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Design of New Chemical Tools (Artificial Enzymes) for Future Biotechnology

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What Are Artificial Enzymes?

Artificial materials which show remarkable catalysis (activity & selectivity) like naturally occurring enzymes.

They catalyze

(1) various reactions which are never catalyzed by naturally occurring enzymes (any model in the nature is unnecessary),

(2) (in some case) even with higher specificity than naturally occurring enzymes.

(3) under non-physiological conditions

Two Kinds of "Artificial Enzymes"

1. Completely man-made enzymes
= Chemically synthesized catalysts
(usually involve no proteins)

2. Semi-artificial enzymes
= Mutant proteins obtained by protein engineering

In this lecture, we focus to the first ones
which can be freely designed according to our need.

Why We Need Artificial Enzymes?

All the livings in the Nature do what is necessary for their existence (not for us).

1. Physiological conditions (pH 7, r.t., 1 atm) are really the best for our practical purposes?

2. All the reactions we need are covered by naturally occurring enzymes?

Necessity of Artificial Enzymes for Future Biotechnology

In order to achieved desired reactions under desired reaction conditions , we have to prepare "tools" for ourselves

|||

Artificial Enzymes

Catalysis proceeds via ES complex and shows both high rates and high specificity.

How to Prepare Artificial Enzymes

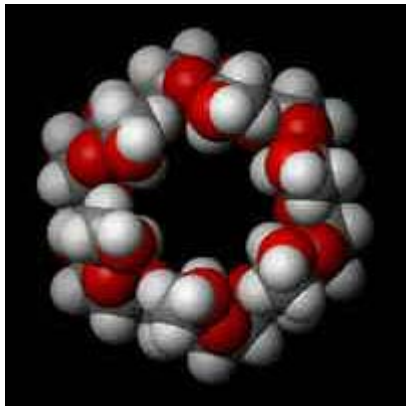
(i) Molecules which bind the substrate
(and place the reaction center near the catalyst)

+

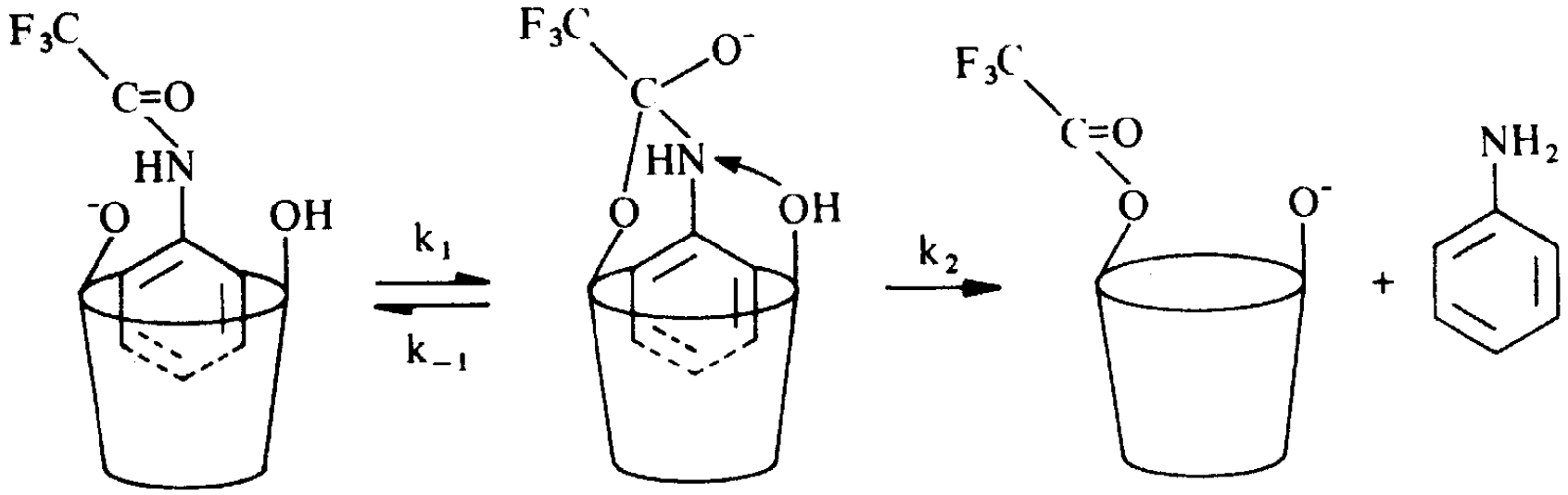
(ii) Catalyst for desired reaction

***Typical examples of the molecules used for (i)**

- **Cyclodextrin**
- **Crown ether**
- **Cyclophane**
- **Calixarene**
- **・・・・・・・・・・**



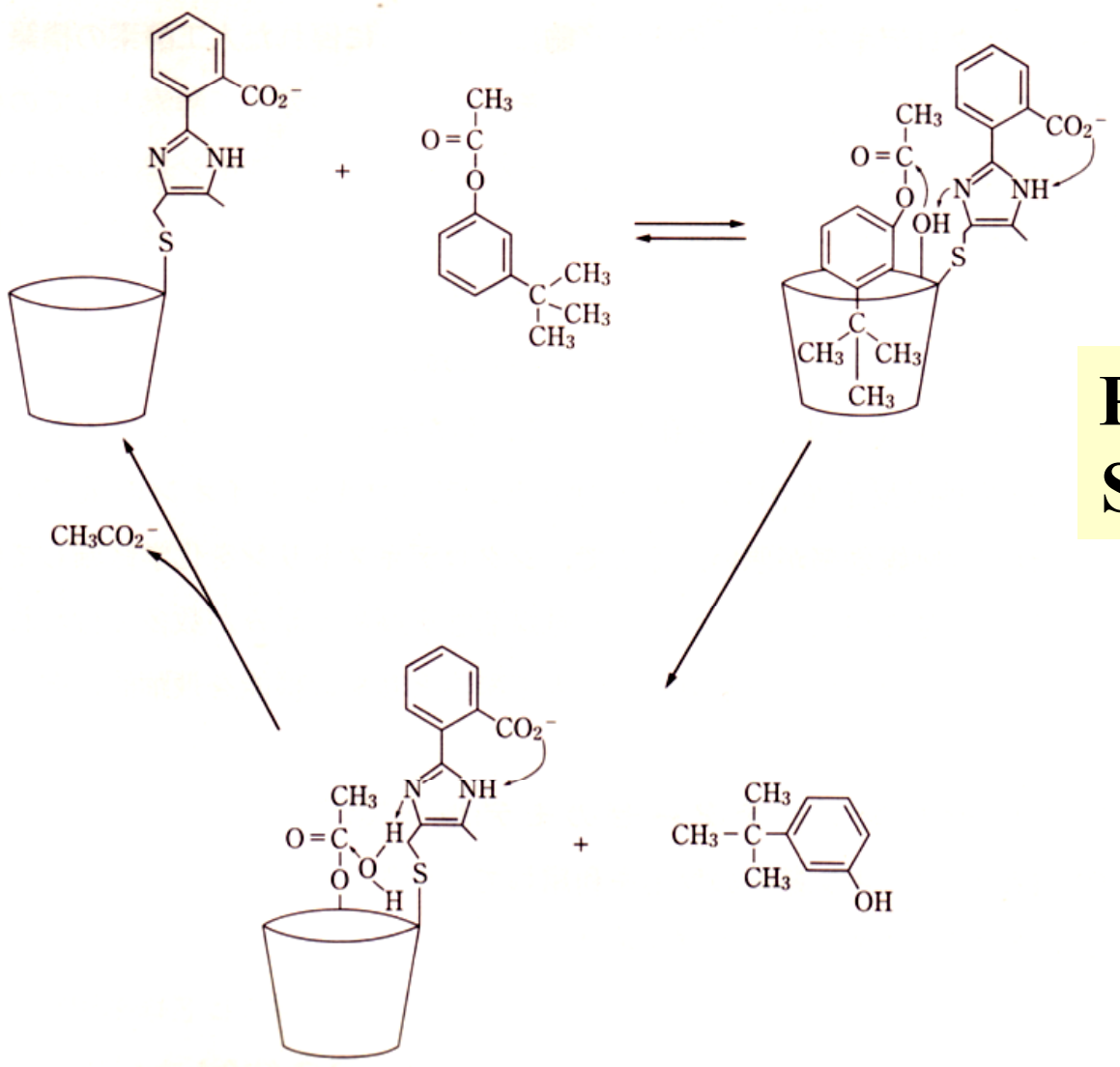
Example 1: Cyclodextrin for amide hydrolysis



Enzyme-Substrate complex

- **Large reaction rate**
- **Substrate-specificity**

Example 2: Chemically modified cyclodextrin as a model of charge-relay system in serine protease



Proton transfer
Ser→His→Asp

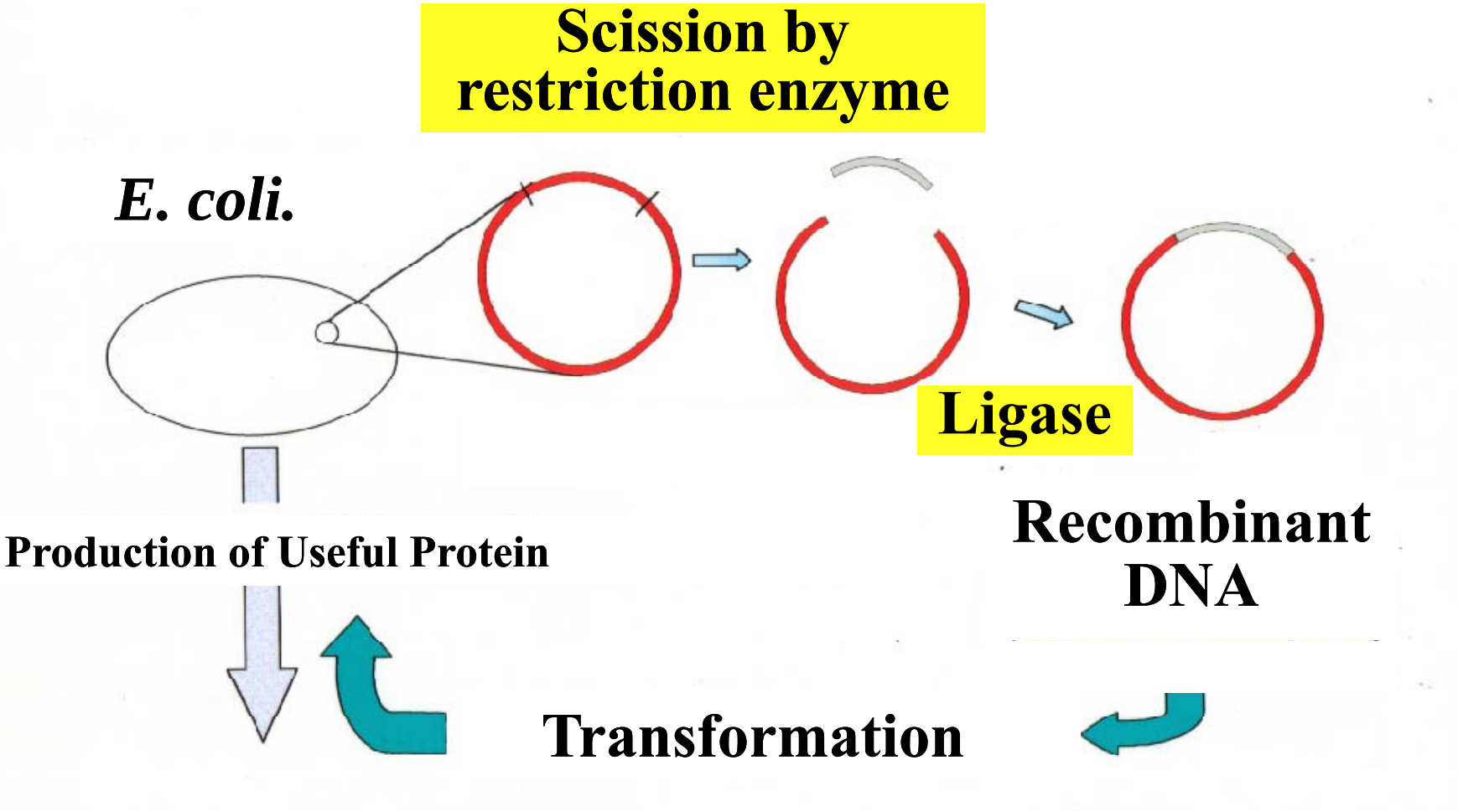
Artificial Restriction DNA Cutter (ARCUT) as Tools for Molecular Biology

Features of our ARCUT

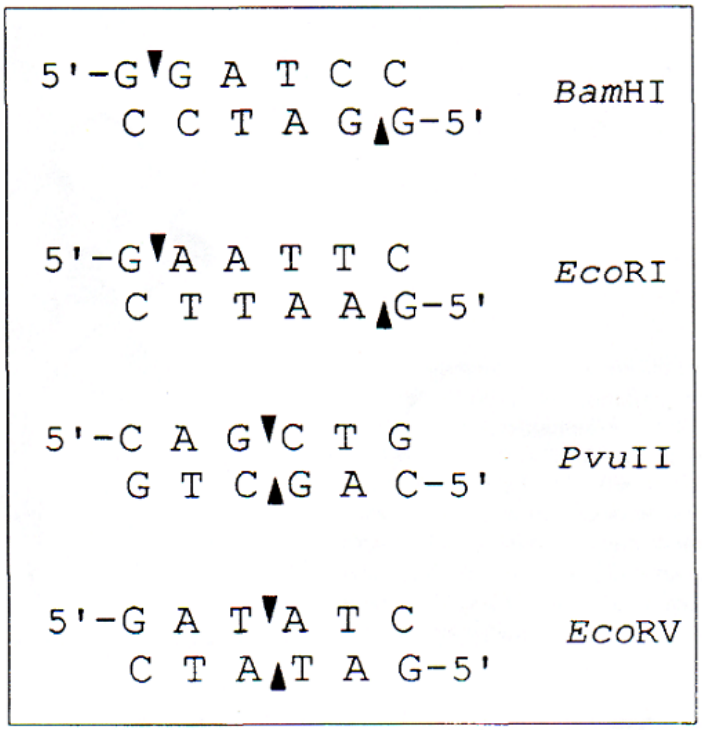
1. The cutters are completely chemistry-based and contain no proteins.
2. Scission occurs by hydrolysis of phosphodiester linkages as in enzymatic scission.
3. Scission-site is determined by Watson-Crick rule and thus the cutter is straightforwardly designed.

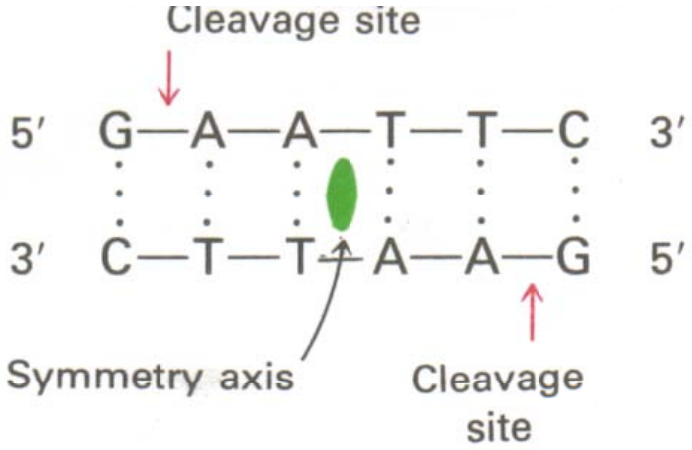
Even huge DNA can be cut at desire site.

