Lecture 5:

Exploring The Genome

Workable Pieces

Course 485

Introduction to Genomics

AIMS

- Review restriction enzymes and plasmids.
- Introduce molecular cloning.
- Introduce Polymerase Chain Reaction.
- Highlight the importance of obtaining workable DNA pieces for genome exploration.

The molecular scissors

Proc. Nat. Acad. Sci. USA
Vol. 68, No. 12, pp. 2913–2917, December 1971

Specific Cleavage of Simian Virus 40 DNA by Restriction Endonuclease of Hemophilus Influenzae*

(gel electrophoresis/electron microscopy/DNA mapping/DNA fragments/tumor virus)

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Communicated by Albert L. Lehninger, September 22, 1971

ABSTRACT **A bacterial restriction endonuclease has been used to produce specific fragments of SV40 DNA. Digestion of DNA from plaque-purified stocks of SV40 with the restriction endonuclease from *Hemophilus influenzae* gave 11 fragments resolvable by polyacrylamide gel electrophoresis, eight of which were equimolar with the original DNA. The fragments ranged from about 6.5 × 10^6 to 7.4 × 10^4 daltons, as determined by electron microscopy, DNA content, or electrophoretic mobility.**

Preparation of Radioactive SV40 DNA from Virions. Confluent BSC-1 monolayers in 100-mm plastic Petri dishes were infected with SV40 at a multiplicity of about 10 PFU/cell. For ^{32}P-labeling, 0.15 mCi of carrier-free [^{32}P]orthophosphate was added to each dish, at 24 hr after infection, in 6 ml of phosphate-free medium containing 4% dialyzed fetal-bovine serum. For labeling with [^{3}H]thymidine of high specific-activity the medium was made 10^{-4}M in FdU 24 hr after in-

J. Mol. Biol. (1970) **51**, 379–391

A Restriction Enzyme from *Hemophilus influenzae*

I. Purification and General Properties

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(*Received 15 September 1969*)

Extracts of *Hemophilus influenzae* strain Rd contain an endonuclease activity which produces a rapid decrease in the specific viscosity of a variety of foreign native DNA's; the specific viscosity of *H. influenzae* DNA is not altered under the same conditions. This "restriction" endonuclease activity has been purified approximately 200-fold. The purified enzyme contains no detectable exo- or endonucleolytic activity against *H. influenzae* DNA. However, with native phage T7 DNA as substrate, it produces about 40 double-strand 5′-phosphoryl, 3′-hydroxyl cleavages. The limit product has an average length of about 1000 nucleotide pairs and contains no single-strand breaks. The enzyme is inactive on denatured DNA and it requires no special co-factors other than magnesium ions.

1. Introduction

A number of bacteria are capable of recognizing and degrading ("restricting") foreign DNA, such as the DNA of a virus grown on another bacterial strain. The DNA of the host is protected by a "host-controlled modification" (Arber, 1965). Recently, Meselson & Yuan (1968) have purified a restriction endonuclease from *Escherichia coli* K12. The enzyme has the interesting properties: (1) that it is site-specific in action, producing only a limited number of double-strand breaks in unmodified DNA, and (2) that it requires adenosine triphosphate and *S*-adenosyl methionine in addition to magnesium ions.

We have made the chance discovery of what appears to be a similar type of enzyme in *Hemophilus influenzae*, strain Rd. In the course of some experiments in which competent *H. influenzae* cells were incubated with radioactively labeled DNA from the *Salmonella* phage P22, we found that this DNA was apparently degraded since it could not be recovered in cesium chloride density gradients. It seemed likely that the effect was one of restriction. We were able to show the presence in crude extracts of an endonuclease activity which produced a rapid decrease in viscosity of foreign DNA preparations and which was without effect on the *H. influenzae* DNA. We describe in this report the purification and properties of the endonuclease. As with the *E. coli* restriction enzyme, our enzyme produces double-strand breaks in a limited number of specific sites. The enzyme requires only magnesium ions as a co-factor, unlike the *E. coli* enzyme. A preliminary report has been published (Smith & Wilcox, 1969).

Restriction Enzymes

- Endo vs. Exo- nucleases.
- Enzymes purified from various bacteria.
- Bacterial “immune system” against viruses.
- Restriction enzymes CUT double stranded DNA at specific restriction sites.

How do bacteria protect itself from restriction enzymes?

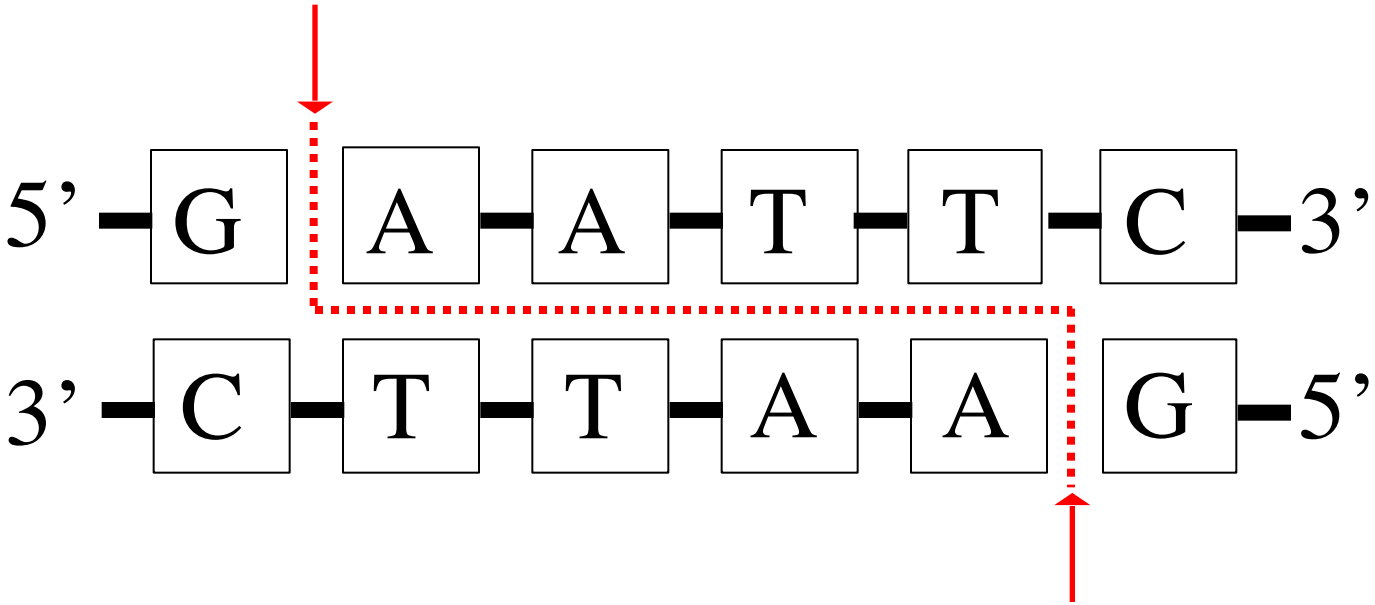
Restriction Enzymes

- Restriction enzymes are categorized into three types: I, **II**, III.
- Each restriction enzyme recognizes specific DNA sequence (**restriction site**).
- Restriction sites (variable length 4, 6, 8 bp)
- Restriction sites are complementary and symmetrical on both DNA strands.
- Restriction sites are found within **palindromes**!

palindrome |ˈpalinˌdrōm|
noun
a word, phrase, or sequence that reads the same backward as forward, e.g., *madam* or *nurses run*.

Palindromes

"Both strands have the same nucleotide sequence but in anti parallel orientation" (Griffiths et. al, 2000)



Nomenclature

Restriction endonucleases are named after the prokaryotic source, which were isolated and purified from.

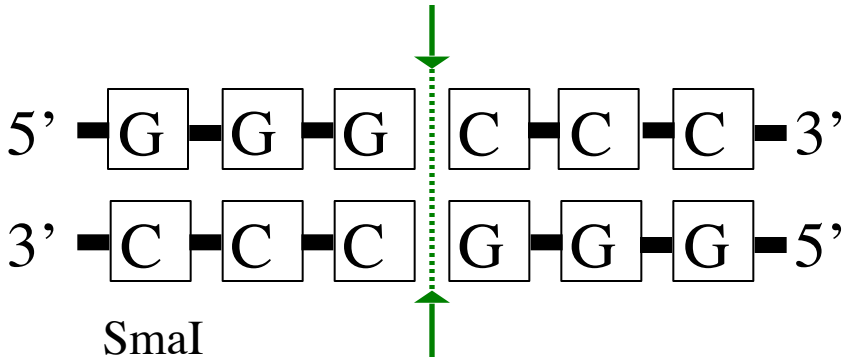
EcoRI

E	*Escherichia* (Genus)
co	*coli* (Species)
R	RY13 (Strain)
I	Order of discovery (Roman numerals)

