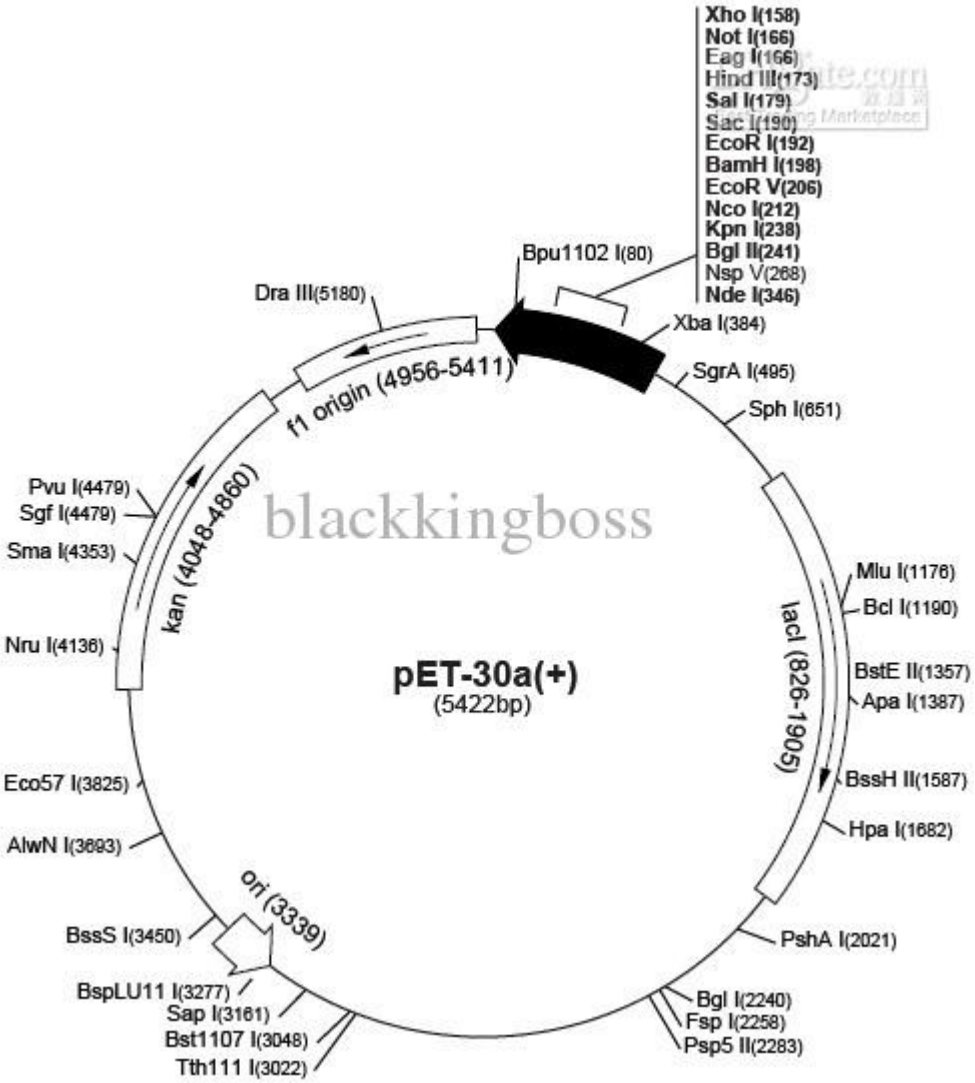
제24강

<pET-30 vector map>



pET-30a-c(+) cloning/expression region

<Primer sequence>

- Forward primer sequence

NcoR1

5′-CGC CATATG ATG AGC ACC GAA AAG TGG-3′

GC ratio: 9/18 = 50 %

- Reverse primer sequence

Xho1

