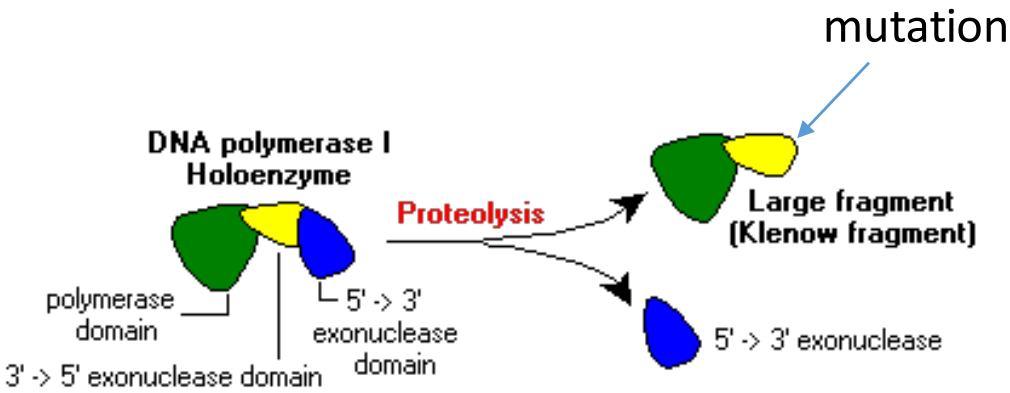
DNA CLONING WITH MODIFICATION OF OVERHANGS

The Exo- Klenow fragment

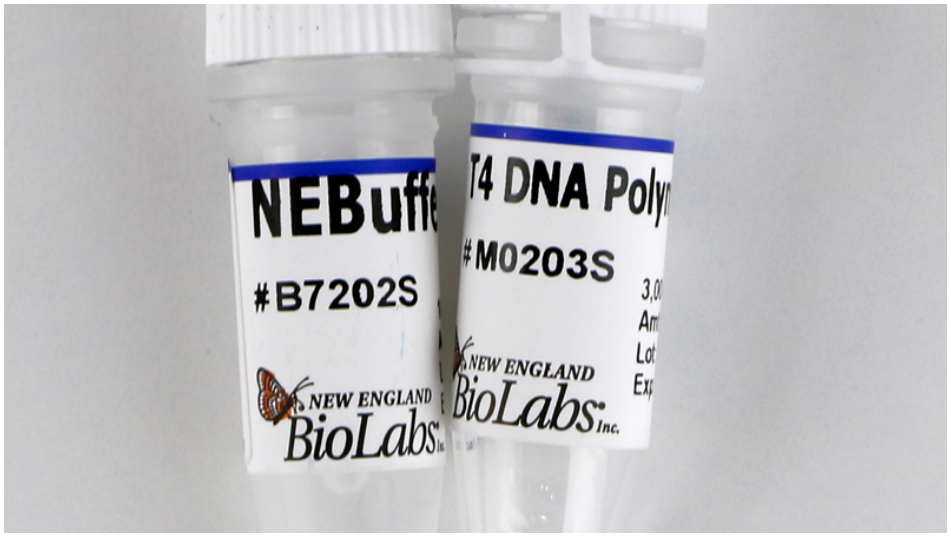
Just as the 5' → 3' exonuclease activity of DNA polymerase I from E.coli can be undesirable, **the 3' → 5' exonuclease activity of Klenow fragment can also be undesirable for certain applications.** This problem can be overcome by **introducing mutations in the gene that encodes Klenow. This results in forms of the enzyme being expressed that retain 5' → 3' polymerase activity, but lack any exonuclease activity (5' → 3' or 3' → 5').** This form of the enzyme is called the exo- Klenow fragment.The exo-Klenow fragment is used in some fluorescent labeling reactions for microarray, and also in dA and dT tailing, an important step in the process of ligating DNA adapters to DNA fragments, frequently used in prepararing DNA libraries for Next-Gen sequencing.



DNA CLONING WITH MODIFICATION OF OVERHANGS

The T4 DNA Polymerase

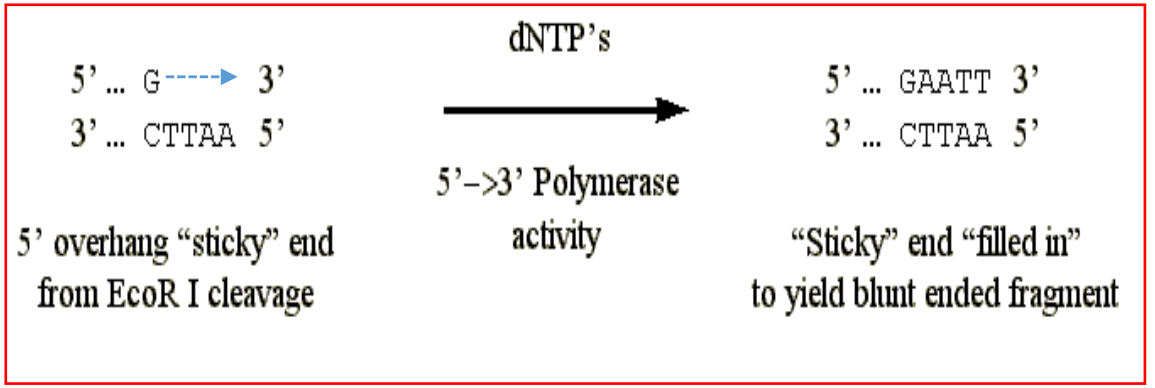
Encodend by T4 phage.
T4 DNA Polymerase catalyzes the synthesis of DNA in the 5´→3´ direction and requires the presence of template and primer. Contains 3'→5' exonuclease activity.
→Gap filling (no strand displacement activity) of 5' overhangs to form blunt ends
→Removal of 3' overhangs
→ No 5' —> 3' exonuclease activity