<Background>
Typical Procedure of Gene Manipulation



Scission Site of Restriction Enzymes

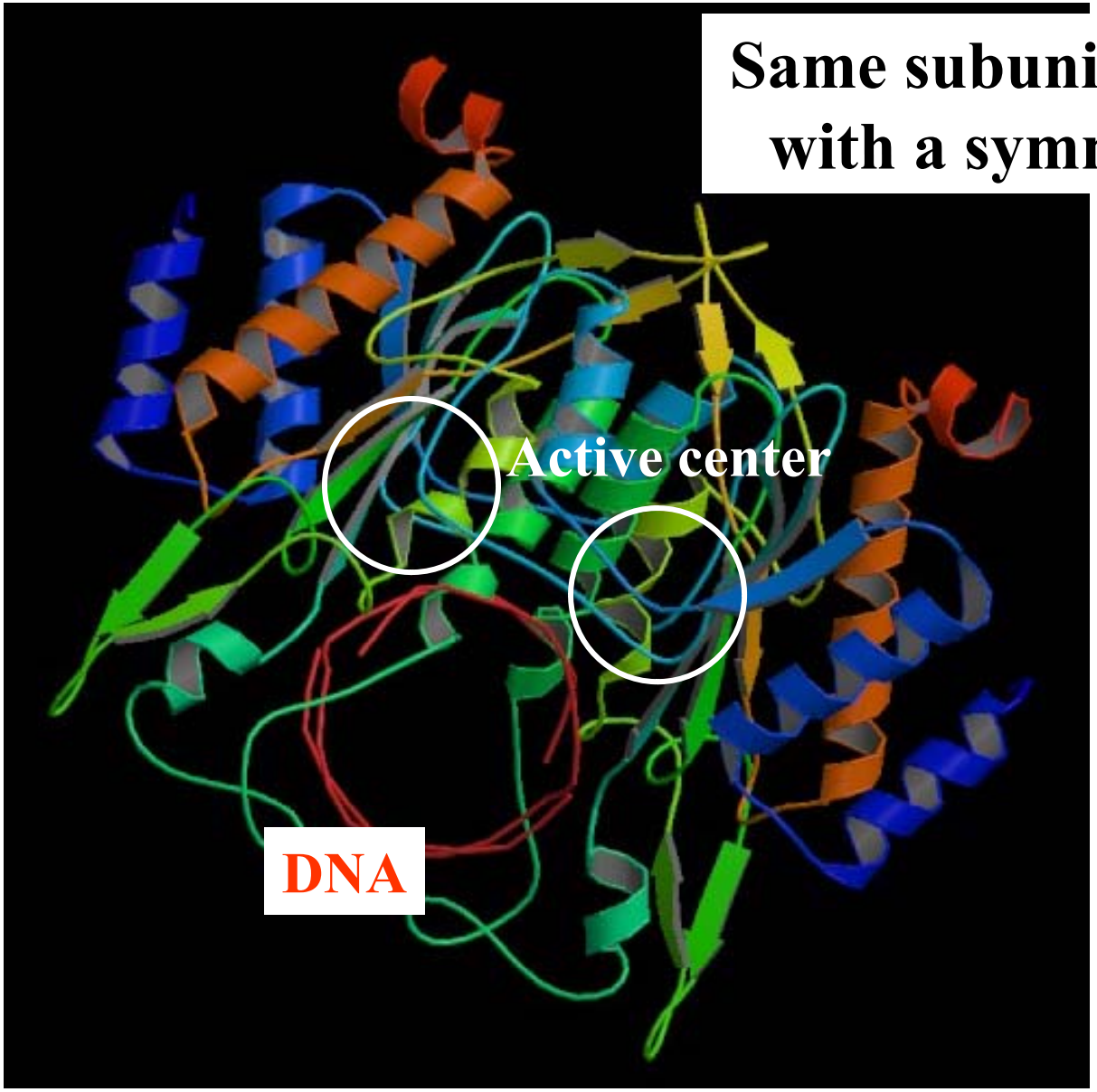




***Eco*RI**

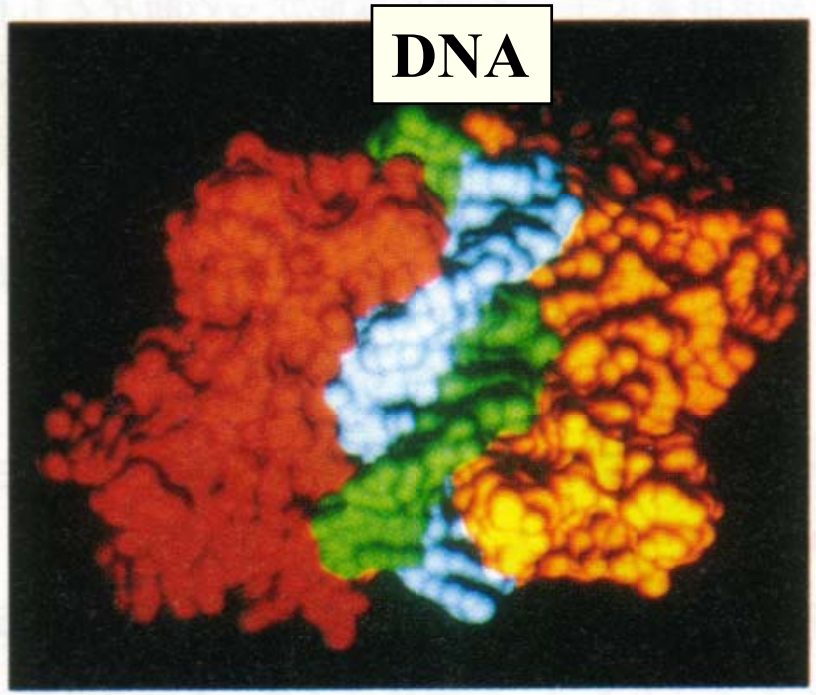
Palindrome site is recognized and cut

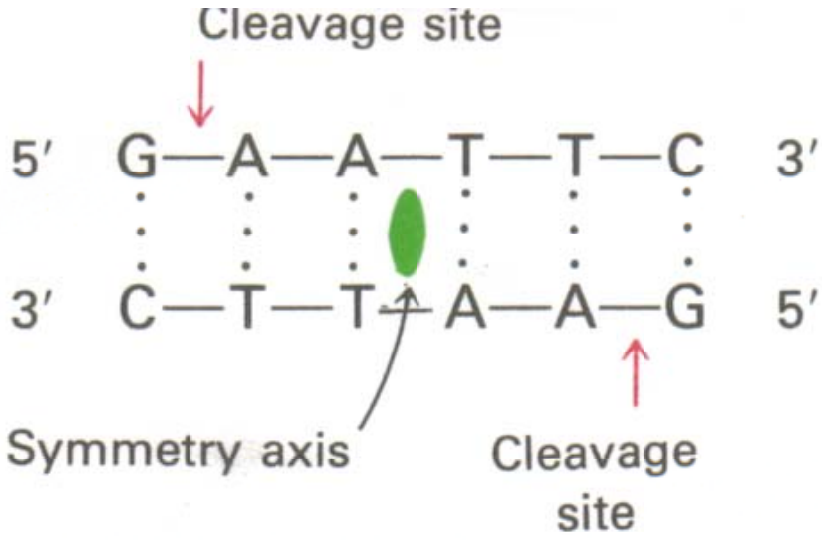
Typical Restriction Enzyme *Eco*RI



Same subunits are combined with a symmetrical center

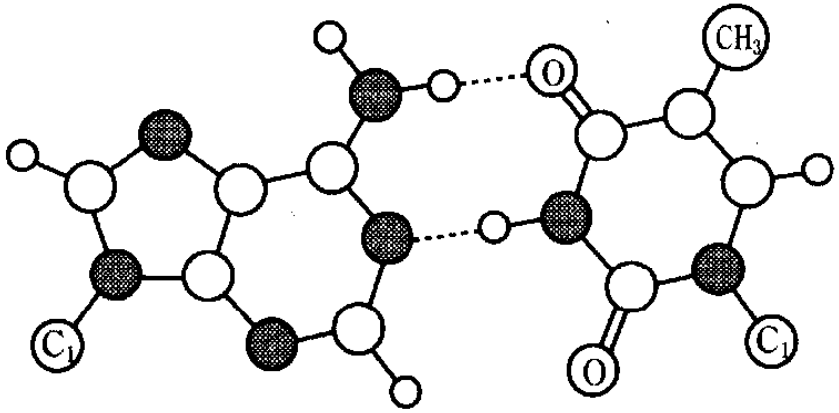
Complex of *Eco*RI with DNA





Major Groove & Minor Groove

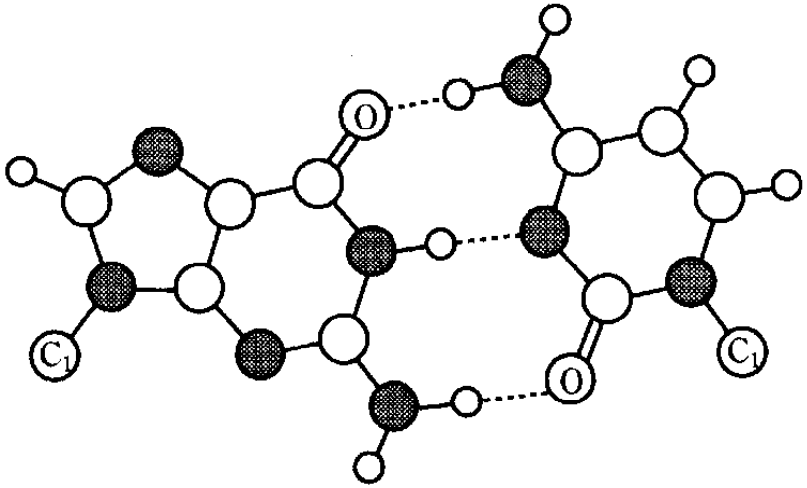
Major Groove



A-T对

Minor Groove

Major Groove

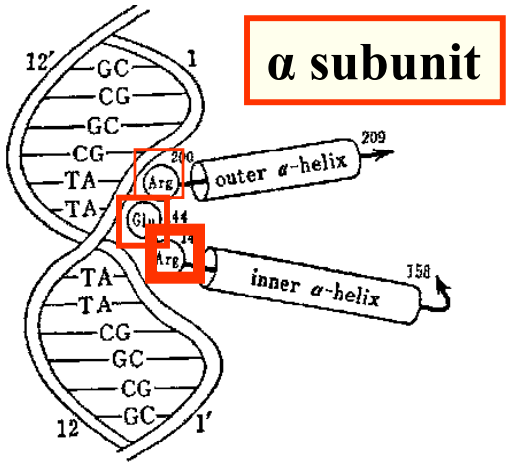


G-C对

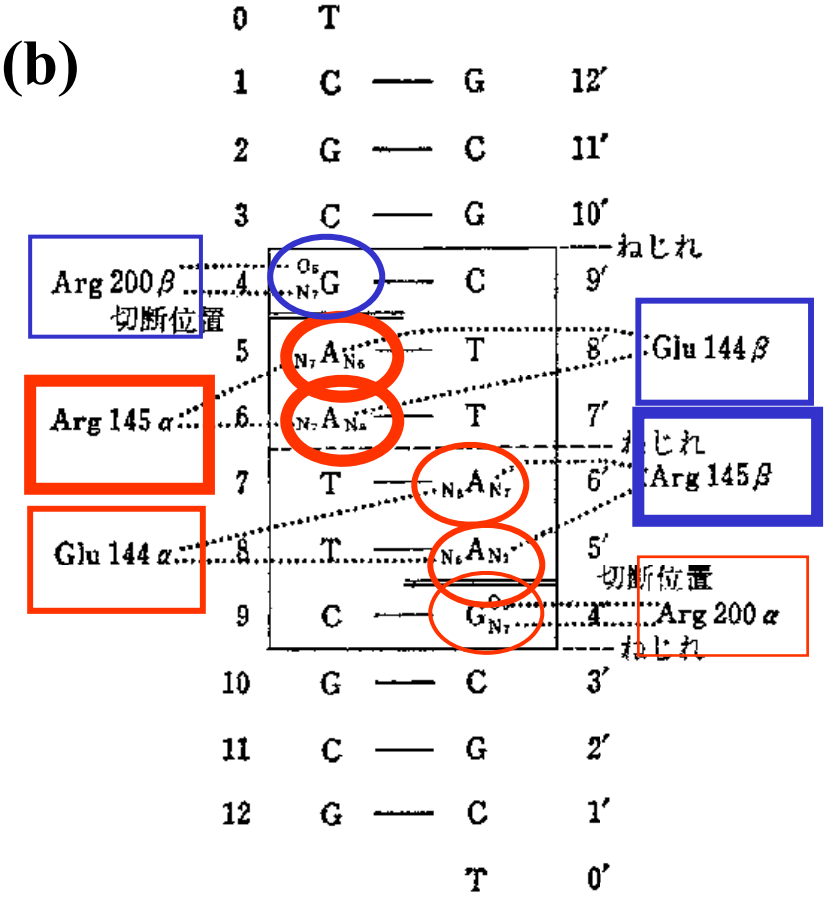
Minor Groove

Mechanism of Sequence-recognition by *Eco*RI

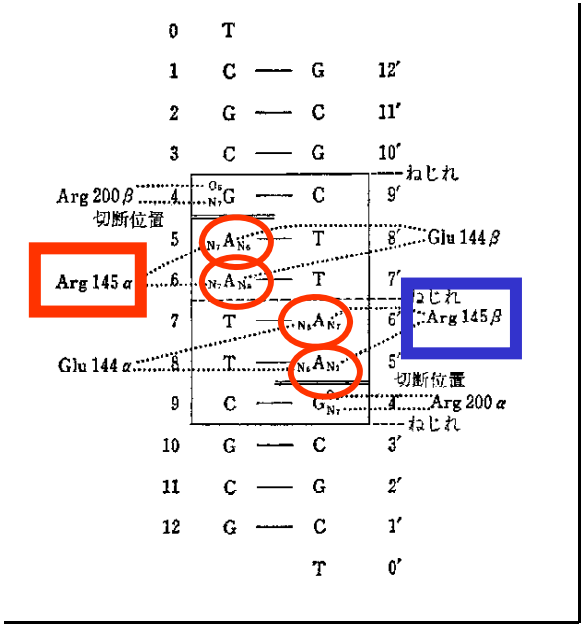
(a)

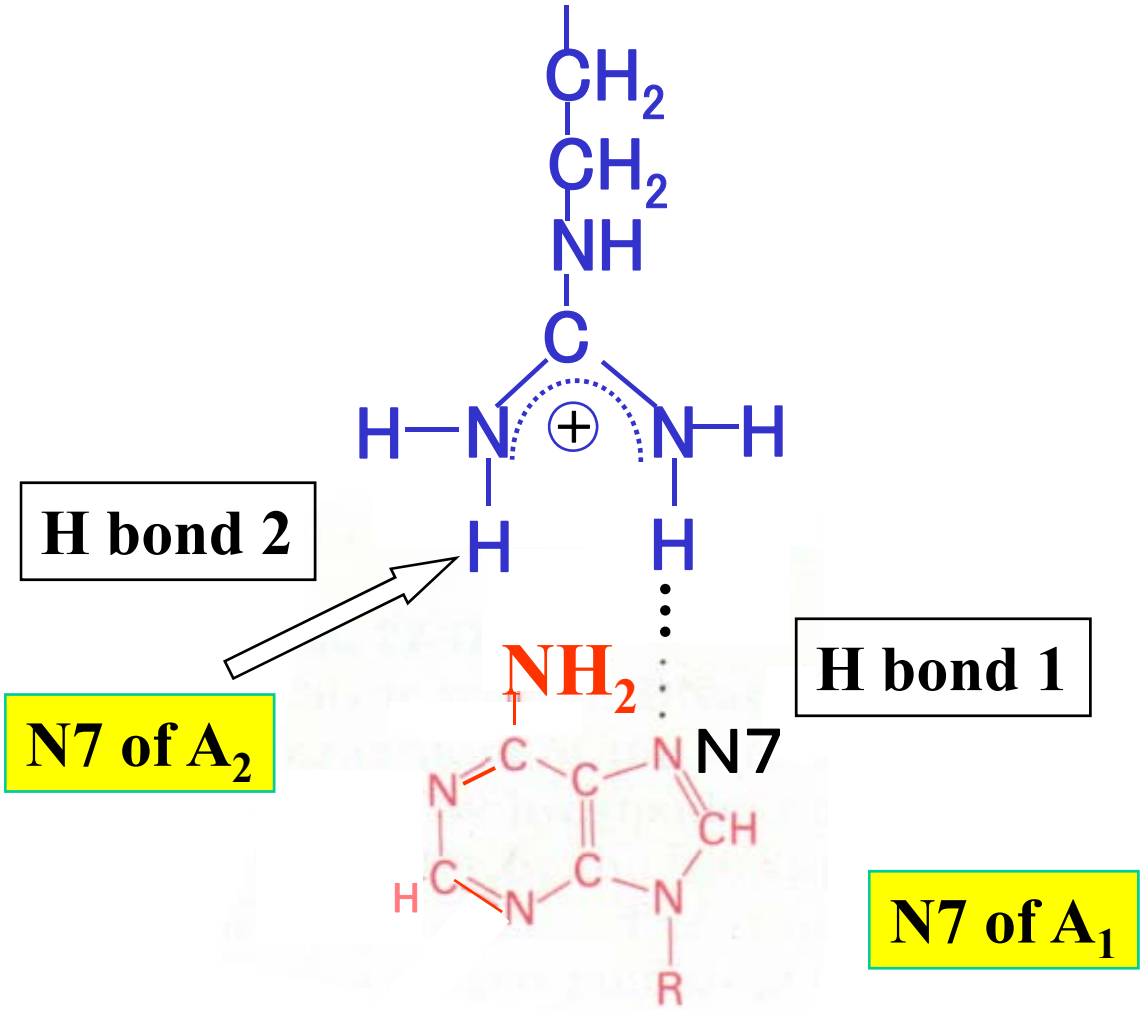


(b)

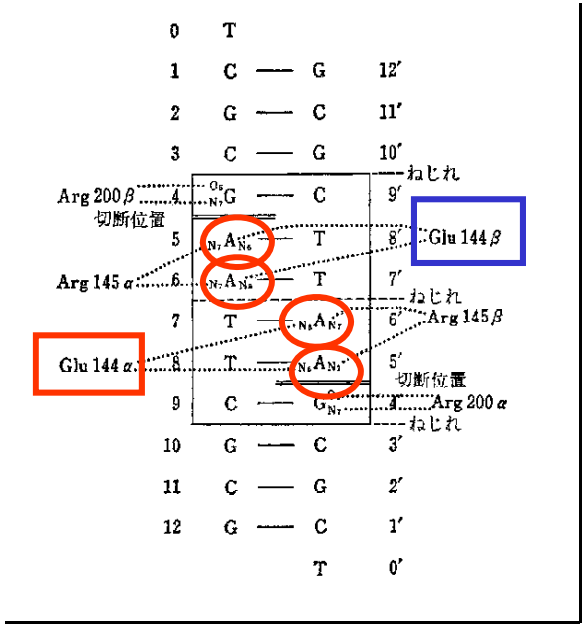


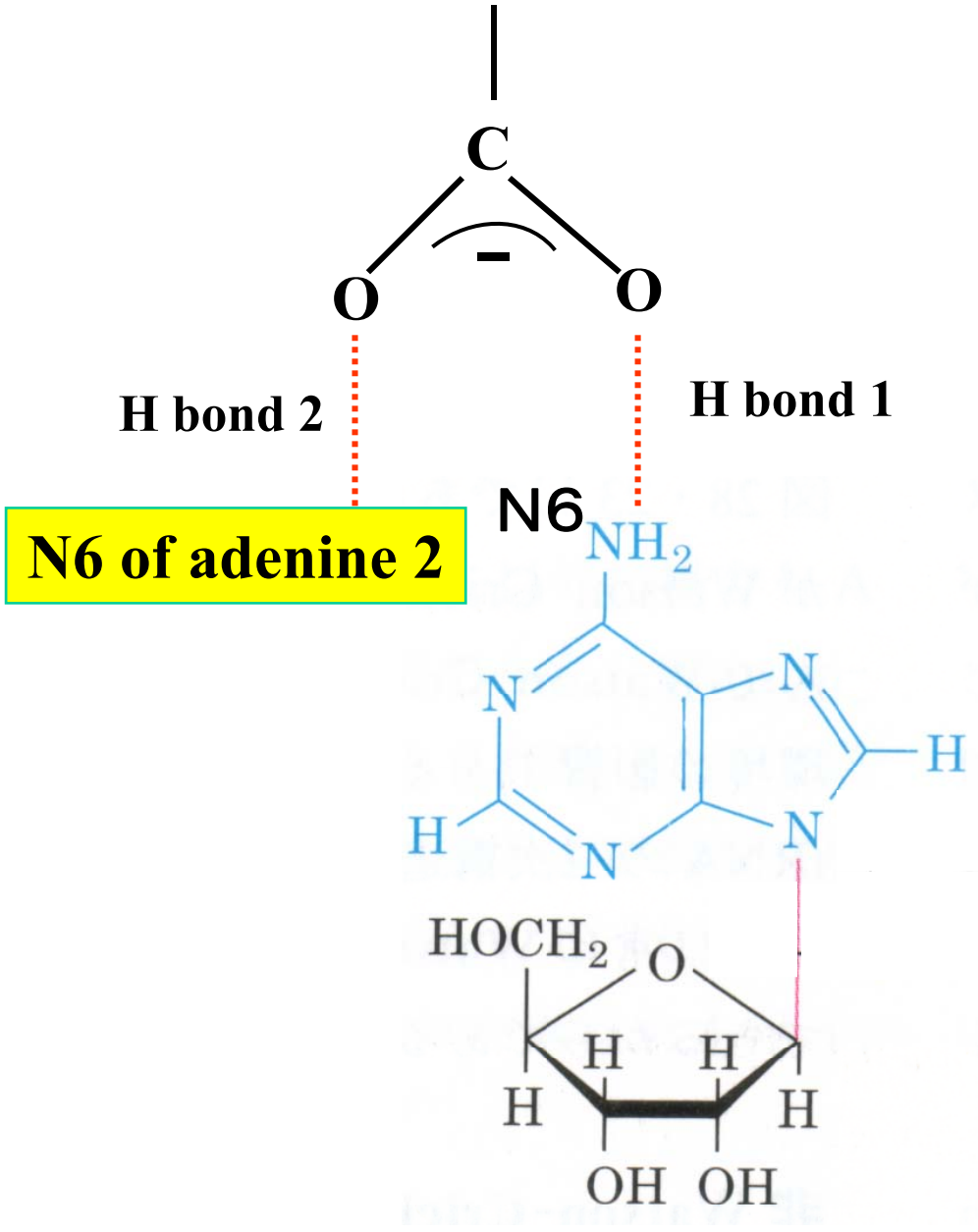
Binding of Arg to Two Adenines



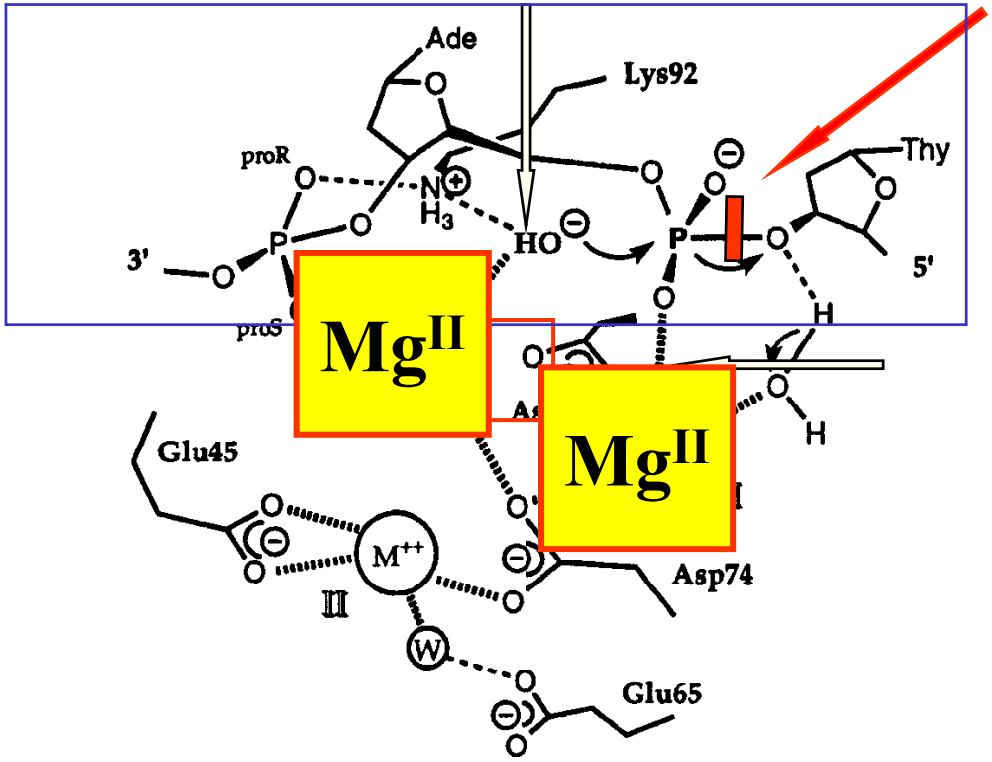


Binding of Glu to Two Adenines





DNA Scission by Restriction Enzyme (EcoRV)