5′- CGC CTCGAG TTA GAC ATT CGC TAG GTT GCC-3′

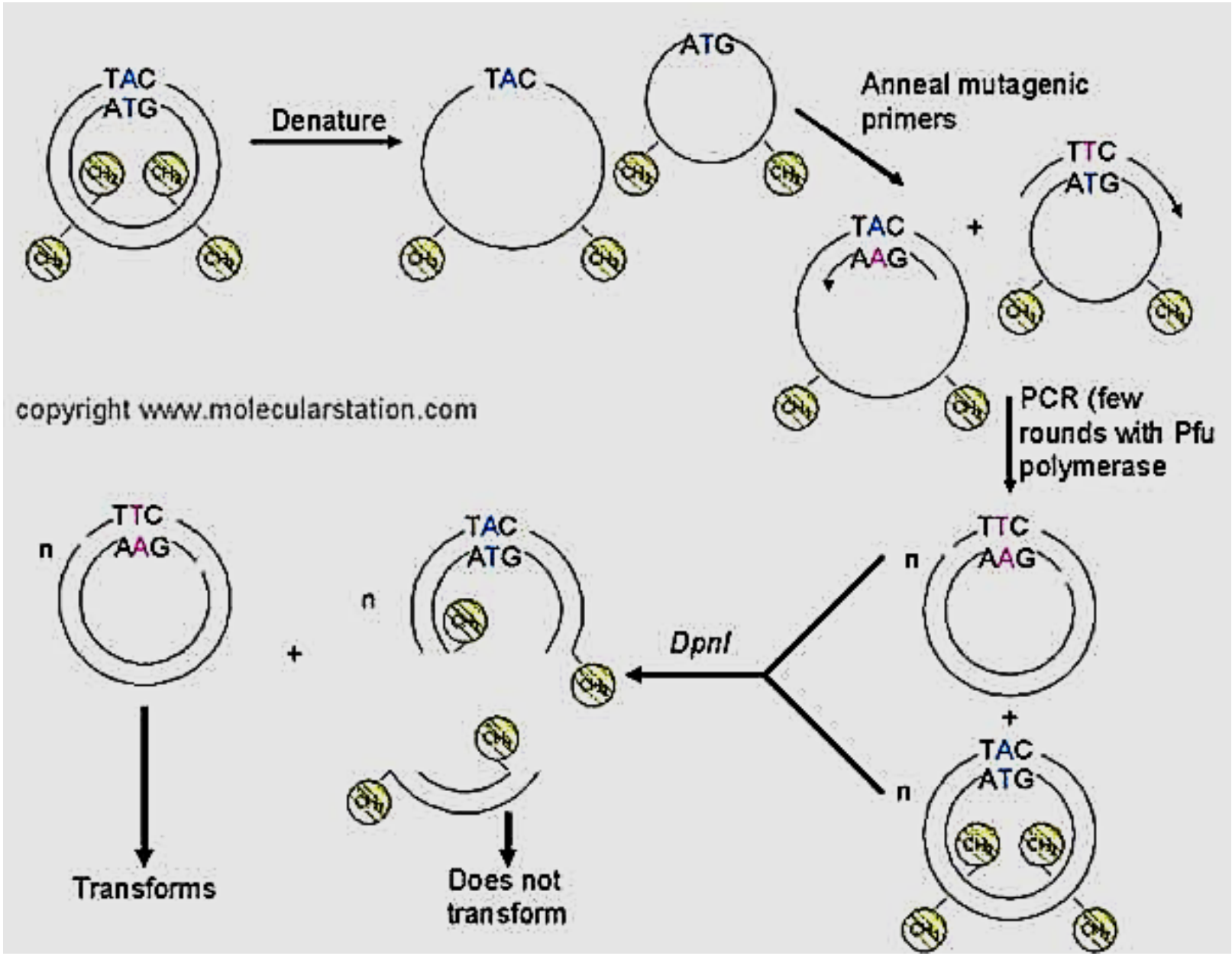
GC ratio: 10/21 = 48 %

<강의 핵심 내용>

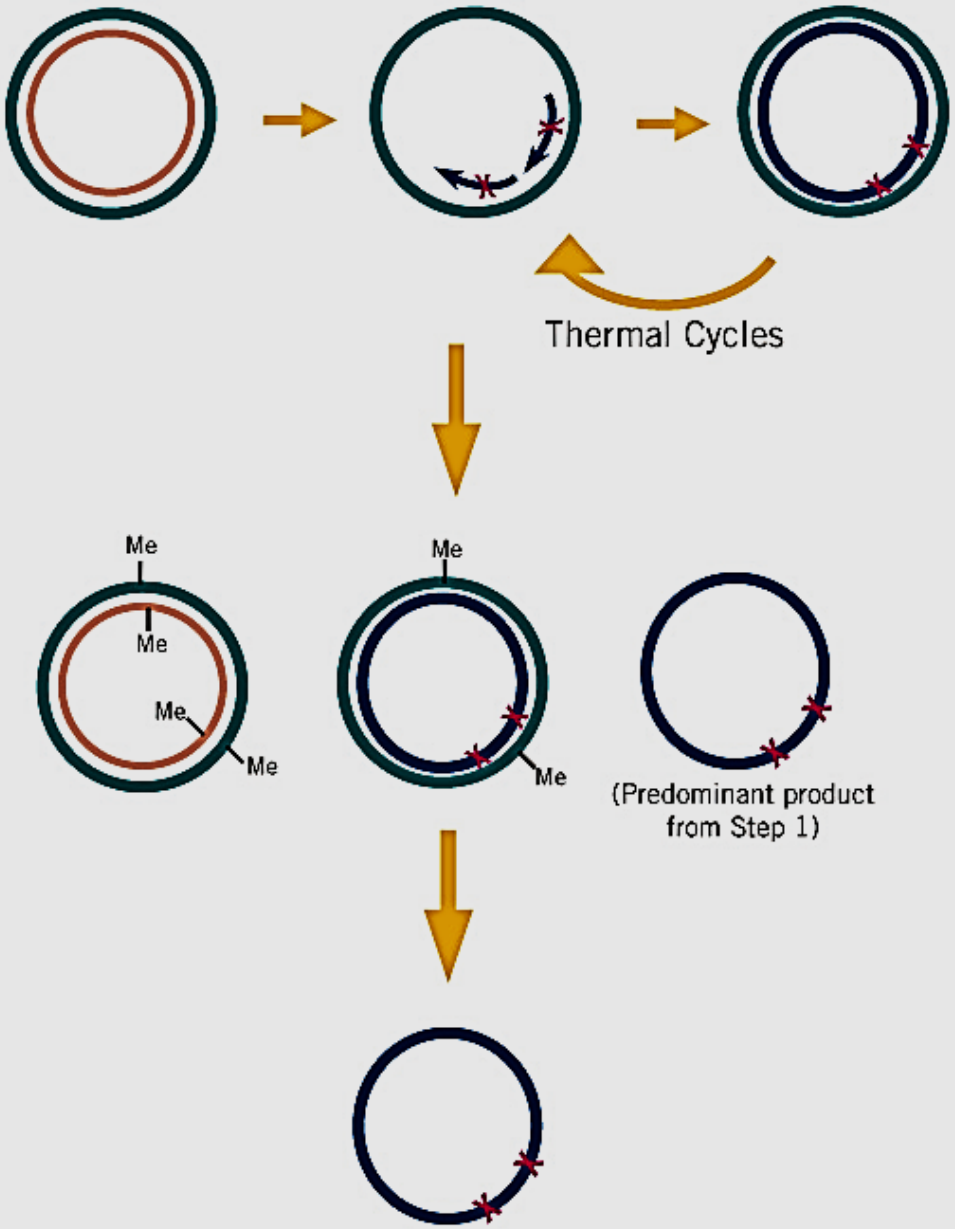
* 특정 염기서열의 결실, 삽입, 치환에 이용되는 site-directed mutagenesis의 원리와 방법에 대해 알아보고, 클로닝하는 전략을 수립한다.

www.flybase.org

www.cit.nih.gov/science.html


TAC
ATG
Denature
TAC
ATG
Anneal mutagenic primers
TAC
AAG
+
TTC
ATG
copyright www.molecularstation.com
PCR (few rounds with Pfu polymerase
n
TTC
AAG
n
TAC
ATG
DpnI
n
TTC
AAG
+
n
TAC
ATG
Transforms
Does not transform