Restriction Enzymes Cut Types

Blunt End

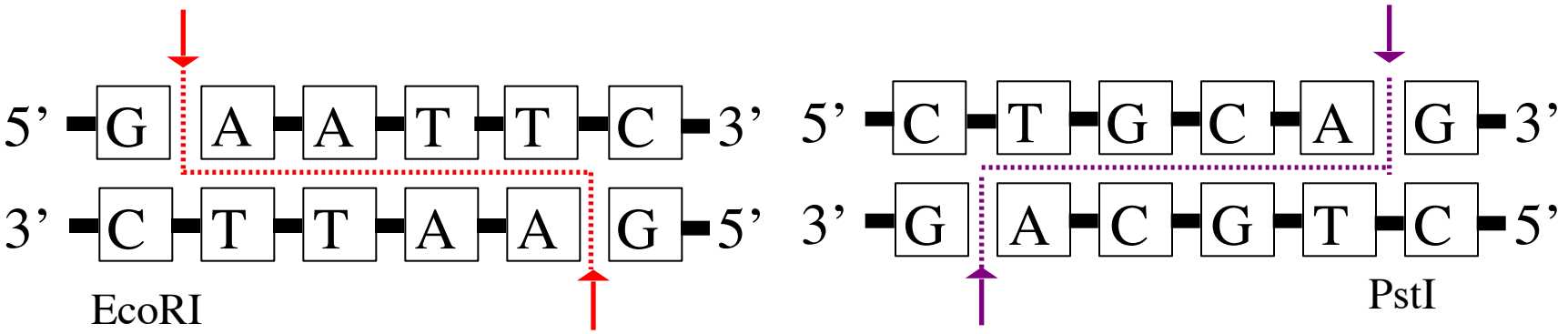
- Restriction enzyme cuts the same position on both strands
- Usefulness for cloning?
- SmaI, HpaI



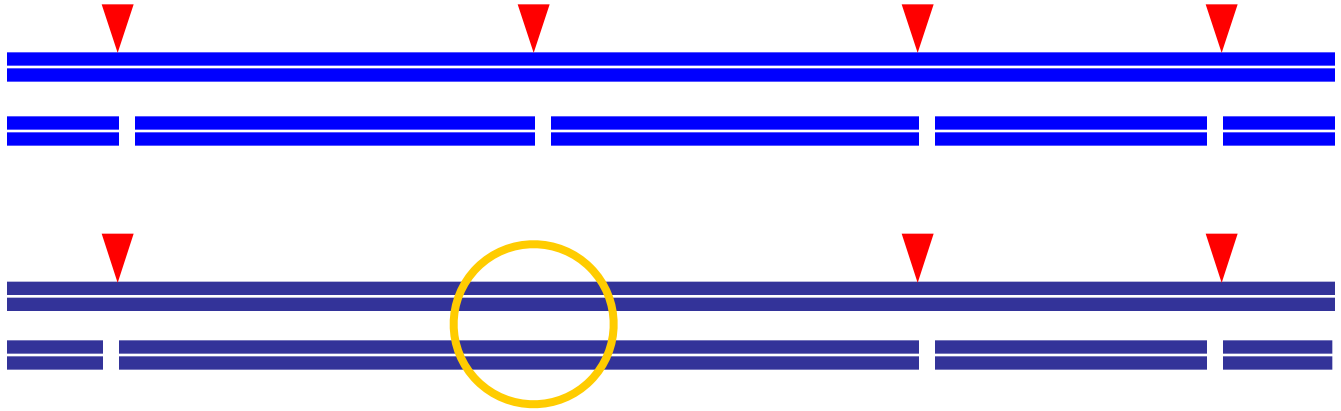
Restriction Enzymes Cut Types

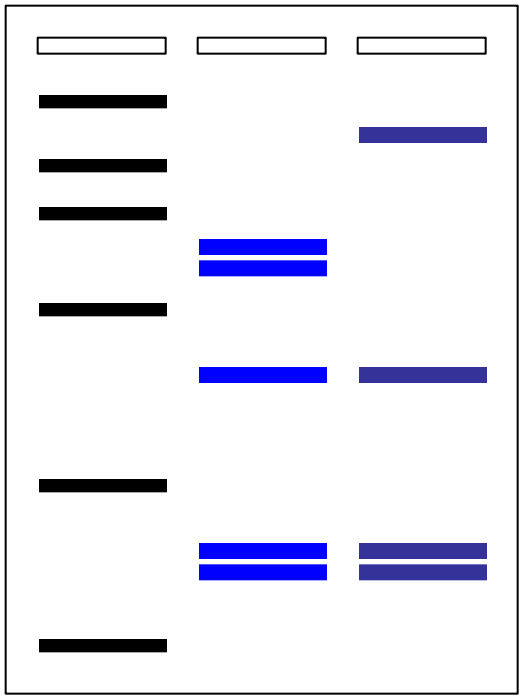
Sticky (Cohesive) End

- Restriction enzyme cuts leaving short single stranded ends
- Usefulness for cloning?



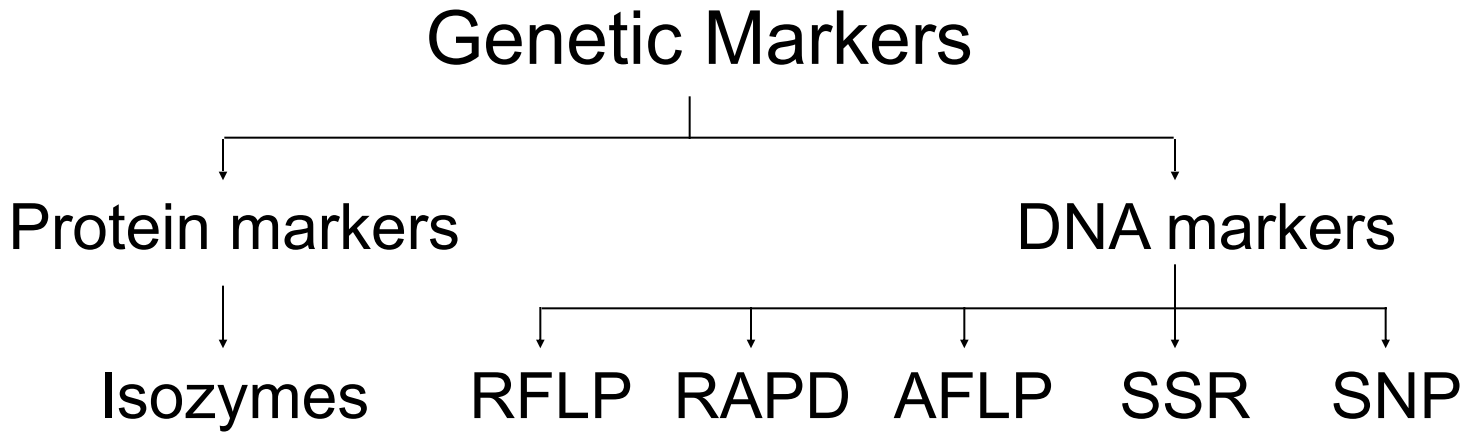
Cutting genomes





What determines the presence or absence of restriction site?

Molecular Markers



Molecular (Genetic) Markers are used to detect variation

We will visit this later

Do you remember what plasmids are?

Can plasmids be cut using restriction enzymes?

Molecular cloning

DNA cloning: A personal view after 40 years

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Edited by Joseph L. Goldstein, University of Texas Southwestern Medical Center, Dallas, TX, and approved August 13, 2013 (received for review August 2, 2013)

In November 1973, my colleagues A. C. Y. Chang, H. W. Boyer, R. B. Helling, and I reported in PNAS that individual genes can be cloned and isolated by enzymatically cleaving DNA molecules into fragments, linking the fragments to an autonomously replicating plasmid, and introducing the resulting recombinant DNA molecules into bacteria. A few months later, Chang and I reported that genes from unrelated bacterial species can be combined and propagated using the same approach and that interspecies recombinant DNA molecules can produce a biologically functional protein in a foreign host. Soon afterward, Boyer's laboratory and mine published our collaborative discovery that even genes from animal cells can be cloned in bacteria. These three PNAS papers quickly led to the use of DNA cloning methods in multiple areas of the biological and chemical sciences. They also resulted in a highly public controversy about the potential hazards of laboratory manipulation of genetic material, a decision by Stanford University and the University of California to seek patents on the technology that Boyer and I had invented, and the application of DNA cloning methods for commercial purposes. In the 40 years that have passed since publication of our findings, use of DNA cloning has produced insights about the workings of genes and cells in health and disease and has altered the nature of the biotechnology and biopharmaceutical industries. Here, I provide a personal perspective of the events that led to, and followed, our report of DNA cloning. PNAS | **September 24, 2013** | vol. 110 | no. 39 | **15521–15529**

Proc. Nat. Acad. Sci. USA
Vol. 70, No. 11, pp. 3240–3244, November 1973

Construction of Biologically Functional Bacterial Plasmids *In Vitro*

(R factor/restriction enzyme/transformation/endonuclease/antibiotic resistance)

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Communicated by Norman Davidson, July 18, 1973

ABSTRACT **The construction of new plasmid DNA species by *in vitro* joining of restriction endonuclease-generated fragments of separate plasmids is described. Newly constructed plasmids that are inserted into *Escherichia coli* by transformation are shown to be biologically functional replicons that possess genetic properties and nucleotide base sequences from both of the parent DNA molecules. Functional plasmids can be obtained by reassociation of endonuclease-generated fragments of larger replicons, as well as by joining of plasmid DNA molecules of entirely different origins.**

*Eco*RI-generated fragments have been inserted into appropriately-treated *E. coli* by transformation (7) and have been shown to form biologically functional replicons that possess genetic properties and nucleotide base sequences of both parent DNA species.

