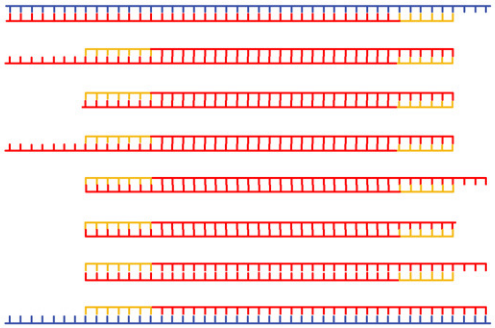
PCR – Polymerase Chain Reaction

New DNA is synthesized



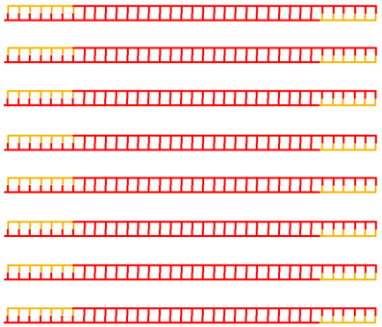
PCR – Polymerase Chain Reaction

In the next cycle, we begin to see DNA molecules whose ends are defined by the primers



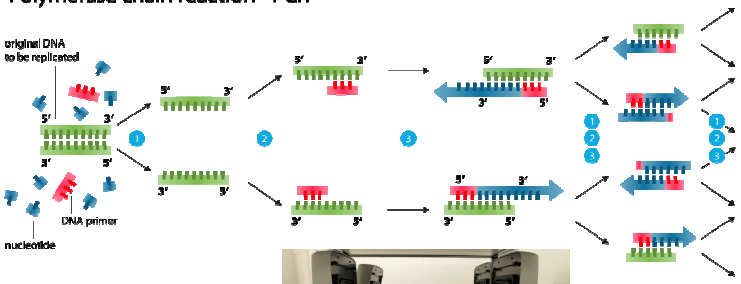
PCR – Polymerase Chain Reaction

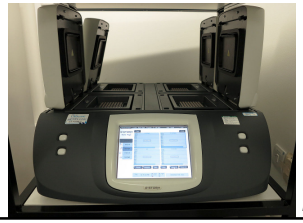
After many cycles of melting, annealing, and replication, the overwhelming majority of DNA molecules in the mixture have ends defined by the primers

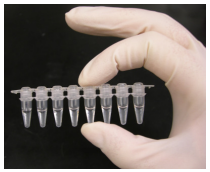


https://www.dnalc.org/view/15475-The-cycles-of-the-polymerase-chain-reaction-PCR-3D-animation.html