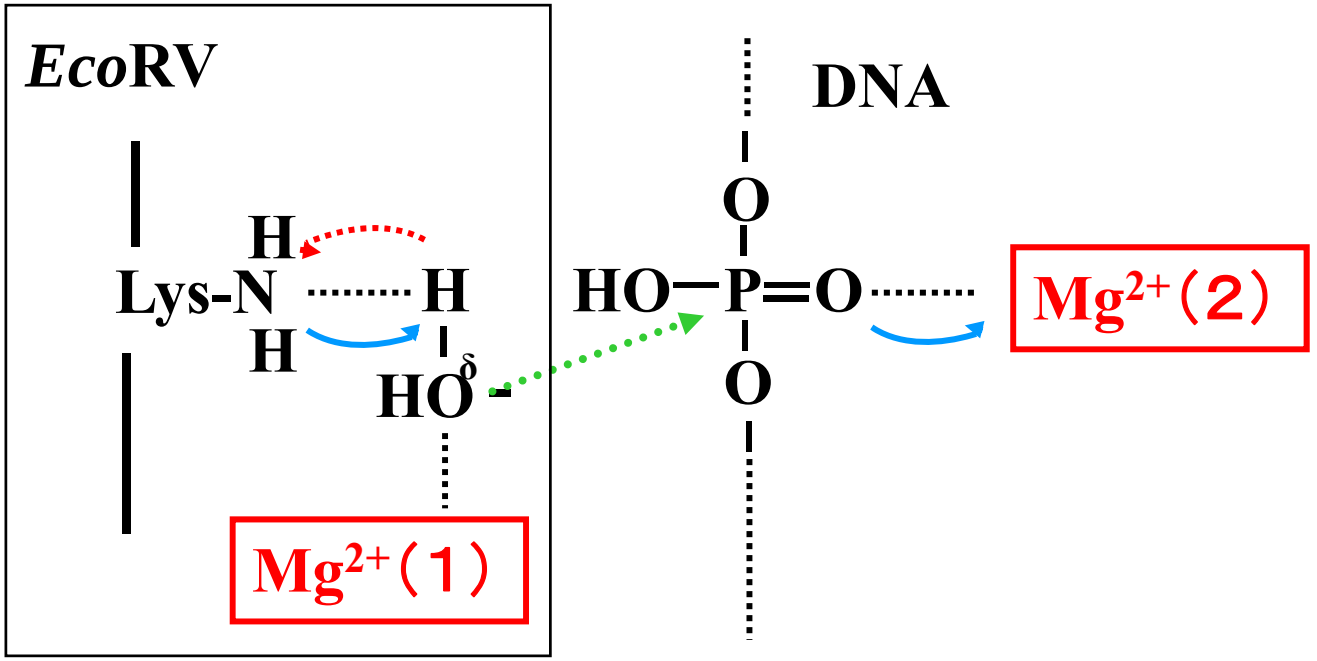


Scissile Phosphodiester Linkage

1）One Mg(II) ion provides OH^- as the nucleophile.

2）Another Mg(II) ion activates the phosphodiester linkage.

Mechanism of DNA Hydrolysis by Restriction Enzyme



Mg^{2+}(1) : Activate water by electron withdrawal

Lys : Proton removal from the water
(General base catalysis)

Mg^{2+}(2) : Electron removal from the phosphodiester

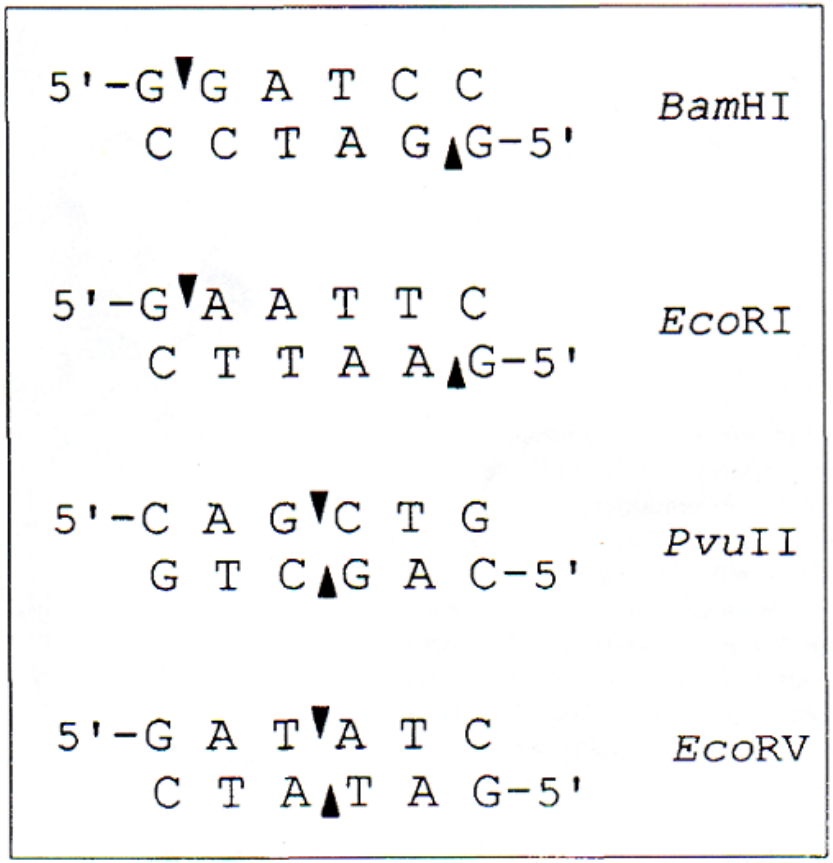
Genomes of higher livings are far larger than plasmid DNA

Plasmid DNA	**4-5 kb**
Phage	**50**
Genome of *E. coli*	**4,600**
Genome of yeast	**13,500**
Human beings	**3,000,000**

In order to pin down one site in human genome, we must recognize 16 or 17 base-sequence!

($4^{16} > 30 \times 10^8$ = the number of base-pairs in human genome)

Most of Naturally Occurring Restriction Enzymes Recognize Mostly 4 or 6 DNA-Base Sequence



Frequency of appearance of scission site
$= 1/4^6$ (= 1/4096)

Note that this number is comparable with the size of plasmid.

Limitations of Naturally Occurring Restriction Enzymes As Tools

（1） **Huge DNA cannot be manipulated.**

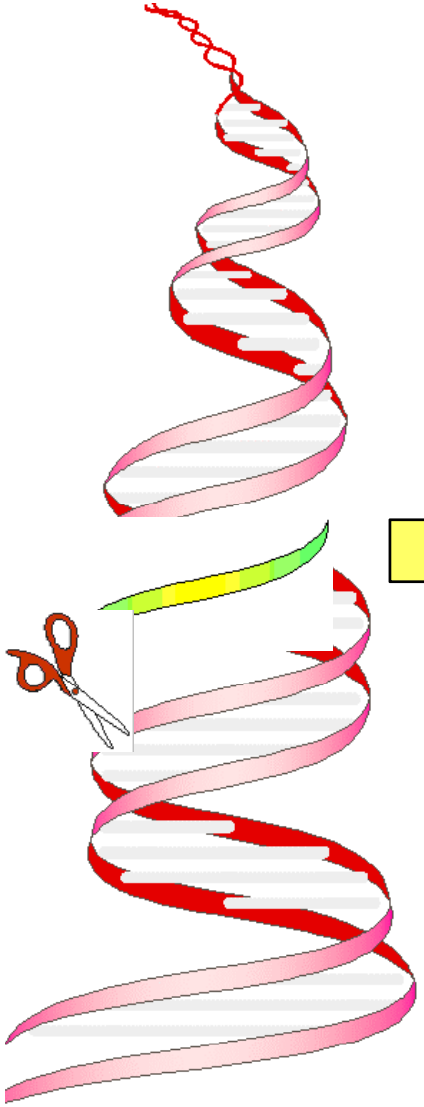
（2） **Scissile sequence is strictly limited.**

（3） ・・・・・・・・・・・・・・・

（4） ・・・・・・・・・・・・・・・

Necessity of new tools

One of the Next Goals of Biotechnology



Site-selective scission

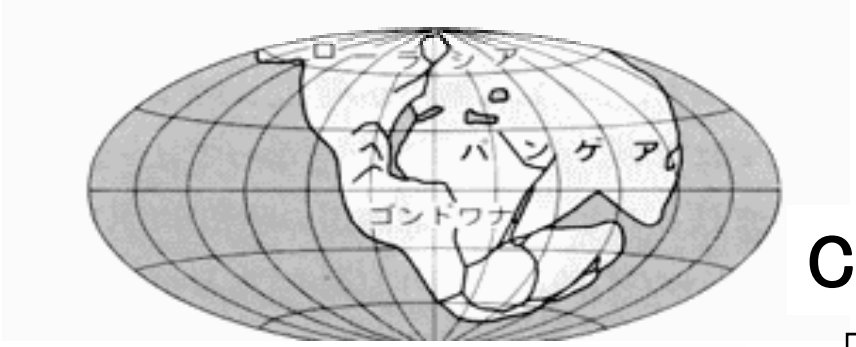
ARCUT

Appropriate ligation

Future Biotechnology

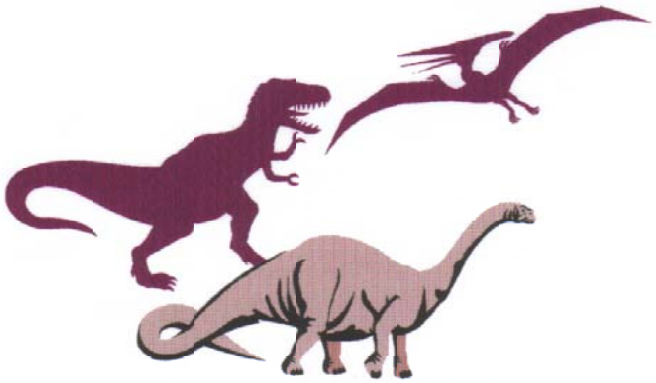
1. Preparation of Catalyst as the First Step Towards Artificial Restriction Enzyme

DNA is unbelievably stable!
(it takes more than 10^8 years to hydrolyze it without enzyme)

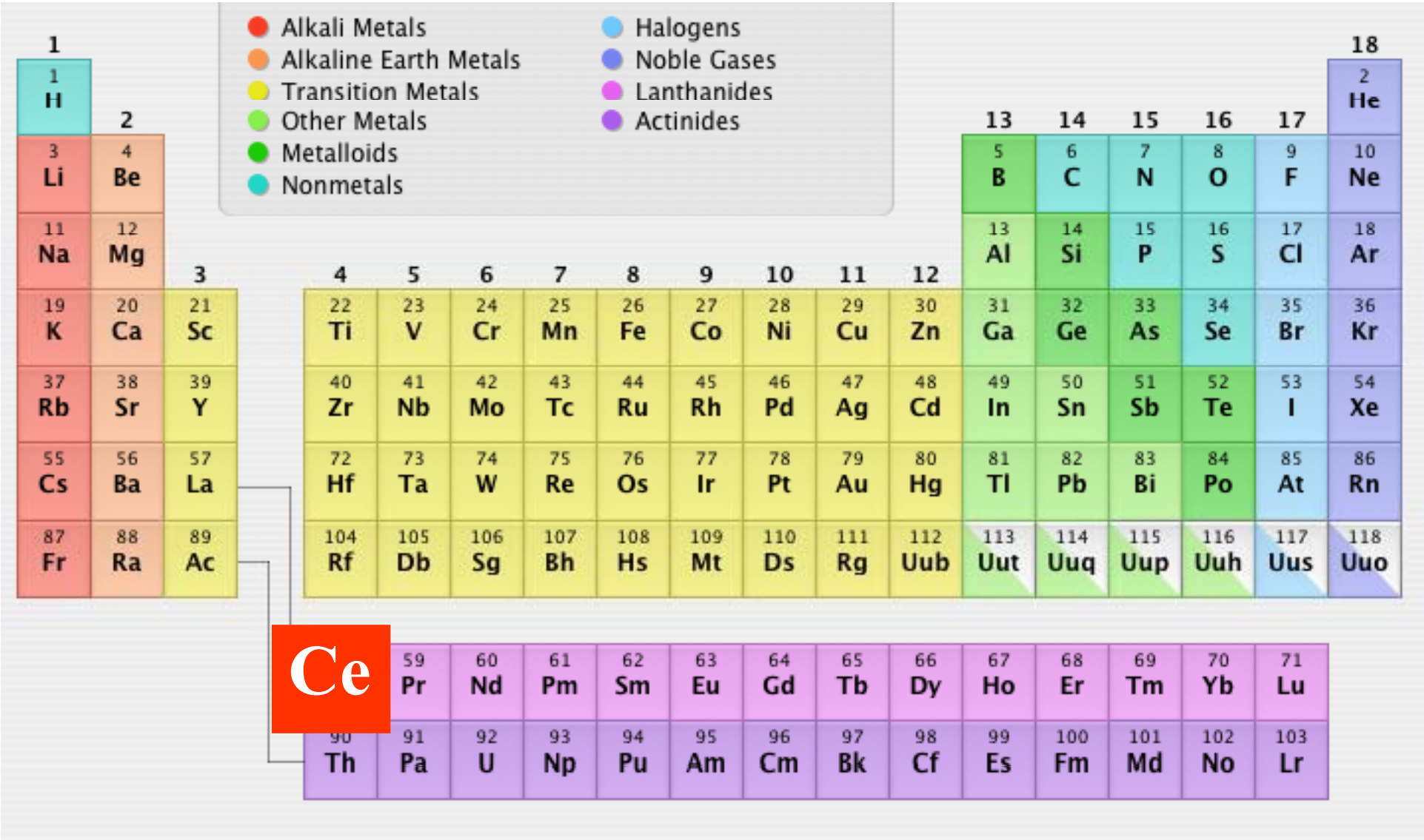


Ce(IV)

Hydrolysis within a few hours under physiological conditions

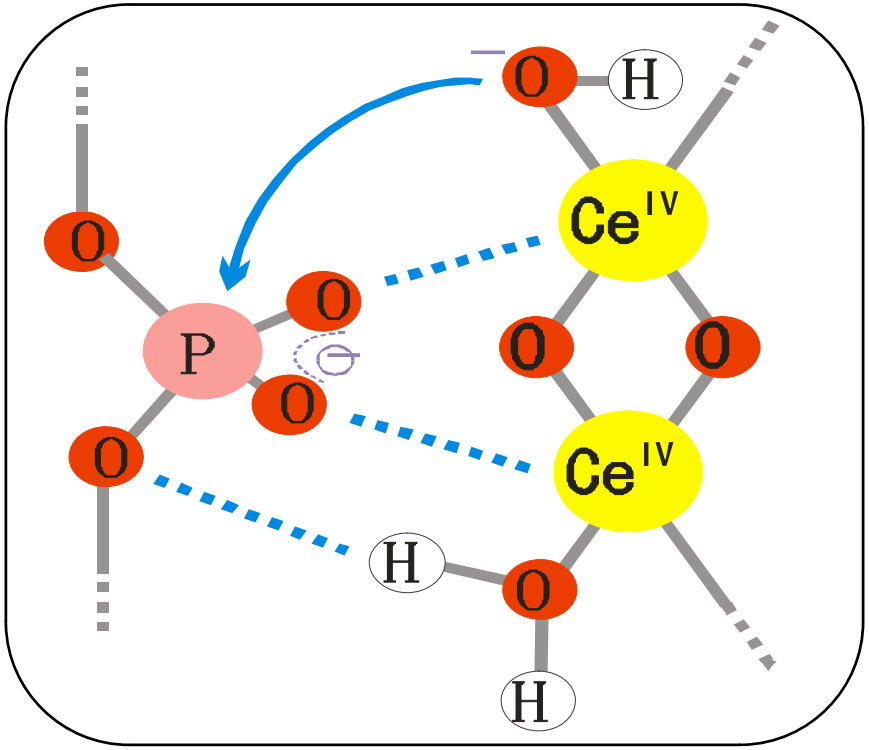


The earth of 10^8 years ago



Electronic configuration of $Ce^{(0)}$ is $4f^15d^16s^2$.

Mechanism of Ce(IV)-Induced DNA Hydrolysis



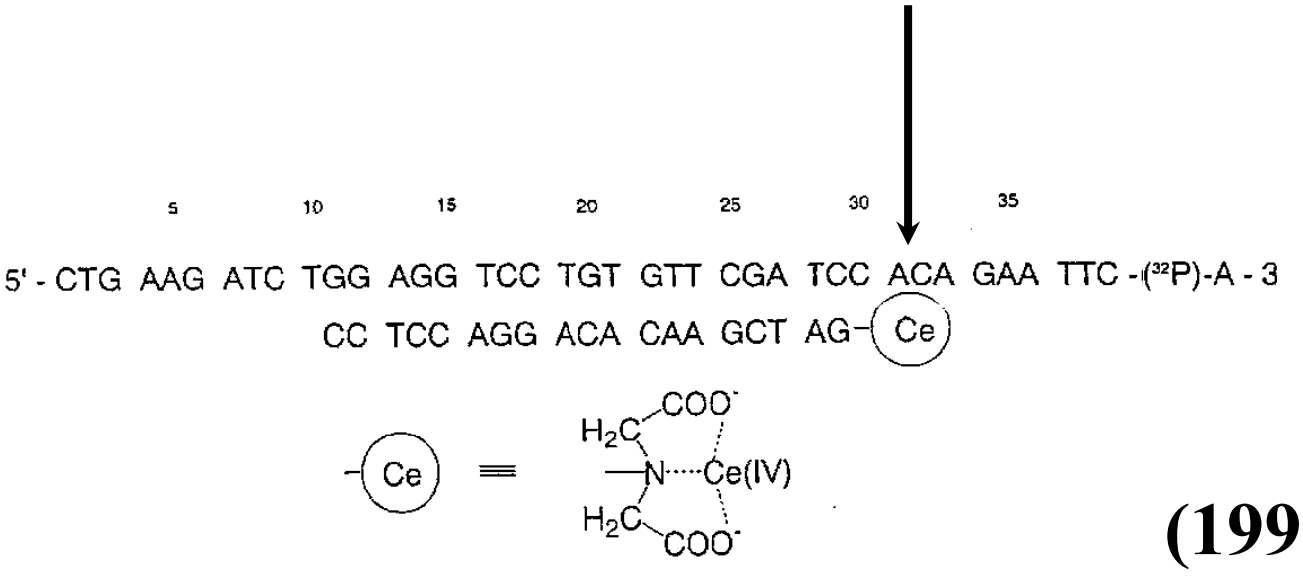
Remarkable electron-withdrawal from the P atom

⇕

the major driving force

Cerium is the sole lanthanide ion whose +4 state is sufficiently stable (Ce(IV) ⇔Ce(III)).