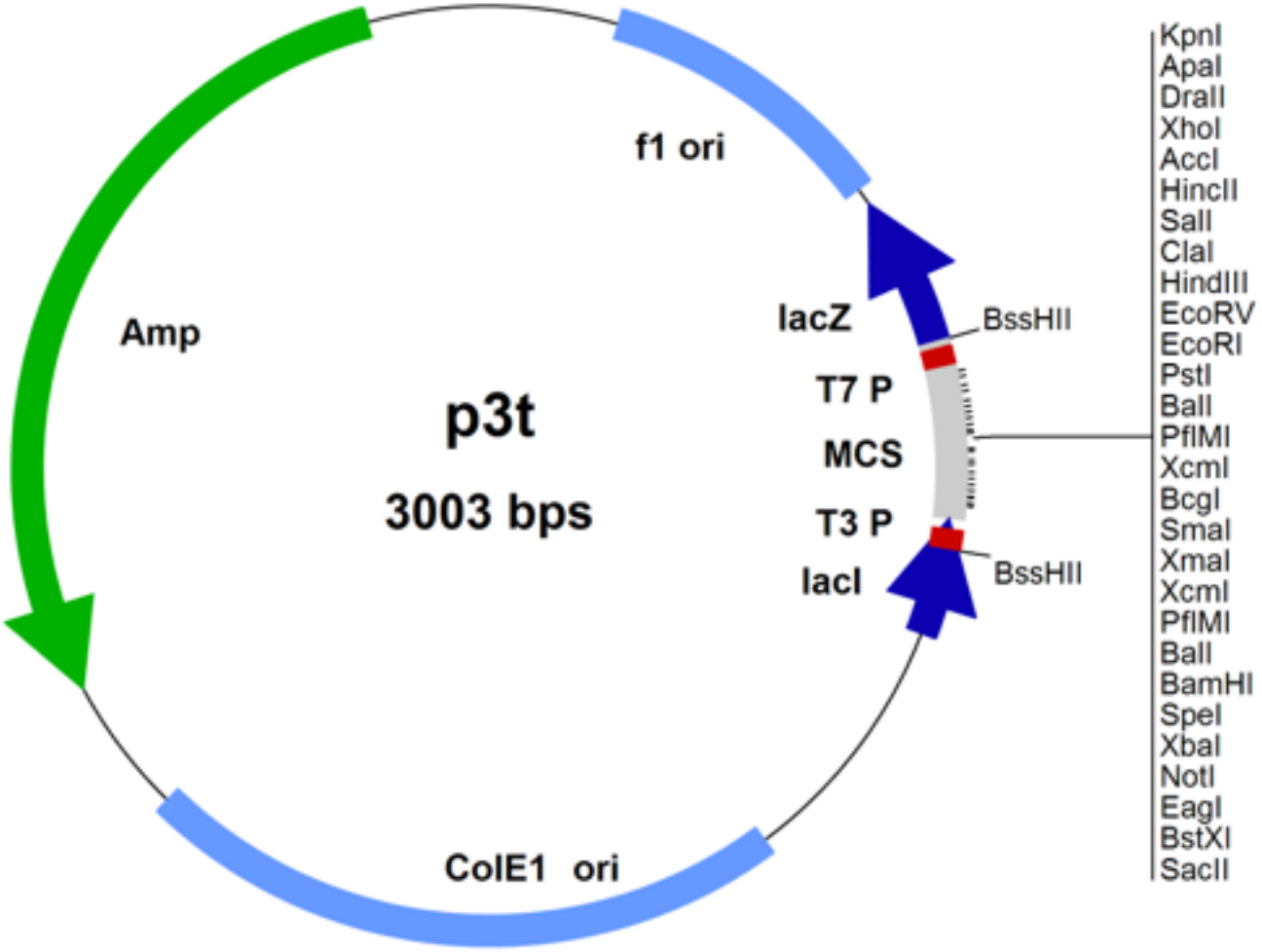
MATERIALS AND METHODS

E. coli strain W1485 containing the RSF1010 plasmid, which carries resistance to streptomycin and sulfonamide, was obtained from S. Falkow. Other bacterial strains and R

What features of plasmids are utilized in molecular cloning?

Cloning vectors

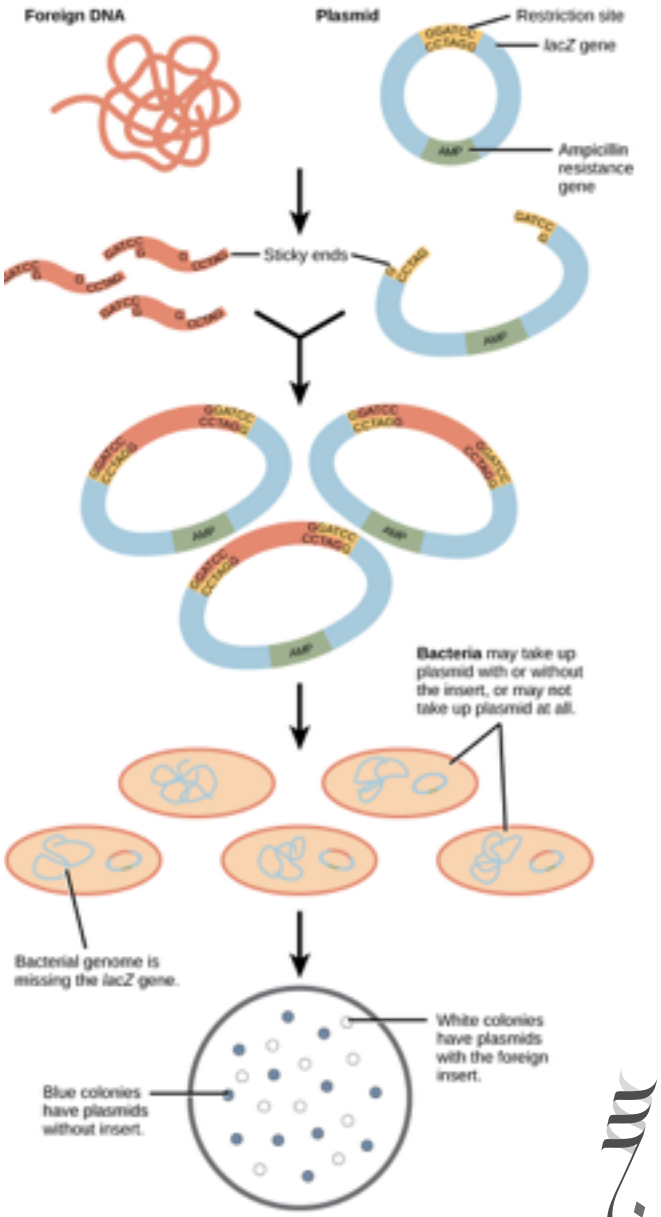
Vectors contain an origin of replication, selection gene, and a cloning site



How does molecular cloning work?

Cloning DNA pieces

- Cut plasmid + cut DNA + glue them + Transform into bacteria.
- Copying unknown DNA using a biological system (bacteria).
- It makes use Endonucleases (restriction enzymes) and plasmids of bacteria to copy a specific unknown piece of DNA.