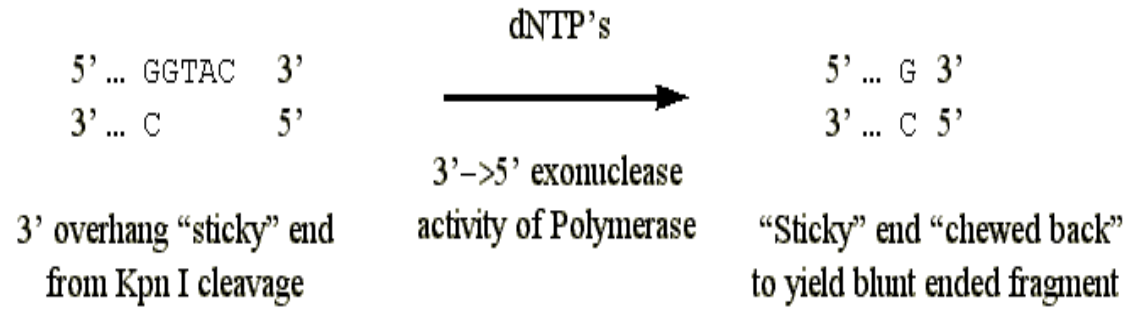


DNA CLONING WITH MODIFICATION OF OVERHANGS

Converting a 5' overhang to blunt end

- Both **Klenow** and **T4 DNA polymerase** can be used to fill in 5' protruding ends with dNTPs
- Polymerase activity: 5' → 3'
- Used in joining DNA fragments with incompatible ends
- Once the ends have been blunted, ligation can proceed





IMPORTANT FOR KLENOW and T4 DNA POLYMERASE REACTION:

dNTPs need to be present in abundance (and be of good quality!)

→ If dNTPs are used up by DNA polymerase actiyivity; exonuclease activity will take over

→ → degradation of plasmid/insert

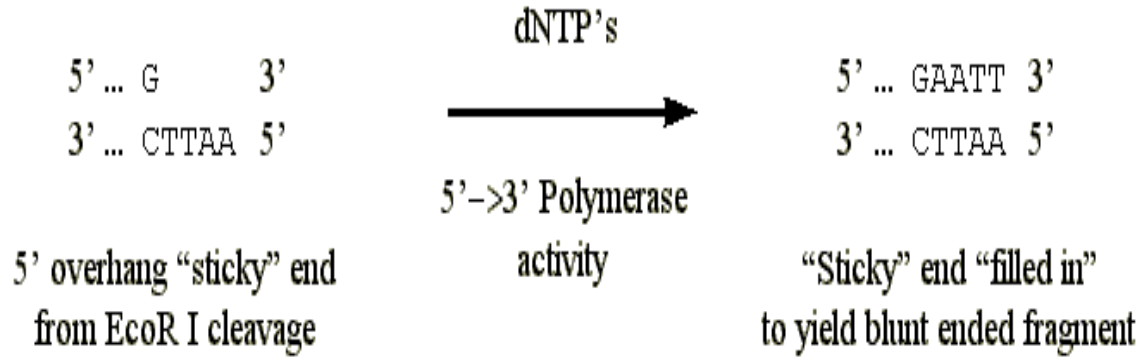
Exo- Klenow fragment is safer in use!

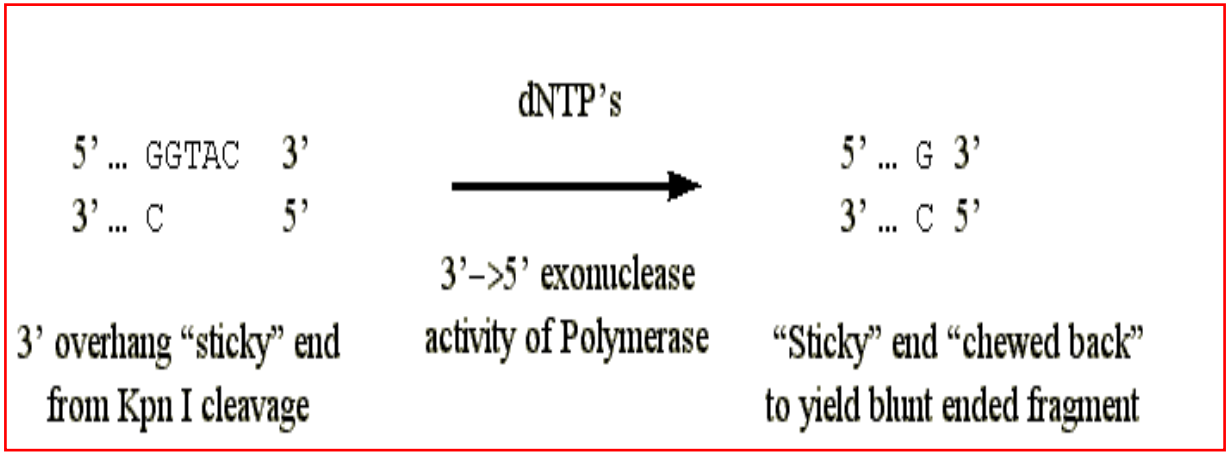
DNA CLONING WITH MODIFICATION OF OVERHANGS

Converting a 3' overhang to a blunt end

- T4 DNA polymerase/Klenow have a 3'→5' exonuclease activity
- In the presence of excess dNTPs will convert a 3' protruding end to a blunt end
- Important 3'exonuclease and 5'→3' DNA polymerase reaction are competing
- Ligation can know proceed

Note: also Klenow fragment has 3'→5' exonuclease activity





IMPORTANT FOR 3'overhang BLUNTING:

dNTPs need to be present in abundance (and be of good quality!)