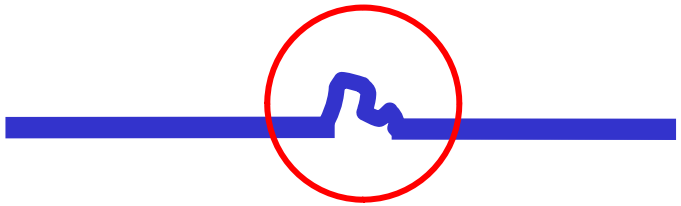
The First-generation Artificial Restriction Enzymes for Sequence-Selective DNA Scission



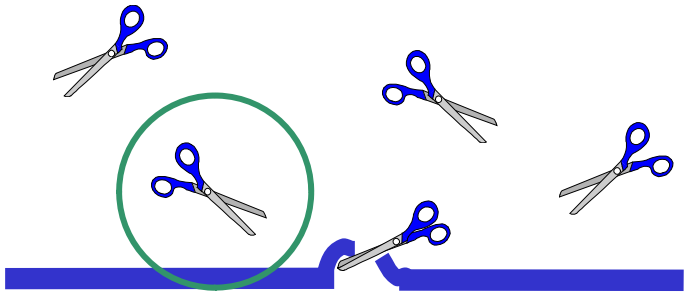
(1994)

Useful new tools were obtained, but double-stranded DNA could not be cleaved.

New Strategy Developed by Our Group for Site-Selective DNA Scission



Differentiation of target site in terms of reactivity



Site-selective scission by catalysts showing high substrate-specificity

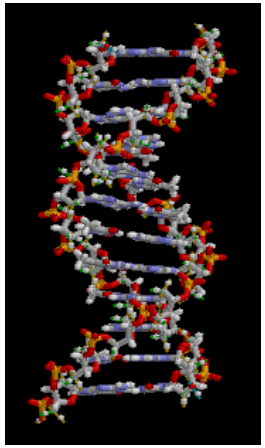
From simple "proximity effect" in the first generation to "site-selective activation of target-site"

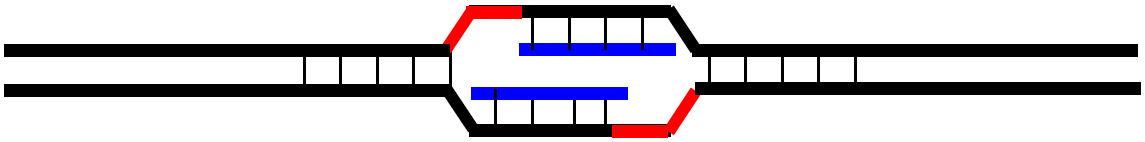
2. Molecular Design of Site-Selective Scission of Double-stranded DNA

Activation of target site in double-stranded DNA

⇩

Scission of this site by Ce(IV) complex





Two Components of ARCUT

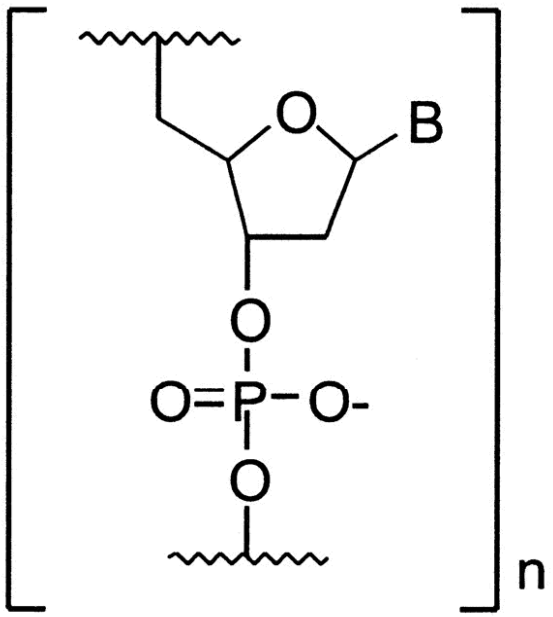
（1）Ce(IV) /EDTA complex

hydrolyzes the hot spot (single-stranded portion)
formed in double-stranded DNA
（intrinsic scission activity： ssDNA >> dsDNA)

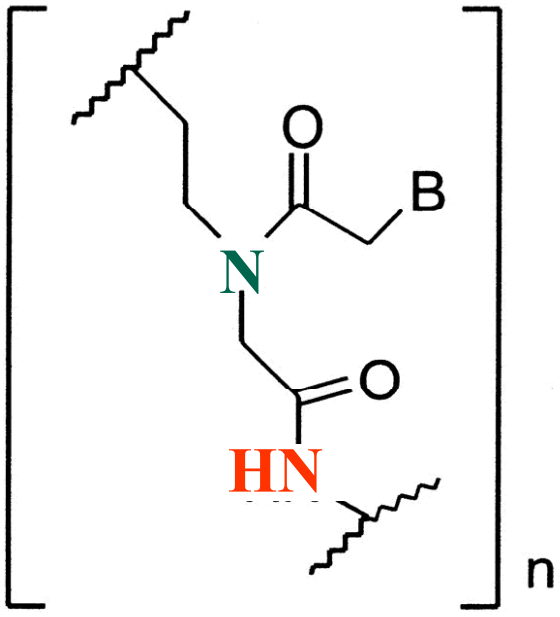
（2）PNA

forms hot spot (single-stranded portion)
at the target site in dsDNA

PNA (Peptide Nucleic Acid) for the Formation of Hot Spot in dsDNA

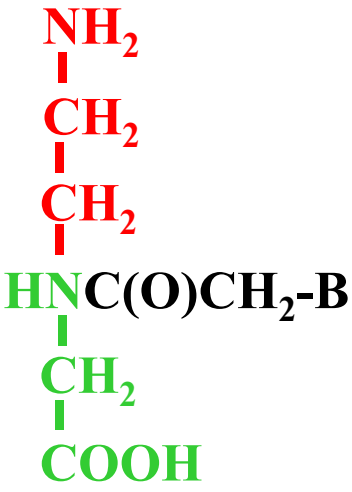


DNA



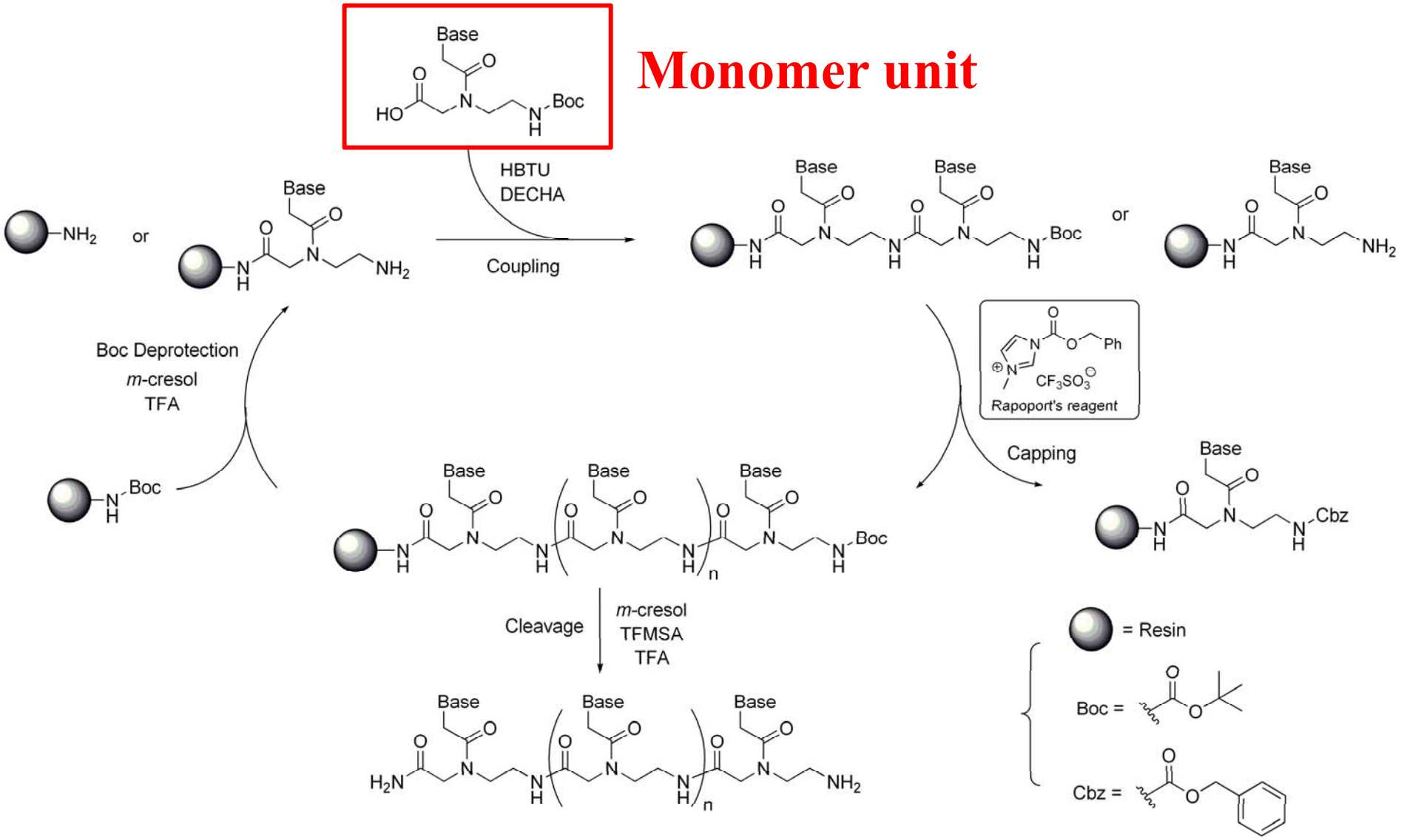
PNA

(Peptide Nucleic Acid)



Condensation polymer of N-aminoethylglycine

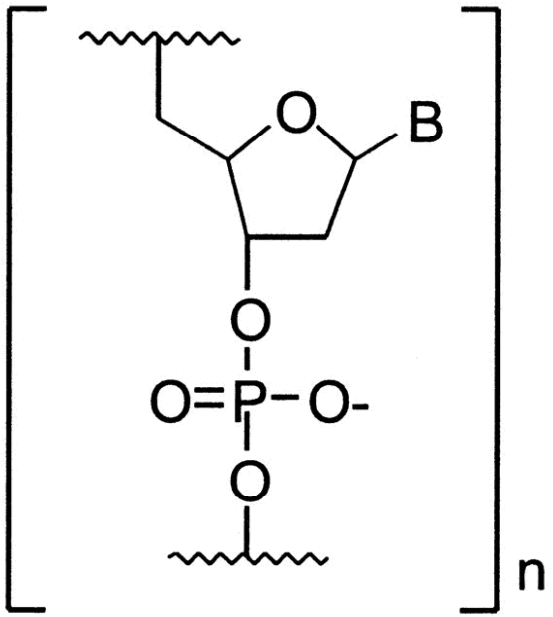
Solid Phase Synthesis of PNA



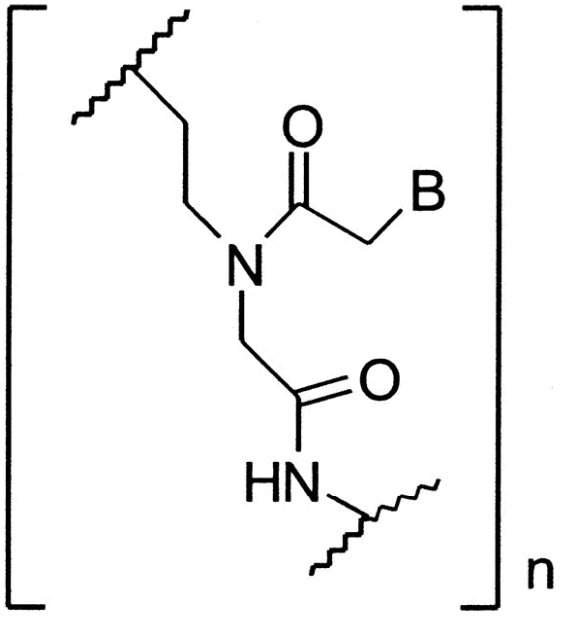
DNA Synthesizer

1 unit～10 min → 60-70 base nucleotide in 10 h

Backbone of PNA Is Neutral
In Contrast to Negative Charges of DNA



DNA

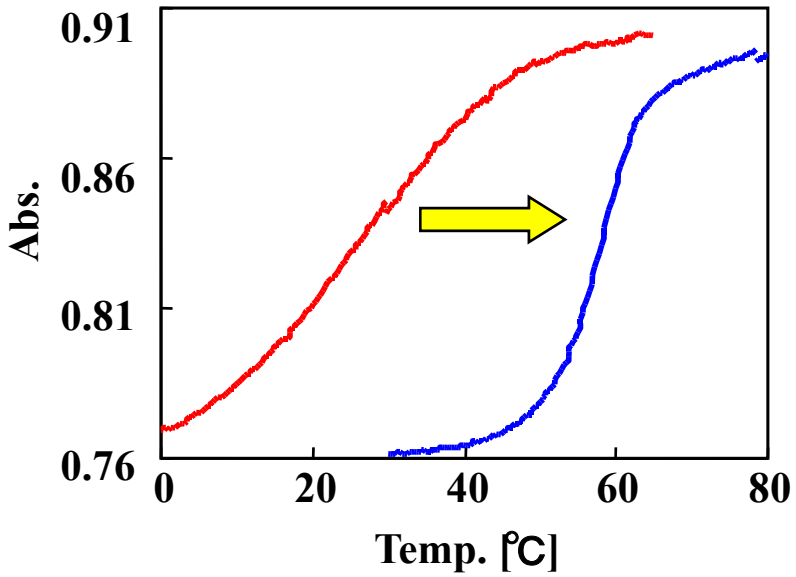


PNA

In DNA/DNA duplexes, electrostatic repulsion is operative between two strands

∴ **Stability of DNA duplexes (T_m) are strongly dependent on ionic strength.**

1. **If you add salt (e.g., KCl) to the solution, electrostatic repulsion between negative charges decreases.**
2. **This factor increases the stability of DNA/DNA duplex, and increases of its Tm.**

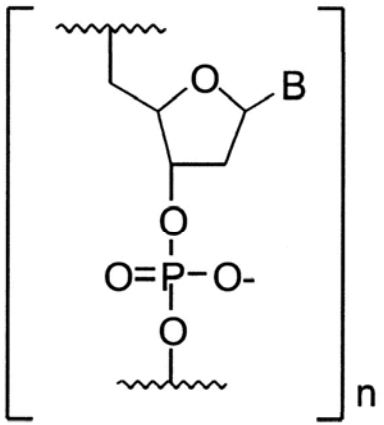


Features of Peptide Nucleic Acid（PNA）

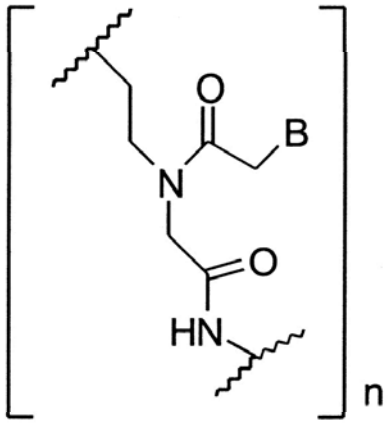
1. Formation of duplexes with complementary DNA

2. Stability： DNA･PNA ＞ DNA･DNA
（absence of electrostatic repulsion）

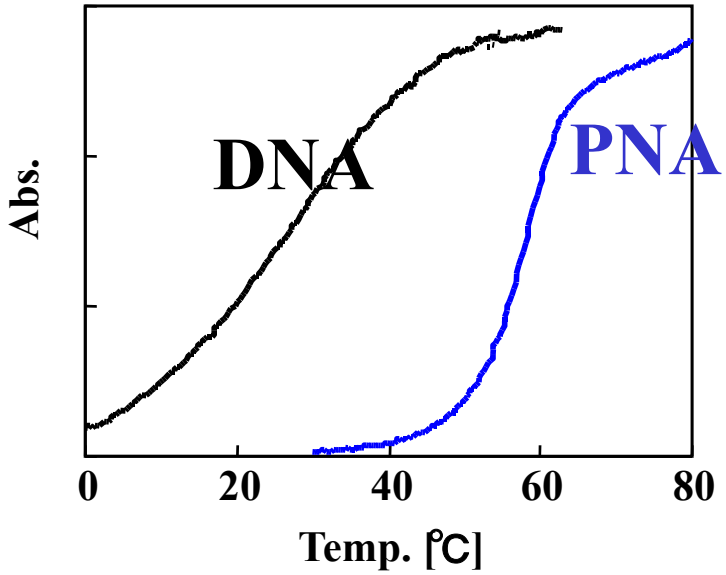
3. Resistance against nucleases



DNA



PNA

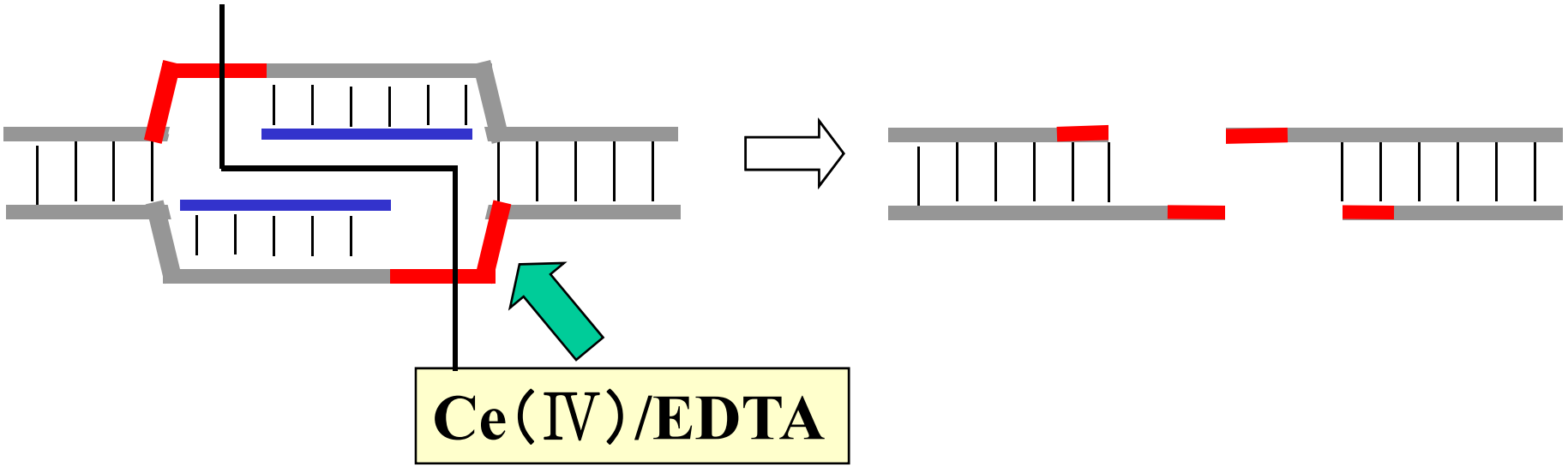


Typical melting curve [36]