Cloning vectors

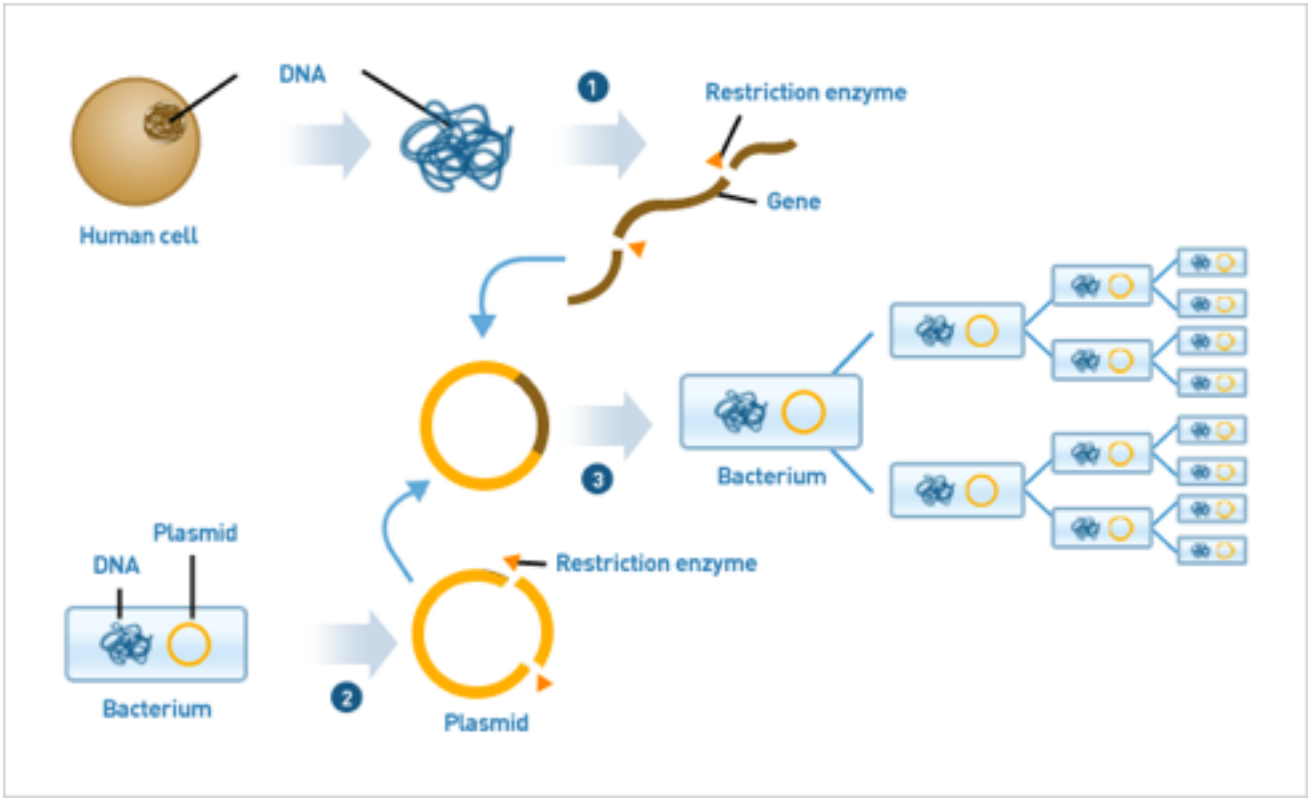
Table 1. Comparison of cloning vectors

Vector	Host	Structure	Insert size
Cosmids	*E. coli*	Circular plasmid	35–45 kb
P1 clones	*E. coli*	Circular plasmid	70–100 kb
BACs	*E. coli*	Circular plasmid	up to 300 kb
PACs	*E. coli*	Circular plasmid	100–300 kb
YACs	*S. cerevisiae*	Linear chromosome	100–2000 kb
MACs	Mammalian cells	Linear chromosome	? >1000 kb

YACs, BACs and MACs: artificial chromosomes as research tools. Trends Biotechnol. 12: 280–6

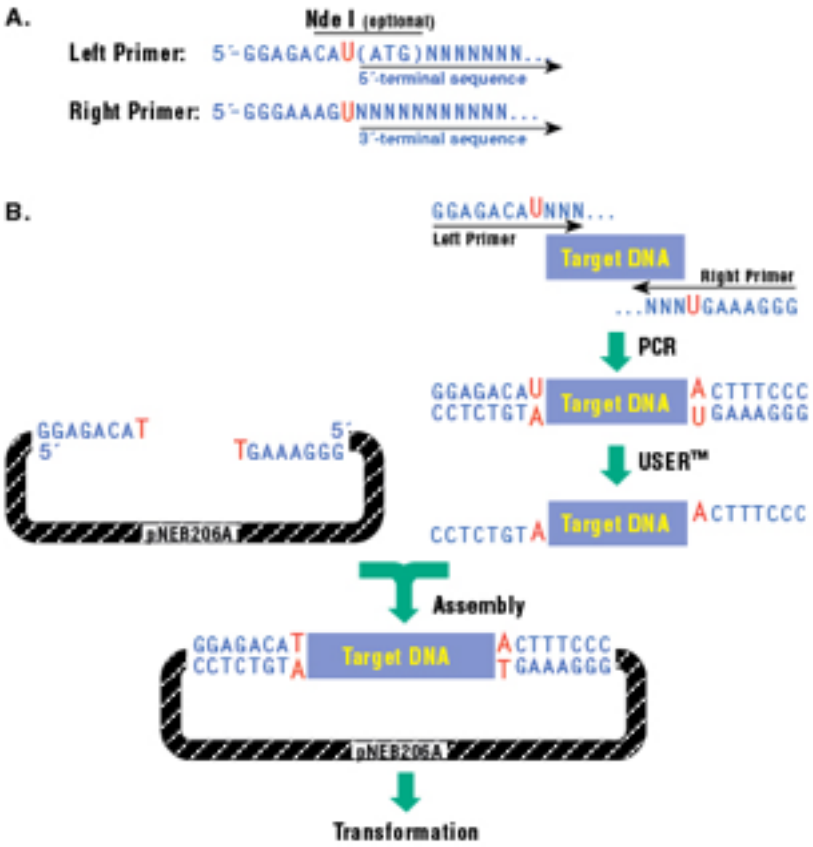
Molecular cloning usefulness

1) Provides enough DNA copies to carryout sequencing reactions.



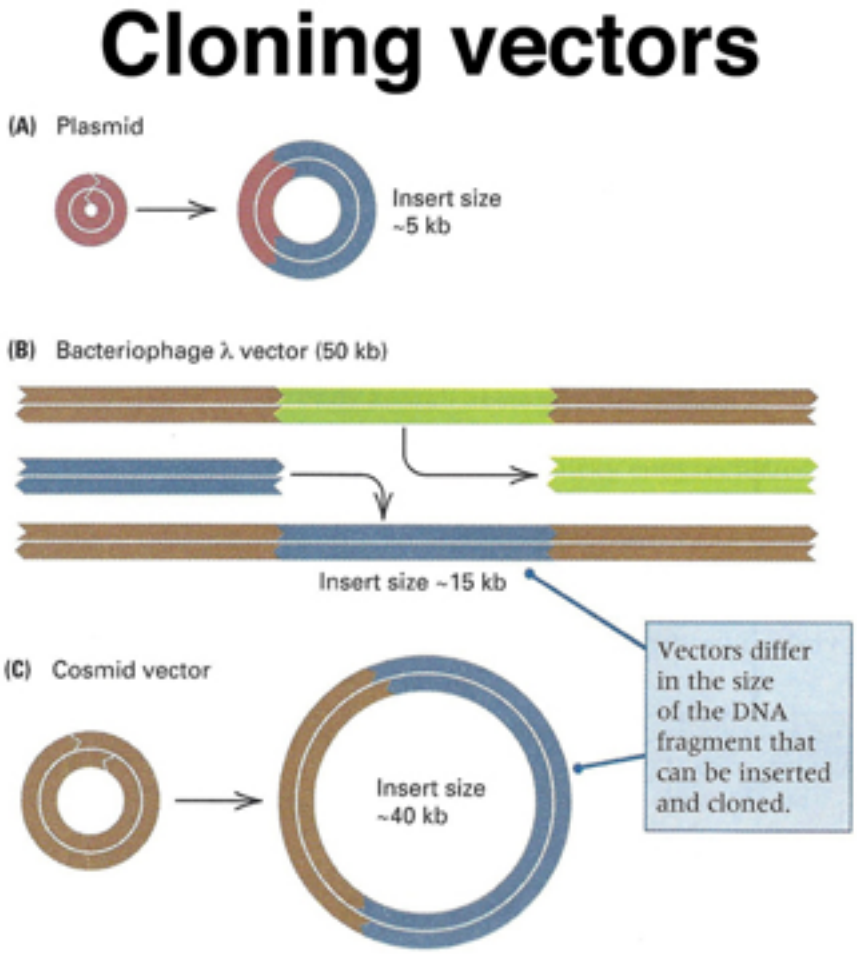
Molecular cloning usefulness

2) The cloning site of the vector is of known sequence, which can be used as primers for the sequencing reactions.



Molecular cloning usefulness

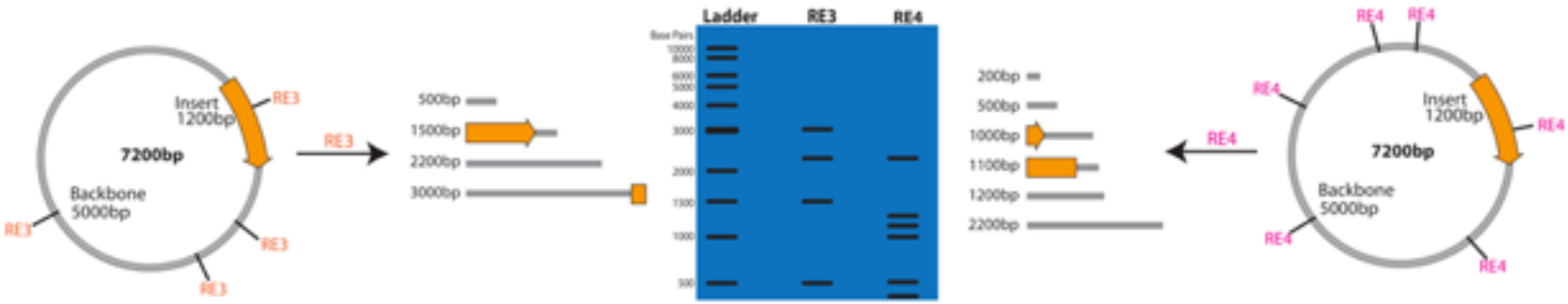
3) Allows coping DNA fragments of different sizes.