Polymerase chain reaction - PCR

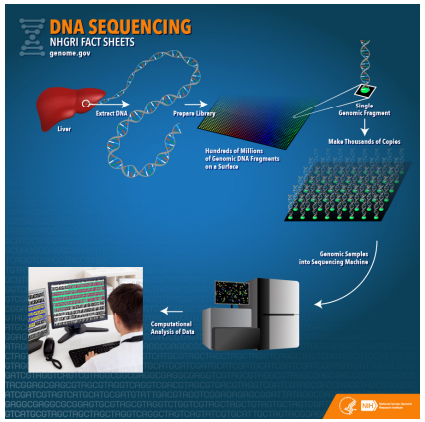


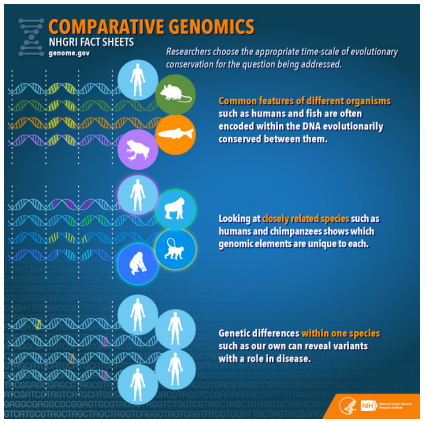




Rror

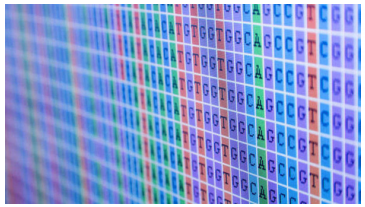
DNA sequencing

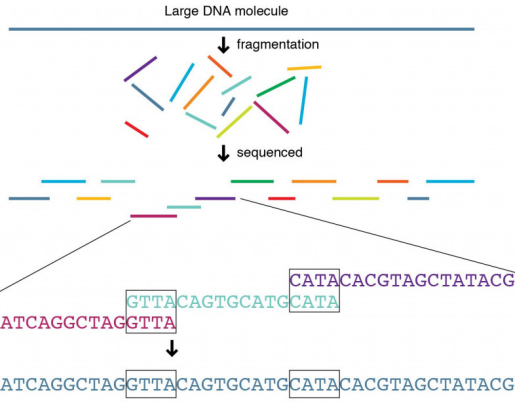




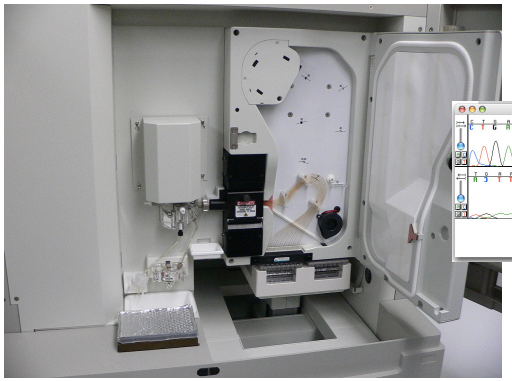
National Library of Medicine, National Institutes of Health

Genome sequencing





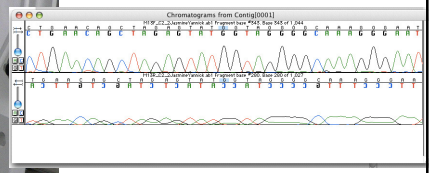
Genome sequencing



Mark Pellegrini

An ABI PRISM 3100 Genetic Analyzer. Such capillary sequencers automated the early efforts of sequencing genomes.

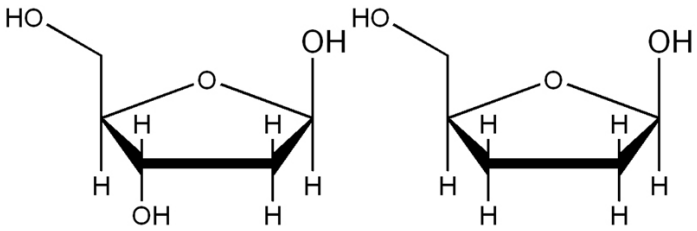
Electropherograms are commonly used to sequence portions of genomes



Tom David

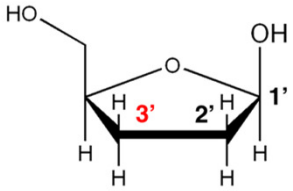
Sanger sequencing

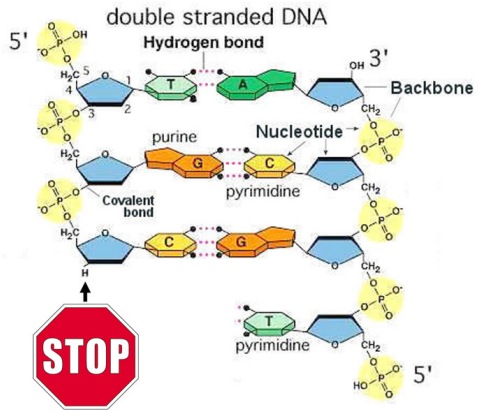
What good is dideoxyribose?



deoxyribose **dideoxyribose**

Sanger sequencing



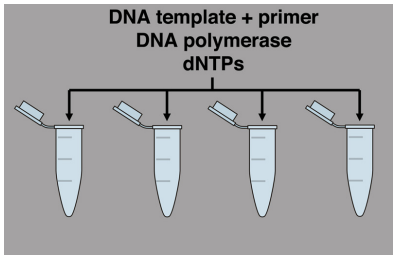


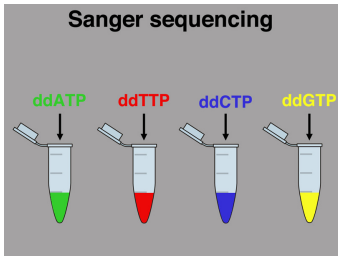
Sanger sequencing

primer

5' 3'
TACGT
ATGCATTAGGGCCTGGCTCTTT
3' 5'