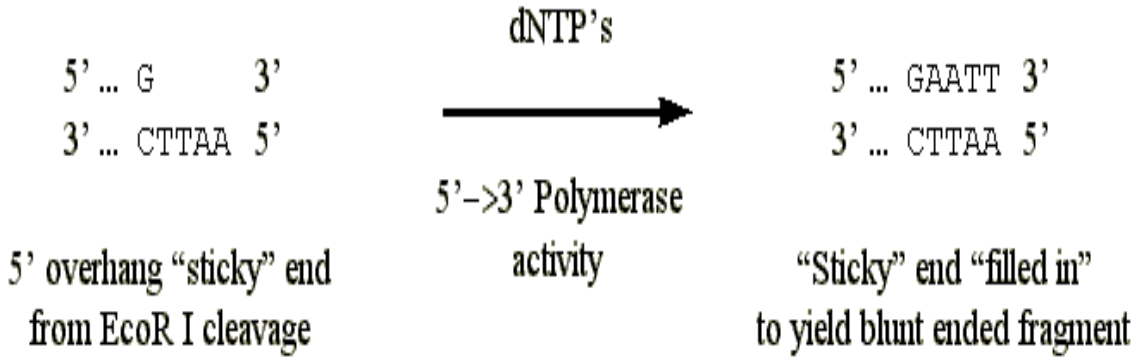
→ If dNTPs are used up by DNA polymerase activity; exonuclease activity will take over

→ → degradation of plasmid/insert

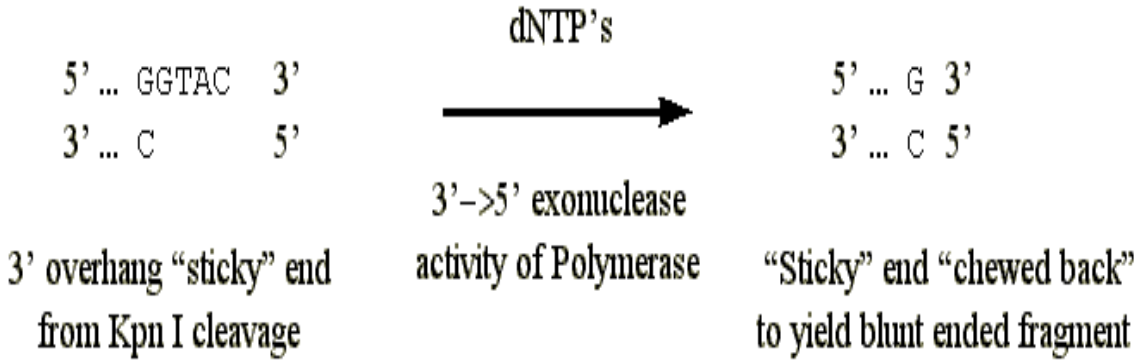
DNA CLONING WITH MODIFICATION OF OVERHANGS

Laboratory reality → ideal use of enzymes

Exo⁻Klenow fragment
5' overhang fill-up



T4 DNA polymerase
3' overhang removal



Note: some researchers use T4 polymerase for 5'overhang blunting AND 3' overhang blunting. Why: only one enzyme; used frequently (always updated on enzyme activity), T4 is stabile, cost extensive; you can blunt a fragment that has 3' and 5' overhang in single reaction

DNA CLONING WITH MODIFICATION OF OVERHANGS

INSERT

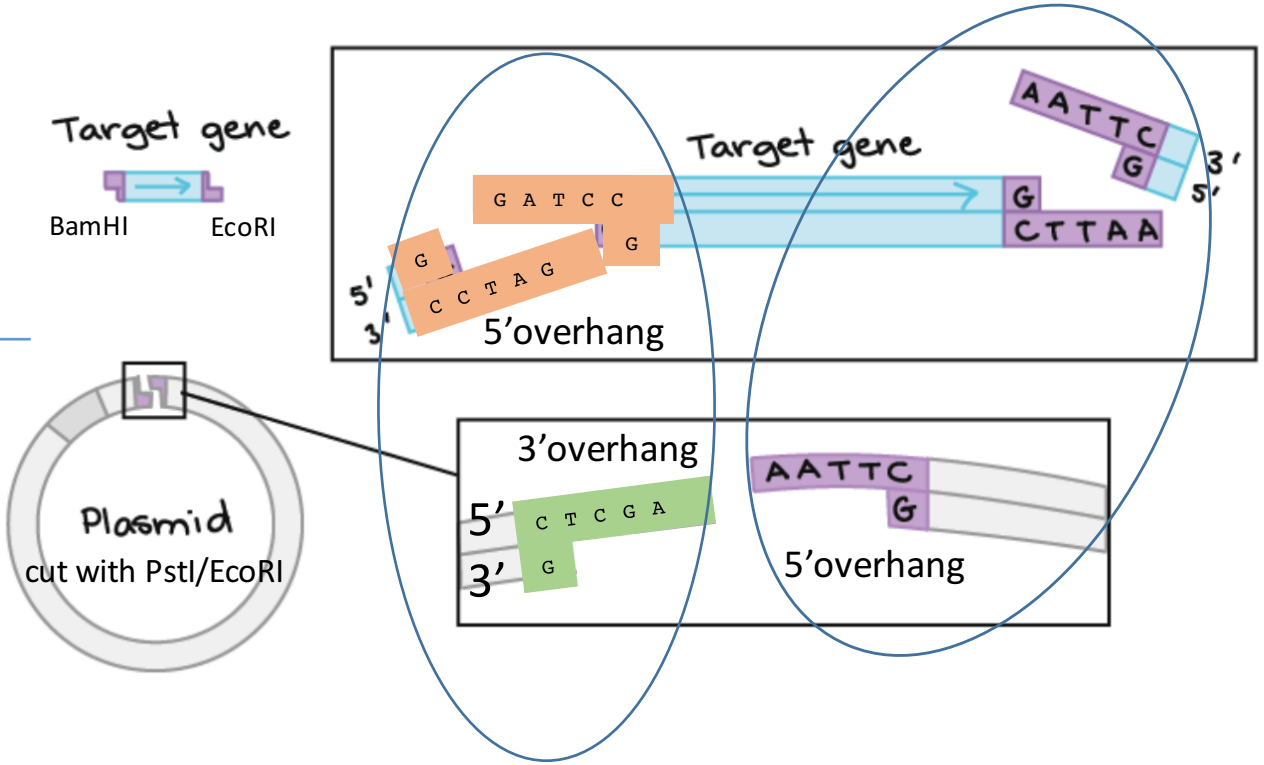
BamHI: G/GATCC
CCTAG/G

EcoRI: G/AATTC
CTTAA/G

VECTOR

PstI: CTCGA/G
G/AGCTC

EcoRI: G/AATTC
CTTAA/G



HOW TO DO?