DNA/PNA duplexes are more stable than DNA/DNA duplexes

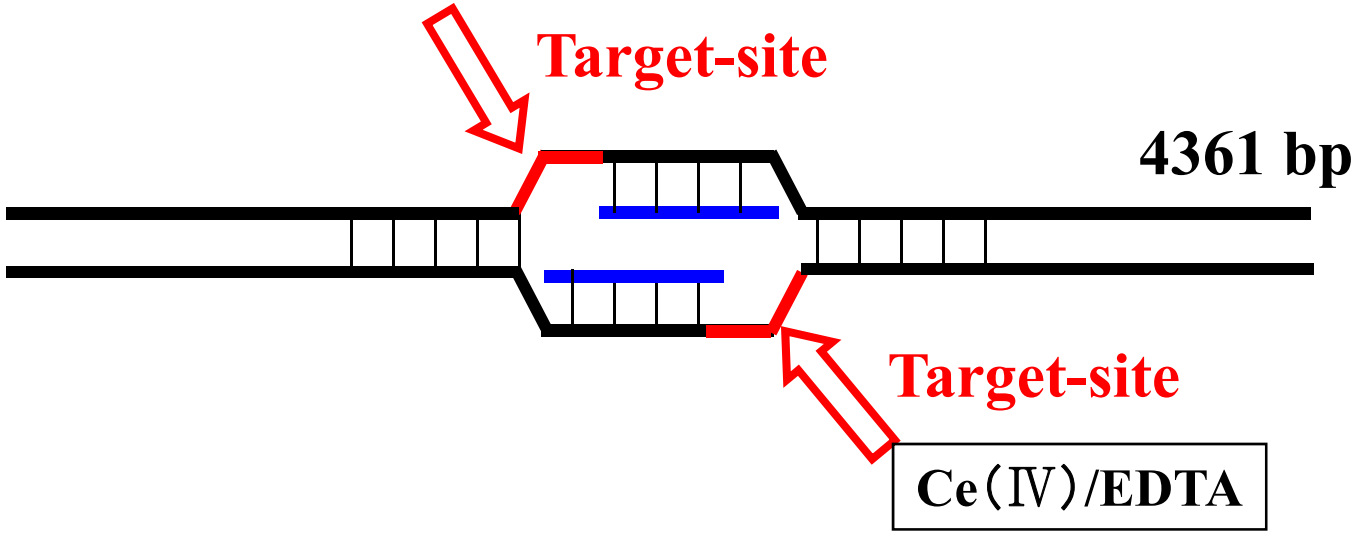
Duplexes		T_m	
DNA/DNA	5'-TGTACGTCACAACTA-3' 3'-ACATGCAGTGTTGAT-5'	53℃	17℃
PNA/DNA	*TGTACGTCACAACTA* 3'-ACATGCAGTGTTGAT-5'	70℃	
DNA/DNA	5'-ACATCATGGTCG-3' 3'-TGTAGTACCAGC-5'	48℃	11℃
PNA/DNA	*ACATCATGGTCG* 3'-TGTAGTACCAGC-5'	59℃	

Invasion of Two PNA Strands to dsDNA for Differentiation of Target Site in ARCUT



Both strands are hydrolyzed at desired site.

Invasion of PNA to Double-stranded DNA



5'-…CGTTCCAGTAACCGGGCATG TTCATCATCAGTAACCCGTA TCGTGAGCATCCTCTCTCGT…-3'

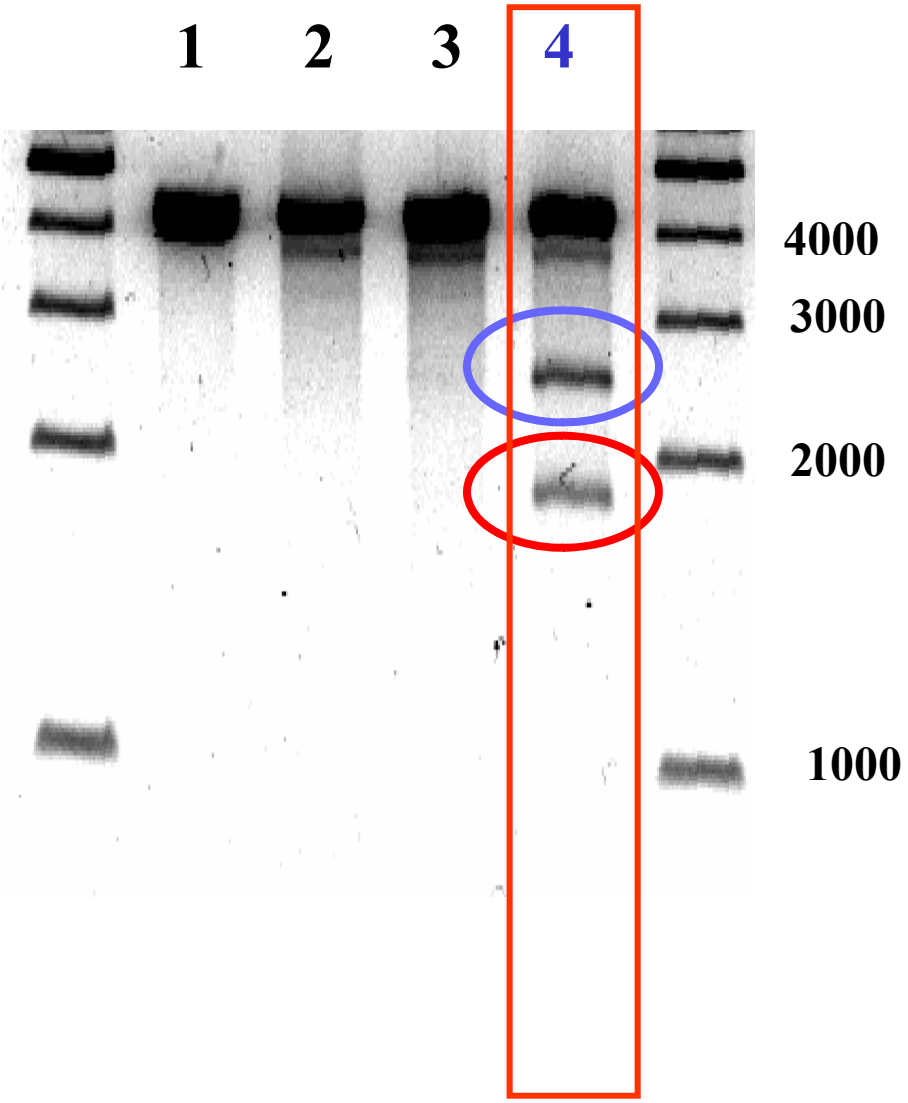
pcPNA1 H_2NCO-(Lys)GUDGUCDUUGGGCDU(Lys)-NH_2

pcPNA2 H_2N-(Lys)UUCDUCDUCDGUDDC(Lys)-$CONH_2$

3'-…GCAAGGTCATTGGCCCGTAC AAGTAGTAGTCATTGGGCAT AGCACTCGTAGGAGAGAGCA…-5'

(U ≒ T, D ≒ A)

ARCUT for Site-selective Scission of Linearized Plasmid DNA

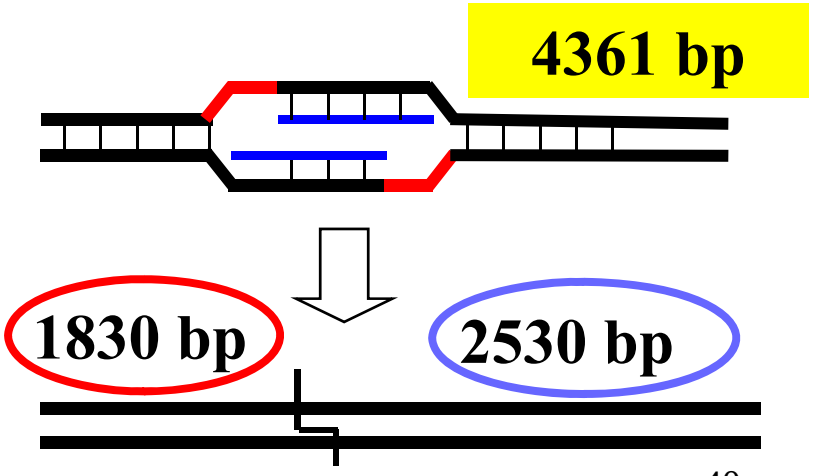


lane 1 control

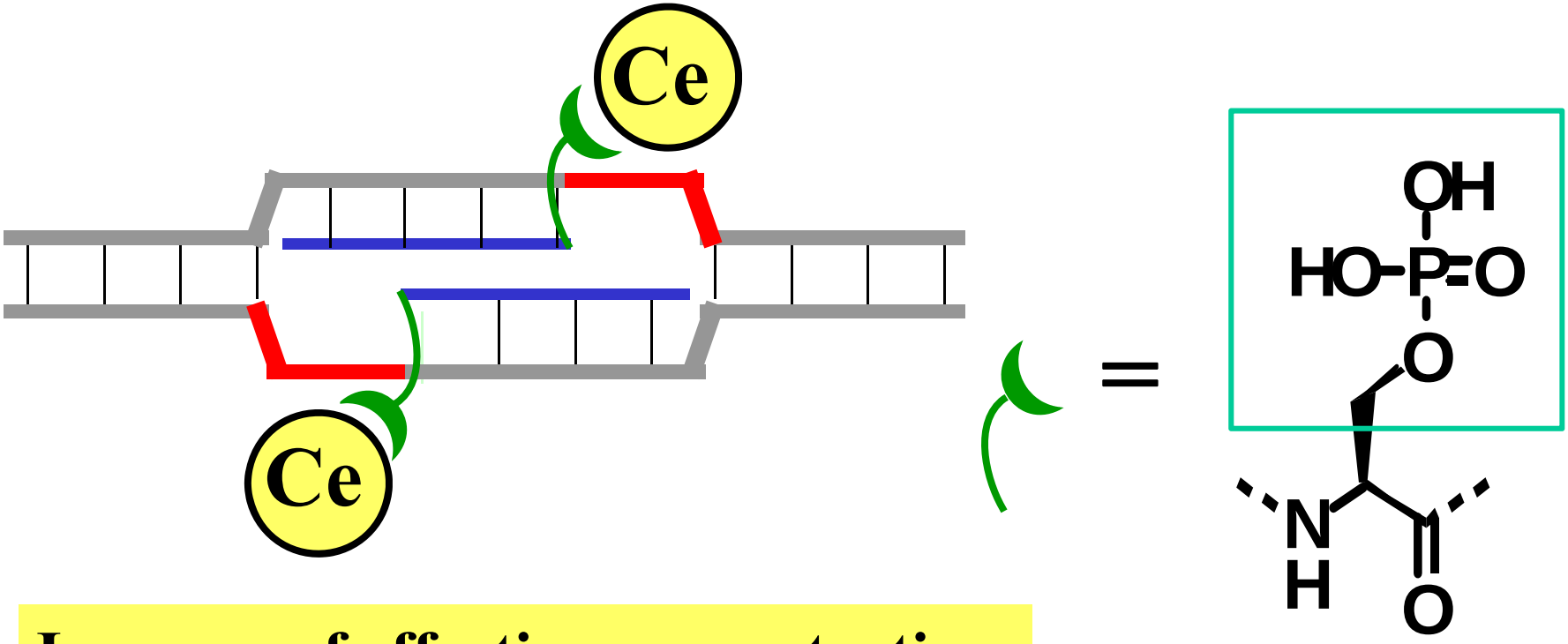
lane 2 Ce(IV)/EDTA only

lane 3 Ce(IV)/EDTA + pcPNA 3&4

lane 4 Ce(IV)/EDTA + pcPNA 1&2



Introduction of Monophosphate to ARCUT for Promotion of Scission Efficiency



Increase of effective concentration of Ce(IV) near the target site

Site-selective DNA Scission by PNA Bearing Monophosphate at the N-Termini

Lane 1 control (DNA only)

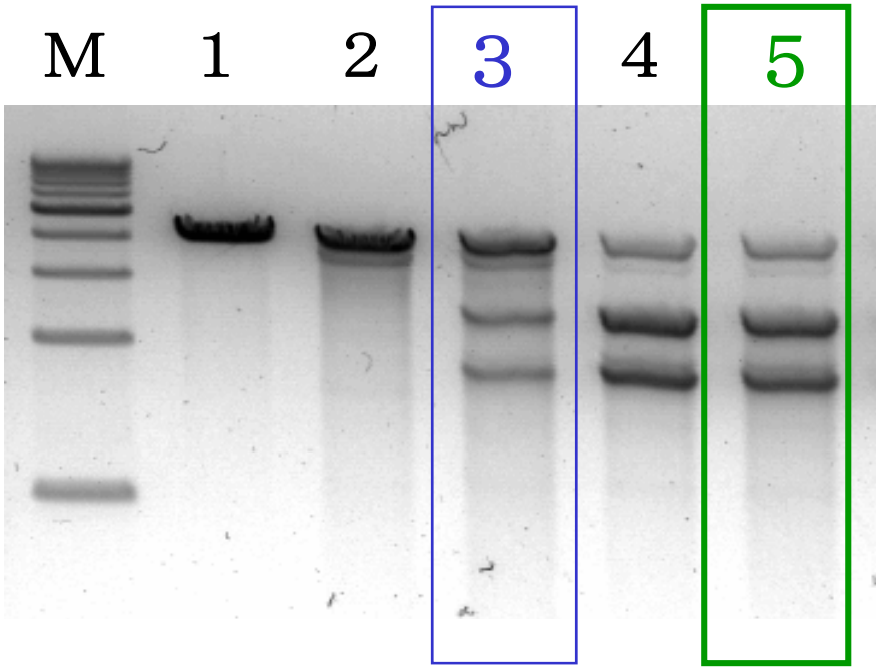
Lane 2 without pcPNA

Lane 3 with PNA 5&6

Lane 4 with PNA 5II&6II

Lane 5 with PNA 5P&6P

Lane M 1kbp ladder marker



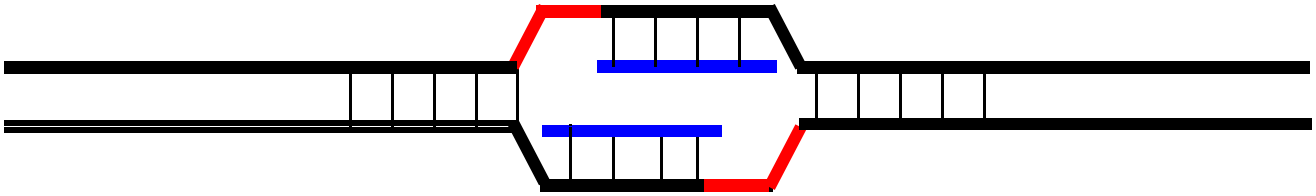
Yields of Desired Fragments = 50-60% in 20 h

Reaction conditions: pBR322 DNA 8 nM, pcPNA 200 nM, HEPES buffer (pH 7) 5 mM, NaCl 100 mM, Ce(IV)/EDTA 200 μM, 50 ℃, 20 h

3. Applications of ARCUT to molecular biology

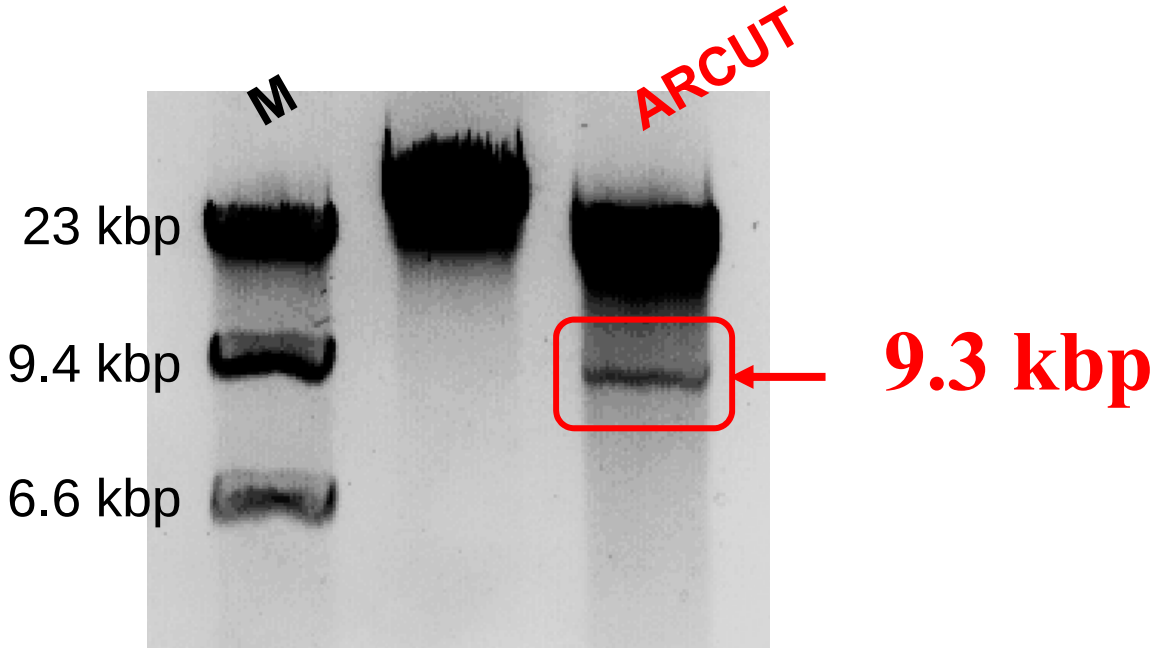
Advantages of ARCUT

1. High site-specificity
   (>> naturally occurring restriction enzyme)
2. Choice of scission site is free.



Length & sequence of PNA: Free!