template

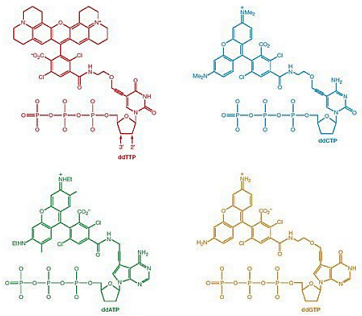


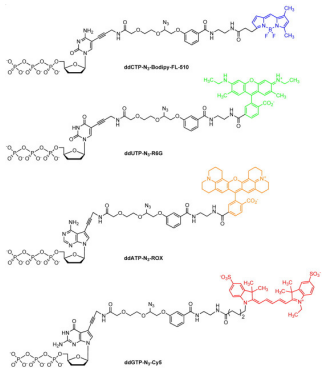


Sanger sequencing

Fluorescent dideoxynucleotides

standard *chemically cleavable (reduction)*





J. Ju *et al. PNAS* **2005**, *102 (17)*, 5926-5931

Sanger sequencing

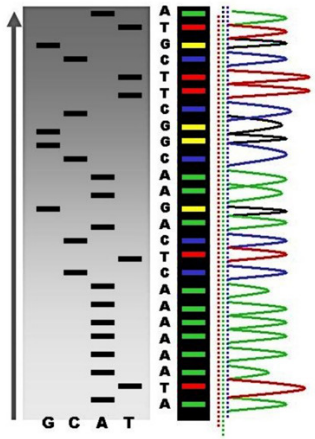
Sequencing Gel

TACGTA
ATGCATTAGGGCCTGGCTCTTT

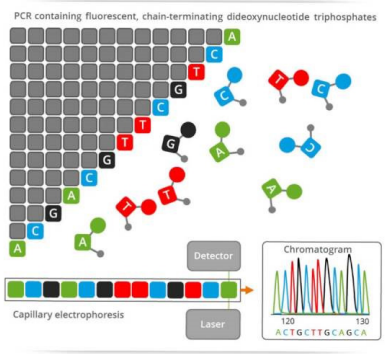
TACGTAA
ATGCATTAGGGCCTGGCTCTTT

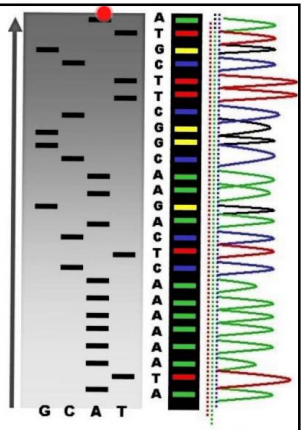
TACGTAATCCCGGA
ATGCATTAGGGCCTGGCTCTTT

TACGTAATCCCGGACCGA
ATGCATTAGGGCCTGGCTCTTT



Sanger Sequencing

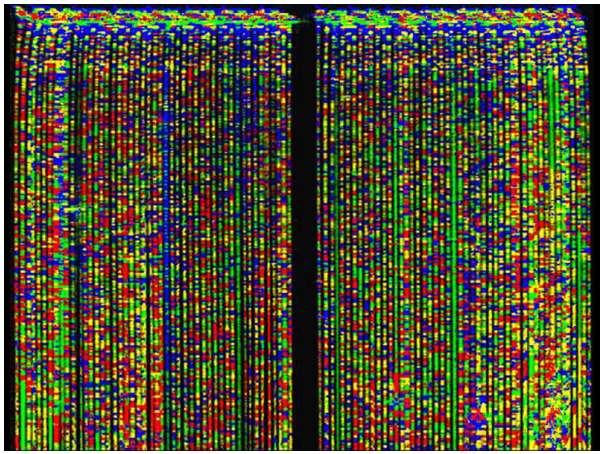




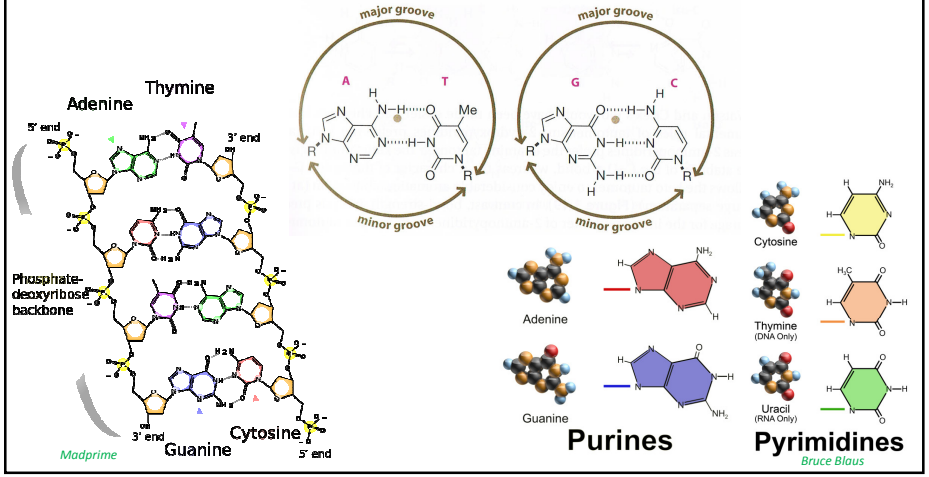
Sanger sequencing uses ddNTPs (dideoxynucleotide triphosphates) which do not have a free 3' OH mixed in with dNTPs
Whenever the DNA polymerase incorporates a ddNTP it won't be able to add any other nucleotides
Then gel electrophoresis is used to separate the DNAvv

https://www.youtube.com/watch?v=ONGdehkB8jU (from 0:50)