Vector:

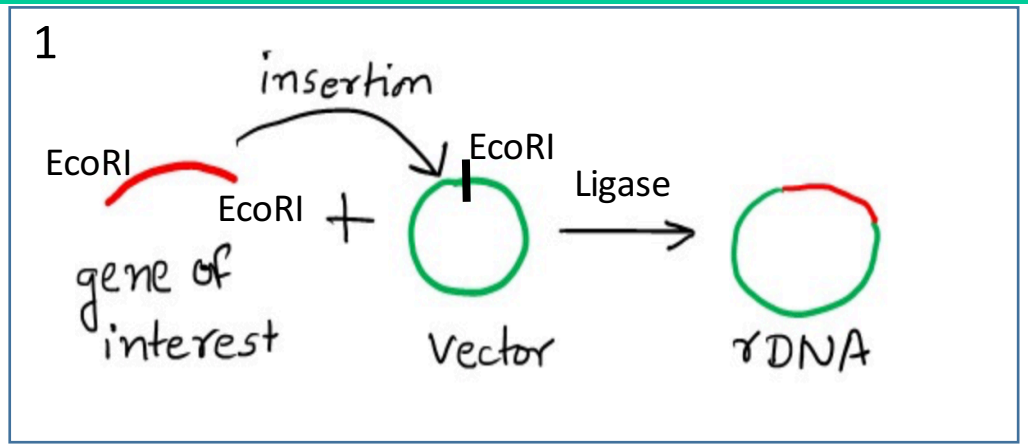
1. Cut PstI
2. Make T4 Polymerase reaction → blunting of 3'overhang
3. Purify DNA from enzymatic reaction (for example column)
4. Cut DNA with EcoRI
5. Run DNA on agarose gel
6. Cut correct band and purify DNA, determine concentration

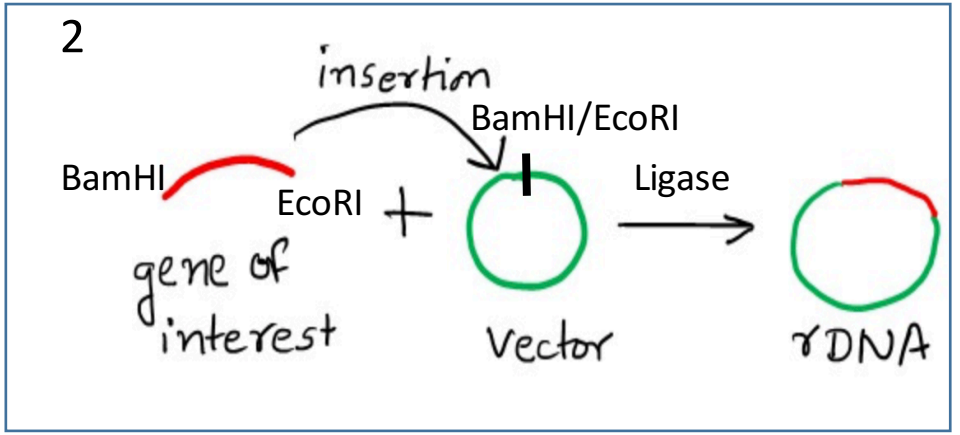
Insert:

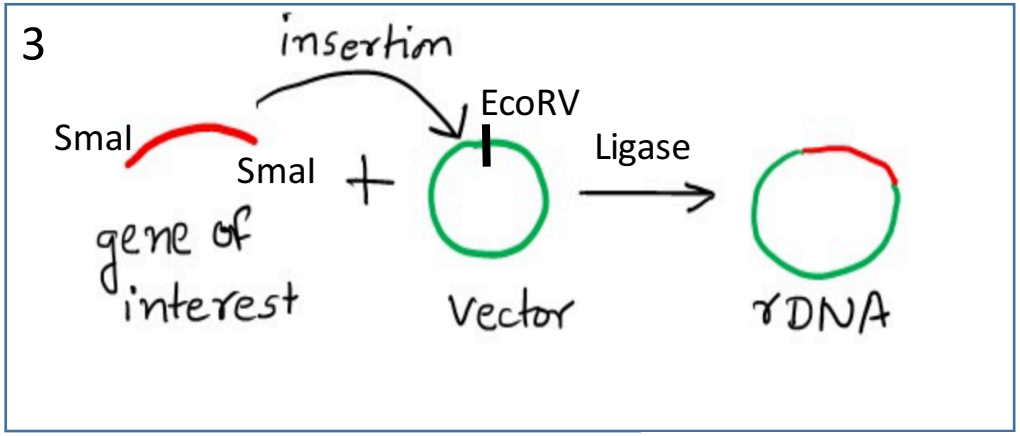
1. Cut DNA with BamHI
2. Make Exo$^-$ Klenow reaction → blunting of 5'overhang
3. Purify DNA from enzymatic reaction (for example column)
4. Cut DNA with EcoRI
5. Run DNA on agarose gel
6. Cut correct band and purify DNA; determine concentration