QuikChange Lightning Multi Method



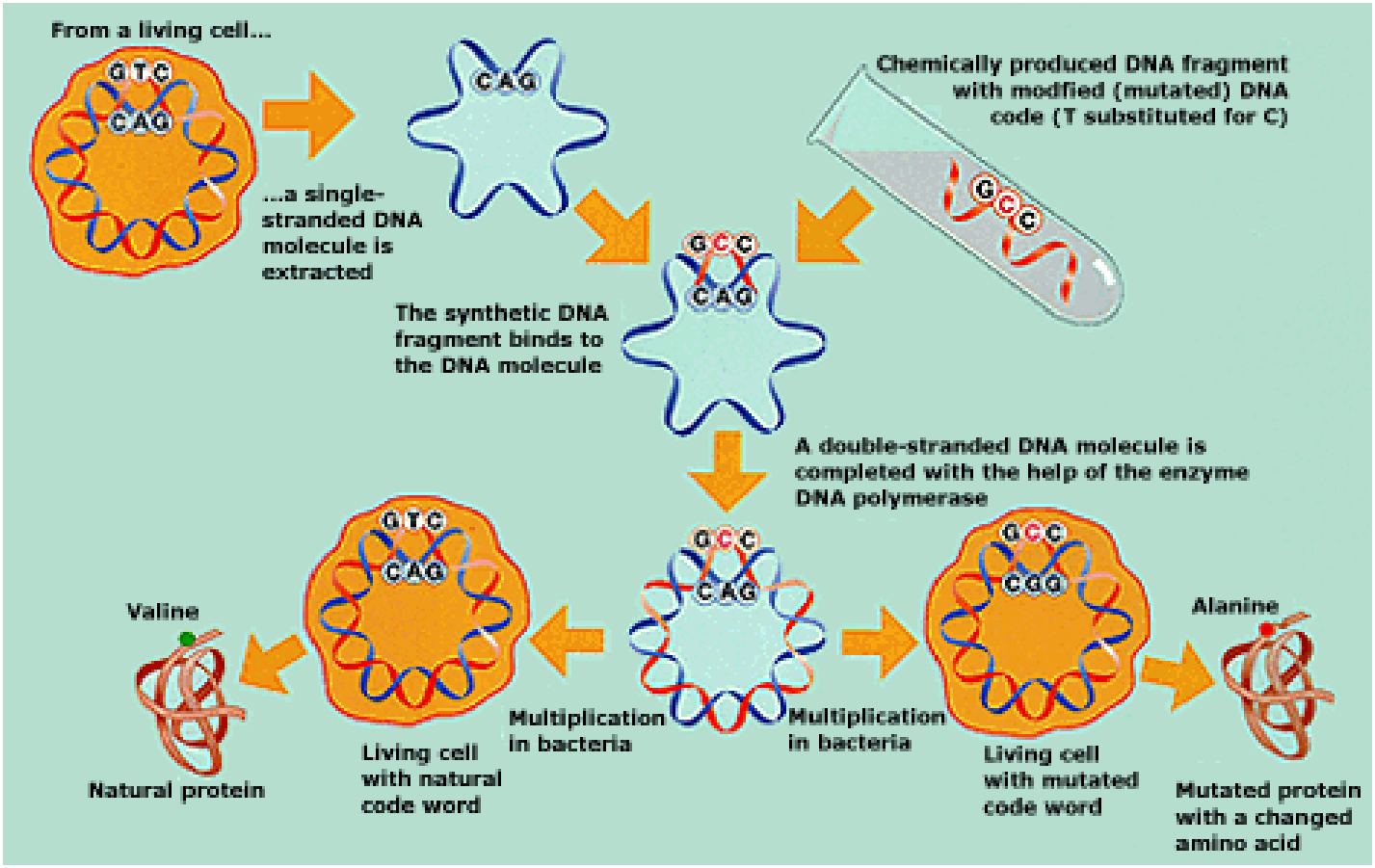
1. **Faster Mutant Strand Synthesis**
   Perform thermal cycling to:
   - Denature DNA template
   - Anneal mutagenic primers
   - Extend primers and ligate nicks with *Pfu* Fusion-based enzyme blend