Sanger sequencing



Canonical nucleobases and Watson-Crick pairing in DNA



Hoogsten base pairing of canonical DNA nucleobases

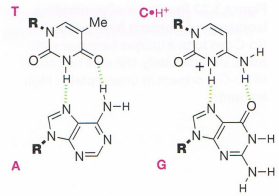
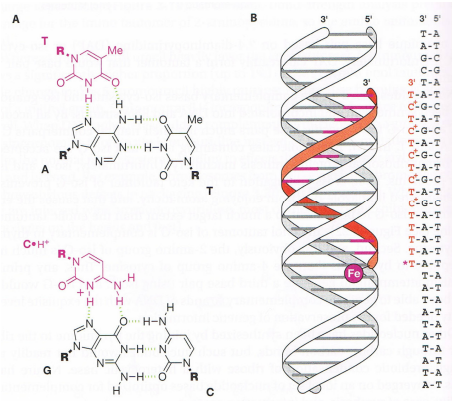
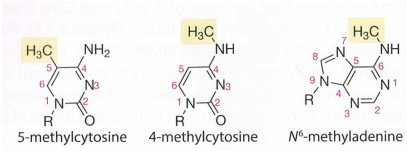


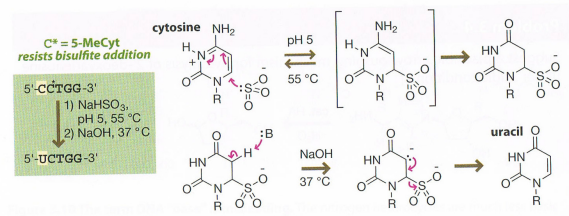
Figure 3.24 Hoogsteen base pairs. Hoogsteen base pairs use a different edge of the purine from a Watson–Crick base pair.



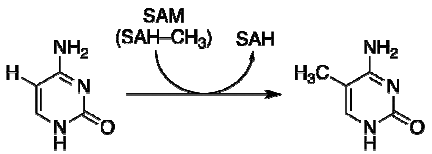
H. E. Moser, P. B. Dervan *Science* **1987**, *238*, 645-650

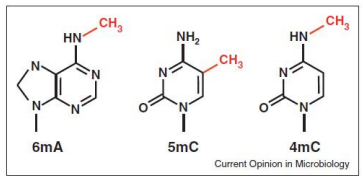
Modifications of nucleobase structures tolerated by polymerases



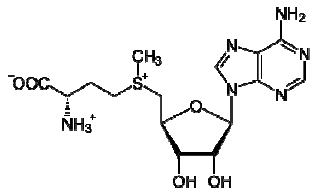


Modifications of nucleobases

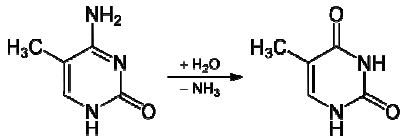




Chemical structures of common modified bases generated by DNA methyltransferases.

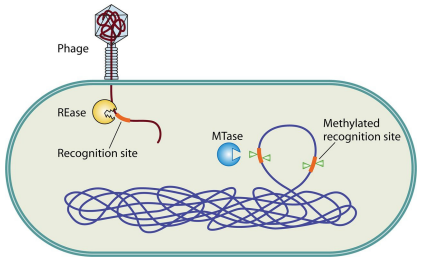


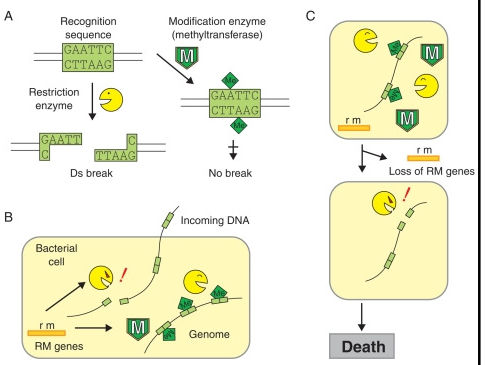
S-Adenosylmethionine (SAM)