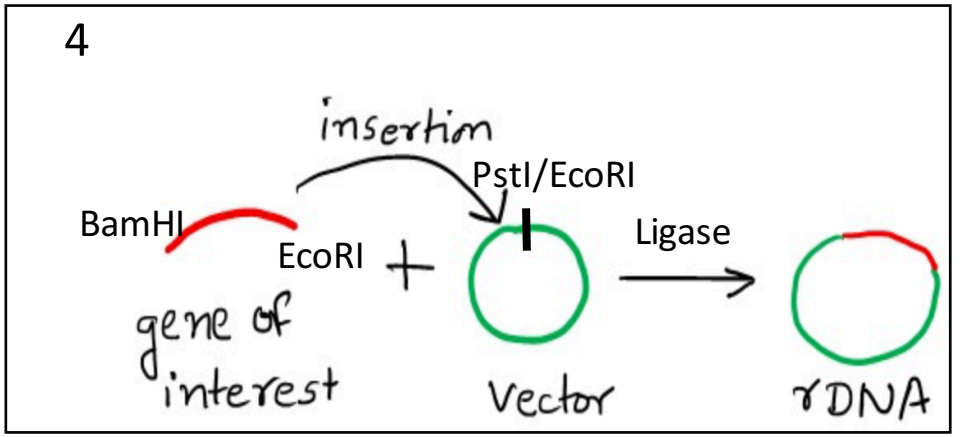
8. Setup ligation (Blunt- Blunt; EcoRI – EcoRI)

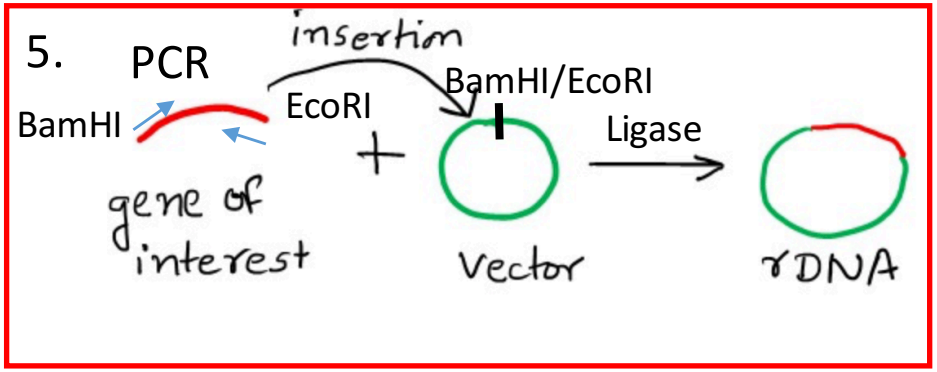
OVERVIEW OVER OTHER CLONING STRATEGIES



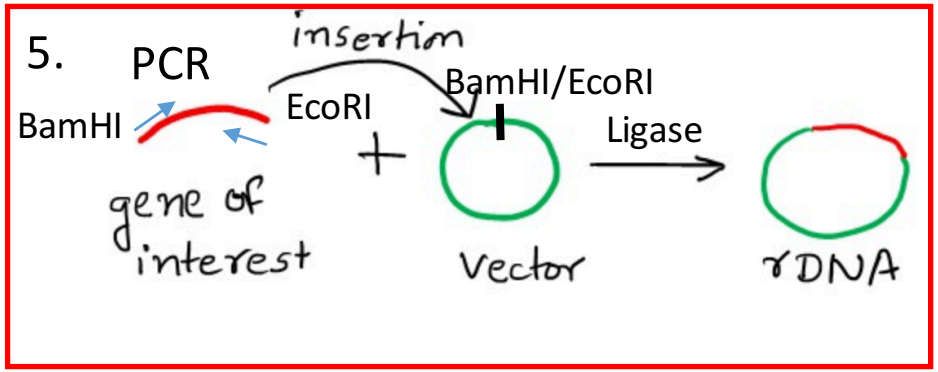








PCR CLONING



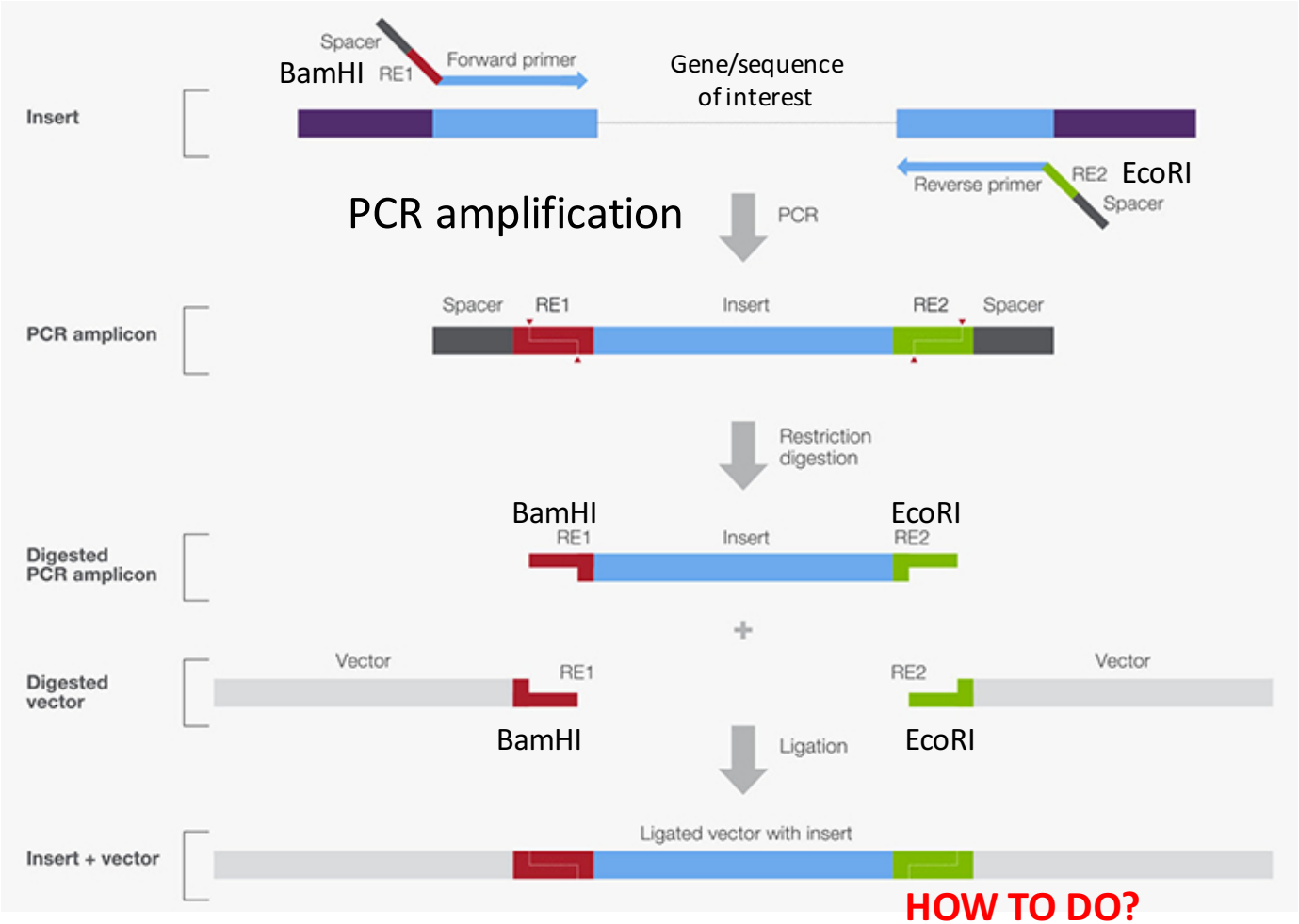
1. DNA of interest is amplified by PCR. PCR oligos contain sequence for restriction enzyme