Molecular cloning usefulness

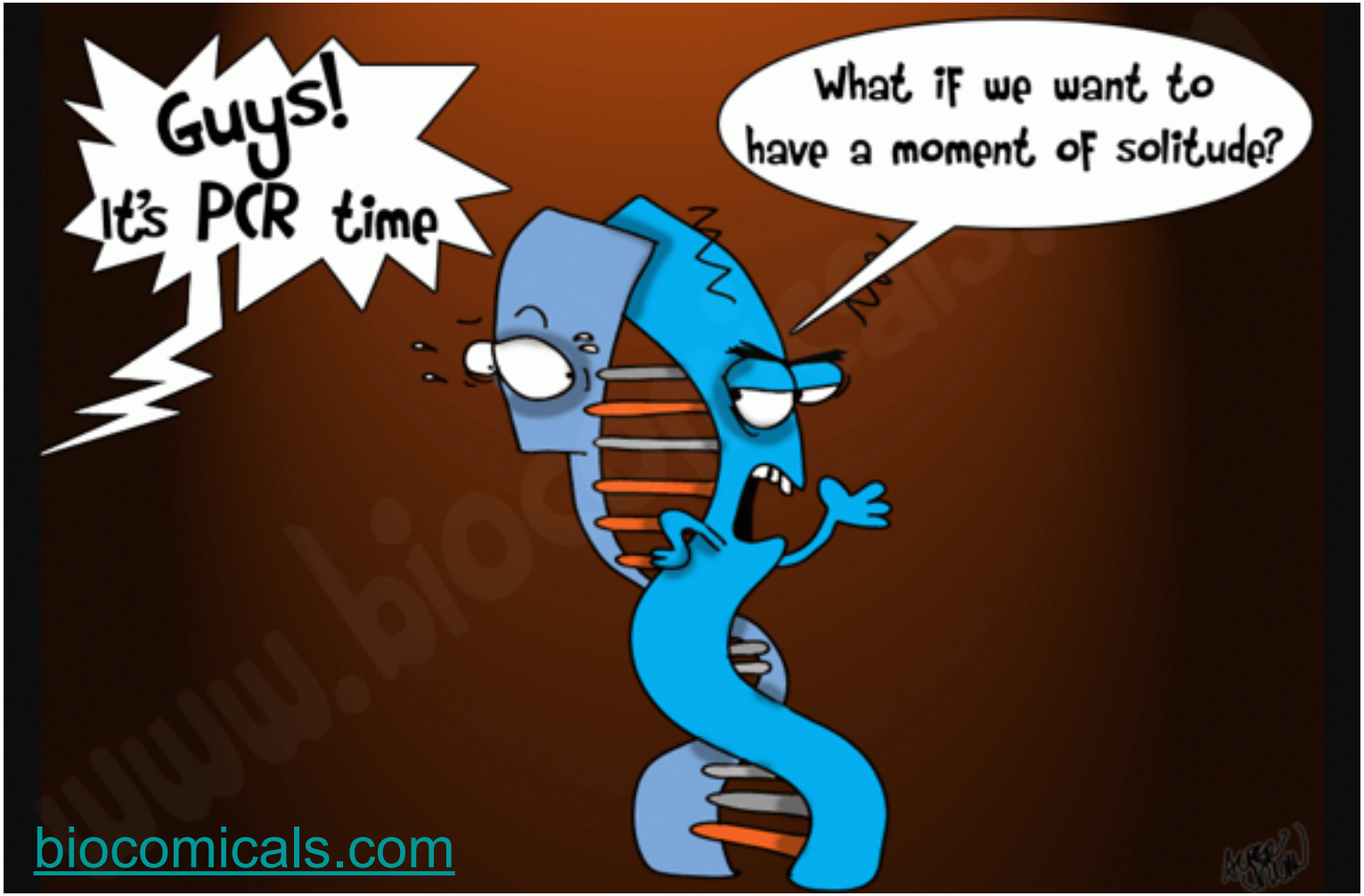
4) Provides information about the size of the unknown fragment.

5) Help in mapping!!



Molecular technologies of the time

Polymerase Chain Reaction



Polymerase chain reaction

• Polymerase Chain Reaction (**PCR**) allows the amplification (copying) of small amounts of DNA millions of copies.

• The method was developed by Kary Mullis (1983) and he was awarded the Nobel Prize for his invention.

Polymerase chain reaction

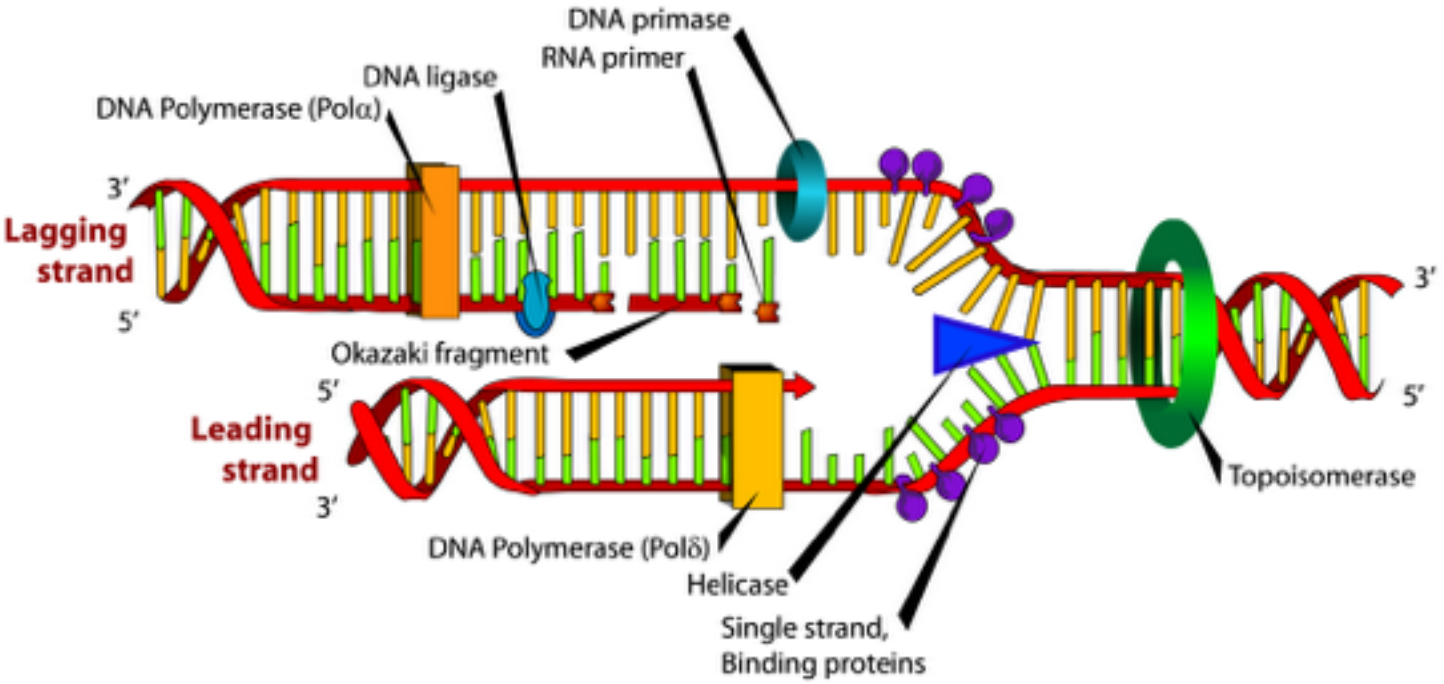
- The process of PCR is similar to the process of DNA replication except it is done in tubes rather than living cells.

- It is considered in many cases the first step before any genetic analysis.

- Many methods and applications involve PCR.

DNA replication and PCR

- DNA replication in the cells involves making an identical copy of the genome (DNA).
- PCR uses the same procedure but to generate millions of copies of a small section of the genome in a tube!



Why PCR?

PCR is used:

1. To amplify small quantities of DNA.
2. For DNA quantification.
3. For genetic profile analyses:
    - RFLP
    - Microsatellite
    - Mitochondrial DNA genotyping and sequencing.
4. For sequencing small section of the genome or the entire genome.