Restriction modification system

„Immune system" of bacteria and archaea against atacking viruses



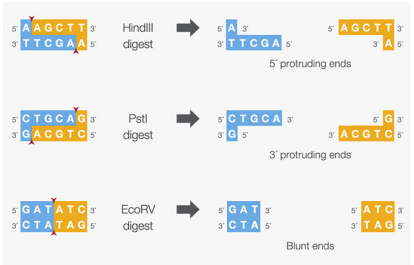


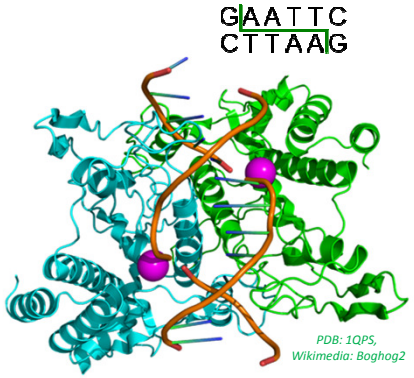
K. Vasu, V. Nagaraja
Microbiol. Mol. Biol. Rev. **2013**, *77(1)*, 53-72

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

EcoI – a typical restriction enzyme

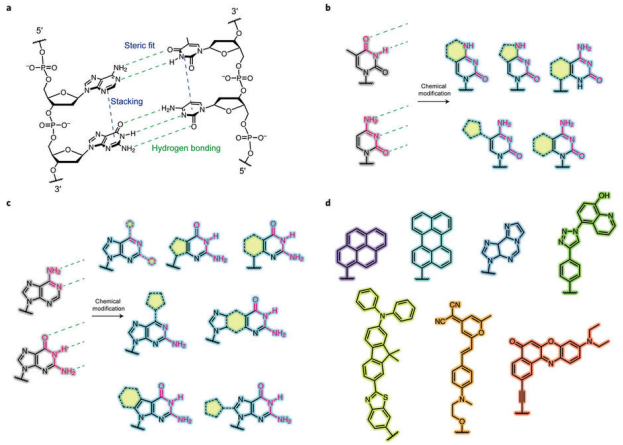
Products of restriction enzymes





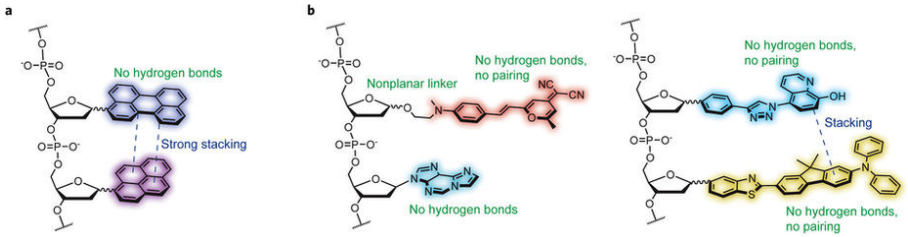
Structure of the homodimeric restriction enzyme EcoRI (cyan and green cartoon diagram) bound to double stranded DNA (brown tubes). Two catalytic magnesium ions (one from each monomer) are shown as magenta spheres and are adjacent to the cleaved sites in the DNA made by the enzyme (depicted as gaps in the DNA backbone).

Non-canonical fluorescent nucleobases



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

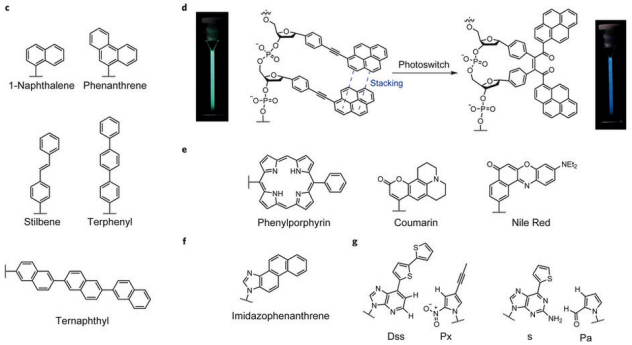
Non-canonical fluorescent nucleobases



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

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

Non-canonical fluorescent nucleobases



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

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

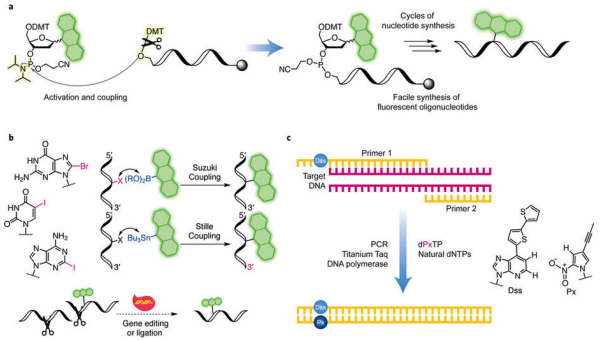
Strategies for incorporating fluorescent nucleobases