(1) Site-selective Scission of λ phage DNA (49 kbp)

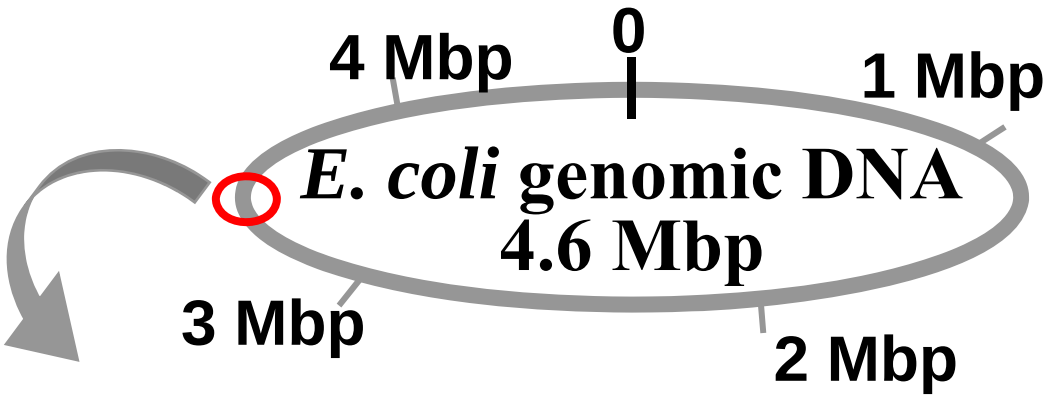


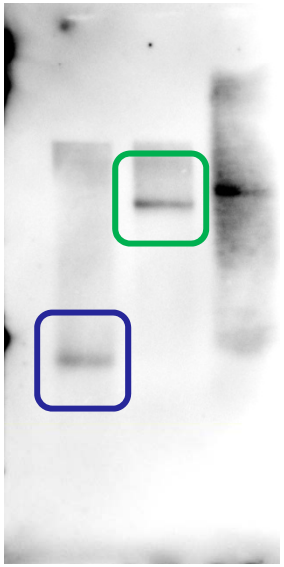
(2) Site-selective Scission of Genome DNA of *E. coli* (4.6 Mbp)

If this huge DNA is treated with conventional restriction enzyme

number of scission sites

$= 4{,}600{,}000/4^6 = 1100$



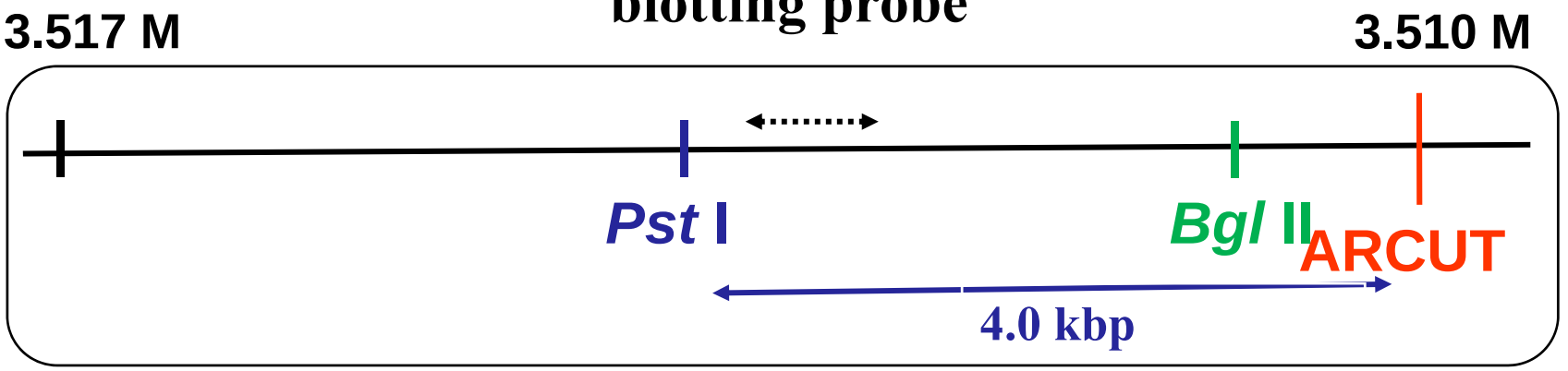


Target Fragment

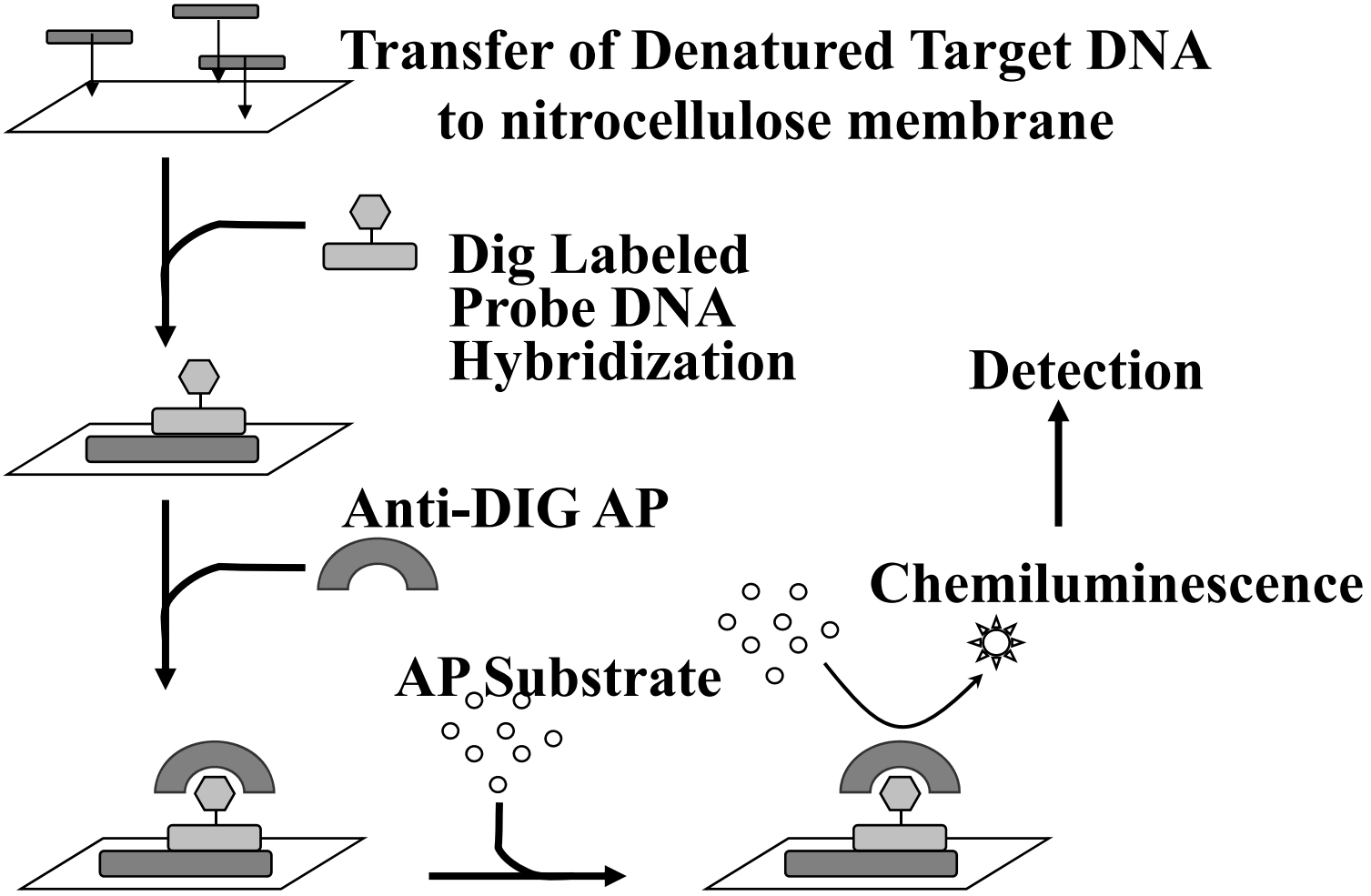
0.9 kbp

4.0 kbp

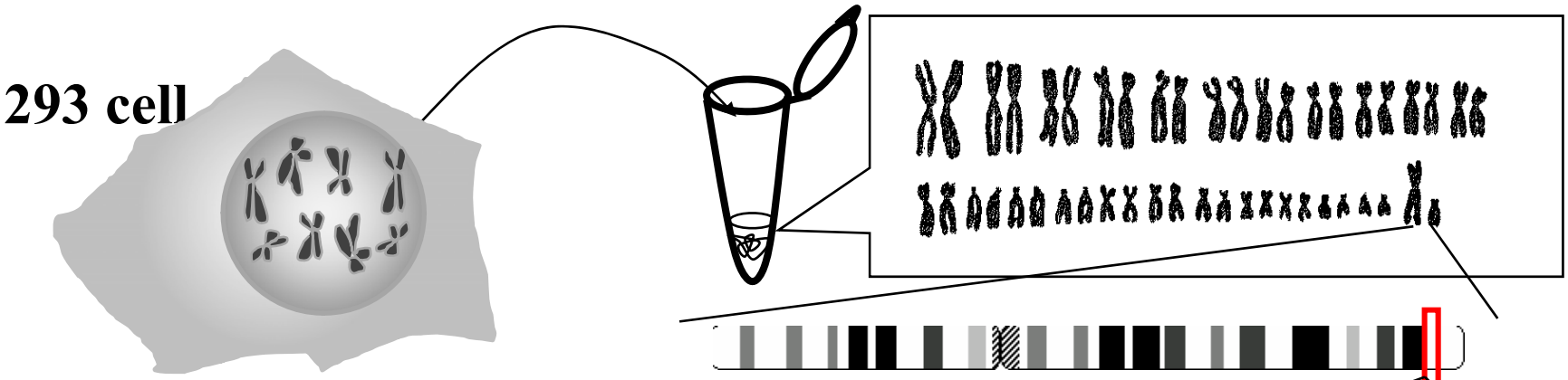
Southern blotting probe

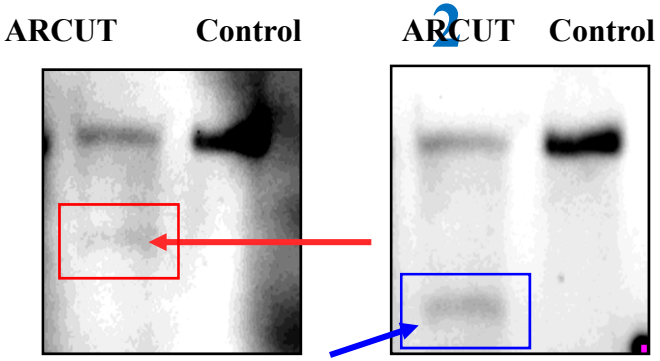


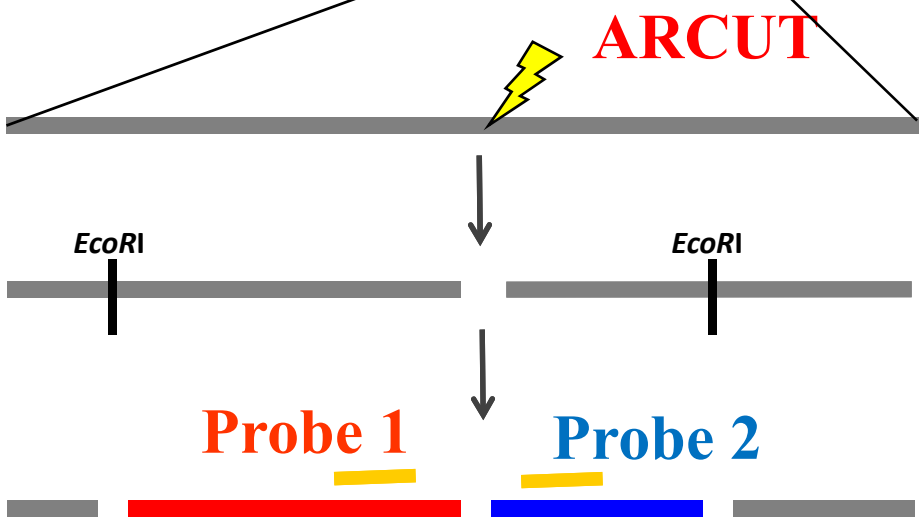
Southern Blotting



(3) Site-selective scission of human genome (3 x 10^9 bp) at one target site







Southern Blotting

Mismatch-recognition by ARCUT in site-selective scission of human genome

Target site (in FMR1 in X chromosome)

5’-AATGGGCGCTTTCTACAAGGT-3’
3’-TTACCCGCGAAAGATGTTCCA-5’

is efficiently hydrolyzed

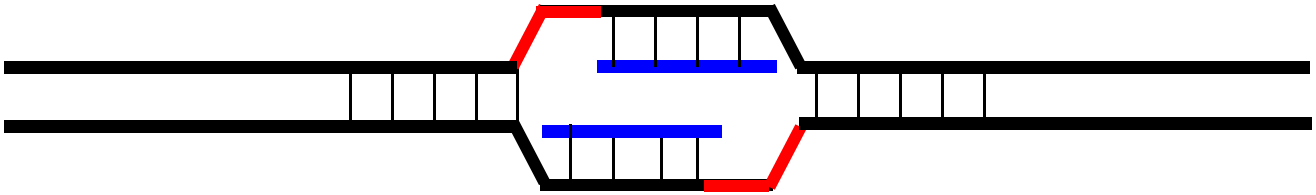
A homologous site (in chromosome 7)

5’-CAGGGGCGCTTTCTACAAGAT-3’
3’-GTCCCCGCGAAAGATGTTCTA-5’

is never hydrolyzed

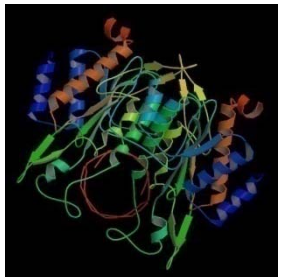
Advantages of ARCUT

2. Choice of scission site is free.

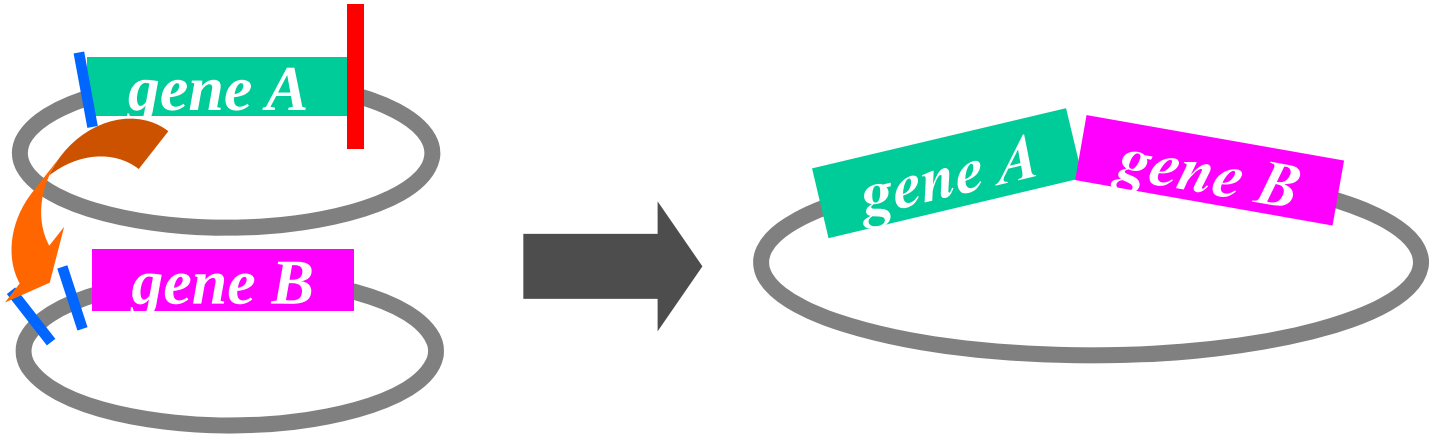


Sequence of PNA: Free!

Cf. ***Eco*RI** **-GAATTC-**



Formation of Fusion Protein by ARCUT



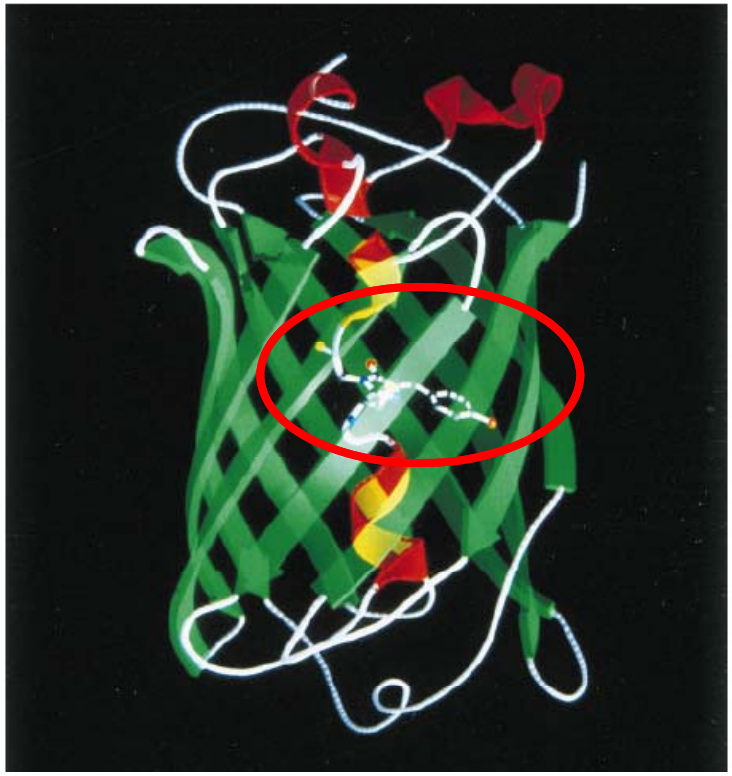
Key points:

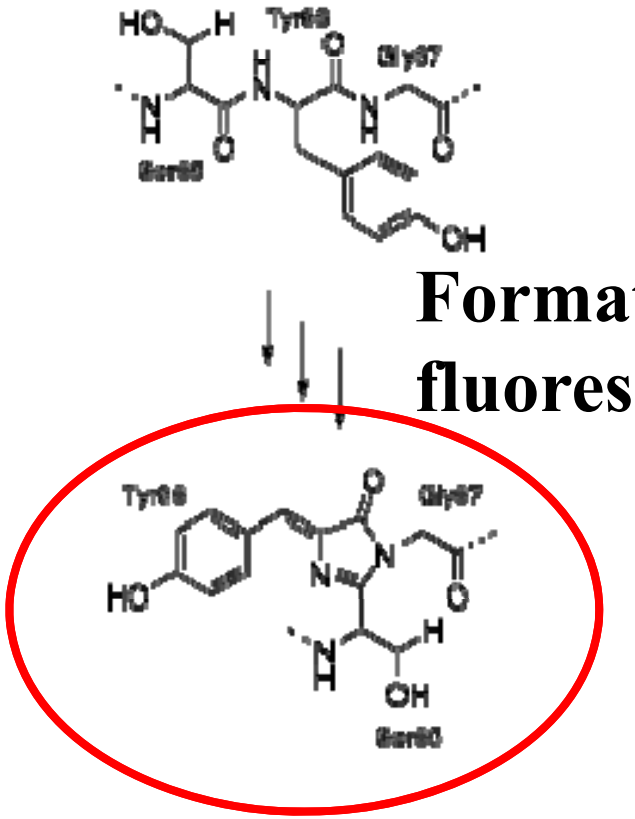
(1) How to cut the DNA just before the stop codon for gene A?

(Mostly, no restriction enzyme is directly available)

(2) The reading frame must be precisely adjusted.