Components

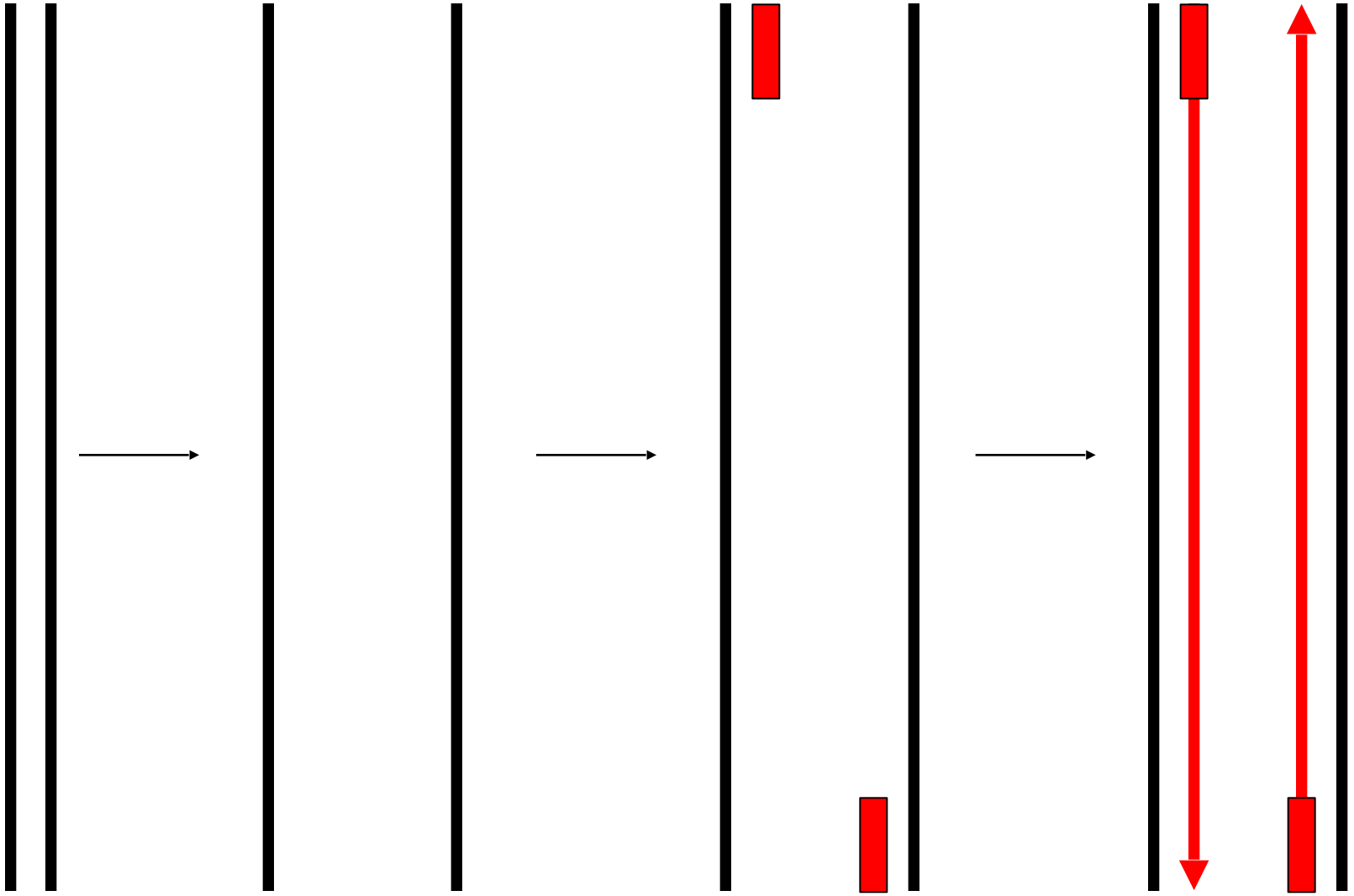
What do we need to replicate (copy) DNA?

1. DNA template.
2. Building block of DNA (dNTPs).
3. DNA copier (an enzyme).
4. 3’OH (primer).

PCR Process

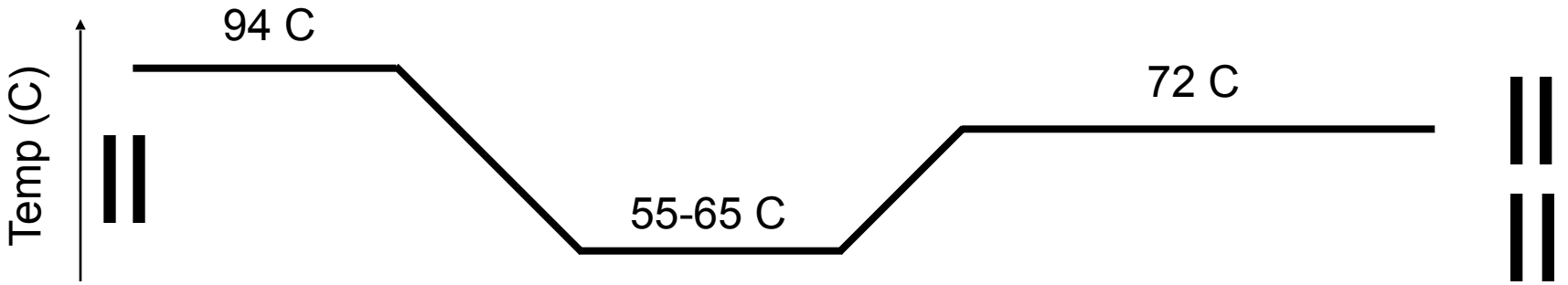
- Three steps are involved in PCR:
    1. **DNA template denaturation:** separation of the two strands of DNA.
    2. **Primers annealing:** small oligonucleotide attaches to each separated strand providing the 3’OH for DNA polymerase.
    3. **DNA polymerization (extension):** DNA polymerase extends the primers on both strands and adds nucleotides.

PCR Process



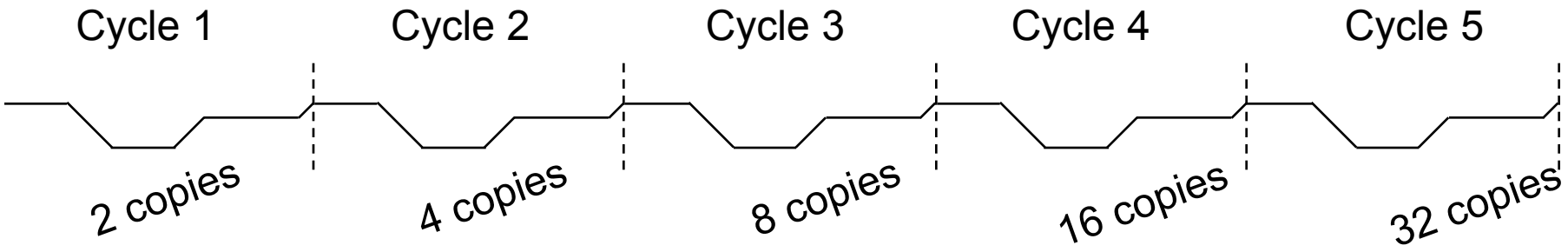
1. DNA denaturation
2. Primers annealing
3. DNA extension

PCR cycles



1. DNA denaturation 2. Primers annealing 3. DNA extension

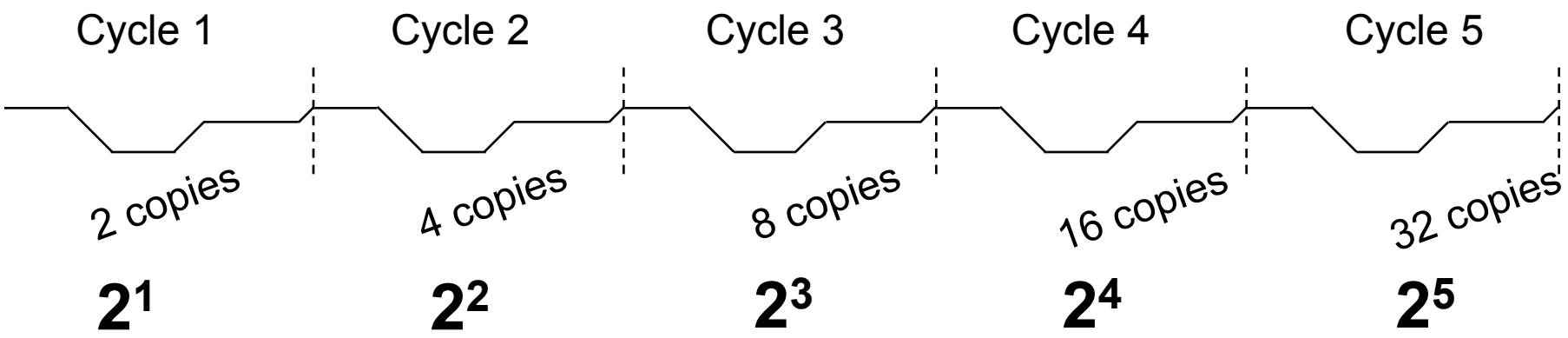
What happens if we repeat this cycle many times?