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

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

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

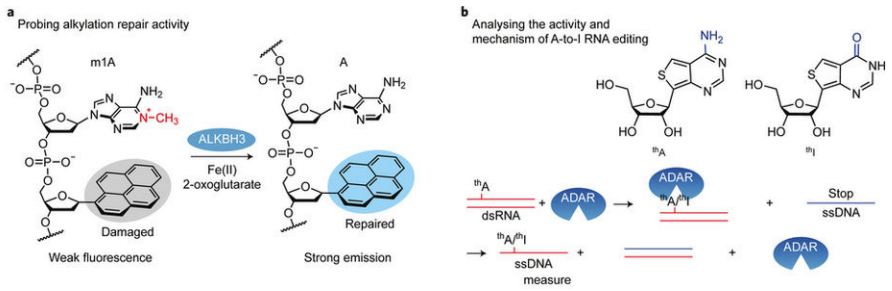
Strategies for incorporating fluorescent nucleobases



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

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

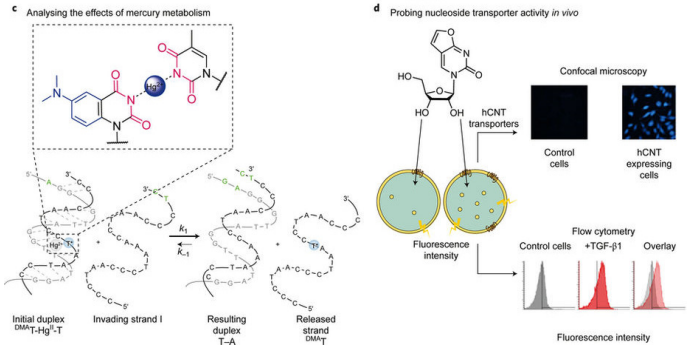
Fluorescent nucleobases for studying DNA and RNA



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

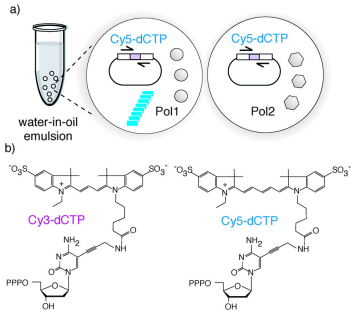
W. Xu, K. M. Chan, E. T. Kool *Nature Chem.* **2017**, *9,* 1043-1055

Fluorescent nucleobases for studying DNA and RNA



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

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

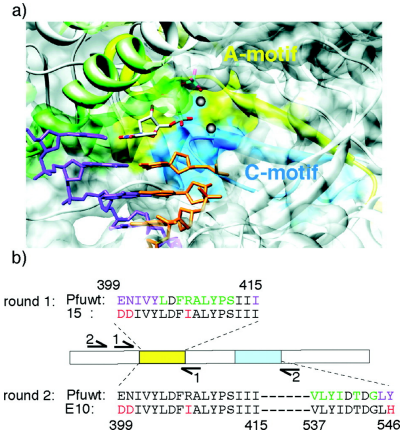


DNA Polymerase is evolutionarily optimized by mutagenesis to accept fluorescent Cy3 or Cy5 nucleotides instead of simple dCTP. Here, the polymerase replicates a short segment of its own encoding gene. Water/oil compartmentalization allows testing many independent mutations in paralell

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

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

CyDNA – synthesis and replication of highly fluorescently-labelled DNA



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

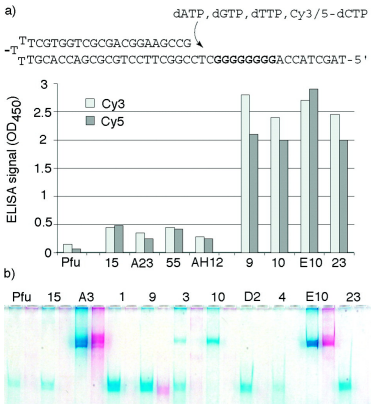
(b) For round 1 selection, diversity was focused on the A-motif and vicinity (399–415) comprising random mutation spike at the core of the A-motif (lime) as well as phylogenetic diversity in the adjacent sequences (purple). For round 2, successful clones from round 1 (e.g., 15) were diversified in the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