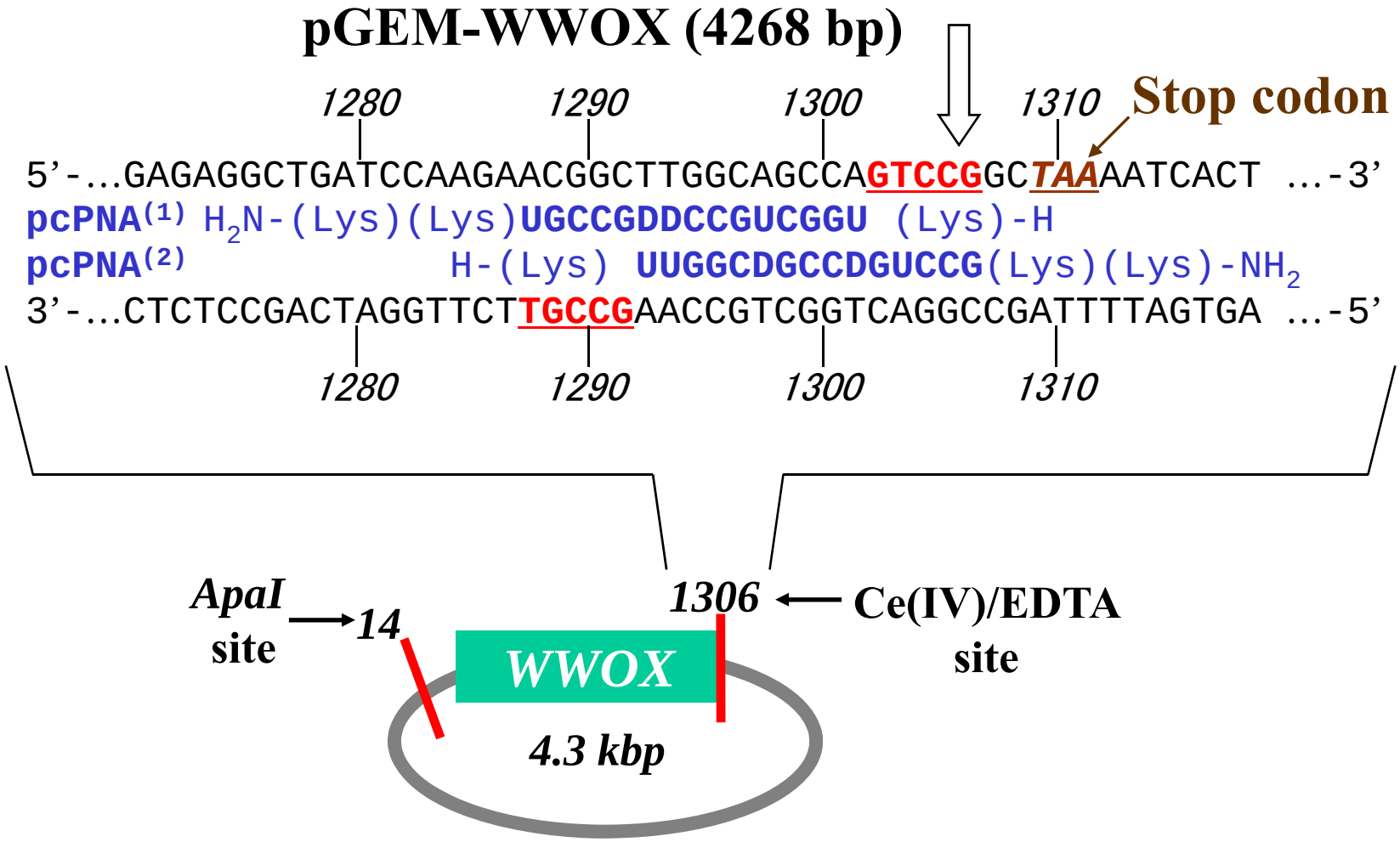
GFP （Green Fluorescent Protein)





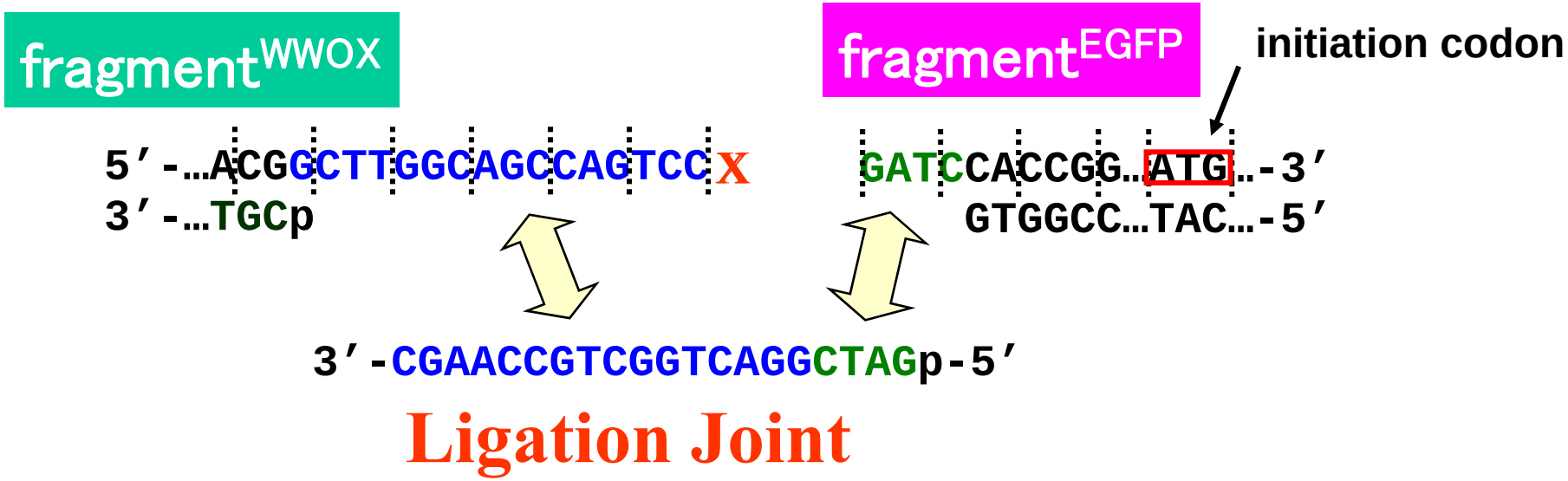
Proc. Natl. Acad. Sci. USA, 94, 2306-2311 (1997)

Selective Scission Just Before the Stop Codon



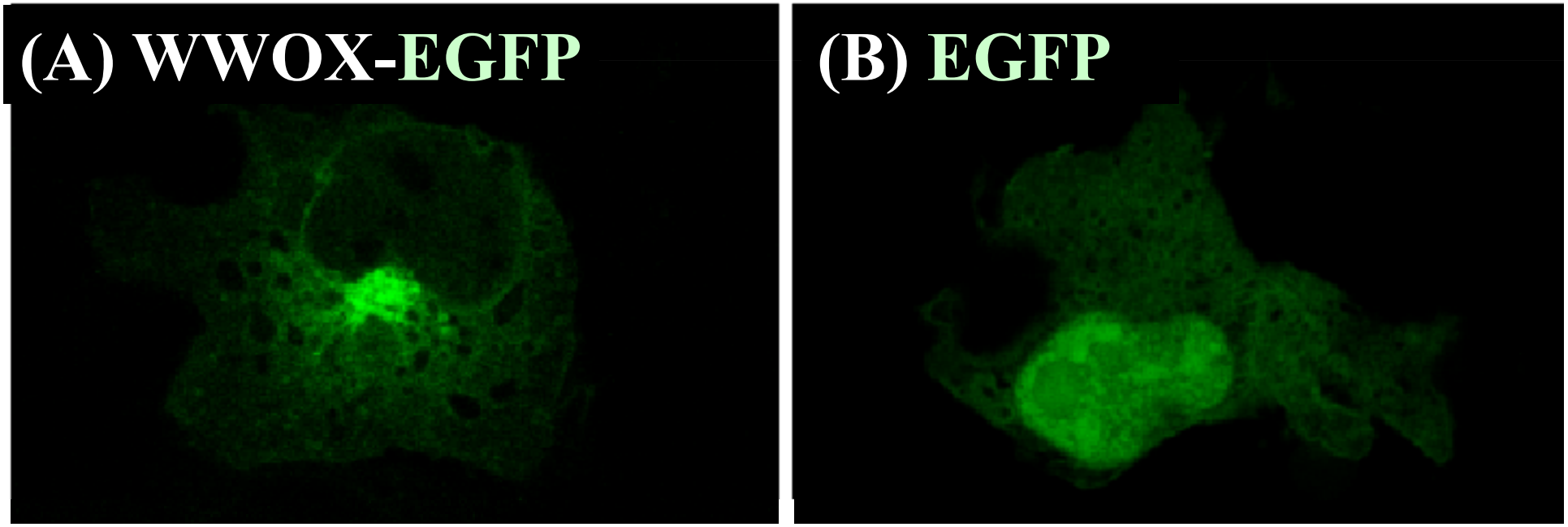
WWOX = WW domain-containing oxidoreductase

Selection of Desired Scission Fragment by Using Appropriate Ligation Joint



f is picked up from the reaction mixture and selectively ligated with f

Fluorescence From Fusion Protein



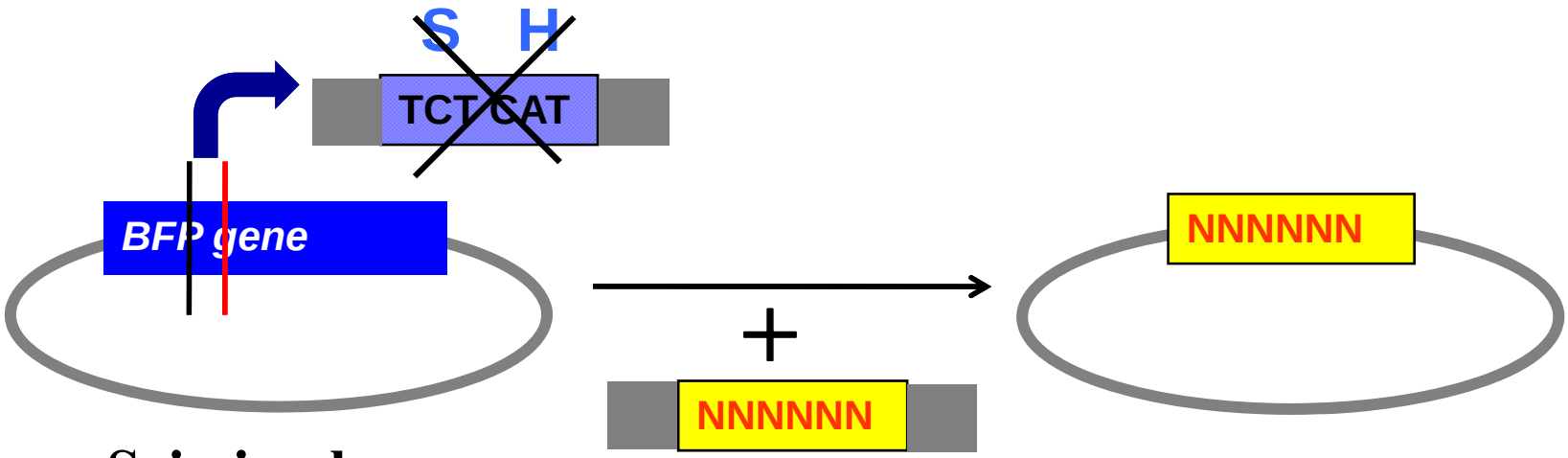
Localized in golgi

Localized in nuclei

DNA is completely kept intact throughout the manipulation

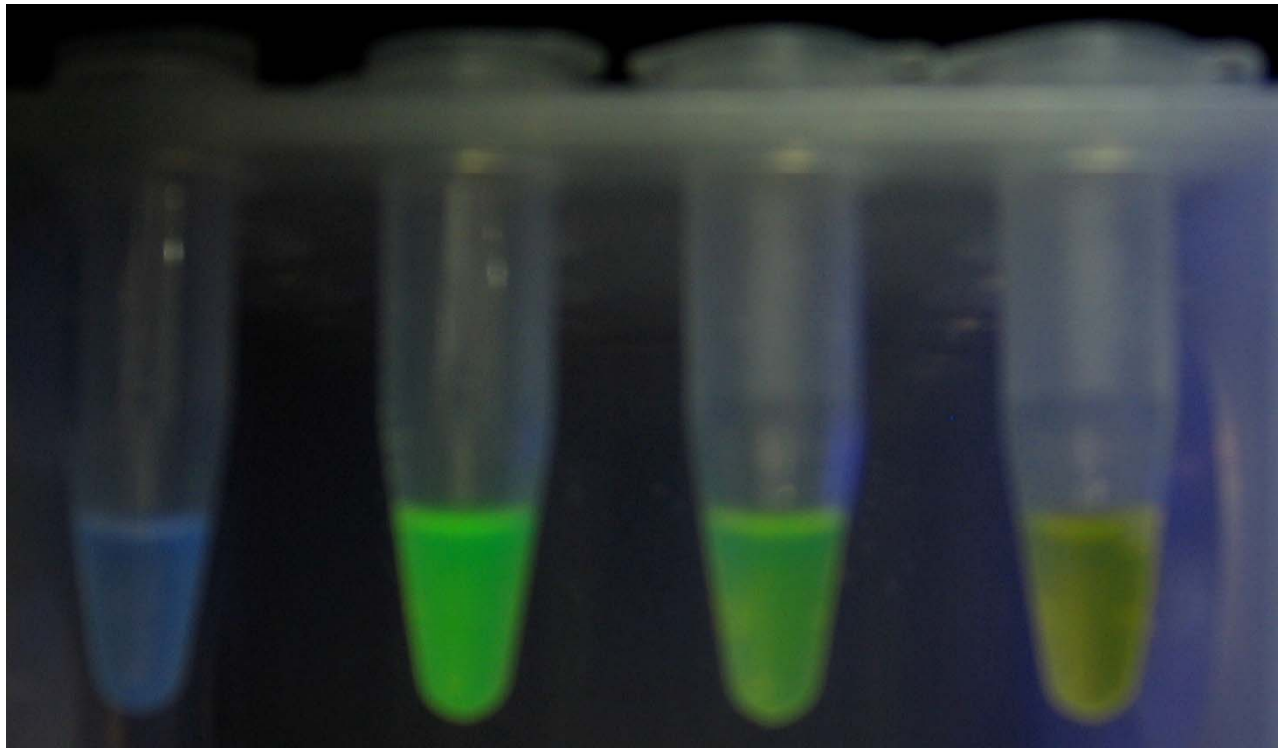
Engineering of Fluorescent Protein Using ARCUT

Randomization at 65-66 of BFP through site-selective scission by ARCUT



Scission by ARCUT &*Spe*I

Recombinant Proteins Obtained



Ser^{65}-His^{66} (BFP)

Cys-Tyr (EGFP)

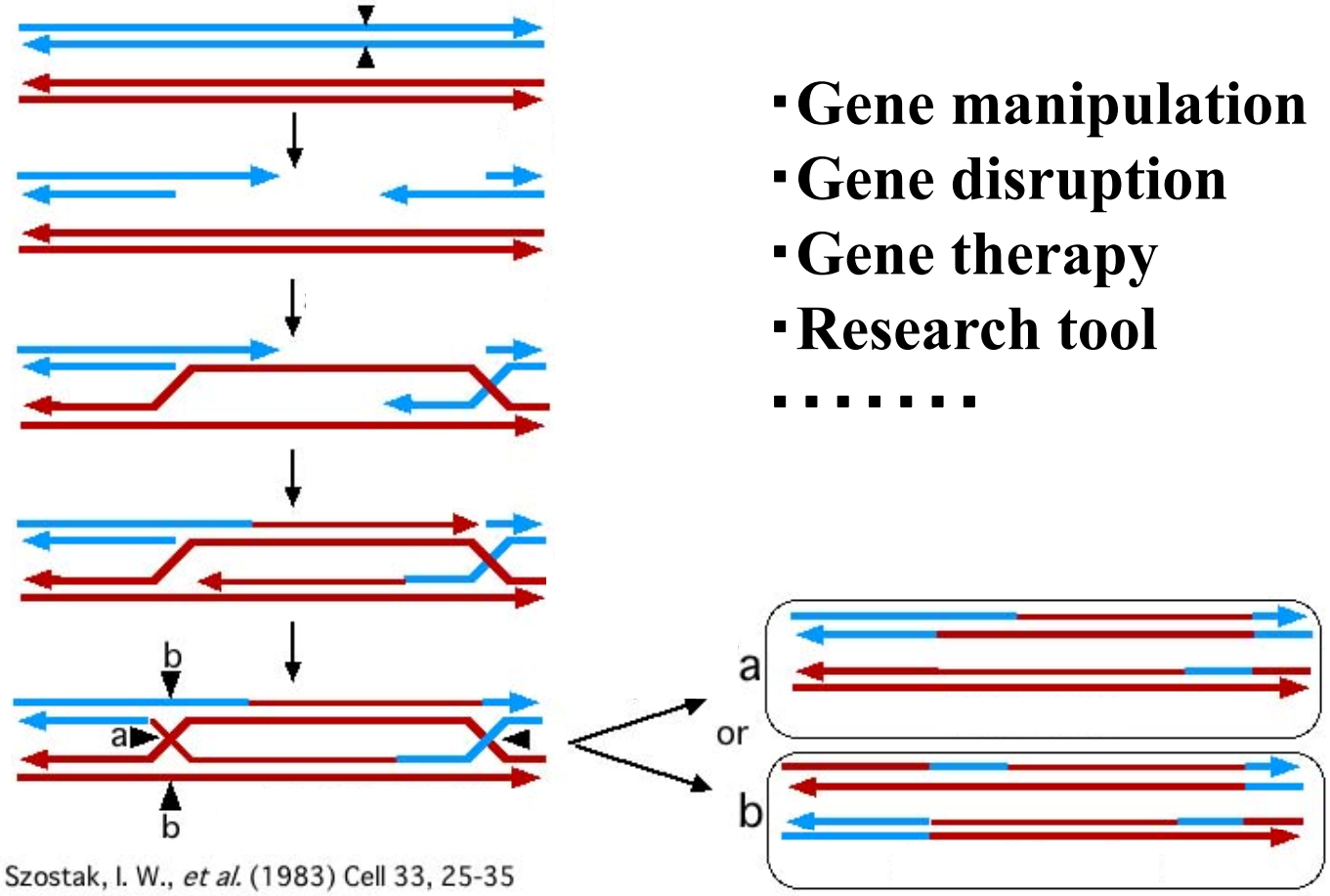
Glu-Tyr

Gly-Tyr

ARCUT is useful to manipulate even small DNA like plasmid

Application to Homologous Recombination for Genetic Manipulation in Human Cells

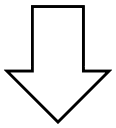
Double-strand break for promotion of HR



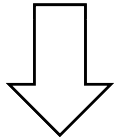
Szostak, I. W., *et al.* (1983) Cell 33, 25-35

- Gene manipulation
- Gene disruption
- Gene therapy
- Research tool
- .....