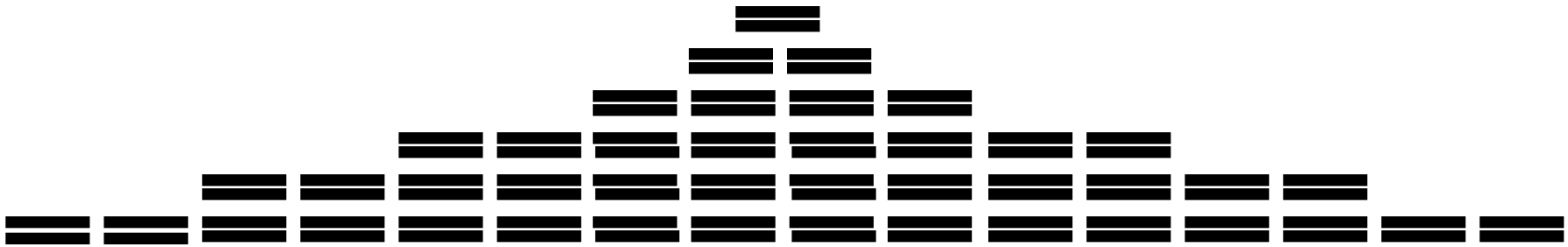
PCR cycles

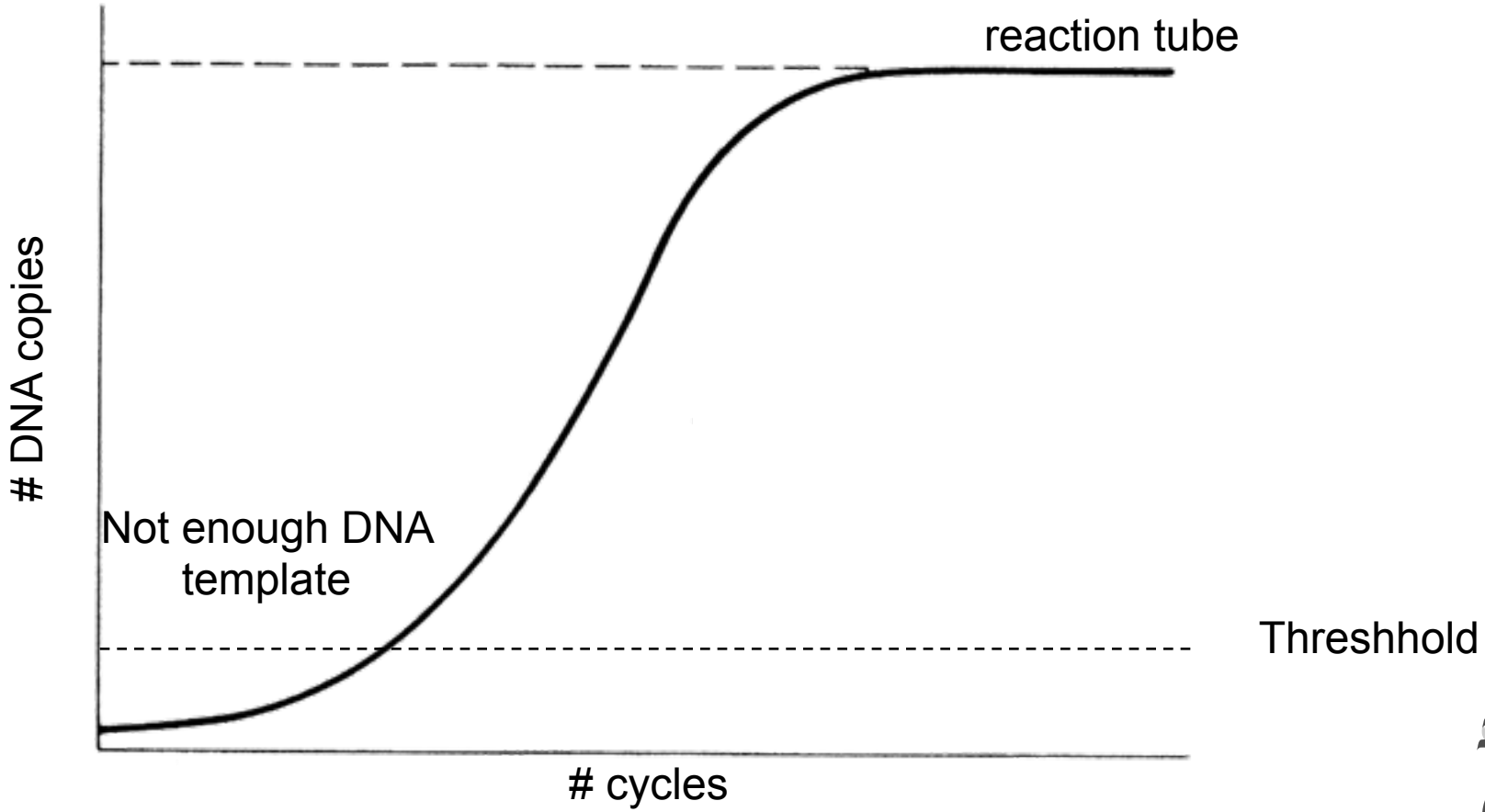
Exponential growth in the number of copies generated.

The number of copies you get at the end of your PCR will be $2^{\#cycles}$ ($2^{36\ cycles}$ = 68 billion copies)



PCR – how many copies?





Problems!

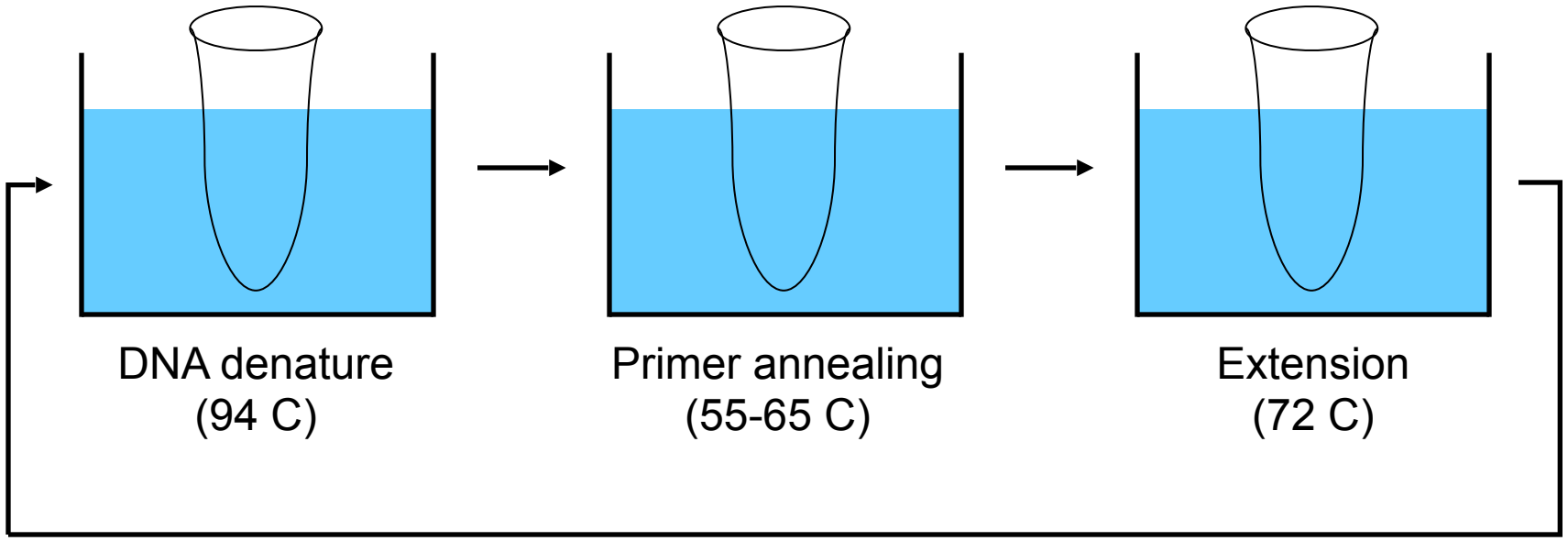
There were some difficulties with this system:

1. Three water-baths with three different temperature.

2. DNA polymerase denatures at 94 C.

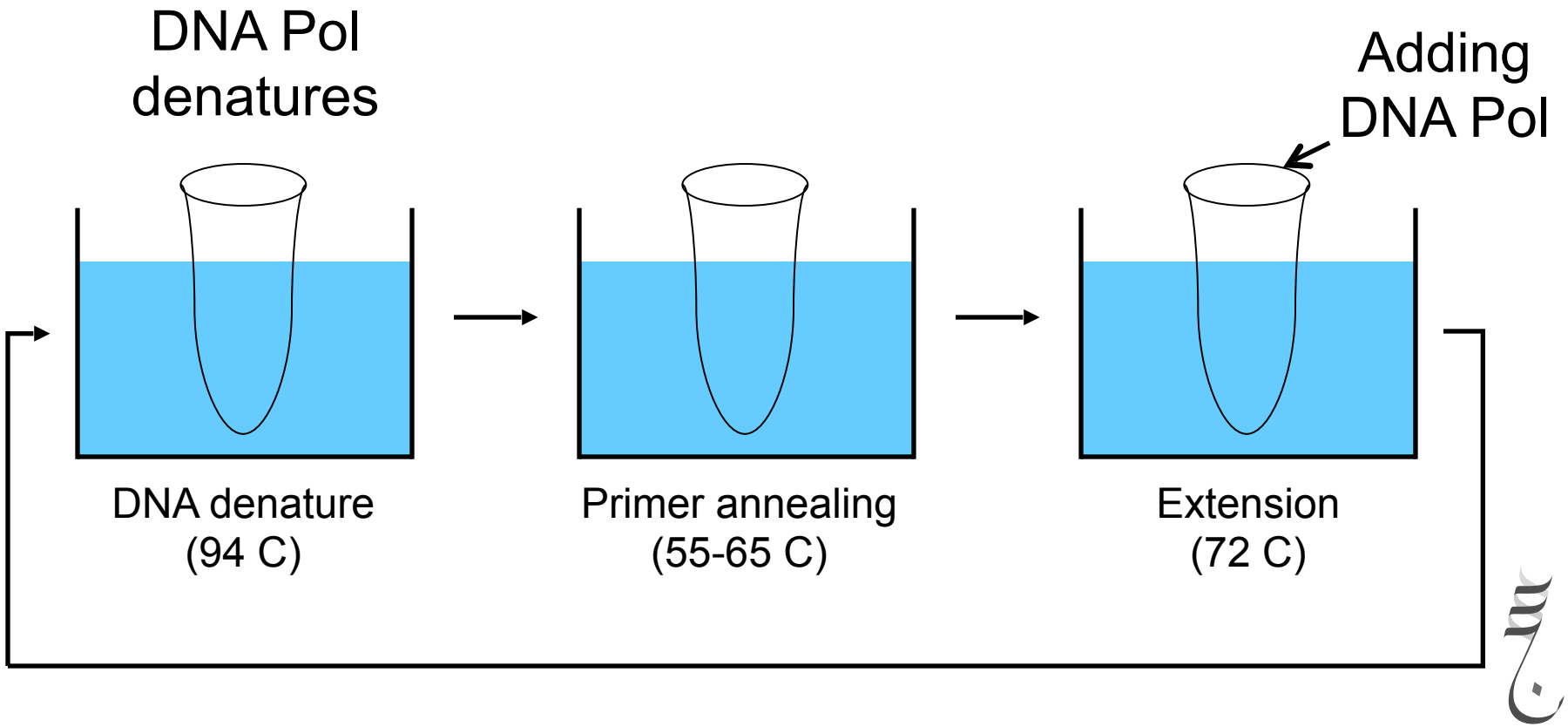
Problems!