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

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

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

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



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

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

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

Fulvio314

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

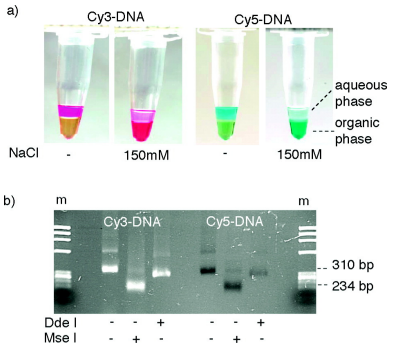
Table 1. Polymerase Fidelity

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

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

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

CyDNA – synthesis and replication of highly fluorescently-labelled DNA

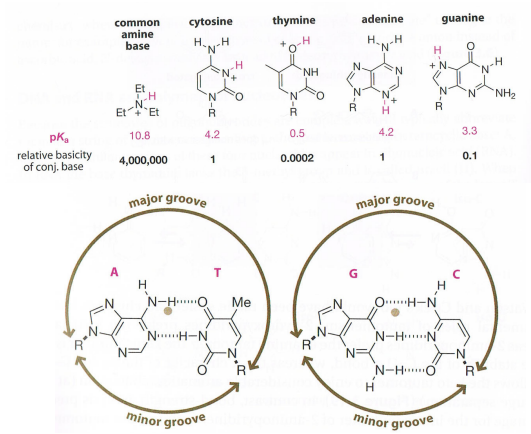


(a) Organic phase partitioning of CyDNA is shown for Cy3-DNA (left) and Cy5-DNA (right). Essentially 100% partitoning occurs in the presence of 150 mM NaCl (the yellow color of the phenol phase is due to addition of 8-hydroxyquinoline to prevent oxidation).

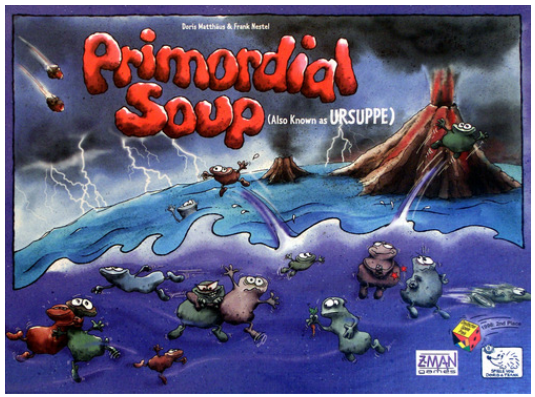
(b) Agarose gel electrophoresis of CyDNA restriction digests. Restriction endonucleases are sensitive probes of noncanonical DNA conformations such as those which occur under torsional strain. Both Cy3- and Cy5-DNA are resistant to cleavage by the restriction endonuclease DdeI (C'TNAG) but are cut by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

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

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



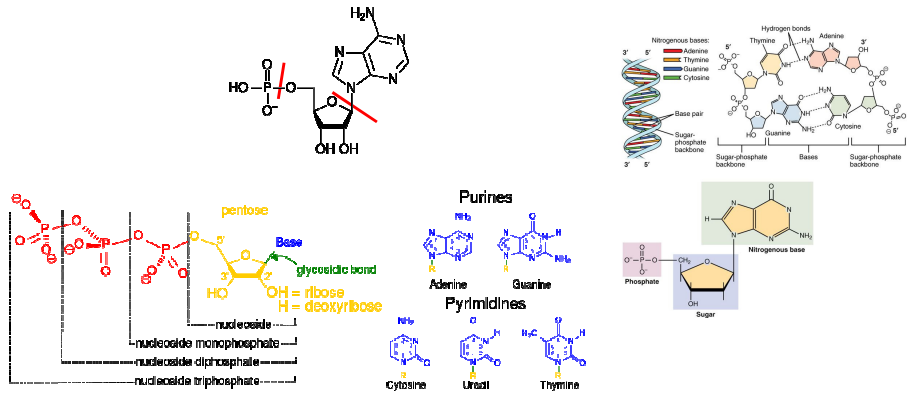
Prebiotic synthesis of nucleotides



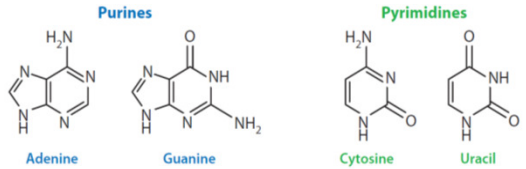
State of the art

Nucleotides - components

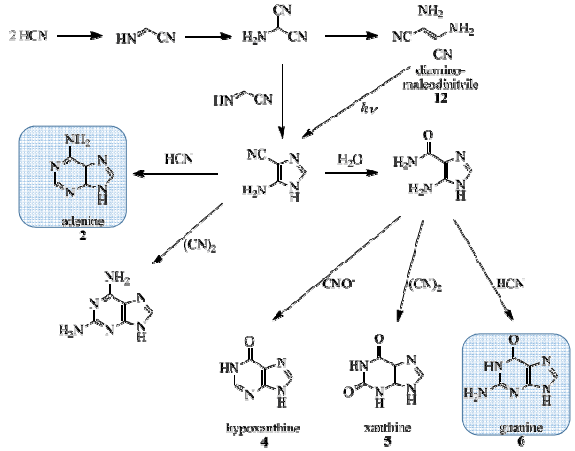
RNA – most likely evolutionarily older („RNA World") than DNA → prebiotic origin of ribose + A, C, G, and U nucleobases