Frequency of homologous recombination is too low to use as tools for gene manipulation

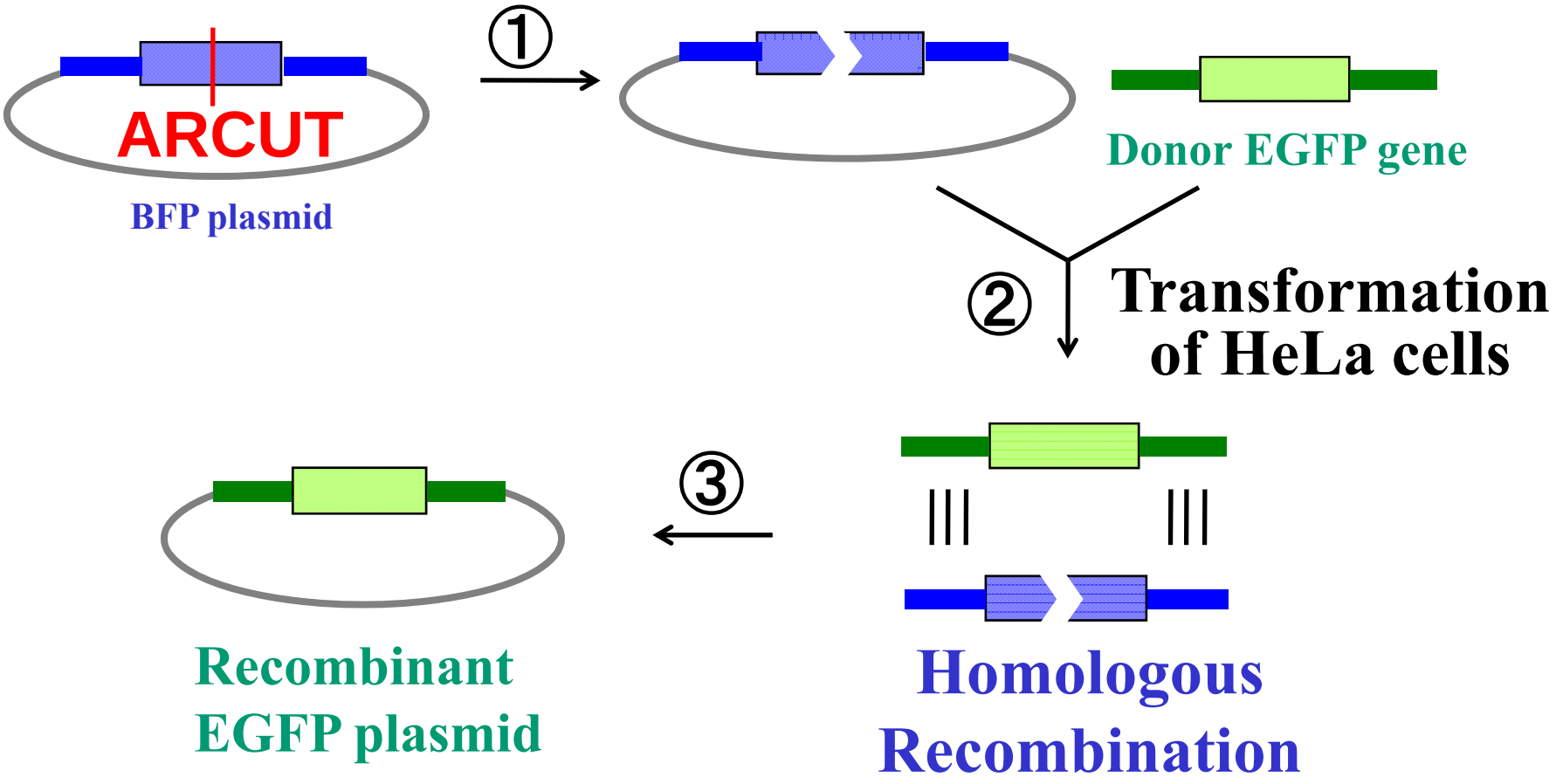


It is greatly promoted when DNA is damages at the target site (repair system is activated)

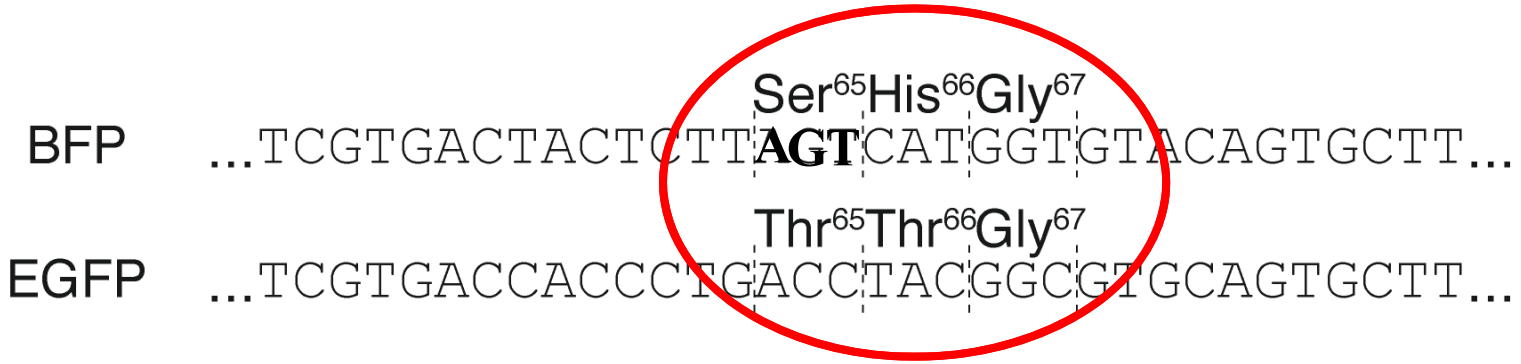


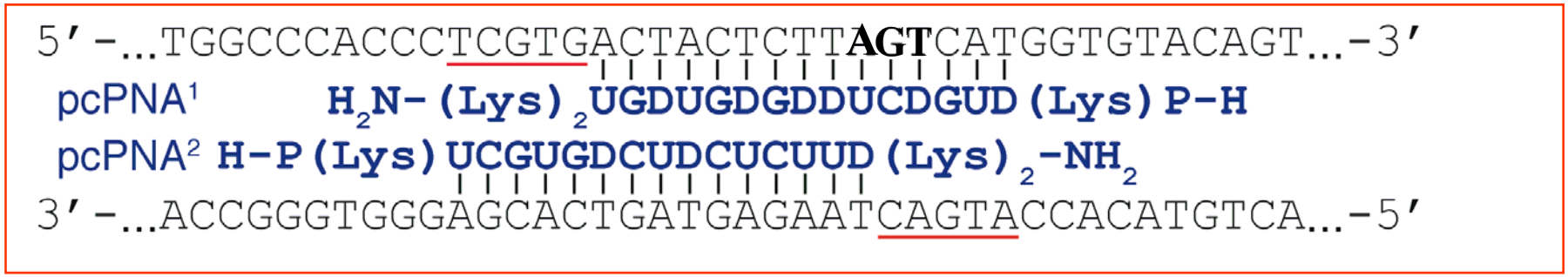
Is ARCUT useful for this purpose?

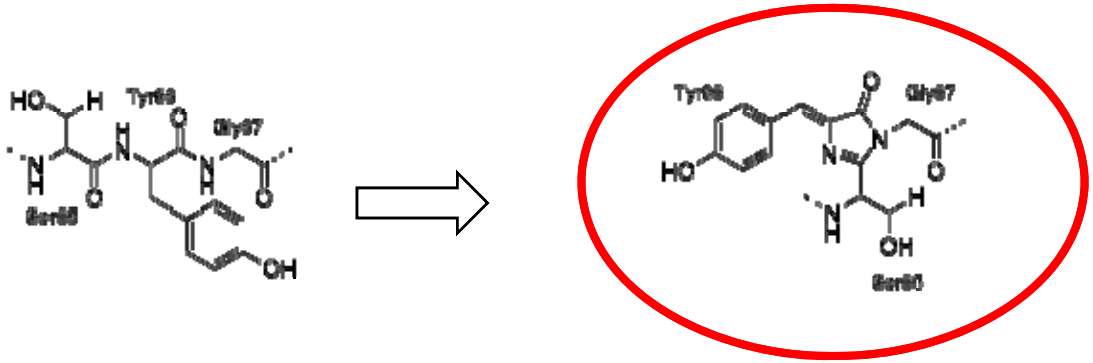
Double-strand break by ARCUT promotes homologous recombination in human cells!



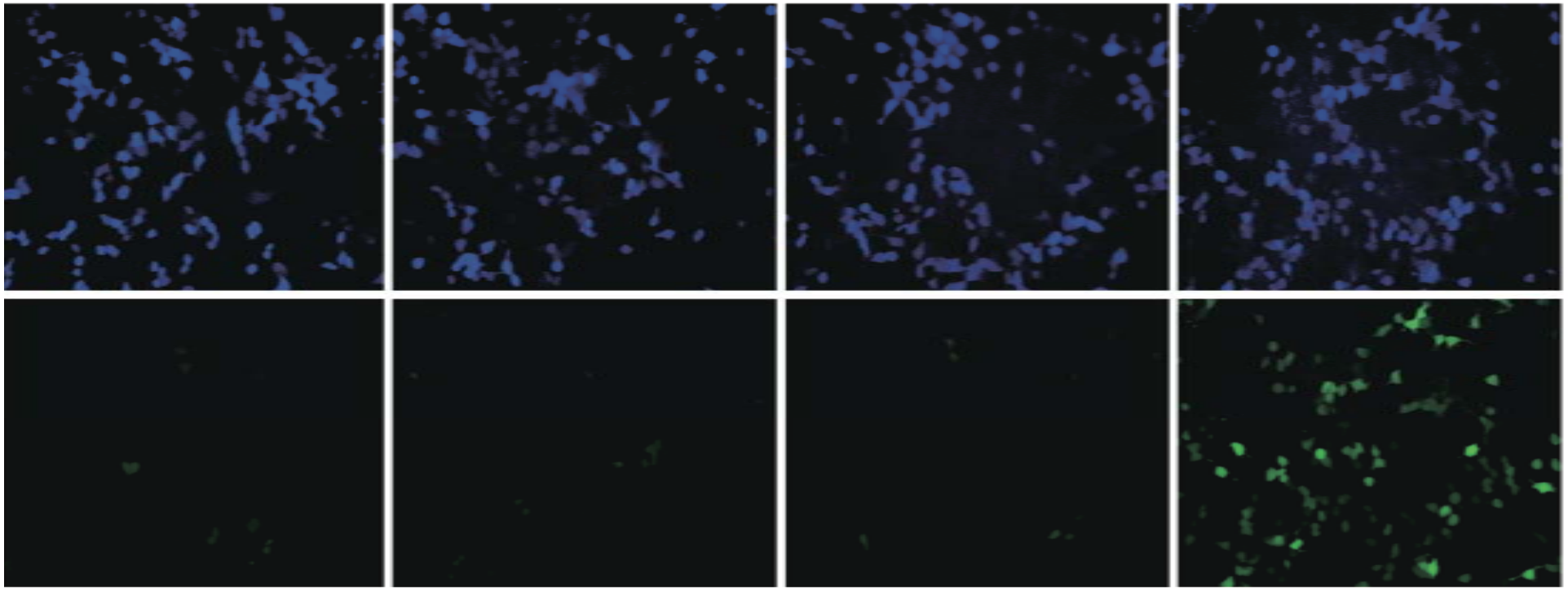
ARCUT used for BFP → EGFP conversion







Promotion of homologous recombination in human cells by ARCUT



Upper: BFP

Lower: GFP (product of recombination)

Substrate gene (BFP)

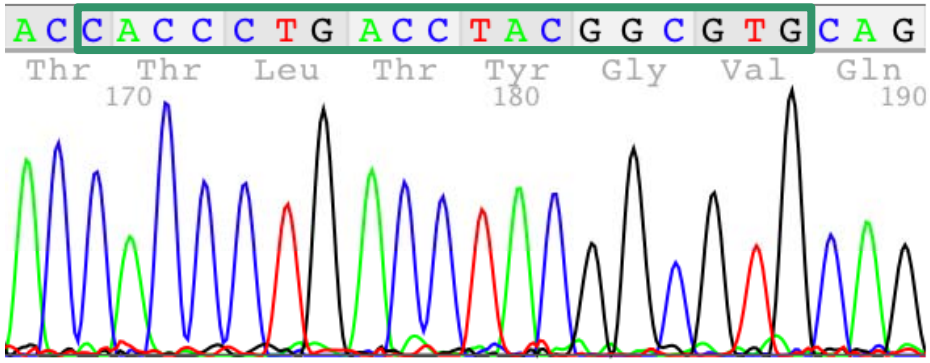
…TCGTGACTACTCTTAGTCATGGTGTACAGTGCTTCA…

Homologous recombination

Recombinant gene

…TCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCA…

(from EGFP)



Homologous recombination at desired site is indicated.

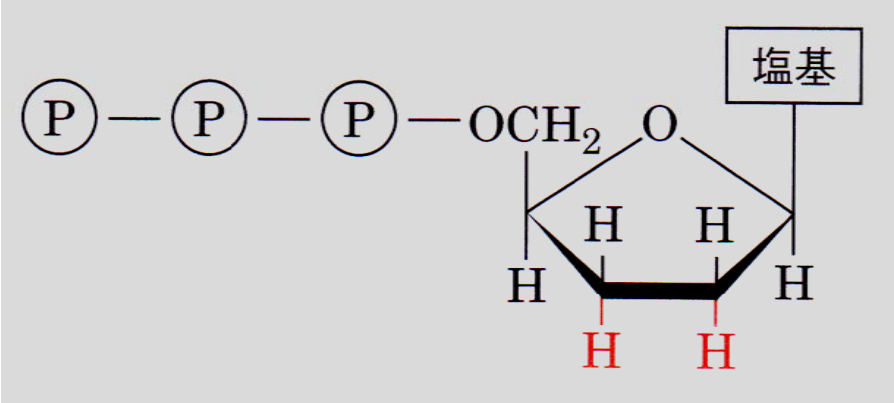
Determination of DNA Sequence
(dideoxy chain termination method)

In DNA polymerase reaction using the sample DNA as template, a small amount of dideoxy-compound is added

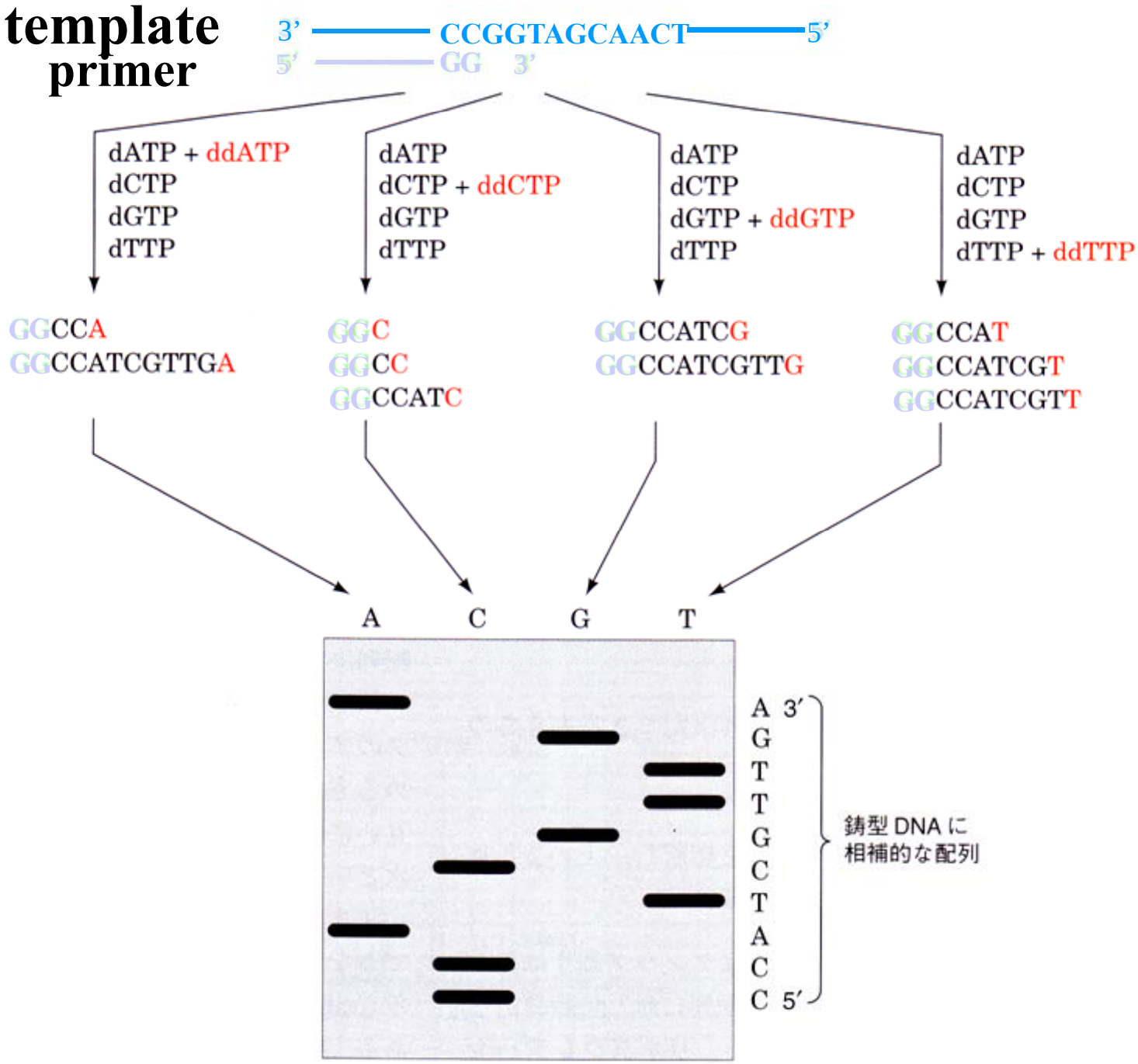
→ the polymerization stops there because of the lack of 3'-OH

→ formation of shorter fragment depending on the sequence

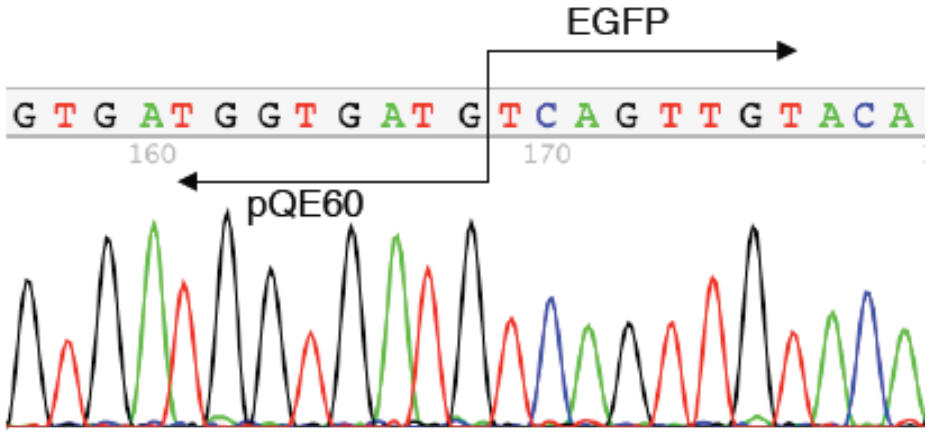
→ sequence is determined from the lengths of the fragments



2',3'-dideoxynucleotide triphosphate


template
primer
3’ CCGGTAGCAACT 5’
5’ GG 3’
dATP + ddATP
dCTP
dGTP
dTTP
dATP
dCTP + ddCTP
dGTP
dTTP
dATP
dCTP
dGTP + ddGTP
dTTP
dATP
dCTP
dGTP
dTTP + ddTTP
GGCCA
GGCCATCGTTGA
GGC
GGCC
GGCCATC
GGCCATCG
GGCCATCGTTG
GGCCAT
GGCCATCGT
GGCCATCGTT
A
C
G
T
A 3’
G
T
T
G
C
T
A
C
C 5’
鋳型 DNA に
相補的な配列

DNA Sequencer



Naturally occurring restriction enzyme vs. ARCUT

Enzyme		ARCUT
(1) DNA recognition Protein	⇨	PNA
(2) Catalyst Mg(II)	⇨	Ce(IV) complex
(3) Scission site & specificity Limited	⇨	Freely designable

Conclusion

1. New tools for site-selective hydrolysis of (huge) double-stranded DNA at any site have been developed.
2. The resultant fragments are enzymatically ligated with various foreign DNAs, and recombinant DNA is expressed in cells.
3. These tools should pave the way to new molecular biology and biotechnology.