2. PCR generates dsDNA that can be cut with restriction enzyme

3. Fragment cloned into vector

PCR CLONING

1. Classic PCR cloning



Forward primer

5'-CGCGGATCCxxxxxxxxxxxxxxxxxx-3'

Spacer BamHI -5 nucelotides)

Sequence pairing with Sequence of interest (min. 18 nucelotides)

Reverse primer

3'-xxxxxxxxxxxxxxxxxxGAATTCCGC-5'

Sequence pairing with Sequence of interest (min. 18 nucelotides)

EcoRI

Spacer 3-5 nucelotides

HOW TO DO?

1. **Design + synthezie oligos**
2. **Make PCR**
3. **Purify PCR product (agarose gel or columns)**
4. **Cut PCR product with BamHI and EcoRI**
5. **Run agarose gel; cut out band; purify DNA; determine concentration**
6. **Setup ligation with vector linearized by EcoRI/BamHI**

PCR CLONING

1. Classic PCR cloning

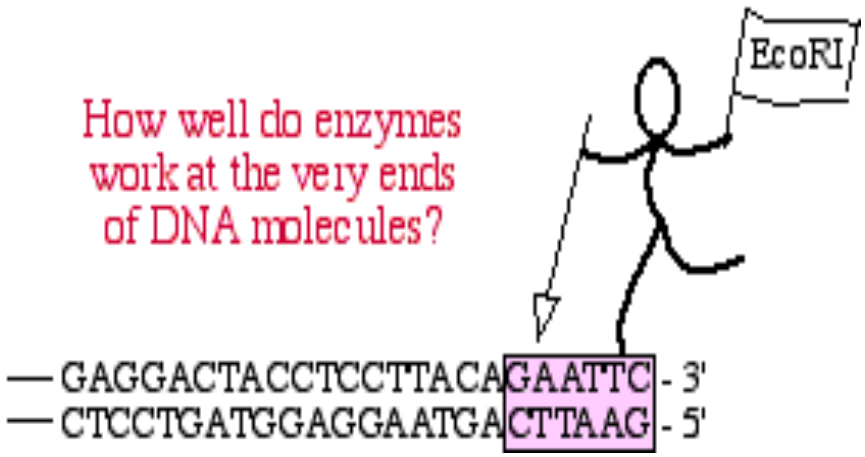
WHY IS A SPACER NEEDED???

Forward primer

5′–CGCGGATCCxxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxxCTTAAGGCG

3′–GCGCCTAGGxxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxxGAATTCCGC–5′

Reverse primer



→ Many resitriction enzymes work poorly on DNA termini

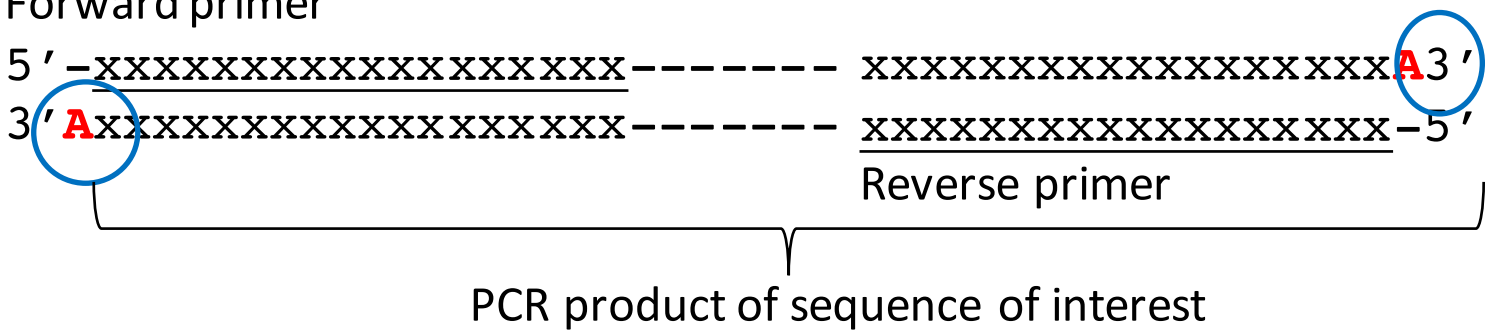
→ Catalogues of enzymes provide data on the cutting efficiency of enzymesat the end of DNA molecules.

→ Generally, enzymes work better if they have a couple of extra nucleotides at the end – improved interaction with DNA

PCR CLONING

2. TA-cloning

ATTENTION: Taq polymerases produce PCR products with A on 3'ends



→ Primers for PCR do not necessarily contain restriction site and spacer!