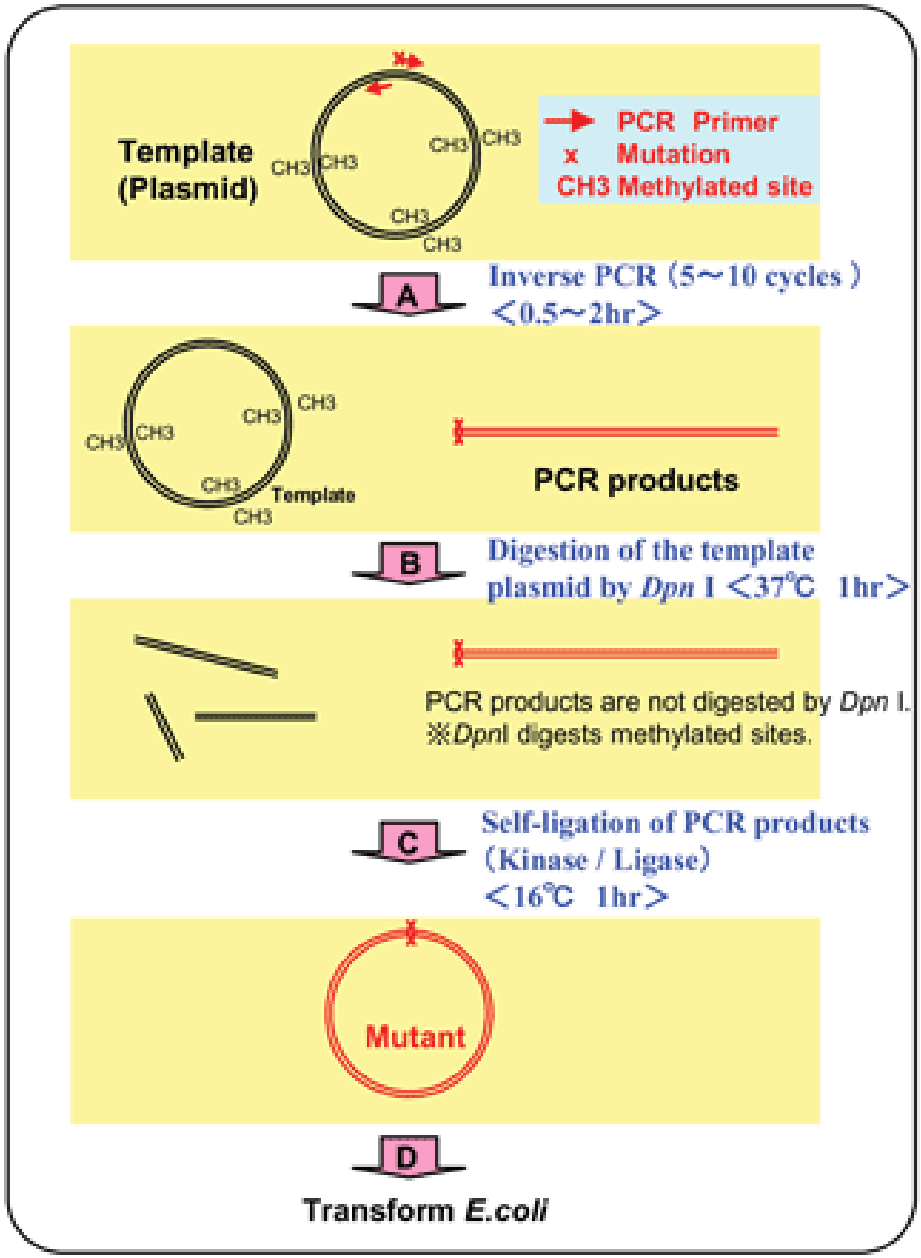
   Total reaction time: 2 hours*

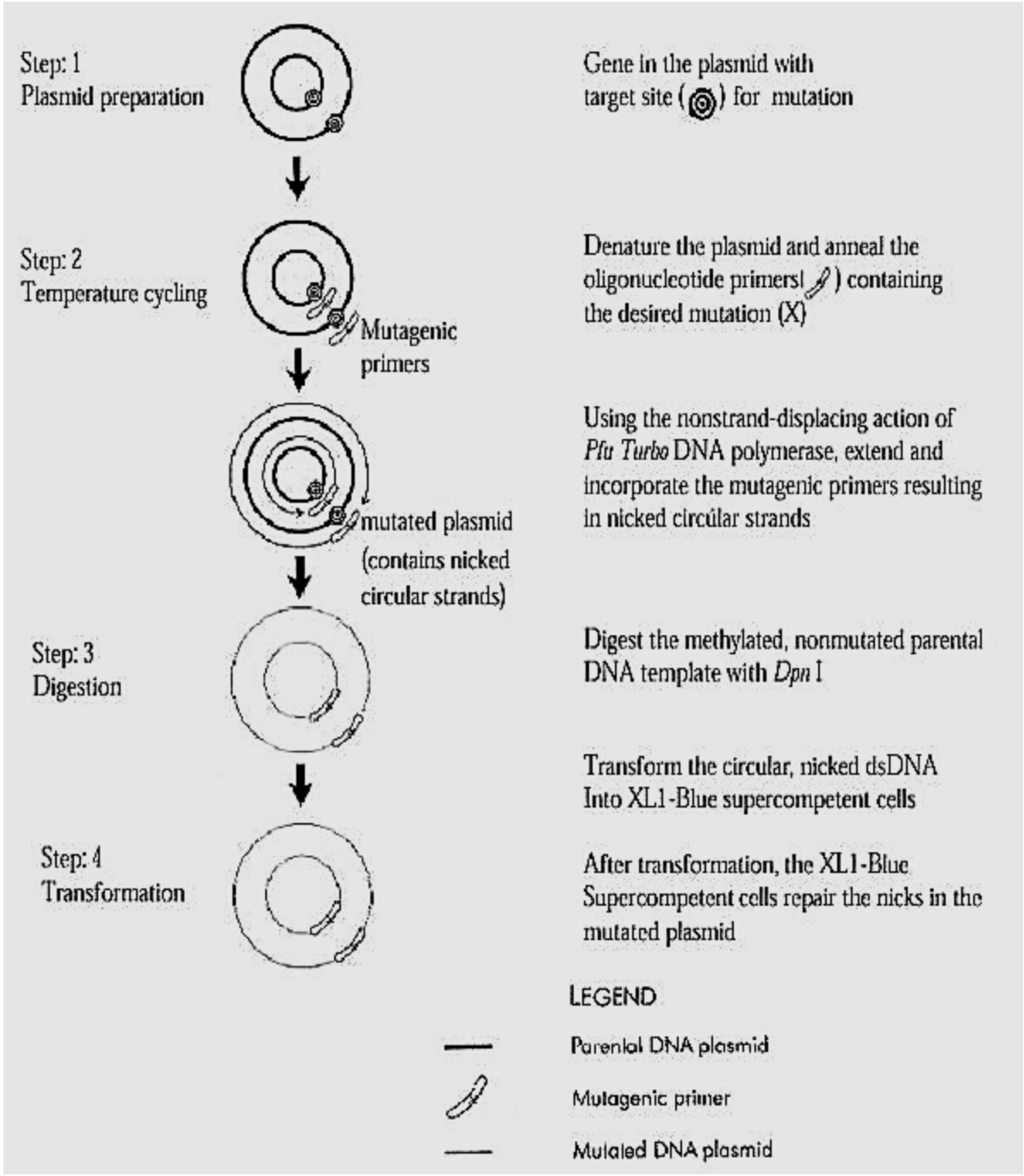
2. **Faster *Dpn* I Digestion of Template**
   Digest methylated and hemimethylated DNA with NEW *Dpn* I enzyme
   Total reaction time: 5 minutes

3. **Transformation**
   Transform mutated ssDNA into XL10 Gold ultracompetent cells
   Total reaction time: 1.5 hours

* Based on a 5-kb plasmid; excludes ramping time.


From a living cell...
GTC
CAG
...a single-stranded DNA molecule is extracted
CAG
Chemically produced DNA fragment with modfied (mutated) DNA code (T substituted for C)
GCC
The synthetic DNA fragment binds to the DNA molecule
GCC
CAG
A double-stranded DNA molecule is completed with the help of the enzyme DNA polymerase
GCC
CAG
GTC
CAG
GCC
CGG
Valine
Natural protein
Multiplication in bacteria
Living cell with natural code word
Multiplication in bacteria
Living cell with mutated code word
Alanine
Mutated protein with a changed amino acid


Template
(Plasmid)
CH3
PCR Primer
x Mutation
CH3 Methylated site
A
Inverse PCR (5~10 cycles)
<0.5~2hr>
Template
PCR products
B
Digestion of the template
plasmid by Dpn I <37℃ 1hr>
PCR products are not digested by Dpn I.