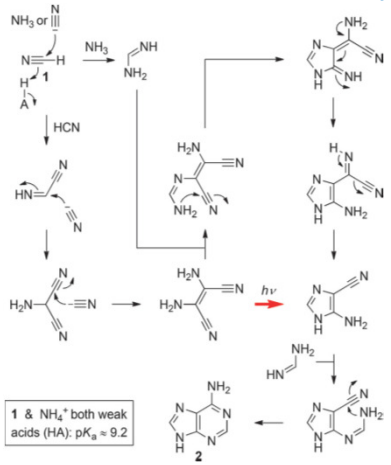
Prebiotic synthesis of nucleobases



Prebiotic synthesis of purines



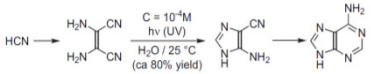
Prebiotic synthesis of adenine



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°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°C) increases the yield of DAMN formation by concentrating HCN between pure ice crystals

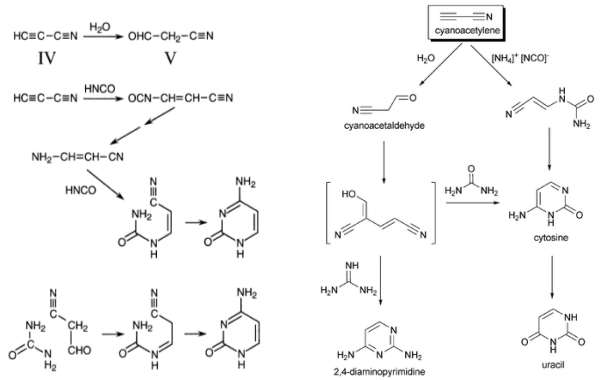


J. Oro *Biochem. Biophys. Res. Commun.* **1960**, *2*, 407.

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

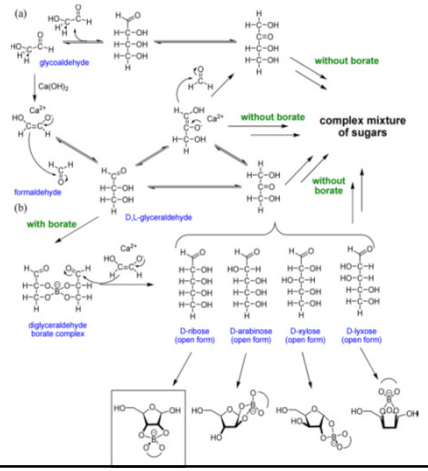
Prebiotic synthesis of pyrimidines

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.

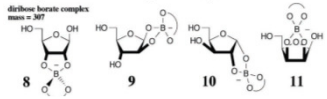
Formose reaction in presence of borates



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.

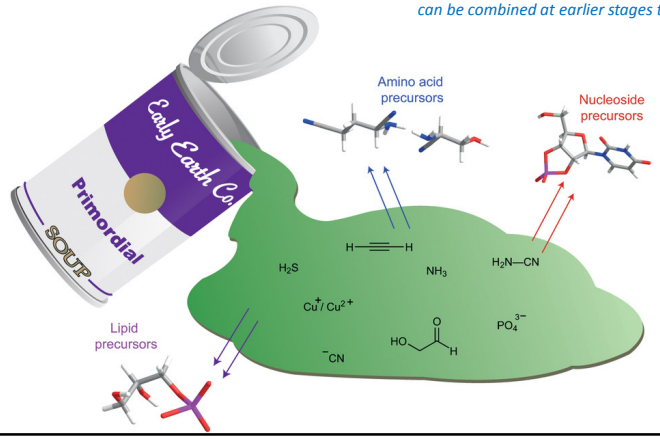


A. Ricardo, M. A. Carrigan, A. N. Olcott, S. A. Benner *Science* **2004**, *303*, 196

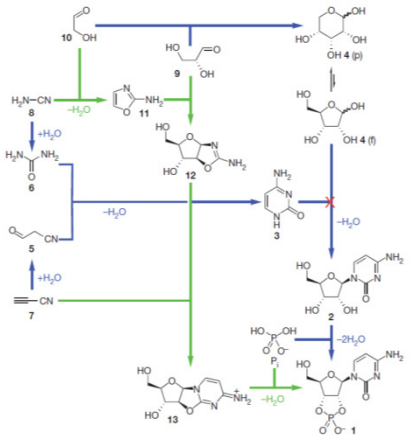
Escosura

Cyanosulfidic chemistry

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

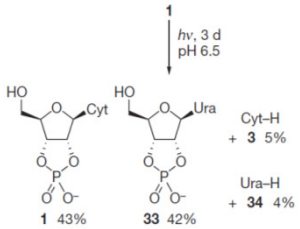


Cyanosulfidic chemistry



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.