→ Primers used to amplify seqeunce of interest

Taq polymerase is a thermostable DNA polymerase named after the thermophilic bacterium Thermus aquaticus from which it was originally isolated.
Taq polymerases are the most frequently used polymerases for PCR

Taq DNA polymerase catalyzes the non-template directed addition of an adenine residue to the 3´-end of both strands of DNA molecules → blunt cloning not possible

This is useful in TA cloning, whereby a cloning vector (such as a plasmid) that has a T (thymine) 3' overhang is used, which complements with the A overhang of the PCR product, thus enabling ligation of the PCR product into the plasmid vector. = **TA cloning**

PCR CLONING

3. TA-Cloning

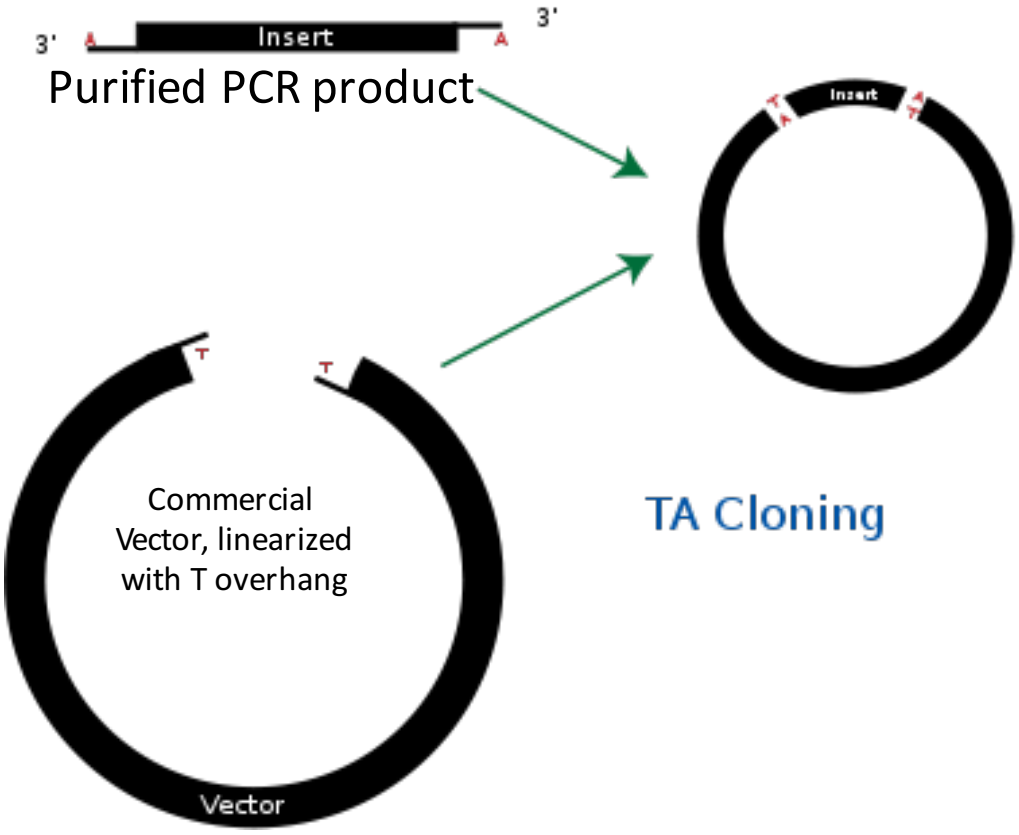
ATTENTION: Taq polymerases produce PCR products with A on 3'ends

Forward primer

5'–CGCGGATCCxxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxxCTTAAGGCGA3'

3'AGCGCCTAGGxxxxxxxxxxxxxxxxxxx-- xxxxxxxxxxxxxxxxxxxGAATTCCGC–5'