※DpnI digests methylated sites.
C
Self-ligation of PCR products
(Kinase / Ligase)
<16℃ 1hr>
Mutant
D
Transform E.coli


Step: 1
Plasmid preparation
Gene in the plasmid with target site ( ) for mutation
Step: 2
Temperature cycling
Mutagenic primers
Denature the plasmid and anneal the oligonucleotide primers( ) containing the desired mutation (X)
mutated plasmid (contains nicked circular strands)
Using the nonstrand-displacing action of *Pfu Turbo* DNA polymerase, extend and incorporate the mutagenic primers resulting in nicked circular strands
Step: 3
Digestion
Digest the methylated, nonmutated parental DNA template with *Dpn* I
Transform the circular, nicked dsDNA Into XL1-Blue supercompetent cells
Step: 4
Transformation
After transformation, the XL1-Blue Supercompetent cells repair the nicks in the mutated plasmid
LEGEND
Parental DNA plasmid
Mutagenic primer
Mutated DNA plasmid

Cloning strategy

1. Open website
   - http://flybase.org/
   - http://helixweb.nih.gov/emboss_lite/
2. Run map & mapsort program for CG17109
3. Generate restriction enzyme map
4. Establish cloning strategy
5. Design primer
   - BLAST search
   - Restriction enzyme site