- The sample has to be transferred into multiple water baths to accommodate the needed temperature.



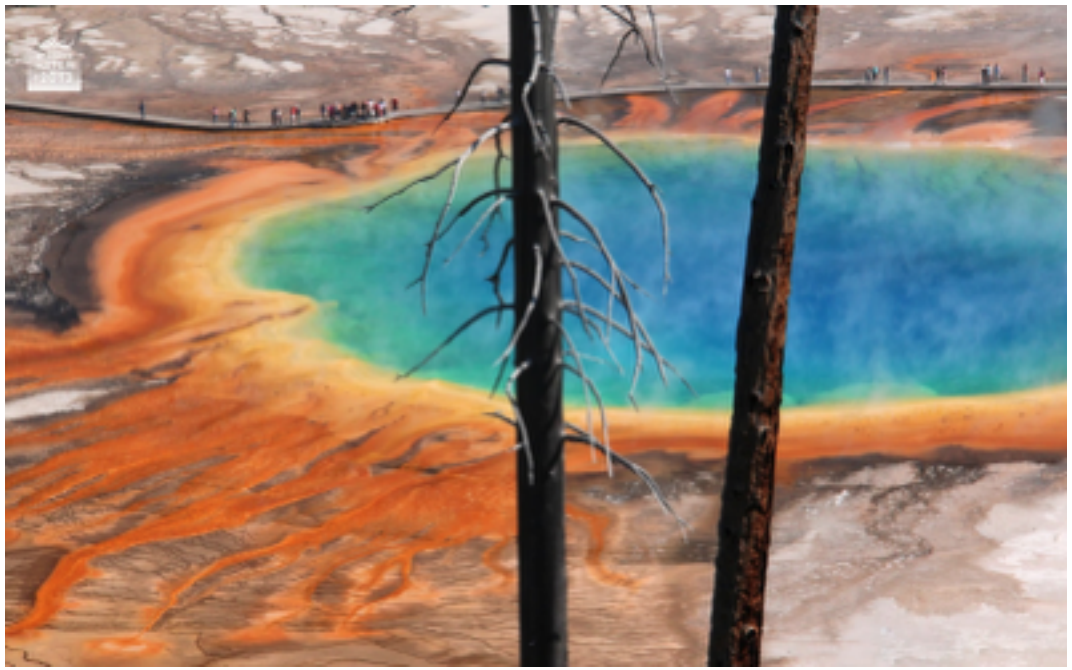
Problems!

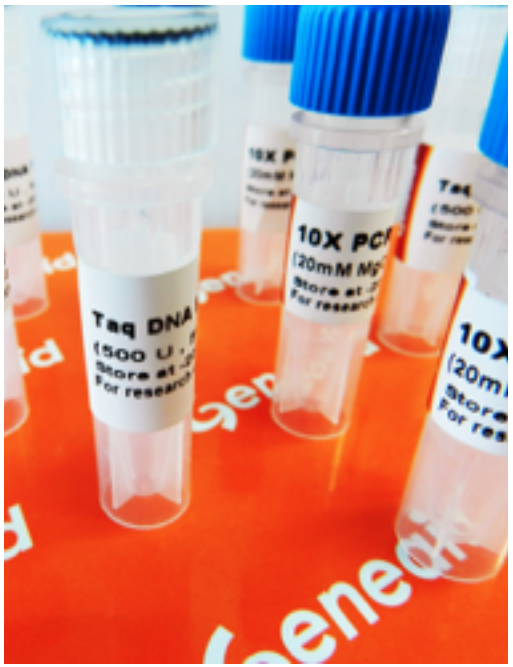
- DNA polymerase needs to be added in every cycle because DNA polymerase denatures at high temperature.



Improvement 1

- Using *Thermus aquaticus* (Taq) polymerase.
- Taq polymerase is heat stable and the cycles can take place without the polymerase being destroyed during the denaturation phase

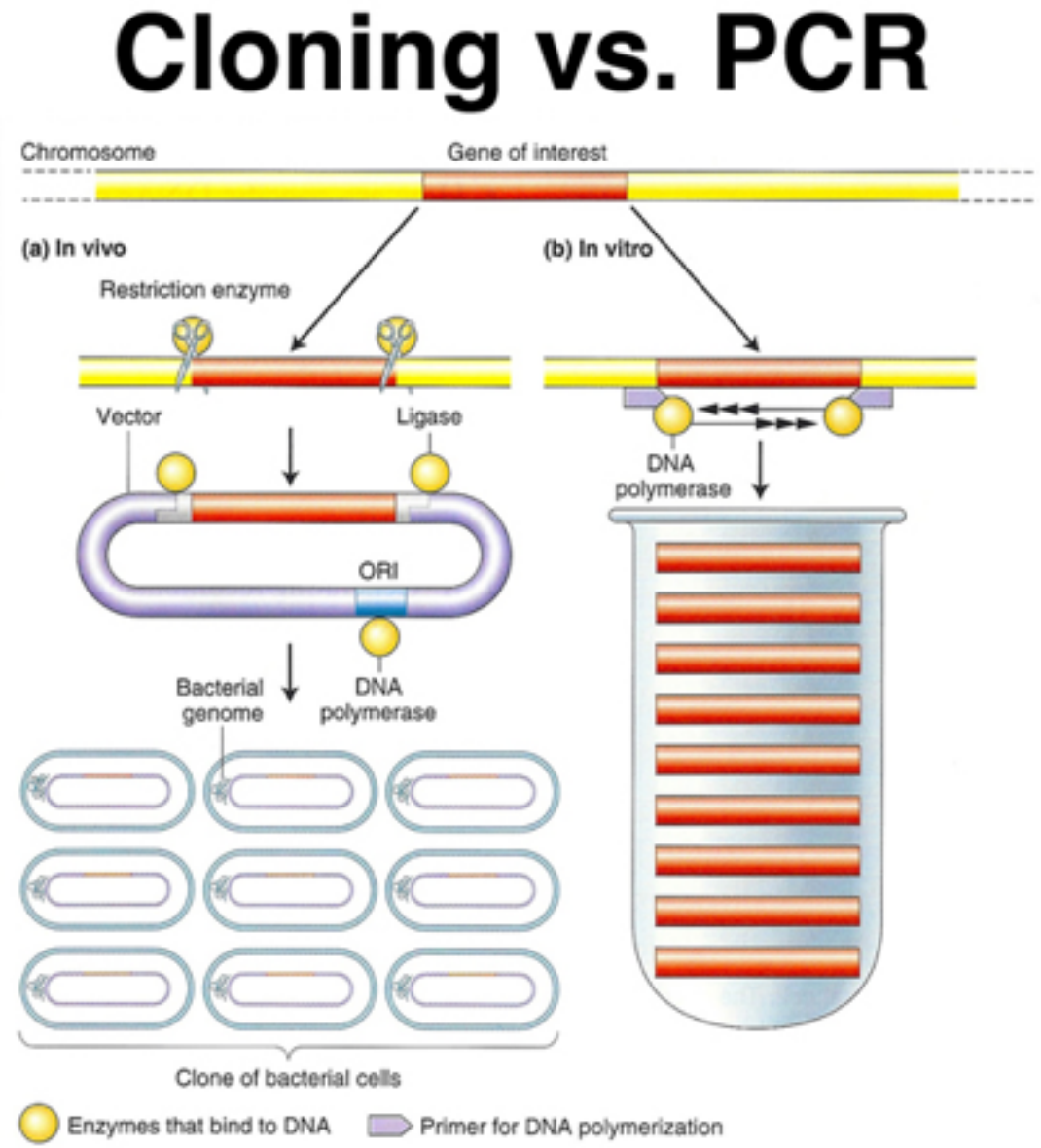




Improvement 2

Replacing old machine (water baths) with a thermocycler

Comparison


Cloning vs. PCR
Chromosome
Gene of interest
(a) In vivo
(b) In vitro
Restriction enzyme
Vector
Ligase
ORI
DNA polymerase
Bacterial genome
DNA polymerase
Clone of bacterial cells
Enzymes that bind to DNA
Primer for DNA polymerization

Why copy DNA?

Why molecular cloning or PCR matters in genome studies?

DNA CANNOT be sequenced using one copy!
Genome is huge!
Sequencing technology!

Expectations

- You understand the ideas behind molecular cloning and PCR.
- Do not focus on details but know the concepts in general.
- This will make more sense when DNA sequencing is covered.

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hhalhaddad@gmail.com