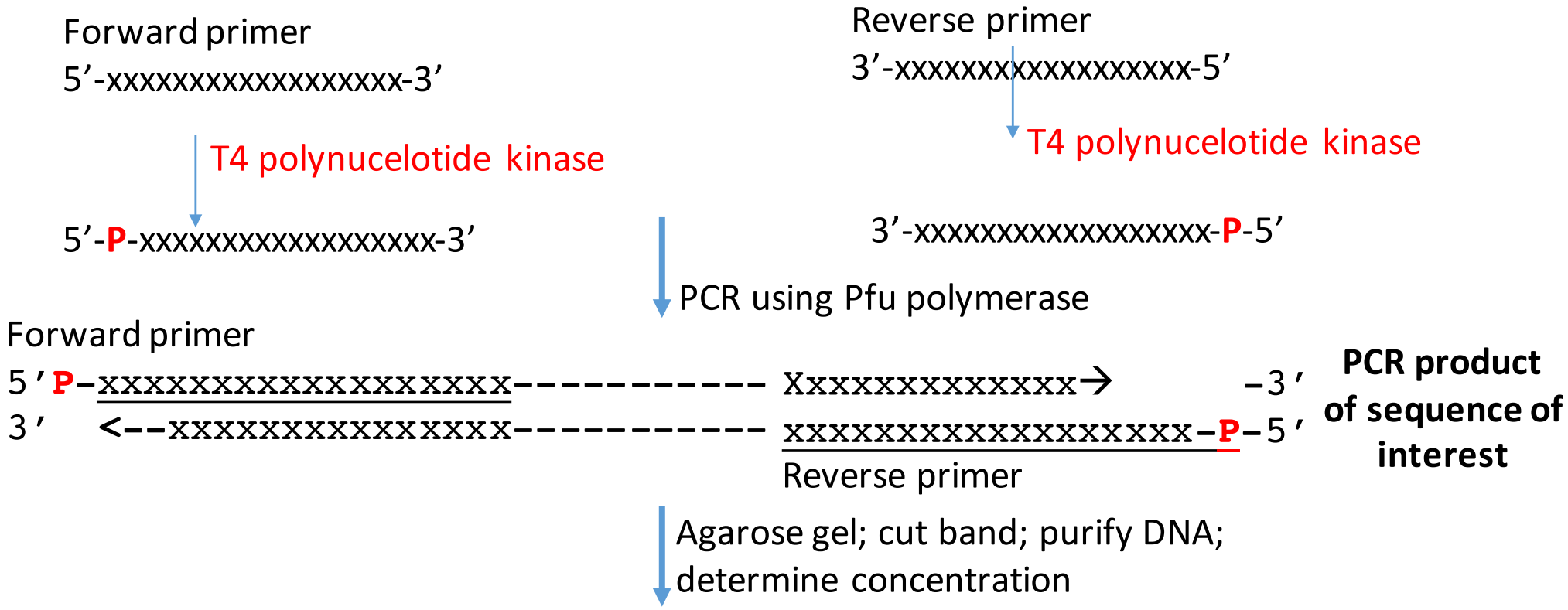
Reverse primer



2. Blunt end cloning using PCR

PCR CLONING

ATTENTION: Other polymerasese do not add A on 3'end: for example: Pfu polymerase creates blunt PCR products



Pfu DNA polymerase is an enzyme found in the hyperthermophilic archaeon *Pyrococcus furiosus*

PCR CLONING

4. Characeristics of DNA polymerases that can be used for DNA cloning

	5′–>3′ Exonuclease	3′–>5′ Exonuclease	Error Rate(x10^{-6})[a]	Strand Displacement	Nick Translation	Thermal Stability	K_m dNTPs	K_m DNA[d]	Extend RNA Primer	Extension from Nick	Primary Applications
Bst DNA Polymerase, Full Length	+	–		_r	+	+			+	+	Labeling, 2nd Strand Synthesis
Bst DNA Polymerase, Large Fragment	–	–		++++	–	+			+	+	Strand Displacement Applications, isothermal amplification
Bsu DNA Polymerase, Large Fragment	–	–		++	–	–			+	+	Labeling, 2nd Strand Synthesis, Strand Displacement
Crimson *Taq* DNA Polymerase	+	–	285	_r	+	++			–	+	PCR (routine)
Deep Vent$_R$™ DNA Polymerase	–	+++		++	–	++++	50 µM[e]	0.01 nM[e]	–	+	PCR (high-fidelity)
Deep Vent$_R$™ (exo–) DNA Polymerase	–	–		+++	–	++++			–	+	PCR (long)
E. coli DNA Polymerase I	+	++	9[h]	_r	+	–	1-2 µM[f]	5 nM[f]	+	+	Nick Translation
Klenow Fragment (3′→5′ exo-)	–	–	100[o]	+++	–	–			+	+	Labeling
DNA Polymerase I, Large (Klenow) Fragment	–	++	18[o]	++	–	–	2 µM[g]		+	+	Polishing Ends
LongAmp® *Taq* DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (routine, long)
LongAmp® Hot Start *Taq* DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (hot start, long)
M-MuLV Reverse Transcriptase	–	–		+++	–	–	18 µM[s]				cDNA Synthesis

PCR CLONING

4. Characeristics of DNA polymerases that can be used for DNA cloning

One*Taq*® DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (routine, difficult)
One*Taq*® Hot Start DNA Polymerase	+	++	~140	_r	+	++			–	+	PCR (hot start, routine, difficult)
phi29 DNA Polymerase	–	++++		+++++	–	–	0.5 µM[q]		+	+	Strand Displacement Applications
Phusion® Hot Start Flex DNA Polymerase*	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity, long)
Phusion® High-Fidelity DNA Polymerase*	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity, long, hot start)
Q5® + Q5® Hot Start DNA Polymerase	–	++++	<0.44	–	–	+++			–	–	PCR (high-fidelity)
Sulfolobus DNA Polymerase IV	–	–		–	–	+					DNA Synthesis Across Template Lesions
T4 DNA Polymerase	–	++++	<1[h]	–	–	–	2 µM[n]		+	–	Polishing Ends, 2nd Strand Synthesis
T7 DNA Polymerase (unmodified)	–	++++	15[b]	–	–	–	18 µM[k]	18 nM[k]	+	–	Site Directed Mutagenesis
Taq DNA Polymerase with Standard *Taq* Buffer	+	–	285[c]	_r	+	++	13 µM[e]	2 nM[e]	–	+	PCR (routine)
Therminator™ DNA Polymerase	–	–		+	–	++++			+	+	Chain Terminator Applications
Vent$_R$® DNA Polymerase	–	++	57[b]	++[e]	–	+++	60 µM[e]	0.1 nM[e]	–	+	PCR (routine, high-fidelity)
Vent$_R$® (exo–) DNA Polymerase	–	–	190[b]	+++[e]	–	+++	40 µM[e]	0.1 nM[e]	–	+	PCR, Sequencing

Phusion Polymerase: trade name for Pfu polymerase that had been engeneered to have improved function

PCR CLONING

4. Characeristics of DNA polymerases that can be used for DNA cloning

What percent of the product molecules contain an error after PCR (30 cycles) with different polymerases?

Polymerase	1 kb template	3 kb template
Phusion High-Fidelity DNA Polymerases (HF Buffer)	1.32%	3.96%
Phusion High-Fidelity DNA Polymerases (GC Buffer)	2.85%	8.55%
Pyrococcus furiosus DNA polymerase	8.4%	25.2%
Taq DNA polymerase	68.4%	205.2%

The table above demonstrates the low error rate of Phusion DNA Polymerase. After 30 cycles of PCR amplifying a 3 kb template, only 3.96 % of the product DNA molecules contain 1 (nucleotide) error each. This means that 96.04 % of the product molecules are entirely error-free. In contrast, after the same PCR protocol performed with *Taq* DNA polymerase, every product molecule contains an average of 2 errors.

PCR is error prone!

→ Keep PCR cycles at the lowest mininum possible